INFLUENCE OF NUTRITIONAL INTERVENTIONS TO OPTIMISE FAT METABOLISM AND EXERCISE PERFORMANCE.

By

ADRIAN HODGSON, BSc

A thesis submitted to

The School of Sport and Exercise Sciences

The University of Birmingham

For the Degree

DOCTOR OF PHILOSOPHY

School of Sport and Exercise Sciences

The University of Birmingham

August 2013

Abstract

This thesis investigated three commonly used nutritional interventions that are often claimed to alter substrate metabolism and improve exercise performance: green tea extract (GTE), coffee and vitamin D.

Prior studies have suggested that endurance exercise capacity may be improved when fat oxidation is increased through an intervention, thereby lowering the reliance on skeletal muscle glycogen. GTE and caffeine have both been hypothesized to increase fat oxidation at rest and during exercise, but the evidence to date remains equivocal. Using a metabolomics approach, we observed that 7 days GTE supplementation resulted in an increase in metabolites related to fat and energy metabolism at rest but not during moderate intensity exercise (56±4% VO_2max). The metabolic effects of GTE were independent of changes in catecholamines, making it less likely that the often cited catechol-O-methyltransferase (COMT) mechanism would be responsible for suggested increases in fat oxidation following GTE *in vivo*. The current thesis also found that endurance exercise performance can be improved to the same extent by either using coffee or caffeine (5 mg/kg body mass). However, these improvements in endurance exercise performance following coffee and caffeine were independent of changes to fat oxidation during exercise. Consequently, it seems unlikely that increasing fat oxidation with nutritional supports such as green tea and coffee can lead to improvements to endurance exercise performance.

It may also be important to prevent the development of nutritional deficiencies which could have a negative impact on exercise performance. Recent evidence has shown that vitamin D deficiency is highly prevalent worldwide, which is known to have deleterious effects on skeletal muscle function (at least in elderly). Yet to date, little is known about the prevalence of vitamin D deficiency in athletes and the impact on exercise performance. Chapters 4 and 5 in this thesis demonstrate that athletes living in Birmingham, United Kingdom (52.4-52.5°N), display a high prevalence of vitamin D deficiency (25-hydroxyvitamin D < 50 nmol/L) during the winter. The poor vitamin D status observed

is likely explained by the high latitude and climate of the test site as well as the observed low dietary intake of vitamin D by the athletes. However, despite the high prevalence of vitamin D deficiency, there was no association between vitamin D status and skeletal muscle function or exercise performance (aerobic, anaerobic, strength). Short term (28 days) vitamin D supplementation (1500IU/d, 3000IU/d or 6000IU/d) at doses above the current recommended daily allowance (200IU) were highly effective in correcting vitamin D deficiency to vitamin D sufficiency (25-hydroxyvitamin D > 75 nmol/L). The current thesis also showed that no further improvement to vitamin D status was observed above 1500IU/d. This data suggests that doses above 1500IU/d do not provide any additional benefit to vitamin D status. The improvements in vitamin D status to sufficiency did not result in exercise performance (aerobic, anaerobic, strength) being improved following supplementation. Taken together vitamin D supplementation, at a dose of at least 1500IU/d, is warranted in athletes in the United Kingdom during the winter to avoid deficiency. However, to date the optimal concentration of vitamin D required to avoid the deleterious effects to skeletal muscle function remains to be a subject of debate. Consequently the impact vitamin D has on exercise performance in athletes remains unclear.

“That which does not destroy us makes us stronger”

Friedrich Nietzsche 1844-1900

“If you want to win something, run 100 metres. If you want to experience something, run a marathon”.

Emil Zátope, Czech Republican Long Distance Runner 1922-2000.

Acknowledgements

There are a number of people I would like to thank who have made this PhD thesis possible. First and foremost I would like to start by thanking Professor Asker Jeukendrup for allowing me to complete a PhD within the school. I have learnt a great deal from you Asker. The drive, ambition and passion that you apply to all areas of your research career has inspired my research path and future. I thank you for the opportunities that you have given to me, the skills in and out of the laboratory, the various road trips and for showing me the attitude and determination that is required to succeed as an academic researcher. I suppose you are also responsible for my PhD induced weight loss from a 105kg rugby player to a 95 kg wannabe cyclist/triathlete, Thanks! I would also like to extend a huge thank you to Dr Sarah Aldred as well as Dr Gareth Wallis, who at the end of my PhD have provided support and advice, thank you to the both of you. I would also like to thank a number of academic researchers within the school who have also helped me and been there as mentors at some point in my PhD including Dr Andrew Blannin, Dr Chris Shaw and Dr Kevin Tipton. I would also like to acknowledge the financial support from Unilever as well as helping in the running of the collaboration with the University of Birmingham. I have learnt a great deal from the experts I have been in contact with, particularly Dr Niels Boon, Dr Silvina Lotito, Dr Doris Jacobs, Dr David Mela and Dr Theo Mulder.

I would like to especially thank my fellow PhD student, Rebecca Randell. You have put up with me for 3 years, including my large head and my persistent talking. Thank you for all your help and support, I think we did a pretty good job. Special thanks must also go to Dr Beate Pfeiffer, who gave me the initial interest to excel in academic research, and for allowing me to work with her on such a great project in my first year. Thank you to the EMRG, past and present, that helped in some capacity towards data collection, trouble shooting lab analysis and making those early morning lab trials that little bit more entertaining. Big thanks to 'Lunch Club' for the banter and cups of tea, you know who you are. I would also like to thank past and present PhD students within the school who have been and will continue to be great friends of mine including Oliver Wilson, Dr Sam Shepherd, Dr Matt Cocks, Dr Dan Crabtree, Dr Leigh Breen, Dr James Turner, Dr Mark Holland, Dr Stuart Bennett and Dr

Andrew Philp. I have also been very lucky to have some great office mates over the past 3 years especially those members of office 127, Dr Andrew Cooke, Dr Nicola Paine, Sally Fenton, Cliff and the other fishes, whose names I can't quite remember. Thank you for putting up with my complaints and stress, while still having a laugh, a beer and a cheer. Also, Sally, thanks for keeping me on my toes in the office with the various moulding cups of tea, frequent milk spillages (mainly on my desk) and milk and cheese disaster dish. Decent from you! Thanks must also go to my undergraduate students in the assistance they have provided in the lab, the lab technicians: Steve Allen, Rob Wheeler, Andy Benham, all of the admin staff and the head of school. I am sure there are many others who at some point helped me out who have not been named, thank you as well.

A solid support team outside of work has also got me through my PhD. Thanks to all the lads and friends at home and in London who have been there for me throughout. Big thanks to my family, especially my Mum and Dad who have listened, supported, helped where they can throughout the 3 years, even if they were not quite sure where I was or what I was doing. You have both been invaluable throughout this whole experience.

Last but certainly not least, a huge thank you must go to my girlfriend Tori. Don't worry, I told you you would make it in the acknowledgements! Without sounding like a wet flannel, without you I would not have got through the last 3 years, words cannot describe the love and support you have provided. You have stood by me in everything I have done, put up with all the lab work at silly o'clock in the morning, been patient where most others would not have been, listened to me drone on about science as well as putting up with my geek status. I am eternally grateful.

List of Publications

Academic Journal papers

Published

1. Pfeiffer B, Stellingwerff T, Zaltas E, **Hodgson AB**, Jeukendrup AE. **Carbohydrate oxidation from a drink during running compared with cycling exercise.** Med Sci Sports Exerc. 2011 Feb;43(2):327-34.
2. Pfeiffer B, Stellingwerff T, **Hodgson AB**, Randell R, Pöttgen K, Res P, Jeukendrup AE. **Nutritional intake and gastrointestinal problems during competitive endurance events.** Med Sci Sports Exerc. 2012 Feb;44(2):344-51.
3. **Hodgson AB**, Randell R, Boon N, Garczarek U, Mela DJ, Jeukendrup AE, Jacobs DM. **Metabolic response to green tea extract during rest and moderate intensity exercise.** J Nutr Biochem. 2013 Jan;24(1):325-34

1. **Hodgson AB**, Randell R, Jeukendrup AE. **Review: The effect of green tea extract on fat oxidation at rest and during exercise: evidence of efficacy and proposed mechanisms.** Adv Nutr. 2013 Mar 1;4(2):129-40
2. Randell R, **Hodgson AB**, Lotito S., Jacobs DM, Mahabir-Jagessar K, Boon N, Jeukendrup AE**. The effect of short versus long term Green Tea Extract supplementation on fat oxidation during exercise** Med Sci Sports Exerc. 2013 May;45(5):883-91.
3. **Hodgson AB**, Randell R, Jeukendrup AE. **The metabolic and performance effects of caffeine compared to coffee during exercise** PLoS One. 2013;8(4):e59561.
4. **Hodgson AB,** Randell R, Jeukendrup AE. **An update: Caffeine and exercise performance.** Sports Cardiovascular Athletic Nutrition, Pulse, Invited review, Accepted March 2013.

Submitted

1. **Hodgson AB**, Sanghera G, Hall T, Aldred S**. High prevalence of vitamin D deficiency and insufficiency in university athletes living in the United Kingdom.** European Journal of Sport Science. Submitted March 2013.

2. **Hodgson AB**, Martin J, Roberts T, Ingham A, Jeukendrup AE. **The effects of short term vitamin D_3 supplementation on vitamin D status and exercise performance in athletes.** Medicine and Science in Sports and Exercise, Submitted Nov 2012.

In preparation

1. **Hodgson AB,** Jeukendrup AE. **Variation of respiratory measures with commercially available breath by breath systems.**
2. **Hodgson AB**, Randell R, Jagesser-T KM-J, Silvina L, Mela DJ, Boon N, Jeukendrup AE, Jacobs DM. **Bioactivity of green tea polyphenols and their association with plasma metabolome.**
3. Randell R, **Hodgson AB**, Lotito S., Jacobs DM, Mahabir-Jagessar K, Boon N, Jeukendrup AE**. The effect of chronic green tea extract supplementation on substrate metabolism during exercise.**

Abstracts

1. **Hodgson AB**, Martin J, Roberts T, Ingram A, Jeukendrup AE. **The effects of short term vitamin D_3 supplementation on athletic performance.** Accepted for poster presentation at the 59th American College of Sports Medicine meeting 2012.
2. **Hodgson AB**, Randell R, Jeukendrup AE. **The effect of caffeine and coffee on substrate metabolism and exercise performance**. Accepted for poster presentation at the 59th American College of Sports Medicine meeting 2012.

3. **Hodgson AB**, Randell R, Boon N, Garczarek U, Mela DJ, Jeukendrup AE, Jacobs DM. **Metabolic response to green tea extract during rest and moderate intensity exercise**. Accepted for poster presentation at European College of Sport Science 2012.
4. **Hodgson AB,** Jeukendrup AE**.** Variation of respiratory measures with commercially available breath by breath systems. Accepted for poster presentation at the 59th American College of Sports Medicine meeting 2011.
5. Randell R, Jeukendrup AE, Pfeiffer B, **Hodgson AB**, Pottgen K, Res P, Stellingwerff T. **Nutritional intake and gastrointestinal problems during competitive endurance events**. Accepted for poster presentation at the 59th American College of Sports Medicine meeting 2011.

Invited talks

1. **Hodgson AB**, Randell R, Jeukendrup AE. **The metabolic and performance effects of caffeine compared to coffee**. November 2012. Invited Speaker at Rank Prize Sports Nutrition Symposium.
2. **Hodgson AB**, **Nutrition of Mother Earth: Green tea.** July 2012. Invited Speaker at the European College of Sport Science, Bruge.
3. **Hodgson AB**, Jeukendrup AE. **A storm in a cup of coffee: The metabolic, ergogenic and health effects of caffeine and coffee**. Invited talk at the Norwegian Coffee Association Coffee Day 2011.

List of Abbreviations

Acetyl-CoA carboxylase	ACC
Activated vitamin D	$1,25(OH)_2D_3$
Acyl CoA synthetase	ACS
Acylcarnitine translocase	ACT
Activated protein 2	AP2
Adenosine diphosphate	ADP
Adenosine monophosphate	AMP
Adenosine monophosphate-activated kinase	AMPK
Adipose triacylgercol lipase	ATGL
Adenosine triphosphate	ATP
Adrenaline	A
Beat per minute	BPM
Body mass	BM
Bone mineral density	BMD
Brown adipose tissue	BAT
Caffeine	CAF
Calcium	Ca^{2+}
Calories	kcal
Carbon dioxide	CO_2
Carnitine palmitoyltransferase 2	CPT2
Carnitine palmitoyl transporter I	CPT1
Carnitine via acylcarnitine translocase	CACT
Catechol-O-methyltransferase	COMT
Ccaat-enhancer binding protein- α	C/EBP-α
Central nervous system	CNS
Chlorogenic acid	5-CQA
Coefficient of Variation	CV
Coffee	COF
Complex cytochrome C oxidase	COX IV
Confidence Interval	CI
Cyclic adenosine monophosphate	cAMP
Day	d
Decaffeinated Coffee	DECAF
Diacylglycerides	DAG
Diet induced thermogenesis	DIT
Deoxyribonucleic acid	DNA
Electron transport chain	ETC
Energy Expenditure	EE
Epicatechin	EC
Epicatechin gallate	ECG
Epigallocatechin	EGC

Epigallocatechin gallate	EGCG
Ethylenediaminetetraacetic acid	EDTA
Excitation-contraction	EC
Extracellular regulated kinase1/2	ERK1/2
Fatty acid binding proteins in the cytosol	FABPc
Fatty acid binding protein found in the sarcolemma	FABPm
Fatty acid synthase	FAS
Fatty acid translocase	FAT/CD36
Fatty acid transport proteins	FATP
Flavin adenine dinucleotide	FADH2
Foods Standard Agency	FSA
Free fatty acids	FA
Gas Chromatography Mass Spectrometry	GC/MS
Glucose-6-phosphate	G6P
Gram	g
Green tea extract	GTE
Heart rate	HR
High density lipoproteins	HDL
High Performance Liquid Chromatography	HPLC
Hormone sensitive lipase	HSL
Hour	hr
1α-Hydroxylase	1-Ohase
25-hydroxyvitamin D	25(OH)D
3-hydroxyacyl-CoA dehydrogenase	HAD
Institute of Medicine	IOM
Intermediate density lipoproteins	IDL
International Units	IU
intramuscular triacylglycerol	IMTG
Kilogram	kg
Kilojoules	kJ
lipoprotein lipase	LPL
Liquid Chromatography Mass Spectrometry	LC/MS
Litres	L
Low density lipoproteins	LDL
Maximal Oxygen Uptake	VO_2 max
Maximal voluntary contraction	MVC
Maximum Watts	W_{max}
Metre	m
Microlitre	µl
Micromole	µM
Milligram	mg
Millilitres	mL
minute	min
Minute carbon dioxide production	VCO2

Minute oxygen consumption	VO2
mitogen activated protein kinase	MAPK
nanomole	nmol
Newton	N
Nicotinamide adenosine dinucleotide	NADH
noradrenaline	NA
parathyroid hormone	PTH
partial least squares/projection to latent structures-discriminant analysis	PLS-DA
PDH kinase	PDK
PDH phosphatase	PDP
peripheral nervous system	PNS
peroxisome proliferator activated receptor	PPAR
peroxisome proliferator activated receptor–γ coactivator	PGC1α
phosphocreatine	PCr
phosphodiesterase	PDE
phosphodiesterase	PDE
phosphofructokinase	PFK
phosphoinositol-3-kinase	PI3-K
Placebo	PLA
Principal component analysis	PCA
Pyruvate dehydrogenase	PDH
Quality Control	QC
Rate of appearance	R_a
Rate of disappearance	R_d
Rating of perceived exertion	RPE
Recommended daily allowance	RDA
Reference Nutrient Intake	RNI
1 Repetition max	1 RM
Respiratory Exchange Ratio	RER
Respiratory Quotient	RQ
Revolution per minute	RPM
sarcoplasmic reticulum	SR
Scientific Advisory Committee on Nutrition	SACN
Seconds	sec
Sodium potassium pump	Na^+-K^+-ATPase
Standard Deviation	SD
Standard Error	SE
Stearoyl-CoA	SCD1
Sterol regulatory element binding protein-1c	SREBP-1c
Sympathetic nervous system	SNS
Time to peak	Tmax
Time trial	TT
Triacylglycerols	TG

Tricarboxylic acid	TCA
Tricarboxylic acid Intermediate	TCAI
Ultraviolet-B radiation	UVB
Very low density lipoproteins	VLDL
Vitamin D binding protein	DBP
Vitamin D receptor-retinoic acid-x-receptor complex	VDR-RXR
Wild type	WT
World Anti-Doping Agency	WADA

List of Tables

List of Figures

TABLE OF CONTENTS

Chapter 1

General Introduction

Content from this chapter has been published:

The Effect of Green Tea Extract on Fat Oxidation at Rest and during Exercise: Evidence of Efficacy and Proposed Mechanisms. Adv Nutr. 2013 Mar 1;4(2):129-40.

Caffeine for exercise performance: An update. Scans Pulse (Accepted 2013)

1.1 Perspective and overview

Today, there is a plethora of nutritional interventions which are often claimed to improve exercise performance or surrogate measures thereof [1]. Amongst these nutritional interventions there are a small number that are regularly talked about or used by athletes. This is perhaps because of the potential health benefits or ergogenic effects they offer. Alternatively, athletes may use nutritional interventions regardless of any scientific supporting evidence, but rather because they are 'trending nutrition hot topics'. Green tea, coffee and vitamin D are three such commonly used nutritional interventions, often cited in the literature and used by athletes to promote health and improve exercise performance. Further, green tea, coffee and vitamin D are readily available to athletes in whole foods and drinks, making them easily accessible. The health promoting effects of green tea have been associated with improvements in conditions including: diabetes, obesity, atherosclerosis and cancer [2]. However, caffeine found in green tea and coffee, is cited to induce negative health effects specifically relating to insulin resistance and hypertension [3]. In contrast the consumption of coffee has a number of health promoting effects similar to green tea [3]. Nonetheless, the metabolic and physiological similarities and differences between caffeine and coffee are yet to be determined, despite coffee being the predominant source of caffeine in an athlete's diet [4]. It has been proposed that green tea and caffeine augments fat metabolism [2, 5], which would be beneficial to health, and may also be important for athletic performance [6]. However, there is very little evidence to show that green tea increases fat oxidation, particularly during exercise. Further, while there is a great deal of evidence that explores the metabolic and ergogenic effects of caffeine, there is a paucity of evidence for the role of coffee improving athletic performance [5].

Vitamin D has been associated with benefits to mortality, diabetes, rheumatoid arthritis and other chronic diseases [7], in addition to its well known role in the maintenance of bone health throughout the life span [8]. Vitamin D has also been associated with improvements in skeletal muscle function, structure and composition [9] and could thus affect exercise performance [10]. However, evidence of the role of vitamin D in exercise performance is further exacerbated by the fact vitamin D deficiency

is high prevalent worldwide today [11]. The prevalence of vitamin D deficiency as well as the role of vitamin D on muscle function and exercise performance in athletes remains to be investigated.

Gaining a better comprehensive understanding of the metabolic and physiological effects of green tea, coffee and vitamin D is important to decide whether or not these nutritional interventions are important for athletes to improve exercise performance. Therefore, the studies in this thesis present data on the effects of green tea, coffee, and vitamin D in the context of endurance exercise.

1.2 Substrate utilisation for endurance exercise performance

Endurance exercise performance is partly determined by the supply and resynthesis of adenosine triphosphate (ATP) through aerobic and anaerobic metabolism. Fat and carbohydrate are the two major substrates that are responsible for the resynthesis of ATP during exercise [12]. This is particularly obvious when exercise duration increases beyond a couple of minutes [13].

It has been known since the turn of the century that both carbohydrate and fat are fuels used in the skeletal muscle at varying contributions at rest and during exercise [12, 14]. This early work showed using the respiratory exchange ratio (RER) that at rest, fat was the predominant substrate, while during exercise there appeared to be an increased contribution of carbohydrate. The contribution of carbohydrate was increased at higher exercise intensities (>65% VO_2 max), over shorter exercise durations and following a high carbohydrate diet [12, 14]. Under these conditions, there was a concomitant decrease in fat oxidation. Numerous other studies have also shown that the utilisation of fat and carbohydrate during exercise is also determined by dietary intake of macronutrients [15-18], gender [19], metabolic health [20] and training status [12, 21-25].

More recently, the early work by Krogh and Lindhard [14] and Christensen and Hansen [12] has been confirmed by Romijn et al [26] and van Loon et al [27] using isotope tracer methodology. Both authors showed that carbohydrate oxidation increased as exercise intensity rose (25-85% VO_2 max), while fat oxidation peaked at moderate exercise intensity (50-65% VO_2 max) when compared to the lower and higher exercise intensities. Similarly, Achten et al [28] performed a well controlled study to

elucidate the exact intensity in which maximal fat oxidation rates occurred (FatMax). It was observed that during graded cycling exercise FatMax was at 64±4 % VO_2 max in moderately trained men, with a steep reduction in fat oxidation and a large increase in carbohydrate oxidation as the exercise intensity increased. However, while carbohydrate oxidation is the predominant substrate during higher exercise intensity, it is important to highlight that the store of glycogen (skeletal muscle and liver) is small when compared to the stores of fat in the human body (1785-2000 kcal and 80,000-140,000 kcal, respectively) [29]. As a result skeletal muscle glycogen depletion can occur when exercise is performed at higher exercise intensities (>70% VO_2 max) [30, 31], which also coincides with the onset of fatigue [6, 32]. Interestingly, altering the provision of skeletal muscle glycogen has been shown to dictate fuel selection and consequently endurance exercise capacity. The seminal work by Bergstrom et al [32] illustrated that carbohydrate feeding was able to increase pre exercise muscle glycogen content which led to a delayed onset of fatigue. Furthermore, Coggan et al [31] showed that carbohydrate feeding during high intensity exercise (70% VO_2 max) to fatigue, significantly increased carbohydrate oxidation, endurance exercise capacity as well as elevated skeletal muscle glycogen at the termination of exercise when compared to placebo. In addition, increasing the supply of fatty acids (FA) during exercise, through intra lipid-heparin infusions [33, 34] or more recently through the feeding of a high fat/low CHO diet [15-18], has been reported to increase fat oxidation and spare skeletal muscle glycogen during high intensity exercise (>70% VO_2 max). Augmenting the availability of FA, while also reducing carbohydrate availability and oxidation, is a highly effective strategy to optimise fat oxidation during exercise. Therefore, identifying nutritional interventions that are capable of augmenting FA availability and oxidation would be of interest to athletes in the hope of improving endurance exercise performance.

1.3 Regulatory factors of fat metabolism during endurance exercise

Fat oxidation is predominantly dependent on the exercise intensity and duration which regulates the utilisation of multiple fat sources. The predominant store of fat is triacylglycerols (TG) found in subcutaneous and visceral adipose tissue, with smaller stores found within skeletal muscle fibres as

intramuscular triacylglycerol (IMTG) and in between muscle fibres (extramuscular TG) [29]. Through the process of lipolysis, FA can be released from TG which is transported in the blood bound to albumin or incorporated into lipoproteins (chylomicrons, very-low-density lipoproteins (VLDL), low-density lipoproteins (LDL), intermediate-density lipoproteins (IDL) and high-density lipoproteins (HDL)). Romijn et al [26] revealed that by using stable isotope methodology ([6,6-$^{2}H_2$] glucose and [$^{2}H_2$] palmitate) FA rate of disappearance (R_d), which was assumed to equal oxidation, peaked at 65% VO_2 max when compared to 25% and 85% VO_2 max. Additionally, it was shown that fat oxidation was mainly driven by the oxidation of FA at low and moderate exercise intensities (25 and 44% VO_2 max, respectively) when compared to other fat sources including intramuscular plus lipoprotein derived TG (IMTG). These data were later confirmed by van Loon et al [27] who also implemented a stable isotope methodology ([6,6-$^{2}H_2$] glucose and [U-^{13}C] palmitate) by accurately measuring FA oxidation from $^{13}CO_2$ in expired air. Similarly, FA oxidation peaked at 57% VO_2 max compared to 44% and 72% VO_2 max, but FA oxidation was significantly reduced at the highest exercise intensity when compared to the other intensities. Hence, the work by van Loon et al [27] and Romijn et al [26] clearly illustrate that sources of fat in the human body are highly regulated by the intensity of exercise. The availability of FA and their subsequent oxidation appears to be the predominant substrate used during exercise, especially at low to moderate exercise intensities. However, before FA can be oxidised in the mitochondria, they must first be released from the adipose tissue, be transported in the circulation to the skeletal muscle, be taken up and transported across the cytoplasm of the skeletal muscle, released from IMTG, before then being transported across the inner and outer mitochondrial membranes [35]. These steps may be regulatory components responsible for the limitations that are observed in fat oxidation during exercise at varying exercise intensity. Having a better understanding of the regulatory components of fat oxidation during exercise, as well as the specific mechanisms of each, may be advantageous when developing novel nutritional interventions that may be able to augment fat oxidation

.

1.3.1 Fatty acid metabolism during exercise

FA metabolism includes the release, transport in the circulation and uptake of FA from adipose tissue into the skeletal muscle. It is these regulatory components that may limit fat oxidation rates across varying exercise intensities, particularly when exercise is performed at high intensity (>70% VO_2 max) [26, 27].

1.3.1.1 Fatty acid release into the circulation during exercise

Lipolysis is the process in which FA and glycerol are liberated from TG through lipase enzymes namely: hormone sensitive lipase (HSL), adipose triacylglycerol lipase (ATGL) and monoacylglycerol lipase (MGL) [36]. The activity of these lipase enzymes are controlled by the sympathetic nervous system (SNS), through the stimulatory action of catecholamines on β-adrenergic receptors [37]. Lipolysis is inhibited by insulin, via its prevention of the phosphorylation of HSL [38]. It has also been shown that lactate, formed as a consequence of increased rates of glycolysis, may also reduce FA release from the adipose tissue. Boyd et al [39] showed that whole body FA and glycerol release during low intensity exercise (40% VO_2 max) was reduced when sodium lactate was infused in males. Additionally, FA release from perfused dog abdominal fat pads when exposed to high lactate concentrations were also shown to be reduced [40].

These regulatory steps to lipolysis provide evidence for the greater R_a of FA from the rest to exercise transition, the continued increase in R_a during graded exercise [26, 27, 41] and the subsequent reduction in FA R_a, firstly at high exercise intensities due to higher glycolytic rate and secondly due to the rise in insulin with carbohydrate feeding [25, 42, 43]. Collectively this supports carbohydrate oxidation having a reciprocal regulation on fat oxidation during exercise. This is in contrast to the traditional glucose-FA cycle hypothesis proposed by Randle and Co-workers [44]. The subsequent reduction in FA release and oxidation at higher exercise intensities or following carbohydrate feeding has been shown to be somewhat diminished when FA concentrations have been restored through lipid-heparin infusions. Romijn et al [33] showed that increasing the availability of FA during high intensity

exercise (85% VO_2 max), to a similar concentration observed at moderate exercise intensities, partially increased fat oxidation by 27%. Horowitz et al [43] illustrated that carbohydrate feeding suppressed lipolysis and fat oxidation during exercise which was rescued by 30% when FA concentrations were increased using an intra-lipid heparin infusion. This evidence shows FA release and availability during exercise appear to be an important factor in maintaining fat oxidation. But other factors inherent in the skeletal muscle have also been identified as responsible for regulating FA oxidation, which will be discussed below.

Increasing lipolysis through greater circulating catecholamine concentrations may be advantageous to increasing fat oxidation during exercise. In support, Mora-Rodriguez et al [45] reported that increasing the adrenaline concentrations during low intensity exercise (25% VO_2 max) resulted in a progressive elevation in lipolysis, measured as glycerol R_a. However, adrenaline infusion at medium and high concentrations during low intensity exercise led to a significant reduction in FA oxidation when compared to a control trial. This firstly illustrates that catecholamine concentrations are highly controlled during exercise to ensure lipolysis matches FA oxidation and secondly supports the fact that catecholamines are also responsible for stimulating glycogenolysis and glycolysis during exercise [46]. Catecholamines only stimulate glycogenolysis under high exercise intensities when combined with an elevation in 5'adenosione monophosphate activated protein kinase (AMPK) [47]. The rise in AMPK occurs when ATP utilisation increases or ATP synthesis reduces leading to an increase in AMP:ATP ratio. *In vivo* this change in energy status ratio occurs under higher exercise intensity and durations [48, 49]. Mora-Rodriguez et al [45] did however see a small significant increase in FA oxidation during the late stages of low intensity exercise when small increases in catecholamines were induced.

Collectively this evidence suggests that identifying nutritional interventions that are capable of augmenting FA availability and inducing changes to the sympathetic nervous system may be effective in augmenting fat oxidation.

1.3.1.2 Fatty acid uptake into the skeletal muscle during exercise

Before FA can enter the mitochondria for oxidation they first must pass through endothelium, interstitial space, plasma membrane, cytoplasm and mitochondrial membranes [50]. It was originally believed that FA could diffuse through membranes and there was a linear relationship between release, uptake and oxidation of FAs [51]. However, it was shown that the uptake of FA into the skeletal muscle at rest and during exercise can become saturated [52], and could potentially limit FA supply for oxidation.

A number of transport proteins found in the plasma membrane of skeletal muscle are responsible for the uptake and transport of FA. However, the exact mechanisms that regulate the transport proteins, particularly at higher exercise intensities when FA oxidation is limited, remains to be elucidated. These transport proteins include: fatty acid binding protein found in the plasma membrane (FABPpm), fatty acid transport proteins (FATP1 and FATP4), fatty acid translocase (FAT/CD36) and fatty acid transport protein in the cytoplasm (FATPc) [50]. It is postulated that all of the transport proteins work in unison to facilitate the uptake of FA and collectively these transport proteins are critical in the uptake of FA. They therefore form an important regulatory component of fat oxidation.

1.3.2 IMTG regulation during exercise

The contribution of FA to fat oxidation intrinsically within the skeletal muscle is sourced from IMTG. This fat source within the skeletal muscle provides a readily available substrate for fat oxidation at rest and during exercise, particularly during low to moderate intensity exercise [26, 27]. Similar to adipose tissue, the rate of IMTG hydrolysis during exercise is principally controlled by the enzyme hormone sensitive lipase (HSL). Adipose tissue triglyceride lipase (ATGL) is also an important enzyme responsible for hydrolysis of triglycerides to diacylglycerides (DAG) in skeletal muscle [53]. Watt et al [54] has shown that HSL is highly active during exercise lasting 1-10 minutes, even as exercise intensity increases from 30-90% VO_2 max. This suggests that IMTG may be an important substrate at the onset of exercise before plasma FA concentrations have begun to increase. During prolonged

moderate intensity exercise IMTG have also been shown to be important. van Loon et al [55] demonstrated a 60% reduction in IMTG in type I muscle fibres following cycling at 60% VO_2 max for 2 hours. Others have reported 50-70% IMTG depletion after 2-3 hours of moderate endurance exercise [56, 57]. Watt et al [57] observed a similar depletion of IMTG after 2 hours of cycling at 57% VO_2 max, but there were no further reductions in IMTG depletion after 4 hours of exercise. This attenuated depletion in the latter half of exercise may be driven by the inhibitory effect of FA-acyl CoA, as a result of the continued increase in adipose tissue lipolysis during exercise [26]. In support, Watt et al [58] reported that nicotinic acid, which is known to suppress plasma FA concentration, caused a significant increase in IMTG degradation during moderate intensity exercise (180 min 60% VO_2 max) despite a significant reduction in total fat oxidation. This data firstly shows that IMTG are greatly utilised during moderate intensity exercise and contribute to fat oxidation. Secondly this highlights the high importance of plasma FA concentration, rather than IMTG hydrolysis alone, during moderate intensity exercise in order to achieve maximal fat oxidation. This further supports the aforementioned notion that nutritional interventions that are capable of increasing plasma FA concentrations may be advantageous to increasing fat oxidation during exercise, particularly at the onset of exercise. Currently there is no evidence to date that suggests that IMTG hydrolysis limits fat oxidation. Hence, integral components within the skeletal muscle, specifically within the mitochondria, may regulate fat oxidation during exercise.

1.3.3 Fatty acid uptake into the mitochondria

Once FA are made available in the cytoplasm they must pass through the outer and inner membranes of the mitochondria before being oxidised [59]. Long chain FAs are dependent on transporters through the mitochondrial membranes. In comparison short and medium chain FAs are able to diffuse freely into the mitochondria [60]. Long chain FAs are firstly activated by the addition of a coenzyme A (CoA) group to form long chain fatty acyl-CoA, catalysed by acyl CoA synthetase (ACS). Carnitine palymitoyl transporter I (CPTI) which is found on the outer membrane of the mitochondria, is then responsible for converting FA-acyl CoA to acyl-carnitine. The acyl-carnitine is then able to pass

through the inner membrane to mitochondrial matrix by free-carnitine via acylcarnitine translocase (ACT). Acyl-carnitine then undergoes transesterification back to FA-acyl CoA and free carnitine by carnitine palmitoyltransferase 2 (CPTII). FA-acyl CoA is then free to undergo β-oxidation in the mitochondria to form acetyl-CoA.

1.3.3.1 Regulation of CPTI activity

The activity of CPTI as well as the availability of free carnitine in the mitochondrial membranes together forms important regulatory components in the transport and oxidation of FA [59, 61]. This is supported by studies that have abolished the activity of CPTI during *ex vivo* muscle contractions, through the use of extomoxir, and observed severe reductions in FA oxidation [62]. Early *in vitro* evidence from rat skeletal muscle reported that at rest the activity of CPTI is allosterically inhibited by malonyl-CoA resulting in alterations to fat oxidation [63]. Malonyl-CoA is produced as a consequence of an accumulation of acetyl-CoA, from accelerated glycolytic flux, which stimulates the activity of acetyl-CoA carboxylase (ACC). Furthermore, a rise in AMPK in animal and humans, from both pharmaceutical activation [64] or alternatively through muscle contractions [65, 66], phosphorylates ACC (thus lowering the activity) and reduces the formation of malonyl-CoA. This gives rationale for the role of nutritional interventions that may be capable of altering energy status (ATP:ADP:AMP) or directly targeting ACC and malonyl-CoA activity.

On the other hand, there is equivocal evidence in humans to support the role of malonyl-CoA in regulating fat oxidation either during moderate intensity exercise [67, 68] or in the recovery period after high intensity exercise [34]. Roepstorff et al [67] reported that malonyl-CoA concentrations decreased in skeletal muscle during prolonged moderate intensity exercise (60% VO_2 max) which was paralleled by an increase in fat oxidation. However, the decrease of malonyl-CoA during exercise was not different between trials that elicited high and low fat oxidation rates. Further, others have demonstrated that malonyl-CoA concentrations in human skeletal muscle did not change between varying exercise intensities [69, 70] or durations [71]. It must be noted, however, that in the studies to

date mixed muscle malonyl-CoA concentration was measured and it cannot be excluded that differences in local malonyl-CoA concentrations are important for the regulation of CPTI (i.e., Malonyl-CoA in and around mitochondria). Although the importance of localized malonyl-CoA concentrations cannot be dismissed, other mechanistic controls may be in place within the skeletal muscle to regulate fat oxidation, specifically CPTI, at rest and during exercise [34].

CPTI may also be regulated by skeletal muscle pH [72, 73]. Bezaire et al [74] showed in isolated intermyofibrillar and subsarcolemmal mitochondria from human vastus lateralis muscles, that reducing the pH from 7.1 to 6.8 reduced CPTI activity by 34-40%. This is supportive of the increased acidic environment that may occur within the skeletal muscle as a consequence of elevated glycolysis and H^+ accumulation through an increase in exercise intensity [26, 27].

1.3.3.2 Regulatory role of carnitine in FA uptake in the mitochondria

The role of carnitine within the skeletal muscle is vital in order for long chain FA to enter the mitochondria. Carnitine is considered as one of the most important regulators of fat oxidation at rest and during exercise. In support, carnitine deficiency within the skeletal muscle (lipid storage myopathy) has been reported to reduce long chain FA oxidation *in vitro* by 75% [75]. The importance of carnitine is further exacerbated by the role it plays in buffering excess acetyl-CoA, when glycolytic flux is in excess of TCA cycle incorporation. Consequently, carnitine accepts an acetyl group, catalysed by carnitine acetyl transferase (CAT), to produce acetyl-carnitine. The production of acetyl-carnitine also maintains the mitochondrial co-enzyme A pool (CoASH) for continued glycolytic flux of pyruvate to acetyl-CoA [27, 57]. Stephens et al [59] showed that at lower exercise intensities ($\leq$ 40% VO_2 max) free carnitine and acetyl-carnitine content does not change when compared to rest. The depletion of free carnitine at higher, but not at lower exercise intensities, is hypothesised to reduce the availability of carnitine for CPTI resulting in a decrease of fat oxidation [76]. This is not only supported by the reduced FA oxidation at higher exercise intensities [26, 27], but also the correlation

between the reductions in fat oxidation and the depletion of free carnitine during increasing exercise intensity [27].

The importance of carnitine in regulating fat oxidation during exercise has meant it has gained a large amount of interest as a potential target for interventions that are able to manipulate its content. Through a series of experiments, Stephens et al [59] have successfully reported that carnitine content can be increased in the skeletal muscle through chronic daily L-carnitine supplementation, only when combined with carbohydrate feeding (80 g). More recently, Wall et al [77] increased carnitine content in the skeletal muscle by 21% by feeding L-carnitine (1.2 g) with carbohydrate polymer (80 g) twice per day for 24 weeks. The increase in carnitine content resulted in a 55% reduction in skeletal muscle glycogen use and a 31% reduction in pyruvate dehydrogenase complex (PDC) activity status during exercise at 50% VO_2 max when compared to the control trial. This evidence suggests that fat oxidation is highly regulated during exercise, principally by carnitine availability within the skeletal muscle. In addition, it is clear that nutritional interventions, such as carnitine supplementation, may be capable of overcoming the regulatory components to fat oxidation. In turn this may increase fat oxidation, spare skeletal muscle glycogen and improve endurance exercise performance. However, to date, carnitine is one of the few nutritional interventions to receive extensive research to investigate the efficacy of increasing fat oxidation during exercise. Future research is therefore required to elucidate novel nutritional interventions that are 1) capable of targeting the regulatory components of fat oxidation, 2) significantly increase fat oxidation during exercise.

1.4 Effects of endurance exercise training on fat metabolism

One of the most effective and documented strategies for increasing the capacity to oxidise fat during exercise is adaptation to chronic endurance exercise training. Both longitudinal training studies [12, 78-85] and cross sectional studies comparing trained and untrained individuals [21-25, 86] illustrate an improvement in fat oxidation at both the same relative and absolute pre-training exercise intensity. The observed increase in fat oxidation following training is explained by adaptations to the regulatory

components of fat metabolism, these include: a greater uptake of FA by a larger number of cytoplasm transport proteins, greater IMTG hydrolysis as well as an improved uptake of FA across the mitochondrial membrane. These adaptations to endurance training are a consequence of the accumulation of specific proteins, induced by an up regulation of gene expression (mRNA). Gene expression is induced by various molecular signals and pathways through physiological and metabolic perturbations from exercise. Recent evidence has accumulated to show that these molecular signals induced by exercise maybe altered by nutrient availability [87].

Thus, the interaction between exercise and nutrition may be important in not only maximising fat oxidation but also optimising the adaptations to endurance training. In turn this may develop nutritional interventions, which when combined with regular endurance exercise, may augment the adaptations to fat metabolism. With this in mind, the following sections will provide a brief summary of the adaptations to fat metabolism following training and the molecular signals responsible for these adaptations.

1.4.1 Fatty acid uptake following endurance training

The increase in fat oxidation following endurance exercise training has been shown to be due to a greater FA uptake and oxidation [21, 23, 84, 88]. In a study conducted by Turcotte et al [21] a plateau in net FA uptake across the exercising leg during was observed in an untrained individual, however net FA uptake continued to increase in the trained individual after 2 hours of exercise. During whole body exercise, Phillips et al [79] observed that 31 days of cycling exercise training led to a reduced whole body and regional lipolysis. This suppressed lipolytic rate is a consequence of a paralleled suppression of catecholamine concentrations following endurance training at the same pre training absolute exercise intensity [79, 89].

The greater oxidation of FA, despite a lower FA release, following training is explained by an improved uptake of FA into the skeletal muscle. In support, FA transport capacity across the plasma membrane and mitochondrial membranes has been well documented. Talanian et al [90] reported that

following 6 weeks of high intensity interval training, FAT/CD36 increased in both whole muscle and mitochondrial membranes. In addition, the authors also showed that FABPpm increased in the cytoplasm and at the mitochondrial membrane. At the same time, it has been shown that endurance training improves the FA transport capacity of mitochondrial membranes, namely CPTI [91]. Tunstall et al [91] showed that CPTI mRNA expression was increased following 9 days of endurance training. This was also accompanied with an elevated FAT/CD36 mRNA expression.

1.4.2 IMTG hydrolysis following endurance training

A number of studies to date have also shown that the contribution of IMTG to fat oxidation following endurance exercise training is increased. This is principally based on studies that have implemented stable isotope methodology before and after endurance training [79, 85, 92, 93] or have compared trained and untrained individuals [22, 24, 25]. In support, Wallis et al [94] reported that net glycerol release from the skeletal muscle increased following 9 weeks of endurance training when using both isotope tracers and arterial femoral difference techniques. Tarnopolsky et al [93] demonstrated that following seven weeks of endurance training, IMTG content was increased due to a 42% increase in lipid droplets which were surrounded by a larger number of mitochondria. More recently, Shaw et al [82] showed that 6 months of endurance training caused a 2 fold increase in IMTG content in both type I and II fibres, which was paralleled with a 1.6 fold increase in mitochondrial content. These findings confirm that in addition to the adaptations to IMTG hydrolysis and contribution to fat oxidation, the endogenous store of fat within the skeletal muscle is increased.

1.4.3 Mitochondrial enzyme and content following endurance training

Endurance exercise training leads to an increase in the number of mitochondria within the skeletal muscle, otherwise known as mitochondrial biogenesis. In addition numerous adaptations to the intermediary metabolism within the matrix of the mitochondria also occur. This has been demonstrated by the improvement in TCA cycle flux following endurance training due to the greater expression and activity of the TCA enzymes: succinate dehydrogenase [95, 96] and citrate synthase

[93, 95, 97, 98]. This ensures a greater production of NADH and $FADH_2$ for the electron transport chain (ETC) as part of oxidative phosphorylation. With the increase in TCAI following endurance training an improvement to VO_2 max [95] and exercise performance [99] have also been reported suggesting that TCA cycle adaptations play a critical role in energy metabolism and performance for endurance trained individuals. In addition, Gollnick and Saltin [100] illustrated that the improved capacity to oxidise fat following endurance training was due to enhanced enzyme activity in β oxidation pathway. It has consistently been shown in humans that endurance training increases the content of 3-hydroxyacyl-CoA dehydrogenase (HAD) an important intermediary and rate limiting enzyme in β oxidation [93, 97, 98, 101]. Endurance training also increases the flux of electrons, from NADH and FADH2, through the electron transfer chain to resynthesise ATP through oxidative phosphorylation in the mitochondrial matrix. More specifically, a number of investigations have demonstrated that endurance training increases the transmembrane protein complex cytochrome C oxidase (COX IV) [102, 103].

Consequently, endurance exercise training improves the capacity to oxidise fat by not only overcoming the regulatory components of fat metabolism, but also increasing the intrinsic machinery required to oxidise fat.

1.4.4 The molecular response to endurance exercise training

The adaptations to fat metabolism following endurance training are explained by alterations in gene transcription that lead to increased expression of specific proteins [104]. The adaptations to fat metabolism are controlled by a number of perturbations which include an increase in AMPK, the transient increase in calcium release from the sarcoplasmic reticulum (SR) as well as disturbances to mechanotransduction [104]. Through these primary messengers induced by exercise, a number of signalling cascades are induced which lead to the up regulation of transcription factors and transcription co-activators, which are responsible for the adaptations to endurance exercise as outlined above. These include: nuclear respiratory factor-1 and 2 (NRF 1/2) [105] and mitochondrial

transcription factor A (Tfam) [106], peroxisome proliferator activated receptor (PPAR) –γ coactivator (PGC1α) [107], and peroxisome proliferator activated receptor (PPAR) [108].

The up regulation of transcription factors and transcription co-activators as a result of endurance exercise is well documented [106]. More recent evidence has suggested that nutrient availability may alter the contraction induced gene expression and molecular signalling [87]. AMPK is known to be a potent activator to numerous transcription factors [104] and has been shown to be critical in inducing the endurance training adaptations [109]. The rise in AMPK occurs with an increase in AMP:ATP ratio, which is achieved when manipulation to carbohydrate availability during training occurs. This is specifically through glycogen depleted exercise training [110] or high intensity exercise training [81], which has resulted in superior adaptations to fat metabolism (3-hydroacyl-CoA dehydrogenase (β-HAD), FAT/CD36, IMTG hydrolysis and citrate synthase) when compared to traditional training. Similar findings have also been documented when endurance exercise training is completed under a low carbohydrate/high fat diet [15, 17, 111, 112]. Therefore, reducing the reliance on endogenous carbohydrate stores, while at the same time, increasing the availability and utilisation of fat at the skeletal muscle may be advantageous to the adaptations to fat metabolism following endurance training.

Altering nutrient availability can also change hormone concentrations which may also have important roles in not only regulating substrate metabolism but also in training induced adaptations. An elevation in catecholamines, especially following high intensity exercise training [113], up regulates cAMP response element binding protein (CREB), which phosphorylates PGC1α [114]. In addition, *in vitro* studies have shown that FA are capable of binding to PPARs and increase the activity of the transcription factor [113]. As already discussed, catecholamines are responsible for increasing plasma FA concentrations during exercise. Therefore, increasing catecholamine concentrations and fat availability may also play a role in augmenting transcription factor activity during exercise.

The nutrient-exercise interactions that have been discussed above are powerful tools to potentially activate the molecular signalling for endurance training adaptations to fat metabolism. Performing exercise that acutely alters nutrient and hormonal concentrations may be of interest to endurance athletes. Hypothetically this may suggest that nutritional interventions that acutely 1) alter energy status (ADP:AMP) of the skeletal muscle during exercise, 2) increase the availability of FA at rest and during exercise 3) up regulate catecholamines at rest and during exercise may optimise the training induced adaptations to fat metabolism [87, 115]. However, to date, very little evidence is available to identify nutritional interventions capable of inducing these metabolic perturbations, increasing the activity of transcription factors or inducing adaptations to fat metabolism with or without endurance exercise training. This demonstrates that future studies are required to elucidate this further.

1.5 Nutritional interventions to augment fat oxidation and exercise performance

A number of bioactive ingredients that originate from plants have become of interest for their potential in increasing fat oxidation [116] as well as interacting with the molecular signals that are responsible for the adaptations to endurance exercise training [87]. There are an abundance of bioactive ingredients that may offer nutritional interventions and support to endurance training on the sports nutrition market today [1, 116, 117]. However, the evidence to support the putative claims are mostly equivocal. In a recent review [116] a variety of bioactive ingredients that have been proposed to increase fat oxidation at rest and during exercise were critically discussed. Of the eight ingredients that were mentioned in the review, green tea was shown to be the most promising ingredient to increase fat oxidation at rest, while there was a paucity of evidence during exercise. Furthermore, caffeine which is found abundantly in coffee as well as in green tea, has been suggested to increase the capacity to oxidise fat during exercise since the first study on this topic by Costill et al in 1978 [118]. Early studies showed an improvement to endurance exercise capacity, which coincided with a sparing of skeletal muscle glycogen [118, 119]. However, to date the evidence that caffeine increases fat oxidation and spares skeletal muscle glycogen is equivocal, with numerous studies showing improvements to endurance exercise performance independent of changes to fat metabolism [5, 120,

121]. Surprisingly though, despite caffeine being a well documented nutritional intervention to improve endurance exercise performance, there is little supporting evidence for the use of coffee [122-125].

The importance of examining the role of green tea and caffeine, particularly when ingested as coffee, is that they are readily available nutritional interventions and supports which are frequently consumed by athletes today and are often cited as nutrition 'trending nutrition hot topics'. In support, tea and coffee are two of the most popular beverages consumed worldwide, with tea being the second most consumed beverage after water [126, 127]. The predominant source of caffeine in the human diet is sourced from coffee [128]. In athletes, similar trends have also been observed. Tunnicliffe et al [129] reported that 70% of Canadian athletes, from a variety of sports, used caffeine before or during training. The average daily intake was 59±6 mg/d, ranging from 0-895 mg/d with coffee being the primary source of caffeine intake by the athletes. Furthermore, Chester et al [130] reported that 97% of UK track athletes and cyclists were found to use caffeine prior to competition, with the major source of caffeine found to be from coffee. Chester et al [130], as well as others [131] have shown that caffeine intake appears to be higher, especially in coffee format, amongst endurance athletes, specifically triathlon, cycling and rowing. Less is known about the prevalence of use of green tea amongst athletes. However, it could be considered that due to the rise in commercial green tea beverages, in the form of ice teas and energy drinks [132], combined with the high accessibility of green tea supplements on the sports nutrition market, the use by athletes may be high.

1.6 Caffeine as a nutritional intervention to augment fat metabolism and exercise performance

1.6.1 Composition of caffeine and metabolism in humans

Caffeine is a trimethylxanthine that is rapidly and completely absorbed through the gastrointestinal tract. It is metabolised in the liver by the cytochrome P450 enzyme, specifically through the isoenzyme CYP1A2 and other isoforms to form three primary dimethlxanthines, namely: paraxanthine, theobromine and theophylline [133]. It is understood that CYP1A2 activity can vary up

to 40-fold within and between individuals [134], altering the bioavailability, clearance and ultimately the physiological effects of plasma caffeine. Following intake, plasma caffeine can appear in the circulation within 15 minutes and result in a time to peak (Tmax) of ~1 h [135], with a half life of 5-30 h [136]. A standard cup of coffee (250 mL) will consist of 60-85 mg caffeine, which will result in an average plasma caffeine concentration of ~7-10 µM [137].

1.6.2 Performance effects of caffeine

1.6.2.1 Time to exhaustion

Caffeine has been recognised for some time to improve endurance exercise performance, with over 40 years of supporting research [138-141]. The seminal work by Costill et al [118, 142] was the first to report the ergogenic effects of caffeine. On two occasions, nine cyclists completed a time to exhaustion (TTE) test (80% VO_2 max) 60-min after consuming decaffeinated coffee (decaf) with added caffeine (330 mg) or decaf only [118]. Following ingestion of caffeine, performance was significantly improved by 15 min when compared to decaf. Costill et al [118] concluded that the improvements to endurance exercise capacity were a consequence of an increase in fat oxidation, which in turn would spare skeletal muscle glycogen. Since this early work, numerous studies have shown similar performance enhancing effects of caffeine using a TTE protocol, for both prolonged sub-maximal exercise (>90 min) and constant higher intensity exercise (20-60 min) (75-90 %VO_2 max) [118-120, 122, 143-150]. A recent meta-analysis, which included 40 double blind studies from a variety of exercise tests (endurance, graded and short), concluded that the overall improvement to exercise performance following caffeine intake was 12% when compared to placebo (95% CI 9.1-15.4). Further, the improvement to performance was larger during endurance (TTE) exercise (20% improvement in performance) compared to graded or short term high intensity exercise tests (2% improvement in performance) [138].It should be noted that not all studies tested whether fat oxidation was augmented following caffeine intake.

1.6.2.2 Time trial performance

The ergogenic effects of caffeine are well documented when using the time to exhaustion test as the predominant performance measure. However, assessing endurance capacity in this manner may lack sufficient ecological validity to translate results to real life endurance sporting events [151]. Taking this into consideration, more recent investigations have implemented the use of time trial (TT) protocols to assess endurance exercise performance [152-155] which has been shown to be a highly reliable [156]. A recent meta-analysis, only including studies that measured endurance exercise performance using a TT, showed that overall caffeine improved performance by 3.2 ± 4.3%, however this improvement was highly variable between studies (-0.3 to 17.3 %) [139]. The ergogenic effects of caffeine were mainly investigated in endurance athletes. More recent evidence has shown that caffeine (6 mg/kg body mass (BM)) is ergogenic in sedentary men when using a time trial protocol [157]. Interestingly, caffeine was predominantly delivered through fluid or capsules with no study using a coffee beverage, despite it being the predominant source of dietary caffeine [128-130, 158].

1.6.3 Dose response and timing of caffeine intake

The dose of caffeine required to improve performance is believed to be between 3-6 mg/kg BM, resulting in plasma caffeine concentrations 30-70 µM [5]. Graham et al [145] illustrated that 3 and 6 mg/kg BM improved TTE (80% VO_2 max) to a similar extent when compared to a water control. However, increasing the dose to 9 mg/kg BM did not result in a significant improvement in performance. Pasman et al [149] confirmed these findings by showing that 5, 9 and 13 mg/kg BM improved TTE (80% VO_2 max) compared to placebo, however no differences were observed between the caffeine doses. These findings have also been confirmed using a TT protocol, whereby 3 and 6 mg/kg BM both significantly improved performance, yet doubling the dose to 6 mg/kg BM did not offer any further benefits [153]. It also appears that using lower doses of caffeine (1-3 mg/kg BM) can be effective in improving endurance exercise performance when using a time trial protocol [152, 154,

159]. Interestingly, caffeine doses as low as 90 mg have been shown to improve time trial performance, resulting in low plasma caffeine concentrations (~8µM) [152].

The timing of caffeine ingestion may be important in observing the ergogenic effects. The majority of studies to date have provided caffeine 1 h prior to exercise to coincide with the Tmax of plasma caffeine [5]. In agreement, a recent study reported that time trial performance was only improved when exercise was performed 1 h after caffeine rather than after an individual's plasma caffeine Tmax (150-180 min), when compared to placebo [155]. In addition, it is evident that the Tmax differs between caffeine consumed as a capsule when compared to coffee consumed as a beverage [160]. Many athletes have been shown to consume caffeine during endurance exercise, particularly during prolonged events [158]. Cox et al [152] performed a well designed study in which cyclists performed a 2 h cycling bout (70% VO_2 max) having consumed either: caffeine 1 h prior to exercise (6 mg/kg BM), caffeine in small doses throughout exercise (6 x 1 mg/kg BM every 20 min), low caffeine in the form of a cola beverage (2 x 5 mL/kg (~13mg/100 mL) at 100 and 120 min) or placebo. Despite the difference in time of intake, TT performance was improved by ~3% across all interventions. Subsequently, Kovacs et al [159] showed that a variety of doses of caffeine (2.1, 3.2, 4.5 mg/kg BM) consumed in combination with carbohydrate (68.8 g/L) during exercise improved time trial performance when compared to placebo and carbohydrate only. Therefore, the timing and form of caffeine intake may determine the ergogenic effects. Studies show that caffeine intake prior to and during endurance exercise is an effective nutritional support to improve exercise performance, however studies investigating whether these effects are greater or less, when caffeine is taken in as coffee, are lacking.

1.6.4 Performance effects of coffee

The performance effects of coffee are less clear, despite coffee being frequently ingested by endurance athletes on a daily basis. Since the work of Costill et al [118, 142], very few studies have investigated the effects of coffee or decaf plus caffeine. Table 1.1 summarises the studies to date. Using a

comparable study design to Costill et al [118], Butts & Crowell et al [125] did not observe an improvement in time to exhaustion (75% VO_2 max) following decaf plus caffeine (330 mg) compared to decaf only. Yet, Wiles et al [124] demonstrated that 1500-m simulated treadmill running time was significantly improved by 4.2 sec following coffee (~2.0-2.5 mg/kg BM) compared to decaf. In addition, the authors also showed that coffee significantly improved running speed in an 'all out sprint' in the final minute of a subsequent 1500-m simulated treadmill run when compared to decaf. These results are difficult to interpret due to the different forms of coffee used (decaf plus caffeine, decaf only) with little comparison to regular coffee or caffeine only. In order to address this question, Graham et al [122] compared the effects of caffeine, regular coffee, decaf, decaf plus caffeine or a placebo, 1 h prior to completing a running TTE test (80% VO_2 max). All caffeinated trials consisted of 4.5 mg/kg BM. The authors observed a significant improvement in TTE with caffeine when compared to the other conditions. Surprisingly, regular coffee and decaf plus caffeine did not improve performance, despite having the same caffeine content. Based on this evidence, the authors concluded that coffee may be inferior to caffeine at improving endurance exercise performance. A difference in the bioavailability of caffeine or paraxanthines was not responsible for this diverse ergogenic effect [122]. It has been proposed that compounds in coffee may interfere with the physiological effects of caffeine. Coffee is composed of ~2% caffeine, with the remainder being made up of chlorogenic acids, ferulic acid, caffeic acid, nicotinic acid as well as other compounds with many currently unidentifiable [136]. However, it has been observed that regular coffee containing caffeine (1.1mg/kg BM) consumed prior to the ingestion of different doses of caffeine (3-7mg/kg BM) does not impair the improvements to exercise performance induced by caffeine [123] . Herein, chapter 3 of this thesis describes a study that aimed to explore the performance effects of coffee compared to caffeine using a time trial protocol.

Table 1.1 Overview of the available literature investigating the effect of coffee on exercise performance

Reference	*Performance protocol*	*Participants*	*Beverages*	*Time before exercise*	*Caffeine dose*	*Performance outcome*	*Coffee ergogenic + / ↔*
Costill et al [118]	80% VO_2 max TTE	7 M 2 F	D+C v D	1 h	300mg (4.4 M, 5.8 F)#	D+C 90.2*; D 75.5 (min)	+
Butts & Crowell [125]	75% VO_2 max TTE	13 M 15 F	D+C v D	1h	300mg (4.0 M, 5.1 F)#	D+C 68.5; D 63.8 (min)	↔
Wiles et al [124]	Treadmill 1500 m run	18 M	Cof v D	1h	3g Cof ~200 mg (2.0-2.5)#	C 286*; D 290 (sec)	+
Wiles et al [124]	Treadmill 1500 m run + 1 min sprint	10 M	Cof v D	1h	3g Cof ~200 mg (2.0-2.5)#	1 min sprint Cof 23.5*; D 22.9 (km/h)*	+
Trice & Haymes [161]	~90% VO_2 max intermittent sprints	8 M	D+C v D	1h	5 mg/kg BM	D+C 77.5; D 61.3 (min)	↔
Graham et al [122]	85% VO_2 max TTE	9 M	C v Cof v D+C v D v Pla	1h	4.45 mg/kg BM	C 41* Cof 32 D+C 32 D 32 Pla 31 (min)	↔
McLellan et al [123]	80% VO_2 max TTE	9 M 4 M	Cof-C$, Pla-Caf, D-Pla, D-C	1.5h	Cof 1.1 mg/kg BM, $C 3,5,7mg/kg BM	Cof-C (3) 25.1*; Cof-C (5) 27.8* ; Cof-C (7) 26.4*; Pla-Caf (5) 26.8*, D-Pla 21.7, D-C (5) 29.0* (min)	+
Lamina et al [162]	20 m shuttle run	12 M	Cof v Pla	1 h	5, 10, 15 mg/kg BM	No ergogenic effect of any dose. *Data not reported.*	↔

Abbreviations: **TTE** Time to Exhaustion, **M** Males, **F** Female, **D+C** Decaffeinated coffee + Caffeine, **C** Caffeine, **D** Decaffeinated coffee, **Cof** Coffee - includes instant and filter varieties, **Pla** Placebo, **BM** Bodyweight, (x) Caffeine dose provided in McLellan et al study.

Absolute doses provided relative to body weight for M and F. **$** Three different Caf doses consumed after Cof *****Significantly different to control (PLA or Decaf). + Improved performance, ↔ No change in performance.

1.6.5 Mechanisms to describe the ergogenic effects of caffeine

Traditionally it was believed that caffeine is capable of augmenting fat oxidation during exercise, which in turn would spare skeletal muscle glycogen [118, 142]. However, due to caffeine's ability to cross numerous biological membranes in the human body [141], caffeine may not just alter substrate metabolism, but also have direct effects on the brain and the skeletal muscle which may delay the onset of central and peripheral fatigue during exercise. These mechanisms of action of caffeine that may explain the ergogenic effects are discussed below.

1.6.5.1 Alterations to fat metabolism following caffeine intake

Costill and colleagues [142, 163], were the first to propose that the mechanism of action of caffeine was due to a sparing of muscle glycogen, as a result of an increase in fat oxidation. A small number of investigations have since also supported this hypothesis [119, 164, 165]. Spriet et al [119] reported that consuming 9 mg/kg BM caffeine significantly improved endurance exercise capacity (80% VO_2 max) which was also accompanied by a 55% reduction in glycogenolysis during the initial 15 minutes of exercise when compared to a placebo. The authors also observed that at rest, but not during exercise, skeletal muscle citrate and acetyl CoA/CoA-SH ratio were increased. The authors concluded this was a consequence of the elevated FA and glycerol concentration. Furthermore, the elevated lipolytic rate may have been induced by the significant increase in adrenaline at rest and throughout exercise due to caffeine ingestion. Essig et al [164] also observed a 42% reduction in glycogen utilisation during 30 minutes of cycling exercise (70% VO_2 max) following the ingestion of coffee compared to decaffeinated coffee. This was also accompanied with a modest rise in plasma FA and glycerol and a 1.5 fold greater IMTG utilisation. However, this only resulted in a 50% increase in fat oxidation during exercise. The finding that caffeine elevates plasma FA is fairly consistent through studies undertaken at rest and during exercise [118-121, 142, 146, 148, 166, 167]. This is both due to the caffeine induced rise in adrenaline stimulating lipolysis [168] and also via caffeine directly antagonizing adenosine receptors A1 at adipocytes to also increase lipolysis [120, 121]. Despite the

greater availability of FA for oxidation during exercise, very few studies have shown an improvement in fat oxidation or a change in RER following caffeine intake [121, 147, 148, 150, 168, 169]. To gain a better insight into the metabolic perturbations that may be occurring following caffeine intake a number of studies employed stable isotope methodology and arterio-venous balance methods. Studies by both Raguso et al [169] and Graham et al [168] showed that caffeine increased arterial lactate and glucose concentrations during exercise, possibly due to a reduced uptake by the skeletal muscle. Both studies observed increases in adrenaline following caffeine ingestion and suggest that caffeine is able to augment the SNS, which consequently may increase lipolysis from adipose tissue whilst also stimulating glycolysis. Alternatively, glucose and lactate may be increased due to caffeine inhibiting the uptake and clearance of glucose and lactate. In both studies, these metabolic perturbations did not result in changes to fat oxidation. A number of other studies have also not shown the glycogen sparing effect following caffeine intake [145-148, 168] with others showing a paradoxical increase in glycogen utilisation [169, 170]. In a more recent review paper, Graham et al [171] combined data from a number of studies that had investigated the effect caffeine intake had on skeletal muscle metabolic changes including glycogen, citrate, acetyl-CoA, glucose-6-phosphate and cAMP. The combined data sets clearly indicated that, despite an increase in SNS activity, caffeine did not alter fat oxidation or inhibit carbohydrate oxidation during exercise.

Thus, these studies are unable to explain the ergogenic effects of caffeine. The studies introduced here have all used anhydrous caffeine rather than coffee. The ergogenic effects of coffee are largely unclear, however as coffee is heavily habitually consumed, it may be interesting to examine whether coffee induces similar metabolic effects when compared to caffeine. With this in mind, in Chapter 3 of this thesis the metabolic effects of caffeine and coffee was be investigated alongside the performance effects of both of these nutritional interventions.

