NUTRITION AND HYDRATION IMPLICATIONS FOR TRAINED ATHLETES

By

SOPHIE CHARLOTTE KILLER

A thesis submitted to Loughborough University for the degree of:

Doctor of Philosophy

Loughborough University

August 2014

© by Sophie Charlotte Killer 2014

ABSTRACT

Lifestyle choices threaten to compromise health and performance of trained athletes. This thesis presents a series of studies which broadly investigated the impact of nutritional and physical challenges to human health and performance.

The potential causes and effects of fluid imbalances on physical health, immune function and athletic performance were investigated. Certain populations experience chronic low-level hypohydration and athletes often fail to rehydrate sufficiently between exercise sessions. The long-term implications of hypohydration are not fully understood, but are suggested to be associated with chronic disease. In this thesis, maintenance of fluid balance was observed in healthy males, despite a caffeine intervention thought to cause diuresis. Furthermore, when mild hypohydration was induced by 24-h fluid restriction, there was little impact on mucosal immunity during endurance exercise compared with euhydration.

The impact of intensified training (IT) on the physical, mental, hormonal and immunological status of well-trained athletes was investigated. A performance-specific nutritional intervention was implemented to investigate the effects of nutrient availability during prolonged exercise training sessions. Phases of IT are a regular feature of a periodised training programme. However, an imbalance between training and recovery can have significant implications for long-term athletic performance and general wellbeing. Changes in neuroendocrine, neurobiological and mucosal immune function were observed during IT and some potential markers of overreaching and were identified. Further research is required before practical application of these markers can be used effectively in the field. A relatively short period of IT resulted in significant disruptions to mood state and sleep quality. Minor changes in exercise performance were observed. Markers of overreaching were highly individual, as were responses to training.

Key Words: Nutrition, Hydration, Immunology, Training, Athlete, Overtraining, Performance, Health.

DEDICATION

To my parents, Stephanie and Terry, and to my brother Stephen. Your love and support knows no limits. Not just during my PhD, but throughout my whole life, you have always been there for me, supported me, believed in me and helped me become the person I am today. You truly are the most wonderful family, thank you, I love you all.

And of course, to Harry. Thank you for your continued love, patience and belief in me. You have made this journey a much happier one for me. I love you with all my heart.

ACKNOWLEDGMENTS

TABLE OF CONTENTS

function in trained cyclists

PUBLICATIONS

Published:

- **Killer SC**, Blannin AK and Jeukendrup AE. No Evidence of Dehydration with Moderate Daily Coffee Intake: A Counterbalanced Cross-Over Study in a Free-Living Population. PLoS ONE 2014 9 (1): e84154. DOI:10.1371/journal.pone.0084154

- Jeukendrup AE and **Killer SC.** Myths Surrounding Pre-Exercise Carbohydrate Feeding. Ann Nutr Metab 2010;57 (suppl 2):18–25. DOI: 10.1159/000322698

- Svendsen IS, **Killer SC** and Gleeson M. Influence of Hydration Status on Changes in Plasma Cortisol, Leukocytes and Antigen-Stimulated Cytokine Production in Whole Blood Culture following Prolonged Exercise. ISRN Nutrition 2014 ID: 561401. DOI: 10.1155/2014/561401

- Ranchordas MK, Rogerson D, Ruddock AR, **Killer SC** and Winter EM. Nutrition for Tennis: Practical Recommendations. JSSM 2013 (12) 211-224

Submitted:

- **Killer SC**, Svendsen IS and Gleeson M. Impact of Short Term Intensified Training on Sleep Quality and Mood State in Well-Trained Cyclists.

- **Killer SC**, Svendsen IS, Randell RK, Carter JM, Jeukendrup AE and Gleeson M. The Physical, Mental and Hormonal Responses to Short-Term Intensified Training in Well-Trained Cyclists with a High Carbohydrate Nutritional Intervention.

- **Killer SC**, Svendsen IS and Gleeson M. Influence of Hydration Status during Prolonged Endurance Exercise on Salivary Antimicrobial Proteins.

In Preparation:

- **Killer SC**, Svendsen IS and Gleeson M. Plasma and Urinary Metabolomics for Monitoring Training Stress in Well-Trained Athletes.

- Svendsen IS, **Killer SC**, Carter JM, Randell RK, Jeukendrup AE and Gleeson M. Effect of Carbohydrate Supplementation during Intensified Training on Markers of Immunity in Highly Trained Cyclists.