1.6.5.2 Effects of caffeine on central and peripheral factors

One popular hypothesis is that caffeine induces its ergogenic effects through its ability to act as an adenosine antagonist, blocking the interaction of adenosine to adenosine receptors (A_1 and A_{2A}) which are found ubiquitously in the human body [172]. Accordingly, caffeine has the potential to alter both central and peripheral components of fatigue. Adenosine is considered a "fine-tuning modulator" of neuronal activity which can inhibit the release of excitatory neurotransmitters in the brain [173]. Secondary to the antagonism of adenosine receptors caffeine has been associated with inducing a dopaminergic response [172]. Dopamine D_2 receptors are found alongside adenosine receptors (A_{2A}) located predominantly on dopaminergic neurons [172]. These neurons influence motor control and behavioural processes, such as mood and motivation. Caffeine increases antagonism of both adenosine and dopamine receptors, thus, has the potential to alter feelings of pain, pleasure/displeasure as well as modifying motor control [174]. Chronic intake of caffeine has been shown *in vitro* to modify the antagonism of caffeine on adenosine receptors, specifically through A_1 receptor blocking [175] and thus reducing the physiological effects of caffeine. However, while this has been shown *in vitro*, in most studies the ergogenic effects of caffeine are similar in users and non users, with or without with drawl from caffeine [166, 176]. Consequently caffeine is believed to affect both the central (CNS) and peripheral nervous system (PNS), through adenosine receptor antagonism, which in turn improves the ability and potential for the skeletal muscle to contract [174].

Caffeine has been shown to alter the CNS which may induce reductions to the central fatigue hypothesis (serotonin induced) and central pain during exercise [174]. Studies both agree [177] and disagree [178], with this hypothesis. These differences may be explained by the intensity of exercise, where it is believed that caffeine offers little benefit during high intensity exercise [178], perhaps due to caffeine's preferential effect in type I rather than type II muscle fibres as discussed below [179]. Moreover, caffeine may also increase the CNS excitability which would increase motor unit recruitment, subsequently improving force during a muscle contraction and power output [174]. Yet, while this paradigm seems logical, particularly based on the central effects of caffeine outlined above,

there is very little supporting evidence to show that caffeine improves motor unit recruitment during maximal voluntary contraction [174].

Concomitantly, caffeine may have effects on the PNS, specifically through direct effects on the skeletal muscle [120, 121, 174]. Tarnopolsky et al [180] showed that *in vivo* electrical stimulation of the peroneal nerve (evoke contraction of dorsiflexors of lower limb) at 20 Hz (similar to endurance exercise muscle contraction), but not 40 Hz, following caffeine intake (6 mg/kg BM) improved force output of the dorsiflexor muscles during a tetanic contraction. The resistance to peripheral fatigue with caffeine has been attributed to caffeine's ability to interfere with excitation-contraction coupling, primarily via an improved release of Ca^{2+} from the SR [181], which may become limited during prolonged moderate- or high intensity exercise [182]. A lack of effect observed at high frequency stimulations (40 Hz as observed above) is believed to cause fatigue proximal to excitation-contraction coupling and may involve K^+ accumulation. In support, caffeine ingestion (6 mg/kg BM) in humans enhanced fatigue resistance during intermittent high intensity exercise through a decrease in potassium (K^+) accumulation in muscle interstitial space [183]. These findings are in line with the consistent improvement to endurance based exercise performance and inconsistent improvement to maximal intensity exercise performance [184]. This is further postulated by recent data by Tallis et al [179] who used the work loop technique on isolated mouse muscles, showed that caffeine (50-140 μM) caused a significant increase in power output at maximal and sub maximal electrically stimulated slow (soleus) and fast twitch (extensor digitorum longus) muscle fibres. But the improvements in power output were greater in the slow twitch fibres when it was sub maximally stimulated. This recent data clearly shows that ergogenic effects of caffeine may be explained by its direct effect on skeletal muscle's ability to contract, independent of changes to substrate oxidation.

1.7 Green tea as a nutritional intervention to augment fat metabolism

Green tea, produced from the plant *Camellia sinensis* L. Species of the Theaceae family, contains naturally occurring flavonoids and caffeine. The compounds found in green tea have shown promise

as an anti-obesity nutritional intervention [185]. A recent meta-analysis has demonstrated that long term intake (3-23 wks) of green tea extract (GTE) (with and without caffeine) reduced body weight in obese and overweight individuals [186]. It is thought that the anti-obesity effects of GTE intake may be attributed to elevated fat oxidation and total energy expenditure [187]. A limited number of studies have investigated the effect of short term and long term GTE supplementation on substrate metabolism and energy expenditure both at rest and during exercise [187-189]. But while GTE has shown promise as a nutritional intervention to augment fat oxidation in some studies, the evidence remains equivocal.

Green tea preserves a high quantity of catechin polyphenol concentration (a sub group of flavonoids) due to the way in which it is manufactured [190]. The four main types of catechins are: (-)-Epigallocatechin-3-gallate (EGCG), (-)-epicatechin-3-gallate (ECG), (-)-epigallocatechin (EGC) and (-)-epicatechin (EC). EGCG is the most abundant and thought to be the most pharmalogically active catechin [191], with the catechins peaking in the plasma ~ 2 h after ingestion, with a half life of ~ 8 h. The type of tea, origin of the leaves, brewing technique and the source of green tea can all influence the catechin content [190]. A standard cup of green tea (250 mL) contains between 100-300 mg of total catechins and 50-90 mg of caffeine [192]. The different amounts of catechins and caffeine in different varieties of tea are shown in Table 1.2.

Table 1.2: Catechin and Caffeine content in a variety of tea products, data taken from [190, 192]

Beverage	***Serving size / brewing technique***	***Total Catechins mg /serving***	***Caffeine mg /serving***
Green Tea leaves	1 g tea leaves / 100 mL of hot water	42-201	25-43
Lipton Green Tea	Teabag / 100 mL of hot water	195-205	32-33
Decaf Green Tea	Teabag / 100 mL of hot water	72-73	<1
Green Tea extract (GTE) supplement	Per capsule	200-210	5-6

In humans, the bioavailability of GTE following ingestion determines the bioactivity [193]. While the bioavailability of catechins is fairly well understood, the bioactivity of each of the catechins *in vivo* remains to be elucidated. Consequently, the action of each catechin, and interaction with caffeine in order to affect fat oxidation, are unclear. The majority (98%) of ingested GTE undergo extensive conjugation in the liver and gut microbiota and appear transiently (60-120 min after ingestion) in the plasma [194]. Conjugated catechins are chemically different to the free catechins (found within GTE beverages) [194]. Therefore, the free and conjugated catechins are likely to have different physiological and biological effects in the human body [195]. Little is known about the bioavailability of the free catechins, that are found at low concentrations in plasma following GTE intake (<2%) [194]. Catechins are also able to pass into the colon where they are catabolised to ring fission products and phenolics acids [196] known as valerolactones. These catabolites of GTE are known to take longer to peak in the plasma (8-15 hours) and their biological effects *in vivo* are unknown [196].

In order to take into account all of the evidence to date that has investigated the effect of GTE on fat oxidation, the following sections have been divided into the effects observed at rest and those observed during exercise. Additionally, due to differences in study designs, particularly the supplementation duration, the following sections will be broken down into shorter and longer term supplementation. These supplementation durations have been defined in Table 1.3.

Table 1.3. Definition of GTE supplement durations for discussion in the sections below.

Shorter term	
Acute	Within 24 hours
Short term	1-7 days
Longer term	
Medium term	1-4 weeks
Long term	>4 weeks
Chronic	>10 weeks

1.7.1 Shorter term green tea intake and its effects on resting fat metabolism.

Dulloo et al [187] were the first to investigate whether GTE could increase fat oxidation at rest. In this double blind cross-over study, 3 doses of encapsulated GTE (270 mg/d EGCG + 150 mg/d caffeine), caffeine (150 mg/d) and placebo (cellulose) were taken over a 24 h period in a respiratory chamber. The authors observed that compared with placebo and caffeine, consumption of GTE significantly increased 24 h fat oxidation (76.2 ± 10.6 g/24hr, 81.9 ± 8.7 g/24hr and 103 ± 13 g/24 h respectively). Interestingly, fat oxidation rates were 20% higher following GTE when compared to caffeine. Thus, the authors concluded that the increase in fat oxidation, following GTE intake, was independent of its caffeine content *per se*. Using a comparable study design, Rumpler et al [197] showed that a catechin rich oolong tea taken over a 24 h period, (662 mg/d catechins, 270 mg/d caffeine) caused a 12% increase in 24 h fat oxidation rates compared to water. However, the authors showed no difference for a lower dose (331 mg/d catechins, 135 mg/d caffeine) suggesting that a higher catechin dose is required to stimulate fat oxidation.

Berube-Parent et al [198] investigated the dose response relationship of GTE on 24 h fat oxidation. On 4 separate occasions, subjects ingested caffeine rich (600 mg/d) GTE capsules differing in doses of EGCG (270, 600, 900, 1200 mg/d EGCG). All GTE doses significantly increased 24 h energy expenditure (EE) by 8% (750 kJ), compared to placebo. However, there were no significant differences between the treatment groups. However, GTE did not increase fat oxidation in any of the GTE groups compared with placebo. Similar results have also been shown following 3 days of GTE (540 mg/d) and caffeine (300 mg/d) compared to placebo [199]. However, Berube-Parent et al [198] speculated that due to the lack of dose response a ceiling effect at 270 mg/d EGCG may be apparent. In addition, the authors also speculated that the relatively high caffeine content (600 mg/d) could mask the effects of GTE on fat oxidation. A dose dependant response of GTE has been found in animals, when caffeine was absent [200]. Based on these animal data, caffeine may only work synergistically with catechins to augment fat oxidation in small doses (<600 mg/d). In support of this notion, Thielecke et al [201] showed an increase in fat oxidation at rest, in the postprandial and fasted state,

when EGCG (300 mg/d) and a small dose of caffeine (200 mg/d) was consumed for 3 days. For this reason, the exact composition of catechins and caffeine may be integral to altering fat oxidation in humans.

To date, the exact composition of GTE catechins required to stimulate fat oxidation is unknown. In order to elucidate this *in vivo*, a recent study [202] compared the effects of combined (~500 mg/13 h total catechins) and individual (EGCG and EGC ~600 mg/ 13 h) catechins + caffeine (150 mg/13 h) on resting metabolism over a 13 h. Subjects received GTE in small and frequent doses (82-214 mg) throughout this period. The authors found no increase in fat oxidation with any of the treatments compared with placebo. As GTE catechins are poorly absorbed [194], ingesting small amounts, in bolus throughout the day, may result in low plasma catechin concentrations which may explain why the authors [202] did not observe a change in fat oxidation. Yet, 2 days of moderate intake of EGCG only in some (300mg/d) [203] but not all cases (405mg/d) [204] has been shown to increase fat metabolism at rest, as estimated from a measured reduction in the RER. Therefore, it currently remains unclear which catechin or catechins is/are required to elicit changes in fat oxidation at rest.

Recently, a meta-analysis was published to address whether acute intake of GTE (with and without caffeine) or caffeine-only has the potential to increase fat oxidation at rest [189]. In total six articles were used following strict controls. It was found that GTE treatment groups yielded significantly greater mean fat oxidation rates (16% higher) compared to placebo. This effect was not seen in caffeine-only studies ($P = 0.11$) supporting the assumption that the effects of GTE are independent of the caffeine content *per se*. In addition, a dose dependant response was observed with a 0.02 g/24 h increase in fat oxidation for each 1 mg increase of ingested catechins. However, due to the limited number of studies and the large variation in dose, the authors were unable to give a lower and upper dose to elicit maximal changes to fat oxidation.

On balance, research has supported an increase in fat oxidation at rest, in response to shorter term GTE intake. However, not all studies showed increases in fat metabolism (or energy expenditure). The

effects of GTE on fat oxidation at rest may be small which are only apparent when tested in larger sample sizes as demonstrated from the conclusive evidence in the meta-analysis. Therefore, the inconsistencies in the studies reported could be related to the small sample sizes implemented, the exact catechin composition and the amount of caffeine present in the GTE. Therefore, further research is required to elucidate the most effective supplementation protocol to enhance fat oxidation during resting conditions.

1.7.2 Longer term green tea intake and its effect on resting fat metabolism

Auvichayapat et al [205], gave GTE (750 mg/d catechins + 87 mg/d caffeine) or placebo for 12 weeks to obese Thai men and women. Resting EE and substrate oxidation were measured at baseline, week 4, 8, and 12. Compared to baseline, longer term GTE intake significantly lowered fasting RQ at week 8 and elevated EE at week 8 and 12. Harada et al [206] administered either a high catechin (593 mg/d catechin + 22 mg/d caffeine) or a low catechin drink (78 mg/d catechin + 81 mg/d caffeine) to 12 Asian males, over a 12 week period. Using a stable isotope method, (^{13}C labelled triglycerides meal), $^{13}CO_2$ excretion was significantly higher in the high catechin group compared to the low catechin group at week 12 compared to baseline. This is indicative of increased dietary fat oxidation. In addition diet induced thermogenesis (DIT), in the 8 h post meal, increased from baseline to week 12 (51.4 kcal and 90.3 kcal respectively) in the high catechin group only. These studies suggest that longer term GTE has the potential to increase fat oxidation and alter energy metabolism.

Long periods of dietary restriction are associated with a suppression of resting EE [207]. Thus, long term GTE intake may prevent this [208]. Diepvens et al [208] investigated the effect of GTE intake (1125 mg/d catechins + 225 mg/d caffeine) for 83 d during dietary restriction versus a placebo in overweight Caucasian women. Resting EE and RQ were measured at day 4 and 32. Compared to placebo, there was no significant difference in fasting EE, DIT or RQ in the GTE group. However, participants used in this study were moderate habitual caffeine consumers (300 mg/day) which may have affected the results as suggested by a recent study by Westerterp-Plantenga et al [209]. The

authors showed that intake of encapsulated GTE (270 mg/day EGCG + 150 mg/day caffeine) or placebo for 12 weeks, following a 4 week low energy diet, only caused a significantly lower fasting RQ for those who had a low habitual caffeine intake (<300 mg/day) compared to a high habitual intake (<300 mg/d). This was also true for reductions in body weight and body fat.

To date there are only a small number of studies that have investigated the effect of longer term GTE ingestion on fat oxidation. Taken together these studies suggest that longer term intake of GTE may potentially augment fat metabolism but only if habitual caffeine intake is low, potentially by overcoming some of the regulatory components of fat oxidation.

1.7.3 Shorter term green tea intake and its effects on fat metabolism during exercise.

During exercise, fat oxidation rates are up to 10-fold higher than at rest, due to the energy demand of the working muscles [210]. But as previously discussed in this chapter, there are numerous regulatory components for the capacity to oxidise fat during exercise. From the limited number of studies to date GTE may be a nutritional intervention that may target some of these regulatory components during exercise. The evidence to support this is outlined below.

In 2008, Venables et al [188] were the first to study the effects of acute (24 h) GTE intake on fat oxidation rates during exercise. In this cross over double blind study, subjects consumed GTE (890 ± 13 mg/day catechins containing 366 ± 5 mg/day EGCG) or placebo before completing a bout of moderate intensity cycling (30 minutes at 50% W_{max}). Average fat oxidation rates during exercise were 17% higher in the GTE condition compared to placebo (0.41 ± 0.03 g/min and 0.35 ± 0.03 g/min, respectively). More recently, during 60 minute cycle at 60% VO_2max, fat oxidation did not increase following 6 days of EGCG (270 mg/day) when compared to caffeine and [211]. Interestingly, more fat was oxidised following caffeine and placebo trials compared to EGCG (0.13 ± 0.05 g/min, 0.16 ± 0.06 g/min, 0.16 ± 0.05 g/min respectively). However, at such low fat oxidation rates it makes it difficult to detect differences because of the variation of the measurement. This is discussed in more detail in the following section.

In conclusion there are only a limited number of studies that have investigated the effect of shorter term GTE intake on fat metabolism during exercise. The results to date remain equivocal. Thus, more research is needed before more firm conclusions can be drawn. In addition, understanding the metabolic differences or similarities of GTE at rest and during exercise would be of great importance. Herein, in order to gain new and comprehensive insights in the metabolic effects of GTE at rest but also during exercise, Chapter 2 of this thesis examines the metabolic response to 7 days of GTE at rest and during moderate intensity exercise using a metabolomics approach.

1.7.4 Longer term green tea intake and its effects on fat metabolism during exercise.

GTE intake, over a more prolonged duration, may result in further increases in fat oxidation rates during exercise. A recent study conducted a 2 hour cycling exercise test (50% W_{max}) following 3 weeks of GTE (159 mg/day catechins) and placebo ingestion, in a randomised cross over study design. Despite longer-term GTE intake, the authors did not observe a difference in average RER during the first or second hour of exercise between the groups (1 h: 0.95 ± 0.01 and 0.96 ± 0.01, 2 h: 0.91 ± 0.01 and 0.91 ± 0.01 for placebo and GTE respectively) [212]. However, subjects only ingested a total of 159 mg/day catechins of which 68 mg was EGCG. This is a much lower dose than that administered in other studies [188].

More promising results have been found when GTE has been ingested chronically (≥ 12 weeks) alongside an exercise training program. Maki et al [213] reported that overweight adults had a greater loss in total and subcutaneous abdominal fat area following a 12 week training program (180 min/week) plus daily consumption of a GTE (625 mg/day catechins, 39 mg/day caffeine) compared to a control beverage (39 mg/day caffeine). Unfortunately fat oxidation was not measured in the study. In animal studies, fat oxidation has been measured following longer term GTE intake in combination with exercise training. Shimotoyodome et al [214] found that mice, after completing 15 weeks of regular exercise in combination with GTE ingestion, had significantly lower RER and increased fat utilisation during exercise, than the exercise only group. Furthermore, a 30% increase in running time

has also been found in mice fed a 0.5% GTE diet in conjunction with exercise training [215]. This increase in endurance capacity was accompanied with increased β oxidation rates and a lower RER, compared with the mice that only exercised. Similarly, more recent evidence has demonstrated that the age related decline in endurance capacity in senescence-accelerated prone mice was prevented when fed a 0.35% GTE diet combined with exercise training. These adaptations to endurance capacity were also paralleled with greater skeletal muscle β oxidation rates following GTE and exercise training [216].

In humans, Ota et al [217] supplemented 14 healthy male subjects with a placebo or GTE beverage rich in catechins (570 mg/day catechins of which 218 mg was EGCG) for 2 months, during which they took part in regular treadmill exercise (5 km/h for 30 min 3 times a week) . It was found that subjects consuming the GTE test beverage had 24% higher fat oxidation rates during exercise than the placebo group. A similar effect was found when the supplementation and exercise training period was extended to 10 weeks [218]. In this study, daily consumption of a test beverage containing 573 mg of GTE catechins plus regular exercise (60 min cycle at 60% VO_2 max 3 times a week) significantly lowered RER compared to placebo. These studies provide an early insight into the possibility that longer term GTE intake in combination with exercise training may be efficacious in elevating fat oxidation.

It appears that in a small number of studies, both shorter and longer term GTE intake has the potential to increase fat oxidation. But this has not been consistently shown. However, no study has directly compared shorter and longer term GTE intake on fat oxidation during exercise. For these reasons, the practical relevance and use of GTE remains ambiguous. The exact mechanisms may differ dependent on the duration of intake.

1.7.5 The mechanisms behind the potential effects of Green tea on substrate utilisation

Several mechanisms have been proposed by which GTE could influence substrate utilisation. The majority of evidence originates from *in vitro* studies, which may have limited application *in vivo.* Such

studies do not consider the bioavailability of catechins in humans, reflected in the dose and form of catechins used *in vitro*. The mechanisms that explain the potential shorter term and longer term effects of GTE ingestion may be distinctly different. Thus, both will be discussed in the following section.

1.7.5.1 Mechanisms behind the shorter-term effects of green tea intake on fat metabolism

For some time now it has been proposed that shorter term intake of GTE, more specifically EGCG as well as caffeine, may target the sympathetic nervous system (~1-2 hours after ingestion).

1.7.5.1.1 GTE and Catechol-O-methyltransferase inhibition

Catechol-*O*- methyltransferase (COMT) is known as an intracellular enzyme and is ubiquitous throughout all mammalian tissues including skeletal muscle and adipose tissue [219]. COMT is a constitutively active enzyme which degrades catechol compounds, such as many of the neurotransmitters, by transferring a methyl group [219]. EGCG has been reported to directly inhibit catechol-*O*- methyltransferase (COMT) [220]. Herein, it is believed that circulatory catecholamine concentrations will be greater, in turn increasing the sympathetic nervous system (SNS) [221] thus stimulating lipolysis via adrenergic receptors and potentially increasing fat oxidation [210]. As a result the COMT mechanism has been extensively referenced in the literature, despite a lack of evidence in humans.

The most cited study to support EGCG inhibitory effects of COMT are reported in an early *in vitro* study by Borchardt [220]. Based on this early work others [222] have reported that COMT is inhibited directly by certain catechins. Interestingly, no specific tea catechin was identified by Borchardt [220]. Therefore, it is difficult to conclude, based on this study alone, whether GTE catechins are capable of inhibiting COMT. More recently it has been suggested that specific tea catechins are substrates and inhibitors of the *O*-methylation of COMT in human, mice and rat liver [223-226]. It has been shown that those catechins that possess a galloyl-type D ring (EGCG and ECG) are 100-1000 more potent at inhibiting COMT *in vitro* [223]. Furthermore, it seems that COMT activity is highly variable within a population which may explain the large individual responses to GTE. This may also explain the

aforementioned ethnic differences in the response to GTE [186], as a higher expression and activity of COMT is apparent in Asian populations [227, 228]. What is currently unclear is the specific site of inhibition or where the accumulation of catecholamines may occur *in vivo*.

To date, there is only one study to show an increase in 24 hour noradrenaline (NA) urinary excretion following shorter term GTE intake at rest in humans [187] with other studies showing no effect [198, 199, 202]. Yet, it would appear that *in vivo* the low circulatory concentrations of catechins are unlikely to inhibit COMT, especially in the conjugated form. In support, the potency of catechins ability to inhibit COMT is lost once they are glucoronidated [223]. Therefore, the inhibitory effects of catechins *in vivo* may be lost once conjugated: methylated, glucuronidated, and sulphated which limits the application of the COMT mechanism *in vivo*. At present even if the COMT enzyme was important in the degradation of catecholamines the link to increasing fat oxidation is an assumption that is often made.

1.7.5.1.2 Synergistic effects of GTE and Caffeine

The metabolic effects of GTE may also be driven by the presence of caffeine in many of the studies to date. As previously discussed in this chapter, caffeine *in vitro* has been shown to affect tissues ubiquitously by indirectly controlling levels of intracellular cyclic adenosine monophosphate (cAMP), calcium and catecholamine release [229]. Caffeine has been proposed to inhibit phosphodiesterase (PDE) activity [229], which is responsible for degrading cAMP a secondary messenger in the sympathetic pathway. The increase of cAMP is thought to promote lipolysis by activating the phosphorylation of hormone sensitive lipase (HSL) [230] which in turn is believed to increase lipolysis and fat oxidation [210] . Once again, there is a wealth of *in vitro* evidence to support the potential mechanisms of caffeine, using supra physiological concentrations (100-6000 µmol/L *in vitro* compared to 20-40 µmol/L in humans) [141]. This clearly limits the application of these results to humans.

It is often hypothesised that the intake of both GTE and caffeine may result in synergistic effects by targeting the SNS, through COMT and PDE. *In vitro*, Dulloo et al [222] investigated the thermogenic effects of GTE and caffeine on brown adipose tissue (BAT). In isolation GTE stimulated BAT thermogenesis greater than caffeine. Therefore, Dulloo et al [222] argued that the combination of both of these ingredients would augment thermogenesis further. In theory, the argument for synergistic effects of GTE and caffeine is sound. However, there is no direct *in vivo* evidence to support it. A number of human studies at rest have shown that GTE and caffeine ingestion has no effect on markers of lipolysis (through changes in plasma FAs and glycerol [2, 208, 231]) or the SNS (represented by no apparent differences in plasma catecholamines [198, 199, 202]. This may be partly explained by the lower doses of caffeine ingested in GTE supplementation protocols (<150 mg ~ 1-2 mg/kg BM) when compared to previous studies that supplement caffeine alone (5-9 mg/kg BM) [119, 168]. Considering that there is little or no evidence that the COMT mechanism is responsible for changes in fat oxidation in humans following GTE (with or without caffeine), Chapter 2 of this thesis also assessed whether 7 days of GTE supplementation was able to augment plasma catecholamine concentrations at rest or during exercise

1.7.5.2 Mechanisms behind the effects of longer term green tea intake

The mechanisms behind the effects of longer term GTE may be different than those that explain the shorter term effects of GTE. The reported increase in fat oxidation, following longer term GTE ingestion, may be explained by alterations to fat metabolism specific gene expression. At present, only animal and *in vitro* evidence is available to support the potential mechanisms following longer term GTE intake.

1.7.5.2.1 Mechanisms behind the effects of longer term green tea intake at rest

A number of animal studies have shown that longer term intake of GTE results in a decrease in adipogenic genes such as peroxisome proliferator-activated receptor- γ (PPAR- γ), Ccaat-enhancer binding protein- α (C/EBP-α), sterol regulatory element binding protein-1c (SREBP-1c), activated

protein 2 (aP2), lipoprotein lipase (LPL), and fatty acid synthase (FAS) [232]. In addition, others have illustrated that longer term GTE intake increases mRNA expression for lipolytic and β oxidation enzymes in the liver and adipose tissue, such as carnitine palmitoyl transporter I (CPTI), HSL and adipose triglyceride lipase (ATGL) [233, 234]. However, this is not supported by all [235]. Recently, 16 weeks of EGCG was found to elevate mRNA expression of fat metabolism enzymes (MCAD, NRF-1, UCP3, and PPARα) in mice skeletal muscle [236]. Alternatively, these alterations to lipolytic and β oxidation enzymes are not always apparent in skeletal muscle [233, 234]. More recently Sae-tan et al [236] was the first to show that 16 weeks of 0.32% dietary EGCG was able to alter mRNA expression specific to skeletal muscle fat metabolism enzymes in mice including: MCAD, NRF-1, UCP3, and PPARα. This demonstrates that longer term GTE intake may have direct effects on increasing gene expression specific to fat metabolism enzymes not only in the liver and adipose tissue but also in the skeletal muscle.

It seems that the alterations to fat metabolism genes may occur in a variety of tissues following longer term GTE intake. What is less clear is the time course of GTE intake required to induce these metabolic alterations at a tissue level. Recently Friedrich et al [237] observed in mice that EGCG (1.0%) consumed for 4 days resulted in lower liver triglyceride and glycogen content in the post-prandial state. These metabolic changes were also accompanied by a down regulation of lipogenic gene mRNA expression for acetyl CoA carboxylase (ACC), FAS and stearoyl-CoA (SCD1) in the liver. In a second experiment the authors showed that 7 days of EGCG (0.25-0.5%) resulted in an increased lipid oxidation. However, the authors did not assess changes in liver, skeletal muscle or adipose tissue gene expression following GTE intake. Taken together these results suggest that GTE ingestion, even as shorter as 4-7 days, may increase lipid oxidation due to decreased lipogenic gene expression.

The precise molecular signalling mechanism by which longer term GTE intake activates fat metabolism gene expression is currently unclear. It is understood these adaptations to fat metabolism may be mediated by peroxisome proliferator receptor-γ co-activator-1-α (PGC-1α) [104] which co-

activates peroxisome proliferator activated receptors (PPAR), both of which are responsible for regulating fat metabolism adaptations [104]. However, Murase et al [238] concluded that increases in fat oxidation following longer term GTE intake were not attributable to changes in PGC-1 α. Further, GTE catechins have been shown in some [239, 240], but not all cases [241] to activate PPARs *in vitro*. Lee et al [240] showed that GTE increased the activation of PPAR- α using a transient transfection assay. It was reported that EGCG and EGC were shown to be most effective. The *in vitro* evidence is also supported by animal data. GTE supplementation of 4-12 weeks has been shown to lead to greater PPAR-α and PPAR-γ expression in the liver, adipose tissue and skeletal muscle [242-244]. However, Chen et al [233] reported no change in liver PPAR expression following 6 months EGCG intake in rats.

At present, longer term GTE may induce changes to fat metabolism enzymes that over time would increase the capacity to oxidise fat. However, at this stage, it is too early to conclude whether these adaptations to fat metabolism are due to GTE induce changes to signalling molecules, such as PGC1-α and PPARs. Future human investigations are warranted. However, what this preliminary evidence demonstrates that potentially up regulating fat oxidation with GTE may augment adaptations to fat metabolism. This has important implications for the adaptations that may occur after exercise.

1.7.5.2.2 Mechanisms behind the effects of longer term green tea intake and exercise training

As mentioned, endurance exercise training is highly effective at increasing fat oxidation [245]. This is due to skeletal muscle adaptations specific to the expression of fat metabolism enzymes and transport proteins following exercise training [104, 210, 246]. These specific adaptations are responsible for improving exercise performance. Few studies have investigated the exact signalling molecules that may explain the adaptations that occur following exercise training with or without longer term GTE intake in human and animal studies. As mentioned above, GTE *in vitro* and in animal models has been suggested to alter PGC1- α and PPAR signalling molecules which may be responsible for inducing the adaptations to fat metabolism. In order for these signalling molecules to be activated it has been

suggested that exercise is required [109]. It would therefore seem that in order for GTE to activate the adaptations to fat metabolism enzymes and proteins, exercise may be required.

In support of this a number of laboratories in Japan have demonstrated that longer term GTE intake in mice, subjected to endurance exercise training, may improve endurance capacity. The performance enhancements were accompanied by increases in fat metabolism specific enzyme gene expression when compared to exercise only mice [238, 241]. Murase et al [238] observed that 12 weeks of GTE (0.1-0.5%) increased mRNA expression for sarcolemmal fatty acid transporter (FAT/CD36) and MCAD above that of exercise alone. These adaptations were accompanied by augmented β oxidation as well as exercise performance, assessed by time to exhaustion test. Similar results have been demonstrated by others that an increase in fat oxidation was explained by skeletal muscle adaptations [214, 238]. In addition these studies also showed that the highest GTE concentration caused a significant increase in plasma FA concentrations at the end of exhaustive exercise [215, 238, 241, 247]. It could be suggested that the combination of exercise training and GTE intake resulted in greater lipolysis which, in addition to the muscle adaptations, explain the increase in β oxidation.

At present the underlying mechanisms to explain the improvements in fat oxidation following longer term GTE intake are incompletely understood. Although based on animal evidence only, it seems that GTE, when ingested on a regular basis, may cause alterations to fat metabolism enzyme gene expression in the liver, adipose tissue and skeletal muscle. It is also important to note that based on allometric scaling the equivalent GTE doses (3-32 cups/d or ~450-4800 mL/d) given to mice may prove difficult to administer to humans [248].

1.8 Nutritional support to avoid nutrient deficiency in athletes

Despite the studies outlined above, suggesting that nutritional interventions may be effective in augmenting fat metabolism and exercise performance, it is rarely suggested that an athlete requires nutritional supports in order to prevent nutritional deficiency. However, emerging evidence over the past decade has alarmingly shown that vitamin D deficiency is highly prevalent worldwide [11], even

in athletes who spend significant time outdoors training and competing [249-251]. Further, it also appears that for many athletes, dietary vitamin D is an often ignored micronutrient, with reports suggesting that athletes consume well below the current RNI [252-254]. With this in mind, the extent of vitamin D deficiency, the sources of vitamin D in athletes and the impact it may have on exercise performance is largely unknown [10]. However, athletes are currently supplementing vitamin D in their diet from supplements in the hope of gaining an ergogenic effect. Therefore, research is required to explore the extent of deficiency and the effect of supplementation on exercise performance in athletes. Chapters 3 and 4 of this thesis explore these unknown questions.

1.9 Vitamin D for athletic performance

1.9.1 Photobiology and metabolism of vitamin D

Vitamin D is a pluripotent fat-soluble secosteroid, produced in the skin via ultraviolet-B (UVB) radiation from sunlight, at a wavelength of 290-315 nm [255]. Sunlight penetrates the skin and converts 7-dehydrocholesterol in the plasma membrane of the epidermis and dermis to pre vitamin D_3 (precholecalciferol) [256]. The pre vitamin D_3 is then converted to stable vitamin D_3 (cholecalciferol), which has been shown to occur within 2 h of exposure to sunlight [257]. The stable vitamin D_3 can either be taken up by the vitamin D binding protein (DBP) into the capillary bed or undergoes photoisomerization to inactive forms [256]. The process of producing inactive forms of vitamin D metabolites ensures vitamin D intoxication does not occur with prolonged exposure to the sun. Vitamin D_3 is then taken up by the liver where it is converted by the enzyme vitamin D-25 hydroxylase (25-OHase) to 25-hydroxyvitamin D (25(OH)D) (calcidiol) which is the stable clinical measure of vitamin D status, with a half life of ~ 3 months [258]. It is evident that in healthy young individuals, who were exposed to sunlight, a significant increase in 25(OH)D occurs within 24 h of exposure [259]. It has been estimated that a single 30 minute exposure in the sun to the whole body can be equivalent to 10,000 IU (250 µg, 1 µg = 40 IU) of vitamin D [258], while exposure to the hands and arms for 15 minutes may provide 800-1500IU of vitamin D. The delivery of vitamin D from the

sun is dependent on the pigment of the skin, the age of the individual and the latitude of exposure [255, 256, 260].

Vitamin D can also be provided through dietary intake as vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol) (Recommended Daily Allowance (RDA) United Kingdom, 200 IU). Vitamin D_2 is a steroid found in plants, sourced from cereals and fungi products [258] whereas the major source of dietary vitamin D is from vitamin D_3 in the form of fish (200-1000 IU/100g serving) , egg yolks (20 IU/serving), butter and fortified foods [258]. Vitamin D can also be found in 'over the counter' supplements in D_2 and D_3, with the majority of vitamin D supplements in the United States being D_2 and in the United Kingdom being D_3. Following intake, dietary vitamin D is absorbed and transported to the liver by chylomicrons in the lymphatic system for hydroxylation [258]. However, vitamin D_2 is less effective than vitamin D_3 at raising 25(OH)D [261], due to the difference in affinity to bind to the 25-OHase [261]. The 25(OH)D can be further hydroxylated in the kidney by the enzyme 25-hydoxyvitamin D-1α-hydroxylase (1-OHase, *CYP27B1*) to the active form $1,25(OH)_2D_3$ (calcitriol) [258].

The traditional role of vitamin D is through calcium and phosphorus homeostasis for bone metabolism and neuromuscular function [9]. Vitamin D regulates intestinal absorption of dietary calcium and phosphorus through $1,25(OH)_2D_3$ interacting with the vitamin D receptor-retinoic acid-x-receptor complex (VDR-RXR) increasing the epithelial calcium channels [258]. $1,25(OH)_2D_3$ also regulates bone metabolism through VDR-RXR in osteoblasts to form mature osteoclasts which causes a release of calcium into the circulation [258]. This process is regulated through circulatory calcium and phosphorus concentrations. When vitamin D concentrations are low, intestinal absorption of calcium and phosphorus is impaired. In order to sustain circulatory concentrations for bone metabolism and neuromuscular function, an increase in the parathyroid hormone (PTH) occurs which stimulates calcium resorption and bone calcium mobilisation, as well as stimulating the production of $1,25(OH)_2D_3$ [258]. When vitamin D is sufficient, PTH is regulated directly by $1,25(OH)_2D_3$ and the increase in circulatory calcium concentrations [258].

Vitamin D status is known to follow a biphasic pattern across the year due to the varying contribution of sunlight exposure throughout each season [262] (Figure 1.1) . Vitamin D is stored in circulation, bound to DBP [256], but also in the adipose tissue and skeletal muscle [263, 264]. The seasonal variation in vitamin D is explained by the cutaneous production of vitamin D which is determined by the zenith angle of the sun altering the amount of UVB from the sun that reaches the earth [258]. During the months of October to March at latitudes above 35°N, little or no cutaneous vitamin D synthesis occurs due to a high zenith angle and low UVB (Figure 1.1) [255, 258]. Apart from the season and latitude, other factors such as time of day, covering the skin with clothes and sun lotion, ageing (through a decrease in 7-dehydrocholesterol in the skin), skin pigment (melanin) are all known to alter cutaneous synthesis of vitamin D and thus increase the prevalence of vitamin D deficiency [258].

1.9.2 Categorisation of vitamin D status

The definition of categorising vitamin D status is complicated due to multiple recommendations currently available in the literature which are based on varying health outcomes. Recently, the Institute of Medicine (IOM) stated that sufficient vitamin D status should be set >50 nmol/L to ensure optimal bone health [265]. In addition, the Scientific Advisory Committee on Nutrition (SACN) and Foods Standard Agency (FSA) in the United Kingdom (UK) define vitamin D deficiency as < 25 nmol/L [266]. However, these recommendations have received a great deal of criticism from the Endocrine Society [267] and other experts in the field [268] with higher values (>75 nmol/L) suggested due to the benefits that vitamin D has on both skeletal and extra skeletal health.

1.9.3 Vitamin D and bone health

The profound effect of vitamin D on bone health has been recognised since the turn of the century [269]. In children and adolescents vitamin D is responsible for preventing the development of rickets, which is defined as the weakening and deformation of the bones due to poor bone mineralisation [258]. In adults, vitamin D is responsible for preventing the development of secondary

hyperparathyroidism, which is associated with the development of osteomalacia, increasing bone turnover and increasing the risk of osteoporosis [258]. Rickets and osteomalacia are usually present when plasma 25(OH)D is < 25 nmol/L [258]. A great deal of research has been conducted in order to establish the optimal concentration of vitamin D that is required to protect against secondary hyperparathyroidism, and thus minimise osteomalacia, osteoporosis and fracture. However, the 25(OH)D concentration that results in a plateau in PTH concentrations in humans remains unclear, which is one of the reasons for the argued vitamin D status guidelines. The IOM states that 25(OH)D concentrations must be >50 nmol/L in order to minimise PTH concentrations. This is principally based on evidence from Preiemel et al [270] who showed that the incidence of osteomalacia in motor vehicle accident victims was not apparent when 25(OH)D was >50 nmol/L. Holick et al [271, 272] has recently provided a review of the evidence to suggest that these recommendations may be in part be misleading and the optimal 25(OH)D may be closer to >75 nmol/L. Further, other large scale studies have also reported 25(OH)D of >50 nmol/L [273] as well as >75 nmol/L [272] in order to result in a PTH plateau. A number of recent reviews have been highly influential in suggesting that PTH plateau occurs at >75 nmol/L, which has led to the general acceptance that for optimal bone health 25(OH)D 50-75 nmol/L should be encouraged [274].

Figure 1.1 Proposed seasonal vitamin D status (dashed black line) based on correction to current seasonal vitamin D status observed in the UK adults (solid black line) to improve exercise performance.

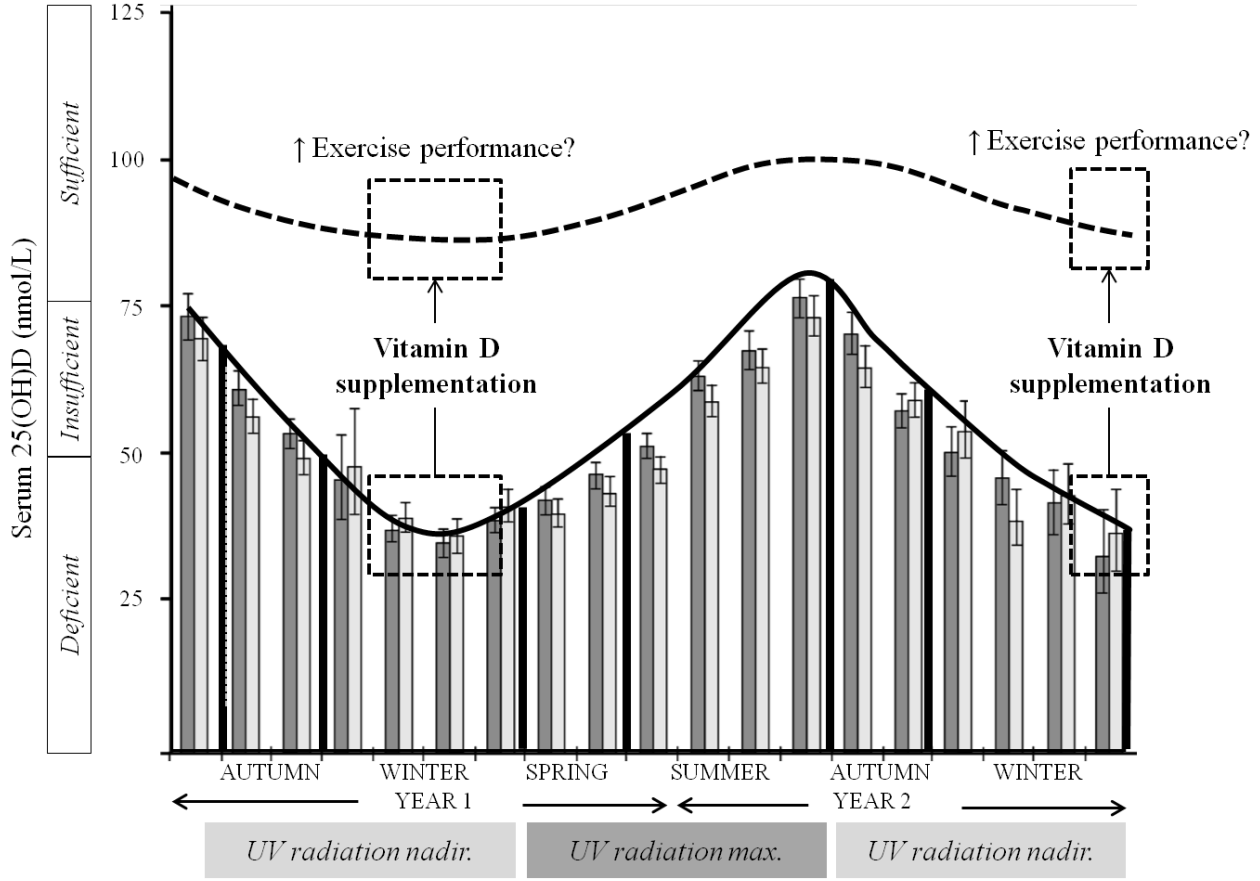


Figure adapted from *Hypponen and Power (2007) Am J Clin Nutr 85 (3) 860-8.* Reproduced with permission from AJCN.

It is often assumed that a reduction in PTH will lead to improved bone health. Yet in the majority of the aforementioned studies markers of bone health or fracture risk were not assessed. Despite this, a large number of population based studies have demonstrated that the risk of fractures and bone loss over time is greatly increased at 25(OH)D <50 nmol/L. Cauley et al [275] showed that the relative risk of hip fractures was greater in women with 25(OH)D < 47.5 nmol/L when compared to those with 25(OH)D >70 nmol/L. Similar findings have also been shown in men as well as young and old individuals [276-280] where the risk of fracture is lower at concentrations >50 nmol/L. Collectively it is well established that vitamin D is vital for bone health and increasing vitamin D status with supplementation can reduce the risk of fractures [265, 281]. Achieving a vitamin D status of 50-75 nmol/L would appear optimal for bone health.

1.9.4 Vitamin D and muscle function

The contention regarding vitamin D status guidelines is also based on well documented evidence that shows that vitamin D is highly important in optimal muscle function and composition. This point is particularly made by the Endocrine Society who argue that the IOM report does not consider the effects that vitamin D has on extra skeletal health.

Muscle weakness, otherwise known as myopathy, features in those individuals with vitamin D deficiency (< 25 nmol/L) [282-284] but may also occur at high vitamin D concentrations 25-50 nmol/L [285]. Myopathy is also accompanied with muscle pain and hypotonia in children and adults [286, 287] resulting in an impaired ability to perform simple physical tasks such as: climbing stairs as well as standing and sitting from a chair. In addition, neuromuscular impairments also occur with vitamin D deficiency [288, 289], as observed by a waddling gait, increased body sway and abnormalities to motor recruitment [9, 290]. The impairment to muscle function and neuromuscular activity may in part be explained by the depleted calcium content and uptake observed in isolated mitochondria and skeletal muscle from animals, respectively [291, 292]. More recently, Sinha et al [293] demonstrated that the skeletal muscle fatigue associated with vitamin D deficiency may be

explained by an attenuated mitochondrial oxidative function as measured by magnetic resonance spectroscopy. Thus it appears that vitamin D is crucial in the maintenance of skeletal muscle structure and functionality.

Evidence exists to show that the predominant impairments to muscle function with vitamin D deficiency are improved with vitamin D supplementation [286, 287, 290]. Furthermore, these improvements are reported to occur at concentrations > 75 nmol/L [267], with Zittermann et al [294] defining optimal concentrations for skeletal muscle function at 25(OH)D 100-250 nmol/L. Morphological changes have also been shown to be a predominant feature of vitamin D deficiency [9, 295]. Biopsy studies have shown atrophy of type II muscle fibres associated with myopathy induced by vitamin D deficiency [296, 297]. Sato et al [296] demonstrated that the age related decline in type II muscle fibres was reversed in those supplemented with vitamin D (1000 IU/d) for 2 years, which correlated with 25(OH)D. Further, Visser et al [298] documented in 331 elderly men and women (>65 y) that the age related decline in muscle mass (sarcopenia) was doubled in those who were vitamin D deficient (<50 nmol/L) and had greater PTH (>3.0 pmol/L) concentrations. More recently, cross sectional evidence in young women showed a 24% greater degree of fat infiltration in the muscle in those with lower 25(OH)D (<50 nmol/L) [299].

1.9.4.1 Evidence from the elderly

These deleterious effects of vitamin D deficiency on skeletal muscle structure and function have also been shown in numerous studies in the elderly to increase the risk of falls and reduce skeletal muscle strength and endurance.

The association between vitamin D and risk of falls may in part be explained by an improved muscle function. A number of large cross sectional studies have examined this potential relationship by using a number of tests including hand grip dynamometer strength, leg strength, sit to stand test, walking speed tests and the timed up and go test. Visser et al [298] monitored 1008 elderly men and women (> 65 y) over a 3 year period and showed that during this time a vitamin D status < 25 nmol/L reduced

hand grip strength twice as much when compared to those with higher vitamin D status. In a similar study design, Wicherts et al [300] showed that in 1234 elderly men and women (>65 y) physical performance (assessed using a variety of tests) was collectively poorer in individuals with vitamin D status < 50 nmol/L as opposed to those with > 75 nmol/L. Furthermore, the authors observed a greater reduction in physical performance in individual who were severely vitamin D deficient (< 25 nmol/L).. In support of these findings Bischoff-Ferrari et al [301] showed that in 4100 elderly individuals had better physical performance test outcomes when concentrations of 25(OH)D were between 22-40 nmol/L, with further improvements observed from 40-94 nmol/L. The study also showed that the benefits of vitamin D on physical performance were observed in both physical active and inactive individuals. Additionally, Bischoff-Ferrari et al [302] previously showed that a higher vitamin D status is also positively associated with improved leg extension power, which is more specific to performance measures that may be used in health adults or athletes. This suggests that the benefits to muscle function may even be observed in individuals with healthy and functional skeletal muscle and in tests that are more specific to athletic performance.

However, not all studies in the elderly have found an association between vitamin D status and muscle function [303-307]. This inconsistency is further evident when looking at the evidence to support the efficacy of vitamin D supplementation to improve muscle function in the elderly. The lack of consistent findings when investigating the effect of vitamin D on muscle function and performance in the elderly is due to the highly variable study design implemented. This includes the variable dose (400-2000IU/d or 8400-14,000IU/wk or 100,000IU/month), composition (Calcium, D_2 or D_3), duration (3 months – 7 years), basal vitamin D status (15-115 nmol/L) and participant characteristics (age, gender and disability). While the evidence is mixed, there are a number of studies that show a significant improvement in muscle function following vitamin D supplementation [296, 308-315]. Lips et al [316] demonstrated that 8400 IU/wk (~1200IU/d) reduced body sway in 226 elderly men and women (>70 y). Gupta et al [317] showed a significant improvement in grip and calf strength in Asian adult men and women (32 y) when supplemented with 60,000IU/ wk of vitamin D3 and

1000mg calcium. However, this effect may only have been observed as the participants were severely deficient prior to supplementation (<25 nmol/L). In agreement other studies have shown no benefit to muscle function when baseline vitamin D status has been insufficient or sufficient (50- >75 nmol/L) [318-320]. Further, a recent meta analysis of 17 randomised controlled trials involving 5072 elderly participants, concluded that there was no significant effect of vitamin D supplementation (range of doses included 400IU/d to 60,000IU/wk) on leg or grip strength in the elderly when baseline 25(OH)D were >25nmol/L [321]. In addition Kenny et al [313] showed no improvement to exercise performance or strength in elderly individuals who were sufficient at baseline, despite a significant increase in 25(OH)D (~87nmol/L) following 1000IU/d for 6 months.

1.9.4.2 Evidence from young adults and adolescents

The effect of vitamin D on muscle function and exercise performance has been examined in adolescents and younger adults [307, 322, 323]. Ward et al [322] tested 99 postmenarchal girls aged 12-14 y and showed that vitamin D status was positively correlated with two leg counter movement jump height, velocity, power and force. Similar findings were observed in Chinese adolescent girls (15 y old) where a positive correlation was found between vitamin D status and hand grip strength [323]. Significantly lower hand grip strength was apparent in those girls who were not vitamin D deficient and severely deficient when compared to those with vitamin D status > 50 nmol/L. More recently, evidence in 200 male and female adults showed that vitamin D sufficiency may be important for endurance performance, as VO_2 max was positively correlated with 25(OH)D [324]. Thus, vitamin D may play a role in muscle function and exercise performance (VO_2 max) in groups of individuals whose muscle morphology has not deteriorated, as observed in the aforementioned elderly studies. Although this observation in healthy adults, let alone in athletes, has not been well defined and requires further investigation. However, to date, the associative evidence between vitamin D and muscle function has not taken into account variables that may contribute towards to positive association. Firstly most of the evidence to date that has found a significant association has mainly been a heterogeneous group. In addition back ground nutrition, such as habitual dietary intake and

dietary supplements, which may contribute to muscle mass growth and development are not taken into account. These are factors that must be considered in more detail in future investigations.

Another variable that may also contribute to explaining the improvements to muscle function, strength and size with higher 25(OH)D is the relationship between vitamin D and testosterone [325, 326]. Testosterone, a steroid hormone from the androgen group, is important in muscle anabolism during growth and development [327]. In addition, serum testosterone release is reduced with age, which has been suggested to be linked with the sarcopenia [328]. Consequently ensuring serum testosterone concentrations are optimal throughout the life span is vital for growth and development.

With this in mind, serum testosterone concentrations (both free and total) have been shown to vary season by season, with higher concentrations seen during the summer compared with the winter [329]. Other studies have failed to observe a seasonal pattern, but attributed the lack of pattern to less time spent outdoors during the summer [330, 331]. This data would suggest that seasonal fluctuations in vitamin D status could alter serum testosterone concentrations. In support, a number of studies have shown that 25(OH)D is associated with serum testosterone concentrations (Free and total) [332-334]. Nimptsch et al [334] illustrated, in 1362 men (aged 40-75 y), that a dose response between 25(OH)D and testosterone (Free and total) was observed, until a plateau was reached between 75-85 nmol/L. This data would suggest that optimal testosterone concentrations are reached when vitamin D sufficient (> 75 nmol/L). In further support, Pilz et al [335] has previously shown in overweight men (25-49 y) that 1 year vitamin D supplementation (3332 IU/d) raised 25(OH)D to sufficiency and significantly increased both free and total testosterone concentration when compared to placebo.

Currently though, it is unknown whether increasing testosterone concentrations in young adults, especially athletes is beneficial to health, athletic performance or training adaptation. It is without doubt that optimal testosterone concentrations are vital for developing children and preserving quality of muscle mass in the elderly. In support, Bischoff-Ferrari et al [336] illustrated that those elderly men and women (> 65 y) with higher serum testosterone had a predicted 60% lower risk of falls, which was

further reduced with 3 years vitamin D (700 IU/d) plus calcium (500 mg/d) supplementation. In contrast, recent data from West et al [337] in young health males, showed that increasing circulatory testosterone during and after acute exercise and throughout a 12 week training programme, did not increase acute muscle protein synthesis [338] or training induced skeletal muscle adaptations [339]. Consequently, the effect of vitamin D and testosterone altering skeletal muscle mass and function, and the benefits it may bring to athletes, remains largely unknown.

On the whole, evidence suggests that vitamin D deficiency may have deleterious effects on the skeletal muscle structure, size and function. Such losses within the skeletal muscle should not to be understated as they are critical in functionality and mobility in the elderly, but in the context of this thesis, may be critical in optimal exercise performance in athletes. However, to date this hypothesis in athletes is unclear. Consequently chapter 4 and 5 of this thesis examined the effect of vitamin D deficiency on muscle function and exercise performance in athletes.

This thesis defines vitamin D status as severe deficiency, deficiency, insufficiency and sufficiency corresponding to 25(OH)D concentrations of <25 nmol/L, <50 nmol/L, 50-75 nmol/L and >75 nmol/L, respectively. These cut offs for vitamin D status are in unison with the majority of guidelines to date [267, 340].

1.9.5 Vitamin D and Immune function

Performance in various athletic events is multi factorial. Besides from the physiological limitations (skeletal muscle mass, contractile function, oxygen kinetics, substrate metabolism), well being and immune function are also important in determining performance. Of late, vitamin D has been shown to have a role in mediating immune function [341]. It is known that exercise, especially of high intensity, has an immunodepressive effect which appears to increase the risk of infection, especially upper respiratory tract infections (URTI) [342]. There are several factors to explain the immunodepressive effects of high intensity exercise which are beyond the scope of this thesis chapter. For an extensive insight into the topic the reader is directed to the recent review by Walsh et al [342]. One factor, in

which vitamin D may play a critical role, is through receptors found on the cell surface membrane of leucocytes known as toll like receptors (TLR) [343]. In brief, TLR are trans-membrane proteins that are critical in detecting microbial pathogens which in turn causes an inflammatory response to initiate proliferation of lymphocytes. However, it has been shown that exercise can acutely reduce the expression of TLR on monocyte cell surface membrane [344] and thus partly explain the immunodepressive effect. Vitamin D has been shown to firstly interact with TLR, as leucocytes possess a VDR and 1-α hydroxylase [341]. In turn this causes the hydroxylation of 25(OH)D to $1,25(OH)_2D_3$, which initiates antibiotic activity through antimicrobial peptide cathelicidin [345]. Therefore, it has been postulated that vitamin D is capable of activating an immune response independent of inflammation or TLR. While the majority of this evidence has stemmed from *in vitro* work, associative evidence has shown a lower risk of infection during the summer months when compared to the autumn and winter in the United Kingdom [346]. More recently Aloia et al [347] illustrated that 3 years of vitamin D supplementation (2 years - 800 IU/d, 1 year – 1000IU/d) reduced incidence of colds and related symptoms during the winter in 34 African-American post-menopausal women. However, since no study has confirmed these findings. Based on the limited data, it could be suggested that vitamin D may be important in optimising immune function, at least in athletes, when immunosuppression maybe prevalent when undergoing training.

1.9.6 Prevalence of vitamin D deficiency in the United Kingdom

In the United Kingdom (UK) (50-60°N), a high prevalence of vitamin D deficiency and insufficiency has been observed. Data from the 1958 British birth cohort study showed that 2850 adults 25(OH)D during the winter and spring season averaged 41.1 nmol/L, of which 47% of adults were deficient (<40 nmol/L), and 16% were clinically deficient (<25 nmol/L) (Figure 1.1) [262]. More recently, Cashman et al [348] showed that during the winter in 1132 men and women living in Ireland (52°N), 59% were vitamin D deficient (<50 nmol/L) and 84% were vitamin D insufficient (<75 nmol/L). Further, the authors showed that even during the summer, 22-36% and 69% of participants tested were vitamin D deficient and insufficient respectively. The high prevalence of vitamin D deficiency and insufficiency

is explained by the fact that the UK has one of the poorest sunny climates worldwide [349] with a high yearly cloud clover [350]. This means that over the course of the year sunlight exposure is severely impaired, reducing the synthesis of vitamin D. In addition, during the winter in the UK, little or no cutaneous vitamin D synthesis occurs due to a high zenith angle and low UVB (Figure 1.1) [255, 258]. Further, it appears that the current UK policy on sun exposure is primarily focused on preventing the development of skin cancer, with previous recommendations stating avoidance of sun exposure (Cancer Research UK and World Health Organisation) [268, 351]. Yet these policies and recommendations neglect the consideration of the health benefits of vitamin D to both bone and muscle [268]. It has since been suggested that 10-15 minutes of sun exposure to just the hands and face in the summer is sufficient to gain adequate vitamin D status [268]. This was further supported by SACN in 2007 who stated that individuals are able to gain enough vitamin D from time spent outdoors with sun bathing not being required, especially to avoid sun burn and higher risk of skin cancer [44]. In contention with the abovementioned statements, evidence has shown that sunlamp treatment (3/wk for 6 wk) to individuals during the winter mimicking the midday summer sun of UK (53.8°N), resulted in only 26% of individuals obtaining sufficient vitamin D status [81]. This data clearly supports the notion that casual sun exposure in the UK is not adequate in the winter, let alone in the summer, to raise and sustain sufficient vitamin D status.

The high prevalence of vitamin D deficiency and insufficiency in the UK may also, to a lesser extent to sun exposure, be explained by the poor dietary intake of vitamin D. Today, very few foods are fortified with vitamin D in the UK and dietary intake from sources such as fish and meats is limited, with current food sources estimated to provide ~120IU/d [268]. Consequently, the high prevalence of vitamin D deficiency and insufficiency in the summer and winter in the UK demonstrates the lack of vitamin D intake from both dietary and sun exposure.

With this in mind, vitamin D supplementation is therefore warranted. However, to date the vitamin D dose required to raise and sustain vitamin D status at sufficient concentrations is currently under debate. The recent IOM report [265] states that in order to achieve a vitamin D status of >50 nmol/L a

vitamin D intake of 600 IU/d is required. Further, the SACN and FSA state a daily recommended value (DRV) of 400 IU/d [266], only if sun exposure is limited. However numerous studies have shown that independent of sun exposure in the UK, an intake of 400-600 IU/d is inadequate to raise vitamin D status to >50 nmol/L [352]. In further support, Hall et al [353] demonstrated that in order to sustain sufficient vitamin D status across the year, summer sun exposure had to be high (90 min/d) and as a result vitamin D supplementation of 1300IU/d was required during the winter. Alternatively, the authors also showed that during low sun exposure in the summer (~20 min/d) it was estimated that 1000IU/d was required during the summer, while during the winter 2550IU/d was required. Yet, the work by Hall et al [353] was conducted in California, which would suggest that far higher doses of vitamin D would be needed in the UK to sustain sufficient vitamin D status all year round due to the poor sun exposure in the UK.

In light of the poor contribution of the sun and the diet to vitamin D status in the UK, it has been proposed that doses above the current RDA in the UK (200 IU/d), between 1000-4000 IU/d, may be required to achieve and sustain vitamin D sufficiency [267, 340]. However, with the higher suggested doses of vitamin D, has bought into question the safety and toxicity of such doses [265, 266, 354-356]. Vitamin D toxicity, categorised by hypercalcaemia, has been reported to only take effect at 25(OH)D concentrations >350 nmol/L which are achieved with vitamin D_3 > 100,000 IU/d [354-356]. However, doses up to 10,000 IU/d have been shown to be efficacious at improving vitamin D status in the absence of toxicity, which questions the current upper limits of vitamin D set [355]. Taken together, vitamin D supplementation would appear to be an effective strategy to ensure vitamin D sufficiency, however to date a vitamin D supplement protocol (dose and duration) is yet to be determined. Herein, to begin establishing an effective supplementation protocol, chapter 6 of this thesis explores the dose response of short term (28 d) vitamin D_3 supplementation, well above the current UK DRV, on achieving vitamin D sufficiency during the winter.

1.9.7 Prevalence of vitamin D deficiency in athletes

Based on the aforementioned evidence, vitamin D deficiency is wide spread in the general population [11]. It can be assumed from large cross sectional data in adults [348, 352, 357], that those vitamin D deficient individuals would likely include physically active and competitive sports men or women. Currently there is a growing appreciation for the prevalence of vitamin D deficiency in athletic populations. However, the prevalence at higher latitudes such as the UK remains unclear (Table 1.4).

Athletes who train and compete indoors, cover their skin, or compete at high latitudes may be more at risk of vitamin D deficiency [10, 252, 260]. In agreement, Hamilton et al [250] showed that athletes who lived and trained in Qatar, who regularly train indoors, cover their skin (for environmental and social factors), or train after sunset observed a high prevalence of vitamin D deficiency (~90%) despite the sunny environment. Similar results were observed in athletes residing in Israel, with 94% of basketball players and dancers being vitamin D insufficient [251]. Interestingly, even outdoor athletes have been shown to suffer from vitamin D deficiency, as demonstrated by French cyclists who train outdoors 16 h/wk [358].

Even at low latitudes, where the climate is less extreme than the abovementioned studies, 33% and 50% of Australian elite gymnasts were vitamin D deficient and insufficient at the end of autumn [359]. At higher latitudes, especially during the winter, a high prevalence of vitamin D deficiency and insufficiency has been reported in athletes [249, 360-364]. Lehtonen-Veromaa et al [362] showed that 68% and 43% of young Finnish girls (60.5°N) competing in gymnastics and running in the winter were vitamin D deficient (<38 nmol/L in the study) and clinically vitamin D deficient (<30 nmol/L) respectively.

However, during the summer, the authors demonstrated an improved vitamin D status, with only 2% of the athletes being vitamin D deficient. Vitamin D deficiency and insufficiency has also been reported amongst elite athletes [361, 363, 364]. Morton et al [363] reported a mean 25(OH)D of 51±19 nmol/L during the winter in the UK elite football players, with 65% of the players being insufficient. Similarly, at the beginning of the football season which corresponded with the end of the summer in

the UK, only 10% of the players were vitamin D insufficient. This was likely due to the majority of the players spending the summer abroad in hot and sunny climates. Further, a recent study in UK elite track and field athletes observed that over the course of the year, 19% and 29% of athletes were vitamin D deficient and insufficient, respectively [364]. However, the authors did not control for the season that the blood sample was collected, therefore little is known about the prevalence of vitamin D status throughout each season in this group of athletes.