- Wadley AJ, **Killer SC**, Svensen IS, and Gleeson M. The impact of intensified training and carbohydrate ingestion on resting and exercise-induced oxidative stress.

Conference Proceedings:

- **Killer SC**, Svendsen IS, Carter JM, Randell RK, Pool S, Sandford GN, Jeukendrup AE and Gleeson M. The Physical, Mental and Hormonal Responses to Short-Term Intensified Training in Well-Trained Cyclists with a High Carbohydrate Nutritional Intervention.
  World Congress of Cycling Science 2014, Leeds, UK.
  Oral Presentation. Journal of Science and Cycling, Conference Proceedings 2014.

- **Killer SC**, Svendsen IS and Gleeson M. The Influence of Hydration Status during Prolonged Endurance Exercise on Salivary Antimicrobial Proteins.
  International Sport and Exercise Nutrition Conference 2013, Newcastle, UK.
  Poster Presentation. IJSNEM 2014 (24) S1-S10.

- **Killer SC**, Svendsen IS, Carter JM, Randell RK, Pool S, Sandford GN, Jeukendrup AE and Gleeson M. Impact of Short Term Intensified Training On Sleep Quality and Mood State in Well Trained Cyclists.
  American College of Sports Medicine Conference 2014, Orlando, USA.
  Poster Presentation. Medicine and Science in Sports and Exercise 2014 S678 (46) 5.

- **Killer SC**, Blannin AK and Jeukendrup AE. The Effects of Coffee on the Hydration Status of Free-Living Individuals.
  International Sport and Exercise Nutrition Conference 2012, Newcastle, UK.
  Oral Presentation. IJSNEM. 2013, 23, S1-S15

- Svendsen IS, **Killer SC** and Gleeson M. Influence of Prolonged Exercise and Hydration Status on Antigen-Stimulated Cytokine Production by Whole Blood Culture.
  International Society of Exercise and Immunology Conference 2013, Newcastle, Australia.
  Oral Presentation. ISEI Abstract – Session theme number 7

Invited Speaker:

- 'The Science of Coffee and Hydration: Separating Fact from Fiction'.
  Association for Science and Information on Coffee (ASIC), 25th International Conference on Coffee. Armenia, Colombia 2014.

- 'The Chronic effects of Coffee Ingestion on Hydration Status: A Counterbalanced Cross-Over Study in a Free-Living Population'.
  Institute for Scientific Information on Coffee (ISIC), Coffee and Health Workshop. Madrid, Spain 2013.

- 'The chronic effects of coffee ingestion on hydration status: a counterbalanced cross-over study in a free-living population'.
  ISIC, Scientific Committee Meeting. Milan, Italy 2012.

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Abbreviation	Meaning
ACSM	American College of Sports Medicine
ACTH	Adrenocorticotrophic Hormone
AMPs	Antimicrobial Proteins
ANOVA	Analysis Of Variance
APE	Atom Percentage Excess
AST	Actual Sleep Time
BDNF	Brain Derived Neurotrophic Factor
BM	Body Mass
BUN	Blood Urea Nitrogen
C	Caffeine Condition
CON	Control Condition (moderate carbohydrate group)
CHO	Carbohydrate
CORT	Cortisol
CREB	Camp Response-Element Binding Protein
CRH	Corticotrophin-Releasing Hormone
D_2O	Deuterium Oxide
DALDA	Daily Analysis of Lifestyle Demands on Athletes

DH	Dehydration
DXA	Dual-energy X-ray Absorptiometry
EFSA	European Food Safety Agency
EH	Euhydration
ELISA	Enzyme-Linked Immunosorbent Assay
EI	Energy Intake
FI	Fragmentation Index
FR	Salivary Flow Rate
FSH	Follicle Stimulating Hormone
GMS	Global Mood State
GR	Glucocorticoid Receptor
HCHO	High Carbohydrate Condition
HPA axis	Hypothalamic-Pituitary-Adrenal Axis
HPLC	High Performance Liquid Chromatography
HR	Heart Rate
IOC	International Olympic Committee
IRMS	Isotope Ratio Mass Spectrometer
IT	Intensified Training
LH	Luteinising Hormone
MT	Mobile Time

MV	First Morning Void
OR	Overreaching
OTS	Overtraining Syndrome
POMS	Profile Of Mood State
PRL	Prolactin
REM	Rapid Eye Movement sleep
RPE	Rate of Perceived Exertion
SE	Sleep Efficiency
SIgA	Salivary Secretory IgA
SLac	Salivary Lactoferrin
SLys	Salivary Lysozyme
SOL	Sleep Onset Latency
ST%	Percentage Sleep Time
SW	Slow Wave sleep
TB	Time in Bed
TBW	Total Body Water
TEST	Testosterone
TMD	Total Mood Disturbance
TT	Time Trial (1h)
TTP	Total Plasma Protein

USG	Urine Specific Gravity
URTI(s)	Upper Respiratory Tract Infection(s)
$\dot{V}O_{2max}$	Maximal Oxygen Uptake
V-SMOW	Vienna Standard Mean Ocean Water
W	Water Condition
W_{max}	Maximal Power Output
WB	Wake Bouts

CHAPTER 1: INTRODUCTION

Nutrition and Hydration Implications for Health and Performance

1.1 Nutrition

1.1.1 Fuel for Performance

Studies in the early 1920s were likely the first to identify the importance of carbohydrate as a fuel for exercise (1,2). By the 1960s, the use of the muscle biopsy technique (3,4) and the subsequent measurement of muscle glycogen enabled a better understanding of the importance of carbohydrate as a substrate for exercise. Muscle glycogen is an essential substrate for both high-intensity short-duration and prolonged endurance exercise. As muscle glycogen stores become depleted in the first 1-2 h of exercise or during periods of increased intensity, blood glucose becomes a more predominant energy fuel. In line with increased muscle glucose uptake, resulting from translocation of GLUT4 glucose transporter to the plasma membrane and subsequent increase in glucose transport and delivery to the skeletal muscle, there is a concomitant increase in liver glucose output to ensure that blood glucose levels remain at (or just above) resting levels (5). During prolonged exercise, liver glycogen levels begin to deplete. At this point the process of gluconeogensis becomes important for energy provision and if the rate of hepatic glucose output falls below the rate of muscle glucose uptake then a state of hypoglycaemia can develop. Clinically, hypoglycaemia is defined as a blood glucose concentration of less than 3 mmol/L; falls in blood glucose to this extent are associated with impairments of central nervous function. Muscle glycogen depletion and hypoglycaemia have been found to be associated with fatigue during exercise (peripheral and central) (6,7). The ingestion of carbohydrate during exercise has repeatedly been shown to sustain carbohydrate oxidation rate and to improve exercise capacity (for reviews see; 8,9).

1.1.2 Sports Nutrition Guidelines

Current sports nutrition guidelines summarise a wealth of data collected over the past 50 years and make recommendations ranging from what to consume before, during and after exercise to the volume, frequency and form of carbohydrate consumption (10,11). These recommendations are based on a vast database of information provided by researchers and

sports nutrition practitioners and to summarise these data is beyond the scope of this introduction. Within this thesis, the interest was in assessing whether the current carbohydrate ingestion guidelines could support both the health and performance of well-trained athletes undergoing a relatively short-term period of intensified training (IT). Specifically, the aim was to gain insight into the physical, metabolic and immunological implications of IT and whether current sports nutrition guidelines may offer any protective effect against early signs of overreaching (OR) and/or the overtraining syndrome (OTS). Previous research has shown high carbohydrate diets help maintain performance during periods of intense exercise training (12,13) and indeed may be required for supercompensation of performance after an appropriate period of tapering (12). It has also been shown that low energy diets (specifically carbohydrate) can negatively impact immune function and exacerbate the increase in circulating stress hormones during and post-exercise (14). However, whilst carbohydrate ingestion to prevent glycogen depletion during heavy training regimens may aid performance and support immune function, it has been suggested that this may delay but does not prevent the development of OR or OTS (6,12,13). Despite this, the aetiology of the OTS is not fully understood, and high carbohydrate availability immediately before, during and post-exercise (as opposed to a general high carbohydrate diet) has been recommended to support higher daily training loads and to promote optimal recovery (15). The emergence of the 'train low' glycogen training hypothesis whereby energy restriction during exercise has been shown to enhance the training response to an exercise session (via enhanced activation of key signalling pathways) is an interesting consideration. 'Train low' may have a place in a well periodised training programme; however, consistently performing exercise sessions in a low carbohydrate state is likely to lead to an inability to complete the desired workload (16,17). Instead of investigating the impact of low carbohydrate availability during IT, which may have resulted in incomplete data sets, we decided to investigate the effects of a high versus moderate carbohydrate consumption during exercise training to identify potential differences between the upper and lower ends of recommendations for carbohydrate intakes. In addition, we provided a small amount of protein immediately post-exercise in the high carbohydrate intervention. A recent study from Witard and colleagues was the first to investigate the impact of supplemental protein ingestion during a period of IT on immune function (18). The authors reported fewer symptoms of upper respiratory tract infections (URTIs) in a high protein (3.0 g/kg) group compared to a control (1.5 g/kg) group during one week of high intensity training. Within this study, the interest was in whether the addition of protein in a