Table 1.4 Overview of studies assessing vitamin D status in athletes across the seasons

Reference	*Country (°N)*	*N*	*Age*	*Season*	*25(OH)D (nmol/L)*	*Prevalence of vitamin D status*
Hamilton et al [250]	Doha, Qatar (25.3°N)	93 *(I:20 O:73)*	13-45 y	Summer	58% < 25 3% < 50	Deficient (<50 nmol/L): 91% Sufficient (50-75 nmol/L):9%
Constantini et al [251]	Jerusalem, Israel (31.8°N)	*98 (I:77 O:21)*	10-30 y	Winter Summer	W-63 ± 21 S-67 ± 43	Deficient (<50 nmol/L): 19% Insufficient (50-75 nmol/L): 48% Sufficient (>75 nmol/L): 27%
Lovell et al [337]	Canberra, Australia (35.3°S)	*18 (I:18 O:0)*	10-17 y	Autumn	56 (range 29-84)	Deficient (<50 nmol/L): 34% Insufficient (50-75 nmol/L): 50% Sufficient (>75 nmol/L): 16%
Lehtonen et al [340]	Turku, Finland (60.5°N)	*131(I:20 O:73)*	9-15 y	Winter Summer	W-34 ± 14 S-63 ± 15	W- Deficient (<37.5 nmol/L): 68% S- Deficient (<37.5 nmol/L): 2%
Barrack et al [343]	California, USA (38.5°N)	26 *(I:0 O:26)*	14-17 y	Autumn	93 ± 3	*NR*
Galan et al [344]	Seville, Spain (37.4°N)	28 *(I:0, O:28)*	27 ± 4 y	Winter Summer	W- 38-111 S- 62-163	W- Insufficient (<75 nmol.L): 64% S- Sufficient (>75 nmol/L): 93%
Halliday et al [249]	Wyoming, USA (41.3°N)	41 *(I:12, O:29)*	20 ± 2 y	Winter Spring Autumn	W-76 ± 24 Sp-105 ± 37 A- 123 ± 42	W-Insufficient (<80 nmol/L): 61% Deficient (<50 nmol/L): 3% Sp- Insufficient (<80 nmol/L): 16% Deficient (<50 nmol/L): 4% A- Insufficient (<80 nmol/L): 10% Deficient (<50 nmol/L): 2%
Ducher et al [345]	Melbourne, Australia (37.8°S)	16 *(I:16, O: 0)*	10-19 y	Winter	50 (range 21-94)	Deficient (<50 nmol/L):56% Insufficient (50-75 nmol/L): 25% Sufficient (>75 nmol/L): 19%
Bescos et al [338]	Barcelona, Spain (41.4°N)	21 *(I:21, O: 0)*	25 ± 4 y	Winter	48 ± 22	Deficient (<50 nmol/L):57% Insufficient 50-75 nmol/L): 33% Sufficient (>75 nmol/L): 10%

Abbreviations: **I** Indoor, **O** Outdoor, **W** Winter, **Sp** Spring, **A** Autumn, **S** Summer **NR** Not reported

Table 1.4 continued

Reference	*Country (°N)*	*N*	*Age*	*Season*	*25(OH)D (nmol/L)*	*Prevalence of vitamin D status*
Morton et al [341]	Liverpool, United Kingdom (53.4°N)	20 (*O: 20*)	26 ± 4 y	Winter Summer	W-104 ± 21 S-51 ± 19	W- Insufficient (<50 nmol/L): 65% Sufficient (>50 nmol/L): 35% S – Insufficient (<50 nmol/L): 10% Sufficient (>50 nmol/L): 90%
Pollock et al [342]	United Kingdom (51-54 °N)	63 (*I: 20, O: 43*)	25 ± 4 y	Winter Summer	W-63 ± 30 S-89 ± 40	Deficient (<50 nmol/L): 19% Insufficient (50-75 nmol/L): 29%
Close et al [339]	Liverpool, United Kingdom (53.4°N)	61(*O: 61*)	22 ± 2 y	Winter	Range 15-125	Deficient (<50 nmol/L) – 62% Insufficient (50-75 nmol/L)– 38%
Close et al [346]	Liverpool, United Kingdom (53.4°N)	30 (*O: 30*)	21 ± 2 y	Winter/Spring	51 ± 26	Severely deficient (<30 nmol/L) – 20% Deficient (<50 nmol/L) – 57%
Wilson et al [347]	Liverpool, United Kingdom (53.4°N)	36 (*F:19, J: 18*)	F 27 ± 5 y J 25 ± 5 y	Winter	F 38 ± 28 J 35 ± 14	Severely deficient (<30 nmol/L) – 37% Deficient (<50 nmol/L) – 31%

Abbreviations: **I** Indoor, **O** Outdoor, **W** Winter, **Sp** Spring, **A** Autumn, **S** Summer **NR** Not reported **F** Flat horse riders **J** Jump horse riders

The most comprehensive study to date investigated vitamin D status, dietary intake of vitamin D and sun exposure habits in 41 athletes, living at latitude of 41.3°N, from a number of indoor and outdoor sports across a season [249]. The authors observed a biphasic response in vitamin D status across the seasons, with a higher prevalence of vitamin D insufficiency in the winter compared to the spring and autumn, 61%, 16% and 12% respectively. It appeared that vitamin D status was higher in outdoor athletes at the end of autumn. Additionally, very few athletes reported consuming the RDA of vitamin D set in the US (<7% in all seasons) or consuming a vitamin D supplement (24-54% of athletes across the season). Yet, multivitamin intake was frequently used by athletes and correlated with vitamin D status in the winter. In agreement, a growing number of studies have illustrated that athletes consume vitamin D well below the DRI and is often an ignored micronutrient by athletes and nutritionists [252-254].

Therefore, vitamin D status in athletes is ultimately determined by a number of risk factors that regulate the cutaneous synthesis of vitamin D from the sun and dietary intake. Nevertheless, despite the work by Halliday et al [249], there is very limited information on the prevalence of vitamin D deficiency in athletes, especially those living and training at high latitudes (>50°N), for example in the UK [361, 363, 364]. Secondly, very little is known about the extent that risk factors are associated with developing vitamin D deficiency in athletes. However, it would appear that the high prevalence of vitamin D deficiency and insufficiency in the winter and autumn months combined with the low dietary vitamin D intake, as previously discussed, warrants the use of vitamin D supplementation by athletes. In light of this discussion, chapters 5 and 6 in this thesis detail studies that explore the prevalence of vitamin D deficiency in a variety of UK athletes, during the winter, while establishing the association that lifestyle, training and dietary factors have with vitamin D status.

1.9.8 Vitamin D and athletic performance

Early evidence from German and Russian athletes suggested the potential benefits and importance of vitamin D for optimal exercise performance [10]. In 1930, reports from Germany stated that exercise

performance could be improved when ultraviolet irradiation from sun lamps was given to athletes, with some athletic associations arguing it provided an unfair athletic advantage [10]. The improvement to performance was observed through the manipulation of vitamin D status by using 8-10 weeks of ultraviolet irradiation. The performance improvements included strength, speed, endurance, cardiovascular fitness and reaction time. However, the reported magnitude of performance improvements was large (8-60%) when compared to placebo, which was paralleled with the fact that many of the studies were often poorly controlled. Coinciding with the early ergogenic effects of vitamin D, consistent literature indicates physical and athletic performance is seasonal; which may be related to the concentration of vitamin D found within the body [10] (Figure 1.1). In support, long distance runners performance and VO_2 max has been shown to improve during the summer compared with winter seasons [370]. Interestingly, Hettinger et al 1956 [371], reported that daily training of the wrist flexor muscles for 7-10 months significantly improved strength gains during the summer and a decline in the winter months. Yet, it is difficult to conclude that the ergogenic effects are solely due to vitamin D due to the lack of control implemented throughout the abovementioned studies [10]. Nevertheless, sustaining sufficient vitamin D status throughout the year, specifically by preventing vitamin D deficiency in the winter and autumn, may be advantageous to an athlete's training and exercise performance (Figure 1.1). Within the past year, this paradigm has undergone preliminary investigation. Barker et al [372] observed that participants who received 200IU/d or 4000IU/d for 28 days did not improve muscle strength when compared to placebo during the winter. However, the lack of finding was likely due to the low latitude of the test site (40°N), which was accompanied by a high prevalence of vitamin D sufficiency prior to supplementation. More recently, a pilot study conducted in a small number of soccer players (n=10) found that 8 weeks of 5000IU/d during the winter in the UK (53°N) significantly improved 10 m sprint times and vertical jump when compared to placebo [361]. The work conducted by Close et al [361] provides early insight into the potential benefits of achieving and sustaining vitamin D sufficiency for athletic performance. However, due to the small subject number recruited and the poor control implemented throughout the study (training regime, diet and supplement compliance) the benefits of vitamin D supplementation in athletes remain unclear. In

addition, the current supplementation protocol (dose and duration) that is necessary to achieve vitamin D sufficiency and consequently provide benefits to exercise performance is also yet to be determined. With this in mind, Close et al [368] recently examined the effect of 20,000 IU/wk and 40,000 IU/wk compared to a placebo control for 12 weeks on muscle function (1-RM bench press, leg press, 20-m sprint and vertical jump height) in rugby and football players. The authors found that 57% of the athletes were vitamin D deficient prior to supplementation which increased to sufficiency in both supplementation groups. Following 12 weeks of supplementation, no difference in vitamin D status was observed between the two doses. However, despite the sufficient concentrations following supplementation, no differences in any muscle function test was observed in the athletes. However, interestingly the authors did demonstrate that high weekly doses of vitamin D (20,000 IU/wk ~ 2800IU/d) are highly effective at increasing vitamin D status in 6 weeks. Therefore, this early data in athletes suggests that high doses of vitamin D, above the RDA, are efficacious at correcting the high prevalence of vitamin D deficiency. However, it is firstly yet to be determined whether or not high daily doses above the vitamin D DRI, which is a more realistic nutritional regime that may be used by many athletes, are effective at increasing vitamin D status. Secondly, it remains unclear if increasing vitamin D status with this supplementation strategy may improve exercise performance. Herein chapter 6 of the thesis focuses on elucidating the optimal dose response with vitamin D supplementation on improving status and whether achieving sufficiency improves exercise performance in UK athletes.

1.9.9 Mechanisms to describe the effects of vitamin D in skeletal muscle

The improvement in skeletal muscle function and structure with vitamin D suggests that it is capable of directly binding and inducing effects within the skeletal muscle. This is firmly supported by the presence of the vitamin D receptor (VDR) within the skeletal muscle which is required to induce the physiological of vitamin D. [373, 374]. In addition, the enzyme 1-OHase has also recently been identified in skeletal muscle [375] allowing 25(OH)D to convert to $1,25(OH)_2D_3$. The interaction and

activation of the VDR in the skeletal muscle is understood to induce genomic (slow) and non-genomic (fast) signalling pathways through the presence of nuclear and non-nuclear VDR [376].

The genomic effects of vitamin D occur by the VDR interacting with a nuclear retinioc acid-x-receptor (RXR) to form a complex which then induces DNA transcription and *de novo* protein synthesis [9]. Vitamin D is capable of targeting more than 1000 vitamin D responsive human genes [377], specifically relating to skeletal muscle calcium handling, proliferation and differentiation [378-382]. Secondly, VDR knockout mice display abnormal skeletal muscle developmental growth, resulting in severely atrophied muscles, impaired functional performance and motor co-ordination [383, 384]. Additionally, these knockout mice have also been shown to have impaired swimming ability and motor co-ordination [383, 385]. Thirdly, vitamin D has also been shown to mediate the synthesis proteins that are vital for skeletal muscle physiology and contractile function including: ATP accumulation [386], increased troponin C [387], as well as actin and sarcoplasmic reticulum expression [388]. Collectively this mechanistic data from animal *in vitro* and *in vivo* studies proposes that vitamin D has pro-anabolic effects within the skeletal muscle. However, the direct examination of these effects within human skeletal muscle is yet to be conducted.

Alternatively, $1,25(OH)_2D_3$ is capable of inducing non-genomic (fast) responses within the skeletal muscle via the VDR by formation of secondary signalling messengers or phosphorylation of intracellular proteins. These signals are thought to occur through manipulations of caveolae microdomains on the plasma membrane of the skeletal muscle which are important in signal transduction [389]. Those characterised signalling pathways include p38 mitogen activated protein kinase (p38 MAPK) [389, 390] phospoinositol-3-kinase (PI3-K)/Akt [391] and extracellular regulated kinase1/2 (ERK 1/2) [392]. All of these signalling proteins are known to be implicated in the upstream regulation of mammalian target of rapamycin (mTOR) complex 1 (mTORC1), which is well documented to be critical in the regulation of skeletal muscle mass [393, 394]. However, the direct interaction between vitamin D, mTORC1 and the regulation of skeletal muscle mass and function in humans is unknown. Interestingly, the benefits to skeletal muscle growth and strength following

amino acid treatment in the elderly were shown to be reduced in those participants who were vitamin D deficient and insufficient [395]. This in part may be explained by reduced non-genomic signalling and blunted mTORC1 activity. But it also may be explained by early evidence which showed greater muscle protein degradation [396] and reduced incorporation of the amino acid leucine, which is known to stimulate mTORC1 [397], in vitamin D deficient rat skeletal muscle [386]. This provides even more evidence for vitamin D possessing a pro anabolic effect within the skeletal muscle.

The effects of vitamin D on muscle function and strength have also been shown to be independent of changes to muscle mass [297, 398]. Early evidence in vitamin D depleted chicks showed the calcium content and uptake by the SR was low [291, 292]. Furthermore, *in vitro* studies have shown that treatment of vitamin D depleted animal myotubes with $1,25(OH)_2D_3$ results in a rapid uptake of calcium [381, 399]. This is due to $1,25(OH)_2D_3$ being able to induce the initiation of calcium mobilisation from the SR, as well as influx from extracellular compartments through voltage gated and store-operated calcium channels [400, 401]. Through the increase in calcium mobilisation, calmodulin and calmodulin-dependent protein kinase II (CAMK II) are also activated [380, 389, 391, 392, 402, 403]. These calmodulin signals are known to be important in skeletal muscle mitochondrial biogenesis [87, 104] which may provide evidence for the improvements to fatigue with correction of vitamin D deficiency [282, 293, 301].

Collectively, the genomic and non-genomic mechanisms of vitamin D illustrate that the importance of vitamin D in skeletal muscle structure and function. It would appear that through the mechanisms, vitamin D is highly important in preventing the development of peripheral fatigue, which is highly important in ageing functionality but also in athletic performance. Additionally, vitamin D may also be critical in optimising the adaptations to exercise training, which is essential in preventing the development of sarcopenia in the elderly but may also be important for athletes. Yet these speculative viewpoints remain to be elucidated critically in human skeletal muscle, both *in vitro* and *in vivo*, in the elderly let alone in athletes.

1.10 Aims of the thesis

This thesis investigates a number of diverse nutritional interventions and supports that may optimise fat metabolism during exercise and improve endurance exercise performance. These interventions and supports include green tea, coffee and vitamin D.

Chapter 2 describes a study where the metabolic effects of short term green tea supplementation were investigated both at rest and during exercise using a metabolomic approach. In addition Chapter 2 investigates the catecholamine response at rest and during exercise in order to test the hypothesis that green tea is capable of inducing alterations to substrate metabolism through the often cited COMT mechanism, which to date has not been comprehensively investigated in humans. **Chapter 3** describes a study where the metabolic and performance effects of caffeine were compared with coffee, in order to establish whether coffee may be used as a nutritional support by athletes to gain the same benefits to performance as previously observed with caffeine.

Based on the recent evidence that has demonstrated a high prevalence of vitamin D deficiency in the general population, there is concern for the extent of deficiency in athletes. **Chapter 4** describes a study whereby a variety of athletes living and training at high latitude were tested to assess the prevalence of vitamin D deficiency while also identifying the potential sources and limitations to vitamin D status. Chapter 4 also identifies whether vitamin D status is associated with exercise performance in athletes. The current vitamin D requirements to prevent deficiency are mixed and under current debate. **Chapter 5** describes a study that explored the effect that a variety of doses of vitamin D had on improving vitamin D status as well as the implications that improving vitamin D status would have on exercise performance.

References

1. Maughan RJ, Greenhaff PL, Hespel P (2011) Dietary supplements for athletes: emerging trends and recurring themes. Journal of sports sciences 29 Suppl 1: S57-66.
2. Thielecke F, Boschmann M (2009) The potential role of green tea catechins in the prevention of the metabolic syndrome - a review. Phytochemistry 70: 11-24.
3. Beaudoin MS, Graham TE (2011) Methylxanthines and human health: epidemiological and experimental evidence. Handbook of experimental pharmacology: 509-548.
4. Tunnicliffe JM, Erdman KA, Reimer RA, Lun V, Shearer J (2008) Consumption of dietary caffeine and coffee in physically active populations: physiological interactions. Appl Physiol Nutr Metab 33: 1301-1310.
5. Graham TE (2001) Caffeine and exercise: metabolism, endurance and performance. Sports Med 31: 785-807.
6. Bergstrom J, Hultman E (1967) A study of the glycogen metabolism during exercise in man. Scandinavian journal of clinical and laboratory investigation 19: 218-228.
7. Wei MY, Giovannucci EL (2010) Vitamin D and multiple health outcomes in the Harvard cohorts. Mol Nutr Food Res 54: 1114-1126.
8. Holick MF (2008) Vitamin D: a D-Lightful health perspective. Nutr Rev 66: S182-194.
9. Ceglia L, Harris SS (2012) Vitamin D and Its Role in Skeletal Muscle. Calcified tissue international.
10. Cannell JJ, Hollis BW, Sorenson MB, Taft TN, Anderson JJ (2009) Athletic performance and vitamin D. Med Sci Sports Exerc 41: 1102-1110.
11. van Schoor NM, Lips P (2011) Worldwide vitamin D status. Best practice & research Clinical endocrinology & metabolism 25: 671-680.
12. Christensen EH (1932) Stoffwechsel und die respiratorischen funktionen bei schwerer korperlicher arbeit. . Scand Arch Physiol 91: 160.
13. Hultman E, Greenhaff PL, Ren JM, Soderlund K (1991) Energy metabolism and fatigue during intense muscle contraction. Biochem Soc Trans 19: 347-353.
14. Krogh A, Lindhard J (1920) The Relative Value of Fat and Carbohydrate as Sources of Muscular Energy: With Appendices on the Correlation between Standard Metabolism and the Respiratory Quotient during Rest and Work. The Biochemical journal 14: 290-363.
15. Burke LM, Angus DJ, Cox GR, Cummings NK, Febbraio MA, et al. (2000) Effect of fat adaptation and carbohydrate restoration on metabolism and performance during prolonged cycling. J Appl Physiol 89: 2413-2421.
16. Hawley JA, Burke LM, Angus DJ, Fallon KE, Martin DT, et al. (2000) Effect of altering substrate availability on metabolism and performance during intense exercise. Br J Nutr 84: 829-838.
17. Stellingwerff T, Spriet LL, Watt MJ, Kimber NE, Hargreaves M, et al. (2006) Decreased PDH activation and glycogenolysis during exercise following fat adaptation with carbohydrate restoration. American journal of physiology Endocrinology and metabolism 290: E380-388.
18. Yeo WK, Lessard SJ, Chen ZP, Garnham AP, Burke LM, et al. (2008) Fat adaptation followed by carbohydrate restoration increases AMPK activity in skeletal muscle from trained humans. J Appl Physiol 105: 1519-1526.
19. Venables MC, Achten J, Jeukendrup AE (2005) Determinants of fat oxidation during exercise in healthy men and women: a cross-sectional study. J Appl Physiol 98: 160-167.
20. Goodpaster BH, Wolf D (2004) Skeletal muscle lipid accumulation in obesity, insulin resistance, and type 2 diabetes. Pediatric diabetes 5: 219-226.
21. Turcotte LP, Richter EA, Kiens B (1992) Increased plasma FFA uptake and oxidation during prolonged exercise in trained vs. untrained humans. American Journal of Physiology 262: E791-799.
22. Sidossis LS, Wolfe RR, Coggan AR (1998) Regulation of fatty acid oxidation in untrained vs. trained men during exercise. The American journal of physiology 274: E510-515.

23. Bergman BC, Brooks GA (1999) Respiratory gas-exchange ratios during graded exercise in fed and fasted trained and untrained men. Journal of applied physiology 86: 479-487.
24. Klein S, Coyle EF, Wolfe RR (1994) Fat metabolism during low-intensity exercise in endurance-trained and untrained men. American Journal of Physiology 267: E934-940.
25. Coggan AR, Raguso CA, Gastaldelli A, Sidossis LS, Yeckel CW (2000) Fat metabolism during high-intensity exercise in endurance-trained and untrained men. Metabolism: clinical and experimental 49: 122-128.
26. Romijn JA, Coyle EF, Sidossis LS, Gastaldelli A, Horowitz JF, et al. (1993) Regulation of endogenous fat and carbohydrate metabolism in relation to exercise intensity and duration. American Journal of Physiology 265: E380-391.
27. van Loon LJ, Greenhaff PL, Constantin-Teodosiu D, Saris WH, Wagenmakers AJ (2001) The effects of increasing exercise intensity on muscle fuel utilisation in humans. J Physiol 536: 295-304.
28. Achten J, Gleeson M, Jeukendrup AE (2002) Determination of the exercise intensity that elicits maximal fat oxidation. Med Sci Sports Exerc 34: 92-97.
29. van Loon LJ (2004) Use of intramuscular triacylglycerol as a substrate source during exercise in humans. J Appl Physiol 97: 1170-1187.
30. Gollnick PD, Piehl K, Saltin B (1974) Selective glycogen depletion pattern in human muscle fibres after exercise of varying intensity and at varying pedalling rates. J Physiol 241: 45-57.
31. Coggan AR, Coyle EF (1987) Reversal of fatigue during prolonged exercise by carbohydrate infusion or ingestion. J Appl Physiol 63: 2388-2395.
32. Bergstrom J, Hultman E (1967) Synthesis of muscle glycogen in man after glucose and fructose infusion. Acta Med Scand 182: 93-107.
33. Romijn JA, Coyle EF, Sidossis LS, Zhang XJ, Wolfe RR (1995) Relationship between fatty acid delivery and fatty acid oxidation during strenuous exercise. J Appl Physiol 79: 1939-1945.
34. Kimber NE, Cameron-Smith D, McGee SL, Hargreaves M (2013) Skeletal muscle fat metabolism after exercise in humans: influence of fat availability. J Appl Physiol.
35. Jeukendrup AE, Saris WH, Wagenmakers AJ (1998) Fat metabolism during exercise: a review. Part I: fatty acid mobilization and muscle metabolism. Int J Sports Med 19: 231-244.
36. Watt MJ, Spriet LL (2004) Regulation and role of hormone-sensitive lipase activity in human skeletal muscle. The Proceedings of the Nutrition Society 63: 315-322.
37. Galster AD, Clutter WE, Cryer PE, Collins JA, Bier DM (1981) Epinephrine plasma thresholds for lipolytic effects in man: measurements of fatty acid transport with [l-13C]palmitic acid. The Journal of clinical investigation 67: 1729-1738.
38. Langin D, Holm C, Lafontan M (1996) Adipocyte hormone-sensitive lipase: a major regulator of lipid metabolism. The Proceedings of the Nutrition Society 55: 93-109.
39. Boyd AE, 3rd, Giamber SR, Mager M, Lebovitz HE (1974) Lactate inhibition of lipolysis in exercising man. Metabolism: clinical and experimental 23: 531-542.
40. Fredholm BB (1971) The effect of lactate in canine subcutaneous adipose tissue in situ. Acta physiologica Scandinavica 81: 110-123.
41. Helge JW, Stallknecht B, Richter EA, Galbo H, Kiens B (2007) Muscle metabolism during graded quadriceps exercise in man. J Physiol 581: 1247-1258.
42. Coyle EF, Jeukendrup AE, Wagenmakers AJ, Saris WH (1997) Fatty acid oxidation is directly regulated by carbohydrate metabolism during exercise. Am J Physiol 273: E268-275.
43. Horowitz JF, Mora-Rodriguez R, Byerley LO, Coyle EF (1997) Lipolytic suppression following carbohydrate ingestion limits fat oxidation during exercise. American Journal of Physiology 273: E768-775.
44. Randle PJ, Newsholme EA, Garland PB (1964) Regulation of glucose uptake by muscle. 8. Effects of fatty acids, ketone bodies and pyruvate, and of alloxan-diabetes and starvation, on the uptake and metabolic fate of glucose in rat heart and diaphragm muscles. The Biochemical journal 93: 652-665.

45. Mora-Rodriguez R, Coyle EF (2000) Effects of plasma epinephrine on fat metabolism during exercise: interactions with exercise intensity. American Journal of Physiology Endocrinology and Metabolism 278: E669-676.
46. Febbraio MA, Lambert DL, Starkie RL, Proietto J, Hargreaves M (1998) Effect of epinephrine on muscle glycogenolysis during exercise in trained men. J Appl Physiol 84: 465-470.
47. Ren JM, Hultman E (1990) Regulation of phosphorylase a activity in human skeletal muscle. J Appl Physiol 69: 919-923.
48. Ferguson RA, Ball D, Krustrup P, Aagaard P, Kjaer M, et al. (2001) Muscle oxygen uptake and energy turnover during dynamic exercise at different contraction frequencies in humans. J Physiol 536: 261-271.
49. Krustrup P, Ferguson RA, Kjaer M, Bangsbo J (2003) ATP and heat production in human skeletal muscle during dynamic exercise: higher efficiency of anaerobic than aerobic ATP resynthesis. J Physiol 549: 255-269.
50. Holloway GP, Luiken JJ, Glatz JF, Spriet LL, Bonen A (2008) Contribution of FAT/CD36 to the regulation of skeletal muscle fatty acid oxidation: an overview. Acta physiologica 194: 293-309.
51. Ahlborg G, Felig P, Hagenfeldt L, Hendler R, Wahren J (1974) Substrate turnover during prolonged exercise in man. Splanchnic and leg metabolism of glucose, free fatty acids, and amino acids. The Journal of clinical investigation 53: 1080-1090.
52. Turcotte LP, Kiens B, Richter EA (1991) Saturation kinetics of palmitate uptake in perfused skeletal muscle. FEBS letters 279: 327-329.
53. Zimmermann R, Strauss JG, Haemmerle G, Schoiswohl G, Birner-Gruenberger R, et al. (2004) Fat mobilization in adipose tissue is promoted by adipose triglyceride lipase. Science 306: 1383-1386.
54. Watt MJ, Heigenhauser GJ, Spriet LL (2003) Effects of dynamic exercise intensity on the activation of hormone-sensitive lipase in human skeletal muscle. J Physiol 547: 301-308.
55. van Loon LJ, Koopman R, Stegen JH, Wagenmakers AJ, Keizer HA, et al. (2003) Intramyocellular lipids form an important substrate source during moderate intensity exercise in endurance-trained males in a fasted state. J Physiol 553: 611-625.
56. Stellingwerff T, Boon H, Jonkers RA, Senden JM, Spriet LL, et al. (2007) Significant intramyocellular lipid use during prolonged cycling in endurance-trained males as assessed by three different methodologies. American Journal of Physiology Endocrinology and Metabolism 292: E1715-1723.
57. Watt MJ, Heigenhauser GJ, Dyck DJ, Spriet LL (2002) Intramuscular triacylglycerol, glycogen and acetyl group metabolism during 4 h of moderate exercise in man. J Physiol 541: 969-978.
58. Watt MJ, Holmes AG, Steinberg GR, Mesa JL, Kemp BE, et al. (2004) Reduced plasma FFA availability increases net triacylglycerol degradation, but not GPAT or HSL activity, in human skeletal muscle. American Journal of Physiology Endocrinology and Metabolism 287: E120-127.
59. Stephens FB, Constantin-Teodosiu D, Greenhaff PL (2007) New insights concerning the role of carnitine in the regulation of fuel metabolism in skeletal muscle. J Physiol 581: 431-444.
60. Saggerson ED, Carpenter CA (1981) Carnitine palmitoyltransferase and carnitine octanoyltransferase activities in liver, kidney cortex, adipocyte, lactating mammary gland, skeletal muscle and heart. FEBS letters 129: 229-232.
61. Sidossis LS, Gastaldelli A, Klein S, Wolfe RR (1997) Regulation of plasma fatty acid oxidation during low- and high-intensity exercise. American Journal of Physiology 272: E1065-1070.
62. Dzamko N, Schertzer JD, Ryall JG, Steel R, Macaulay SL, et al. (2008) AMPK-independent pathways regulate skeletal muscle fatty acid oxidation. J Physiol 586: 5819-5831.
63. McGarry JD, Stark MJ, Foster DW (1978) Hepatic malonyl-CoA levels of fed, fasted and diabetic rats as measured using a simple radioisotopic assay. J Biol Chem 253: 8291-8293.

64. Steinberg GR, Smith AC, Van Denderen BJ, Chen Z, Murthy S, et al. (2004) AMP-activated protein kinase is not down-regulated in human skeletal muscle of obese females. J Clin Endocrinol Metab 89: 4575-4580.
65. Winder WW, Wilson HA, Hardie DG, Rasmussen BB, Hutber CA, et al. (1997) Phosphorylation of rat muscle acetyl-CoA carboxylase by AMP-activated protein kinase and protein kinase A. J Appl Physiol 82: 219-225.
66. Wojtaszewski JF, Nielsen P, Hansen BF, Richter EA, Kiens B (2000) Isoform-specific and exercise intensity-dependent activation of 5'-AMP-activated protein kinase in human skeletal muscle. J Physiol 528 Pt 1: 221-226.
67. Roepstorff C, Halberg N, Hillig T, Saha AK, Ruderman NB, et al. (2005) Malonyl-CoA and carnitine in regulation of fat oxidation in human skeletal muscle during exercise. American Journal of Physiology Endocrinology and Metabolism 288: E133-142.
68. Holloway GP, Bezaire V, Heigenhauser GJ, Tandon NN, Glatz JF, et al. (2006) Mitochondrial long chain fatty acid oxidation, fatty acid translocase/CD36 content and carnitine palmitoyltransferase I activity in human skeletal muscle during aerobic exercise. J Physiol 571: 201-210.
69. Odland LM, Howlett RA, Heigenhauser GF, Hultman E, Spriet LL (1998) Skeletal muscle malonyl-CoA content at the onset of exercise at varying power outputs in humans. Am J Physiol 274: 1080-1085.
70. Dean D, Daugaard JR, Young ME, Saha A, Vavvas D, et al. (2000) Exercise diminishes the activity of acetyl-CoA carboxylase in human muscle. Diabetes 49: 1295-1300.
71. Odland LM, Heigenhauser GJ, Lopaschuk GD, Spriet LL (1996) Human skeletal muscle malonyl-CoA at rest and during prolonged submaximal exercise. American Journal of Physiology 270: E541-544.
72. Bezaire V, Heigenhauser GJ, Spriet LL (2004) Regulation of CPT I activity in intermyofibrillar and subsarcolemmal mitochondria from human and rat skeletal muscle. American Journal of Physiology Endocrinology and Metabolism 286: E85-91.
73. Starritt EC, Howlett RA, Heigenhauser GF, Spriet LL (2000) Sensitivity of CPTI to malonyl-CoA in trained and untrained human skeletal muscle. American Journal of Physiology Endocrinology and Metabolism 278: 462-468.
74. Bezaire V, Bruce CR, Heigenhauser GJ, Tandon NN, Glatz JF, et al. (2006) Identification of fatty acid translocase on human skeletal muscle mitochondrial membranes: essential role in fatty acid oxidation. American Journal of Physiology Endocrinology and Metabolism 290: E509-515.
75. Engel AG, Angelini C (1973) Carnitine deficiency of human skeletal muscle with associated lipid storage myopathy: a new syndrome. Science 179: 899-902.
76. Wall B, Stephens FB, Van Loon L, Constantin D, Macdonald AI, et al. (2013) Reduced fat oxidation during high intensity, submaximal exercise: is the availability of carnitine important? European Journal of Sport Science 13: 191-199.
77. Wall BT, Stephens FB, Constantin-Teodosiu D, Marimuthu K, Macdonald IA, et al. (2011) Chronic oral ingestion of L-carnitine and carbohydrate increases muscle carnitine content and alters muscle fuel metabolism during exercise in humans. J Physiol 589: 963-973.
78. Coggan AR, Kohrt WM, Spina RJ, Bier DM, Holloszy JO (1990) Endurance training decreases plasma glucose turnover and oxidation during moderate-intensity exercise in men. J Appl Physiol 68: 990-996.
79. Phillips SM, Green HJ, Tarnopolsky MA, Heigenhauser GF, Hill RE, et al. (1996) Effects of training duration on substrate turnover and oxidation during exercise. J Appl Physiol 81: 2182-2191.
80. Coggan AR, Swanson SC, Mendenhall LA, Habash DL, Kien CL (1995) Effect of endurance training on hepatic glycogenolysis and gluconeogenesis during prolonged exercise in men. American Journal of Physiology 268: E375-383.

81. Shepherd SO, Cocks M, Tipton KD, Ranasinghe AM, Barker TA, et al. (2012) Sprint interval and traditional endurance training increase net intramuscular triglyceride breakdown and expression of perilipin 2 and 5. J Physiol
82. Shaw CS, Shepherd SO, Wagenmakers AJ, Hansen D, Dendale P, et al. (2012) Prolonged exercise training increases intramuscular lipid content and perilipin 2 expression in type I muscle fibres of patients with type 2 diabetes. American Journal of Physiology Endocrinology and Metabolism.
83. Friedlander AL, Jacobs KA, Fattor JA, Horning MA, Hagobian TA, et al. (2007) Contributions of working muscle to whole body lipid metabolism are altered by exercise intensity and training. merican Journal of Physiology Endocrinology and Metabolism 292: E107-116.
84. Friedlander AL, Casazza GA, Horning MA, Buddinger TF, Brooks GA (1998) Effects of exercise intensity and training on lipid metabolism in young women. American Journal of Physiology 275: E853-863.
85. Martin WH, 3rd, Dalsky GP, Hurley BF, Matthews DE, Bier DM, et al. (1993) Effect of endurance training on plasma free fatty acid turnover and oxidation during exercise. American Journal of Physiology 265: E708-714.
86. Achten J, Jeukendrup AE (2003) Maximal fat oxidation during exercise in trained men. Int J Sports Med 24: 603-608.
87. Hawley JA, Burke LM, Phillips SM, Spriet LL (2011) Nutritional modulation of training-induced skeletal muscle adaptations.J Appl Physiol 110: 834-845.
88. Kiens B, Essen-Gustavsson B, Christensen NJ, Saltin B (1993) Skeletal muscle substrate utilization during submaximal exercise in man: effect of endurance training. J Physiol 469: 459-478.
89. Winder WW, Hickson RC, Hagberg JM, Ehsani AA, McLane JA (1979) Training-induced changes in hormonal and metabolic responses to submaximal exercise. J Appl Physiol 46: 766-771.
90. Talanian JL, Holloway GP, Snook LA, Heigenhauser GJ, Bonen A, et al. (2010) Exercise training increases sarcolemmal and mitochondrial fatty acid transport proteins in human skeletal muscle. American Journal of Physiology, Endocrinology Metabolism 299: E180-188.
91. Tunstall RJ, Mehan KA, Wadley GD, Collier GR, Bonen A, et al. (2002) Exercise training increases lipid metabolism gene expression in human skeletal muscle. American Journal of Physiology, Endocrinology Metabolism 283: E66-72.
92. Schrauwen P, van Aggel-Leijssen DP, Hul G, Wagenmakers AJ, Vidal H, et al. (2002) The effect of a 3-month low-intensity endurance training program on fat oxidation and acetyl-CoA carboxylase-2 expression. Diabetes 51: 2220-2226.
93. Tarnopolsky MA, Rennie CD, Robertshaw HA, Fedak-Tarnopolsky SN, Devries MC, et al. (2007) Influence of endurance exercise training and sex on intramyocellular lipid and mitochondrial ultrastructure, substrate use, and mitochondrial enzyme activity. American journal of physiology Regulatory, integrative and comparative physiology 292: R1271-1278.
94. Wallis GA, Friedlander AL, Jacobs KA, Horning MA, Fattor JA, et al. (2007) Substantial working muscle glycerol turnover during two-legged cycle ergometry. American Journal of Physiology, Endocrinology Metabolism 293: E950-957.
95. Blomstrand E, Krustrup P, Sondergaard H, Radegran G, Calbet JA, et al. (2011) Exercise training induces similar elevations in the activity of oxoglutarate dehydrogenase and peak oxygen uptake in the human quadriceps muscle. Pflugers Archiv : European Journal of Physiology 462: 257-265.
96. Turcotte LP, Swenberger JR, Tucker MZ, Yee AJ (1999) Training-induced elevation in FABP(PM) is associated with increased palmitate use in contracting muscle. J Appl Physiol 87: 285-293.
97. Hansen AK, Fischer CP, Plomgaard P, Andersen JL, Saltin B, et al. (2005) Skeletal muscle adaptation: training twice every second day vs. training once daily. J Appl Physiol 98: 93-99.

98. Perry CG, Lally J, Holloway GP, Heigenhauser GJ, Bonen A, et al. (2010) Repeated transient mRNA bursts precede increases in transcriptional and mitochondrial proteins during training in human skeletal muscle. J Physiol 588: 4795-4810.
99. Burgomaster KA, Hughes SC, Heigenhauser GJ, Bradwell SN, Gibala MJ (2005) Six sessions of sprint interval training increases muscle oxidative potential and cycle endurance capacity in humans. J Appl Physiol 98: 1985-1990.
100. Gollnick PD, Saltin B (1982) Significance of skeletal muscle oxidative enzyme enhancement with endurance training. Clinical physiology 2: 1-12.
101. Yeo WK, Paton CD, Garnham AP, Burke LM, Carey AL, et al. (2008) Skeletal muscle adaptation and performance responses to once a day versus twice every second day endurance training regimens. J Appl Physiol 105: 1462-1470.
102. Gibala MJ, Little JP, van Essen M, Wilkin GP, Burgomaster KA, et al. (2006) Short-term sprint interval versus traditional endurance training: similar initial adaptations in human skeletal muscle and exercise performance. J Physiol 575: 901-911.
103. Henriksson J, Reitman JS (1977) Time course of changes in human skeletal muscle succinate dehydrogenase and cytochrome oxidase activities and maximal oxygen uptake with physical activity and inactivity. Acta physiologica Scandinavica 99: 91-97.
104. Coffey VG, Hawley JA (2007) The molecular bases of training adaptation. Sports Med 37: 737-763.
105. Lin J, Wu H, Tarr PT, Zhang CY, Wu Z, et al. (2002) Transcriptional co-activator PGC-1 alpha drives the formation of slow-twitch muscle fibres. Nature 418: 797-801.
106. Baar K (2004) Involvement of PPAR gamma co-activator-1, nuclear respiratory factors 1 and 2, and PPAR alpha in the adaptive response to endurance exercise. The Proceedings of the Nutrition Society 63: 269-273.
107. Philp A, Belew MY, Evans A, Pham D, Sivia I, et al. (2011) The PGC-1alpha-related coactivator promotes mitochondrial and myogenic adaptations in C2C12 myotubes. Am J Physiol Regul Integr Comp Physiol 301: R864-872.
108. Wang YX, Zhang CL, Yu RT, Cho HK, Nelson MC, et al. (2004) Regulation of muscle fibre type and running endurance by PPARdelta. PLoS biology 2: e294.
109. Narkar VA, Downes M, Yu RT, Embler E, Wang YX, et al. (2008) AMPK and PPARdelta agonists are exercise mimetics. Cell 134: 405-415.
110. Hulston CJ, Venables MC, Mann CH, Martin C, Philp A, et al. (2010) Training with low muscle glycogen enhances fat metabolism in well-trained cyclists. Med Sci Sports Exerc 42: 2046-2055.
111. Goedecke JH, Christie C, Wilson G, Dennis SC, Noakes TD, et al. (1999) Metabolic adaptations to a high-fat diet in endurance cyclists. Metabolism 48: 1509-1517.
112. Cameron-Smith D, Burke LM, Angus DJ, Tunstall RJ, Cox GR, et al. (2003) A short-term, high-fat diet up-regulates lipid metabolism and gene expression in human skeletal muscle. Am J Clin Nutr 77: 313-318.
113. Philp A, Burke LM, Baar K (2011) Altering endogenous carbohydrate availability to support training adaptations. Nestle Nutrition Institute workshop series 69: 19-31; discussion 31-17.
114. Akimoto T, Sorg BS, Yan Z (2004) Real-time imaging of peroxisome proliferator-activated receptor-gamma coactivator-1alpha promoter activity in skeletal muscles of living mice. Am J Physiol Cell Physiol 287: C790-796.
115. Jeukendrup AE, Randell R (2011) Fat burners: nutrition supplements that increase fat metabolism. Obes Rev 12: 841-851.
116. Jeukendrup AE, Randell R (2011) Fat burners: nutrition supplements that increase fat metabolism. Obesity reviews : an official journal of the International Association for the Study of Obesity 12: 841-851.
117. Maughan RJ, Burke LM (2011) Practical nutritional recommendations for the athlete. Nestle Nutrition Institute workshop series 69: 131-149.

118. Costill DL, Dalsky GP, Fink WJ (1978) Effects of caffeine ingestion on metabolism and exercise performance. Med Sci Sports 10: 155-158.
119. Spriet LL, MacLean DA, Dyck DJ, Hultman E, Cederblad G, et al. (1992) Caffeine ingestion and muscle metabolism during prolonged exercise in humans. American Journal of Physiology, 262: E891-898.
120. Mohr T, Van Soeren M, Graham TE, Kjaer M (1998) Caffeine ingestion and metabolic responses of tetraplegic humans during electrical cycling. J Appl Physiol 85: 979-985.
121. Van Soeren M, Mohr T, Kjaer M, Graham TE (1996) Acute effects of caffeine ingestion at rest in humans with impaired epinephrine responses. J Appl Physiol 80: 999-1005.
122. Graham TE, Hibbert E, Sathasivam P (1998) Metabolic and exercise endurance effects of coffee and caffeine ingestion. J Appl Physiol 85: 883-889.
123. McLellan TM, Bell DG (2004) The impact of prior coffee consumption on the subsequent ergogenic effect of anhydrous caffeine. Int J Sport Nutr Exerc Metab 14: 698-708.
124. Wiles JD, Bird SR, Hopkins J, Riley M (1992) Effect of caffeinated coffee on running speed, respiratory factors, blood lactate and perceived exertion during 1500-m treadmill running. Br J Sports Med 26: 116-120.
125. Butts N, Crowell D (1985) Effect of caffeine ingestion on cardiorespiratory endurance in men and women. Res Q Exerc Sport 56: 301-305.
126. Cabrera C, Artacho R, Gimenez R (2006) Beneficial effects of green tea--a review. J Am Coll Nutr 25: 79-99.
127. Chu Y-F (2012) Coffee: Emerging Health Effects and Disease Prevention. Wiley-Blackwell IFT press.
128. Frary CD, Johnson RK, Wang MQ (2005) Food sources and intakes of caffeine in the diets of persons in the United States. Journal of the American Dietetic Association 105: 110-113.
129. Tunnicliffe JM, Shearer J (2008) Coffee, glucose homeostasis, and insulin resistance: physiological mechanisms and mediators. Appl Physiol Nutr Metab 33: 1290-1300.
130. Chester N, Wojek N (2008) Caffeine consumption amongst British athletes following changes to the 2004 WADA prohibited list. Int J Sports Med 29: 524-528.
131. Del Coso J, Munoz G, Munoz-Guerra J (2011) Prevalence of caffeine use in elite athletes following its removal from the World Anti-Doping Agency list of banned substances. Appl Physiol Nutr Metab 36: 555-561.
132. Burke LM (2008) Caffeine and sports performance. Appl Physiol Nutr Metab 33: 1319-1334.
133. Miners JO, Birkett DJ (1996) The use of caffeine as a metabolic probe for human drug metabolizing enzymes. General pharmacology 27: 245-249.
134. Kashuba AD, Bertino JS, Jr., Kearns GL, Leeder JS, James AW, et al. (1998) Quantitation of three-month intraindividual variability and influence of sex and menstrual cycle phase on CYP1A2, N-acetyltransferase-2, and xanthine oxidase activity determined with caffeine phenotyping. Clinical pharmacology and therapeutics 63: 540-551.
135. Goldstein ER, Ziegenfuss T, Kalman D, Kreider R, Campbell B, et al. (2010) International society of sports nutrition position stand: caffeine and performance. J Int Soc Sports Nutr 7: 5.
136. Crozier TW, Stalmach A, Lean ME, Crozier A (2012) Espresso coffees, caffeine and chlorogenic acid intake: potential health implications. Food Funct 3: 30-33.
137. Perera V, Gross AS, McLachlan AJ (2010) Caffeine and paraxanthine HPLC assay for CYP1A2 phenotype assessment using saliva and plasma. Biomedical chromatography : BMC 24: 1136-1144.
138. Doherty M, Smith PM (2004) Effects of caffeine ingestion on exercise testing: a meta-analysis. Int J Sport Nutr Exerc Metab 14: 626-646.
139. Ganio MS, Klau JF, Casa DJ, Armstrong LE, Maresh CM (2009) Effect of caffeine on sport-specific endurance performance: a systematic review. J Strength Cond Res 23: 315-324.
140. Graham TE (2001) Caffeine, coffee and ephedrine: impact on exercise performance and metabolism. Can J Appl Physiol 26 Suppl: S103-119.

141. Magkos F, Kavouras SA (2005) Caffeine use in sports, pharmacokinetics in man, and cellular mechanisms of action. Critical reviews in food science and nutrition 45: 535-562.
142. Ivy JL, Costill DL, Fink WJ, Lower RW (1979) Influence of caffeine and carbohydrate feedings on endurance performance. Med Sci Sports 11: 6-11.
143. Bell DG, McLellan TM (2002) Exercise endurance 1, 3, and 6 h after caffeine ingestion in caffeine users and nonusers. J Appl Physiol 93: 1227-1234.
144. Bell DG, McLellan TM (2003) Effect of repeated caffeine ingestion on repeated exhaustive exercise endurance. Med Sci Sports Exerc 35: 1348-1354.
145. Graham TE, Spriet LL (1995) Metabolic, catecholamine, and exercise performance responses to various doses of caffeine. J Appl Physiol 78: 867-874.
146. Graham TE, Spriet LL (1991) Performance and metabolic responses to a high caffeine dose during prolonged exercise. J Appl Physiol 71: 2292-2298.
147. Greer F, Friars D, Graham TE (2000) Comparison of caffeine and theophylline ingestion: exercise metabolism and endurance. J Appl Physiol 89: 1837-1844.
148. Jackman M, Wendling P, Friars D, Graham TE (1996) Metabolic catecholamine, and endurance responses to caffeine during intense exercise. J Appl Physiol 81: 1658-1663.
149. Pasman WJ, van Baak MA, Jeukendrup AE, de Haan A (1995) The effect of different dosages of caffeine on endurance performance time. Int J Sports Med 16: 225-230.
150. Van Soeren MH, Graham TE (1998) Effect of caffeine on metabolism, exercise endurance, and catecholamine responses after withdrawal. J Appl Physiol 85: 1493-1501.
151. Currell K, Jeukendrup AE (2008) Validity, reliability and sensitivity of measures of sporting performance. Sports medicine 38: 297-316.
152. Cox GR, Desbrow B, Montgomery PG, Anderson ME, Bruce CR, et al. (2002) Effect of different protocols of caffeine intake on metabolism and endurance performance. J Appl Physiol 93: 990-999.
153. Desbrow B, Biddulph C, Devlin B, Grant GD, Anoopkumar-Dukie S, et al. (2012) The effects of different doses of caffeine on endurance cycling time trial performance. Journal of sports sciences 30: 115-120.
154. Jenkins NT, Trilk JL, Singhal A, O'Connor PJ, Cureton KJ (2008) Ergogenic effects of low doses of caffeine on cycling performance. Int J Sport Nutr Exerc Metab 18: 328-342.
155. Skinner TL, Jenkins DG, Taaffe DR, Leveritt MD, Coombes JS (2012) Coinciding exercise with peak serum caffeine does not improve cycling performance. Journal of science and medicine in sport / Sports Medicine Australia.
156. Jeukendrup A, Saris WH, Brouns F, Kester AD (1996) A new validated endurance performance test. Med Sci Sports Exerc 28: 266-270.
157. Laurence G, Wallman K, Guelfi K (2012) Effects of caffeine on time trial performance in sedentary men. Journal of sports sciences 30: 1235-1240.
158. Desbrow B, Leveritt M (2006) Awareness and use of caffeine by athletes competing at the 2005 Ironman Triathlon World Championships. Int J Sport Nutr Exerc Metab 16: 545-558.
159. Kovacs EM, Stegen J, Brouns F (1998) Effect of caffeinated drinks on substrate metabolism, caffeine excretion, and performance. J Appl Physiol 85: 709-715.
160. Liguori A, Hughes JR, Grass JA (1997) Absorption and subjective effects of caffeine from coffee, cola and capsules. Pharmacology, biochemistry, and behavior 58: 721-726.
161. Trice I, Haymes EM (1995) Effects of caffeine ingestion on exercise-induced changes during high-intensity, intermittent exercise. Int J Sport Nutr Exerc Metab 5: 37-44.
162. Lamina S, Musa DI (2009) Ergogenic effect of varied doses of coffee-caffeine on maximal aerobic power of young African subjects. African health sciences 9: 270-274.
163. Costill DL, Coyle E, Dalsky G, Evans W, Fink W, et al. (1977) Effects of elevated plasma FFA and insulin on muscle glycogen usage during exercise. J Appl Physiol 43: 695-699.
164. Essig D, Costill DL, Van Handel PJ (1980) Effects of Caffeine Ingestion on Utilization of Muscle Glycogen and Lipid During Leg Ergometer Cycling. Int J Sports Med 01: 86-90.

165. Erickson MA, Schwarzkopf RJ, McKenzie RD (1987) Effects of caffeine, fructose, and glucose ingestion on muscle glycogen utilization during exercise. Med Sci Sports Exerc 19: 579-583.
166. Van Soeren MH, Sathasivam P, Spriet LL, Graham TE (1993) Caffeine metabolism and epinephrine responses during exercise in users and nonusers. J Appl Physiol 75: 805-812.
167. Greer F, McLean C, Graham TE (1998) Caffeine, performance, and metabolism during repeated Wingate exercise tests. J Appl Physiol 85: 1502-1508.
168. Graham TE, Helge JW, MacLean DA, Kiens B, Richter EA (2000) Caffeine ingestion does not alter carbohydrate or fat metabolism in human skeletal muscle during exercise. J Physiol 529 Pt 3: 837-847.
169. Raguso CA, Coggan AR, Sidossis LS, Gastaldelli A, Wolfe RR (1996) Effect of theophylline on substrate metabolism during exercise. Metabolism: clinical and experimental 45: 1153-1160.
170. Vergauwen L, Richter EA, Hespel P (1997) Adenosine exerts a glycogen-sparing action in contracting rat skeletal muscle. Am J Physiol 272: E762-768.
171. Graham TE, Battram DS, Dela F, El-Sohemy A, Thong FS (2008) Does caffeine alter muscle carbohydrate and fat metabolism during exercise? Applied physiology, nutrition, and metabolism = Physiologie appliquee, nutrition et metabolisme 33: 1311-1318.
172. Ferre S (2008) An update on the mechanisms of the psychostimulant effects of caffeine. Journal of neurochemistry 105: 1067-1079.
173. Ribeiro JA, Sebastiao AM, de Mendonca A (2002) Adenosine receptors in the nervous system: pathophysiological implications. Progress in neurobiology 68: 377-392.
174. Tarnopolsky MA (2008) Effect of caffeine on the neuromuscular system--potential as an ergogenic aid. Appl Physiol Nutr Metab 33: 1284-1289.
175. Karcz-Kubicha M, Antoniou K, Terasmaa A, Quarta D, Solinas M, et al. (2003) Involvement of adenosine A1 and A2A receptors in the motor effects of caffeine after its acute and chronic administration. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 28: 1281-1291.
176. Irwin C, Desbrow B, Ellis A, O'Keeffe B, Grant G, et al. (2011) Caffeine withdrawal and high-intensity endurance cycling performance. J Sports Sci 29: 509-515.
177. Doherty M, Smith PM (2005) Effects of caffeine ingestion on rating of perceived exertion during and after exercise: a meta-analysis. Scandinavian Journal of Medicine & Science in Sports 15: 69-78.
178. Astorino TA, Cottrell T, Talhami Lozano A, Aburto-Pratt K, Duhon J (2012) Effect of caffeine on RPE and perceptions of pain, arousal, and pleasure/displeasure during a cycling time trial in endurance trained and active men. Physiology & behavior 106: 211-217.
179. Tallis J, James RS, Cox VM, Duncan MJ (2012) The effect of physiological concentrations of caffeine on the power output of maximally and submaximally stimulated mouse EDL (fast) and soleus (slow) muscle. J Appl Physiol 112: 64-71.
180. Tarnopolsky M, Cupido C (2000) Caffeine potentiates low frequency skeletal muscle force in habitual and nonhabitual caffeine consumers. Journal of applied physiology 89: 1719-1724.
181. Ortenblad N, Nielsen J, Saltin B, Holmberg HC (2011) Role of glycogen availability in sarcoplasmic reticulum Ca2+ kinetics in human skeletal muscle. J Physiol 589: 711-725.
182. Allen DG, Lee JA, Westerblad H (1989) Intracelluar calcium and tension during fatigue in isolated single muscle fibres from Xenopus laevis. J Physiol 415: 433-458.
183. Mohr M, Nielsen JJ, Bangsbo J (2011) Caffeine intake improves intense intermittent exercise performance and reduces muscle interstitial potassium accumulation. J Appl Physiol 111: 1372-1379.
184. Davis JK, Green JM (2009) Caffeine and anaerobic performance: ergogenic value and mechanisms of action. Sports Med 39: 813-832.
185. Thavanesan N (2011) The putative effects of green tea on body fat: an evaluation of the evidence and a review of the potential mechanisms. The British journal of nutrition 106: 1297-1309.
186. Hursel R, Viechtbauer W, Westerterp-Plantenga MS (2009) The effects of green tea on weight loss and weight maintenance: a meta-analysis. International journal of obesity 33: 956-961.

187. Dulloo AG, Duret C, Rohrer D, Girardier L, Mensi N, et al. (1999) Efficacy of a green tea extract rich in catechin polyphenols and caffeine in increasing 24-h energy expenditure and fat oxidation in humans. Am J Clin Nutr 70: 1040-1045.
188. Venables MC, Hulston CJ, Cox HR, Jeukendrup AE (2008) Green tea extract ingestion, fat oxidation, and glucose tolerance in healthy humans. Am J Clin Nutr 87: 778-784.
189. Hursel R, Viechtbauer W, Dulloo AG, Tremblay A, Tappy L, et al. (2011) The effects of catechin rich teas and caffeine on energy expenditure and fat oxidation: a meta-analysis. Obesity reviews : an official journal of the International Association for the Study of Obesity 12: e573-581.
190. Henning SM, Fajardo-Lira C, Lee HW, Youssefian AA, Go VL, et al. (2003) Catechin content of 18 teas and a green tea extract supplement correlates with the antioxidant capacity. Nutrition and cancer 45: 226-235.
191. Kao YH, Hiipakka RA, Liao S (2000) Modulation of endocrine systems and food intake by green tea epigallocatechin gallate. Endocrinology 141: 980-987.
192. Astill C, Birch MR, Dacombe C, Humphrey PG, Martin PT (2001) Factors affecting the caffeine and polyphenol contents of black and green tea infusions. Journal of agricultural and food chemistry 49: 5340-5347.
193. Holst B, Williamson G (2008) Nutrients and phytochemicals: from bioavailability to bioefficacy beyond antioxidants. Current opinion in biotechnology 19: 73-82.
194. Kroon PA, Clifford MN, Crozier A, Day AJ, Donovan JL, et al. (2004) How should we assess the effects of exposure to dietary polyphenols in vitro? Am J Clin Nutr 80: 15-21.
195. Selma MV, Espin JC, Tomas-Barberan FA (2009) Interaction between phenolics and gut microbiota: role in human health. Journal of agricultural and food chemistry 57: 6485-6501.
196. Aura AM (2008) Microbial metabolism of dietary phenolic compounds in the colon. Phytochem Rev 7: 22.
197. Rumpler W, Seale J, Clevidence B, Judd J, Wiley E, et al. (2001) Oolong Tea Increases Metabolic Rate and Fat Oxidation in Men. J Nutr 131: 5.
198. Berube-Parent S, Pelletier C, Dore J, Tremblay A (2005) Effects of encapsulated green tea and Guarana extracts containing a mixture of epigallocatechin-3-gallate and caffeine on 24 h energy expenditure and fat oxidation in men. Br J Nutr 94: 432-436.
199. Rudelle S, Ferruzzi MG, Cristiani I, Moulin J, Mace K, et al. (2007) Effect of a thermogenic beverage on 24-hour energy metabolism in humans. Obesity (Silver Spring) 15: 349-355.
200. Murase T, Nagasawa A, Suzuki J, Hase T, Tokimitsu I (2002) Beneficial effects of tea catechins on diet-induced obesity: stimulation of lipid catabolism in the liver. Int J Obes Relat Metab Disord 26: 1459-1464.
201. Thielecke F, Rahn G, Bohnke J, Adams F, Birkenfeld AL, et al. Epigallocatechin-3-gallate and postprandial fat oxidation in overweight/obese male volunteers: a pilot study. Eur J Clin Nutr.
202. Gregersen NT, Bitz C, Krog-Mikkelsen I, Hels O, Kovacs EM, et al. (2009) Effect of moderate intakes of different tea catechins and caffeine on acute measures of energy metabolism under sedentary conditions. Br J Nutr 102: 1187-1194.
203. Boschmann M, Thielecke F (2007) The effects of epigallocatechin-3-gallate on thermogenesis and fat oxidation in obese men: a pilot study. J Am Coll Nutr 26: 389S-395S.
204. Lonac MC, Richards JC, Schweder MM, Johnson TK, Bell C (2011) Influence of short-term consumption of the caffeine-free, epigallocatechin-3-gallate supplement, Teavigo, on resting metabolism and the thermic effect of feeding. Obesity 19: 298-304.
205. Auvichayapat P, Prapochanung M, Tunkamnerdthai O, Sripanidkulchai BO, Auvichayapat N, et al. (2008) Effectiveness of green tea on weight reduction in obese Thais: A randomized, controlled trial. Physiol Behav 93: 486-491.
206. Harada U, Chikama A, Saito S, Takase H, Nagao T, et al. (2004) Effects of the Long-Term Ingestion of Tea Catechins on Energy Expenditure and Dietary Fat Oxidation in Healthy Subjects. Journal of Health Science 51: 7.

207. Astrup A, Gotzsche PC, van de Werken K, Ranneries C, Toubro S, et al. (1999) Meta-analysis of resting metabolic rate in formerly obese subjects. Am J Clin Nutr 69: 1117-1122.
208. Diepvens K, Kovacs EM, Nijs IM, Vogels N, Westerterp-Plantenga MS (2005) Effect of green tea on resting energy expenditure and substrate oxidation during weight loss in overweight females. Br J Nutr 94: 1026-1034.
209. Westerp-Plantenga MS, Lejeune MPGM, E.M.R K (2005) Body weight loss and weight management in relation to habitual caffeine intake and green tea supplementation. Obesity Research 13.
210. Achten J, Jeukendrup AE (2004) Optimizing fat oxidation through exercise and diet. Nutrition 20: 716-727.
211. Dean S, Braakhuis A, Paton C (2009) The effects of EGCG on fat oxidation and endurance performance in male cyclists. Int J Sport Nutr Exerc Metab 19: 624-644.
212. Eichenberger P, Colombani PC, Mettler S (2009) Effects of 3-week consumption of green tea extracts on whole-body metabolism during cycling exercise in endurance-trained men. Int J Vitam Nutr Res 79: 24-33.
213. Maki KC, Reeves MS, Farmer M, Yasunaga K, Matsuo N, et al. (2009) Green tea catechin consumption enhances exercise-induced abdominal fat loss in overweight and obese adults. J Nutr 139: 264-270.
214. Shimotoyodome A, Haramizu S, Inaba M, Murase T, Tokimitsu I (2005) Exercise and green tea extract stimulate fat oxidation and prevent obesity in mice. Med Sci Sports Exerc 37: 1884-1892.
215. Murase T, Haramizu S, Shimotoyodome A, Tokimitsu I, Hase T (2006) Green tea extract improves running endurance in mice by stimulating lipid utilization during exercise. Am J Physiol Regul Integr Comp Physiol 290: R1550-1556.
216. Murase T, Haramizu S, Ota N, Hase T (2008) Tea catechin ingestion combined with habitual exercise suppresses the aging-associated decline in physical performance in senescence-accelerated mice. Am J Physiol Regul Integr Comp Physiol 295: R281-289.
217. Ota N, Soga S, Shimotoyodome A, Haramizu S, Inaba M, et al. (2005) Effects of combination of regular exercise and tea catechins intake on energy expenditure in humans. Journal of Health Science 51: 233-236.
218. Ichinose T, Nomura S, Someya Y, Akimoto S, Tachiyashiki K, et al. (2011) Effect of endurance training supplemented with green tea extract on substrate metabolism during exercise in humans. Scand J Med Sci Sports 21: 598-605.
219. Mannisto PT, Kaakkola S (1999) Catechol-O-methyltransferase (COMT): biochemistry, molecular biology, pharmacology, and clinical efficacy of the new selective COMT inhibitors. Pharmacological reviews 51: 593-628.
220. Borchardt RT, Huber JA (1975) Catechol O-methyltransferase. 5. Structure-activity relationships for inhibition by flavonoids. J Med Chem 18: 120-122.
221. Goldstein DS, Eisenhofer G, Kopin IJ (2003) Sources and significance of plasma levels of catechols and their metabolites in humans. The Journal of pharmacology and experimental therapeutics 305: 800-811.
222. Dulloo AG, Seydoux J, Girardier L, Chantre P, Vandermander J (2000) Green tea and thermogenesis: interactions between catechin-polyphenols, caffeine and sympathetic activity. Int J Obes Relat Metab Disord 24: 252-258.
223. Chen D, Wang CY, Lambert JD, Ai N, Welsh WJ, et al. (2005) Inhibition of human liver catechol-O-methyltransferase by tea catechins and their metabolites: structure-activity relationship and molecular-modeling studies. Biochem Pharmacol 69: 1523-1531.
224. Zhu BT (2002) Catechol-O-Methyltransferase (COMT)-mediated methylation metabolism of endogenous bioactive catechols and modulation by endobiotics and xenobiotics: importance in pathophysiology and pathogenesis. Current drug metabolism 3: 321-349.