post-exercise recovery beverage would increase total dietary protein intake across the period of IT (or whether there would be subconscious compensation of extra protein during CON) and whether it may contribute to differences between interventions.

1.2 Hydration

1.2.1 Overview of Fluid Balance

Maintenance of fluid homeostasis is essential for life; water ingestion from food and beverages balances fluid losses from the body to ensure adequate hydration of bodily tissues. The body is in constant flux and it is therefore normal for small fluctuations in fluid balance to occur throughout a 24 h cycle without causing any noticeable effects on health or performance (19). However, in many cases fluid balance is not maintained and there is evidence that certain populations experience chronic low level hypohydration (19). Despite a lack of research into the long term implications of mild hypohydration, it has been suggested that low habitual fluid intakes may be associated with a range of chronic diseases such as urolithiasis (urinary tract stone infection), constipation, asthma, cardiovascular disease, diabetic hyperglycaemia and certain cancers (19). Furthermore, studies that have increased daily fluid intakes across certain populations have seen reduced caloric intakes, increased satiety and increased weight loss (for a thorough review see (20)). Within athletic populations, large sweat rates during exercise challenge fluid balance and additional fluid consumption is required to maintain euhydration. As within the sedentary population, athletes and those who are recreationally active also experience wide variability in sweat losses and fluid intakes. Fluid losses of between 10-15 L/day have been reported in athletes training in hot environments (equal to 25-30% of total body water) (21), although such high sweat rates are uncommon.

1.2.2 Hydration Guidelines

The current European Food Safety Authority (EFSA) dietary reference value for water intakes for male adults is 2.5 L/day (22). However, other published guidelines within the same population range from 1.5 L/day (23) to 3.7 L/day (24). Within sports the hydration guidelines become even more complicated, with top hydration researchers expressing

conflicting opinions on how much an athlete should drink and when. The current consensus from the American College of Sports Medicine (ACSM) is that an athlete should consume 5-7 mL/kg BM of fluids 4 h before exercise and an additional 3-5 mL/kg BM 2 h before exercise (25). During exercise, athletes are regularly advised to adopt a 'paced' strategy to ensure sufficient fluid intakes across the duration of the event to minimise fluid deficits (26). Furthermore, recommendations for athletes to limit body mass losses during exercise to no greater than 1-2% of pre-competition body mass are commonplace (27). Despite this, larger body mass losses during prolonged exercise are not uncommon and may be well tolerated by certain athletes who may gain a performance advantage. For example, a large reduction in body mass (~5%) may be sufficient to reduce the energy cost of exercise, especially if power/weight ratio is a significant performance factor, during the later stages of the event which would potentially present a performance advantage (21). However, the implications of such weight losses during exercise are not fully understood and hence the general opinion is that athletes should drink sufficient fluids to limit body mass losses to 1-2% and should plan an individual hydration strategy in advance of the event (11,26). Despite this, there is an alternative view suggesting that drinking *ad libitum* is sufficient to ensure fluid balance and that fluid needs should be dictated by thirst, not a pre-prepared strategy (28).

1.2.3 Hydration Challenges

In light of the potentially harmful implications of dehydration on general health and exercise performance, it is important to consider the possible causes of chronic dehydration and likely substances that could challenge hydration status on a daily basis. The research literature (29) and lay-media (30) provide guidelines for recommended fluid intakes. Despite coffee being one of the most widely consumed beverages across the world, with an estimated 1.6 billion cups consumed worldwide each day (102), it has been suggested by numerous authorities that caffeinated beverages should not be included within daily fluid requirement guidelines (29) and that a glass of water should be consumed with each cup of coffee to maintain fluid balance (30). The advice stems from the understanding that caffeine (1, 3, 7-trimethylxanthine) behaves as an acute diuretic through its action as an adenosine receptor antagonist reducing sodium reabsorption in the kidney's nephrons. The consumption of caffeine in relatively high doses of ≥500 mg, has been shown to elicit a diuretic effect (31-33). Despite general health guidelines advising individuals about the effects of coffee on hydration status, only two studies have specifically investigated the effects of caffeine in the

form of coffee on fluid balance. One study examined the effects of a high dose of coffee (six cups providing 624 mg caffeine/day) on urine excretion following 5 days of caffeine deprivation (33) and reported a 41% increase in urine output. The second study investigated a moderate dose of caffeine (3.1 mg/kg/day) in caffeine habituated males, provided in coffee and other caffeinated beverages (including colas) and reported no effect of coffee on a wide range of hydration markers (34). Both of these studies are limited by their design (see chapter 2 for a thorough overview) and neither can answer the simple question of whether coffee can contribute to daily fluid requirement in caffeine 'habituated' adults. Therefore we were interested in understanding the true implications of moderate coffee consumption within a caffeine-habituated population by assessing hydration status with a wide range of validated techniques. With water advocated for its hydrating properties, we were interested in directly comparing the effects of coffee consumption with equal amounts of water. We believe this is an important question due to coffee's global popularity and the potential effects of chronic dehydration on health. Furthermore, within the athletic population, caffeine is ever growing in its popularity as an ergogenic aid. In a recent review of the prevalence of caffeine's use in sport, Del Coso and colleagues reported that 3 out of 4 athletes that had provided a urine sample between 2004-2008 (20,686 samples in total) had consumed caffeine before or during sports competition (35). Coffee is one of the largest sources of caffeine used by athletes prior to competition (36) therefore it is of interest to understand the implications of coffee on fluid balance, not only within the general population, but also within athletic populations whereby the potential for fluid balance disruption is potentially even greater.

1.2.4 Hydration & Immune Function

It is not uncommon for athletes to commence exercise in a state of fluid deficit (26). Failure to rehydrate between sessions or aggressive weight making strategies makes dehydration a real risk within athletic populations. Negative effects of dehydration on exercise performance include: increased cardiovascular strain (increased heart rate (HR)) (37), core temperature, perception of effort (38) and result in significant increases in plasma cortisol levels (39) compared to exercise in a euhydrated state. Indeed, a pre-exercise fluid deficit of as little as 1.5-2.0% body mass has been suggested to negatively impact performance and has been shown to reduce running capacity in events lasting from ~4 to 30 min (21,11). Furthermore, the potential for a disturbed immune response when exercising in a state of modest dehydration is significantly augmented. Fluid restriction studies have reported significant

reductions in salivary flow rates in participants who were dehydrated to 3% body mass loss (40). Exercise-induced fluid losses have also been reported to result in reductions in saliva flow rate at as little as 1.3% body mass loss (41). Reductions in saliva flow rate are of particular importance to immune function as saliva contains numerous antimicrobial proteins (AMPs) that play an important role in mucosal immunity. Unfortunately, there is a lack of data exploring the interactions between exercise and AMPs, especially within the context of fluid balance. However a recent study from Fortes and colleagues attempted to shed light on this area (40). Salivary IgA (SIgA) and lysozyme (SLys) were assessed following exercise-induced dehydration and subsequent overnight fluid restriction. The authors reported a significant reduction in salivary flow rate at body mass losses of 3% during exercise which remained attenuated until rehydration was permitted the following morning. Dehydration also resulted in a significant decrease in SIgA concentration, with no change in secretion rate and conversely, no change in SLys concentrations but a significant reduction in secretion rate. Rehydration the following morning returned all saliva markers of mucosal immunity to baseline, highlighting the transient nature of the immune response. To our knowledge, no study has investigated the effects of commencing endurance exercise in a state of moderate dehydration on mucosal immunity, specifically across a range of salivary AMPs. With immune function at mucosal surfaces acting as an essential factor for the maintenance of health and well-being (42), it is important to understand the implications of hydration status prior to an exercise challenge.