225. Zhu BT, Patel UK, Cai MX, Lee AJ, Conney AH (2001) Rapid conversion of tea catechins to monomethylated products by rat liver cytosolic catechol-O-methyltransferase. Xenobiotica; the fate of foreign compounds in biological systems 31: 879-890.
226. Nagai M, Conney AH, Zhu BT (2004) Strong inhibitory effects of common tea catechins and bioflavonoids on the O-methylation of catechol estrogens catalyzed by human liver cytosolic catechol-O-methyltransferase. Drug Metab Dispos 32: 497-504.
227. Klemetsdal B, Straume B, Giverhaug T, Aarbakke J (1994) Low catechol-O-methyltransferase activity in a Saami population. Eur J Clin Pharmacol 46: 231-235.
228. McLeod HL, Fang L, Luo X, Scott EP, Evans WE (1994) Ethnic differences in erythrocyte catechol-O-methyltransferase activity in black and white Americans. J Pharmacol Exp Ther 270: 26-29.
229. Leijten PA, van Breemen C (1984) The effects of caffeine on the noradrenaline-sensitive calcium store in rabbit aorta. J Physiol 357: 327-339.
230. Stralfors P, Belfrage P (1983) Phosphorylation of hormone-sensitive lipase by cyclic AMP-dependent protein kinase. J Biol Chem 258: 15146-15152.
231. Sale C, Harris RC, Delves S, Corbett J (2006) Metabolic and physiological effects of ingesting extracts of bitter orange, green tea and guarana at rest and during treadmill walking in overweight males. Int J Obes (Lond) 30: 764-773.
232. Lee MS, Kim CT, Kim Y (2009) Green tea (-)-epigallocatechin-3-gallate reduces body weight with regulation of multiple genes expression in adipose tissue of diet-induced obese mice. Ann Nutr Metab 54: 151-157.
233. Chen N, Bezzina R, Hinch E, Lewandowski PA, Cameron-Smith D, et al. (2009) Green tea, black tea, and epigallocatechin modify body composition, improve glucose tolerance, and differentially alter metabolic gene expression in rats fed a high-fat diet. Nutr Res 29: 784-793.
234. Wolfram S, Raederstorff D, Wang Y, Teixeira SR, Elste V, et al. (2005) TEAVIGO (epigallocatechin gallate) supplementation prevents obesity in rodents by reducing adipose tissue mass. Ann Nutr Metab 49: 54-63.
235. Kim HJ, Jeon SM, Lee MK, Jung UJ, Shin SK, et al. (2009) Antilipogenic effect of green tea extract in C57BL/6J-Lep ob/ob mice. Phytother Res 23: 467-471.
236. Sae-Tan S, Grove KA, Kennett MJ, Lambert JD (2011) (-)-Epigallocatechin-3-gallate increases the expression of genes related to fat oxidation in the skeletal muscle of high fat-fed mice. Food & function 2: 111-116.
237. Friedrich M, Petzke KJ, Raederstorff D, Wolfram S, Klaus S (2011) Acute effects of epigallocatechin gallate from green tea on oxidation and tissue incorporation of dietary lipids in mice fed a high-fat diet. International journal of obesity.
238. Murase T, Haramizu S, Shimotoyodome A, Nagasawa A, Tokimitsu I (2005) Green tea extract improves endurance capacity and increases muscle lipid oxidation in mice. Am J Physiol Regul Integr Comp Physiol 288: R708-715.
239. Danesi F, Di Nunzio M, Boschetti E, Bordoni A (2009) Green tea extract selectively activates peroxisome proliferator-activated receptor beta/delta in cultured cardiomyocytes. Br J Nutr 101: 1736-1739.
240. Lee K (2004) Transactivation of peroxisome proliferator-activated receptor alpha by green tea extracts. J Vet Sci 5: 325-330.
241. Murase T, Haramizu S, Shimotoyodome A, Tokimitsu I (2006) Reduction of diet-induced obesity by a combination of tea-catechin intake and regular swimming. Int J Obes (Lond) 30: 561-568.
242. Li RW, Douglas TD, Maiyoh GK, Adeli K, Theriault AG (2006) Green tea leaf extract improves lipid and glucose homeostasis in a fructose-fed insulin-resistant hamster model. J Ethnopharmacol 104: 24-31.
243. Serisier S, Leray V, Poudroux W, Magot T, Ouguerram K, et al. (2008) Effects of green tea on insulin sensitivity, lipid profile and expression of PPARalpha and PPARgamma and their target genes in obese dogs. Br J Nutr 99: 1208-1216.

244. Qin B, Polansky MM, Harry D, Anderson RA Green tea polyphenols improve cardiac muscle mRNA and protein levels of signal pathways related to insulin and lipid metabolism and inflammation in insulin-resistant rats. Mol Nutr Food Res 54 Suppl 1: S14-23.
245. Jeukendrup AE, Saris WH, Wagenmakers AJ (1998) Fat metabolism during exercise: a review--part II: regulation of metabolism and the effects of training. Int J Sports Med 19: 293-302.
246. Kiens B (2006) Skeletal muscle lipid metabolism in exercise and insulin resistance. Physiological reviews 86: 205-243.
247. Haramizu S, Ota N, Hase T, Murase T (2011) Catechins attenuate eccentric exercise-induced inflammation and loss of force production in muscle in senescence-accelerated mice. J Appl Physiol
248. Schneider K, Oltmanns J, Hassauer M (2004) Allometric principles for interspecies extrapolation in toxicological risk assessment--empirical investigations. Regulatory toxicology and pharmacology : RTP 39: 334-347.
249. Halliday TM, Peterson NJ, Thomas JJ, Kleppinger K, Hollis BW, et al. (2011) Vitamin D status relative to diet, lifestyle, injury, and illness in college athletes. Med Sci Sports Exerc 43: 335-343.
250. Hamilton B, Grantham J, Racinais S, Chalabi H (2010) Vitamin D deficiency is endemic in Middle Eastern sportsmen. Public health nutrition 13: 1528-1534.
251. Constantini NW, Arieli R, Chodick G, Dubnov-Raz G (2010) High prevalence of vitamin D insufficiency in athletes and dancers. Clin J Sport Med 20: 368-371.
252. Willis KS, Peterson NJ, Larson-Meyer DE (2008) Should we be concerned about the vitamin D status of athletes? Int J Sport Nutr Exerc Metab 18: 204-224.
253. Braun H, Koehler K, Geyer H, Kleiner J, Mester J, et al. (2009) Dietary supplement use among elite young German athletes. Int J Sport Nutr Exerc Metab 19: 97-109.
254. Knez WL, Peake JM (2010) The prevalence of vitamin supplementation in ultraendurance triathletes. Int J Sport Nutr Exerc Metab 20: 507-514.
255. Webb AR, Holick MF (1988) The role of sunlight in the cutaneous production of vitamin D3. Annual review of nutrition 8: 375-399.
256. Holick MF (1994) McCollum Award Lecture, 1994: vitamin D--new horizons for the 21st century. Am J Clin Nutr 60: 619-630.
257. Tian XQ, Chen TC, Matsuoka LY, Wortsman J, Holick MF (1993) Kinetic and thermodynamic studies of the conversion of previtamin D3 to vitamin D3 in human skin. The Journal of biological chemistry 268: 14888-14892.
258. Holick MF (2007) Vitamin D deficiency. N Engl J Med 357: 266-281.
259. Holick MF, Matsuoka LY, Wortsman J (1989) Age, vitamin D, and solar ultraviolet. Lancet 2: 1104-1105.
260. Holick MF, Chen TC (2008) Vitamin D deficiency: a worldwide problem with health consequences. The Am J Clin Nutr 87: 1080S-1086S.
261. Tripkovic L, Lambert H, Hart K, Smith CP, Bucca G, et al. (2012) Comparison of vitamin D2 and vitamin D3 supplementation in raising serum 25-hydroxyvitamin D status: a systematic review and meta-analysis. The Am J Clin Nutr 95: 1357-1364.
262. Hypponen E, Power C (2007) Hypovitaminosis D in British adults at age 45 y: nationwide cohort study of dietary and lifestyle predictors. Am J Clin Nutr 85: 860-868.
263. Mawer EB, Backhouse J, Holman CA, Lumb GA, Stanbury SW (1972) The distribution and storage of vitamin D and its metabolites in human tissues. Clinical science 43: 413-431.
264. Heaney RP, Horst RL, Cullen DM, Armas LA (2009) Vitamin D3 distribution and status in the body. Journal of the American College of Nutrition 28: 252-256.
265. (2010) IOM Report wwwiomedu/Reports/2010/Dietary-Reference-Intakes-for-Calcium-and-Vitamin-D/Report-Briefaspx.
266. Ashwell M, Stone EM, Stolte H, Cashman KD, Macdonald H, et al. (2010) UK Food Standards Agency Workshop Report: an investigation of the relative contributions of diet and sunlight to vitamin D status. The British journal of nutrition 104: 603-611.

267. Holick MF, Binkley NC, Bischoff-Ferrari HA, Gordon CM, Hanley DA, et al. (2011) Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society clinical practice guideline. The Journal of clinical endocrinology and metabolism 96: 1911-1930.
268. Gillie O (2010) Sunlight robbery: a critique of public health policy on vitamin D in the UK. Molecular nutrition & food research 54: 1148-1163.
269. Girgis CM, Clifton-Bligh RJ, Hamrick MW, Holick MF, Gunton JE (2012) The Roles of Vitamin D in Skeletal Muscle: Form, Function, and Metabolism. Endocrine reviews.
270. Priemel M, von Domarus C, Klatte TO, Kessler S, Schlie J, et al. (2010) Bone mineralization defects and vitamin D deficiency: histomorphometric analysis of iliac crest bone biopsies and circulating 25-hydroxyvitamin D in 675 patients. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research 25: 305-312.
271. Holick MF (2012) Evidence-based D-bate on health benefits of vitamin D revisited. Dermatoendocrinol 4: 183-190.
272. Holick MF (2011) The D-batable parathyroid hormone plateau. Am J Med 124: 1095-1096.
273. Saliba W, Lavi I, Rennert HS, Rennert G (2012) Vitamin D status in primary hyperparathyroidism. Eur J Intern Med 23: 88-92.
274. Dawson-Hughes B, Heaney RP, Holick MF, Lips P, Meunier PJ, et al. (2005) Estimates of optimal vitamin D status. Osteoporos Int 16: 713-716.
275. Cauley JA, Lacroix AZ, Wu L, Horwitz M, Danielson ME, et al. (2008) Serum 25-hydroxyvitamin D concentrations and risk for hip fractures. Ann Intern Med 149: 242-250.
276. Cauley JA, Parimi N, Ensrud KE, Bauer DC, Cawthon PM, et al. (2010) Serum 25-hydroxyvitamin D and the risk of hip and nonspine fractures in older men. J Bone Miner Res 25: 545-553.
277. Looker AC, Mussolino ME (2008) Serum 25-hydroxyvitamin D and hip fracture risk in older U.S. white adults. J Bone Miner Res 23: 143-150.
278. Melhus H, Snellman G, Gedeborg R, Byberg L, Berglund L, et al. (2010) Plasma 25-hydroxyvitamin D levels and fracture risk in a community-based cohort of elderly men in Sweden. J Clin Endocrinol Metab 95: 2637-2645.
279. Gerdhem P, Ringsberg KA, Obrant KJ, Akesson K (2005) Association between 25-hydroxy vitamin D levels, physical activity, muscle strength and fractures in the prospective population-based OPRA Study of Elderly Women. Osteoporos Int 16: 1425-1431.
280. Sai AJ, Walters RW, Fang X, Gallagher JC (2011) Relationship between vitamin D, parathyroid hormone, and bone health. J Clin Endocrinol Metab 96: E436-446.
281. Bischoff-Ferrari HA, Dawson-Hughes B, Willett WC, Staehelin HB, Bazemore MG, et al. (2004) Effect of Vitamin D on falls: a meta-analysis. JAMA 291: 1999-2006.
282. Schott GD, Wills MR (1976) Muscle weakness in osteomalacia. Lancet 1: 626-629.
283. Ziambaras K, Dagogo-Jack S (1997) Reversible muscle weakness in patients with vitamin D deficiency. West J Med 167: 435-439.
284. Irani PF (1976) Electromyography in nutritional osteomalacic myopathy. J Neurol Neurosurg Psychiatry 39: 686-693.
285. Peacock M, Liu G, Carey M, McClintock R, Ambrosius W, et al. (2000) Effect of calcium or 25OH vitamin D3 dietary supplementation on bone loss at the hip in men and women over the age of 60. J Clin Endocrinol Metab 85: 3011-3019.
286. Marsden CD, Reynolds EH, Parsons V, Harris R, Duchen L (1973) Myopathy associated with anticonvulsant osteomalacia. British medical journal 4: 526-527.
287. Prineas JW, Mason AS, Henson RA (1965) Myopathy in Metabolic Bone Disease. British medical journal 1: 1034-1036.
288. Floyd M, Ayyar DR, Barwick DD, Hudgson P, Weightman D (1974) Myopathy in chronic renal failure. The Quarterly journal of medicine 43: 509-524.
289. Tague SE, Clarke GL, Winter MK, McCarson KE, Wright DE, et al. (2011) Vitamin D deficiency promotes skeletal muscle hypersensitivity and sensory hyperinnervation. The

Journal of neuroscience : the official journal of the Society for Neuroscience 31: 13728-13738.
290. Glerup H, Mikkelsen K, Poulsen L, Hass E, Overbeck S, et al. (2000) Hypovitaminosis D myopathy without biochemical signs of osteomalacic bone involvement. Calcif Tissue Int 66: 419-424.
291. Curry OB, Basten JF, Francis MJ, Smith R (1974) Calcium uptake by sarcoplasmic reticulum of muscle from vitamin D-deficient rabbits. Nature 249: 83-84.
292. Pleasure D, Wyszynski B, Sumner A, Schotland D, Feldman B, et al. (1979) Skeletal muscle calcium metabolism and contractile force in vitamin D-deficient chicks. J Clin Invest 64: 1157-1167.
293. Sinha A, Hollingsworth KG, Ball S, Cheetham T (2013) Improving the Vitamin D Status of Vitamin D Deficient Adults Is Associated With Improved Mitochondrial Oxidative Function in Skeletal Muscle. J Clin Endocrinol Metab.
294. Zittermann A (2003) Vitamin D in preventive medicine: are we ignoring the evidence? Br J Nutr 89: 552-572.
295. Hamilton B (2010) Vitamin D and human skeletal muscle. Scand J Med Sci Sports 20: 182-190.
296. Sato Y, Iwamoto J, Kanoko T, Satoh K (2005) Low-dose vitamin D prevents muscular atrophy and reduces falls and hip fractures in women after stroke: a randomized controlled trial. Cerebrovasc Dis 20: 187-192.
297. Sorensen OH, Lund B, Saltin B, Andersen RB, Hjorth L, et al. (1979) Myopathy in bone loss of ageing: improvement by treatment with 1 alpha-hydroxycholecalciferol and calcium. Clinical science 56: 157-161.
298. Visser M, Deeg DJ, Lips P (2003) Low vitamin D and high parathyroid hormone levels as determinants of loss of muscle strength and muscle mass (sarcopenia): the Longitudinal Aging Study Amsterdam. J Clin Endocrinol Metab 88: 5766-5772.
299. Gilsanz V, Kremer A, Mo AO, Wren TA, Kremer R (2010) Vitamin D status and its relation to muscle mass and muscle fat in young women. J Clin Endocrinol Metab 95: 1595-1601.
300. Wicherts IS, van Schoor NM, Boeke AJ, Visser M, Deeg DJ, et al. (2007) Vitamin D status predicts physical performance and its decline in older persons. J Clin Endocrinol Metab 92: 2058-2065.
301. Bischoff-Ferrari HA, Dietrich T, Orav EJ, Hu FB, Zhang Y, et al. (2004) Higher 25-hydroxyvitamin D concentrations are associated with better lower-extremity function in both active and inactive persons aged > or =60 y. Am J Clin Nutr 80: 752-758.
302. Bischoff HA, Stahelin HB, Urscheler N, Ehrsam R, Vonthein R, et al. (1999) Muscle strength in the elderly: its relation to vitamin D metabolites. Arch Phys Med Rehabil 80: 54-58.
303. Chan R, Chan D, Woo J, Ohlsson C, Mellstrom D, et al. (2012) Not all elderly people benefit from vitamin D supplementation with respect to physical function: results from the Osteoporotic Fractures in Men Study, Hong Kong. Journal of the American Geriatrics Society 60: 290-295.
304. Ceglia L, Chiu GR, Harris SS, Araujo AB (2011) Serum 25-hydroxyvitamin D concentration and physical function in adult men. Clin Endocrinol (Oxf) 74: 370-376.
305. Annweiler C, Beauchet O, Berrut G, Fantino B, Bonnefoy M, et al. (2009) Is there an association between serum 25-hydroxyvitamin D concentration and muscle strength among older women? Results from baseline assessment of the EPIDOS study. J Nutr Health Aging 13: 90-95.
306. Marantes I, Achenbach SJ, Atkinson EJ, Khosla S, Melton LJ, 3rd, et al. (2011) Is vitamin D a determinant of muscle mass and strength? Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research 26: 2860-2871.
307. Allali F, El Aichaoui S, Khazani H, Benyahia B, Saoud B, et al. (2009) High prevalence of hypovitaminosis D in Morocco: relationship to lifestyle, physical performance, bone markers, and bone mineral density. Semin Arthritis Rheum 38: 444-451.

308. Houston DK, Tooze JA, Hausman DB, Johnson MA, Nicklas BJ, et al. (2011) Change in 25-hydroxyvitamin d and physical performance in older adults. J Gerontol A Biol Sci Med Sci 66: 430-436.
309. Pfeifer M, Begerow B, Minne HW, Abrams C, Nachtigall D, et al. (2000) Effects of a short-term vitamin D and calcium supplementation on body sway and secondary hyperparathyroidism in elderly women. J Bone Miner Res 15: 1113-1118.
310. Pfeifer M, Begerow B, Minne HW, Suppan K, Fahrleitner-Pammer A, et al. (2009) Effects of a long-term vitamin D and calcium supplementation on falls and parameters of muscle function in community-dwelling older individuals. Osteoporosis international : a journal established as result of cooperation between the European Foundation for Osteoporosis and the National Osteoporosis Foundation of the USA 20: 315-322.
311. Bischoff HA, Stahelin HB, Dick W, Akos R, Knecht M, et al. (2003) Effects of vitamin D and calcium supplementation on falls: a randomized controlled trial. J Bone Miner Res 18: 343-351.
312. Dhesi JK, Jackson SH, Bearne LM, Moniz C, Hurley MV, et al. (2004) Vitamin D supplementation improves neuromuscular function in older people who fall. Age Ageing 33: 589-595.
313. Kenny AM, Biskup B, Robbins B, Marcella G, Burleson JA (2003) Effects of vitamin D supplementation on strength, physical function, and health perception in older, community-dwelling men. J Am Geriatr Soc 51: 1762-1767.
314. Latham NK, Anderson CS, Lee A, Bennett DA, Moseley A, et al. (2003) A randomized, controlled trial of quadriceps resistance exercise and vitamin D in frail older people: the Frailty Interventions Trial in Elderly Subjects (FITNESS). J Am Geriatr Soc 51: 291-299.
315. Brunner RL, Cochrane B, Jackson RD, Larson J, Lewis C, et al. (2008) Calcium, vitamin D supplementation, and physical function in the Women's Health Initiative. J Am Diet Assoc 108: 1472-1479.
316. Lips P, Binkley N, Pfeifer M, Recker R, Samanta S, et al. (2010) Once-weekly dose of 8400 IU vitamin D(3) compared with placebo: effects on neuromuscular function and tolerability in older adults with vitamin D insufficiency. Am J Clin Nutr 91: 985-991.
317. Gupta R, Sharma U, Gupta N, Kalaivani M, Singh U, et al. (2010) Effect of cholecalciferol and calcium supplementation on muscle strength and energy metabolism in vitamin D deficient Asian Indians: A randomized controlled trial. Clin Endocrinol (Oxf).
318. Kukuljan S, Nowson CA, Bass SL, Sanders K, Nicholson GC, et al. (2009) Effects of a multi-component exercise program and calcium-vitamin-D3-fortified milk on bone mineral density in older men: a randomised controlled trial. Osteoporosis international : a journal established as result of cooperation between the European Foundation for Osteoporosis and the National Osteoporosis Foundation of the USA 20: 1241-1251.
319. Witham MD, Crighton LJ, Gillespie ND, Struthers AD, McMurdo ME (2010) The effects of vitamin D supplementation on physical function and quality of life in older patients with heart failure: a randomized controlled trial. Circulation Heart failure 3: 195-201.
320. Grady D, Halloran B, Cummings S, Leveille S, Wells L, et al. (1991) 1,25-Dihydroxyvitamin D3 and muscle strength in the elderly: a randomized controlled trial. J Clin Endocrinol Metab 73: 1111-1117.
321. Stockton KA, Mengersen K, Paratz JD, Kandiah D, Bennell KL (2011) Effect of vitamin D supplementation on muscle strength: a systematic review and meta-analysis. Osteoporosis international : a journal established as result of cooperation between the European Foundation for Osteoporosis and the National Osteoporosis Foundation of the USA 22: 859-871.
322. Ward KA, Das G, Berry JL, Roberts SA, Rawer R, et al. (2009) Vitamin D status and muscle function in post-menarchal adolescent girls. J Clin Endocrinol Metab 94: 559-563.
323. Foo LH, Zhang Q, Zhu K, Ma G, Hu X, et al. (2009) Low vitamin D status has an adverse influence on bone mass, bone turnover, and muscle strength in Chinese adolescent girls. J Nutr 139: 1002-1007.

324. Ardestani A, Parker B, Mathur S, Clarkson P, Pescatello LS, et al. (2011) Relation of vitamin D level to maximal oxygen uptake in adults. The American journal of cardiology 107: 1246-1249.
325. Anagnostis P, Karras S, Goulis DG (2013) Vitamin D in human reproduction: a narrative review. Int J Clin Pract 67: 225-235.
326. Smith RP, Coward RM, Kovac JR, Lipshultz LI (2013) The evidence for seasonal variations of testosterone in men. Maturitas.
327. Mauras N (2001) Growth hormone and sex steroids. Interactions in puberty. Endocrinol Metab Clin North Am 30: 529-544.
328. Maggio M, Lauretani F, Ceda GP (2013) Sex hormones and sarcopenia in older persons. Curr Opin Clin Nutr Metab Care 16: 3-13.
329. Smals AG, Kloppenborg PW, Benraad TJ (1976) Circannual cycle in plasma testosterone levels in man. J Clin Endocrinol Metab 42: 979-982.
330. Svartberg J, Barrett-Connor E (2004) Could seasonal variation in testosterone levels in men be related to sleep? Aging Male 7: 205-210.
331. Svartberg J, Jorde R, Sundsfjord J, Bonaa KH, Barrett-Connor E (2003) Seasonal variation of testosterone and waist to hip ratio in men: the Tromso study. J Clin Endocrinol Metab 88: 3099-3104.
332. Blomberg Jensen M, Bjerrum PJ, Jessen TE, Nielsen JE, Joensen UN, et al. (2011) Vitamin D is positively associated with sperm motility and increases intracellular calcium in human spermatozoa. Hum Reprod 26: 1307-1317.
333. Wehr E, Pilz S, Boehm BO, Marz W, Obermayer-Pietsch B (2010) Association of vitamin D status with serum androgen levels in men. Clin Endocrinol (Oxf) 73: 243-248.
334. Nimptsch K, Platz EA, Willett WC, Giovannucci E (2012) Association between plasma 25-OH vitamin D and testosterone levels in men. Clin Endocrinol (Oxf) 77: 106-112.
335. Pilz S, Frisch S, Koertke H, Kuhn J, Dreier J, et al. (2011) Effect of vitamin D supplementation on testosterone levels in men. Horm Metab Res 43: 223-225.
336. Bischoff-Ferrari HA, Orav EJ, Dawson-Hughes B (2008) Additive benefit of higher testosterone levels and vitamin D plus calcium supplementation in regard to fall risk reduction among older men and women. Osteoporos Int 19: 1307-1314.
337. West DW, Phillips SM (2010) Anabolic processes in human skeletal muscle: restoring the identities of growth hormone and testosterone. Phys Sportsmed 38: 97-104.
338. West DW, Kujbida GW, Moore DR, Atherton P, Burd NA, et al. (2009) Resistance exercise-induced increases in putative anabolic hormones do not enhance muscle protein synthesis or intracellular signalling in young men. J Physiol 587: 5239-5247.
339. West DW, Burd NA, Tang JE, Moore DR, Staples AW, et al. (2010) Elevations in ostensibly anabolic hormones with resistance exercise enhance neither training-induced muscle hypertrophy nor strength of the elbow flexors. J Appl Physiol 108: 60-67.
340. Heaney RP, Holick MF (2011) Why the IOM recommendations for vitamin D are deficient. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research 26: 455-457.
341. O'Brien MA, Jackson MW (2012) Vitamin D and the immune system: Beyond rickets. Veterinary journal.
342. Walsh NP, Gleeson M, Pyne DB, Nieman DC, Dhabhar FS, et al. (2011) Position statement. Part two: Maintaining immune health. Exerc Immunol Rev 17: 64-103.
343. Gleeson M, McFarlin B, Flynn M (2006) Exercise and Toll-like receptors. Exerc Immunol Rev 12: 34-53.
344. Lancaster GI, Khan Q, Drysdale P, Wallace F, Jeukendrup AE, et al. (2005) The physiological regulation of toll-like receptor expression and function in humans. J Physiol 563: 945-955.
345. Liu PT, Stenger S, Li H, Wenzel L, Tan BH, et al. (2006) Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response. Science 311: 1770-1773.

346. Cannell JJ, Vieth R, Umhau JC, Holick MF, Grant WB, et al. (2006) Epidemic influenza and vitamin D. Epidemiology and infection 134: 1129-1140.
347. Aloia JF, Li-Ng M (2007) Re: epidemic influenza and vitamin D. Epidemiology and infection 135: 1095-1096; author reply 1097-1098.
348. Cashman KD, Hill TR, Lucey AJ, Taylor N, Seamans KM, et al. (2008) Estimation of the dietary requirement for vitamin D in healthy adults. Am J Clin Nutr 88: 1535-1542.
349. Coyle EF, Jeukendrup AE, Oseto MC, Hodgkinson BJ, Zderic TW (2001) Low-fat diet alters intramuscular substrates and reduces lipolysis and fat oxidation during exercise. American journal of physiology Endocrinology and metabolism 280: E391-398.
350. Coyle EF, Jeukendrup AE, Wagenmakers AJ, Saris WH (1997) Fatty acid oxidation is directly regulated by carbohydrate metabolism during exercise. American Journal of Physiology 273: E268-275.
351. Shepherd SO, Cocks M, Tipton KD, Ranasinghe AM, Barker TA, et al. (2012) Preferential utilization of perilipin 2-associated intramuscular triglycerides during 1 h of moderate-intensity endurance-type exercise. Experimental physiology 97: 970-980.
352. Muldowney S, Lucey AJ, Hill TR, Seamans KM, Taylor N, et al. (2012) Incremental Cholecalciferol Supplementation up to 15 mug/d Throughout Winter at 51-55{degrees} N Has No Effect on Biomarkers of Cardiovascular Risk in Healthy Young and Older Adults. The Journal of nutrition 142: 1519-1525.
353. Coggan AR, Raguso CA, Williams BD, Sidossis LS, Gastaldelli A (1995) Glucose kinetics during high-intensity exercise in endurance-trained and untrained humans. J Appl Physiol 78: 1203-1207.
354. Heaney RP, Vieth R, Hollis BW (2011) Vitamin D efficacy and safety. Archives of internal medicine 171: 266; author reply 267.
355. Vieth R (2007) Vitamin D toxicity, policy, and science. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research 22 Suppl 2: V64-68.
356. Vieth R, Chan PC, MacFarlane GD (2001) Efficacy and safety of vitamin D3 intake exceeding the lowest observed adverse effect level. Am J Clin Nutr 73: 288-294.
357. Cashman KD, Muldowney S, McNulty B, Nugent A, Fitzgerald AP, et al. (2012) Vitamin D status of Irish adults: findings from the National Adult Nutrition Survey. The British journal of nutrition 10: 1-9.
358. Maimoun L, Manetta J, Couret I, Dupuy AM, Mariano-Goulart D, et al. (2006) The intensity level of physical exercise and the bone metabolism response. Int J Sports Med 27: 105-111.
359. Lovell G (2008) Vitamin D status of females in an elite gymnastics program. Clin J Sport Med 18: 159-161.
360. Bescos Garcia R, Rodriguez Guisado FA (2011) Low levels of vitamin D in professional basketball players after wintertime: relationship with dietary intake of vitamin D and calcium. Nutr Hosp 26: 945-951.
361. Close GL, Russell J, Cobley JN, Owens DJ, Wilson G, et al. (2012) Assessment of vitamin D concentration in non-supplemented professional athletes and healthy adults during the winter months in the UK: implications for skeletal muscle function. Journal of sports sciences.
362. Lehtonen-Veromaa M, Mottonen T, Irjala K, Karkkainen M, Lamberg-Allardt C, et al. (1999) Vitamin D intake is low and hypovitaminosis D common in healthy 9- to 15-year-old Finnish girls. European journal of clinical nutrition 53: 746-751.
363. Morton JP, Iqbal Z, Drust B, Burgess D, Close GL, et al. (2012) Seasonal variation in vitamin D status in professional soccer players of the English Premier League. Applied physiology, nutrition, and metabolism = Physiologie appliquee, nutrition et metabolisme 37: 798-802.
364. Pollock N, Dijkstra P, Chakraverty R, Hamilton B (2012) Low 25(OH) vitamin D concentrations in international UK track and field athletes. S Afr J SM 24: 55-59.

365. Barrack MT, Van Loan MD, Rauh MJ, Nichols JF (2010) Physiologic and behavioral indicators of energy deficiency in female adolescent runners with elevated bone turnover. Am J Clin Nutr 92: 652-659.
366. Galan F, Ribas J, Sanchez-Martinez PM, Calero T, Sanchez AB, et al. (2012) Serum 25-hydroxyvitamin D in early autumn to ensure vitamin D sufficiency in mid-winter in professional football players. Clinical nutrition 31: 132-136.
367. Ducher G, Kukuljan S, Hill B, Garnham AP, Nowson CA, et al. (2011) Vitamin D status and musculoskeletal health in adolescent male ballet dancers a pilot study. Journal of dance medicine & science : official publication of the International Association for Dance Medicine & Science 15: 99-107.
368. Close GL, Leckey J, Patterson M, Bradley W, Owens DJ, et al. (2013) The effects of vitamin D3 supplementation on serum total 25[OH]D concentration and physical performance: a randomised dose-response study. Br J Sports Med.
369. Wilson G, Fraser WD, Sharma A, Eubank M, Drust B, et al. (2012) Markers of Bone Health, Renal Function, Liver Function, Anthropometry and Perception of Mood: A Comparison between Flat and National Hunt Jockeys. Int J Sports Med.
370. Svedenhag J, Sjodin B (1985) Physiological characteristics of elite male runners in and off-season. Canadian journal of applied sport sciences 10: 127-133.
371. Hettinger T, Muller EA (1956) [Seasonal course of trainability of musculature.]. Int Z Angew Physiol 16: 90-94.
372. Barker T, Martins TB, Hill HR, Kjeldsberg CR, Henriksen VT, et al. (2012) Different doses of supplemental vitamin D maintain interleukin-5 without altering skeletal muscle strength: a randomized, double-blind, placebo-controlled study in vitamin D sufficient adults. Nutrition & metabolism 9: 16.
373. Bischoff HA, Borchers M, Gudat F, Duermueller U, Theiler R, et al. (2001) In situ detection of 1,25-dihydroxyvitamin D3 receptor in human skeletal muscle tissue. Histochem J 33: 19-24.
374. Bischoff-Ferrari HA, Borchers M, Gudat F, Durmuller U, Stahelin HB, et al. (2004) Vitamin D receptor expression in human muscle tissue decreases with age. J Bone Miner Res 19: 265-269.
375. Adams JS, Hewison M (2012) Extrarenal expression of the 25-hydroxyvitamin D-1-hydroxylase. Archives of biochemistry and biophysics 523: 95-102.
376. Capiati D, Benassati S, Boland RL (2002) 1,25(OH)2-vitamin D3 induces translocation of the vitamin D receptor (VDR) to the plasma membrane in skeletal muscle cells. J Cell Biochem 86: 128-135.
377. Tavera-Mendoza LE, White JH (2007) Cell defenses and the sunshine vitamin. Sci Am 297: 62-65, 68-70, 72.
378. Okuno H, Kishimoto KN, Hatori M, Itoi E (2012) 1alpha,25-dihydroxyvitamin D(3) enhances fast-myosin heavy chain expression in differentiated C2C12 myoblasts. Cell Biol Int 36: 441-447.
379. Srikuea R, Zhang X, Park-Sarge OK, Esser KA (2012) VDR and CYP27B1 are expressed in C2C12 cells and regenerating skeletal muscle: potential role in suppression of myoblast proliferation. American journal of physiology Cell physiology 303: C396-405.
380. Buitrago CG, Arango NS, Boland RL (2012) 1alpha,25(OH)2D3-dependent modulation of Akt in proliferating and differentiating C2C12 skeletal muscle cells. J Cell Biochem 113: 1170-1181.
381. Giuliani DL, Boland RL (1984) Effects of vitamin D3 metabolites on calcium fluxes in intact chicken skeletal muscle and myoblasts cultured in vitro. Calcif Tissue Int 36: 200-205.
382. Drittanti L, de Boland AR, Boland R (1989) Modulation of DNA synthesis in cultured muscle cells by 1,25-dihydroxyvitamin D-3. Biochim Biophys Acta 1014: 112-119.
383. Burne TH, McGrath JJ, Eyles DW, Mackay-Sim A (2005) Behavioural characterization of vitamin D receptor knockout mice. Behav Brain Res 157: 299-308.

384. Endo I, Inoue D, Mitsui T, Umaki Y, Akaike M, et al. (2003) Deletion of vitamin D receptor gene in mice results in abnormal skeletal muscle development with deregulated expression of myoregulatory transcription factors. Endocrinology 144: 5138-5144.
385. Kalueff AV, Lou YR, Laaksi I, Tuohimaa P (2004) Impaired motor performance in mice lacking neurosteroid vitamin D receptors. Brain research bulletin 64: 25-29.
386. Birge SJ, Haddad JG (1975) 25-hydroxycholecalciferol stimulation of muscle metabolism. J Clin Invest 56: 1100-1107.
387. Pointon JJ, Francis MJ, Smith R (1979) Effect of vitamin D deficiency on sarcoplasmic reticulum function and troponin C concentration of rabbit skeletal muscle. Clin Sci (Lond) 57: 257-263.
388. de Boland AR, Albornoz LE, Boland R (1983) The effect of cholecalciferol in vivo on proteins and lipids of skeletal muscle from rachitic chicks. Calcif Tissue Int 35: 798-805.
389. Buitrago C, Boland R (2010) Caveolae and caveolin-1 are implicated in 1alpha,25(OH)2-vitamin D3-dependent modulation of Src, MAPK cascades and VDR localization in skeletal muscle cells. The Journal of steroid biochemistry and molecular biology 121: 169-175.
390. Morelli S, Buitrago C, Boland R, de Boland AR (2001) The stimulation of MAP kinase by 1,25(OH)(2)-vitamin D(3) in skeletal muscle cells is mediated by protein kinase C and calcium. Mol Cell Endocrinol 173: 41-52.
391. Buitrago C, Gonzalez Pardo V, de Boland AR (2002) Nongenomic action of 1 alpha,25(OH)(2)-vitamin D3. Activation of muscle cell PLC gamma through the tyrosine kinase c-Src and PtdIns 3-kinase. Eur J Biochem 269: 2506-2515.
392. Buitrago C, Costabel M, Boland R (2011) PKC and PTPalpha participate in Src activation by 1alpha,25OH2 vitamin D3 in C2C12 skeletal muscle cells. Molecular and cellular endocrinology 339: 81-89.
393. Drummond MJ, Fry CS, Glynn EL, Dreyer HC, Dhanani S, et al. (2009) Rapamycin administration in humans blocks the contraction-induced increase in skeletal muscle protein synthesis. The Journal of physiology 587: 1535-1546.
394. Bodine SC, Stitt TN, Gonzalez M, Kline WO, Stover GL, et al. (2001) Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo. Nat Cell Biol 3: 1014-1019.
395. Fuller JC, Jr., Baier S, Flakoll P, Nissen SL, Abumrad NN, et al. (2011) Vitamin D status affects strength gains in older adults supplemented with a combination of beta-hydroxy-beta-methylbutyrate, arginine, and lysine: a cohort study. JPEN J Parenter Enteral Nutr 35: 757-762.
396. Wassner SJ, Li JB, Sperduto A, Norman ME (1983) Vitamin D Deficiency, hypocalcemia, and increased skeletal muscle degradation in rats. J Clin Invest 72: 102-112.
397. Atherton PJ, Smith K, Etheridge T, Rankin D, Rennie MJ (2010) Distinct anabolic signalling responses to amino acids in C2C12 skeletal muscle cells. Amino acids 38: 1533-1539.
398. Gloth FM, 3rd, Smith CE, Hollis BW, Tobin JD (1995) Functional improvement with vitamin D replenishment in a cohort of frail, vitamin D-deficient older people. J Am Geriatr Soc 43: 1269-1271.
399. Selles J, Boland R (1991) Rapid stimulation of calcium uptake and protein phosphorylation in isolated cardiac muscle by 1,25-dihydroxyvitamin D3. Mol Cell Endocrinol 77: 67-73.
400. Vazquez G, de Boland AR, Boland RL (1998) 1alpha,25-dihydroxy-vitamin-D3-induced store-operated Ca2+ influx in skeletal muscle cells. Modulation by phospholipase c, protein kinase c, and tyrosine kinases. J Biol Chem 273: 33954-33960.
401. Vazquez G, de Boland AR, Boland R (1997) Stimulation of Ca2+ release-activated Ca2+ channels as a potential mechanism involved in non-genomic 1,25(OH)2-vitamin D3-induced Ca2+ entry in skeletal muscle cells. Biochem Biophys Res Commun 239: 562-565.
402. Buitrago CG, Ronda AC, de Boland AR, Boland R (2006) MAP kinases p38 and JNK are activated by the steroid hormone 1alpha,25(OH)2-vitamin D3 in the C2C12 muscle cell line. J Cell Biochem 97: 698-708.

403. Boland RL (2011) VDR activation of intracellular signaling pathways in skeletal muscle. Molecular and cellular endocrinology.

Chapter 2

The Metabolic Response to Green Tea Extract during Rest and Moderate Intensity Exercise.

Published article

Adrian B. Hodgson[†], Rebecca Randell[†], Niels Boon[††], Ursula Garczarek[††], David J. Mela[††], Asker E. Jeukendrup[†], Doris M. Jacobs[††]

J Nutr Biochem. 2013 Jan;24(1):325-34.

A.B.H Conceived and designed the experiment, performed the experiment, interpreted the data and wrote the manuscript, R.K.R Performed the experiment and assisted in the interpretation of the data, N.B Conceived and designed the experiment, U.G. Analysed the data at Metabonomics Health, D.M. Assisted in the interpretation of data and editing of manuscript, A.E.J Conceived and designed the experiment, D.J Interpreted the data, analysed the data at Metabonomics Health

† School of Sport and Exercise Sciences, University of Birmingham, United Kingdom

†† Unilever R&D, Olivier van Noortlaan 120, 3130 AC Vlaardingen, The Netherlands

2.1 Abstract

Background: Green tea catechins have been hypothesized to increase energy expenditure and fat oxidation by inhibiting catechol-O-methyltransferase (COMT) and thus promoting more sustained adrenergic stimulation. Metabolomics may help to clarify the mechanisms underlying their putative physiological effects. The study investigated the effects of seven days ingestion of green tea extract (GTE) on the plasma metabolite profile at rest and during exercise. **Methods**: In a placebo-controlled, double-blind, randomized, parallel study, 27 healthy physically active males consumed either GTE (n= 13, 1200mg catechins, 240 mg caffeine / day) or placebo (n= 14, PLA) drinks for seven days. After consuming a final drink (day 8) participants rested for two hours and then completed 60 minutes moderate intensity cycling exercise (56±4% VO_2max). Blood samples were collected before and during exercise. Plasma was analyzed using untargeted 4-phase metabolite profiling and targeted profiling of catecholamines. **Results:** Using the metabolomic approach we observed that GTE did not enhance adrenergic stimulation (adrenaline and noradrenaline) during rest or exercise. At rest, GTE lead to changes in metabolite concentrations related to fat metabolism (β-hydroxybutyrate), lipolysis (glycerol) and TCA cycle intermediates (citrate) when compared to PLA. GTE during exercise caused reductions in β-hydroxybutyrate concentrations as well as increases in pyruvate, lactate and alanine concentrations when compared to PLA. **Conclusions**: GTE supplementation resulted in marked metabolic differences during rest and exercise. These metabolic differences were not related to the adrenergic system, which questions the *in vivo* relevance of the COMT inhibition mechanism of action for GTE.

2.2 Introduction

The concept of "challenging homeostasis" has been proposed as a novel approach to define biomarkers for nutritional related health [1]. It is thought that responses to a challenge of homeostasis will affect multiple pathways and provide information other than static homeostatic and disease relevant end-point measures. These dynamic responses may be appropriate means to assess effects and mechanisms of nutritional interventions that may remain hidden under the large variation within a healthy population. Exercise is a physiological challenge to homeostasis of the human body. Metabolomics based on gas- or liquid chromatography – mass spectrometry (GC/MS, LC/MS) is capable of measuring the multi-factorial, integrative metabolic responses to an exercise challenge and/or a nutritional intervention or supplement [2-6].

Green tea has been reported to have a number of health promoting effects, including anti-obesity properties [7-10]. These health benefits are generally attributed to its polyphenol content, particularly to catechins that are often enriched in GTE (green tea extract). These are comprised of epigallocatechin gallate (EGCG), epicatechin gallate, and gallocatechin gallate, among others. EGCG is thought to be the most pharmacologically active of the catechins. GTE also contains caffeine. The anti-obesity properties of GTE are attributed to both acute [11] and chronic [7,12] GTE supplementation augmenting energy expenditure (EE) and fat oxidation under resting conditions. However, this has not been consistently reported [13,14]. During moderate intensity exercise, EE is elevated several times when compared to rest, and absolute rates of lipolysis and fat oxidation are 3-10 fold higher. The administration of GTE could at least in theory have an additive effect on fat metabolism above and beyond what is seen with exercise alone. Indeed, it has been observed that fat metabolism is up regulated during exercise following both acute [15] and chronic [16] GTE supplementation. So far, differences in the metabolic effects of GTE between rest and exercise have not been investigated in a single study. It is also currently unknown, whether the effects of GTE on fat metabolism or any other related metabolic effects are more prominent following acute or chronic GTE supplementation.

It has previously been observed that certain catechins in GTE target specific control points of the sympathetic nervous system [17]. EGCG has been suggested to directly inhibit catechol-*O*-methyltransferase (COMT) an enzyme that is responsible for degrading catecholamines (noradrenaline (NA) and adrenaline (A)) [18]. Inhibiting the degrading action of COMT would lead to elevated catecholamine concentrations resulting in greater sympathetic nerve stimulation and thus higher rates of lipolysis. This is thought to elevate fat oxidation and EE at rest and during exercise [10]. The COMT pathway is likely to take effect following acute GTE supplementation. Alternatively, it has been suggested that chronic GTE supplementation may lead to an up- or down-regulation of various proteins and enzymes involved in fat metabolism [19,20]. However, there is no convincing evidence to support the acute or chronic mechanisms of GTE *in vivo*.

Based on the mixed study outcomes on fat metabolism following GTE supplementation, and the lack of mechanistic evidence *in vivo,* the aim of the present study was to examine the effects of 7 days GTE supplementation (1200 mg total catechins and 240 mg caffeine/day) on human metabolism at rest and during moderate intensity exercise (56±4% VO_2(max)). These effects were assessed in human plasma using GC-MS- and LC-MS/MS-based 4-phases metabolite profiling [21]. This comprehensive metabolite profiling approach provided an unbiased and systemic investigation into the multi-factorial metabolic response following GTE supplementation at rest and during exercise. We hypothesized that GTE ingestion for 7 days would induce metabolic changes consistent with increased lipolysis and that these changes could be maintained or enhanced during exercise after a single bolus intake of GTE. In addition, we tested the hypothesis that GTE stimulates the adrenergic system by measuring targeted profiles including various catecholamines.

2.3 Methods and Materials

2.3.1 Study design

2.3.1.1 Participants

27 healthy physically active male participants were recruited for the purposes of the study. Inclusion criteria were: < 4 cups of tea or coffee / day (thus less than ~ 400 mg caffeine / day), exercise 3-5 times / week, 30-90 min / session. In addition, participants had to be 18-55 y and BMI < 30 $kg.m^{-2}$ to be included in the study. Participants were randomly allocated into either a GTE group (n=13, age 22 ± 5 y, weight 77.6 ± 12.0 kg, BMI 24.3 ± 3.0 kg/m^2) or a placebo (PLA) group (n=14, age 22 ± 8 y, weight 78.8 ± 10.2 kg, BMI 24.7 ± 2.7 kg/m^2). All participants gave written informed consent to participate in the study. The study was approved by the University of Birmingham Ethics Committee.

2.3.1.2 Preliminary Testing

One week prior to the first experimental trial, all participants visited the human performance lab for familiarisation, assessment of health and an incremental exercise test using an electronically braked cycle ergometer (Lode Excalibur Sport, Groningen, Netherlands) to volitional exhaustion [22]. Participants started by cycling at 95 *W* for 3 minutes and work rate was increased by 35 W (W_{inc}) every 3 minutes (t_{inc}) until they reached exhaustion. The W_{max} was calculated by using the following equation [23]:

$$W_{max} = W_{out} + (t/t_{inc})\, W_{inc}$$

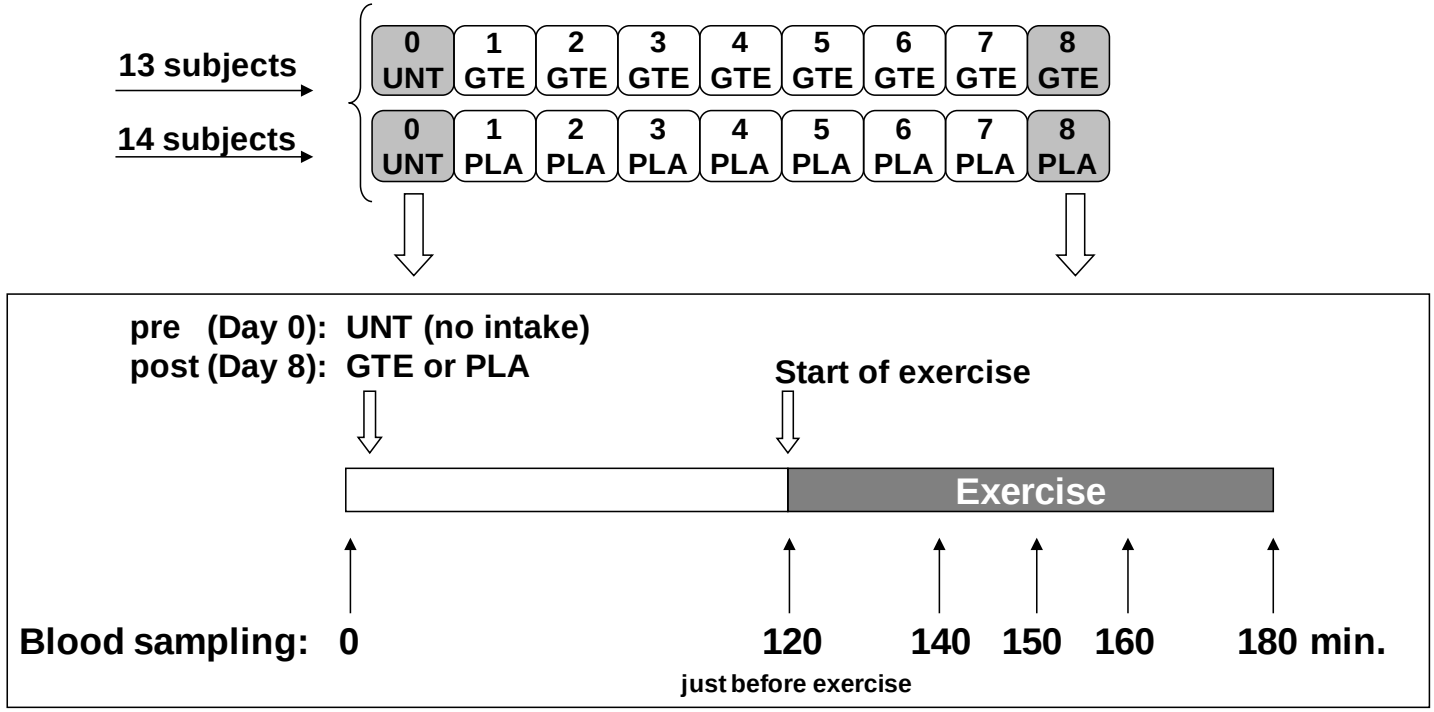
W_{out} is the power output of the last completed stage (in W), and *t* is the time (in minutes) spent in the final stage. W_{max} values were used to determine the workload of 50% W_{max} (~55% VO_2(max)) used in the experimental trials. Respiratory measures of oxygen consumption ($\dot{V}O_2$) and carbon dioxide production ($\dot{V}CO_2$) were assessed using an online gas analysis system (Oxycon Pro, Jaeger). Heart rate (HR) was measured continuously (Polar S625X; Polar Electro Oy, Kempele, Finland). $\dot{V}O_2$ was

considered to be maximal if 2 of the 3 following conditions were met: *1*) a levelling off of ($\dot{V}O_2$) with further increasing workloads; *2*) a HR within 10 beats/minutes of the age predicted maximum (220 bpm - age); and *3*) a respiratory exchange ratio of >1.

2.3.1.3 Experimental Design

The study was designed as a placebo-controlled, double-blind, randomized, parallel study. Subjects in the assigned groups consumed either a GTE drink (1200 mg catechins, 240 mg caffeine) or a PLA drink for seven days (Figure 2.1). On day 0 (D0), all participants arrived at the Human Performance Lab between 0600 and 0800 following a 10 hour overnight fast and 24 hour controlled diet on D0. Upon arrival a flexible 20-gauge Teflon catheter (Venflon; Becton Dickinson, Plymouth, United Kingdom) was then inserted into an antecubital vein. A 3-way stopcock (PVB Medizintechnik, Kirchseean, Germany) was attached to the catheter to allow repeated blood sampling during the trial. A resting blood sample (5 mL) was taken (t=0 min). Participants then rested for 2 hours in a seated position. At the end of the rest period a second resting blood sample (5 mL) was taken (t=120 min). Following this, participants began cycling at 50% W_{max} for 60 minutes. Throughout the exercise blood samples (5 mL) were taken at t=140, 150, 160, 180 minutes (Figure 2.1). The catheter was kept patent by flushing it with 3-4 mL isotonic saline (0.9%; Baxter, Norfolk, United Kingdom) after each blood sample. The experimental trial was then repeated 8 days later. The only difference in the trials on D0 and D8 was the consumption of a single bolus of GTE (600 mg catechins, 120 mg caffeine) or PLA after the collection of the resting blood sample and prior to the resting period.

Figure. 2.1. Study design schematic



2.3.1.4 Diet and Supplement

After the initial trial, participants were randomly assigned to either ingest GTE or PLA for 7 days (Figure 2.1). The GTE and PLA supplements were provided in the form of a drink. Each drink was provided in a 330 mL can. The GTE (Taiyo International, Japan) and PLA compositions are displayed in Table 2.1.

Table 2.1. Content of the GTE and PLA drink

	Treatment	**GTE**	**PLA**
mg / 330 mL can	Caffeine	120.4	10
	GC	55.1	0
	EGC	5.5	0
	C	181.1	0
	EC	47	0
	EGCG	207.5	0
	GCG	31	0
	ECG	25.6	0
	GC	0	0
Total mg / 330 mL can	Catechins	559	0
	Caffeine	120.4	10
Total mg / day	Catechins	1119	0
	Caffeine	240.8	20

Abbreviations: GC, gallocatechin; EGC, epigallocatechin; C, catechin; EC, epicatechin; EGCG, epigallocatechin gallate; GCG, gallocatechingallate; ECG, epicatechingallate; GC, gallocatechin.

Participants were instructed to consume two drinks per day, one drink an hour prior to breakfast and another drink an hour prior to dinner. The supplementation period started on the day following the pre-supplementation trial. Subjects received daily reminders in the form of text messages in the morning and the evening to ensure compliance. Participants returned to the human performance lab on D8 having consumed the drinks for 7 days. The 24 hour period prior to D0 trial participants were asked to record a food diary which was replicated prior to D8 trial.

2.3.1.5 Sample collection

All blood samples were stored on ice for no longer than 35 minutes. Subsequently, plasma was separated by centrifugation (1500xg, 10 minutes, 4°C), aliquoted in 1 mL samples and stored at -80°C.

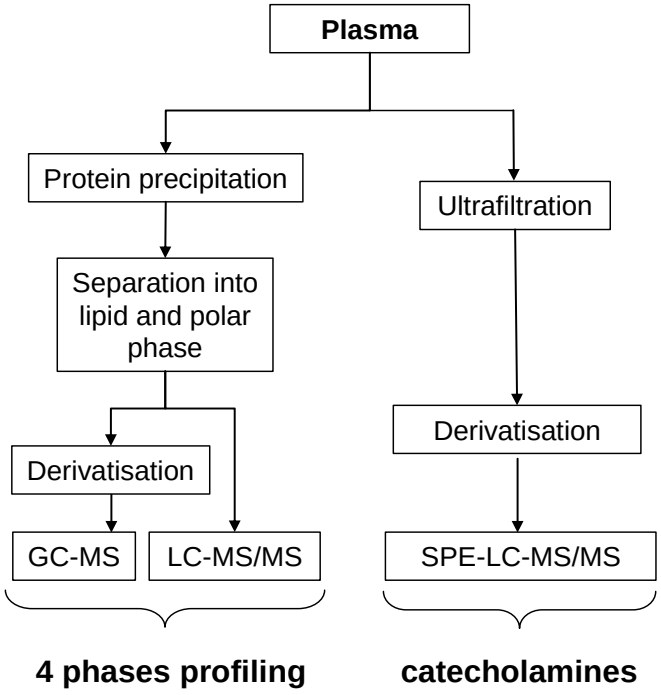
2.3.2 Data acquisition

2.3.2.1 Metabolite Profiling

Four-phase metabolite profiling and quantification of catecholamines (Figure 2.2) were performed on human plasma samples at Metanomics Health GmbH, Berlin, Germany. Three types of mass spectrometry analyses were applied. GC-MS (gas chromatography-mass spectrometry; Agilent 6890 GC coupled to an Agilent 5973 MS-System, Agilent, Waldbronn, Germany) and LC-MS/MS (liquid chromatography-MS/MS; Agilent 1100 HPLC-System (Agilent, Waldbronn, Germany) coupled to an Applied Biosystems API4000 MS/MS-System (Applied Biosystems, Darmstadt, Germany)) were used for broad profiling, as described by van Ravenzwaay *et al.* [24]. SPE-LC-MS/MS (solid phase extraction-LC-MS/MS; Symbiosis Pharma (Spark, Emmen, Netherlands) coupled to an Applied Biosystems API4000 MS/MS-System (Applied Biosystems, Darmstadt, Germany)) was used for the determination of catecholamine concentrations. 228 metabolites fulfilled the quality criteria for relative quantification, and absolute quantification was performed for an additional 10 metabolites. From a total of 238 metabolites, 163 were known metabolites and 75 were not chemically identified with sufficient certainty (i.e. thus considered in the present study to be unknown analytes).

Technical reference samples were measured in parallel with the study samples in order to allow the relative quantification of metabolites in the study samples. These technical reference samples were generated by pooling aliquots of plasma from all study samples. A relative quantification for each metabolite was obtained by normalizing peak intensity in the study samples to the median peak intensity of the corresponding metabolite in the technical reference samples measured in the same batch. The relative quantification were log-transformed before ANOVA analysis and were back transformed (10^x) to obtain the ratios that are presented in the tables below.

Figure 2.2. Sample workflow: 4 phases profiling and platform of catecholamines



2.3.3 Metabolite Profiling by GC-MS and LC-MS/MS

Proteins were removed from plasma samples (60 µl) by precipitation. Subsequently polar and non-polar plasma fractions were separated for both GC-MS and LC-MS/MS analysis by adding water and a mixture of ethanol and dichloromethane. For GC-MS analyses, the non-polar fraction was treated with methanol under acidic conditions to yield the fatty acid methyl esters derived from both free fatty acids and hydrolyzed complex lipids. The polar and non-polar fractions were further derivatized with

O-methyl-hydroxyamine hydrochloride (20 mg/mL in pyridine, 50 µL) to convert oxo-groups to O-methyloximes and subsequently with a silylating agent (MSTFA, 50 µl) before GC-MS analysis [25]. For LC-MS/MS analyses, both fractions were reconstituted in appropriate solvent mixtures. High performance LC (HPLC) was performed by gradient elution using methanol/water/formic acid on reversed phase separation columns. Mass spectrometric detection technology was applied as described in the patent US 7196323, which allows targeted and high sensitivity "Multiple Reaction Monitoring" profiling in parallel to a full screen analysis.

For the lipid phase, the broad profiling technology determines e.g. fatty acid concentrations after acid/methanol treatment which is essential for derivatization preceding GC-MS analysis. As a consequence, complex lipids are hydrolyzed to components of the lipid backbone (i.e. glycerol) and fatty acids. Hence, the concentration of a fatty acid determined by this procedure represents the sum of its occurrence in free and in lipid-bound form. Components of the backbone can be recognized by the term ("lipid fraction ") added to the metabolite name. As an example "glycerol, lipid fraction" represents glycerol liberated from complex lipids – in contrast, "glycerol, polar fraction" represents glycerol which had been present originally in the biological sample. The use of "additional" indicates that quantification can be affected by the co-occurrence of metabolites exhibiting identical characteristics in the analytical methods. Literature data and/or comparison with alternative methods (e.g. LC-MS/MS, GC-MS) suggest that such metabolites are present at minor concentrations only.

Quantification of catecholamines

Catecholamines and their related metabolites were measured by online SPE-LC-MS/MS, as described by Yamada *et al.* [26]. Quantification was performed using stable isotope-labelled standards.

2.3.4 Data analysis

The data set comprised profiles from 318 plasma samples that were analyzed both using multivariate and univariate statistical methods. (Six samples were not considered due to labelling errors.)

Multivariate analysis

Multivariate analysis including PCA [principal component analysis] and PLS-DA [partial least squares/projection to latent structures-discriminant analysis] was performed using SIMCA P+ version 12 software (Umetrics, Umea, Sweden). One subject was completely excluded from the analysis due to abnormal metabolite profiles. For another subject, one specific time point (D0, t=160 min) was discarded. All metabolite data were log-transformed to better match normal distribution. Data were further centred and scaled to unit variance. Scaling to unit variance introduces a common scale for all metabolites independent on their absolute amount of variance. Thereby, the resulting models obtain robustness, i.e., they cannot be dominated by a single or few high-variance metabolites. The explorative unsupervised multivariate analysis method PCA was used for the detection of trends, patterns and groupings among samples and variables. The supervised projection method PLS-DA was used to display the maximum co-variance of metabolic data with a defined Y variable (class, categorical) in the dataset. The cross-validated cumulative Q^2 value was used as a measure of the predictive value of the PLS-DA model. A Q^2 value of 1 indicates maximum predictive power, whereas Q^2 values close to or below 0 indicate a lack of predictive power. As a rule of thumb, models with $Q^2_{cum} > 50\%$ are considered to be of good predictive power in this context. To account for the fact that several samples for each subject were analyzed, cross-validation was performed by the leave-on-subject out method. This ensured that cross-validation was not inappropriately facilitated by the presence of related samples (here: different time point from the same subject) in the test dataset given the usually lower intra-individual variability compared to inter-individual variability.

Univariate analysis

ANOVA analysis was performed using R-software package nlme [27,28]. Treatment factors “UNT” comprised all samples on D0, “GTE” all samples collected on D8 from subjects consuming GTE, and “PLA” all samples collected on D8 from subjects consuming placebo, respectively. The ANOVA mixed-effects model included the categorical fixed effects factors “treatment” (UNT, PLA, GTE) and

"time" (0, 120, 140, 150, 160, 180) and their interaction and random intercept effects for "day" and "subject". The combination of the binary factors "day" (0, 8) and the ternary factor "treatment" (UNT, GTE, PLA) allowed for setting up the following mixed-effects model without third-order interactions. The model specification (in R notation) was:

Fixed effects: metabolite ~ (treatment + time)^2,

Random effects: metabolite ~1 | day / subject.

The correlation between samples of the same subject at different times was handled by a model of type AR(p=1) = ARMA(p=1,q=0) with equidistant time steps time.int (1,2,…6). All study-relevant contrasts were readout in the form of treatment contrasts by selecting different factor reference levels. The t-statistics results of the ANOVA models comprised estimates, standard deviations, t-values, and p-values. Model diagnostics were conducted in four ways to ensure adequateness of model structure: (a) Residuals were inspected visually by scatter plots of standardized residuals versus fitted values: generally, residuals did not correlate with fitted values and showed homogenous variance. (b) Random subject intercepts were tested by t-Test comparing the two study arms GTE and PLA on D0. The number of significant metabolites was on false positive level. (c) Random day effects were negligible relative to other variance components. Accordingly, they do not influence other effect estimates. (Note that the random day effect was introduced only due to technical/algorithmic reasons.) (d) The AR(1) correlation coefficient ("phi") was found to vary in a reasonable range with mean 0,14 and interquartile range from P25%=0.03 to P75%=0.24. The t-statistics results of significantly changed metabolites are summarized in a colour coded format in the tables. The resulting numbers of significantly changed metabolites were evaluated by binominial test to account for false positives due to multiple hypotheses testing (data not shown).

2.4 Results

The 4-phases metabolite profiling using both GC-MS and LC-MS/MS (Figure 2.2) comprised in total of 238 metabolites including 32 amino acids and amino acid derivatives, 11 carbohydrates and related

metabolites, 10 metabolites related to energy metabolism, 3 nucleobases and related metabolites, 10 vitamin cofactors and related metabolites, 12 miscellaneous metabolites, 73 complex lipids, fatty acids and related metabolites, and 75 unknown analytes. In addition, 10 catecholamines and other monoamines were in most instances quantitatively measured using a targeted profiling method.

Initially, multivariate statistics (PCA and PLS-DA) was performed to identify outliers and to get a general idea on the variability of the data. PLS-DA clearly discriminated between exercise and resting conditions (Q^2_{cum}=80.5%). Furthermore, a clear separation was observed between pre- and post–GTE-supplementation. (Q^2_{cum}=51.5% including all time points; Q^2_{cum}=49.2% including time points 140-180 min and subjects of the GTE study arm). Other models discriminating between the GTE and the PLA interventions under resting and exercise conditions, respectively, were not significant indicating that inter-individual differences might dominate the discrimination over treatment and/or exercise. Based on this overall impression, a univariate ANOVA mixed-effects model was calculated and clearly identified statistically significant changes in individual metabolites that could be attributed to the effect induced by exercise or the GTE supplement.