Health and Wellbeing of the Elite Athlete

1.3 Sleep

1.3.1 Overview of Sleep Research

Sleep is an essential component of post-exercise recovery for an athlete (43,44). Sleep can be divided into two main components; slow wave (SW) and rapid eye movement (REM), and both are of equal importance for athletic recovery. Throughout the night, sleep will alternate between these two stages allowing for the restorative benefits of both to take place (45). The brain remains relatively active during REM sleep and it has therefore been proposed that this

phase of sleep is essential for motor skill and memory consolidation (46). At the beginning of the night the occurrence of SW sleep, whereby brain activity is significantly reduced, enables the release of growth hormone from the pituitary gland (45). Growth hormone is responsible for stimulating protein synthesis and therefore has a key role in repair and adaptation of muscle and bone (47).

Despite a lack of data in 'healthy sleepers' within the general population and almost no data in well-trained athletes, sleep studies have suggested that sleep loss results in performance attenuation. It has also been proposed that athletic performance is highly dependent on the sleep-wakefulness circadian cycle (45). In a meta-analysis from 1996, Pilcher & Huffcutt reported that partial sleep deprivation (<5 h in 24 h period) has a profound effect on human functioning (cognitive and motor performance) (48). Furthermore, data collected within athletic populations, albeit in the context of sleep deprivation or significant disruption, have reported increased cognitive errors, impaired decision making, reduced maximal power and increased fatigued during exercise (49).

1.3.2 Relationship between Exercise and Sleep

There is a small collection of theories that outline the relationship between exercise and sleep. Perhaps the most commonly cited theory is the restorative theory of energy conservation (50) and body restoration (51,52). According to this theory if sleep provides the opportunity for body restitution (as opposed to brain), then a change in exercise load should directly impact sleeping behaviours. Furthermore, it is suggested that the duration of sleep and proportion of SW sleep is dependent on energy expenditure during the previous period of wakefulness (50). It has been proposed that one can only achieve such low levels of energy expenditure during sleep and that quiet rest alone will not suffice (50).

Modern neuroscientific theories suggest that complex interactions between the brain and neuroendocrine systems may play a key role in homeostatic sleep regulation (44). Specifically, a study from Dworak and colleagues observed an increase in total brain adenosine (shown to have sleep-promoting properties (53)) in rats following high intensity exercise (54).

A further theory is the thermogenic hypothesis. It has been proposed that declining body temperatures initiate sleep and may facilitate entrance to deeper stages of sleep (55). Indeed,

some studies have reported associations between peripheral heat loss (56) and lowered body temperature (57) with sleep onset. In light of the physiological basis of this theory, it is reasonable to assume that exercise may have an impact on sleep, especially when carried out in relatively close proximity to sleep onset.

1.3.3 Exercise: Timing and Type

It has been proposed that exercise performed late in the evening may increase arousal and result in poor sleep hygiene (58). However, it is also understood that exercise can reduce anxiety and stress (59), which could have the opposite effect. Exercise in the evenings has been shown to reduce sleep onset latency compared with when exercising in the morning or early evening (60) but have no effect on sleep quality compared to a no-exercise control within a population of physically fit young adults (44). The potential benefits of exercising in the evenings may be related to the thermogenic hypothesis whereby exercising in the late evening raises body temperature and therefore encourages body cooling. Epidemiological studies, which have the benefit of relatively large samples sizes but the disadvantage of self-report data, have consistently shown improvements in self-report sleep quality with both acute and regular exercise (61). However, exercise that is prolonged and high intensity is suggested to reduce sleep quality, with reports of decreased REM sleep and increased wakefulness (61).

1.3.4 Training and Competition

Whilst a moderate level of regular physical activity has been shown to improve self-reported sleep compared with a sedentary lifestyle, it is understood that intensive exhaustive exercise may be detrimental to sleep quality. There is a paucity of data on the effects of exercise training on sleep physiology in athletic populations (62). Two studies have attempted to monitor sleep across an athletic season and have reported conflicting results. Taylor and colleagues monitored sleep patterns in female swimming using polysomnography at various time points throughout the season, including beginning, peak and taper periods (63). The authors observed increased SW sleep during the busiest periods of the season and, consistent with the restorative theory of sleep, suggested that the athletes experienced heightened sleep quality during these intense exercise periods. Conversely, Fietze and colleagues reported increased sleep disturbance in professional ballerinas in the lead up to a Premier show (64).