2.4.1 Effect of exercise on human metabolism

Table 2.2 illustrates the metabolite changes induced by moderate intensity exercise in all subjects pre-supplementation (UNT). The changes during exercise (140, 150, 160, 180 min) were compared to resting conditions at the end of the two hour rest period (120 min). They comprised of increases in indicators of glycolysis (lactate and pyruvate), indicators of lipolysis (glycerol), TCA cycle intermediates (citrate, fumarate, malate, succinate), indicators of adenine catabolism (uric acid), accelerated protein synthesis (uridine, pseudouridine), indicators of hormonal acitivity (cortisol, dehyroepinandrosterone sulfate), catecholamines (DOPAC, DOPA, DOPEG, HMPG, 5-HIAA, HVA, noradrenaline), vitamins and related metabolites (4-pyridoxic acid, panthenic acid, cryptoxanthin), certain fatty acids (e.g. arachidonic acid, linoleic acid), certain phospholidids (e.g. choline plasmalogen, lysophosphatidylcholine (C18:0)), certain sphingolipids (e.g. ceramide (C18:1,C24:0),

Table 2.2. ANOVA estimate for effect of exercise on metabolome derived from data from pre-supplementation (UNT).

time / min METABOLITE	140 vs 120	150 vs 120	160 vs 120	180 vs 120
Alanine	1.30	1.33	1.37	1.33
Cysteine (additional: Cystine)	1.07	1.06	1.10	1.11
Methionine	1.05	1.03	1.08	1.12
Phenylalanine	1.05	1.06	1.07	1.11
Tyrosine	1.04	1.05	1.07	1.13
4-Hydroxyphenylpyruvate	1.15	1.20	1.27	1.30
Ketoleucine	1.27	1.23	1.18	1.18
trans-4-Hydroxyproline	0.91	0.89	0.89	0.87
Urea	0.99	1.07	1.02	1.08
Erythrol	1.09	1.08	1.12	1.16
Glucosamine	1.16	1.12	1.12	1.18
Glucose	1.04	1.02	1.04	1.02
Mannosamine	1.14	1.12	1.17	1.17
Mannose	0.94	0.82	0.77	0.70
myo-Inositol	1.04	1.03	1.08	1.12
Sucrose	0.90	0.72	0.87	0.81
Cholestenol No 02	1.04	1.06	1.04	1.00
Cholesterol, total	1.06	1.07	1.05	1.04
Cholesterylester C16:0	1.10	0.97	1.01	1.00
Cholesterylester C18:1	1.02	1.10	1.10	1.09
Cholesterylester C18:2	1.03	1.09	1.07	1.12
Cholesterylester C20:4	1.02	1.04	1.02	1.08
Glycerol, lipid fraction	1.00	0.96	0.94	0.93
Pentadecanol	1.02	1.02	1.13	1.13
Taurochenodeoxycholic acid (additional: Taurodeoxycholic acid)	1.25	1.67	1.14	0.96
Arachidonic acid (C20:cis[5,8,11,14]4)	1.08	1.05	1.04	1.04
Behenic acid (C22:0)	1.08	1.04	1.05	1.02
conjugated Linoleic acid (C18:trans[9,11]2) (add.: conjugated Linoleic acid (C18:cis[9]trans[11]2))	1.06	1.07	1.08	1.07
dihomo-gamma-Linolenic acid	1.09	1.07	1.08	1.04
Docosahexaenoic acid (C22:cis[4,7,10,13,16,19]6)	1.09	1.05	1.04	1.05
Eicosapentaenoic acid (C20:cis[5,8,11,14,17]5)	1.08	1.04	1.01	1.01
gamma-Linolenic acid (C18:cis[6,9,12]3)	1.08	1.04	1.06	1.03
Isopalmitic acid (C16:0)	1.06	1.05	1.04	1.03
Lignoceric acid (C24:0)	1.05	1.01	1.03	1.03
Linoleic acid (C18:cis[9,12]2)	1.06	1.04	1.04	1.02
Myristic acid (C14:0)	1.05	1.06	1.09	1.17
Nervonic acid (C24:cis[15]1)	1.09	1.03	1.06	1.04
Palmitic acid (C16:0)	1.04	1.03	1.04	1.04
Palmitoleic acid (C16:cis[9]1)	1.06	1.06	1.07	1.10
Stearic acid (C18:0)	1.03	1.02	1.01	1.02
Tricosanoic acid (C23:0)	1.10	1.06	1.05	1.03
DAG (C18:1,C18:2)	1.09	1.09	1.10	1.12
TAG (C16:0,C16:1)	0.98	0.89	0.88	0.89
TAG (C16:0,C18:2)	0.97	0.91	0.93	0.93
TAG (C18:1,C18:2)	0.95	0.89	0.93	0.90
TAG (C18:2,C18:2)	0.98	0.97	0.97	0.93
Galactose, lipid fraction	1.09	1.04	1.07	1.05
Glucose, lipid fraction	1.20	1.15	1.26	1.10
myo-Inositol, lipid fraction	1.05	1.06	1.05	1.05
Cortisol	1.11	1.11	1.06	1.10
Dehydroepiandrosterone sulfate (additional:Testosterone-17-sulfate)	1.18	1.10	1.14	1.20
Pseudouridine	1.02	1.05	1.03	1.08
Uric acid	1.03	1.03	1.07	1.07
Uridine	1.04	1.04	1.09	1.10

time / min METABOLITE	140 vs 120	150 vs 120	160 vs 120	180 vs 120
Choline plasmalogen (C18,C20:4)	1.05	1.03	1.05	1.03
Glycerol phosphate, lipid fraction	1.11	1.08	1.06	1.01
Lysophosphatidylcholine (C18:0)	1.13	1.07	1.02	1.02
Lysophosphatidylcholine (C18:1)	0.98	0.95	0.99	0.90
Lysophosphatidylcholine (C18:2)	1.02	1.00	1.03	0.92
Lysophosphatidylcholine (C20:4)	0.99	0.97	0.94	0.91
Phosphatidylcholine (C16:0,C16:0)	1.04	1.04	1.02	1.03
Phosphatidylcholine (C16:1,C18:2)	1.01	1.03	1.06	1.03
Phosphatidylcholine (C18:0,C18:1)	1.02	1.02	1.01	1.00
Phosphatidylcholine (C18:0,C18:2)	0.99	1.00	0.99	1.00
Phosphatidylcholine (C18:0,C20:4)	1.00	1.00	1.01	1.01
Phosphatidylcholine (C18:0,C22:6)	1.04	1.03	1.02	1.03
Phosphatidylcholine (C18:2,C20:4) (additional: Phosphatidylcholine (C16:0,C22:6))	1.00	1.00	1.01	1.00
1-Hydroxy-2-amino-(cis,trans)-3,5-octadecadiene (from sphingolipids)	1.11	1.08	1.03	1.06
3-O-Methylsphingosine (add.: Sphingolipids, erythro-Sphingosine, threo-Sphingosine)	1.13	1.09	1.03	1.06
5-O-Methylsphingosine (add.: Sphingolipids, erythro-Sphingosine, threo-Sphingosine)	1.12	1.07	1.03	1.09
Ceramide (d18:1,C24:0)	1.11	1.07	1.05	1.05
Ceramide (d18:1,C24:1) (additional: Ceramide (d18:2,C24:0))	1.14	1.06	1.11	1.07
erythro-C16-Sphingosine	1.09	1.07	0.96	1.03
erythro-Dihydrosphingosine	1.10	1.06	1.02	1.05
erythro-Sphingosine (additional: Sphingolipids)	1.10	1.07	1.03	1.06
Sphingomyelin (d18:1,C16:0)	1.06	1.05	1.08	1.11
Sphingomyelin (d18:1,C24:0)	1.05	1.02	1.04	0.99
threo-Sphingosine (additional: Sphingolipids)	1.11	1.07	1.04	1.07
2-Hydroxybutyrate	0.81	0.75	0.72	0.74
3-Hydroxybutyrate	0.84	0.95	1.14	1.54
Citrate (additional: Isocitrate)	0.98	1.02	1.09	1.14
Fumarate	1.17	1.20	1.17	1.14
Lactate	**2.73**	**2.52**	**2.46**	**2.06**
Malate	1.85	1.89	1.84	1.79
Pyruvate (additional: Phosphoenolpyruvate (PEP))	**2.29**	**2.22**	**2.32**	**2.24**
Succinate	**2.08**	**2.16**	**2.33**	**2.32**
3,4-Dihydroxyphenylacetic acid (DOPAC)	1.10	1.21	1.24	1.40
3,4-Dihydroxyphenylalanine (DOPA)	1.05	1.11	1.12	1.14
3,4-Dihydroxyphenylglycol (DOPEG)	1.32	1.53	1.61	1.81
4-Hydroxy-3-methoxyphenylglycol (HMPG)	1.05	1.17	1.26	1.49
5-Hydroxy-3-indoleacetic acid (5-HIAA)	1.05	1.09	1.11	1.16
Homovanillic acid (HVA)	1.09	1.17	1.25	1.43
Noradrenaline (Norepinephrine)	**2.61**	**2.81**	**2.82**	**3.09**
Serotonin (5-HT)	1.16	0.96	1.20	1.49
Canthaxanthin	1.03	1.11	1.11	1.29
Creatine	1.08	1.12	1.17	1.26
Cryptoxanthin	1.13	1.07	1.09	1.06
Glycerol, polar fraction	1.44	1.62	1.79	**2.24**
Hippuric acid	1.17	1.21	1.30	1.28
Phosphocreatine	0.86	0.92	0.77	0.87
Taurine	1.04	1.04	1.13	1.20
4-Pyridoxic acid	1.11	1.11	1.18	1.20
alpha-Tocopherol	1.06	1.00	0.98	1.02
Coenzyme Q10	1.07	0.99	1.01	0.99
Nicotinamide	0.78	0.77	0.77	0.81
Pantothenic acid	1.31	1.27	1.35	1.37
Threonic acid	1.07	1.06	1.05	1.14

Data presented here is the relative concentration expressed as ratios. This data was log transformed prior to ANOVA statistical analysis. Colour coding: ratio > 1 & p-value < 0.01 (■) ratio > 1 & 0.01 ≤ p-value < 0.05 (■) ratio > 1 & 0.05 ≤ p-value < 0.10 (■) ratio < 1 & 0.05 ≤ p-value < 0.10 (■) ratio < 1 & 0.01 ≤ p-value < 0.05 (■) ratio < 1 & p-value < 0.01 (■) Font coding: black & bold: (ratio < 0.5) or (ratio >2) black & normal: (0.5 ≤ ratio < 0.9) or (1.1 < ratio ≤ 2) black & italic: 0.9 ≤ ratio ≤ 1.1.

erthro-sphingosine), certain cholesterylester (e.g. cholesterylester C18:2), several amino acids (alanine, cysteine, methionine, phenylalanine, tyrosine), and miscellaneous metabolites (creatine, taurine, hippuric acid). Only a small number of these metabolites increased considerably during exercise with a ratio > 2 (Table 2.2). These included lactate, pyruvate, succinate, noradrenaline, and glycerol. On the other hand, some metabolites were reduced during exercise, such as 2-hydroxybutyrate, trans-4-hydroxyproline, mannose, certain triacylglycerides (TG) and nicotinamide. The onset of exercise caused a significant reduction in 3-hydroxybutyrate, followed by a subsequent increase towards the end of exercise indicating an initial increase in fat oxidation at the onset of exercise with a delay in fatty acid mobilisation and availability late throughout exercise. The onset of exercise also increased the levels of certain fatty acids and sphingolipids. Other metabolites such as pseudourine, uric acid, uridine, taurine, serotonin, and certain cholesterylesters only showed changes towards the end of exercise.

2.4.2 Effect of GTE under resting condition

The effect of GTE under resting conditions was assessed by comparing the metabolite profiles from post-GTE-supplementation (GTE–D8) versus pre-supplementation (UNT– D0) and from post-GTE-supplementation (GTE –D8) versus post-PLA-supplementation (PLA–D8). As a control, the profiles from post-PLA-supplementation (PLA–D8) were compared to pre-supplementation (UNT–D0) (Table 2.3). To assess the impact of 7-days supplementation of GTE under resting fasted conditions only the profiles recorded at time point 0 on each intervention day were taken into account.

Caffeine, hippuric acid, homovanillic acid and 3,4-dihydroxyphenylacetate were elevated post-GTE-supplementation. Caffeine was present in the GTE supplement and hence the presence of caffeine in plasma on D8 was considered as a measure of compliance. The relative concentrations of caffeine are displayed in Figure 2.3. The appearance of hippuric acid, homovanillic acid and 3,4-dihydroxyphenylacetate are known metabolites from gut-microbial degradation of polyphenols [29]. Besides these exogenous effects, GTE induced reductions in di- and triacylglycerides (DAG, TAG), certain fatty acids, certain phosphatidylcholines, alanine, tryptophan, creatine, urea, serotonin, and 5-

hydroxy-3-indoleactetic acid (5-HIAA). Higher concentrations were observed for certain cholesterylesters, 3-hydyroxybutyrate, citrate, lactaldehyde, and glycerol (Figure 2.4).

Table 2.3. ANOVA estimate for effect of GTE on metabolome under fasting and resting condition.

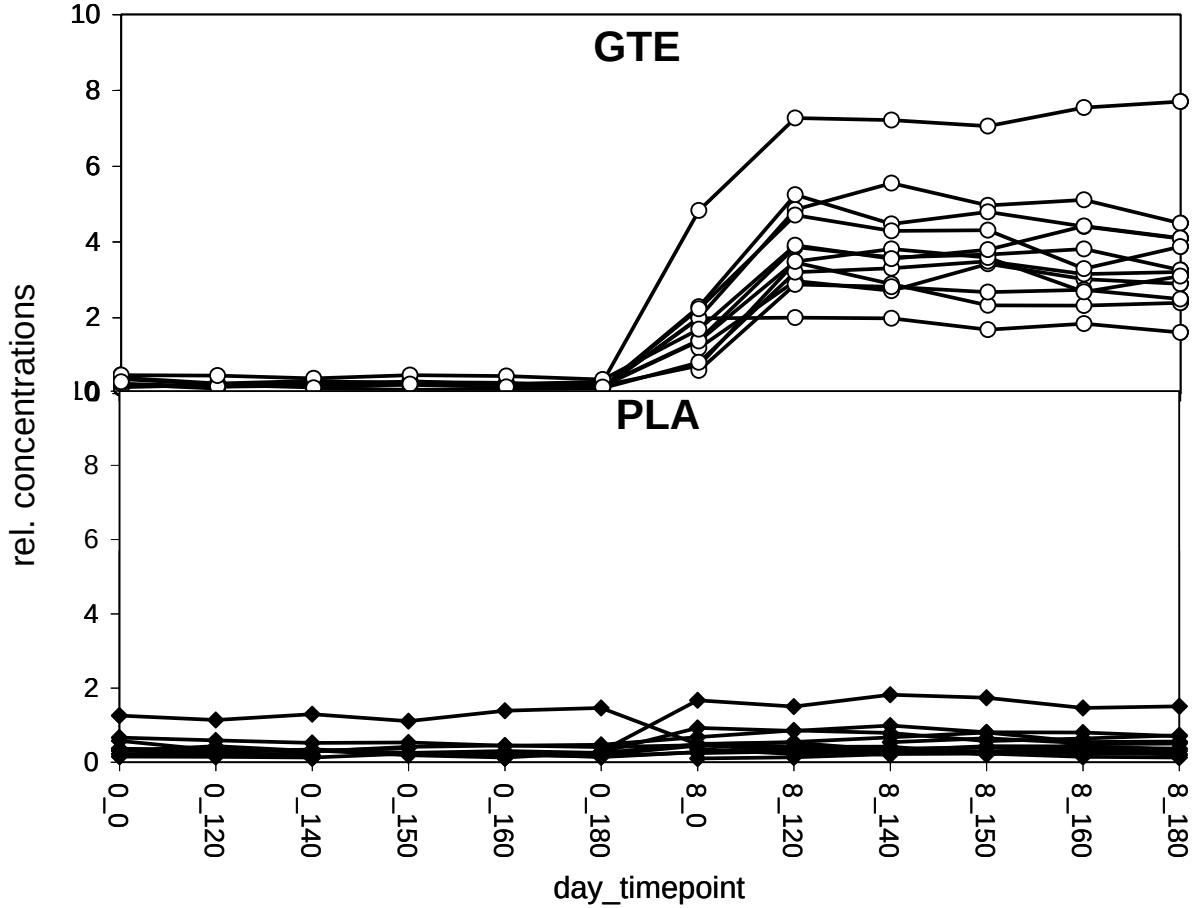
METABOLITE	PLA-D8 vs UNT-D0	GTE-D8 vs UNT-D0	GTE-D8 vs PLA-D8
Alanine	*1.10*	*0.93*	0.85
Asparagine	1.10	*1.01*	*0.91*
Threonine	*1.09*	*1.00*	*0.92*
Tryptophan	*1.04*	*0.93*	0.89
Ketoleucine	0.88	*0.97*	1.11
Urea	*1.00*	0.82	0.81
Cholesterylester C18:1	*0.93*	1.13	1.22
Cholesterylester C18:2	*0.91*	1.13	1.24
Cholesterylester C20:4	*0.95*	1.11	1.17
Glycerol, lipid fraction	1.15	0.87	0.76
Pentadecanol	*1.06*	0.88	0.84
Docosahexaenoic acid (C22:cis[4,7,10,13,16,19]6)	1.14	0.83	0.73
Eicosapentaenoic acid (C20:cis[5,8,11,14,17]5)	1.31	0.84	0.64
Isopalmitic acid (C16:0)	1.13	*0.92*	0.81
Myristic acid (C14:0)	1.23	*1.04*	0.85
DAG (C18:1,C18:2)	*1.06*	0.88	0.83
TAG (C16:0,C16:1)	1.27	0.87	0.69
TAG (C16:0,C18:2)	1.13	0.88	0.78
TAG (C18:2,C18:2)	*1.09*	0.82	0.75
TAG (C18:2,C18:3)	1.26	0.68	0.54
Phosphatidylcholine (C18:0,C22:6)	*1.05*	0.87	0.82
Phosphatidylcholine No 02	*1.06*	*0.94*	0.88
Sphingomyelin (d18:1,C16:0)	*0.91*	*1.07*	1.18
3-Hydroxybutyrate	0.74	1.63	**2.21**
Citrate (additional: Isocitrate)	*1.02*	1.22	1.20
Lactaldehyde	*0.93*	1.13	1.22
3,4-Dihydroxyphenylacetic acid (DOPAC)	1.20	1.61	1.34
5-Hydroxy-3-indoleacetic acid (5-HIAA)	*1.09*	*0.95*	0.87
Homovanillic acid (HVA)	*1.07*	1.50	1.41
Serotonin (5-HT)	1.39	0.73	0.52
Caffeine	1.45	**5.64**	**3.88**
Creatine	1.12	0.86	0.77
Glycerol, polar fraction	0.82	1.11	1.36
Hippuric acid	0.80	1.64	**2.04**
Ascorbic acid (additional: Glucose)	1.13	*1.03*	*0.91*

Data presented here is the relative concentration expressed as ratios. This data was log transformed prior to ANOVA statistical analysis. Colour coding: ratio > 1 & p-value < 0.01 (■) ratio > 1 & 0.01 ≤ p-value < 0.05 (■) ratio > 1 & 0.05 ≤ p-value < 0.10 (■) ratio < 1 & 0.05 ≤ p-value < 0.10 (■) ratio < 1 & 0.01 ≤ p-value < 0.05 (■) ratio < 1 & p-value < 0.01 (■) Font coding: black & bold: (ratio < 0.5) or (ratio >2) black & normal: (0.5 ≤ ratio < 0.9) or (1.1 < ratio ≤ 2) black & italic: 0.9 ≤ ratio ≤ 1.1.

2.4.3 Effect of GTE during exercise

The ANOVA mixed-effects model segregated the effects induced by GTE or PLA during exercise from those induced by exercise alone. Table 2.4 lists the metabolite changes solely caused by GTE or PLA during exercise. The following comparisons were made: Post-GTE-supplementation (GTE–D8) versus pre-supplementation (UNT–D0), post-GTE-supplementation (GTE–D8) versus post-PLA- supplementation (PLA–D8), and - as a control - post-PLA-supplementation (PLA–D8) versus pre-supplementation (UNT–D0) The effects of GTE or PLA during exercise were assessed after 20, 30, 40 and 60 minutes of exercise. The data were normalized to the end of the 2 hour rest period (t= 120 min) (Figure 2.1) to focus on the effects induced during exercise and to eliminate any effects that accounted for resting conditions and were apparent in shifts in baseline levels at time points 0 and 120 min. Our study design included the 7 days (chronic) supplementation as well as the single bolus (acute) intake of either GTE or PLA on D8. It is thought that 7 days GTE supplementation would mostly affect plasma metabolites that were measured under resting conditions at time point 0, whereas the single bolus intake prior to the trial on D8 would determine the effects during exercise. The reason for this is that the main components of GTE would have accumulated in plasma after the two hours of rest [29]. Thus, by normalizing the metabolite changes to the end of the 2 hour rest period (120 min), the effects during exercise may at first instance be ascribed to the main components in GTE boosted by the single bolus intake. Overall, the metabolite changes induced by GTE during exercise were weaker than the effects induced by exercise itself. Nevertheless, significant metabolite changes were observed both when comparing the effect of GTE – D8 versus UNT – D0 and the effect of GTE – D8 versus PLA – D8 (Table 2.4). These changes comprised of the increase in alanine, cysteine, histidine, certain cholesterylesters, lactate, malate, pyruvate, hippurate, and phosphocreatine. In addition, reductions in 3-hydroxybutyrate, certain fatty acids, certain TAGs, and DOPEG were apparent during exercise (Figure 2.4). Other changes could not fully be attributed to the GTE supplementation. These may be due to day-to-day variations or the PLA supplementation. For example, glycerol, glutamate, TAG (C16:0,C16:1), noradrenaline, serotonin and taurine were reduced in GTE – D8 when compared to PLA – D8. However, these changes were not seen when comparing GTE - D8 versus UNT – D0. On the other hand, these changes were increased when comparing the effect of PLA – D8 versus UNT – D0.

Figure 2.3 Relative concentrations of caffeine



	PLA-D8 vs UNT-D0				GTE-D8 vs UNT-D0				GTE-D8 vs PLA-D8			
time / min METABOLITE	140 vs 120	150 vs 120	160 vs 120	180 vs 120	140 vs 120	150 vs 120	160 vs 120	180 vs 120	140 vs 120	150 vs 120	160 vs 120	180 vs 120
Alanine	*0.97*	*1.00*	*0.94*	*0.97*	1.12	1.14	1.11	1.18	1.16	1.14	1.19	1.22
Aspartate	1.15	1.18	1.26	1.36	1.17	1.13	1.44	1.22	*1.02*	*0.96*	1.14	0.90
Cysteine (additional: Cystine)	*1.00*	*1.00*	*0.95*	*0.94*	1.16	1.13	*1.07*	1.10	1.16	1.14	1.13	1.17
Glutamate	1.13	1.26	*1.08*	1.21	*0.94*	*1.02*	0.85	*0.93*	0.83	0.81	0.79	0.77
Histidine	*0.95*	*0.99*	*0.98*	*0.97*	*1.08*	*1.07*	*1.08*	*1.09*	1.14	*1.09*	1.10	1.12
Urea	*0.97*	0.86	0.89	0.86	*1.02*	0.88	*0.95*	0.88	*1.05*	*1.03*	*1.07*	*1.02*
Sucrose	1.71	1.36	1.48	1.75	1.51	1.75	1.24	1.12	0.88	1.29	0.84	0.64
Cholesterylester C18:2	*1.00*	*1.04*	*1.00*	*0.93*	*1.06*	*1.04*	*1.06*	*1.05*	*1.05*	*1.00*	*1.07*	1.13
Cholesterylester C20:4	*0.96*	*0.97*	*0.98*	*0.95*	*1.00*	*1.00*	*1.06*	*1.03*	*1.03*	*1.03*	*1.08*	*1.09*
conjugated Linoleic acid (C18:trans[9,11]2) (add.: conjugated Linoleic acid (C18:cis[9]trans[11]2))	*0.93*	*0.97*	*0.98*	*0.95*	*0.91*	*0.93*	*0.91*	*0.96*	*0.98*	*0.96*	*0.93*	*1.01*
dihomo-gamma-Linolenic acid (C20:cis[8,11,14]3)	*0.93*	*0.97*	*0.96*	*0.98*	*0.95*	*0.98*	*0.94*	*0.99*	*1.03*	*1.01*	*0.99*	*1.01*
Eicosanoic acid (C20:0)	*1.02*	*1.09*	*1.07*	*1.09*	*1.01*	*1.06*	*0.98*	*1.08*	*0.99*	*0.97*	*0.92*	*0.99*
gamma-Linolenic acid (C18:cis[6,9,12]3)	0.89	*0.91*	*0.92*	0.88	*0.94*	*0.98*	*0.91*	*0.98*	*1.05*	*1.09*	*0.99*	1.11
Heptadecanoic acid (C17:0)	*0.98*	*1.08*	*1.10*	*1.03*	*0.96*	*0.97*	*0.99*	*0.92*	*0.99*	0.90	0.90	0.89
Linoleic acid (C18:cis[9,12]2)	*0.98*	*0.98*	*1.01*	*1.01*	*0.95*	*0.95*	*0.93*	*0.99*	*0.97*	*0.97*	*0.92*	*0.98*
Myristic acid (C14:0)	*0.95*	*0.99*	*1.02*	*0.93*	0.89	*0.95*	*0.91*	*0.93*	*0.94*	*0.96*	0.89	*1.00*
Oleic acid (C18:cis[9]1)	*0.99*	*1.01*	*1.04*	*1.02*	*0.92*	*0.93*	*0.92*	*0.98*	*0.93*	*0.93*	0.89	*0.97*
Palmitic acid (C16:0)	*0.98*	*0.99*	*1.02*	*1.01*	*0.94*	*0.96*	*0.93*	*0.99*	*0.96*	*0.97*	*0.91*	*0.98*
Palmitoleic acid (C16:cis[9]1)	*0.99*	*1.02*	*1.06*	*1.04*	0.87	*0.91*	0.88	*0.95*	0.88	0.89	0.84	*0.91*
TAG (C16:0,C16:1)	*1.09*	1.19	1.14	1.15	*0.98*	*1.02*	*0.96*	*0.97*	*0.90*	0.86	0.84	0.85
TAG (C16:0,C18:2)	*1.09*	*1.09*	*1.06*	*1.06*	*0.99*	*0.97*	*0.96*	*0.94*	*0.91*	0.90	0.90	0.89
TAG (C18:2,C18:2)	*1.01*	*1.04*	*1.07*	*1.02*	*0.96*	*0.91*	*0.92*	*0.94*	*0.95*	0.88	0.86	*0.91*
TAG (C18:2,C18:3)	1.11	1.12	*0.96*	*1.07*	*0.91*	*0.93*	0.79	*0.92*	0.82	0.83	0.82	0.86
myo-Inositol, lipid fraction	*0.96*	*0.96*	*0.95*	*0.94*	*0.94*	*0.95*	*0.91*	*0.92*	*0.99*	*0.99*	*0.96*	*0.98*
Choline plasmalogen (C18,C20:4)	*1.02*	*1.03*	*0.99*	*1.01*	*0.95*	*0.98*	*0.95*	*0.99*	*0.94*	*0.95*	*0.96*	*0.98*
Lysophosphatidylcholine (C18:2)	*0.98*	*0.95*	0.87	*0.95*	*0.96*	*0.91*	0.87	0.88	*0.98*	*0.96*	*1.00*	*0.93*
Phosphatidylcholine (C16:0,C20:4) (additional: Phosphatidylcholine (C18:2,C18:2))	*0.99*	*1.00*	*0.99*	*0.99*	*1.00*	*1.01*	*1.01*	*1.01*	*1.01*	*1.01*	*1.02*	*1.02*
Phosphatidylcholine (C18:0,C20:4)	*0.99*	*1.00*	*0.99*	*0.99*	*1.01*	*1.02*	*0.98*	*1.03*	*1.02*	*1.03*	*1.00*	*1.04*
Phosphatidylcholine (C18:0,C22:6)	*1.00*	*1.02*	*1.00*	*0.99*	*0.98*	*0.96*	*0.99*	*0.97*	*0.98*	*0.95*	*0.99*	*0.98*
Phosphatidylcholine (C18:2,C20:4) (additional: Phosphatidylcholine (C16:0,C22:6))	*1.01*	*1.02*	*1.01*	*1.01*	*0.99*	*1.00*	*0.99*	*1.00*	*0.97*	*0.98*	*0.99*	*1.00*
Phosphatidylcholine No 02	*1.02*	*0.99*	*1.01*	*1.05*	1.10	*1.05*	1.13	*1.10*	*1.08*	*1.05*	1.11	*1.05*
Ceramide (d18:1,C24:1) (additional: Ceramide (d18:2,C24:0))	*1.04*	1.14	*1.02*	*1.06*	*0.91*	*0.99*	*0.95*	*1.01*	0.88	0.87	*0.93*	*0.95*
Sphingomyelin (d18:1,C16:0)	*1.00*	*0.98*	*0.98*	0.83	*0.92*	*0.95*	*0.98*	*0.99*	*0.92*	*0.96*	*1.00*	1.19
3-Hydroxybutyrate	*1.08*	1.18	1.11	*1.06*	0.77	0.73	0.73	0.69	0.71	0.62	0.66	0.65
Lactaldehyde	*1.09*	*1.03*	*1.06*	1.14	*1.04*	0.87	*1.03*	1.24	*0.96*	0.85	*0.97*	*1.09*
Lactate	*1.03*	1.11	*1.03*	1.10	1.38	1.54	1.45	1.39	1.34	1.39	1.41	1.26
Malate	*1.02*	*1.07*	1.11	1.21	1.15	1.21	1.40	1.26	1.12	1.12	1.26	*1.04*
Pyruvate (add.: Phosphoenolpyruvate (PEP))	*1.03*	*1.10*	*1.00*	*1.07*	1.31	1.42	1.34	1.32	1.27	1.30	1.34	1.24
Succinate	*0.93*	*1.03*	*0.90*	*0.99*	1.15	1.12	*1.07*	*1.03*	1.23	*1.09*	1.18	*1.03*
3,4-Dihydroxyphenylglycol (DOPEG)	*1.08*	*1.02*	*1.09*	*1.05*	*0.94*	*0.92*	0.88	0.90	0.87	0.90	0.81	0.86
Noradrenaline (Norepinephrine)	*1.09*	1.16	1.27	1.31	*1.02*	*1.06*	1.12	*1.00*	*0.93*	*0.92*	0.88	0.77
Serotonin (5-HT)	1.11	**2.12**	1.42	**2.86**	0.75	0.86	0.88	0.83	0.67	**0.41**	0.62	**0.29**
Cortisol	1.16	1.22	1.14	*1.08*	*0.94*	*0.94*	*0.96*	*1.03*	0.81	0.77	0.84	*0.96*
Dehydroepiandrosterone sulfate (additional: Testosterone-17-sulfate)	*0.99*	*1.04*	*1.05*	*1.05*	0.85	0.87	0.81	*0.97*	0.85	0.84	0.78	*0.93*
Canthaxanthin	*1.08*	1.22	*1.09*	1.20	*0.95*	0.90	0.87	0.88	0.88	0.73	0.80	0.73
Glycerol, polar fraction	1.18	1.27	1.21	1.24	*0.96*	*0.99*	*1.00*	*1.03*	0.81	0.78	0.83	0.83
Hippuric acid	*1.03*	1.24	*1.06*	*1.00*	1.27	1.37	1.32	1.57	1.23	1.10	1.24	1.57
Phosphocreatine	0.85	0.71	1.17	*0.93*	1.43	1.24	1.51	1.34	1.68	1.75	1.29	1.44
Taurine	*1.07*	1.13	*1.06*	1.22	*0.96*	*0.98*	*0.95*	*0.99*	0.90	0.87	0.90	0.81
4-Pyridoxic acid	0.86	0.90	0.81	0.82	*1.05*	*1.10*	*0.98*	*1.00*	1.22	1.22	1.21	1.21
beta-Carotene	*1.03*	*1.10*	*1.03*	*0.98*	0.89	*1.06*	*1.07*	1.14	0.86	*0.96*	*1.04*	1.17

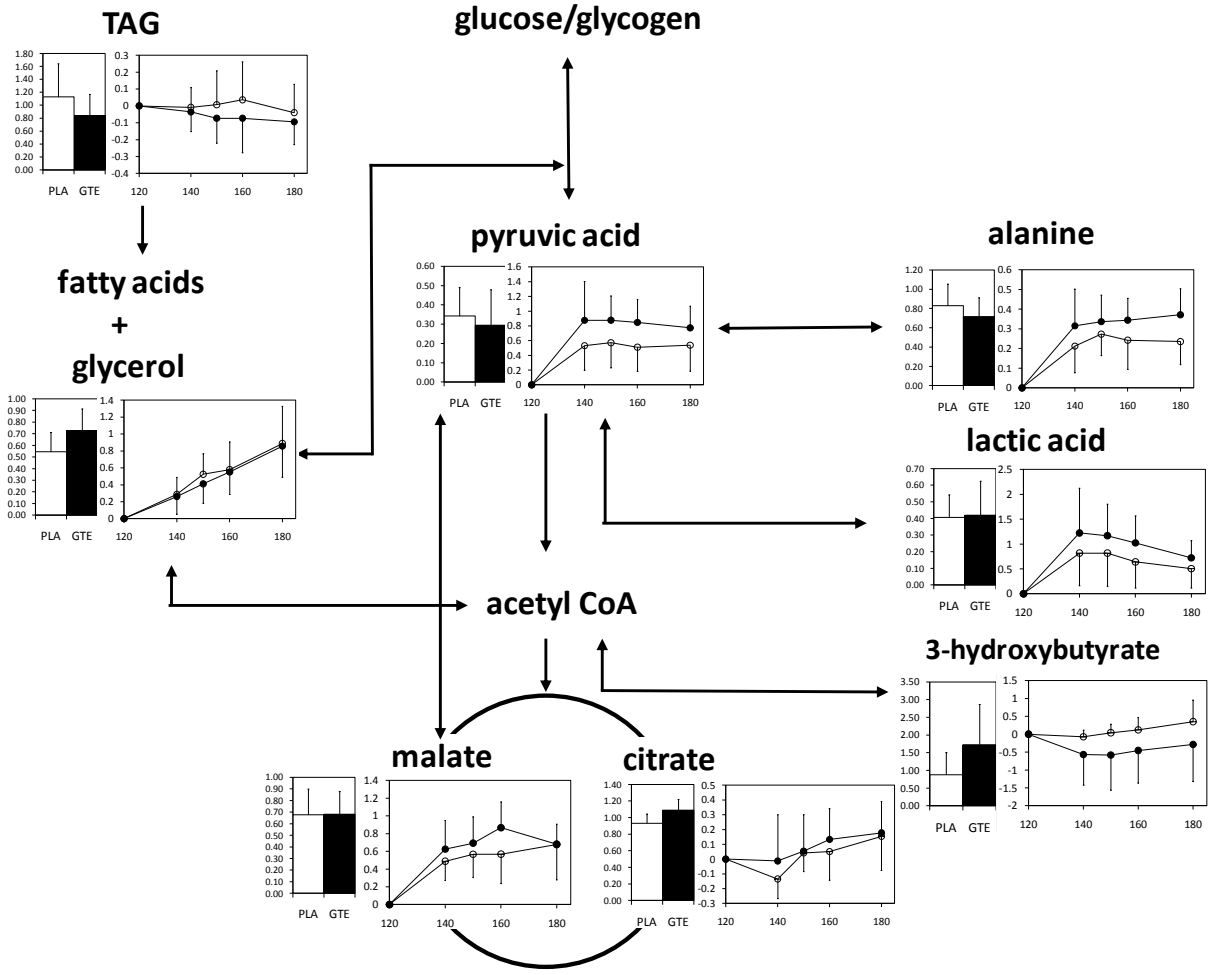
Table 2.4. ANOVA estimate for effect of GTE on metabolome during exercise.

Data presented here is the relative concentration expressed as ratios. This data was log transformed prior to ANOVA statistical analysis. Colour coding: ratio > 1 & p-value < 0.01 (■) ratio > 1 & 0.01 ≤ p-value < 0.05 (■) ratio > 1 & 0.05 ≤ p-value < 0.10 (■) ratio < 1 & 0.05 ≤ p-value < 0.10 (■) ratio < 1 & 0.01 ≤ p-value < 0.05 (■) ratio < 1 & p-value < 0.01 (■) Font coding: black & bold: (ratio < 0.5) or (ratio >2) black & normal: (0.5 ≤ ratio < 0.9) or (1.1 < ratio ≤ 2) black & italic: 0.9 ≤ ratio ≤ 1.1.

2.5 Discussion

By applying untargeted GC-MS- and LC-MS-based metabolite profiling to human plasma, we have been able to comprehensively investigate the metabolic responses following GTE supplementation at rest and during moderate intensity exercise. The main finding of the current study was that 7 days GTE supplementation resulted in different metabolic effects during rest and exercise when compared to PLA and UNT. In particular, GTE altered metabolite concentrations involved in the intermediary metabolism (Figure 2.4). At rest GTE induced metabolite changes indicative of enhanced lipolysis (increase in glycerol, reduction in TAGs, increased fat oxidation (increase in 3-hydroxybutyrate), activated TCA cycle (increase in citrate), reduced amino acid catabolism (reduction in urea and creatine), reduced Cori-cycle (reduction in alanine), and enhanced glycolysis (increase in lactaldehyde). These metabolite changes suggest that GTE has the potential to enhance aerobic energy and fat metabolism at rest. Metabolic effects related to oxidative energy metabolism have previously been observed using a ^{1}H-NMR metabolic profiling in human urine following 2 days of GTE supplementation [30]. Our results are also in line with a number of studies that have demonstrated increases in whole body EE and fat oxidation during resting conditions following GTE supplementation [7,11,12]. However, a number of studies have reported no difference in FFA or glycerol concentrations despite changes in whole body measures of fat oxidation [13,31,32]. In the current study fat oxidation rates were not measured during the rest period. Therefore, it cannot finally be concluded whether the metabolite changes related to fat metabolism (glycerol or 3-hydroxybutyrate) would have induced changes in fat oxidation.

Figure 2.4 Plasma metabolite concentrations of GTE versus PLA during rest (bar graph) and during exercise (line graph) illustrated as part of the intermediary metabolism. Black circles and white circles represent GTE and PLA respectively. Data are means ± SD. The x-axis of the line graphs indicate time in minutes



.

In comparison, GTE supplementation resulted in contrasting effects during exercise. GTE caused metabolite changes that are associated with enhanced glycolysis (increase in pyruvate, lactate), enhanced Cori-cycle (increase in alanine), and decreased fat oxidation (reduction in 3-hydyroxybutyrate) when compared to PLA and UNT. These changes suggest an enhancement in anaerobic energy metabolism. This result is somewhat unexpected, since several previous studies have reported that acute [15] and chronic [16] GTE supplementation have the ability to induce greater fat oxidation rates over and above that of exercise alone, potentially through stimulating lipolysis as illustrated by higher concentrations of glycerol [15]. In the current study no change in lipolytic metabolites (glycerol) were observed during exercise. This may be explained in part by the increase in lactate concentration considering that lactate is known to inhibit lipolysis and limit fat oxidation rates during exercise [33]. The high lactate concentrations were unexpected due to the moderate exercise intensity implemented in the study. The accumulation of pyruvate and lactate and the reduction in 3-hydroxybutyrate indicate a lower pyruvate oxidation through acetyl-CoA (Figure 2.4). It is notable that we observed no difference in fat oxidation rates during exercise following GTE supplementation when compared to PLA and UNT (data not shown) suggesting that these metabolite changes were too weak to cause changes in physiological parameters.

In the current study, we also used targeted profiling of catecholamines in order to elucidate whether GTE supplementation is able to stimulate the adrenergic system. Fat metabolism can be activated by stimulating the adrenergic response. Not surprisingly, in the present study exercise alone induced significant changes of 14 metabolites involved in the catecholamine metabolism. EGCG, a major catechin in GTE, has been suggested to directly inhibit COMT (catechol-O-methyltransferase) [18] an enzyme that is responsible for degrading catecholamines (NA and A). The current hypothesis is that degrading these catecholamines leads to attenuation of sympathetic nervous system (SNS) activity. The inhibition of COMT and thus reduced degradation of NA would result in an increase in EE, lipolysis and potentially an increase in fat oxidation [10]. A limited number of studies have shown that GTE supplementation increases catecholamine concentrations following acute GTE supplementation

[14,34,35]. However, the actual contribution of this mechanism to reported effects of GTE in humans remains unclear. In our study GTE did not increase NA and directly related metabolites either at rest or during exercise. This result suggests that GTE supplementation may not alter the activity of COMT and thus questions the efficacy of the widely referenced COMT mechanism in humans. Interestingly during the resting condition metabolites indicative of lipolysis were elevated, which may occur through a cascade of events activated by catecholamines of the adrenergic system [33]. However, since no differences in NA and A were observed also during resting conditions, this could suggest that GTE stimulated lipolysis via non-adrenergic mechanisms. The non-adrenergic mechanisms could include the up-regulation of lipid-metabolizing enzymes by NF-κB (29). Alternatively, augmented lipolysis may be explained by the action of caffeine rather than the effects of green tea catechins. Caffeine has been shown to stimulate lipolysis independent of changes in catecholamines [36] via antagonizing adenosine receptors directly on adipocytes [37]. However, it is not known whether green tea catechins are able to elicit direct effects on adipose tissue similar to that of caffeine.

This is the first study to directly compare the metabolic responses to GTE supplementation between resting and during moderate intensity exercise conditions. Significant differences in metabolic responses at rest and during exercise following GTE supplementation were observed. Similar results were observed in a previous study showing that the ingestion of capsinoids - which are non-pungent analogues of capsaicin (8-methyl-N-vanillyl-6-nonenamide) naturally present in sweet pepper - increased adrenergic activity and energy expenditure at rest but had little effect during exercise [38]. It could be argued that during exercise GTE was not potent enough to further enhance an already stimulated environment. In comparison, GTE may offer stimulatory effects to human metabolism under resting conditions. The apparent difference in the metabolic response at rest and during exercise may be rooted in differences between chronic and acute GTE supplementation. In the current study, the metabolic effects observed during rest can be largely attributed to the chronic effects of GTE (7 days). In comparison the metabolic effects observed during exercise could be argued to be induced by the acute effects (single dose) post GTE supplementation. Thus, an increase in EE and fat oxidation

and their related metabolic effects may only be present after chronic GTE supplementation. Chronic supplementation may induce a progressive regulation of various proteins and enzymes at certain sites in the body resulting in a systemic shift on the endogenous metabolism after long-term supplementation. For example, chronic GTE supplementation (8-24 wk) has been demonstrated to alter the gene expression related to energy metabolism and lipid homeostasis in an animal model [39]. However, the authors acknowledge that while the study design is capable of detecting chronic effects (7 days) at rest, the metabolic effects during exercise are induced both by acute and chronic GTE supplementation combined. Therefore, future research is required to tease out the differential metabolic effects between acute and chronic GTE supplementation both at rest and during exercise.

Alternatively, the apparent difference in the metabolic effects at rest and during exercise may be attributed to different bioactive components in GTE and/or the presence of caffeine known to be crucial factors ultimately influencing the rates of fat or carbohydrate oxidation [40]. The single dose of GTE or PLA consumed two hours before the exercise test during the post- supplementation trial increased the concentrations of directly absorbed components in GTE such as catechins, in particular EGCG, and caffeine (Figure 2.3) and may be responsible for the effects observed during exercise. In comparison, phenolic metabolites originating from gut microbial degradation of polyphenols usually only appear in circulation 3-4 hours after polyphenol consumption [29] and are unlikely to be responsible for the effect during exercise. However, they could have contributed to the chronic effects observed at rest. Following 7-days GTE supplementation, some phenolic acids such as hippuric acid, homovanillic acid and 3,4-dihydroxyphenylacetic acid were increased in plasma at rest due to the slow bioconversion of polyphenols by gut microbiota. Nutrient – gut microbiota interactions have previously been discussed to play an important role in polyphenol bioavailability, human obesity, and insulin resistance [41]. Further investigations are needed to assign acute and chronic effects to certain bioactive species in GTE and to specify the role of gut microbiota.

The current study also illustrated novel metabolic responses of GTE at rest and during exercise. GTE induced reductions in metabolites from the tryptophan pathway (tryptophan, serotonin and 5-HIAA) at

rest and in 3,4-dihydroxyphenylglycol (DOPEG), serotonin and NA during exercise. DOPEG is known to inhibit the enzyme monoamine oxidase (MAO) causing the oxidative deamination of NA, serotonin, and other amines. Inhibition of this enzyme would lead to increasing concentrations of NA and serotonin. Yet these two metabolites were reduced at the end of the exercise period suggesting a complex relationship between DOPEG, NA and serotonin as observed in plasma. This data provides evidence that GTE is capable of altering activity of certain catecholamines. However, the underlying mechanisms as well as their relation to physiological outcomes remain to be elucidated.

Finally, changes in certain TAG's and fatty acids were observed post-GTE- supplementation under resting and exercise conditions. This may suggest that GTE affects the response of exogenous and endogenous lipids. Manipulating lipids could be an interesting target for protecting against insulin resistance. Elevation in circulating lipids (DAG, TAG, FFA) has commonly been associated with impaired insulin-mediated glucose uptake [42,43] and elevated insulin resistance [44] usually coexisting with obesity and type 2 diabetes [45]. Exercise is one way of providing protection by having beneficial effects on glycaemic control [46] and by increasing utilisation of intramuscular lipids by reducing DAG and ceramides [47]. Recent evidence has shown that chronic GTE supplementation suppresses insulin resistance mainly in animal models [39,48] and improves insulin sensitivity in young physically active males [15]. However, the protective effects against insulin resistance with GTE in humans is currently not conclusive [49]. It is interesting to consider the possibility that GTE may provide additive protection against insulin resistance when combined with exercise, by increasing the turnover of intramuscular lipids rather than increasing storage of lipids associated with the metabolic syndrome.

In summary, our comprehensive metabolite profiling approach broadened our understanding on the mode of action of exercise and GTE beyond the physiological outcomes. Moderate intensity exercise stimulated multiple metabolic pathways including lipolysis, glycolysis, as well as the activation of the TCA cycle and the adrenergic system. The metabolite changes induced by GTE were more subtle and affected fewer pathways when compared to those induced by exercise alone. In agreement with

previous studies, we illustrated that 7-days GTE supplementation mainly enhanced metabolites indicative of lipolysis and fat oxidation under resting conditions, when compared to PLA. This effect was not enhanced during exercise. This suggests that a single dose of GTE may not be potent enough to stimulate further the metabolism that was already up-regulated by exercise. Furthermore, GTE did not stimulate the adrenergic system during rest and exercise since no increase in NA and related catecholamines were observed. This challenges COMT inhibition as the putative mechanism of action of GTE *in vivo*. Yet GTE stimulated lipolysis under resting conditions suggesting non-adrenergic mechanisms. It is clear, that the effects observed in plasma only provide limited information on the underlying mechanisms of action, since the metabolite concentrations in plasma reflect spill-over effects that may occur in several tissues including brain, skeletal muscle, liver, gut and adipose tissue. Further investigations are required to elucidate the potential targets, interactions and bioactivity of green tea catechins to understand the exact mechanisms of GTE and the physiological outcomes in the human body.

2.6 References

1. van Ommen B, Keijer J, Heil SG, Kaput J. Challenging homeostasis to define biomarkers for nutrition related health. Mol Nutr Food Res 2009;53:795-804.
2. Burke MF, Dunbar RL, Rader DJ. Could exercise metabolomics pave the way for gymnomimetics? Sci Transl Med 2010;2:41ps35.
3. Chorell E, Moritz T, Branth S, Antti H, Svensson MB. Predictive metabolomics evaluation of nutrition-modulated metabolic stress responses in human blood serum during the early recovery phase of strenuous physical exercise. J Proteome Res 2009;8:2966-77.
4. Lewis GD, Farrell L, Wood MJ, Martinovic M, Arany Z, Rowe GC, Souza A, Cheng S, McCabe EL, Yang E et al. Metabolic signatures of exercise in human plasma. Sci Transl Med 2010;2:33ra37.
5. Miccheli A, Marini F, Capuani G, Miccheli AT, Delfini M, Di Cocco ME, Puccetti C, Paci M, Rizzo M, Spataro A. The influence of a sports drink on the postexercise metabolism of elite athletes as investigated by NMR-based metabolomics. J Am Coll Nutr 2009;28:553-64.
6. Yan B, A J, Wang G, Lu H, Huang X, Liu Y, Zha W, Hao H, Zhang Y, Liu L et al. Metabolomic investigation into variation of endogenous metabolites in professional athletes subject to strength-endurance training. J Appl Physiol 2009;106:531-8.
7. Auvichayapat P, Prapochanung M, Tunkamnerdthai O, Sripanidkulchai BO, Auvichayapat N, Thinkhamrop B, Kunhasura S, Wongpratoom S, Sinawat S, Hongprapas P. Effectiveness of green tea on weight reduction in obese Thais: A randomized, controlled trial. Physiol Behav 2008;93:486-91.
8. Ikeda I. Multifunctional effects of green tea catechins on prevention of the metabolic syndrome. Asia Pac J Clin Nutr 2008;17 Suppl 1:273-4.
9. Kovacs EM, Mela DJ. Metabolically active functional food ingredients for weight control. Obes Rev 2006;7:59-78.
10. Westerterp-Plantenga MS. Green tea catechins, caffeine and body-weight regulation. Physiol Behav 2010;100:42-6.
11. Dulloo AG, Duret C, Rohrer D, Girardier L, Mensi N, Fathi M, Chantre P, Vandermander J. Efficacy of a green tea extract rich in catechin polyphenols and caffeine in increasing 24-h energy expenditure and fat oxidation in humans. Am J Clin Nutr 1999;70:1040-5.
12. Harada U, Chikama A, Saito S, Takase H, Nagao T, Hase T, Tokimitsu I. Effects of the Long-Term ingestion of tea catechins on energy expenditure and dietary fat oxidation in healthy subjects. J of Health sc 2005;51:248-52.
13. Diepvens K, Kovacs EM, Nijs IM, Vogels N, Westerterp-Plantenga MS. Effect of green tea on resting energy expenditure and substrate oxidation during weight loss in overweight females. Br J Nutr 2005;94:1026-34.
14. Gregersen NT, Bitz C, Krog-Mikkelsen I, Hels O, Kovacs EM, Rycroft JA, Frandsen E, Mela DJ, Astrup A. Effect of moderate intakes of different tea catechins and caffeine on acute measures of energy metabolism under sedentary conditions. Br J Nutr 2009;102:1187-94.
15. Venables MC, Hulston CJ, Cox HR, Jeukendrup AE. Green tea extract ingestion, fat oxidation, and glucose tolerance in healthy humans. Am J Clin Nutr 2008;87:778-84.
16. Ichinose T, Nomura S, Someya Y, Akimoto S, Tachiyashiki K, Imaizumi K. Effect of endurance training supplemented with green tea extract on substrate metabolism during exercise in humans. Scand J Med Sci Sports 2010.

17. Rains TM, Agarwal S, Maki KC. Antiobesity effects of green tea catechins: a mechanistic review. J Nutr Biochem 2011;22:1-7.
18. Borchardt RT, Huber JA. Catechol O-methyltransferase. 5. Structure-activity relationships for inhibition by flavonoids. J Med Chem 1975;18:120-2.
19. Murase T, Haramizu S, Shimotoyodome A, Nagasawa A, Tokimitsu I. Green tea extract improves endurance capacity and increases muscle lipid oxidation in mice. Am J Physiol Regul Integr Comp Physiol 2005;288:R708-R715.
20. Watanabe J, Kawabata J, Niki R. Isolation and identification of acetyl-CoA carboxylase inhibitors from green tea (Camellia sinensis). Biosci Biotechnol Biochem 1998;62:532-4.
21. Mutch DM, Fuhrmann JC, Rein D, Wiemer JC, Bouillot JL, Poitou C, Clement K. Metabolite profiling identifies candidate markers reflecting the clinical adaptations associated with Roux-en-Y gastric bypass surgery. PLoS One 2009;4:e7905.
22. Achten J, Gleeson M, Jeukendrup AE. Determination of the exercise intensity that elicits maximal fat oxidation. Med Sci Sports Exerc 2002;34:92-7.
23. Kuipers H, Verstappen FT, Keizer HA, Geurten P, van KG. Variability of aerobic performance in the laboratory and its physiologic correlates. Int J Sports Med 1985;6:197-201.
24. van Ravenzwaay B, Cunha GC, Leibold E, Looser R, Mellert W, Prokoudine A, Walk T, Wiemer J. The use of metabolomics for the discovery of new biomarkers of effect. Toxicol Lett 2007;172:21-8.
25. Roessner U, Wagner C, Kopka J, Trethewey RN, Willmitzer L. Technical advance: simultaneous analysis of metabolites in potato tuber by gas chromatography-mass spectrometry. Plant J 2000;23:131-42.
26. Yamada H, Yamahara A, Yasuda S, Abe M, Oguri K, Fukushima S, Ikeda-Wada S. Dansyl chloride derivatization of methamphetamine: a method with advantages for screening and analysis of methamphetamine in urine. J Anal Toxicol 2002;26:17-22.
27. Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., and the R Core team. nlme: Linear and Nonlinear Mixed Effects Models. R package version 3.1-91 . 2009.
28. R Development Core Team. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing, 2008.
29. Crozier A, Del RD, Clifford MN. Bioavailability of dietary flavonoids and phenolic compounds. Mol Aspects Med 2010;31:445-67.
30. Van Dorsten FA, Daykin CA, Mulder TP, Van Duynhoven JP. Metabonomics approach to determine metabolic differences between green tea and black tea consumption. J Agric Food Chem 2006;54:6929-38.
31. Sale C, Harris RC, Delves S, Corbett J. Metabolic and physiological effects of ingesting extracts of bitter orange, green tea and guarana at rest and during treadmill walking in overweight males. Int J Obes (Lond) 2006;30:764-73.
32. Thielecke F, Rahn G, Bohnke J, Adams F, Birkenfeld AL, Jordan J, Boschmann M. Epigallocatechin-3-gallate and postprandial fat oxidation in overweight/obese male volunteers: a pilot study. Eur J Clin Nutr 2010;64:704-13.
33. Jeukendrup AE, Saris WH, Wagenmakers AJ. Fat metabolism during exercise: a review--part II: regulation of metabolism and the effects of training. Int J Sports Med 1998;19:293-302.
34. Berube-Parent S, Pelletier C, Dore J, Tremblay A. Effects of encapsulated green tea and Guarana extracts containing a mixture of epigallocatechin-3-gallate and caffeine on 24 h energy expenditure and fat oxidation in men. Br J Nutr 2005;94:432-6.

35. Rudelle S, Ferruzzi MG, Cristiani I, Moulin J, Mace K, Acheson KJ, Tappy L. Effect of a thermogenic beverage on 24-hour energy metabolism in humans. Obesity (Silver Spring) 2007;15:349-55.
36. Van SM, Mohr T, Kjaer M, Graham TE. Acute effects of caffeine ingestion at rest in humans with impaired epinephrine responses. J Appl Physiol 1996;80:999-1005.
37. Beaudoin MS, Graham TE. Methylxanthines and human health: epidemiological and experimental evidence. Handb Exp Pharmacol 2011;509-48.
38. Josse AR, Sherriffs SS, Holwerda AM, Andrews R, Staples AW, Phillips SM. Effects of capsinoid ingestion on energy expenditure and lipid oxidation at rest and during exercise. Nutr Metab (Lond) 2010;7:65-75.
39. Chen N, Bezzina R, Hinch E, Lewandowski PA, Cameron-Smith D, Mathai ML, Jois M, Sinclair AJ, Begg DP, Wark JD et al. Green tea, black tea, and epigallocatechin modify body composition, improve glucose tolerance, and differentially alter metabolic gene expression in rats fed a high-fat diet. Nutr Res 2009;29:784-93.
40. Hursel R, Westerterp-Plantenga MS. Thermogenic ingredients and body weight regulation. Int J Obes (Lond) 2010;34:659-69.
41. Diamant M, Blaak EE, de Vos WM. Do nutrient-gut-microbiota interactions play a role in human obesity, insulin resistance and type 2 diabetes? Obes Rev 2011;12:272-81.
42. Frayn KN, Coppack SW, Humphreys SM, Clark ML, Evans RD. Periprandial regulation of lipid metabolism in insulin-treated diabetes mellitus. Metabolism 1993;42:504-10.
43. Timmers S, Schrauwen P, de VJ. Muscular diacylglycerol metabolism and insulin resistance. Physiol Behav 2008;94:242-51.
44. Itani SI, Ruderman NB, Schmieder F, Boden G. Lipid-induced insulin resistance in human muscle is associated with changes in diacylglycerol, protein kinase C, and IkappaB-alpha. Diabetes 2002;51:2005-11.
45. Reaven GM. Banting lecture 1988. Role of insulin resistance in human disease. Diabetes 1988;37:1595-607.
46. Manders RJ, Van Dijk JW, van Loon LJ. Low-intensity exercise reduces the prevalence of hyperglycemia in type 2 diabetes. Med Sci Sports Exerc 2010;42:219-25.
47. van Loon LJ, Koopman R, Stegen JH, Wagenmakers AJ, Keizer HA, Saris WH. Intramyocellular lipids form an important substrate source during moderate intensity exercise in endurance-trained males in a fasted state. J Physiol 2003;553:611-25.
48. Hininger-Favier I, Benaraba R, Coves S, Anderson RA, Roussel AM. Green tea extract decreases oxidative stress and improves insulin sensitivity in an animal model of insulin resistance, the fructose-fed rat. J Am Coll Nutr 2009;28:355-61.
49. Brown AL, Lane J, Coverly J, Stocks J, Jackson S, Stephen A, Bluck L, Coward A, Hendrickx H. Effects of dietary supplementation with the green tea polyphenol epigallocatechin-3-gallate on insulin resistance and associated metabolic risk factors: randomized controlled trial. Br J Nutr 2009;101:886-94.

Chapter 3

The metabolic and performance effects of caffeine compared to coffee during endurance exercise

Paper Published

Adrian B. Hodgson†, Rebecca Randell†, Asker Jeukendrup†

PLoS One. 2013;8(4):e59561

A.B.H Conceived and designed the experiment, performed the experiment, interpreted the data and wrote the manuscript, R.R Assisted in the data collection. A.J. Assisted with designing the experiment.

† School of Sport and Exercise Sciences, University of Birmingham, United Kingdom

3.1 Abstract

Background: There is consistent evidence supporting the ergogenic effects of caffeine for endurance based exercise. However, whether caffeine ingested through coffee has the same effects is still subject to debate. The primary aim of the study was to investigate the performance enhancing effects of caffeine and coffee using a time trial performance test, while also investigating the metabolic effects of caffeine and coffee. **Methods**: In a single-blind, crossover, randomised counter-balanced study design, eight trained male cyclists/triathletes (Mean ± SD: Age 41±7y, Height 1.80±0.04m, Weight 78.9±4.1kg, VO_2 max 58±3 $mL \bullet kg^{-1} \bullet min^{-1}$) completed 30 min of steady-state (SS) cycling at approximately 55% VO_2max followed by a 45 min energy based target time trial (TT). One hour prior to exercise each athlete consumed drinks consisting of caffeine (5 mg/kg BM), instant coffee (5 mg/kg BM), instant decaffeinated coffee or placebo. The set workloads produced similar relative exercise intensities during the SS for all drinks, with no observed difference in carbohydrate or fat oxidation. **Results:** Performance times during the TT were significantly faster (~5.0%) for both caffeine and coffee when compared to placebo and decaf (38.35±1.53, 38.27±1.80, 40.23±1.98, 40.31±1.22 min respectively, $p<0.05$). The significantly faster performance times were similar for both caffeine and coffee. Average power for caffeine and coffee during the TT was significantly greater when compared to placebo and decaf (294±21W, 291±22W, 277±14W, 276±23W respectively, $p<0.05$). No significant differences were observed between placebo and decaf during the TT. **Conclusions**: The present study illustrates that both caffeine (5 mg/kg BM) and coffee (5 mg/kg BM) consumed 1 h prior to exercise can improve endurance exercise performance.

3.2 Introduction

Numerous studies to date have shown that caffeine ingested prior to [1-7] and during [8] prolonged sub-maximal and high intensity exercise can improve performance. Since the seminal work by Costill and colleagues [9] it is often cited that caffeine induces its ergogenic effects by an increase in fat oxidation through the sympathetic nervous system, and a subsequent sparing of muscle glycogen [2]. However, there is very little support for an increase in fat oxidation [10,11] or an enhancement to the sympathetic nervous system [12] being the principal mechanism of caffeine's ergogenic effect. Since, recent investigations have elucidated that the principal mechanism of caffeine's ergogenic effects is through its ability to act as an adenosine receptor antagonist to induce effects on both central and peripheral nervous system [13] to reduce pain and exertion perception [14], improve motor recruitment [13] and excitation-contraction coupling [15-17].

In the literature to date, the ergogenic effects are well documented with the time to exhaustion test at a fixed power output being the predominant performance measure used [1,2,9-11]. It was questioned whether assessing endurance capacity in this way would have sufficient ecological validity to translate results to real life events [18]. However, since then a number of studies have confirmed the ergogenic effects of caffeine using time trial protocols [3-5,7,8], which involves completing an energy based target or set distance in as fast as time possible, thus simulating variable intensities that are likely to occur during competitive events. In most of these studies pure (anhydrous) caffeine was ingested through capsules or dissolved in water. Based on this research it is often assumed that ingesting caffeine in a variety of dietary sources, such as coffee, will result in the same ergogenic effect.

Very few studies, however, have shown a positive effect of coffee on exercise performance. Coffee improved performance in some [9,19-21], but not all studies [22-24]. This may seem surprising as reports have shown that coffee is the most concentrated dietary source of caffeine as well as being one of the largest sources of caffeine used by athletes prior to competition [25]. Amongst the current studies, only two investigations have actually used coffee rather than decaffeinated coffee plus

anhydrous caffeine [21,22], with only one of these studies showing an ergogenic effect of the coffee [21]. This further identifies the equivocal evidence surrounding the performance effects of coffee. The most cited study is perhaps a study by Graham et al [22], who showed that running time to exhaustion (85% VO_2 max) was only improved when runners ingested pure caffeine (4.5mg/kg BM), prior to exercise, but not when they ingested either regular coffee (4.5mg/kg BM), decaffeinated coffee plus caffeine (4.5 mg/kg BM), decaffeinated coffee and a placebo control. The authors reported that the difference in performance could not be explained by the caffeine or methylxanthine plasma concentrations 1h following intake or at end of exercise, as no difference was observed between trials that contained caffeine.

Graham et al [22] suggested that other components in coffee known as chlorogenic acids, may have antagonised the physiological responses of caffeine. However, in this study [22] chlorogenic acids in the coffee or in the plasma were not measured. Chlorogenic acids are a group of phenolic compounds that possess a quinic acid ester of hydroxycinnamic acid [26]. The consumption of chlorogenic acids varies significantly in coffee ranging from 20-675mg per serving [26]. It has previously been shown *in vitro* that chlorogenic acids antagonize adenosine receptor binding of caffeine [27] and causes blunting to heart rate and blood pressure in rats [28]. For this reason, it is unclear what role chlorogenic acids, found in coffee, will have on the physiological and metabolic effects of coffee and caffeine during exercise in humans. Therefore, due to the large variation of chlorogenic acids between coffee beverages and the unclear performance effects of coffee to date, it is yet to be determined if coffee causes differences in the performance and metabolic effects during exercise when compared to caffeine alone.

Therefore, the primary aim of the present study was to investigate whether acute intake of instant coffee (5 mg/kg BM) and anhydrous caffeine (5 mg/kg BM) are ergogenic to cycling performance compared to decaffeinated coffee or placebo beverages when using a validated 45-minute time trial performance test.. In addition, completing a steady state exercise bout prior to the time trial performance test is a routine protocol used in our laboratory [18,29]. For this reason it provided an

opportunity to also investigate the effect of acute anhydrous caffeine or coffee intake on substrate oxidation and plasma metabolite responses during 30-min steady state exercise (55% VO_2 max). The study hypothesis was that despite the previous work by Graham et al [22], 5 mg/kg BM regardless of the form of administration (anhydrous or coffee) would be ergogenic to performance similarly when compared to decaffeinated coffee or placebo, but this effect would not be mediated through changes in fat metabolism.

3.3 Materials and Methods

3.3.1 Participants

Eight trained cyclists/triathletes (Mean ± SD: Age 41±7y, Height 1.80±0.04m, Weight 78.9±4.1kg, $\dot{V}O_2$ max 58±3 mL•kg^{-1}•min^{-1}) were recruited from local Birmingham cycling and triathlon clubs. Inclusion criteria included participants who trained 3 or more times per week (>90-min/session), had been training for >2 years, and had a low habitual caffeine intake of ≤ 300mg/d (approximately ≤ 3 cups coffee/d). All participants were fully informed of the experimental trials and all risks and discomforts associated before providing written informed consent to participate in the study. All procedures and protocols were approved by the Life and Environmental Sciences Ethical Review Committee at the University of Birmingham.

3.3.2 General Study design

The study design was a cross over, randomised and single blinded. Maximal oxygen uptake ($\dot{V}O_2$ max) and power (Wmax) was assessed during a preliminary trial. Following this each participant completed 4 experimental trials, each separated by 7 days. Each trial consisted of consuming: caffeine (5mg/kg BM) (CAF), instant coffee (5mg/kg BM) (COF), instant decaffeinated coffee (DECAF) or placebo (PLA, Quinine Sulphate) in the overnight fasted state (8hrs) 1 h before completing a 30 min steady state cycle exercise bout (SS) (55% $\dot{V}O_2$ max). Following this each participant was instructed to

complete a time trial lasting approximately 45-min. Each participant returned to the lab 7 days later to repeat the trial, with a different supplement.

3.3.3 Preliminary Trial

Before the experimental trial, participants visited the Human Performance Laboratory at the University of Birmingham on two separate occasions separated by 7 days. During the first visit participants completed an incremental exercise test on an electronically braked cycle ergometer (Lode Excalibur Sport, Groningen, Netherlands) to volitional exhaustion ($\dot{V}O_2$ max test). Prior to beginning the test participants firstly had weight (OHaus, Champ II scales, USA) and height (Seca stadiometer, UK) recorded. Participants mounted the cycle ergometer, which was followed by a 5-min warm up at 75 W, participants then started the test at 95 W for 3-min. The resistance was increased every 3-min, in incremental steps of 35W, until they reached voluntary exhaustion. Wmax was calculated using the following equation:

$$W_{max} = W_{out} + [(t/180) \text{ X } 35]$$

Where W_{out} is the power output of the last stage completed during the test, and t is the time spent, in seconds, in the final stage. Throughout the test respiratory gas measurements ($\dot{V}O_2$ and $\dot{V}CO_2$) were collected continuously using an Online Gas Analyser (Oxycon Pro, Jaeger). $\dot{V}O_2$ was considered maximal if 2 out of the 4 following criteria were met: 1) if $\dot{V}O_2$ levelled off even when workload increased 2) a respiratory exchange ratio (RER) of >1.05 3) a heart rate within 10 beats/min of age predicted maximal heart rate 4) a cadence of 50 rpm could not be maintained. Heart rate (HR) was recorded during each stage of the test using a HR monitor (Polar, Warwick, UK). Wmax was used to determine the work load for the steady state exercise bouts throughout all subsequent experimental trials (50% W_{max}). Wmax was also used to calculate the total amount of work to be completed during the 45-min time trial and the linear factor, both calculated according to the formula derived by

Jeukendrup et al [18]. The bike position was recorded following the test to be replicated in all other trials.

Approximately 7 days later participants reported back to the lab, for the second preliminary trial, between 0600 and 0800 having undergone an 8 hr fast. The purpose of the trial was to familiarise each subject to the experimental trial and time trial protocol. All participants completed 30 min SS at 50% W_{max} (55% $\dot{V}O_2$ max). Expired breath samples were collected every 10-min for measures of $\dot{V}O_2$, $\dot{V}CO_2$, and RER (Oxycon Pro, Jaeger). Immediately following all participants completed a time trial lasting ~45 min. The data collected during the familiarisation trial was not used for any of the final analysis.

3.3.4 Experimental Trial

All participants reported to the Human Performance Lab between 0600 and 0800 having completed an 8 h overnight fast. On arrival weight was recorded (Seca Alpha, Hamburg, Germany) and a flexible 20-gauge Teflon catheter (Venflon; Becton Dickinson, Plymouth, United Kingdom) was inserted into an antecubital vein. A 3-way stopcock (Connecta; Becton Dickinson) was attached to the catheter to allow for repeated blood sampling during the experimental period. An initial 15 mL fasting blood sample was collected (EDTA-containing tubes, BD vacutainers). Following this all participants consumed one of the treatment beverages and rested for 1h, with further samples taken at 30 min and 60 min (10 mL EDTA). After the rest period, participants then mounted the cycle ergometer, in an identical bike position as recorded during the preliminary trial, and began a 30 min SS at 50% W_{max} (55% $\dot{V}O_2$ max). Blood samples (15 mL) and 4 min respiratory breath samples, VO_2, VCO_2 and RER, (Oxycon Pro, Jaeger) were collected every 10 min during the exercise. The catheter was kept patent during both the rest and exercise period by flushing it with 5 mL isotonic saline (0.9% w/v; B Braun) after every blood sample. In addition, heart rate (HR) was recorded (Polar RS800CX) every 15 min at rest and every 10 min during SS. Ratings of Perceived Exertion (RPE) scale were recorded every 10

min during SS using the 6-20 Borg scale [425]. Upon completion of the SS, the subject was instructed to stop exercising for ~1 min, and the cycle ergometer was set in the linear mode. The participants were instructed to complete an energy-based target amount of work at 70% W_{max} in the quickest time possible. The total amount of work (650 ± 37KJ) was calculated for the 45 min time trial. A linear factor, 70% W_{max} divided by $(90rpm)^2$ was entered into the cycle ergometer. Participants received a countdown prior to starting the time trial and received no verbal or visual feedback regarding performance time or physiological measures throughout the test. No additional measures, blood or respiratory, were taken through the test. Participants received no feedback about their performance until they had completed all 4 experimental trials. Following the completion of the TT, each participant completed a questionnaire to guess the test beverage consumed prior to the commencement of the trial, as well as report any GI distress experienced during the trial.

3.3.5 Treatment Beverages

During each visit to the lab, participants ingested one of four treatment beverages. This included caffeine (5 mg/kg BM), regular coffee (5 mg/kg BM), decaffeinated coffee and placebo. Therefore, decaffeinated coffee and placebo acted as controls to both of the caffeinated trials. Caffeine (Anhydrous caffeine, 99.8% pure, Blackburn Distributions Ltd, Nelson, United Kingdom) was weighed (394.4±7.0 mg) prior to the trial, and was immediately dissolved and vortexed for 15 min in 600 mL of water prior to consumption and served in an opaque sports drinks bottle. Coffee was prepared using instant coffee (Nescafe Original). In order to select the correct weight of coffee to equal 5 mg/kg BM, Nescafe states that Nescafe Original instant coffee provides 3.4g caffeine/100 g of instant coffee. This information was confirmed using a HPLC method (see below), and based on the analysis it was calculated that 0.15 g coffee/kg BM equalled 5 mg/kg BM. Therefore, prior to each trial, coffee was weighed (11.8±1.0 g) and dissolved in 600 mL hot water (94±2 °C) and served in a mug. Both coffee and decaffeinated coffee were served hot, while caffeine and placebo were served cold.

DECAF (Nescafe Original Decaffeinated coffee, <97% caffeine free) was prepared in an identical fashion, with the same amount of decaffeinated coffee as the COF beverage. Using a HPLC method, decaffeinated coffee provided minimal caffeine throughout each of the prepared beverages (0.17 mg/kg BM or mean intake of 13.41±0.70mg). In order to blind the participants from the taste of the caffeine trial, the placebo trial consisted of 8mg of Quinine sulphate (Sigma, UK). Quinine sulphate is a food ingredient found in tonic water to give a bitter taste. The quinine sulphate was dissolved in 600 mL of water, vortexed for 15 min, and served in an opaque sports drinks bottle. The exact dose of quinine sulphate was preliminary tested in our lab. A dose of 8 mg was sufficient to prevent the blinded researchers from distinguishing between the placebo and caffeine trial.

The coffee and decaffeinated coffee samples were further analysed externally for chlorogenic acids (5-CQA) (Eurofins Scientific, Italy). Based on the analysis, total 5-CQA was 33.91 mg/g and 28.29 mg/g for COF and DECAF respectively. This was then used to calculate the average concentration of total 5-CQA and related isomers for each participants drinks, based on the weight of instant COF and DECAF. The average chlorogenic acid intake in COF and DECAF are presented in Table 3.1.

All of the beverages were prepared in 600 mL of water. This was firstly to avoid any difference in uptake and bioavailability of each of the ingested ingredients, as well as replicating the format of coffee consumption from everyday life. Secondly 600 mL of coffee dissolved in water was not only considered tolerable, based on taste testing by researchers within our laboratory, but also matched similar volumes as used by Graham et al [216]. Once participants received each of the beverages at the beginning of the trial, they had 15 min to consume the entire 600 mL.

3.3.6 Diet and Exercise Control

Participants were instructed to record their food intake the day prior to the preliminary familiarisation trial. Participants had to replicate this diet in the 24 h prior to each experimental trial, as well as refraining from any exercise, consume no alcohol and withdraw from any caffeinated products.

3.3.7 Calculations

Substrate metabolism was measured during the SS. From the respiratory output measurements of $\dot{V}O_2$ and $\dot{V}CO_2$ (L/min), carbohydrate [1] (CHO) and fat oxidation [2] was calculated every 10 min during the SS. In order to calculate CHO and fat oxidation stoichiometric equations [31] were used, which assume that each of the participants were exercising at a steady state and that protein oxidation was negligible

$$[1] \text{ Carbohydrate oxidation} = 4.210\ \dot{V}CO_2 - 2.962\ \dot{V}O_2$$

$$[2] \text{ Fat Oxidation} = 1.65\ \dot{V}O_2 - 1.701\ \dot{V}CO_2$$

3.3.8 Blood Analysis

Following collection, all tubes were placed in ice until the end of the experimental trial. Following this each tube was centrifuged at 3500 rpm for 15 min at 4°C. Aliquots of plasma were immediately frozen in liquid nitrogen and stored at -80°C for later analysis. Each blood sample taken throughout each experimental trial were analysed for plasma glucose (Glucose Oxidase; Instrumentation Laboratories, England), fatty acids (FA) [NEFA-C; Randox, England], glycerol (Glycerol; Randox, England) and lactate [Lactate, Randox, England] using an ILAB 650 (Instrumentation Laboratory, Cheshire, United Kingdom).

3.3.9 Plasma Caffeine and Chlorogenic Acid analysis

Plasma caffeine were analysed externally (City Hospital, Dudley, Birmingham) using a reversed-HPLC-UV method. The sample preparation included: 200 µL of plasma were added to 100 µL internal standard (Proxyphylline, Sigma, United Kingdom) before mixing, heating and adding 500 µL of acetic acid. The supernatant was the injected (5 µL) onto a Phenomenex Prodigy 150 x 4.60mm 5 µ Octadecyl Silane (ODS) column using an auto sampler and detected at a UV of 273 nm.

Table 3.1: Mean caffeine and chlorogenic acid (Total 5-QCA) concentration in each treatment beverage serving.

Treatment beverage	Serving Volume (mL)	Caffeine content (mg/serving)	Total 5-CQA (mg/serving)	% of Total 5-CQA								
				CQA	5-CQA	4-QCA	5-FQA	4-FQA	3,5-diCQA	3,4-diCQA	4,5-diCQA	4,5-CFQA
CAF	600	394.4±7.0	-	-	-	-	-	-	-	-	-	-
COF	600	394.4±7.0	393.3±7.3	21%	32%	22%	8%	7%	3%	2%	3%	2%
DECAF	600	13.4±0.2	328.1±6.1	23%	33%	24%	7%	7%	3%	1%	2%	1%
PLA	600	-	-	-	-	-	-	-	-	-	-	-

Means ± SE n=8 **Abbreviations: CQA** Caffeoylquinic acid**, 5-QCA** 5-O-Caffeoylquinic acid**, 4-QCA** 4-O-Caffeoylquinic acid**, 5-FQA** 5-O-Feruloylquinic acid**, 4-FQA** 4-O-Feruloylquinic acid**, 3,5-diCQA** 3,5-O-Dicaffeoylquinic acid**, 3,4-diCQA** 3,4-O-Dicaffeoylquinic acid**, 4,5-diCQA** 4,5-O-Dicaffeoylquinic acid**, 4,5-CFQA** 4,5-O-Dicaffeoylquinic acid

Caffeine concentrations were quantified using one point calibration from a calibrator that had previously been internally validated against a 9 point calibration curve (City Hospital, Dudley, Birmingham). With each batch two QC (High and Low) were run, with reference to the internal standard to account for any loses.

The caffeine content of coffee and decaffeinated coffee were confirmed at the School of Sport and Exercise Sciences. In brief, coffee and decaf coffee samples were prepared (identical to preparation described above) and cooled before 5µL of each sample were injected onto a Phenomenex Luna 10 µ C_{18} (2) column using an auto sampler (WPS 3000, Dionex, United Kingdom). The mobile phase consisted of 0.1 M acetic acid in water and 0.1M acetic acid in acetonitrile. Caffeine concentrations were quantified using a 10-point caffeine calibration curve in water.

Coffee and Decaf coffee were analysed for chlorogenic acids. The analysis was conducted externally (Eurofins Scientific, Italy) using a reverse HPLC methodology at 325 nm (Water Symmetry C18, 250 x 4.6 mm, 5 um) with external 5-QCA standards for quantification on a 3 point calibration (10-250 mg/kg). The mobile phase consisted of aqueous 0.5 % formic acid and acetonitrile.

3.3.10 Statistical Analysis

Data analysis was performed using SPSS for WINDOWS software (version 17; SPSS Inc, Chicago, IL). Data are expressed as means ± SEMs, unless otherwise stated. A repeated measure ANOVA was used to assess differences in respiratory, substrate metabolism, plasma metabolite and caffeine concentration as well as time trial performance measurements during each trial. In order to detect differences across time and between treatments a Fisher protected least significant differences post hoc test was used. Significance was set at $P < 0.05$.

3.4 Results

3.4.1 Steady State Exercise

3.4.1.1 Whole body respiratory measures, HR and RPE.

The selected workload of 50% Wmax during the SS (171±7W) resulted in similar oxygen uptake ($\dot{V}O_2$) (2590±79, 2595±89, 2465±79, 2522±71 mL/min for CAF, COF, DECAF and PLA respectively P=0.278). As a result the relative exercise intensity during the SS was similar throughout each trial (58±2%, 58±2%, 55±1% and 55±1% for CAF, COF, DECAF and PLA respectively P=0.337). Energy expended during SS was also shown to be similar (1607±49KJ, 1611±54KJ, 1531±50 KJ, 1565±43) for CAF, COF, DECAF and PLA respectively P=0.248). In addition no significant difference was observed in average HR during exercise (119±4, 119±4, 119±4, 120±5 bpm for CAF, COF, DECAF and PLA respectively P=0.281) or RPE values (10±0, 10±0, 11±0 and 11±0 for CAF, COF, DECAF and PLA respectively P=0.091) during SS between trials.

3.4.1.2 Carbohydrate and Fat oxidation

Carbohydrate oxidation rates during SS significantly reduced in all treatments across time (P=0.001). However, there was no significant difference in carbohydrate oxidation between each of the treatments (Figure 3.1A P=0.288). Similarly, fat oxidation rates significantly increased during SS in all treatments (P=0.001). No significant difference in fat oxidation was observed between each of the treatments (Figure 3.1B P=0.445). Accordingly the contribution of carbohydrate and fat to total energy expenditure during SS was not significantly different between any of the treatments (P=0.463).

3.4.1.3 Plasma metabolite concentrations

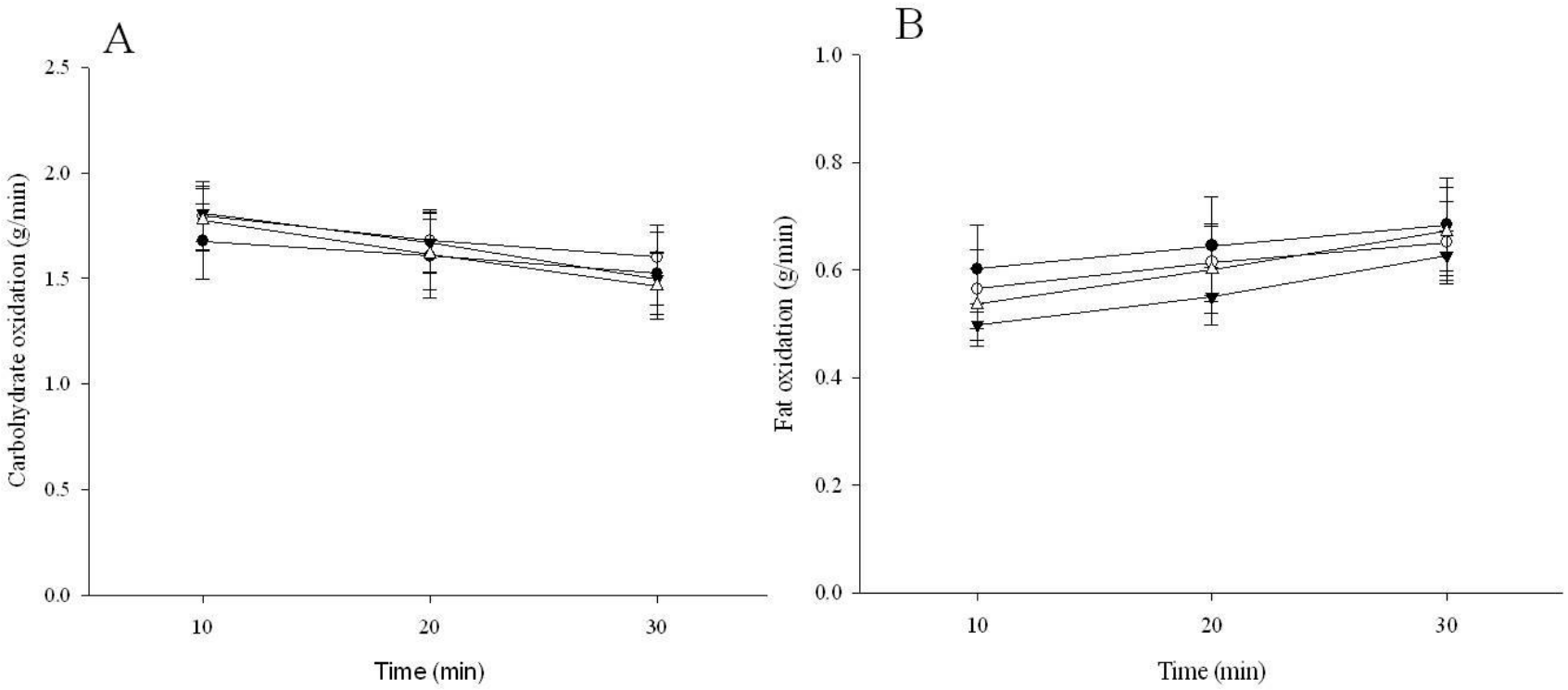
Plasma metabolite responses at rest and exercise are displayed in Figure 3.2 A-D. Plasma glucose concentrations (Figure 3.2A) were significantly elevated at the end of rest compared to the beginning of rest following CAF and COF ($p<0.05$ for both), while no significant difference occurred following DECAF or PLA (P=0.676 and 0.188 respectively). The elevation in glucose concentrations with CAF

following the rest period was significantly higher compared to DECAF only (P<0.05). During exercise, plasma glucose increased over time following CAF and COF, however only COF reached statistical significance (P<0.05). DECAF and PLA glucose concentrations fell during the onset of exercise with a significant increase in both treatments later in exercise (T=10-30 P<0.05 for both). As a result, CAF had significantly higher glucose concentrations within the first 20 minutes of exercise compared to DECAF and PLA (P<0.05 for both), while at end of exercise CAF and COF had significantly higher glucose concentrations compared to PLA only (P<0.05 for both). Plasma FAs concentration (Figure 3.2B) were significantly elevated at the end of rest compared to beginning of rest following CAF (P=0.010), while DECAF and PLA had reduced FA concentration, with only DECAF reaching statistical significance (P=0.007 and P=0.072 respectively). Therefore, CAF had significantly higher FAs concentration compared to DECAF and PLA at end of rest period (P<0.05 for both). During the beginning of exercise, CAF continued to have significantly elevated FAs concentration compared to DECAF only (P=0.037). FA concentration significantly increased during exercise (T=10-30 P=0.030) with no significant differences observed between treatments (P=0.231).

Plasma glycerol concentrations (Figure 3.2C) did not significantly change at rest for CAF (P=0.066), COF (P=0.392) and DECAF (P=0.104) but PLA significantly fell (P=0.022). DECAF was significantly lower at end of rest compared to CAF, COF and PLA (P<0.05 for all), with no significant differences observed between any other beverage. During exercise there was a significant increase in glycerol concentrations for all treatments over time (P=0.001), with significantly higher concentrations observed for CAF (P=0.027) and COF (P=0.003) at beginning of exercise compared to DECAF only.

Plasma lactate concentrations (Figure 3.2D), were significantly increased following the consumption of CAF and COF only during the rest period compared to DECAF (P=0.050 and P=0.003 respectively) and PLA (P=0.002 and P=0.001 respectively). In addition DECAF had significantly elevated lactate compared to PLA at end of rest period (P=0.012).

Figure 3.1: Carbohydrate oxidation (g/min) (A) and fat oxidation (g/min) (B) rates during 30 min steady state exercise (55% VO2 max) 1 hour following ingestion of caffeine, coffee, decaf or placebo beverages



Data represented seen as Closed circles – Caffeine Open circles – Caffeinated Coffee Closed triangles – Decaffeinated coffee Open triangles – Placebo. Means ± SE n = 8

All treatments lactate concentrations significantly increased at the onset of exercise (P=0.004) with CAF and COF being significantly higher compared to PLA (P=0.037 and P=0.010 respectively). CAF and COF had sustained lactate concentrations at the end of exercise, with significantly higher concentrations compared to DECAF (P=0.008 and P=0.028 respectively) and PLA (P=0.050 and P=0.005 respectively).

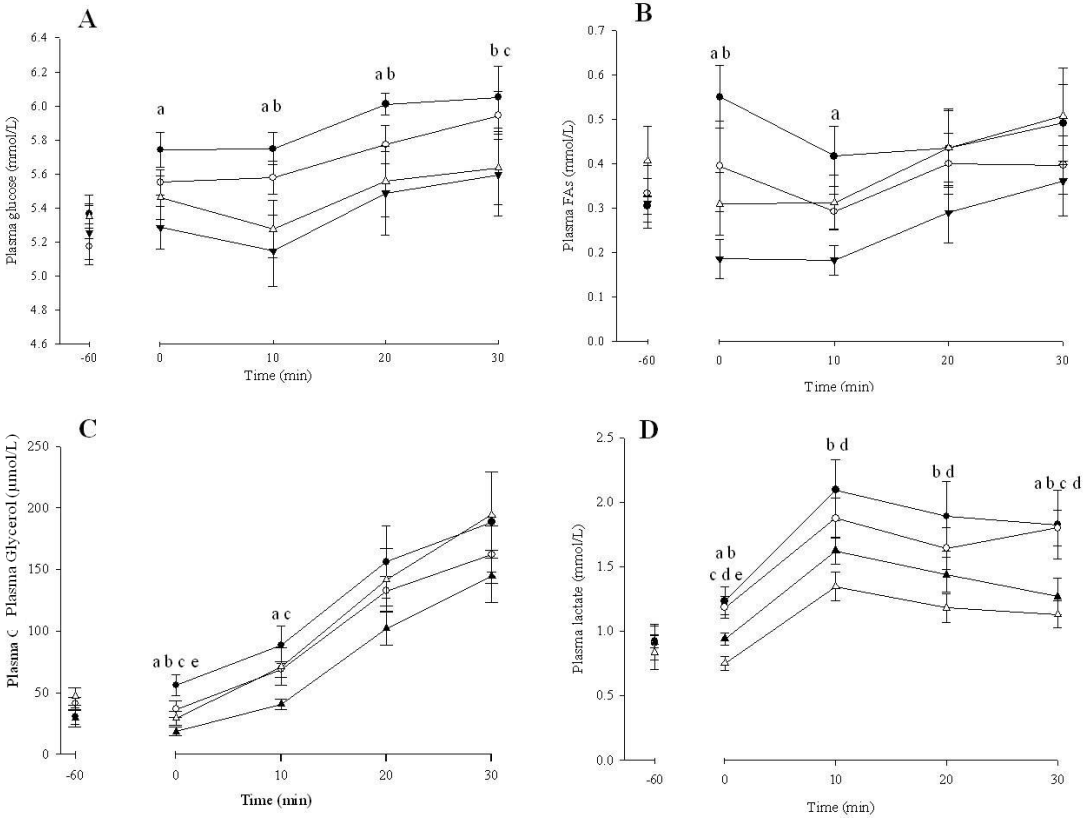
3.4.2 Plasma caffeine concentrations

The plasma caffeine concentrations following each beverage are displayed in Figure 3.3. At baseline plasma caffeine concentrations were very low for all treatments (<3 µM), with no significant differences observed (P=0.478). Plasma caffeine significantly increased following CAF and COF when compared to DECAF (P=0.000 and P=0.009 respectively) and PLA (P=0.000 and P= 0.010 respectively), with peak concentrations observed 60 min after intake (38.2±2.8 µM and 33.5±5.0 µM respectively). No significant difference was observed in the plasma caffeine concentrations between CAF or COF (P=0.156) and DECAF or PLA (P=0.558) throughout the trials

3.4.3 Time trial performance

CAF and COF significantly improved TT finishing times when compared to both DECAF and PLA (Figure 3.4). As a result mean power output during the TT was significantly greater for both CAF and COF compared to DECAF and PLA (294±6, 291±7, 276±7, 277±4 W, respectively). However, no significant differences were seen in average heart rate during the TT (170±3, 167±4, 164±3, 165±4 BPM, respectively). CAF significantly improved TT performance by 4.9 % (95% confidence interval (CI) = 2.3-6.8 %) and 4.5 % (95% confidence interval (CI) = 2.3-6.2 %) compared to PLA and DECAF respectively ($p<0.05$). Equally, COF significantly improved TT performance by 4.7% (95% confidence interval (CI) = 2.3-6.7 %) and 4.3 % (95 % confidence interval (CI) = 2.5-7.1 %) compared to PLA and DECAF respectively ($p<0.05$) (Table 3.2). In addition there were no significant differences in TT performance between CAF and COF or PLA and DECAF.

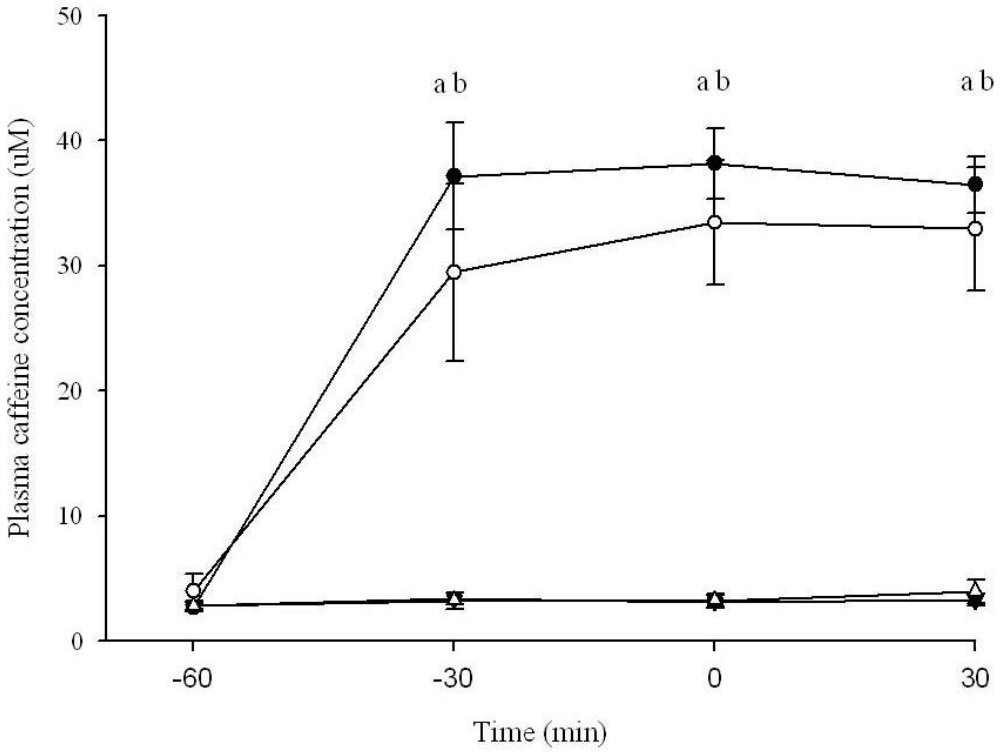
Figure 3.2: Plasma metabolite responses at rest (t=-60-0) and during 30 min steady state exercise (55 % VO_2 max) (t=0-30) following ingestion of caffeine, coffee, decaf or placebo beverages.



A Glucose. **B** Fatty acids (FA). **C** Glycerol. **D** Lactate. Data represented seen as Closed circles – Caffeine Open circles – Caffeinated Coffee Closed triangles – Decaffeinated coffee Open triangles – Placebo. a Sig. different between CAF and DECAF ($p<0.05$) b Sig. different between CAF and PLA ($p<0.05$) c Sig. different between COF and DECAF ($p<0.05$) d Sig. different between COF and PLA ($p<0.05$). Means ± SE n = 7

.

Figure 3.3: Plasma caffeine concentrations following ingestion of caffeine, coffee, decaf or placebo beverages.



a CAF significantly different to DECAF and PLA ($p<0.001$) b COF significantly different to DECAF and PLA ($p<0.001$). Data represented seen as Closed circles – Caffeine Open circles – Caffeinated Coffee Closed triangles – Decaffeinated coffee Open triangles – Placebo Means ± SE n = 7.

Following the completion of the TT, 3/8 participants were able to successfully guess the correct order of test beverages consumed prior to the trial. The correct guesses were more consistent for detecting CAF compared to the other drinks, with 6/8 of the participants guessing correctly. None of the participants reported any serious symptoms of GI distress at the end of any of the trials.

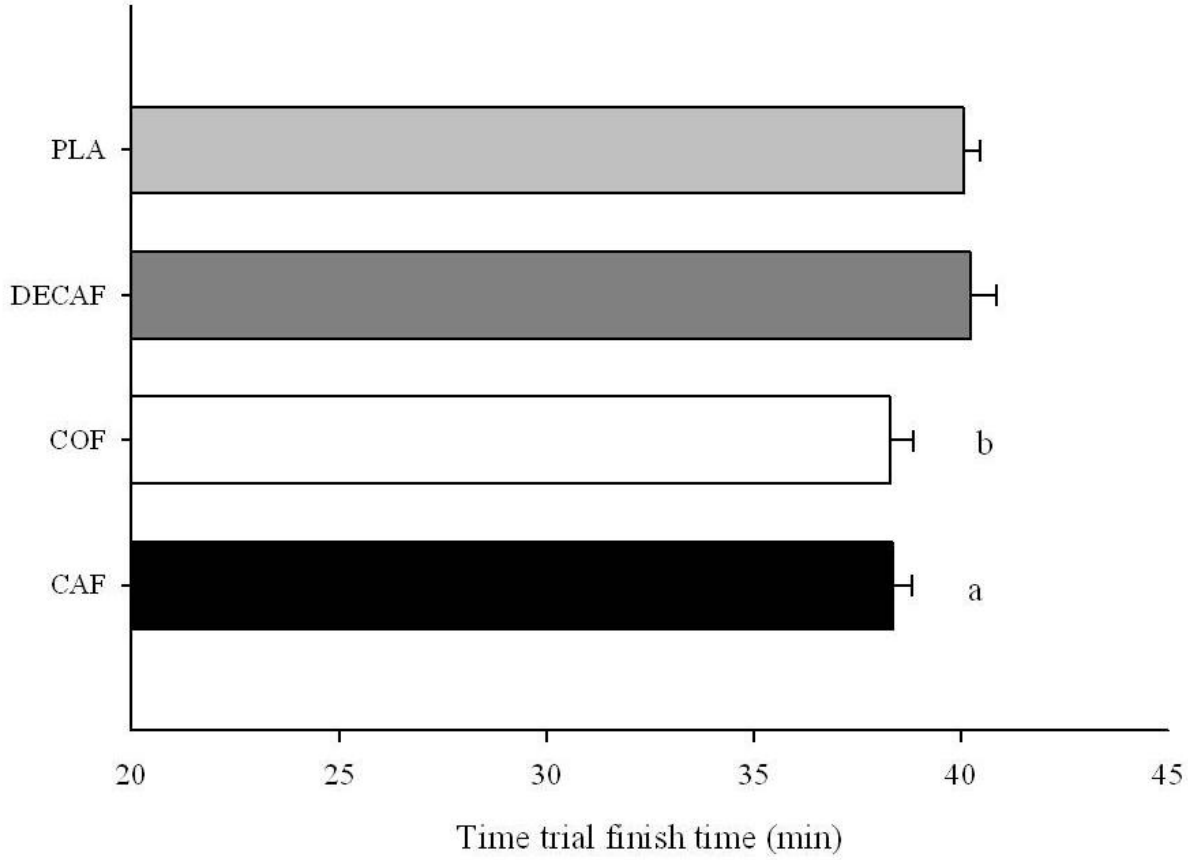
Table 3.2: Time trial performance data for each treatment. Means ± SE n = 8 **a** significantly different to DECAF and PLA ($p<0.05$) **b** significantly different to DECAF and PLA ($p<0.05$)

Treatment	TT finish time (min)	Improvement compared to PLA % (95 % confidence intervals)	P value	Improvement compared to DECAF % (95 % confidence intervals)	P value
CAF	38.35±0.48[a]	4.9 (2.3-6.8)	0.001	4.5 (2.3-6.2)	0.002
COF	38.27±0.57[b]	4.7 (2.3-6.7)	0.002	4.3 (2.5-7.1)	0.002
DECAF	40.23±0.63	-0.4 (-4.0-3.1)	0.784	-	-
PLA	40.06±0.39	-	-	0.3(-0.3-3.9)	0.784

3.5 Discussion

The present study examined the effects of acute intake of coffee (5 mg/kg BM) and caffeine (5 mg/kg BM) on time trial cycling performance, as well as substrate utilisation during SS exercise. Numerous studies to date have shown the efficacy of acute caffeine ingestion for improving prolonged endurance exercise performance [1-7]. The effects of caffeine on time trial endurance performance (>5 min) have recently been reviewed in a well conducted meta-analysis [7]. The authors concluded that of the 12 studies that investigated caffeine intake (1-6 mg/kg BM), performance was improved by ~3%. Fewer studies have investigated the ergogenic effects of coffee, with results being mixed thus far. In agreement with the literature, the current study found an improvement in performance following caffeine intake of 4.9% and 4.5% when compared to decaf coffee and placebo, respectively (Table 3.2).

Figure 3.4: Time trial finishing time (min) for caffeine, coffee, decaf or placebo beverages



a CAF significantly different to DECAF and PLA ($p<0.05$) b COF significantly different to DECAF and PLA ($p<0.05$). Data represented seen as Closed bar– Caffeine Open bar – Caffeinated Coffee Dark grey bar– Decaffeinated coffee Light grey bar– Placebo. Means ± SE n = 8.

Interestingly, the current study also showed that coffee improved performance to the same extent as caffeine when compared to decaf coffee and placebo, 4.7 % and 4.3 % respectively. Thus, this is the first study to date to demonstrate that coffee consumed 1 h prior to exercise, at a high caffeine dose (5 mg/kg BM), is equally as effective as caffeine at improving endurance exercise performance. Our findings are in line with a number of studies that have shown improvements to performance following coffee intake [9,19-21]. Costill et al [9] were the first to show that decaf coffee plus caffeine (330 mg), improved exercise time to exhaustion (80 % VO_2 max) compared with decaffeinated coffee (~18 %). More recently, Wiles et al [19] showed that coffee was able to improve 1500 m treadmill running performance when compared to decaffeinated coffee (~3 %). However, the current study results are in contrast to a number of other studies [22-24]. For example, the work conducted by Graham et al [22] showed that coffee (4.5 mg/kg BM), regardless of the format of intake (regular coffee or decaffeinated coffee plus caffeine) did not result in an improvement to running time to exhaustion (75 % VO_2 max), where as caffeine (4.5 mg/kg BM) significantly improved performance. Therefore, it was concluded by Graham et al [22] that the performance effects of coffee may be inferior to caffeine. Despite this evidence, the current study clearly demonstrates that coffee is as effective as caffeine at improving endurance exercise performance.

The discrepancy in the performance effects of caffeine and coffee between the present study and Graham et al [22] might be explained by the type of performance test implemented. Time to exhaustion tests have been shown to be highly variable from day to day, with a coefficient of variation (CV) ~27 % in one study [18]. It is possible that this large variability may have contributed to the lack of performance effects found by Graham et al [22]. Whereas using a time trial performance measure, as used in the current study, has previously been shown to be highly reproducible (CV ~ 3 %) and could detect smaller differences in performance [18]. Also the number of comparisons in the study by Graham et al [22] was greater than in the present study with a similar subject number, indicating that their statistical power was smaller. Perhaps for these reasons, the current study was able to detect

similar changes in performance following caffeine and coffee intake (~5 %) (Table 3.2), whereas Graham et al [22] did not.

The composition and preparation of coffee in each of the studies [9,19-24] may also explain the discrepancies in the ergogenic effects of coffee. Coffee is ~2% caffeine, with the remainder composed of chlorogenic acids, ferulic acid, caffeic acid, nicotinic acid as well as other unidentifiable compounds [26]. It is evident that the source of coffee beans, roasting, storage and preparation (brewing and filtering) dramatically alters the caffeine and chlorogenic acid content of the coffee [26]. In accordance, recent evidence has shown that the chlorogenic acid content of commercially available espresso coffees range from 24-422 mg/serving [26]. In support, the current study observes a high chlorogenic acid content in both coffee and decaffeinated coffee samples (Table 3.1). Graham et al [22] speculated that chlorogenic acids found in coffee may have blunted the physiological effects of caffeine, preventing an improvement in exercise performance. However, the authors did not report measurements of chlorogenic acids in coffee or in plasma to support this speculation. Despite the compounds present in coffee, the authors reported that the bioavailability of plasma caffeine and paraxanthines did not differ to caffeine [22], which is in line with the present study (Figure 3.4). Further, *in vitro* studies suggest that chlorogenic acids antagonize adenosine receptor binding of caffeine [27] and cause blunting to heart rate and blood pressure in rats [28]. Yet, *in vivo* there is no evidence to suggest that chlorogenic acids, especially at the low nanomolar concentration typically observed [32], impact on the mechanisms of action of caffeine that lead to the ergogenic effects. In support of this notion, and in agreement with the current study, regular coffee (1.1 mg/kg BM) consumed prior to the ingestion of different doses of caffeine (3-7 mg/kg BM) has been shown not to affect the ergogenic effects of caffeine [21].

The improvement in performance in the current study is unlikely to be explained by alterations to fat oxidation, as no difference during the SS exercise bout was observed (Figure 3.1B). This is in agreement with a number of investigations that do not support the thesis that caffeine improves exercise performance by augmenting fat metabolism [33,34]. In addition these effects are apparent

despite consistent increases in adrenaline though activation of the SNS [33,34] and a subsequent elevation in FA appearance in the circulation following caffeine intake [33,34]. It is evident that the improvement in performance is likely through caffeine's direct antagonism of adenosine receptors (A_1 and A_{2A}) on the skeletal muscle membrane to improve excitation-contraction coupling [13] via a greater release of Ca^{2+} from the SR [16] and/or improved Na^+/K^+ ATPase pump activity [15]. In support of this notion, Mohr et al [12] observed that tetrapelegic patients, who have an impaired sympathoadrenal response [35], showed that caffeine improved exercise performance, while RER did not change during an electrically stimulated cycling test. Further, the authors also observed a significant increase in FA and glycerol at rest and during exercise following caffeine intake, despite a lack of an adrenaline response. This is due to the fact that adenosine has been shown to inhibit lipolysis [36] and enhance insulin stimulated glucose uptake in contracting skeletal muscle *in vitro* [37]. In support, the current study observed a significant increase in plasma glucose, FA and glycerol concentrations following caffeine (Figure 3.2 A, B, C). In addition, the consistently reported elevation in adrenaline concentrations [33] combined with adenosine receptor antagonism following caffeine intake during exercise may work synergistically to activate glycogenolysis in exercising and non-exercising tissues [34] as well as adipose tissue/skeletal muscle lipolysis [33]. This supports the fact that the current study (Figure 3.2 D) and others have shown that caffeine increases plasma lactate concentrations at rest and during exercise [33,34]. Though to date there is little supporting evidence that caffeine stimulates exercising skeletal muscle glycogenolysis [33,38], with early studies showing a paradoxical glycogen sparing effect with caffeine [2]. The elevated lactate concentrations are more likely due to a reduced clearance by the exercising muscle and a greater release by non exercising tissues [33]. Consequently, due to the healthy participants tested in the current study it is likely that the adenosine receptor antagonism by caffeine plays a crucial role in inducing the ergogenic effects of caffeine while regulating the metabolite response synergistically with the SNS.

Interestingly, despite coffee producing similar ergogenic effects as caffeine, the metabolite responses were not identical (Figure 3.2). The current study observed that the significant increase in plasma

glucose, FA and glycerol with caffeine was paralleled with an attenuated response for coffee, and a significantly blunted response with decaf coffee when compared to placebo (Figure 3.2 A, B, C). This is likely due to the compounds in coffee [39] inducing subtle effects on antagonism of adenosine receptors (A_1 and A_{2A}) in a variety of exercising and non exercising tissues. In accordance, Graham et al [22] previously showed that coffee resulted in a blunted adrenaline response when compared to caffeine at rest in humans, which was attributed to chlorogenic acids antagonizing adenosine receptor binding of caffeine [27]. In addition nicotinic acid, a fatty acid ester found in coffee known to inhibit lipolysis, has been shown to lower FA concentrations in patients suffering from hyperlipidemia [40]. Chlorogenic acids are also believed to improve glucose uptake at the skeletal muscle when compared to caffeine [41], also by altering the antagonism of adenosine receptors. More recently, caffeic acid has been found to stimulate skeletal muscle glucose transport, independent of insulin, when accompanied with an elevation in AMPK *in vitro* [42]. Despite the aforementioned evidence, it remains unclear why compounds in coffee appear to modulate the metabolite response but not the ergogenic effects of coffee in the current study.

The current study provided a large bolus of caffeine in the form of anhydrous caffeine or coffee one hour prior to exercise (5 mg/kg BM). The chlorogenic acid content of the coffee beverages was different, which is worth highlighting as a potential limitation of the current study. Previous studies have failed to make comparisons between coffee and decaf coffee and instead have used decaf plus anhydrous caffeine [9,19-21,23,43]. In addition these studies did not examine the chlorogenic acid content of the test beverages. Thus, the novelty of the current study was that the performance effects were investigated between caffeine and coffee, independent of the combined effects of decaffeinated coffee plus caffeine. Adding a decaf plus caffeine trial would have been successful in controlling for chlorogenic acid content of the beverage. However, firstly, investigating the effect of chlorogenic acids on the metabolic and performance effects of caffeine was not the primary aim of the current study. Secondly, and more importantly, the low nanomolar concentration of chlorogenic acids *in vivo* [32] is unlikely to impact on the mechanisms of action of caffeine when compared to the physiological

effects observed *in vitro* from supra physiological concentrations of chlorogenic acids [27,28]. Yet, as differences in the metabolic effects of caffeine compared to coffee were observed in the current study, it may be important for future studies to control for chlorogenic acid content in coffee beverages or additionally increase the dose of chlorogenic acids to raise the bioavailability *in vivo*. In turn this will provide further insights into the metabolic differences between caffeine and coffee. In conclusion, the present study showed that caffeine and coffee (5 mg/kg BM) were both able to improve exercise performance to the same extent, when compared to both decaffeinated coffee and placebo. Our data does not support the notion that chlorogenic acids found in coffee impair the ergogenic effects of caffeine. However, the compounds found in coffee may alter the metabolic effects, as the current study observed differences between caffeine and coffee at rest and during exercise. It is yet to be determined if lower doses of caffeine, when ingested as coffee, offer the same ergogenic effects. This would offer a more applicable and realistic nutritional strategy for athletes.

3.6 References

1. Graham TE, Spriet LL (1995) Metabolic, catecholamine, and exercise performance responses to various doses of caffeine. J Appl Physiol 78: 867-874.
2. Spriet LL, MacLean DA, Dyck DJ, Hultman E, Cederblad G, et al. (1992) Caffeine ingestion and muscle metabolism during prolonged exercise in humans. Am J Physiol 262: E891-898.
3. Jenkins NT, Trilk JL, Singhal A, O'Connor PJ, Cureton KJ (2008) Ergogenic effects of low doses of caffeine on cycling performance. Int J Sport Nutr Exerc Metab 18: 328-342.
4. Irwin C, Desbrow B, Ellis A, O'Keeffe B, Grant G, et al. (2011) Caffeine withdrawal and high-intensity endurance cycling performance. J Sports Sci 29: 509-515.
5. Desbrow B, Biddulph C, Devlin B, Grant GD, Anoopkumar-Dukie S, et al. (2012) The effects of different doses of caffeine on endurance cycling time trial performance. J Sports Sci 30: 115-120.
6. Doherty M, Smith PM (2004) Effects of caffeine ingestion on exercise testing: a meta-analysis. Int J Sport Nutr Exerc Metab 14: 626-646.
7. Ganio MS, Klau JF, Casa DJ, Armstrong LE, Maresh CM (2009) Effect of caffeine on sport-specific endurance performance: a systematic review. J Strength Cond Res 23: 315-324.
8. Cox GR, Desbrow B, Montgomery PG, Anderson ME, Bruce CR, et al. (2002) Effect of different protocols of caffeine intake on metabolism and endurance performance. J Appl Physiol 93: 990-999.
9. Costill DL, Dalsky GP, Fink WJ (1978) Effects of caffeine ingestion on metabolism and exercise performance. Med Sci Sports 10: 155-158.
10. Chesley A, Howlett RA, Heigenhauser GJ, Hultman E, Spriet LL (1998) Regulation of muscle glycogenolytic flux during intense aerobic exercise after caffeine ingestion. Am J Physiol 275: R596-603.
11. Graham TE, Spriet LL (1991) Performance and metabolic responses to a high caffeine dose during prolonged exercise. J Appl Physiol 71: 2292-2298.
12. Mohr T, Van Soeren M, Graham TE, Kjaer M (1998) Caffeine ingestion and metabolic responses of tetraplegic humans during electrical cycling. J Appl Physiol 85: 979-985.
13. Tarnopolsky MA (2008) Effect of caffeine on the neuromuscular system--potential as an ergogenic aid. Appl Physiol Nutri Metab 33: 1284-1289.
14. Doherty M, Smith PM (2005) Effects of caffeine ingestion on rating of perceived exertion during and after exercise: a meta-analysis. Scand J Med Sci Sports 15: 69-78.
15. Mohr M, Nielsen JJ, Bangsbo J (2011) Caffeine intake improves intense intermittent exercise performance and reduces muscle interstitial potassium accumulation. J Appl Physiol 111: 1372-1379.
16. Tallis J, James RS, Cox VM, Duncan MJ (2012) The effect of physiological concentrations of caffeine on the power output of maximally and submaximally stimulated mouse EDL (fast) and soleus (slow) muscle. J Appl Physiol 112: 64-71.
17. Tarnopolsky M, Cupido C (2000) Caffeine potentiates low frequency skeletal muscle force in habitual and nonhabitual caffeine consumers. J Appl Physiol 89: 1719-1724.
18. Jeukendrup A, Saris WH, Brouns F, Kester AD (1996) A new validated endurance performance test. Med Sci Sports Exerc 28: 266-270.
19. Wiles JD, Bird SR, Hopkins J, Riley M (1992) Effect of caffeinated coffee on running speed, respiratory factors, blood lactate and perceived exertion during 1500-m treadmill running. Br J Sports Med 26: 116-120.
20. Trice I, Haymes EM (1995) Effects of caffeine ingestion on exercise-induced changes during high-intensity, intermittent exercise. Int J Sport Nutri 5: 37-44.

21. McLellan TM, Bell DG (2004) The impact of prior coffee consumption on the subsequent ergogenic effect of anhydrous caffeine. Int J Sport Nutr Exerc Metab 14: 698-708.
22. Graham TE, Hibbert E, Sathasivam P (1998) Metabolic and exercise endurance effects of coffee and caffeine ingestion. J Appl Physiol 85: 883-889.
23. Lamina S, Musa DI (2009) Ergogenic effect of varied doses of coffee-caffeine on maximal aerobic power of young African subjects. African health sciences 9: 270-274.
24. Butts N, D. C (1985) Effect of caffeine ingestion on cardiorespiratory endurance in men and women. Res Q Exerc Sport 56: 301-305.
25. Desbrow B, Leveritt M (2006) Awareness and use of caffeine by athletes competing at the 2005 Ironman Triathlon World Championships. Int J Sport Nutr Exerc Metab 16: 545-558.
26. Crozier TW, Stalmach A, Lean ME, Crozier A (2012) Espresso coffees, caffeine and chlorogenic acid intake: potential health implications. Food Funct 3: 30-33.
27. de Paulis T, Schmidt DE, Bruchey AK, Kirby MT, McDonald MP, et al. (2002) Dicinnamoylquinides in roasted coffee inhibit the human adenosine transporter. European journal of pharmacology 442: 215-223.
28. Tse SY (1992) Cholinomimetic compound distinct from caffeine contained in coffee. II: Muscarinic actions. Journal of pharmaceutical sciences 81: 449-452.
29. Currell K, Jeukendrup AE (2008) Validity, reliability and sensitivity of measures of sporting performance. Sports medicine 38: 297-316.
30. Borg G (1982) Psychophysical bases of perceived exertion. Med Sci Sports 14: 377-381.
31. Jeukendrup AE, Wallis GA (2005) Measurement of substrate oxidation during exercise by means of gas exchange measurements. Int J Sports Med 26 Suppl 1: S28-37.
32. Stalmach A (2012) Bioavailability of Coffee Chlorogenic Acids, in Coffee: Emerging Health Effects and Disease Prevention (ed Y.-F. Chu). Wiley-Blackwell, Oxford, UK
33. Graham TE, Helge JW, MacLean DA, Kiens B, Richter EA (2000) Caffeine ingestion does not alter carbohydrate or fat metabolism in human skeletal muscle during exercise. J Physiol 529 Pt 3: 837-847.
34. Raguso CA, Coggan AR, Sidossis LS, Gastaldelli A, Wolfe RR (1996) Effect of theophylline on substrate metabolism during exercise. Metabolism: clinical and experimental 45: 1153-1160.
35. Van Soeren M, Mohr T, Kjaer M, Graham TE (1996) Acute effects of caffeine ingestion at rest in humans with impaired epinephrine responses. J Appl Physiol 80: 999-1005.
36. Kather H, Bieger W, Michel G, Aktories K, Jakobs KH (1985) Human fat cell lipolysis is primarily regulated by inhibitory modulators acting through distinct mechanisms. The Journal of clinical investigation 76: 1559-1565.
37. Vergauwen L, Hespel P, Richter EA (1994) Adenosine receptors mediate synergistic stimulation of glucose uptake and transport by insulin and by contractions in rat skeletal muscle. J Clin Invest 93: 974-981.
38. Graham TE, Battram DS, Dela F, El-Sohemy A, Thong FS (2008) Does caffeine alter muscle carbohydrate and fat metabolism during exercise? Appl Physiol Nutri Metab 33: 1311-1318.
39. Beaudoin MS, Graham TE (2011) Methylxanthines and human health: epidemiological and experimental evidence. Handbook of experimental pharmacology: 509-548.
40. Wahlberg G, Walldius G (1992) Effects of nicotinic acid treatment on fatty acid composition of plasma lipids and adipose tissue in hyperlipidaemia. Scandinavian journal of clinical and laboratory investigation 52: 547-553.
41. Ong KW, Hsu A, Tan BK (2012) Chlorogenic acid stimulates glucose transport in skeletal muscle via AMPK activation: a contributor to the beneficial effects of coffee on diabetes. PloS one 7: e32718.

42. Tsuda S, Egawa T, Ma X, Oshima R, Kurogi E, et al. (2012) Coffee polyphenol caffeic acid but not chlorogenic acid increases 5'AMP-activated protein kinase and insulin-independent glucose transport in rat skeletal muscle. Journal of Nutritional Biochemistry.
43. Butts N, Crowell D (1985) Effect of caffeine ingestion on cardiorespiratory endurance in men and women. Res Q Exerc Sport 56: 301-305.

Chapter 4

High prevalence of vitamin D deficiency and insufficiency in university athletes living in the United Kingdom.

Paper in submission

Adrian B. Hodgson†, Gurimder Sanghera†, Thomas Hall†, Sarah Aldred†.

European Journal of Sport Science

A.B.H Conceived and designed the experiment, performed the experiment, interpreted the data and wrote the manuscript, G.S and T.H Assisted in the data collection. S.A Assisted with designing the experiment.

† School of Sport and Exercise Sciences, University of Birmingham, United Kingdom

4.1 Abstract

Background: The current study aims were threefold: 1) investigate vitamin D status in athletes living in the United Kingdom (52.4-52.5°N), 2) identify the potential sources and limitations of vitamin D status in athletes and 3) examine the effect vitamin D status may have on muscle function. **Methods:** Forty-eight indoor and outdoor athletes from a variety of sports were recruited during the winter. Vitamin D status (25(OH)D) was assessed from a single blood sample, followed by completion of a questionnaire pack that assessed sun exposure and vitamin D intake. In addition muscle function was assessed using a mechanography vertical jump and hand grip maximum voluntary contraction. **Results:** Mean 25(OH)D was 51±24 nmol/L with 10%, 44%, 29% and 17% of athletes being severely vitamin D deficient (<25 nmol/L), deficient (<50 nmol/L), insufficient (50-75 nmol/L) and sufficient (>75 nmol/L), respectively. No significant differences in 25(OH)D were observed between gender or indoor and outdoor athletes. No correlation was found between 25(OH)D and time spent outdoors during training or leisure time, while a positive association was found between 25(OH)D and vitamin D supplement intake ($r=0.353$ $p<0.005$) but not dietary intake. Further, vitamin D status had no association with muscle function performance. **Conclusions:** The current study illustrates a high prevalence of vitamin D deficiency in UK athletes during the winter. Reported seasonal sun exposure and dietary vitamin D intake had little effect on winter vitamin D status. Future work should explore the efficacy of vitamin D supplements as an effective nutritional intervention to correct vitamin D deficiency during the winter.

4.2 Introduction

Vitamin D is a secosteroid traditionally involved in optimal bone health which is obtained through endogenous cutaneous synthesis from ultraviolet radiation (UVB) as well as from dietary sources (oily fish, eggs, butter and fortified foods) [1]. Vitamin D is also critical in the maintenance of optimal skeletal muscle function, whereby myopathy [2] and neuromuscular impairments [3] are prominent features in children and adults with severe vitamin D deficiency (clinical measure of vitamin D, 25-hydroxyvitamin D (25(OH)D) < 25nmol/L). The role of vitamin D in the skeletal muscle is confirmed by the vitamin D receptor (VDR) being abundantly expressed in the skeletal muscle [4], allowing the active form of vitamin D ($1,25(OH)_2D_3$) to regulate over 1000 vitamin D responsive human genes [5].

Alarmingly, accumulating evidence has shown a high prevalence of worldwide vitamin D deficiency [6] especially at high latitudes, such as in the United Kingdom (UK) [7]. This suggests that the deleterious effects of vitamin D deficiency on skeletal muscle function are possibly prominent in our population today. In support of this notion, numerous studies to date have shown that vitamin D deficiency (< 50 nmol/L) in elderly men and women are associated with a greater risk of falls [8], development of fatigue [9], and reduced skeletal muscle strength [10]. In addition, skeletal muscle strength and function has also been shown to be negatively associated with vitamin D status in healthy adolescent girls [11]. Moreover, vitamin D supplementation has shown promise as a nutritional support in the correction of vitamin D deficiency [12] and the observed impairment to skeletal muscle function in the elderly.

A growing number of studies have begun to assess the vitamin D status in athletes worldwide. Collectively these studies show a high prevalence of vitamin D deficiency in athletes worldwide [13-17]. Hamilton et al 2010 reported that athletes who live and train in Qatar had a high prevalence of vitamin D deficiency (~90%) despite the sunny environment and low latitude. Close et al [14] has recently shown that outdoor athletes from a variety of sports residing from Liverpool, UK (53.4^0N) display a high prevalence of vitamin D deficiency (~60%). However, to date a complete understanding

of the extent of the prevalence of vitamin D deficiency in UK athletes remains unclear. Despite this, these findings are of great concern and relevance for optimal athletic performance.

The factors that compromise vitamin D intake include poor dietary sources as well as factors that limit endogenous cutaneous synthesis from the sun. These include: poor UVB at high latitudes and in cloudy climates, dark skin pigmentation, use of sun lotion, clothing, time of day, and time spent outdoors [1]. For these reasons, the high prevalence of vitamin D deficiency in athletes is likely explained by those who live and train indoors as well as at high latitudes and poor climates, as observed in the UK (50-60^{o}N) [18]. Yet while athletes have been shown to consume sufficient quantities of many micronutrients (based on reference nutrient intake (RNI)), it appears that athletes consume vitamin D well below the RNI (200 IU/d in the UK, 5 µg/d) [19]. This would suggest that athletes may benefit from vitamin D supplementation to avoid deficiency. However, the estimation of vitamin D intake (sun, food and supplements) in athletes is largely unknown.

Interestingly, despite the importance of vitamin D in the human body, the categorisation of an optimal 25(OH)D concentrations remains under continued debate. The recent Institute of Medicine (IOM) report states that inadequate vitamin D status is defined at 25(OH)D <50 nmol/L [20], while the Scientific Advisory Committee on Nutrition (SACN) in the UK define vitamin D deficiency as 25(OH)D <25 nmol/L [21]. These guidelines are solely based on the benefits of vitamin D on bone health [22]. However, they have received a great deal of criticism, with other experts defining sufficient vitamin D status as >75 nmol/L [23] which include the benefits to skeletal muscle function and not just bone health. In contrast, the Endocrinology Society [24] state that vitamin D status is deficient, insufficient and sufficient when 25(OH)D concentrations are <50 nmol/L, 50-75 nmol/L and > 75 nmol/L respectively. In support, benefits to skeletal muscle strength and performance in the elderly have been observed at 25(OH)D concentrations >100 nmol/L [25] whereas in athletes benefits to exercise performance have recently been observed when 25(OH)D was increased >75 nmol/L (range 75-139 nmol/L) following vitamin D supplementation [14]. Currently, the majority of evidence

has been documented in the elderly with an optimal vitamin D status required to elicit benefits to skeletal muscle function still being unknown.

Hence, the aim of the current study was three fold. Firstly, to assess whether or not a high prevalence of vitamin D deficiency in university UK athletes, at the current test site (high latitude 52.4-52.5°N**)** during the winter (December-March), was comparable to the current trends in athletes to date. In an attempt to account for the differing vitamin D status guidelines, the current study categorised severe vitamin D deficiency, deficiency, insufficiency and sufficiency as serum total 25(OH)D of <25 nmol/L, <50 nmol/L, 50-75 nmol/L and >75 nmol/L, respectively. Secondly, the study also aimed to estimate vitamin D intake (sun, food and supplements) and assess the contribution of intake to vitamin D status. Finally the current study aimed to provide preliminary insight into the association between 25(OH)D and skeletal muscle function in a small number of athletes, when using basic assessments of skeletal muscle function.

4.3 Method

4.3.1 Study Participants

Fifty male and female athletes (Mean ± SD, age: 25 ± 8 y, height: 1.75 ± 0.08 m, weight: 73.8 ± 13.6 kg, body mass index (BMI) 27.3 ± 3.8 kg/m^2) were recruited from the University of Birmingham, Birmingham in the UK (52.4-52.5°N) between December and March. To be eligible to participate, participants were required to have trained in a specific sport for at least 2 years and train at least twice per week, for 60-90 minutes per session. The study was approved by the University of Birmingham's Science, technology engineering and mathematics ethical review committee.

4.3.2 Experimental trial

4.3.2.1 Vitamin D status

Participants arrived at the laboratory following a 3 h fast between 0800 and 1600 where height (Seca stadiometer, UK) and weight (OHaus, Champ II scales, USA) were recorded. A resting venous blood

sample (Venisystem Butterfly, NU Care, UK) was taken from each participant, which was immediately placed into silica-polymer gel tubes (Vacutainer, BD, USA) and left at room temperature for 1 h to clot. Samples were then centrifuged at 3500rpm and 4°C for 15 min, before serum was extracted and stored at -80°C. Serum total 25(OH)D was later analysed using a 25-OH vit D enzymeimmunoassay (Immunodiagnostik Systems) which measures both $25(OH)D_2$ and $25(OH)D_3$ together. The intra and inter assay coefficient of variation (%CV) was 4.7 %CV and 8.4 %CV across a working range of 6.8-380 nmol/L. This assay has been shown to be comparable to other methodologies as approved by the Vitamin D External Quality Assessment Scheme (DEQAS www.deqas.org [26].

Following this, each participant completed a questionnaire pack which estimated sources and limitations of vitamin D. Sun exposure was estimated by asking each participant whether they were an indoor or outdoor athlete as well as estimating sun exposure by the amount of time they spent outdoors in each season across the previous year for training/competing and in leisure time (h/wk). This was estimated from the frequency (d/wk), duration (<30 min, 1 h, 1-3 h, 3-5 h or > 5 h) and time of day (early morning, mid morning, lunchtime, afternoon or evening) spent outdoors for each season.

Dietary vitamin D intake was estimated using an adapted food frequency questionnaire [27]. A list of common vitamin D rich foods found in the UK diet and other less common foods were listed. Participants were asked to state how often they consumed each of the foods as 100g servings, unless otherwise stated for more conventional serving sizes for juices, milks and spreads (never or <1 /month, 1-3 /month, 1 /wk, 2-4 /wk, 5-6 /wk, 1 /d and 2-3 /d, 4-5 /d or >6 /d). The vitamin D concentration of each food was gathered from the McCance and Widdowson's Composition of Foods Integrated dataset (CoF IDS) from the Department of Health [28]. Vitamin D content in each food item, multiplied by the median of the frequency selected, provided an estimation of the vitamin D content consumed per day (IU/d). Vitamin D supplement use was estimated by the frequency of intake of multivitamins and/or vitamin D only supplements (never or <1 /month, 1-3 /month, 1 /wk, 2-4 /wk, 5-6 /wk, 1 /d and 2-3 /d, 4-5 /d or >6 /d). Participants were asked to provide the brand and product details for the

multivitamin and vitamin D supplement. The dose of vitamin D intake from the supplement was multiplied by the median of the frequency of use to give an estimation of intake (IU/d).

4.3.2.2 Skeletal muscle function tests

Two skeletal muscle function performance tests were completed. Vertical jump height and force was assessed for each participant on a multi-component force platform (Kistler, 9281CA, Winterthur, Switzerland) with integrated Bioware software (Kistler, version 3.2.6, Winterthur, Switzerland). Participants were instructed to perform a total of three maximal countermovement jumps with hands on hips. Between each repeat each participant rested for 60 seconds. Jump height was calculated by taking the velocity of each jump (calculated using the flight time method [29]) squared and dividing it by gravitational forces [29]. Jump force was measured by the integrated force platform in Newtons (N), which was normalised for weight. The maximal jump height of the three attempts was used for analysis.

In addition participants completed a hand grip maximal voluntary contraction (MVC). In brief, a bespoke dynometer integrated with a computer system (Spike version 2.0, Cambridge Electronic Design Ltd., Cambridge, UK) measured MVC in Newtons (N). Participants performed four maximal contractions (MVC) each followed by 60s rest. MVC was classified as the largest of the four repeated contractions. The protocol was repeated for both the dominant and non-dominant hand.

4.3.3 Statistical Analysis

Data analysis was performed using SPSS for WINDOWS software (version 17; SPSS Inc, Chicago, IL). All data is expressed as Mean ± SD, unless stated otherwise. Prior to statistical analysis all data was tested for normality using the Kolmogorov-Smirnov test. Data that deviated from normality was log transformed. In order to detect differences in serum 25(OH)D, hours spent outdoors training/competing and during leisure time, dietary and supplement vitamin D intake between groups a one way ANOVA was used. A Bonferroni post hoc test was used to correct for multiple comparisons. Pearson correlations assessed associations between serum 25(OH)D and body weight, BMI, vitamin D

intake and performance data. All Pearson correlations were controlled for by body weight and sex. Whereas Spearman correlations assessed associations between serum 25(OH)D and specific vitamin D rich foods, vitamin D supplement intake, training time outdoors and tanning bed use. Significance was set at $P < 0.05$.

4.4 Results

4.4.1 Participant characteristics

Fifty participants were recruited to take part in the study, with two participants choosing to withdraw from the study with no specific reason. Forty eight participants (45 Caucasian and 3 Indian) were therefore tested, of which 28 were men (Mean ± SD, age: 26 ± 10 y, height: 1.79 ± 0.06 m, weight: 80.2 ± 11.7 kg, body mass index (BMI) 27.7 ± 3.4 kg/m^2) and 20 were women (Mean ± SD, age: 22 ± 4 y, height: 1.69 ± 0.06 m, weight: 64.8 ± 10.8 kg, body mass index (BMI) 26.6 ± 4.3 kg/m^2). The participants performed in a variety of sports (Table 4.1). Based on these sports, twenty one of the participants were classed as indoor athletes, and twenty seven were classed as outdoor athletes.

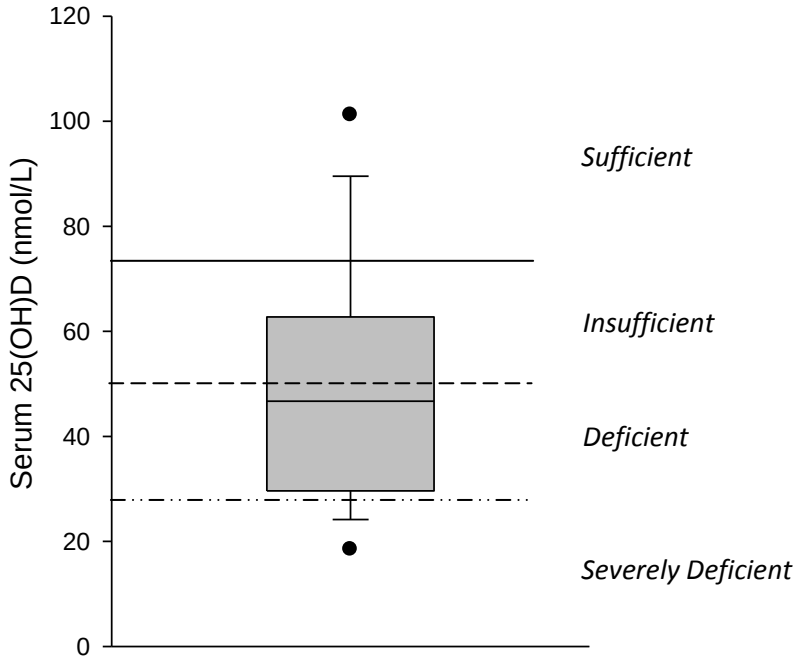
Table 4.1: List of sports included in the study (I, Indoor, O, Outdoor)

Sport	Number of athletes
Football (O)	2
Rugby (O)	5
Field Hockey (O)	2
Netball (I)	1
Badminton (I)	2
Basketball (I)	3
Gymnastics (I)	2
Weight lifters (I)	10
Athletics (O)	2
Triathlon (O)	9
Cricket (O)	1
Squash (I)	1
Running (O)	4
Volleyball (I)	1
Dancing (I)	2
Golf (O)	1

4.4.2 Vitamin D status

Mean serum total 25(OH)D was 51±24 nmol/L (range 18 - 112 nmol/L) with 10% (5/48), 44% (21/48), 29% (14/48) and 17% (8/48) of athletes being severely vitamin D deficient (<25 nmol/L), deficient (<50 nmol/L), insufficient (50-75 nmol/L) and sufficient (>75 nmol/L), respectively. Moreover, only 4% (2/48) of the athletes tested had 25(OH)D >100nmol/L. (Figure 4.1). No significant differences were observed between men and women (P=0.089) or indoor and outdoor (P=0.339) athletes despite total serum 25(OH)D appearing higher for women and outdoor sports (Table 4.2). Men had a higher prevalence of vitamin D deficiency than women, especially at 25(OH)D concentrations that are categorised as severely vitamin D deficient (<25nmol/L). On the other hand women had a higher prevalence of insufficiency than men. Further, the prevalence of deficiency, insufficiency and sufficiency were similar between indoor and outdoor athletes (Table 4.2).

Figure 4.1: Winter serum 25(OH)D concentrations from a variety of indoor and outdoor athletes living and training at high latitude in the United Kingdom. (Mean±SD)



Circles above and below the box plot represent outliers (below and above the 5th and 95th percentile, respectively).

4.4.3 Sun exposure during training and leisure time

Outdoor athletes reported spending significantly more time outdoors training/competing in all seasons compared to indoor athletes ($p<0.001$) (Figure 4.2). However, no differences were found between men and women in any season. The majority of participants reported training/competing outdoors early in the morning during the autumn (47%) and winter (50%), whereas during the spring (41%) and summer (47%) the majority of participants trained outdoors at lunchtime/early afternoon. Time spent training/competing outdoors did not correlate with serum 25(OH)D in autumn ($r=0.061$ $P=0.686$), spring ($r=0.128$ $P=0.391$) or summer ($r=-0.027$ $P=0.856$). However, time spent training/competing outdoors in winter positively correlated with serum 25(OH)D ($r=0.344$ $p=0.017$).

Table 4.2: Mean serum total 25(OH)D (nmol/L) for each group and prevalence (% within each group) of each vitamin D status group. Mean ± SD.

		Prevalence of each vitamin D status group			
Group	Serum Total25(OH)D (nmol/L)	Severely deficient (<25nmol/L)	Deficient (<50nmol/L)	Insufficient (50-75nmol/L)	Sufficient (>75nmol/L)
Men (n=28)	47±23	18%	50%	18%	14%
Women (n=20)	58±24	0%	35%	45%	20%
Indoor (n=21)	48±24	9%	48%	29%	14%
Outdoor (n=27)	55±24	10%	41%	30%	19%

In addition participants reported spending 3.4 ± 2.5 h/wk, 3.2 ± 2.6 h/wk, 5.0 ± 2.7 h/wk and 6.1 ± 2.3 h/wk outdoors during leisure time in the autumn, winter, spring and summer respectively. No correlations were found between serum 25(OH)D and leisure time spent outdoors in autumn ($r=0.107$ $P=0.475$), winter ($r=-0.105$ $P=0.484$), spring ($r=-0.152$ $P=0.308$) and summer ($r=-0.104$ $P=0.489$).

4.4.4 Other sources and limitations to sun exposure

No participant reported ever using sun beds. In total, 42% of participants reported using sun lotion in the summer, with only 4% reporting using sun lotion in the spring. The average sun protection factor

(SPF) used by participants was 18 ± 9 (range 5-50) with no correlation between the SPF and serum 25(OH)D (r=0.080 p=0.598). Only 17% of participants reported wearing no shirt outdoors in the summer. For each season, the total amount of clothes worn was calculated by adding the responses to each individual piece of clothing. No significant differences were observed for total clothes worn in each season when comparing between indoor and outdoor athletes, or men and women.

4.4.5 Vitamin D intake

Total vitamin D intake (diet and supplements) was estimated at 317 ± 647 IU/d (max: 3040 IU/d). Only 11/48 (23%) participants consumed vitamin D greater than prescribed reference nutrient intake (RNI) [30]. Total vitamin D intake was positively correlated with serum 25(OH)D but failed to reach significance (r=0.275 P=0.064). Dietary vitamin D intake was estimated to be 106 ± 131 IU/d (max: 633 IU/d). However, only 3/48 (6%) of the participants actually met the RNI. No correlation was found between dietary vitamin D intake and serum 25(OH)D (r=0.081 P=0.594).

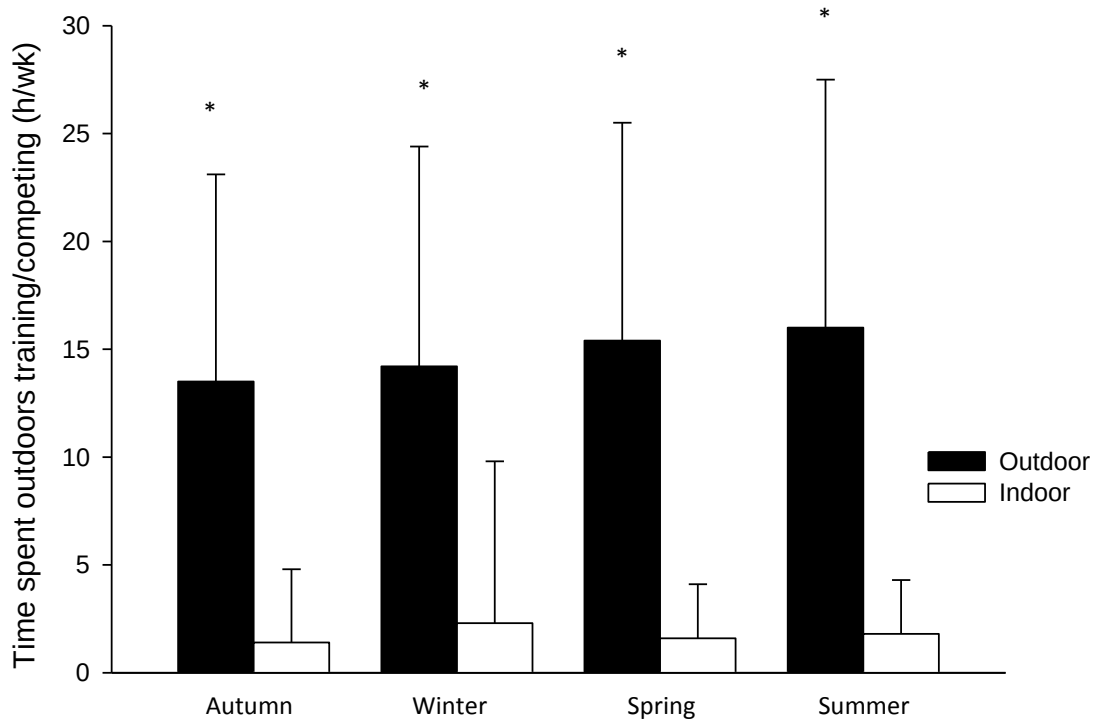
Vitamin D supplements were used by 2/48 (4%) participants, while 10/48 (21%) participants consumed a multivitamin containing vitamin D. The average vitamin D supplement (including multivitamin supplements) was 694 ± 1008 IU/d (max: 3000IU/d), with only 3 participants consuming a supplement that was above 400 IU/d, of which all were men. Vitamin D supplement dose was positively correlated with serum 25(OH)D (r=0.353 P<0.005).

4.4.5.1 Muscle function performance tests and vitamin D status

No significant correlation was observed between jump force (r= 0.048 P=0.755), jump height (r= -0.275 P=0.071), dominant hand grip (r= -0.088 P=0.570) or dominant hand grip force (r= 0.12 P=0.939) and serum 25(OH)D.

Figure 4.2: Seasonal differences in estimation of time spent outdoors (h/wk) training/competing for indoor and outdoor athletes

(Mean±SD) * Significantly different to indoor athletes (P<0.05).



4.5 Discussion

The current study demonstrates a high prevalence of vitamin D deficiency and insufficiency during the winter in UK athletes, with 10% (5/48) 44% (21/48) and 29% (14/48) of the athletes tested being severely vitamin D deficient, deficient and insufficient, respectively. These findings are in line with previous studies conducted in athletes, at both high [13-15, 17] and low latitudes [16]. Morton et al 2012 reported that 65% of elite football players were vitamin D deficient (< 50 nmol/L) during the winter in the UK (53°N). More recently, the same research group recruited 61 outdoor athletes from a variety of sports who were living and training at high latitude (53°N). It was reported that during the winter, 64% of a variety of outdoor athletes were vitamin D deficient [14]. Similar findings have also been found in athletes living and training at higher latitudes to the current study test site (60.5°N) [17]. Hence this data firstly brings to light the potential negative implications to athletic performance, considering that vitamin D deficiency is closely associated with sub-optimal skeletal muscle function and structure [2, 9, 10]. Secondly the data also highlights the opportunity for continued research into the physiological consequences of vitamin D deficiency at the current test site.

The high prevalence of vitamin D deficiency and insufficiency observed in the current study is not surprising. This is because during the winter, at high latitudes, cutaneous vitamin D synthesis reaches nadir [1]. In addition the high cloud cover that the UK experiences most of the year also limits cutaneous vitamin D synthesis [31]. These factors are also likely to explain why no significant differences were observed between indoor and outdoor athletes vitamin D status (Table 4.2), which is in agreement with others [27]. This was evident despite outdoor athletes reporting spending more time outdoors training/competing during the summer and spring when compared to indoor athletes (Figure 4.2). Yet the current study is unable to comment on the vitamin D status of the participants during the other seasons. However, [32] calculated that in California, summer sun exposure had to be high (90 min/d) followed by vitamin D supplementation of 1300IU/d during the winter to sustain sufficient vitamin D status across the year. Consequently, in order for UK athletes to sustain vitamin D

sufficiency it may be that sun exposure should be encouraged during the summer in combination with vitamin D supplementation.

In contrast to this suggestion, the current study illustrates that very few participants reported spending time outdoors with no top on (17%) during the summer with a high proportion of athletes frequently using sun lotion in the summer. Collectively these factors are known to impair cutaneous synthesis of vitamin D [1]. As little as 30 minutes in the midday sun wearing minimal clothing (to achieve a whole body minimal erythemal dose) has been shown to be equivalent to 10,000IU of vitamin D which will raise 25(OH)D considerably [1]. However, the current recommendations for sun exposure and sun protection are undefined in the UK [30]. This is mainly due to the difficulty of accurately measuring sun exposure, the incomplete knowledge of the suns contribution to vitamin D status and the risk of developing skin cancer [21]. Collectively, the poor climate of the UK combined with the low reported time spent outdoors during the summer would suggest that athletes require a greater dietary intake of vitamin D to sustain sufficient vitamin D status.

The estimated vitamin D intake in the current study (106 ± 131 IU/d) was below the RNI (400IU) for the UK [30]. These estimations are in agreement with previous investigations in UK adults [7] and in athletes worldwide [17, 27]. However, it is argued that dietary vitamin D intake at the current RNI is inadequate to sustain a sufficient vitamin D status when in the absence of sunlight [1]. Despite this, these are the current recommendations for vitamin D intake in the UK [30]. In support, recent evidence from UK and Ireland (50-60°N) has shown that 400 IU/d of dietary vitamin D was only able to sustain 25(OH)D at 25 nmol/L across the year [7] and unable to maintain summer 25(OH)D during the winter [33]. Hall et al [32] also showed that during low sun exposure in the Californian summer (~20 min/d) it was estimated that an additional 1000IU/d was required to sustain sufficient vitamin D status. More strikingly, during the winter the authors calculated that 2550IU/d was required to sustain sufficient vitamin D status. Considering that sun exposure is severely reduced in the UK during the summer when compared to California, combining this data with the current study findings clearly

suggests that higher doses (1000-2500IU/d) of dietary vitamin D are essential to achieve vitamin D sufficiency.

Increasing vitamin D intake in the diet is often difficult from food sources alone. Vitamin D supplements have been shown to be effective tools to improve the vitamin D content in the diet while also raising vitamin D status. However, the current study shows that very few athletes (25%) reported using a supplement containing vitamin D. Others have also shown a poor use of vitamin D supplements amongst athletes [19], even though athletes frequently use an array of dietary supplements [34]. Furthermore, the estimated vitamin D supplement intake in the current study was correlated with 25(OH)D ($r=0.353$ $P<0.005$) supporting the use of high doses of vitamin D supplements to increase vitamin D status. However, the current guidelines for vitamin D supplement use is unclear and currently under debate [20, 24]. It is generally well accepted that in order to raise 25(OH)D to sufficient concentrations, doses above the RNI in the UK and RDA (600IU/d) in the United States doses are required (1000-4000IU/d) [23, 24]. In support, Close et al [13, 14] has recently demonstrated in two separate studies that daily vitamin D intake at 5000IU/d for 8 weeks [14] or larger weekly doses (20,000IU and 40,000IU) for 8 weeks [13] are both highly effective at improving vitamin D status to sufficient concentrations. Taken together vitamin D supplements would be an effective nutritional strategy for UK athletes to ensure vitamin D sufficiency can be achieved during the winter. Yet to date the optimal vitamin D supplementation protocol (dose, duration, and frequency) has not been established. Therefore, future dose response intervention studies are required to elucidate this further.

It is important to highlight that the current studies estimations for vitamin D intake, from both sun exposure and dietary vitamin D intake, are not without its limitations. The current study implemented food frequency questionnaire based quantification of vitamin D intake. This was primarily due to the ease of gaining an insight into the dietary habits of the athletes tested. The authors however acknowledge that using this approach may result in an underestimation of vitamin D intake due to athletes under reported serving sizes, frequency and dose of supplement use or the declared use of

fortified foods. Future studies should aim to use more accurate measurements of vitamin D intake through the use of diet diaries, diet recalls and UV dosimeters.

Despite vitamin D deficiency being shown in the elderly to have deleterious effects on skeletal muscle function and structures [2, 9, 10], the current study showed that vitamin D status was not significantly associated with skeletal muscle function (vertical jump and hand grip MVC). This data suggests that vitamin D status may not be important in determining skeletal muscle function in athletes. In contention, Ward et al [11] showed that in young adolescent girls vitamin D status was positively correlated with jump height, velocity, power and force. Recently, Close et al [14]showed that soccer players significantly improved 10 m sprint times and vertical jump following 8 weeks of 5000IU/d during the winter in the UK (53°N) when compared to placebo. The improvements in performance were observed when vitamin D status was improved to sufficiency. However, these findings have not been replicated by the same research group [13]. Therefore, it remains unclear what role vitamin D has on skeletal muscle function in athletes and thus requires further investigation.

The lack of association between vitamin D status and muscle function in the current study may be due to the small number of participants as well as the heterogeneous group tested. Previous associative studies have observed small to medium effect sizes (r = 0.22-0.32) for the association between 25(OH)D and skeletal muscle function and performance in larger sample sizes (99- >300 participants). It is also important to point out that in trained athletes skeletal muscle function and structure may be superior when compared to untrained adults, adolescents and the elderly. Thus, it may be speculated that the effect of vitamin D may have smaller effects in athletes when compared to those previously researched groups. Consequently, in order to effectively address the current research question large sample sizes (>300) may be necessary to pick up the small effect sizes potentially observed in athletes.

In summary, we have demonstrated that the prevalence of vitamin D deficiency and insufficiency is high in a variety of indoor and outdoor athletes living in the UK during the winter. In addition we observed that athletes from the UK had a low estimated dietary intake of vitamin D, well below the

current RNI. However, the significant positive correlation between vitamin D supplement intake and 25(OH)D support the use of vitamin D supplements as an effective strategy to improve vitamin D status in athletes. Yet, it remains unclear whether improving vitamin D status in athletes has any beneficial effects on performance or other determinants of performance (immunity, illness, recovery). Therefore, further investigations using vitamin D supplements are required to test the currently speculative benefits of vitamin D in athletes.

4.6 References:

1. Holick MF (2007) Vitamin D deficiency. N Engl J Med 357: 266-281.
2. Schott GD, Wills MR (1976) Muscle weakness in osteomalacia. Lancet 1: 626-629.
3. Irani PF (1976) Electromyography in nutritional osteomalacic myopathy. J Neurol Neurosurg Psychiatry 39: 686-693.
4. Bischoff-Ferrari HA, Borchers M, Gudat F, Durmuller U, Stahelin HB, et al. (2004) Vitamin D receptor expression in human muscle tissue decreases with age. J Bone Miner Res 19: 265-269.
5. Tavera-Mendoza LE, White JH (2007) Cell defenses and the sunshine vitamin. Sci Am 297: 62-65, 68-70, 72.
6. van Schoor NM, Lips P (2011) Worldwide vitamin D status. Best practice & research Clinical endocrinology & metabolism 25: 671-680.
7. Cashman KD, Hill TR, Lucey AJ, Taylor N, Seamans KM, et al. (2008) Estimation of the dietary requirement for vitamin D in healthy adults. Am J Clin Nutr 88: 1535-1542.
8. Suzuki T, Kwon J, Kim H, Shimada H, Yoshida Y, et al. (2008) Low serum 25-hydroxyvitamin D levels associated with falls among Japanese community-dwelling elderly. J Bone Miner Res 23: 1309-1317.
9. Wicherts IS, van Schoor NM, Boeke AJ, Visser M, Deeg DJ, et al. (2007) Vitamin D status predicts physical performance and its decline in older persons. J Clin Endocrinol Metab 92: 2058-2065.
10. Bischoff HA, Stahelin HB, Urscheler N, Ehrsam R, Vonthein R, et al. (1999) Muscle strength in the elderly: its relation to vitamin D metabolites. Arch Phys Med Rehabil 80: 54-58.
11. Ward KA, Das G, Berry JL, Roberts SA, Rawer R, et al. (2009) Vitamin D status and muscle function in post-menarchal adolescent girls. J Clin Endocrinol Metab 94: 559-563.
12. Heaney RP, Davies KM, Chen TC, Holick MF, Barger-Lux MJ (2003) Human serum 25-hydroxycholecalciferol response to extended oral dosing with cholecalciferol. Am J Clin Nutr 77: 204-210.
13. Close GL, Leckey J, Patterson M, Bradley W, Owens DJ, et al. (2013) The effects of vitamin D3 supplementation on serum total 25[OH]D concentration and physical performance: a randomised dose-response study. Br J Sports Med 47: 692-696.
14. Close GL, Russell J, Cobley JN, Owens DJ, Wilson G, et al. (2012) Assessment of vitamin D concentration in non-supplemented professional athletes and healthy adults during the winter months in the UK: implications for skeletal muscle function. J Sports Sci 31: 344-353.
15. Morton JP, Iqbal Z, Drust B, Burgess D, Close GL, et al. (2012) Seasonal variation in vitamin D status in professional soccer players of the English Premier League. Appl Physiol Nutri Metab 37: 798-802.
16. Hamilton B, Grantham J, Racinais S, Chalabi H (2010) Vitamin D deficiency is endemic in Middle Eastern sportsmen. Public health nutrition 13: 1528-1534.
17. Lehtonen-Veromaa M, Mottonen T, Irjala K, Karkkainen M, Lamberg-Allardt C, et al. (1999) Vitamin D intake is low and hypovitaminosis D common in healthy 9- to 15-year-old Finnish girls. Europ Clin Nutr 53: 746-751.
18. Cashman KD, Muldowney S, McNulty B, Nugent A, Fitzgerald AP, et al. (2012) Vitamin D status of Irish adults: findings from the National Adult Nutrition Survey. Br J Nutr 10: 1-9.
19. Carlsohn A, Cassel M, Linne K, Mayer F (2011) How much is too much? A case report of nutritional supplement use of a high-performance athlete. Br J Nutr [E pub ahead of print]: 1-5.
20. IOM Report wwwiomedu/Reports/2010/Dietary-Reference-Intakes-for-Calcium-and-Vitamin-D/Report-Briefaspx.
21. Ashwell M, Stone EM, Stolte H, Cashman KD, Macdonald H, et al. (2010) UK Food Standards Agency Workshop Report: an investigation of the relative contributions of diet and sunlight to vitamin D status. Br J Nutr 104: 603-611.
22. Priemel M, von Domarus C, Klatte TO, Kessler S, Schlie J, et al. (2010) Bone mineralization defects and vitamin D deficiency: histomorphometric analysis of iliac crest bone biopsies and

circulating 25-hydroxyvitamin D in 675 patients. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research 25: 305-312.
23. Heaney RP, Holick MF (2011) Why the IOM recommendations for vitamin D are deficient. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research 26: 455-457.
24. Holick MF, Binkley NC, Bischoff-Ferrari HA, Gordon CM, Hanley DA, et al. (2011) Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society clinical practice guideline. J Clin Endocrinol Metab 96: 1911-1930.
25. Dam TT, von Muhlen D, Barrett-Connor EL (2009) Sex-specific association of serum vitamin D levels with physical function in older adults. Osteoporos Int 20: 751-760.
26. Roth HJ, Schmidt-Gayk H, Weber H, Niederau C (2008) Accuracy and clinical implications of seven 25-hydroxyvitamin D methods compared with liquid chromatography-tandem mass spectrometry as a reference. Annals of clinical biochemistry 45: 153-159.
27. Halliday TM, Peterson NJ, Thomas JJ, Kleppinger K, Hollis BW, et al. (2011) Vitamin D status relative to diet, lifestyle, injury, and illness in college athletes. Med Sci Sports Ex 43: 335-343.
28. Food.Standards.Agency McCance and Widdowson's Composition of Foods Integrated dataset (CoF IDS). wwwfoodgovuk
29. Linthorne NP (2001) Analysis of standing vertical jumps using a force platform. American Journal of Physics 69: 1198-1204.
30. Department.of.Health (1998) Nutrition and bone health with particular reference to calcium and vitamin D: Report of the Subgroup on Bone Health (Working Group on the Nutritional Status of the Population) of the Committee on Medical Aspects of Food and Nutrition Policy. wwwdhgovuk The Staionery Office.
31. Engelsen O, Brustad M, Aksnes L, Lund E (2005) Daily duration of vitamin D synthesis in human skin with relation to latitude, total ozone, altitude, ground cover, aerosols and cloud thickness. Photochemistry and photobiology 81: 1287-1290.
32. Hall LM, Kimlin MG, Aronov PA, Hammock BD, Slusser JR, et al. (2010) Vitamin D intake needed to maintain target serum 25-hydroxyvitamin D concentrations in participants with low sun exposure and dark skin pigmentation is substantially higher than current recommendations. J Nutr 140: 542-550.
33. Muldowney S, Lucey AJ, Hill TR, Seamans KM, Taylor N, et al. (2012) Incremental Cholecalciferol Supplementation up to 15 mug/d Throughout Winter at 51-55{degrees} N Has No Effect on Biomarkers of Cardiovascular Risk in Healthy Young and Older Adults. J Nutr 142: 1519-1525.
34. Maughan RJ, Greenhaff PL, Hespel P (2011) Dietary supplements for athletes: emerging trends and recurring themes. J Sport Sci 29 Suppl 1: S57-66.

Chapter 5

The effects of short term vitamin D_3 supplementation on vitamin D status and exercise performance in athletes.

Paper in submission

Adrian B. Hodgson†, Jonathon Martin†, Timothy Roberts†, Andrew Ingham††, Asker Jeuekendrup†

Medicine and Science in Sports and Exercise

A.B.H Conceived and designed the experiment, performed the experiment, interpreted the data and wrote the manuscript, J.M and T.R Assisted in the data collection. A.I For making and providing the placebo tablets for the study, A.J. Assisted in the designing of the experiment and interpretation of the data.

† School of Sport and Exercise Sciences, University of Birmingham, United Kingdom

††Pharmacy School, School of Life and Health Sciences, Aston University, Aston, Birmingham,

5.1 Abstract

Background: Vitamin D deficiency in the elderly has been demonstrated to impair musculoskeletal performance, while restoring vitamin D status may avoid this impairment. However, it is currently unclear whether this also applies to trained athletes. The aim of the current study was to investigate the effects of 28 d vitamin D_3 supplementation on a number of indicators of aerobic, anaerobic and strength performance. **Methods:** Thirty six endurance trained male cyclists/triathletes were recruited and randomly assigned into one of four supplementation groups (n=9): Placebo (PLA), 1500IU, 3000IU and 6000IU lasting for 28 d, as part of a double blind parallel study design. Athletes reported to the lab for 2 days of performance testing pre and post supplementation, including VO_2 max test, unilateral leg strength test (1RM), maximal voluntary contraction hand grip test, 1 h time trial and repeated Wingate tests. All data collection was performed at latitude of 52.4-52.5°N, between December and March. **Results:** At baseline, 8%, 53% and 33% of athletes were severely vitamin D deficient (<25 nmol/L), deficient (<50 nmol/L) insufficient (50-75 nmol/L), respectively. Vitamin D supplementation caused 25(OH)D to increase to a sufficient status in all subjects (>100nmol/L), however no significant difference was observed between any dose. Vitamin D supplementation did not alter performance measures and vitamin D status was not associated with performance. **Conclusions:** In summary the study shows that 28 d vitamin D supplementation, above the RNI, is efficacious at improving vitamin D status in athletes. However, this was not associated with improvements in exercise performance.

5.2 Introduction

Besides from the traditional role of vitamin D for optimal bone health, it has more recently been highlighted for its importance for optimal skeletal muscle function [1]. Myopathy [2], skeletal muscle atrophy [3] and neuromuscular impairments [4] feature in those individuals with vitamin D deficiency (< 50 nmol/L). As a consequence those who have vitamin D deficiency, especially from data in the elderly and in adolescent girls, have shown severe impairments to simple physical tasks and skeletal muscle strength [5-8]. This evidence is supported by the more recent evidence to show the presence of the vitamin D receptor (VDR) at the skeletal muscle [9], which allows activated vitamin D ($1,25(OH)_2D_3$) to bind to the VDR and target more than 1000 vitamin D responsive human genes [10]. The VDR target genes relate to skeletal muscle calcium handling, proliferation and differentiation [11] which is apparent in VDR knockout mice who display severe skeletal muscle atrophy, impaired functional performance and motor co-ordination [12].

The deleterious effects of vitamin D deficiency to the skeletal muscle are alarming for athletes who aim for peak physical conditioning for optimal exercise performance. This is further exacerbated by the accumulating evidence in the literature, and from the data presented in Chapter 4, to show a high prevalence of vitamin D deficiency and insufficiency in athletes [13-15]. Early evidence from German and Russian athletes reported that manipulation of vitamin D status, using ultraviolet irradiation from sun lamps, improved exercise performance [16]. Since this early work, there is little evidence in athletes to suggest that vitamin D is ergogenic to performance. Despite this, evidence in the elderly has demonstrated that long term vitamin D supplementation (8 wk – 3 y) reduces the risk of falls [17], improves muscle function [18] and attenuates skeletal muscle atrophy [3]. Though the optimal 25(OH)D concentration required to prevent deleterious effects to skeletal muscle function is largely unknown, with the IOM suggesting that 25(OH)D >50 nmol/L is sufficient [19], the Endocrine Society acknowledge that 25(OH)D >100 nmol/L may be required for optimal skeletal muscle function [20], while others believe 25(OH)D concentrations between 100-250 nmol/L are required [21].

The categorisation of vitamin D status as well as the recommended dose of vitamin D to ensure vitamin D sufficiency is under continued debate. A recent report from the Institute of Medicine (IOM) [19] established that the recommended daily allowance (RDA) of vitamin D for adults in the United States is 600IU/d, where as in the UK the daily recommended value (RNI) is 200IU/d [22]. The IOM report has received a great deal of criticism [20, 23], with others suggesting higher doses of vitamin D (1000-2000IU) are required to correct and prevent vitamin D deficiency [20]. However, with higher doses of vitamin D, questions have been asked regarding safety of intake [24]. There is also considerable debate about the upper limits. Numerous researchers argue however that the current upper limits (UL 4000IU/d) are too low, with far higher doses shown to be safe for use (10,000IU/d) [24]. Recent evidence in athletes has found that 8 weeks of 5000IU/d vitamin D_3 during the winter in the UK (53°N) successfully improved mean vitamin D status from 29±25 to 103±25 nmol/L [14] while the same authors also showed that 12 weeks of 20,000 IU/wk and 40,000 IU/wk vitamin D_3 increased mean baseline vitamin D status of 51±24 nmol/L to 85±10 nmol/L and 91±24 nmol/L [13]. Despite the authors observing significant improvements in vitamin D status to sufficient concentrations they reported improvements to performance only in one study [14]. Based on the equivocal data, further investigation into the effect of vitamin D supplementation on improving vitamin D status and the effect of exercise performance is required.

Therefore, the purpose of the study was to assess the prevalence of vitamin D status in athletes living and training in the UK before and after short term (28 days) vitamin D_3 supplementation at different doses (1500IU, 3000IU and 6000IU). In addition the effect that short term vitamin D_3 supplementation had on a number of indicators of exercise performance was also investigated. To account for the differing vitamin D status guidelines, we categorised vitamin D status as severe deficiency, deficiency, insufficiency and sufficiency as serum total 25(OH)D of <25 nmol/L, <50 nmol/L, 50-75 nmol/L and >75 nmol/L, respectively.

5.3 Method

5.3.1 Participants

Forty male Caucasian endurance trained cyclists/triathletes (Mean ± SD, age: 39 ± 8 y, height: 1.80 ± 0.66 m, weight: 76.3 ± 6.2 kg, maximal oxygen uptake (VO_2 max) 60.0 ± 3.8 $mL.kg^{-1}.min^{-1}$) were recruited for the study from local cycling and triathlon clubs. All participants were healthy according to results of a general health questionnaire. To be eligible to participate, participants were required to maintain the volume and load of training that had been completed in the month prior to the study in order to minimise the effect of differences in training load. In addition each participant completed a vitamin D food frequency questionnaire [297]. This was to estimate vitamin D intake from vitamin D rich foods typically consumed in the UK and the contribution of supplements. Vitamin D intake concentration (IU/d) was quantified using data from the McCance and Widdowson's Composition of Foods Integrated dataset (CoF IDS) from the Department of Health [436]. To be eligible for the study vitamin D intake from a supplement source had to be lower than 600IU/d, which had to be ceased for the duration of the study. Each participant also had to have not travelled to a destination with a lower latitude than the UK (50-60°N) three months prior to the study or for the duration of the study. Written informed consent was given by each participant prior to study commencement. The study was approved by the University of Birmingham's Science, technology engineering and mathematics ethical review committee.

5.3.2 General study design

Each participant completed a double blind parallel study design, conducted between December 2010 and March 2011, during which cutaneous vitamin D_3 synthesis reaches nadir [27]. Participants underwent two days of exercise performance tests before and after a 28 d supplementation period. Participants were randomly assigned to one of four supplementation groups consisting of either placebo (PLA), 1500IU, 3000IU or 6000IU. Serum total 25(OH)D and PTH concentrations were assessed pre and post supplementation in order to measure vitamin D status.

5.3.3 Familiarisation trial

One week before the beginning of the study each participant visited the human performance lab to assess specific inclusion criteria as well as familiarise each participant to the lab and exercise test protocols. Each participant had the experimental protocol described to them in detail, and completed a shortened time-trial and 4 Wingate tests to minimise the variation and avoid a learning effect between test repetitions. The time trial protocol was identical to what has been described previously [28]. However, a shortened adapted protocol was used (~20 min). The energy target was calculated at 5kJ/kg of bodyweight (BM). The linear factor was set by calculating work rate at 70% of estimated Wmax divided by $(90\ rpm)^2$. Participants were informed the 20 min familiarisation time trial was equivalent to one third of the time trial performed during the experimental trial. Following 15 min of rest, each participant completed the Wingate tests (described in detail below).

5.3.4 Experimental Protocol

The experimental trial was completed over a two day period. Day 1 consisted of 3 performance tests: VO_2 max test, 1 repetition max strength test (1RM), hand grip maximal voluntary contraction (MVC) strength test. Day 2 consisted of 2 performance tests: 1 h time trial (TT) and 4 Wingate tests. Participants reported on both performance test days to the Human Performance Laboratory (HPL) between 0600 and 0800 after a 10 h overnight fast.

5.3.4.1 Pre supplementation trials

Day 1

On arrival to the human performance lab, measures of height and weight were recorded. Participants then had a single 10 mL blood sample taken (Venipuncture, Nu Care Butterfly) prior to commencing the performance tests.

VO_2 max test

Each participant began the trial with an incremental exercise test performed on an electronically braked cycle ergometer (Lode Excalibur Sport, Groningen, Netherlands), to volitional exhaustion. Participants completed a warm up at 75 W for 5 min. Following this the test began at 95 W and increased by 35 W (W_{inc}) increments every 3 min (t_{inc}) until the participant reached exhaustion. The Wmax was calculated by using the following equation:

$$Wmax = W_{out} + (t/t_{inc})\ W_{inc}$$

W_{out} is the power output that was reached in the final completed stage, and t is the time (in seconds) spent in the final stage. Respiratory measures of oxygen consumption (VO_2) and carbon dioxide production (VCO_2) were assessed using an online gas analysis system (Oxycon Pro, Jaeger) throughout the test. Heart rate (HR) was measured continuously (Polar S625X; Polar Electro Oy, Kempele, Finland). VO_2 max was considered if 2 of the 3 conditions were met: 1) a levelling off of VO_2 with further increasing workloads; 2) a HR within 10 beats/min of the age predicted maximum (220 bpm - age); and 3) a respiratory exchange ratio of >1.1. The bike position following all tests using the cycle ergometer was recorded in order to replicate for all future trials.

1 repetition max leg strength test (1RM)

On completion of the VO_2 max test, participants rested for 15 min before beginning the 1RM. The 1RM was performed unilaterally on both dominant and non-dominant legs for leg press and leg extension exercises (Cybex VR3 Leg-Press machine, Cybex International, Inc, Medway, MA, USA) using the multiple repetitions testing procedure [29].

Hand grip (MVC) strength test

The hand grip test was performed immediately after the 1RM. Hand grip MVC was assessed using a dynometer with Spike data acquisition software (version 2.0, Cambridge Electronic Design Ltd., Cambridge, UK). The software package recorded peak force achieved during each attempt. MVC was

considered when there was less than 5% between repeats. Participants completed 3-4 attempts. The protocol was repeated on both dominant and non-dominant hands.

Day 2

Participants reported back to the lab the following day. On arrival to the human performance lab measures of height and weight were recorded.

1 hour time trial

Participants completed a 5 min warm up at 100W (Lode Excalibur Sport, Groningen, Netherlands) before beginning the time trial test. The time trial consisted of completing an energy-based target amount of work at 70% W_{max} in the quickest time possible. The total amount of work (971±62 kJ) was calculated for the 60 min time trial. A linear factor, 70% W_{max} divided by $(90rpm)^2$ was entered into the cycle ergometer. Participants received a countdown prior to starting the time trial and received no verbal or visual feedback regarding performance time or physiological measures throughout the test. Participants received no feedback about their performance until they had completed all experimental trials. On completion, participants rested for 15 min before moving onto the final performance test.

Wingate Test

The Wingate test consisted of 4 repeated 30 second "all out" efforts on an electronically braked cycle ergometer (Lode Excalibur Sport, Groningen, Netherlands) against a resistance equivalent to 0.075 kg/kg body mass (Wingate software version 1.11, Lode). The "all out" efforts were completed in the seated position, with all participants starting at 90 RPM. Subjects were verbally encouraged to continue pedalling as fast as possible throughout the 30 second test. Following the 30 seconds "all out" effort, subjects completed a recovery period of 4 min 30 sec. During each of the four 30 second "all out" efforts, peak power (W), mean power (W), and time to fatigue (W/sec) were measured and recorded online.

5.3.4.2 Post supplementation trials

Both pre supplementation performance days (Day 1 and 2) were repeated in an identical fashion following 28 d vitamin D supplementation.

5.3.5 Diet and Supplement control

Participants were instructed in the 24-h period prior to the pre supplementation trial day 1 and day 2 to record all food using a food diary. This included all food and drink consumed over the 2 days. This was replicated before the post supplementation trial day 1 and 2.

After completing the pre supplementation performance tests participants were randomly allocated to one of four supplement groups in a double-blind fashion. The intervention groups included: placebo, 1500IU, 3000IU and 6000IU (Boots Pharmacy, UK). The vitamin D doses were selected based on anecdotal evidence from athletes who are taking vitamin D supplements in this dose range in the hope of an ergogenic effect. All of the 'over the counter' vitamin D tablets were analysed externally (LC-MS/MS, Birmingham City Hospital, Dudley, Birmingham, UK) for total vitamin D to ensure the correct dose was being provided in each intervention group. The placebo tablet was specially made (Aston University, School of Pharmacy) to match the appearance (shape and size) and composition (99% w/w Sucrose monohydrate BP and 1% w/w Lactose monohydrate BP) of the vitamin D tablets, minus the presence of vitamin D. Participants were instructed to take 6 tablets/d with water, 1 h before consuming breakfast. Each of the interventions was stored in white sealed pots with 50 tablets in each. This was enough for 1 wk supplementation, participants returned to the lab to collect further tablets. Intervention number, week number and instructions were printed on the label of each pot for participant and researcher purposes. Compliance was strictly monitored throughout the intervention period. Participants were instructed to record the time and date of consumption using a supplement consumption diary sheet. In addition participants received daily *sms* messages to remind them to take the supplement. During the visit following 2 wk supplementation participants completed a compliance questionnaire to check if any of the supplements had not been taken. Compliance was met if

participants had missed less than 2 days of supplements. Participants also returned the empty pots during this visit and at end of the intervention period. Surplus tablets were counted to check that the appropriate number of tablets had been taken.

5.3.6 Blood Analysis

Blood samples taken pre and post supplementation were immediately placed into silica-polymer gel tube (Vacutainer, BD, USA). These blood samples were left to rest at room temperature for 1 h. Samples were then centrifuged at 3500rpm and 4°C for 15 min, before serum was separated and stored at -80°C.

Blood serum concentrations of 25(OH)D_2 and D_3 as well as parathyroid hormone (PTH) was analysed. In brief, 25-hydroxyvitamin D_2 and D_3 was extracted from serum using a single reagent containing 26,27–hexadeuterium-25-hydroxyvitamin D_3 internal standard. Vitamin D_2 and D_3 was measured separately using Waters Quattro Premier XE MS detector with ACQUITY Ultra-Performance LC. PTH was measured using Roche Parathyroid hormone intact assay on a Roche modular vertical P analyser (Roche, Diagnostics Limited, East Sussex UK). All analysis took place at Birmingham City Hospital, Dudley, Birmingham, UK.

5.3.7 Statistical analysis

Data analysis was performed by using SPSS for WINDOWS software (version 17; SPSS Inc, Chicago, IL). Data are expressed as means ± SD unless otherwise stated. Between group differences (PRE to POST) for performance measure outcomes and change in blood measures were determined using univariate ANCOVA. Within group differences in blood measures and performance measures (PRE to POST), as well as subject characteristics were determined using paired sampled t-test with Bonferroni correction. Pre supplementation blood measure differences were determined using one way ANOVA. In order to look at differences in Wingate power output across time, a repeated measure ANOVA was used to compare pre and post supplementation within each group. The association between vitamin D

status and performance measures pre supplementation and the change in vitamin D status and performance was assessed using partial regression model controlling for body weight. Significance was set at $P < 0.05$.

5.4 Results

From the 40 athletes that were recruited, six subjects were removed from the analysis. Four subjects were removed for non-compliance to the supplement, and the other two for visiting foreign destinations, of lower latitude than the test site, during the supplementation period. This resulted in 34 athletes in total with 7 athletes in PLA, 7 athletes in 1500IU, and 10 athletes in both 3000IU and 6000IU.

5.4.1 Estimated vitamin D intake

Estimated dietary vitamin D intake was 116 ± 162 IU/d. Only 3 out of the 34 athletes consumed >400IU/d. All vitamin D supplements were ceased during the study, however only 13 out of the 34 athletes reported using a vitamin D supplement prior to the study.

5.4.2 Vitamin D status pre supplementation

Mean total 25(OH)D, 25(OH)D_2, 25(OH)D_3 and PTH for all groups PRE and POST are summarised in Table 5.1. Pre supplementation, 8% (3/36), 53% (18/36), 33%, (11/36) and 14% (5/36) of athletes were severely vitamin D deficient (<25 nmol/L), deficient (>50 nmol/L), insufficient (50-75 nmol/L), and sufficient (75-100 nmol/L) respectively. Mean PTH concentrations pre supplementation were 32.7± 10.1 pg/mL. No significant difference between groups was reported pre supplementation for total 25(OH)D, 25(OH)D2, 25(OH)D_3, and PTH concentrations.

Table 5.1: Vitamin D status, including total 25(OH)D, 25(OH)D2, 25(OH)D3 (nmol/L) and PTH (pg/mL), pre and post supplementation.

Data presented as mean ± SD. * Significantly different to baseline ($p<0.05$). † Significantly different to PLA ($p<0.05$).

	PRE SUPPLEMENT				**POST SUPPLEMENT**			
Group	**Total 25(OH)D** (nmol/L)	**D_3** (nmol/L)	**D_2** (nmol/L)	**PTH** (pg/mL)	**Total 25(OH)D** (nmol/L)	**D_3** (nmol/L)	**D_2** (nmol/L)	**PTH** (pg/mL)
PLA	**39.8 ± 12.5**	34.7 ± 13.1	5.1 ± 5.2	32.7 ± 6.4	**44.4 ± 16.5**	43.7 ± 21.2	3.5 ± 2.0	34.4 ± 4.1 *
1500IU	**59.7 ± 24.4**	55.9 ± 23.4	3.9 ± 1.4	30.3 ± 12.5	**103.6 ± 26.2*†**	100.8 ± 26.2*†	2.8 ± 0.0	30.3 ± 10.5
3000IU	**52.3 ± 24.2**	49.0 ± 24.6	3.3 ± 1.1	34.7 ± 11.9	**125.6 ± 34.3*†**	120.5 ± 34.4*†	5.0 ± 5.1	30.2 ± 10.1*
6000IU	**45.2 ± 18.8**	42.1 ± 18.9	3.0 ± 0.5	32.5 ± 10.7	**130.5 ± 37.5*†**	127.7 ± 37.5*†	2.8 ± 0.0	27.4 ± 6.9

5.4.3 Vitamin D status post supplementation

Following supplementation, total serum 25(OH)D increased significantly for all groups receiving vitamin D_3 ($p<0.05$) (Figure 5.1). Vitamin D_3 supplementation caused significant increases in 25(OH)D_3 concentration when compared to baseline, 43.9 ± 17.2 nmol/L, 73.2 ± 42.1 nmol/L, and 85.3 ± 37.1 nmol/L for 1500IU, 3000IU and 6000IU respectively ($p<0.05$) (Figure 5.2). Yet no significant difference was observed in the change in 25(OH)D_3 concentration between 1500IU, 3000IU and 6000IU. In addition, while 25(OH)D_3 significantly increased, no significant difference was observed in 25(OH)D_2 concentration following vitamin D_3 supplementation or PLA (Table 5.1). There was a small but significant increase in PTH concentrations post supplementation in PLA whereas 1500IU did not significantly change. Those athletes receiving 3000IU had a significant decline in PTH concentration pre to post supplementation (34.7±11.9 pg/mL and 30.2±10.1 pg/mL respectively, $p<0.05$). Whereas those receiving 6000IU had a lower PTH concentration post supplementation, but this failed to reach significance (32.5±10.7pg/mL and 27.4±6.9pg/mL respectively, $p=0.087$).

5.4.4 Exercise performance measures

Following supplementation, body weight did not significantly differ PRE to POST for PLA, 1500IU, 3000IU or 6000IU (77.2±8.6 kg and 76.9±8.5 kg, 76.4±5.3 kg and 76.8±5.4 kg, 75.2±6.1 kg and 74.7±6.0 kg, 76.9±5.9 kg and 77.4±5.8 kg respectively).

5.4.4.1 Aerobic exercise

Vitamin D supplementation did not change any measure from the VO_2 max test PRE to POST within any of the groups or in any of the vitamin D groups compared to PLA (Table 5.2). Time trial (TT) performance did not change PRE to POST in any groups or in any of the vitamin D groups compared to PLA (Table 5.2). However, a trend for an improved TT performance time was observed for 3000IU and 6000IU, but this failed to reach significance.

Figure 5.1: Total 25(OH)D concentration pre and post supplementation for each intervention group. Data presented as median and range (25% and 75% percentiles). * Significantly different to baseline ($p<0.05$).

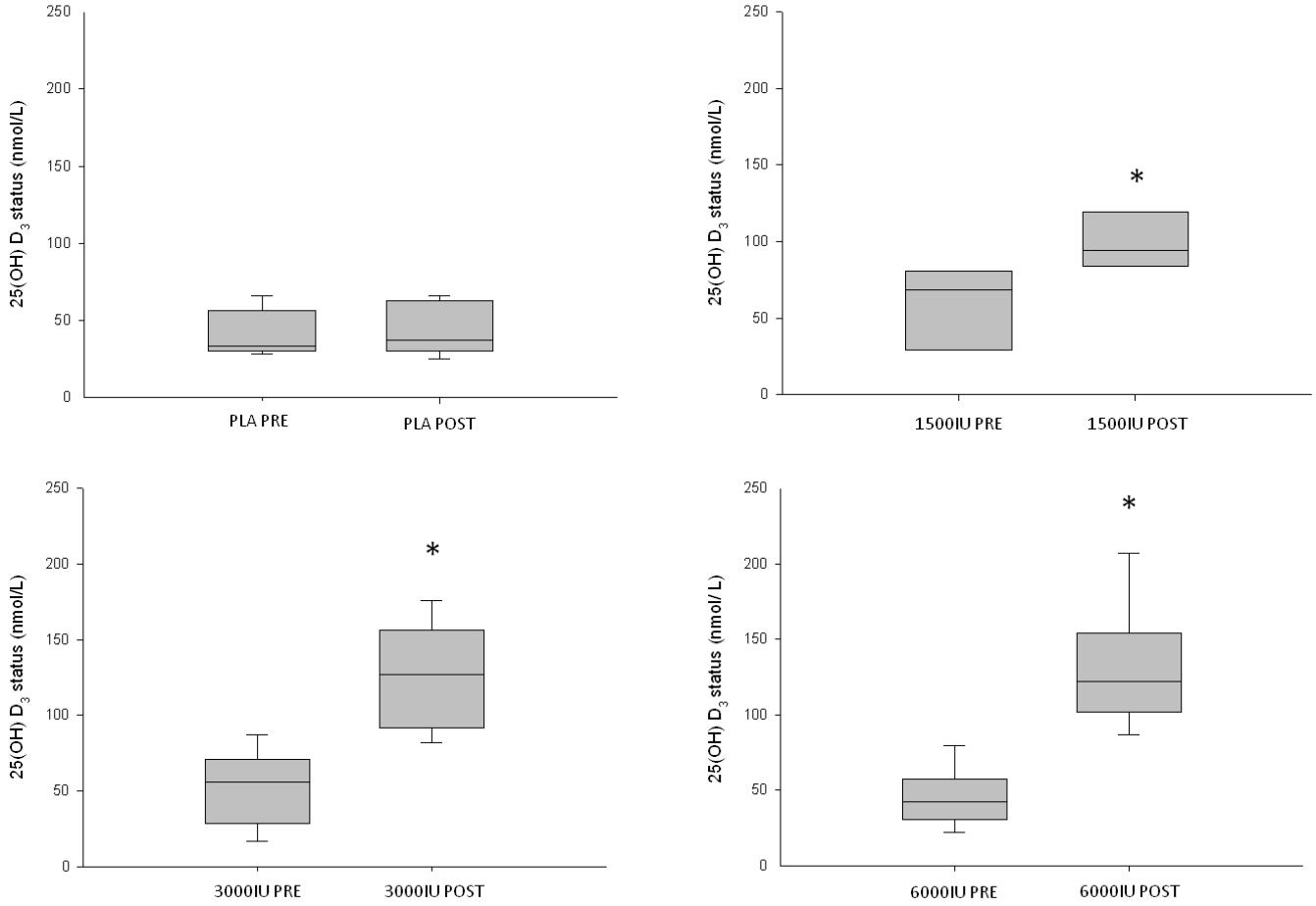
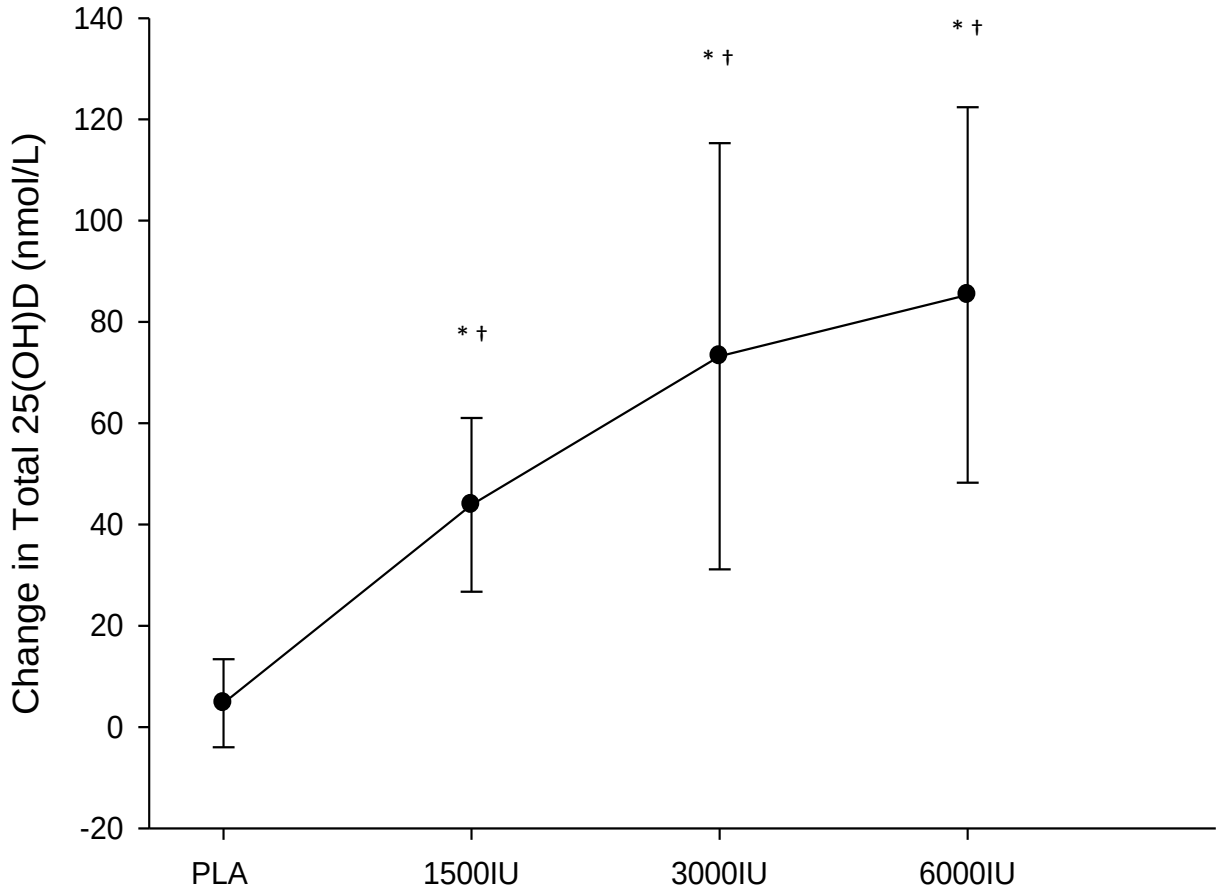


Figure 5.2: Change in total 25(OH)D (nmol/L) pre to post supplementation for each intervention. * Significantly different to baseline ($p<0.05$). † Significantly different to PLA ($p<0.05$). Data presented as mean ± SD



5.4.4.2 Anaerobic exercise

The mean of the four Wingate attempts for peak power, mean power and rate to fatigue are summarised in Table 5.3. There were no significant differences in any of the Wingate performance outcomes within each group PRE to POST or compared to PLA.

Table 5.2: Summary of aerobic exercise performance measures pre and post supplementationVO_2 max test and 1 hour time trial test, pre to post supplementation. Data presented as mean ± SD. * Significantly different to baseline ($p<0.05$). † Significantly different to PLA ($p<0.05$).

	Group							
	PLA		**1500IU**		**3000IU**		**6000IU**	
Measure	**PRE**	**POST**	**PRE**	**POST**	**PRE**	**POST**	**PRE**	**POST**
VO_2 max (L/min)	4.78 ±0.39	4.85 ±0.50	4.52 ±0.28	4.50 ±0.30	4.38 ±0.40	4.31 ±0.34	4.54 ±0.32	4.62 ±0.30
W_{max} (W)	385.1 ±39.0	382.8 ±42.1	344.8 ±23.3	338.6 ±30.8	348.6 ±29.1	348.6 ±29.1	372.1 ±29.2	374.1 ±29.3
HR_{max} (bpm)	172 ±17	171 ±20	177 ±9	176 ±7	178 ±9	178 ±12	182 ±7	182 ±10
TT finish time (min)	60.06 ±2.30	59.50 ±2.05	59.02 ±2.41	59.57 ±2.65	64.16 ±4.75	62.51 ±4.55	60.00 ±3.08	59.08 ±3.00
TT power (W)	291.8 ±40.2	290.4 ±41.6	268.5 ±24.4	264.9 ±24.2	249.8 ±29.8	257.2 ±30.4	287.2 ±36.1	295.5 ±34.9

Table 5.3: Summary of mean Wingate performance pre and post supplementation: Peak power, Average power and Rate to fatigue. Data presented as mean ± SD. * Significantly different to baseline ($p<0.05$). † Significantly different to PLA ($p<0.05$).

	Peak Power (W)		**Average Power** (W)		**Rate to Fatigue** (W/sec)	
Group	**PRE**	**POST**	**PRE**	**POST**	**PRE**	**POST**
PLA	1155±157	1158±161	650±68	639±64	28±7	28±6
1500IU	1073±177	1096±162	593±33	607±16	27±7	27±8
3000IU	1137±163	1165±173	617±70	615±66	27±6	29±6
6000IU	1157±182	1211±187	632±54	638±44	29±8	31±9

5.4.4.3 Muscle strength

Single leg 1 repetition max (1RM), on dominant and non-dominant leg press (LP), did not significantly differ in the change PRE to POST for any of the groups or when the difference was

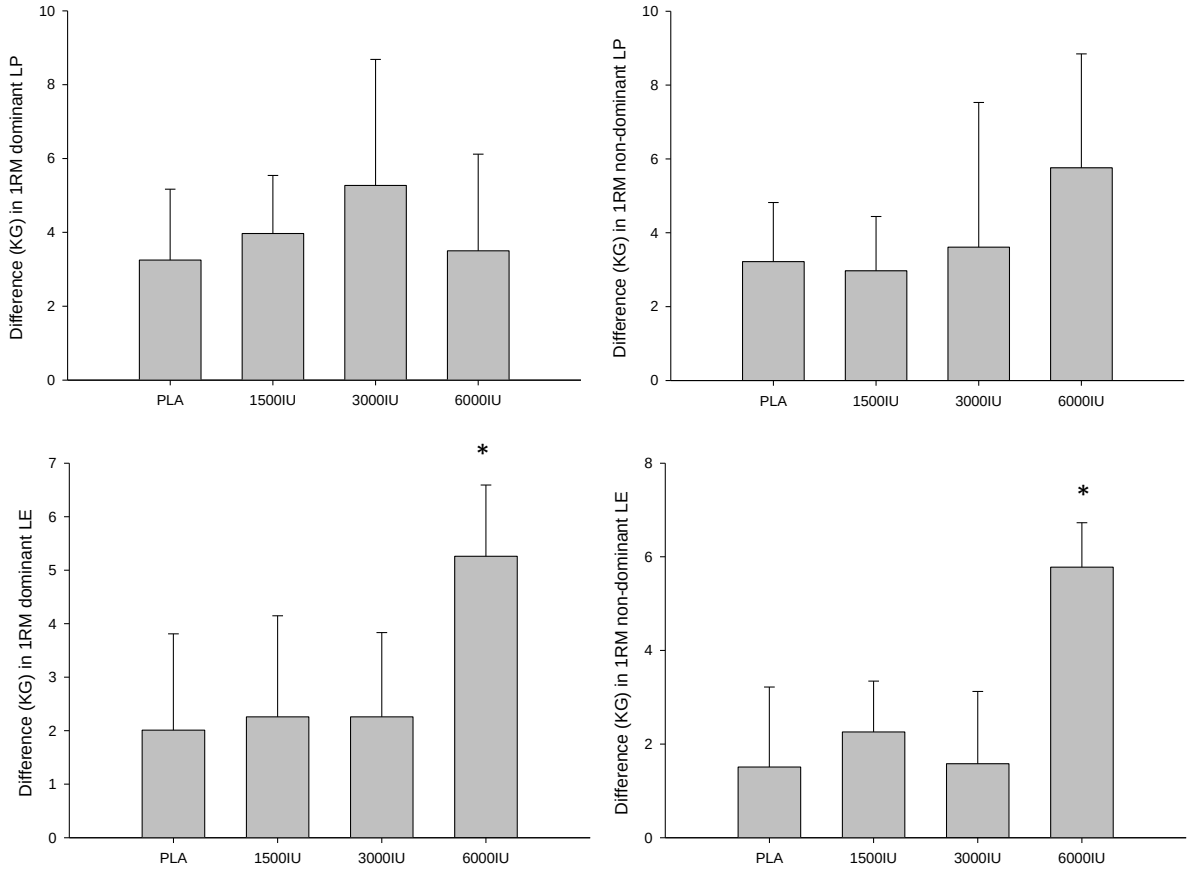
compared to PLA (Figure 5.4). Similarly, leg extension (LE) did not significantly differ in the change PRE to POST for PLA, 1500IU and 3000IU, however 6000IU caused a significant increase in 1RM LE when comparing pre to post supplementation on the dominant (64±11 kg and 69±9 kg respectively ($p<0.05$)) and non-dominant (66±11 kg and 71±10 kg respectively ($p<0.02$)) legs. However, the significant increase in LE PRE to POST was not significantly different to PLA, 1500IU or 3000IU (Figure 5.4). Hand grip maximal voluntary contraction (HG) appeared to not significantly change pre to post supplementation for PLA (570±85 kg and 596±76 kg (D) respectively, 532±89 kg and 555±81 kg (ND) respectively), 1500IU (636± 66 kg and 619±64 kg (D) respectively, 586±90 kg and 595±109 kg (ND) respectively), 3000IU (581± 54 kg and 601±54 kg (D) respectively, 554±62 kg and 566±52 kg (ND) respectively) or 6000IU (596± 75 kg and 594±65 kg (D) respectively, 588±69 kg and 574±63 kg (ND) respectively).

5.4.5 Vitamin D association to exercise performance

Pre supplementation total 25(OH)D did not correlate with any of the performance measures. Following vitamin D supplementation, despite a significant increase in total 25(OH)D, no correlation was found between total 25(OH)D and performance measures. In addition we calculated the change in both total 25(OH)D and performance measures following supplementation, however this also showed no correlation. There was also no correlation between PTH and performance measure or body weight and total 25(OH)D.

Figure 5.3: Absolute change (kg) in 1RM single leg press (LP) and extension (LE) following supplementation.

Data presented as mean ± SD * significantly different to baseline ($p<0.05$). † Significantly different to PLA ($p<0.05$).



5.5 Discussion

By understanding the prevalence of vitamin D deficiency in athletes and the vitamin D dose and supplementation protocol to avoid deficiency, the implications vitamin D may have on skeletal muscle function and performance in athletes can be explored. For these reasons, the current study had three aims: firstly to assess vitamin D status in endurance trained athletes, secondly to investigate the effect of vitamin D supplementation on improving vitamin D status and thirdly to examine the effect vitamin D status had on performance pre and post supplementation.

The present investigation confirms the high prevalence of vitamin D deficiency in athletes, with 8%, 53% and 33% being severely vitamin D deficient, deficient and insufficient, respectively. These figures are in agreement with the limited number of studies in athletes to date [30]. More specifically, similar prevalence of vitamin D deficiency and insufficiency have been demonstrated at similar latitude to the current test site (52.4-52.5°N) [14, 15]. These results are not surprising, and as discussed in Chapter 4, are likely explained by poor cutaneous vitamin D synthesis and poor dietary intake. These findings were shown by the low vitamin D intake (116 ± 162 IU/d), prior to supplementation, which was well below the current RNI in the UK (200IU/d) [22], as assessed by food frequency questionnaire.

The current study clearly illustrates that all participants receiving a vitamin D supplement improved vitamin D status to a sufficient concentration (>75nmol/L) in 28 d during the winter. Those participants receiving 1500IU, 3000IU and 6000IU resulted in a significant increase in 25(OH)D from baseline to 43.9±17.2, 73.2±42.1 and 85.3±37.1 nmol/L, respectively (Figure 5.1 and 5.2). We also observed a significant and non significant reduction in PTH following 3000IU and 6000IU, respectively (Table 5.1), which is in agreement with the inverse relationship observed between 25(OH)D and PTH through negative feedback regulation [31].These changes in 25(OH)D are similar to previous studies following longer term (8-20 wk) vitamin D_3 supplementation [32, 33]. Barger-Lux et al [32] illustrated that 8 weeks supplementation of 1,000IU/d and 10,000IU/d to healthy adults

during the winter led to a 29 nmol/L and 146 nmol/L increase in 25(OH)D. Similarly, Heaney et al [33] supplemented 1000IU/d, 5000IU/d and 10,000IU/d to healthy men during the winter for a total of 20 weeks, with blood samples taken every 3 weeks throughout the supplementation period. The authors showed a ~12 nmol/L, 91 nmol/L and 158 nmol/L increase in 25(OH)D following 20 weeks of 1000IU/d, 5000IU/d and 10,000IU/d, respectively. It should be pointed out that no further change in 25(OH)D was observed after ~8 weeks, with others showing a plateau in 25(OH)D following 1000IU [34], 50,000IU and 100,000IU [35, 36] for 1-2 months. Heaney et al (REF) calculated that for every 40IU ingested an increase of 0.7 nmol/L occurred, which is in line with the current studies response to each vitamin D dose.

Interestingly, despite the significant increase in 25(OH)D from baseline, the differences between each of the vitamin D groups following supplementation did not reach statistical significance (Figure 5.1 and 5.2). This would suggest that 1500IU taken for 28 d is an adequate dose to improve vitamin D status to sufficient concentrations. It should be noted that despite the higher doses of vitamin D, above the current RNI, the risk of vitamin D toxicity is low. Vitamin D toxicity, categorised by hypercalcaemia, has been reported to only take effect at 25(OH)D concentrations of 220-600nmol/L [37]. In the current study plasma calcium was not measured, however 25(OH)D following supplementation in all vitamin D groups was well below the 25(OH)D concentrations related to toxicity (Table 1). In addition vitamin D intake above the current RNI of up to 10,000IU/d has been shown to not cause hypercalcaemia [37]. For these reasons, it would be suggested that short term supplementation of high vitamin D_3 doses, as used in the current study, are efficacious at improving vitamin D status whilst posing little concern regarding vitamin D toxicity.

The lack of significant difference in 25(OH)D between each of the vitamin D supplement groups may be explained by the variation in response to the vitamin D supplements (Figure 5.2). It has been shown previously that the response to a vitamin D supplement may be altered by baseline vitamin D status, age of participant, body composition and genetic factors [32, 33]. In the current study no significant differences in body mass between group characteristics at baseline were observed. Alternatively, small

non-significant variations in vitamin D status at baseline were apparent (Table 5.1). It is evident that the response to vitamin D supplementation is biphasic [38], with larger increases in 25(OH)D achieved with smaller doses of vitamin D when compared to larger doses when vitamin D deficient [32]. Therefore, an identical vitamin D dose may not produce the same response in 25(OH)D, when individuals baseline vitamin D status varies. This suggests that the varied response in the current study may be explained by the response to a supplement based on participant's baseline vitamin D status.

Despite elevating 25(OH)D to prevent insufficiencies in all participants in the vitamin D intervention groups, we did not observe any changes in aerobic or anaerobic performance. In addition we also showed that pre and post supplementation 25(OH)D and PTH concentration did not correlate with any of the aerobic, anaerobic or strength performance measures. This suggests that vitamin D status is not a limiting factor to exercise performance in endurance trained athletes. These findings are in contrast to the previous causative evidence in elderly [39-41] and young adults [6, 42]. Studies conducted in the elderly have shown an improvement in strength based tests following vitamin D supplementation [43]. Interestingly we observed a significant increase in 1 RM for dominant and non dominant leg extension but not for leg press, in those receiving 6000IU only. When comparing the change in 1RM leg extension between groups no significant difference was apparent (Figure 5.3). In addition, due to the lack of association between the change in vitamin D and change in performance, it is difficult to attribute the improvement in 1RM leg extension following 6000IU to the vitamin D supplement. However, it has been acknowledged by the authors that the lack of test re-test of the performance measures in the current study may have explained large variances in performance outcomes. Despite this, Barker et al [44] recently showed no change in leg strength or power, assessed using a leg isometric dynometer, following 28 d of 200IU/d or 4000IU/d vitamin D_3 supplementation in healthy male participants. Whilst it may be argued that the lack of finding by Barker et al [44] may have been explained by the fact that most participants already had relatively high levels of vitamin D prior to supplementation (63% of participants were sufficient). In contrast, Close et al [14] recently reported that 8 weeks of 5000IU/d vitamin D_3 supplementation to a small group of soccer players (n=5)

significantly improved 10-metre sprint times and vertical jump heights when compared to a placebo (n=5). Whereas, 1RM bench press and back squat did not significantly differ following vitamin D supplementation.

Based on the abovementioned evidence, it appears that the exact 25(OH)D concentration that impairs or improves musculoskeletal performance is unknown. It would seem that vitamin D supplementation may only provide benefits to musculoskeletal performance if an individual is vitamin D deficient in the first place (<50 nmol/L). A large proportion of the participants in the current study were vitamin D deficient (53%), with few (8%) being severely vitamin D deficient (<25nmol/L). A recent meta analysis concluded that there was no significant effect of vitamin D supplementation (range of doses included 400IU/d to 60,000IU/wk) on leg or grip strength in the elderly when baseline 25(OH)D was >25nmol/L [45]. In addition Kenny et al [18] showed that no improvements to musculoskeletal performance was apparent in elderly individuals who were sufficient at baseline, despite a significant increase in 25(OH)D (~87nmol/L) following 1000IU/d for 6 months. In further support of this notion, the improvements in exercise performance in athletes observed by Close et al [14] were predominantly deficient at baseline, with 3/5 of the participants in the vitamin D group being severely deficient (<25 nmol/L). Thus it is possible that vitamin D supplementation may only have effects in individuals with severe deficiency.

On the other hand, the optimal sufficient concentration of 25(OH)D following supplementation to improve musculoskeletal performance is unknown. Despite the relatively high 25(OH)D concentrations following supplementation in the current study (> 100 nmol/L) (Table 5.1) we did not detect a change in any measure of exercise performance. It has been shown in elderly studies that the risk of falls and fractures is lower when 25(OH)D is above 75nmol/L [43]. Dam et al [46] reported that lower body muscular performance in elderly women was impaired when 25(OH)D <80nmol/L compared to >115nmol/L. In athletes, Close et al [14] reported improvements to musculoskeletal performance at 25(OH)D concentrations > 75 nmol/L (range 75-140 nmol/L), which interestingly are in the range of the concentrations observed following vitamin D supplementation in the present study.

However, improvements to muscular maximal voluntary contraction have also been observed at 35nmol/L following intra muscular administration of 100,000IU vitamin D_3. Yet within this study severely deficient (7nmol/L) veiled Arab women were recruited [47]. Therefore, it remains to be determined whether it is the extent of deficiency prior to supplementation or the concentration following supplementation that dictates the improvements to musculoskeletal performance that have been documented previously.

In summary, the present study has shown a high prevalence of vitamin D deficiency and insufficiency in endurance athletes based in the UK. Vitamin D supplementation, during the winter, above the current RNI is highly effective in increasing vitamin D status to sufficient concentrations in 28 d. It appeared that 1500IU for 28 d was an adequate dose to raise status in deficient individuals, when compared to higher doses. However, at present the exact dosing protocol and duration of supplementation required to sustain sufficient vitamin D status has not been established. Our data also suggests that vitamin D status pre and post supplementation was not a limiting factor to exercise performance in endurance trained athletes. The role of vitamin D and the optimal 25(OH)D concentration that may elicit impairments or improvements to muscle function and performance are yet to be determined.

5.6 References

1. Lips P (2006) Vitamin D physiology. Prog Biophys Mol Biol 92: 4-8.
2. Ceglia L, Harris SS (2013) Vitamin D and Its Role in Skeletal Muscle. Calcif Tissue Int 92: 151-162.
3. Hamilton B (2010) Vitamin D and human skeletal muscle. Scand J Med Sci Sports 20: 182-190.
4. Willis KS, Peterson NJ, Larson-Meyer DE (2008) Should we be concerned about the vitamin D status of athletes? Int J Sport Nutr Exerc Metab 18: 204-224.
5. Cannell JJ, Hollis BW, Sorenson MB, Taft TN, Anderson JJ (2009) Athletic performance and vitamin D. Med Sci Sports Exerc 41: 1102-1110.
6. Pollock N, Dijkstra P, Chakraverty R, Hamilton B (2012) Low 25(OH) vitamin D concentrations in international UK track and field athletes. S Afr J SM 24: 55-59.
7. Dawson-Hughes B (2008) Serum 25-hydroxyvitamin D and functional outcomes in the elderly. Am J Clin Nutr 88: 537S-540S.
8. Dawson-Hughes B (2012) Serum 25-hydroxyvitamin D and muscle atrophy in the elderly. Proc Nutr Soc 71: 46-49.
9. Dirks-Naylor AJ, Lennon-Edwards S (2011) The effects of vitamin D on skeletal muscle function and cellular signaling. The Journal of steroid biochemistry and molecular biology 125: 159-168.
10. Ward KA, Das G, Berry JL, Roberts SA, Rawer R, et al. (2009) Vitamin D status and muscle function in post-menarchal adolescent girls. J Clin Endocrinol Metab 94: 559-563.
11. Ardestani A, Parker B, Mathur S, Clarkson P, Pescatello LS, et al. (2011) Relation of vitamin D level to maximal oxygen uptake in adults. The American journal of cardiology 107: 1246-1249.
12. Barker T, Martins TB, Hill HR, Kjeldsberg CR, Henriksen VT, et al. (2012) Different doses of supplemental vitamin D maintain interleukin-5 without altering skeletal muscle strength: a randomized, double-blind, placebo-controlled study in vitamin D sufficient adults. Nutrition & metabolism 9: 16.
13. Kenny AM, Biskup B, Robbins B, Marcella G, Burleson JA (2003) Effects of vitamin D supplementation on strength, physical function, and health perception in older, community-dwelling men. J Am Geriatr Soc 51: 1762-1767.
14. van Schoor NM, Lips P (2011) Worldwide vitamin D status. Best practice & research Clinical endocrinology & metabolism 25: 671-680.
15. Powers S, Nelson WB, Larson-Meyer E (2011) Antioxidant and Vitamin D supplements for athletes: Sense or nonsense? J Sports Sci 29 S47-55.
16. Morton JP, Iqbal Z, Drust B, Burgess D, Close GL, et al. (2012) Seasonal variation in vitamin D status in professional soccer players of the English Premier League. Appl Physiol Nutr Metab 37: 798-802.
17. Close GL, Russell J, Cobley JN, Owens DJ, Wilson G, et al. (2012) Assessment of vitamin D concentration in non-supplemented professional athletes and healthy adults during the winter months in the UK: implications for skeletal muscle function. J Sports Sci 31: 344-353.
18. IOM Report wwwiomedu/Reports/2010/Dietary-Reference-Intakes-for-Calcium-and-Vitamin-D/Report-Briefaspx.
19. Ashwell M, Stone EM, Stolte H, Cashman KD, Macdonald H, et al. (2010) UK Food Standards Agency Workshop Report: an investigation of the relative contributions of diet and sunlight to vitamin D status. Br J Nutr 104: 603-611.
20. Holick MF, Binkley NC, Bischoff-Ferrari HA, Gordon CM, Hanley DA, et al. (2011) Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society clinical practice guideline. J Clin Endocrinol Metab 96: 1911-1930.
21. Department.of.Health (1998) Nutrition and bone health with particular reference to calcium and vitamin D: Report of the Subgroup on Bone Health (Working Group on the Nutritional

Status of the Population) of the Committee on Medical Aspects of Food and Nutrition Policy. wwwdhgovuk The Staionery Office.
22. Heaney RP, Holick MF (2011) Why the IOM recommendations for vitamin D are deficient. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research 26: 455-457.
23. Heaney RP, Vieth R, Hollis BW (2011) Vitamin D efficacy and safety. Archives of internal medicine 171: 266; author reply 267.
24. Halliday TM, Peterson NJ, Thomas JJ, Kleppinger K, Hollis BW, et al. (2011) Vitamin D status relative to diet, lifestyle, injury, and illness in college athletes. Med Sci Sports Ex 43: 335-343.
25. Food.Standards.Agency McCance and Widdowson's Composition of Foods Integrated dataset (CoF IDS). wwwfoodgovuk
26. Webb AR, Holick MF (1988) The role of sunlight in the cutaneous production of vitamin D3. Annual review of nutrition 8: 375-399.
27. Currell K, Jeukendrup AE (2008) Validity, reliability and sensitivity of measures of sporting performance. Sports medicine 38: 297-316.
28. Witard OC, Tieland M, Beelen M, Tipton KD, van Loon LJ, et al. (2009) Resistance exercise increases postprandial muscle protein synthesis in humans. Med Sci Sports Ex 41: 144-154.
29. Lehtonen-Veromaa M, Mottonen T, Irjala K, Karkkainen M, Lamberg-Allardt C, et al. (1999) Vitamin D intake is low and hypovitaminosis D common in healthy 9- to 15-year-old Finnish girls. European journal of clinical nutrition 53: 746-751.
30. Carlsohn A, Cassel M, Linne K, Mayer F (2011) How much is too much? A case report of nutritional supplement use of a high-performance athlete. Br J Nutr: 1-5.
31. Barger-Lux MJ, Heaney RP, Dowell S, Chen TC, Holick MF (1998) Vitamin D and its major metabolites: serum levels after graded oral dosing in healthy men. Osteoporosis international : a journal established as result of cooperation between the European Foundation for Osteoporosis and the National Osteoporosis Foundation of the USA 8: 222-230.
32. Heaney RP, Davies KM, Chen TC, Holick MF, Barger-Lux MJ (2003) Human serum 25-hydroxycholecalciferol response to extended oral dosing with cholecalciferol. Am J Clin Nutr 77: 204-210.
33. Vieth R (2007) Vitamin D toxicity, policy, and science. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research 22 Suppl 2: V64-68.
34. Heaney RP, Armas LA, Shary JR, Bell NH, Binkley N, et al. (2008) 25-Hydroxylation of vitamin D3: relation to circulating vitamin D3 under various input conditions. Am J Clin Nutr 87: 1738-1742.
35. Bischoff-Ferrari H (2009) Vitamin D: what is an adequate vitamin D level and how much supplementation is necessary? Best Pract Res Clin Rheumatol 23: 789-795.
36. Stockton KA, Mengersen K, Paratz JD, Kandiah D, Bennell KL (2011) Effect of vitamin D supplementation on muscle strength: a systematic review and meta-analysis. Osteoporosis international : a journal established as result of cooperation between the European Foundation for Osteoporosis and the National Osteoporosis Foundation of the USA 22: 859-871.
37. Dam TT, von Muhlen D, Barrett-Connor EL (2009) Sex-specific association of serum vitamin D levels with physical function in older adults. Osteoporosis international : a journal established as result of cooperation between the European Foundation for Osteoporosis and the National Osteoporosis Foundation of the USA 20: 751-760.
38. Glerup H, Mikkelsen K, Poulsen L, Hass E, Overbeck S, et al. (2000) Hypovitaminosis D myopathy without biochemical signs of osteomalacic bone involvement. Calcif Tissue Int 66: 419-424.

Chapter 6

General Discussion

This thesis investigated three commonly used nutritional interventions and supports by athletes that are often cited to optimise fat metabolism during exercise and improve endurance exercise performance. These interventions and supports included green tea, coffee and vitamin D.

More specifically the purpose of this thesis was four fold:

1. **To describe the metabolic effects of green tea at rest and during moderate intensity exercise.**
2. **To compare the metabolic and performance effects of caffeine and coffee during endurance exercise.**
3. **To provide insight into the prevalence of vitamin D deficiency in athletes and the potential impact on exercise performance.**
4. **To determine the influence of vitamin D supplementation on improving vitamin D status and exercise performance in athletes.**

6.1 Part A. Green Tea and Caffeine

Chapter 2 examined the metabolic effects of green tea extract (GTE) on human metabolism at rest and during moderate intensity exercise. These effects were assessed in plasma using a metabolomics approach, which can be used to systematically assess patterns and changes in plasma metabolites following exercise and dietary interventions. This approach allowed a comprehensive unbiased and systemic investigation into the diverse metabolic responses of GTE. This is the first study to directly compare the metabolic responses to GTE supplementation at rest and during moderate intensity exercise. GTE resulted in different metabolic effects during rest and exercise when compared to placebo: At rest GTE induced metabolite changes indicative of enhanced lipolysis (increase in glycerol, reduction in TAGs, increased fat oxidation (increase in 3-hydroxybutyrate), and activated TCA cycle (increase in citrate). These metabolic changes suggest that GTE has the potential to augment fat metabolism at rest, potentially by increasing lipolysis, which is in agreement with studies

that have shown increases in whole body fat oxidation at rest in humans [1-4]. However, previous studies have also shown no change in fat oxidation at rest following GTE in humans [5-8]. During exercise, GTE caused metabolite changes that were associated with enhanced glycolysis (increase in pyruvate, lactate) and decreased fat oxidation (reduction in 3-hydyroxybutyrate). These findings were somewhat unexpected as previous evidence has demonstrated that short [9] and long term [10, 11] GTE intake increases fat oxidation during exercise in humans. Although the reasons for these differential effects remain unclear, these data suggest that GTE has little effect on fat metabolism during exercise, which may limit its use as a nutritional support in that occasion. This is further supported by the fact that whole body fat oxidation was not altered in the same study following 7 days GTE, which has been reported in a separate manuscript [12].

It is often cited in the literature that the short term metabolic effects of GTE are induced through a stimulatory effect on the SNS through the enzyme catechol-*O*- methyltransferase (COMT) [13]. COMT is a constitutively active enzyme, ubiquitous throughout all mammalian tissues including skeletal muscle and adipose tissue [14]. EGCG has been reported to directly inhibit catechol-*O*-methyltransferase (COMT) [15]. Herein, it is believed that circulatory catecholamine concentrations will be greater thus stimulating lipolysis via adrenergic receptors and potentially increasing fat oxidation [16]. However, the evidence to support the COMT mechanism is limited to a small number of *in vitro* studies [15, 17]. In order to investigate this mechanism *in vivo*, **Chapter 2** used a targeted metabolomics approach to assess catecholamine response, specifically including adrenaline and noradrenaline. Not surprisingly, **Chapter 2** showed that moderate intensity exercise alone induced significant changes of 14 metabolites involved in the catecholamine metabolism. However, in **Chapter 2** we found no change in plasma catecholamines at rest or during moderate intensity exercise for GTE compared to PLA.

Therefore, the metabolic effects that were observed at rest and during exercise in **Chapter 2** appear to be independent of any significant changes in catecholamine concentration. As a result, this questions the efficacy of the widely referenced COMT mechanism to explain changes in substrate metabolism in

humans. **Chapter 2** is supported by a small number of investigations that shows no change in plasma catecholamines following GTE when compared to PLA [5-7]. Thus, the mechanisms to describe the increase in fat oxidation at rest and during exercise following short term GTE intake remain unclear. Previous investigations in animals have shown that the improvements to fat oxidation following GTE maybe explained by increases in mRNA expression of specific fat metabolism enzymes. But this data is purely limited to longer term GTE intake [18-20]. On the other hand, Murase et al [21] has recently reported that treatment of rat L6 myoblasts with EGCG transiently increased AMPK and ACC activity. Both of these enzymes in the skeletal muscle have been proposed to acutely regulate fat metabolism [22]. The authors also showed that the increase in AMPK activity was due to the activation of the upstream kinase LKB1. The increase in AMPK and ACC was also confirmed following the acute feeding of EGCG (200 mg/kg BM) to mice, which was paralleled with an increase in fat oxidation at rest. However, while these research findings show promise in explaining the previously reported increase in fat oxidation following short term GTE intake [1, 9] they are currently limited due to the lack of supporting human evidence. Therefore, future research is required to elucidate the mechanisms of short term GTE intake on fat oxidation, potentially focusing on the role of AMPK, LKB1 and other fat metabolism regulating signalling molecules

Alternatively, caffeine, ingested mainly at high doses (>5 mg/kg BM) has previously been shown to consistently cause an increase in catecholamines at rest and during exercise [23-26] which has also increases plasma FA and glycerol concentrations [26-28]. Therefore, the presence of caffeine in GTE may be responsible for augmenting the SNS and stimulating lipolysis. But regardless of the presence of caffeine in the GTE drink in **Chapter 2**, no difference in plasma catecholamine response was observed. This is likely explained by the lower dose of caffeine (120 mg) ingested in **Chapter 2** compared to previous caffeine studies [5-7]. Further, despite the increase in the SNS and lipolysis following moderate to high caffeine intake (>5 mg/kg BM) very few have observed an increase in fat oxidation [29-32]. This is supported by the findings in **Chapter 3**, whereby caffeine (5 mg/kg BM) consumed 1 hour prior to exercise caused a significant increase in plasma fatty acid and glycerol

concentrations at rest, but did not cause any difference in whole body fat oxidation when compared to coffee, placebo or decaf coffee. Collectively this evidence suggests that the metabolic significance of increasing catecholamines at rest and during exercise with GTE and/or caffeine is unclear. In support, previous evidence has shown that increasing circulating adrenaline concentrations (within a physiological range), through infusions, has not led to an increase in fat oxidation, despite an increase in lipolysis [33]. Furthermore, increasing the availability of FA during exercise following GTE and caffeine, as observed in **Chapter 2 and 3**, also suggests a lack of metabolic significance for increasing fat oxidation. Previous studies have shown that plasma FA oxidation is the predominant fat source oxidised during low to moderate intensity exercise, with a concomitant reduction at higher exercise intensities [34, 35]. However, increasing the availability of FA through intralipid plus heparin infusions during exercise under high glycolytic conditions, such as high intensity exercise or carbohydrate feeding, partly rescues the blunted fat oxidation rates [36, 37]. However, it would appear that the magnitude of plasma FA concentration change is crucial in order to augment fat oxidation. The magnitude of FA change with GTE and caffeine, shown in **Chapter 2 and 3** and other previous caffeine [26, 38] and GTE [1, 9, 12], may not be large enough to cause significant changes in fat oxidation. In addition, **Chapter 2 and 3** studied the effects of GTE and Caffeine, respectively, during moderate intensity exercise which has previously been shown not to limit FA availability or oxidation [34, 35]. Therefore, increasing fat oxidation during exercise may only be apparent when FA availability and oxidation is limited, such as at high exercise intensities (>75 % VO_2 max) [36] or in individuals who have impaired metabolic flexibility [39], as seen in obesity and non-insulin – dependent diabetes mellitus.

The metabolic effects of GTE, with caffeine, during exercise has been rarely studied [9]. When the findings of **Chapter 2 and 3** are taken together, the increase in lactate observed, may suggest the presence of caffeine in GTE could possibly alter the metabolic response during exercise when compared to GTE alone. These findings are in contrast to previous hypotheses that state the GTE and caffeine may have a synergistic effect [13]. The duration of GTE intake may also be a significant

factor in determining not only the effects on fat metabolism but also the mechanisms of action (as previously discussed). In **Chapter 2**, the metabolic effects observed during exercise can largely be attributed to the acute (single dose) post-GTE-supplementation, while the effects at rest are due to chronic (7 days) GTE supplementation. Firstly, the different metabolic effects induced at rest and during exercise in the current thesis may have been explained by the chronic and acute feeding of GTE, respectively. Secondly, considering that longer term GTE intake in human [40-43] and in animals [20] has shown promise at increasing fat oxidation, it remains to be determined whether longer term intake is more effective then shorter term intake at altering fat metabolism. Therefore, future research is required to examine the longer term effects of GTE supplementation on whole body fat metabolism at rest and during exercise. In addition, evidence in animal studies have shown that longer term GTE intake, when combined with endurance exercise training, results in superior adaptations to fat metabolism when compared to exercise alone [18, 19, 44]. Considering the well documented evidence to show that endurance exercise training is highly effective at augmenting fat oxidation and improving endurance exercise capacity [45-48], future research should include the effects of GTE on skeletal muscle adaptations at rest and following endurance exercise training in humans. In turn this may provide evidence for the use of GTE as a nutritional support to endurance exercise training as well as improve our understanding of the mechanisms of actions to describe the metabolic effects of GTE.

In **Chapter 3**, the ergogenic effects of coffee compared to caffeine were examined. It had been suggested that caffeine resulted in increases in exercise capacity but coffee (including caffeine) did not [49]. **Chapter 3** offers new evidence to support the use of coffee as nutritional support for endurance athletes. Coffee (5mg/kg BM) consumed 1 hour prior to exercise was equally as effective at improving endurance exercise performance when compared to caffeine (5mg/kg BM). Prior to the presented work, only two studies have used both caffeine and coffee combinations within the same study [49, 50], with contradicting results regarding the ergogenic effect of coffee. Graham et al [49] found that anhydrous caffeine (4.5 mg/kg BM consumed as a capsule) significantly improved running time to

exhaustion when compared to placebo (consumed as a capsule) and decaf coffee (consumed as a beverage). However, coffee (4.5 mg/kg BM consumed as a beverage) did not result in a significant improvement to running time to exhaustion when compared to placebo or decaf coffee. On the other hand, McLellan et al [50] reported that consuming coffee (1.1mg/kg BM) prior to the ingestion of different doses of caffeine (3-7mg/kg BM) did not to affect the ergogenic effects of caffeine when using a time to exhaustion test. The discrepancies in findings may be explained by the mode of delivery of caffeine, coffee, decaf and placebo or maybe explained by the performance tests selected in each study. In both of the aforementioned studies a time to exhaustion test was used. However, it has been questioned whether assessing performance using a time to exhaustion test is reliable or has sufficient ecological validity [51]. With this in mind, **Chapter 3** follows a well designed and controlled study by only using beverages as the format of caffeine administration, using a double placebo control (decaf coffee and placebo) and using a highly validated time trial protocol.

In contrast to the similar performance effects of caffeine and coffee in **Chapter 3**, differences in the metabolite responses at rest and during exercise were observed between the beverages. However, these differing metabolite responses did not alter fat oxidation during exercise. This firstly demonstrates that the performance benefits of caffeine, either delivered as anhydrous caffeine or coffee, is not explained by changes to fat oxidation. During the steady state exercise bout, caffeine caused a significant increase in plasma glucose, FA and glycerol whereas coffee had a small non-significant attenuated response to each metabolite. Further, decaf coffee resulted in a lower plasma FA and glycerol concentration when compared to placebo. The mechanisms to describe the varying metabolite responses following each beverage are difficult to explain. Previous evidence has shown an increase in plasma FA and glycerol concentrations in spinal cord injured individuals, who have an impaired sympathoadrenal response, following caffeine intake [52]. The metabolite response induced by caffeine was independent of adrenaline and explained by the antagonism of adenosine receptors on adipose tissue or skeletal muscle. This is because adenosine has been shown to inhibit lipolysis [53] and enhance insulin stimulated glucose uptake in contracting skeletal muscle *in vitro* [54]. Therefore,

it is likely that compounds in coffee may induce subtle effects on antagonism of adenosine receptors (A_1 and A_{2A}) in a variety of tissues [55]. This is partly supported by the findings of Graham et al [49] who showed that coffee lead to a blunted adrenaline concentration following coffee intake when compared to caffeine. These conclusions are limited due to the lack of research. However, while the differing metabolic effects of caffeine and coffee do not alter the ergogenic effects, they may have implications on health outcomes [55]. Therefore, future research is required to understand the metabolic effects of compounds found in coffee and the significance they have on health outcomes.

6.2 Future research questions – Green tea and caffeine:

Following on from **Chapter 2 and 3**, a number of key questions still remain to be answered:

1. Does longer term (> 7 days) GTE supplementation augment fat metabolism at rest and during exercise when compared to shorter term (< 7 days) GTE supplementation?
2. Can GTE supplementation provide additive adaptations to fat metabolism when combined with endurance exercise training in humans?
3. What are the mechanisms to describe the metabolic effects of longer term GTE supplementation within the skeletal muscle in humans?
4. Which of the green tea catechins are bioactive in humans following short and long term GTE supplementation?
5. What is the metabolic effect of GTE at rest and during exercise in individuals who have an impaired ability to oxidise fat?
6. Can coffee be used as a nutritional support to improve endurance exercise performance at lower doses (1-3 mg/kg BM)?
7. What are the physiological and metabolic effects of compounds found in coffee *in vivo*?

6.3 Part B: Vitamin D

Vitamin D has been discussed extensively to date in relation to bone health [56]. However, it also has an important role on optimal skeletal muscle function, structure and composition, particularly in the

elderly skeletal muscle [57]. This evidence in the elderly could suggest that vitamin D may have an important role in determining exercise performance [58]. This is alarming when considering the high prevalence of vitamin D deficiency that is observed worldwide today [59]. Taking together the importance of vitamin D in elderly skeletal muscle combined with the high prevalence of vitamin D deficiency in our population could translate into negative impacts on exercise performance in healthy skeletal muscle. These deleterious effects of vitamin D deficiency to skeletal muscle function would be highly important to athletes. Yet despite this, little is known about the prevalence of vitamin D deficiency in athletes or the impact it may have on exercise performance.

Currently, the definition of categorising vitamin D status is complicated due to multiple recommendations currently available in the literature which are based on varying health outcomes. This thesis defines vitamin D status as severe deficiency, deficiency, insufficiency and sufficiency corresponding to 25(OH)D concentrations of <25 nmol/L, <50 nmol/L, 50-75 nmol/L and >75 nmol/L, respectively. These cut offs for vitamin D status are in unison with the majority of guidelines to date [60, 61]. **Chapters 4 and 5** assessed vitamin D status in athletes living at high latitude during the winter, both of which are known to impede upon cutaneous vitamin D synthesis [62]. **Chapter 4** showed that 44% (21/48) and 29% (14/48) of university athletes from a variety of indoor and outdoor sports were vitamin D deficient and insufficient, respectively. Similarly **Chapter 5**, found that 53% (19/36) and 33% (12/36) of endurance trained cyclists were also vitamin D deficient and insufficient, respectively. The extent of deficiency and insufficiency reported in these studies is in line with recent evidence in athletes living and training at slightly higher latitude in the UK (53°N), whereby 64% of a variety of outdoor athletes [63] and 65% of elite football players were vitamin D deficient (<50 nmol/L) [64]. Moreover, in the results presented in **Chapter 4** even outdoor athletes had high prevalence of vitamin D deficiency and insufficiency, despite athletes retrospectively reporting that they spent large amounts of time outdoors training/competing during the summer and spring where cutaneous synthesis of vitamin D is high [65]. This was also true of the endurance cyclists in **Chapter 5**, who all trained/competed outdoors regularly. These findings are in keeping with evidence from

outdoor athletes both at high [66-69] and low [63, 64, 70, 71] latitudes. However, despite the similar trends in vitamin D status between the current thesis work and others, it is difficult to compare between studies due to the different methods used to quantify vitamin D status [72]. The differences between methods include the ability to extract 25(OH)D from the sample, the traceability and the accuracy of the assay. Previous studies have shown a lack of correlation between commercially available vitamin D assays [73] which are likely attributed to the lack of a standardised reference material. The thesis took these methodological issues into account by using a commercially available kit in **Chapter 4** that has been shown to be highly comparable to other methodologies as approved by the Vitamin D External Quality Assessment Scheme (DEQAS www.deqas.org [73]. Furthermore, in an attempt to further improve the accuracy of measuring vitamin D status in athletes, **Chapter 5** used liquid chromatography tandem mass spectrometry (LC-MS/MS). Recent reports have shown that LC-MS/MS produces more accurate and sensitive measurements of 25(OH)D when compared to immunoassay methods [72]. With these methodological justifications in mind, the data in **Chapter 4 and 5** clearly demonstrate that vitamin D deficiency is highly prevalent in both indoor and outdoor athletes living and training in the UK during the winter.

The data presented in **Chapter 4 and 5** also highlighted the importance of dietary vitamin D intake. Both studies observed that UK athlete's vitamin D intake was below the current RNI (106 ± 131 IU/d and 116 ± 162 IU/d), which supports previous evidence in athletes [63, 67] but also in UK adults [74]. Studies have shown that in order to prevent vitamin D deficiency and insufficiency, far higher doses of vitamin D above the RNI (400IU) are required in the diet [75]. These recommendations are evident in the strong correlation observed in **Chapter 4** between vitamin D supplement intake, which would increase dietary vitamin D intake, and 25(OH)D concentrations In further support, **Chapter 5** demonstrated the efficacy of short term (28 days) vitamin D supplementation at high doses in improving vitamin D status to sufficient concentrations. This was achieved by using 1500IU/d, 3000IU/d and 6000IU/d in just 28 days. However, no difference in changes to 25(OH)D following

supplementation was observed between vitamin D groups. This suggests that doses above 1500IU/d do not provide any additional benefit to vitamin D status.

The effect that vitamin D deficiency and sufficiency may have on athletic performance is currently unclear. In **Chapter 4,** results showed that muscle function was not positively correlated with 25(OH)D when using basic assessments of leg and forearm muscle function (vertical jump and MVC hand grip). In addition, in **Chapter 5,** 25(OH)D was not correlated with any aerobic, anaerobic or strength performance measurement during the winter in endurance athletes. However, while the majority of athletes were vitamin D deficient and insufficient, very few were severely deficient (< 25 nmol/L). It has been suggested that the deleterious effects of vitamin D deficiency on skeletal muscle function may only occur at concentrations below 25 nmol/L [76-78]. Alternatively, **Chapter 5** examined the paradigm that improving vitamin D status to sufficient concentrations with vitamin D supplementation may improve muscle function and exercise performance compared to those who remain vitamin D deficient. The results presented show that despite elevating 25(OH)D to sufficient vitamin D status in all participants in the supplemented groups (> 100 nmol/L), no difference in aerobic, anaerobic or strength performance measurements were observed when compared to placebo. These findings are in contrast to causative and longitudinal evidence in the young adults [79, 80] and the elderly [81-85] that show an association between vitamin D status and physical performance. Yet, the findings from **Chapter 4 and 5** bring into question the optimal concentration of 25(OH)D that impairs or improves skeletal muscle function. It would be argued that the benefits to skeletal muscle function following vitamin D supplementation would only be apparent when deficient in the first place. In support, a recent meta analysis concluded that there was no significant effect of vitamin D supplementation (range of doses included 400IU/d to 60,000IU/wk) on leg or grip strength in the elderly when baseline 25(OH)D was >25nmol/L [86]. Further, in the limited number of studies in athletes, it has been shown that improvements to exercise performance only occurred when the majority of athletes were severely vitamin D deficient [71] while no improvement was observed when sufficient [87] prior to supplementation. On the other hand the target concentration of 25(OH)D

following supplementation may also be important, with many suggesting that 25(OH)D > 100 nmol/L may be required to improve exercise performance in athletes [71, 88, 89]. However, **Chapter 5** shows no benefit to exercise performance when endurance athletes 25(OH)D concentration was predominantly above 100 nmol/L. Therefore, it remains to be determined whether it is the extent of deficiency prior to supplementation or the concentration following supplementation that dictates the improvements to musculoskeletal performance.

The data presented in **Chapter 4 and 5** does not support the hypothesis that vitamin D status influences muscle function or exercise performance in athletes. This may be explained by the large number of factors that may alter vitamin D status and the response to supplementation to include: latitude, season, ethnicity, habitual diet, physical activity levels and body composition [56, 90]. Future research is yet to be conducted to elucidate the role these factors have on vitamin D metabolism and physiological outcomes. In addition, given the large number of factors that may alter vitamin D status and response to supplementation, future research should focus on larger sample sizes than used in the current thesis. The limited sample sizes, relative to the numerous factors that may have reduced the ability to detect changes in skeletal muscle function and exercise performance, used in **Chapter 4 and 5** is recognised as a limitation of the work.

The effect of vitamin D on skeletal muscle function, especially within an athletic population, is timely and novel. In order to gain further insights into the potential benefits of vitamin D for athletes, future long term intervention studies are required in combination with muscle biopsy methods. In turn this will identify the mechanisms of actions, the time course and optimal 25(OH)D concentration required to induce the specific mechanisms within the skeletal muscle. In addition, considering the limited evidence to date that illustrates that long term vitamin D supplementation when combined with exercise training may lead to superior adaptations to the skeletal muscle [91-98], it may also be of interest to study this hypothesis in athletes. In support, Carrillo et al [97] demonstrated that 12 weeks of resistance training, combined with vitamin D (4000IU/d), in overweight and obese adults showed significant improvements to upper body peak power above the placebo group. In the elderly it has

been shown that the response to training was attenuated when 25(OH)D was <47.5 nmol/L [98]. More recently it has been shown that elderly individuals supplemented with HMB, arginine and lysine significantly improved lean muscle mass, but the improvements to strength only occurred when vitamin D status was sufficient [99]. Therefore, vitamin D may be an important nutritional support when undergoing exercise training by inducing pro anabolic effects within the skeletal muscle [84, 100, 101]. However, the pro anabolic effects of vitamin D on healthy skeletal muscle adaptations, as seen in athletes when compared to the elderly [102], are yet to be investigated.

6.4 Future research questions - Vitamin D:

Following on from the studies presented in **chapters 4 and 5**, a number of key questions still remain to be answered:

1. Is the prevalence of vitamin D deficiency and insufficiency in athletes dependent on season of the year in the UK?
2. What is an effective supplementation protocol (dose and duration) to achieve vitamin D sufficiency in all seasons of the year?
3. What is the optimal 25(OH)D concentration to elicit benefits to muscle function?
4. Does long term vitamin D supplementation augment muscle function and performance in athletes?
5. What are the mechanisms to describe the improvements in skeletal muscle function and adaptations following vitamin D supplementation in humans?
6. What is the combined and independent effect of exercise training and vitamin D supplementation on performance and training adaptation outcomes?

6.5 Conclusions

Chapter 2 illustrated that GTE increases metabolites related to fat and energy metabolism at rest, but not during moderate intensity exercise when using a metabolomics approach. The alterations to energy and fat metabolism at rest were independent of changes to catecholamines, which questions the relevance of the COMT mechanism *in vivo*. **Chapter 3** revealed that coffee is as effective as caffeine in improving exercise performance but the ergogenic effects were independent of changes in fat metabolism. **Chapter 4 and 5** highlighted that UK athletes require nutritional support to avoid vitamin D deficiency. **Chapter 4** showed a high prevalence of vitamin D deficiency and insufficiency in UK university athletes from a variety of sports during the winter. The poor vitamin D status was likely explained by the high latitude and climate of the test site as well as low dietary intake of vitamin D by the athletes. It was also shown that vitamin D status was not associated with muscle function in the athletes tested. **Chapter 5** also showed a high prevalence of vitamin D deficiency and insufficiency, which was corrected when high doses of vitamin D (1500IU, 3000IU or 6000IU) above the RNI (400IU) were supplemented for 28 days. The results showed that 1500IU/d improved vitamin D status to sufficient concentrations, with the higher doses of vitamin D providing no further increases. Despite all vitamin D groups becoming vitamin D sufficient, exercise performance (aerobic, anaerobic and strength) was not altered following supplementation in endurance trained athletes.

6.6 References:

1. Dulloo AG, Duret C, Rohrer D, Girardier L, Mensi N, Fathi M, Chantre P, Vandermander J (1999) Efficacy of a green tea extract rich in catechin polyphenols and caffeine in increasing 24-h energy expenditure and fat oxidation in humans. Am J Clin Nutr 70: 1040-1045.
2. Rumpler W, Seale J, Clevidence B, Judd J, Wiley E, Yamamoto S, Komatsu T, Sawaki T, Ishikura Y, Hosoda K (2001) Oolong Tea Increases Metabolic Rate and Fat Oxidation in Men. J Nutr 131: 5.
3. Thielecke F, Rahn G, Bohnke J, Adams F, Birkenfeld AL, Jordan J, Boschmann M (2010) Epigallocatechin-3-gallate and postprandial fat oxidation in overweight/obese male volunteers: a pilot study. Eur J Clin Nutr.
4. Boschmann M, Thielecke F (2007) The effects of epigallocatechin-3-gallate on thermogenesis and fat oxidation in obese men: a pilot study. J Am Coll Nutr 26: 389S-395S.
5. Berube-Parent S, Pelletier C, Dore J, Tremblay A (2005) Effects of encapsulated green tea and Guarana extracts containing a mixture of epigallocatechin-3-gallate and caffeine on 24 h energy expenditure and fat oxidation in men. Br J Nutr 94: 432-436.
6. Rudelle S, Ferruzzi MG, Cristiani I, Moulin J, Mace K, Acheson KJ, Tappy L (2007) Effect of a thermogenic beverage on 24-hour energy metabolism in humans. Obesity (Silver Spring) 15: 349-355.
7. Gregersen NT, Bitz C, Krog-Mikkelsen I, Hels O, Kovacs EM, Rycroft JA, Frandsen E, Mela DJ, Astrup A (2009) Effect of moderate intakes of different tea catechins and caffeine on acute measures of energy metabolism under sedentary conditions. Br J Nutr 102: 1187-1194.
8. Lonac MC, Richards JC, Schweder MM, Johnson TK, Bell C (2011) Influence of short-term consumption of the caffeine-free, epigallocatechin-3-gallate supplement, Teavigo, on resting metabolism and the thermic effect of feeding. Obesity 19: 298-304.
9. Venables MC, Hulston CJ, Cox HR, Jeukendrup AE (2008) Green tea extract ingestion, fat oxidation, and glucose tolerance in healthy humans. Am J Clin Nutr 87: 778-784.
10. Ichinose T, Nomura S, Someya Y, Akimoto S, Tachiyashiki K, Imaizumi K (2011) Effect of endurance training supplemented with green tea extract on substrate metabolism during exercise in humans. Scand J Med Sci Sports 21: 598-605.
11. Ota N, Soga S, Shimotoyodome A, Haramizu S, Inaba M, Murase T, Tokimitsu I (2005) Effects of combination of regular exercise and tea catechins intake on energy expenditure in humans. Journal of Health Science 51: 4.
12. Randell RK, Hodgson AB, Lotito SB, Jacobs DM, Boon N, Mela DJ, Jeukendrup AE (2012) No Effect of 1 or 7 Days Green Tea Extract Ingestion on Fat Oxidation during Exercise. Med Sci Sports Exerc.
13. Dulloo AG, Seydoux J, Girardier L, Chantre P, Vandermander J (2000) Green tea and thermogenesis: interactions between catechin-polyphenols, caffeine and sympathetic activity. Int J Obes Relat Metab Disord 24: 252-258.
14. Mannisto PT, Kaakkola S (1999) Catechol-O-methyltransferase (COMT): biochemistry, molecular biology, pharmacology, and clinical efficacy of the new selective COMT inhibitors. Pharmacological reviews 51: 593-628.
15. Borchardt RT, Huber JA (1975) Catechol O-methyltransferase. 5. Structure-activity relationships for inhibition by flavonoids. J Med Chem 18: 120-122.
16. Achten J, Jeukendrup AE (2004) Optimizing fat oxidation through exercise and diet. Nutrition 20: 716-727.
17. Chen D, Wang CY, Lambert JD, Ai N, Welsh WJ, Yang CS (2005) Inhibition of human liver catechol-O-methyltransferase by tea catechins and their metabolites: structure-activity relationship and molecular-modeling studies. Biochem Pharmacol 69: 1523-1531.
18. Murase T, Haramizu S, Shimotoyodome A, Nagasawa A, Tokimitsu I (2005) Green tea extract improves endurance capacity and increases muscle lipid oxidation in mice. Am J Physiol Regul Integr Comp Physiol 288: R708-715.

19. Murase T, Haramizu S, Shimotoyodome A, Tokimitsu I, Hase T (2006) Green tea extract improves running endurance in mice by stimulating lipid utilization during exercise. Am J Physiol Regul Integr Comp Physiol 290: R1550-1556.
20. Shimotoyodome A, Haramizu S, Inaba M, Murase T, Tokimitsu I (2005) Exercise and green tea extract stimulate fat oxidation and prevent obesity in mice. Med Sci Sports Exerc 37: 1884-1892.
21. Murase T, Misawa K, Haramizu S, Hase T (2009) Catechin-induced activation of the LKB1/AMP-activated protein kinase pathway. Biochem Pharmacol 78: 78-84.
22. Kiens B, Alsted TJ, Jeppesen J (2011) Factors regulating fat oxidation in human skeletal muscle. Obesity reviews : an official journal of the International Association for the Study of Obesity 12: 852-858.
23. Van Soeren MH, Sathasivam P, Spriet LL, Graham TE (1993) Caffeine metabolism and epinephrine responses during exercise in users and nonusers. J Appl Physiol 75: 805-812.
24. Van Soeren MH, Graham TE (1998) Effect of caffeine on metabolism, exercise endurance, and catecholamine responses after withdrawal. J Appl Physiol 85: 1493-1501.
25. Greer F, Friars D, Graham TE (2000) Comparison of caffeine and theophylline ingestion: exercise metabolism and endurance. J Appl Physiol 89: 1837-1844.
26. Graham TE, Helge JW, MacLean DA, Kiens B, Richter EA (2000) Caffeine ingestion does not alter carbohydrate or fat metabolism in human skeletal muscle during exercise. J Physiol 529 Pt 3: 837-847.
27. Jackman M, Wendling P, Friars D, Graham TE (1996) Metabolic catecholamine, and endurance responses to caffeine during intense exercise. J Appl Physiol 81: 1658-1663.
28. Raguso CA, Coggan AR, Sidossis LS, Gastaldelli A, Wolfe RR (1996) Effect of theophylline on substrate metabolism during exercise. Metabolism: clinical and experimental 45: 1153-1160.
29. Chesley A, Howlett RA, Heigenhauser GJ, Hultman E, Spriet LL (1998) Regulation of muscle glycogenolytic flux during intense aerobic exercise after caffeine ingestion. Am J Physiol 275: R596-603.
30. Graham TE, Spriet LL (1991) Performance and metabolic responses to a high caffeine dose during prolonged exercise. J Appl Physiol 71: 2292-2298.
31. Graham TE (2001) Caffeine and exercise: metabolism, endurance and performance. Sports Med 31: 785-807.
32. Graham TE, Battram DS, Dela F, El-Sohemy A, Thong FS (2008) Does caffeine alter muscle carbohydrate and fat metabolism during exercise? Appl Physiol Nutr Metab 33: 1311-1318.
33. Mora-Rodriguez R, Coyle EF (2000) Effects of plasma epinephrine on fat metabolism during exercise: interactions with exercise intensity. American journal of physiology Endocrinology and metabolism 278: E669-676.
34. Romijn JA, Coyle EF, Sidossis LS, Gastaldelli A, Horowitz JF, Endert E, Wolfe RR (1993) Regulation of endogenous fat and carbohydrate metabolism in relation to exercise intensity and duration. Am J Physiol 265: E380-391.
35. van Loon LJ, Greenhaff PL, Constantin-Teodosiu D, Saris WH, Wagenmakers AJ (2001) The effects of increasing exercise intensity on muscle fuel utilisation in humans. J Physiol 536: 295-304.
36. Romijn JA, Coyle EF, Sidossis LS, Zhang XJ, Wolfe RR (1995) Relationship between fatty acid delivery and fatty acid oxidation during strenuous exercise. J Appl Physiol 79: 1939-1945.
37. Horowitz JF, Mora-Rodriguez R, Byerley LO, Coyle EF (1997) Lipolytic suppression following carbohydrate ingestion limits fat oxidation during exercise. Am J Physiol 273: E768-775.
38. Spriet LL, MacLean DA, Dyck DJ, Hultman E, Cederblad G, Graham TE (1992) Caffeine ingestion and muscle metabolism during prolonged exercise in humans. Am J Physiol 262: E891-898.

39. Goodpaster B, Kelley D (2002) Skeletal muscle triglyceride: Marker or mediator of obesity-induced insulin resistance in type 2 diabetes mellitus? Current Diabetes Reports 2: 216-222.
40. Auvichayapat P, Prapochanung M, Tunkamnerdthai O, Sripanidkulchai BO, Auvichayapat N, Thinkhamrop B, Kunhasura S, Wongpratoom S, Sinawat S, Hongprapas P (2008) Effectiveness of green tea on weight reduction in obese Thais: A randomized, controlled trial. Physiol Behav 93: 486-491.
41. Diepvens K, Kovacs EM, Nijs IM, Vogels N, Westerterp-Plantenga MS (2005) Effect of green tea on resting energy expenditure and substrate oxidation during weight loss in overweight females. Br J Nutr 94: 1026-1034.
42. Harada U, Chikama A, Saito S, Takase H, Nagao T, Hase T, Tokimitsu I (2004) Effects of the Long-Term Ingestion of Tea Catechins on Energy Expenditure and Dietary Fat Oxidation in Healthy Subjects. Journal of Health Science 51: 7.
43. Westerp-Plantenga MS, Lejeune **M**PGM, E.M.R K (2005) Body weight loss and weight management in relation to habitual caffeine intake and green tea supplementation. Obesity Research 13.
44. Murase T, Haramizu S, Shimotoyodome A, Tokimitsu I (2006) Reduction of diet-induced obesity by a combination of tea-catechin intake and regular swimming. Int J Obes (Lond) 30: 561-568.
45. Phillips SM, Green HJ, Tarnopolsky MA, Heigenhauser GF, Hill RE, Grant SM (1996) Effects of training duration on substrate turnover and oxidation during exercise. J Appl Physiol 81: 2182-2191.
46. Yeo WK, Paton CD, Garnham AP, Burke LM, Carey AL, Hawley JA (2008) Skeletal muscle adaptation and performance responses to once a day versus twice every second day endurance training regimens. J Appl Physiol 105: 1462-1470.
47. Hulston CJ, Venables MC, Mann CH, Martin C, Philp A, Baar K, Jeukendrup AE (2010) Training with low muscle glycogen enhances fat metabolism in well-trained cyclists. Med Sci Sports Exerc 42: 2046-2055.
48. Kiens B, Essen-Gustavsson B, Christensen NJ, Saltin B (1993) Skeletal muscle substrate utilization during submaximal exercise in man: effect of endurance training. J Appl Physiol 469: 459-478.
49. Graham TE, Hibbert E, Sathasivam P (1998) Metabolic and exercise endurance effects of coffee and caffeine ingestion. J Appl Physiol 85: 883-889.
50. McLellan TM, Bell DG (2004) The impact of prior coffee consumption on the subsequent ergogenic effect of anhydrous caffeine. Int J Sport Nutr Ex Metab 14: 698-708.
51. Jeukendrup A, Saris WH, Brouns F, Kester AD (1996) A new validated endurance performance test. Med Sci Sports Exerc 28: 266-270.
52. Mohr T, Van Soeren M, Graham TE, Kjaer M (1998) Caffeine ingestion and metabolic responses of tetraplegic humans during electrical cycling. J Appl Physiol 85: 979-985.
53. Kather H, Bieger W, Michel G, Aktories K, Jakobs KH (1985) Human fat cell lipolysis is primarily regulated by inhibitory modulators acting through distinct mechanisms. The Journal of clinical investigation 76: 1559-1565.
54. Vergauwen L, Hespel P, Richter EA (1994) Adenosine receptors mediate synergistic stimulation of glucose uptake and transport by insulin and by contractions in rat skeletal muscle. J Clin Invest 93: 974-981.
55. Beaudoin MS, Graham TE (2011) Methylxanthines and human health: epidemiological and experimental evidence. Handbook of experimental pharmacology: 509-548.
56. Holick MF (2007) Vitamin D deficiency. N Engl J Med 357: 266-281.
57. Ceglia L, Harris SS (2012) Vitamin D and Its Role in Skeletal Muscle. Calcified tissue international.
58. Cannell JJ, Hollis BW, Sorenson MB, Taft TN, Anderson JJ (2009) Athletic performance and vitamin D. Med Sci Sports Exerc 41: 1102-1110.
59. van Schoor NM, Lips P (2011) Worldwide vitamin D status. Best practice & research Clinical endocrinology & metabolism 25: 671-680.

60. Holick MF, Binkley NC, Bischoff-Ferrari HA, Gordon CM, Hanley DA, Heaney RP, Murad MH, Weaver CM (2011) Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society clinical practice guideline. The Journal of clinical endocrinology and metabolism 96: 1911-1930.
61. Heaney RP, Holick MF (2011) Why the IOM recommendations for vitamin D are deficient. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research 26: 455-457.
62. Webb AR, Holick MF (1988) The role of sunlight in the cutaneous production of vitamin D3. Annual review of nutrition 8: 375-399.
63. Lehtonen-Veromaa M, Mottonen T, Irjala K, Karkkainen M, Lamberg-Allardt C, Hakola P, Viikari J (1999) Vitamin D intake is low and hypovitaminosis D common in healthy 9- to 15-year-old Finnish girls. European journal of clinical nutrition 53: 746-751.
64. Morton JP, Iqbal Z, Drust B, Burgess D, Close GL, Brukner PD (2012) Seasonal variation in vitamin D status in professional soccer players of the English Premier League. Appl Physiol Nutr Metab 37: 798-802.
65. Hypponen E, Power C (2007) Hypovitaminosis D in British adults at age 45 y: nationwide cohort study of dietary and lifestyle predictors. Am J Clin Nutr 85: 860-868.
66. Constantini NW, Arieli R, Chodick G, Dubnov-Raz G (2010) High prevalence of vitamin D insufficiency in athletes and dancers. Clin J Sport Med 20: 368-371.
67. Halliday TM, Peterson NJ, Thomas JJ, Kleppinger K, Hollis BW, Larson-Meyer DE (2011) Vitamin D status relative to diet, lifestyle, injury, and illness in college athletes. Med Sci Sports Exerc 43: 335-343.
68. Hamilton B, Grantham J, Racinais S, Chalabi H (2010) Vitamin D deficiency is endemic in Middle Eastern sportsmen. Public health nutrition 13: 1528-1534.
69. Lovell G (2008) Vitamin D status of females in an elite gymnastics program. Clin J Sport Med 18: 159-161.
70. Bescos Garcia R, Rodriguez Guisado FA (2011) Low levels of vitamin D in professional basketball players after wintertime: relationship with dietary intake of vitamin D and calcium. Nutricion hospitalaria : organo oficial de la Sociedad Espanola de Nutricion Parenteral y Enteral 26: 945-951.
71. Close GL, Russell J, Cobley JN, Owens DJ, Wilson G, Gregson W, Fraser WD, Morton JP (2012) Assessment of vitamin D concentration in non-supplemented professional athletes and healthy adults during the winter months in the UK: implications for skeletal muscle function. J Sport Sci.
72. Fraser WD (2009) Standardization of vitamin D assays: art or science? Annals of clinical biochemistry 46: 3-4.
73. Roth HJ, Schmidt-Gayk H, Weber H, Niederau C (2008) Accuracy and clinical implications of seven 25-hydroxyvitamin D methods compared with liquid chromatography-tandem mass spectrometry as a reference. Annals of clinical biochemistry 45: 153-159.
74. Cashman KD, Hill TR, Lucey AJ, Taylor N, Seamans KM, Muldowney S, Fitzgerald AP, Flynn A, Barnes MS, Horigan G, Bonham MP, Duffy EM, Strain JJ, Wallace JM, Kiely M (2008) Estimation of the dietary requirement for vitamin D in healthy adults. Am J Clin Nutr 88: 1535-1542.
75. Coggan AR, Raguso CA, Williams BD, Sidossis LS, Gastaldelli A (1995) Glucose kinetics during high-intensity exercise in endurance-trained and untrained humans. J Appl Physiol 78: 1203-1207.
76. Schott GD, Wills MR (1976) Muscle weakness in osteomalacia. Lancet 1: 626-629.
77. Ziambaras K, Dagogo-Jack S (1997) Reversible muscle weakness in patients with vitamin D deficiency. West J Med 167: 435-439.
78. Irani PF (1976) Electromyography in nutritional osteomalacic myopathy. J Neurol Neurosurg Psychiatry 39: 686-693.

79. Ward KA, Das G, Berry JL, Roberts SA, Rawer R, Adams JE, Mughal Z (2009) Vitamin D status and muscle function in post-menarchal adolescent girls. J Clin Endocrinol Metab 94: 559-563.
80. Ardestani A, Parker B, Mathur S, Clarkson P, Pescatello LS, Hoffman HJ, Polk DM, Thompson PD (2011) Relation of vitamin D level to maximal oxygen uptake in adults. The American journal of cardiology 107: 1246-1249.
81. Wicherts IS, van Schoor NM, Boeke AJ, Visser M, Deeg DJ, Smit J, Knol DL, Lips P (2007) Vitamin D status predicts physical performance and its decline in older persons. J Clin Endocrinol Metab 92: 2058-2065.
82. Houston DK, Cesari M, Ferrucci L, Cherubini A, Maggio D, Bartali B, Johnson MA, Schwartz GG, Kritchevsky SB (2007) Association between vitamin D status and physical performance: the InCHIANTI study. The journals of gerontology Series A, Biological sciences and medical sciences 62: 440-446.
83. Houston DK, Tooze JA, Hausman DB, Johnson MA, Nicklas BJ, Miller ME, Neiberg RH, Marsh AP, Newman AB, Blair SN, Kritchevsky SB (2011) Change in 25-hydroxyvitamin d and physical performance in older adults. J Gerontol A Biol Sci Med Sci 66: 430-436.
84. Sato Y, Iwamoto J, Kanoko T, Satoh K (2005) Low-dose vitamin D prevents muscular atrophy and reduces falls and hip fractures in women after stroke: a randomized controlled trial. Cerebrovasc Dis 20: 187-192.
85. Pfeifer M, Begerow B, Minne HW, Suppan K, Fahrleitner-Pammer A, Dobnig H (2009) Effects of a long-term vitamin D and calcium supplementation on falls and parameters of muscle function in community-dwelling older individuals. Osteoporosis international : a journal established as result of cooperation between the European Foundation for Osteoporosis and the National Osteoporosis Foundation of the USA 20: 315-322.
86. Stockton KA, Mengersen K, Paratz JD, Kandiah D, Bennell KL (2011) Effect of vitamin D supplementation on muscle strength: a systematic review and meta-analysis. Osteoporosis international : a journal established as result of cooperation between the European Foundation for Osteoporosis and the National Osteoporosis Foundation of the USA 22: 859-871.
87. Barker T, Martins TB, Hill HR, Kjeldsberg CR, Henriksen VT, Dixon BM, Schneider ED, Dern A, Weaver LK (2012) Different doses of supplemental vitamin D maintain interleukin-5 without altering skeletal muscle strength: a randomized, double-blind, placebo-controlled study in vitamin D sufficient adults. Nutrition & metabolism 9: 16.
88. Close GL, Leckey J, Patterson M, Bradley W, Owens DJ, Fraser WD, Morton JP (2013) The effects of vitamin D3 supplementation on serum total 25[OH]D concentration and physical performance: a randomised dose-response study. Br J Sports Med.
89. Zittermann A (2003) Vitamin D in preventive medicine: are we ignoring the evidence? Br J Nutr 89: 552-572.
90. Willis KS, Peterson NJ, Larson-Meyer DE (2008) Should we be concerned about the vitamin D status of athletes? Int J Sport Nutr Exerc Metab 18: 204-224.
91. Holm L, Olesen JL, Matsumoto K, Doi T, Mizuno M, Alsted TJ, Mackey AL, Schwarz P, Kjaer M (2008) Protein-containing nutrient supplementation following strength training enhances the effect on muscle mass, strength, and bone formation in postmenopausal women. J Appl Physiol 105: 274-281.
92. Kukuljan S, Nowson CA, Sanders K, Daly RM (2009) Effects of resistance exercise and fortified milk on skeletal muscle mass, muscle size, and functional performance in middle-aged and older men: an 18-mo randomized controlled trial. J Appl Physiol 107: 1864-1873.
93. Kukuljan S, Nowson CA, Sanders KM, Nicholson GC, Seibel MJ, Salmon J, Daly RM (2011) Independent and combined effects of calcium-vitamin D3 and exercise on bone structure and strength in older men: an 18-month factorial design randomized controlled trial. J Clin Endocrinol Metab 96: 955-963.
94. Josse AR, Atkinson SA, Tarnopolsky MA, Phillips SM (2011) Increased consumption of dairy foods and protein during diet- and exercise-induced weight loss promotes fat mass loss

and lean mass gain in overweight and obese premenopausal women. The Journal of nutrition 141: 1626-1634.
95. Josse AR, Tang JE, Tarnopolsky MA, Phillips SM (2010) Body composition and strength changes in women with milk and resistance exercise. Med Sci Sports Exerc 42: 1122-1130.
96. Siddiqui SM, Chang E, Li J, Burlage C, Zou M, Buhman KK, Koser S, Donkin SS, Teegarden D (2008) Dietary intervention with vitamin D, calcium, and whey protein reduced fat mass and increased lean mass in rats. Nutrition research 28: 783-790.
97. Carrillo AE, Flynn MG, Pinkston C, Markofski MM, Jiang Y, Donkin SS, Teegarden D (2012) Impact of vitamin D supplementation during a resistance training intervention on body composition, muscle function, and glucose tolerance in overweight and obese adults. Clinical nutrition.
98. Okuno J, Tomura S, Yabushita N, Kim MJ, Okura T, Tanaka K, Yanagi H (2010) Effects of serum 25-hydroxyvitamin D(3) levels on physical fitness in community-dwelling frail women. Archives of gerontology and geriatrics 50: 121-126.
99. Fuller JC, Jr., Baier S, Flakoll P, Nissen SL, Abumrad NN, Rathmacher JA (2011) Vitamin D status affects strength gains in older adults supplemented with a combination of beta-hydroxy-beta-methylbutyrate, arginine, and lysine: a cohort study. JPEN J Parenter Enteral Nutr 35: 757-762.
100. Birge SJ, Haddad JG (1975) 25-hydroxycholecalciferol stimulation of muscle metabolism. J Clin Invest 56: 1100-1107.
101. Wassner SJ, Li JB, Sperduto A, Norman ME (1983) Vitamin D Deficiency, hypocalcemia, and increased skeletal muscle degradation in rats. J Clin Invest 72: 102-112.
102. Koopman R, van Loon LJ (2009) Aging, exercise, and muscle protein metabolism. J Appl Physiol 106: 2040-2048.