This study involved the use of actigraphy to track changes in sleeping patterns and found reduced sleep quality (decreased sleep efficiency and duration) during the period of increased training volume. Interestingly, mood state was not monitored during this time and therefore the potential interaction of anxiety with sleep in the lead up to the show cannot be ignored and the ultimate cause for reduced sleep quality cannot be determined.

A recent study investigated the impact of 3 weeks of intensified training on sleep quality in a group of moderately trained athletes (65). Training volume was increased by 30% over a three week period and by the end of the trial, half of the participants were diagnosed as OR. Interestingly, the athletes that were diagnosed as OR reported some signs of sleep disruption, including reduced sleep efficiency, actual sleep time and increased immobile time, however time in bed, sleep latency and the sleep fragmentation index did not change. No changes in sleep quality were observed in athletes who were not diagnosed as OR. Unfortunately, it is difficult to know whether the athletes described as overreached were actually experiencing overreaching, due to the methods of classification used by the authors. However, it is interesting to consider that it may not be training load per se that impacts sleep quality, but perhaps the individual response of the athlete to the change in training load.

No studies to date have investigated the impact of short-term intensified training on sleep. Our aim is to monitor changes in sleep quality over the course of 9 days of IT in a population of well-trained athletes. We anticipate that these data will provide an insight into the effects of a training camp style programme on sleep and well being.

1.3.5 Actigraphy for Sleep Analysis

The use of wrist watch actigraphy dates back to the early 1950s, with wind-up watches being used to assess hyperactivity in children (66). These days 'actiwatches' are small, sophisticated devices that are widely available and frequently used within sleep research in medicine, chronobiology, psychiatry and psychopharmacology (67). Modern day actigraphy allows objective measures of sleep to be collected over extended periods of time with very little inconvenience to the participant (68). During data collection, each movement is recorded and translated into digital counts that accumulate over a pre-determined epoch interval and are stored within the actiwatch before being downloaded onto a computer and analysed against specific algorithms. Known limitations of actigraphy include: inaccuracy in assessing onset latency and precise timing of sleep and wake epochs, inability to accurately

diagnose sleeping disorders (but may be useful in screening and assessment of treatment), general inaccuracy in cases of insomnia and quiet wakefulness and potential variation between different actiwatch devices (68). However, in light of these limitations, actigraphy has been recommended as an acceptable method of estimating sleep patterns in normal, healthy adult populations (69). The use of actigraphy within this study will provide a non-invasive method of analysing the changes in sleep in a group of well trained athletes undergoing a training camp-style intervention. To the author's knowledge, this is something that has not previously been investigated.

1.4 Immunology

1.4.1 Exercise Immunology

Exercise immunology, a sub-discipline of exercise physiology, is a relatively new science, with interest in the area starting to increase around the 1970s (14). Around this time, epidemiology studies began to confirm the anecdotal reports from coaches and athletes that with increased training came increased susceptibility to infection. Following this, a plethora of studies have investigated both the acute and chronic impact of training on immune function (103,104). It has been suggested that the volume and/or intensity of training is related to susceptibility to URTIs. In 1994, Nieman proposed a model for this relationship, based on a J-shaped curve; suggesting low volume physical activity reduced the relative risk of infection below that of a sedentary individual, but with increased volume/intensity of training came an increased risk of immunosuppression (70,71) and incidence of URTI. Indeed, many studies have supported this theory, across a wide range of sporting disciplines. Despite this, the J-shaped curve relationship is not always found to hold true, with studies failing to observe relationships between exercise training load and infection risk in elite athletes (72-74). In 2006, an updated model was proposed (75). The S-shaped relationship between training load and infection rate proposes that the immune system of an elite athlete is able to withstand infection during periods of intensified training and/or psychological stress (75). This physiological response extreme may in itself be one of the contributing factors to elite success.

1.4.2 Overview of Mucosal Immunity

Mucosal immunity is involved in the first line of defence of the immune system and is considered to be the largest component of immune function (14). The mucosal immune system is specifically involved in protecting mucosal surfaces. Within the context of this PhD, we were specifically interested in the saliva, which provides protection of the salivary glands and is regulated by the autonomic nervous system. It is understood that humans produce ~1500 mL of saliva per day which protects the oral mucosa by its mechanical washing effect (14). In addition, the saliva contains numerous antimicrobial peptides and proteins (AMPs) that are involved in innate mucosal immune function. The innate component of immune function is largely responsible for defence against pathogens entering the body and for the initiation of a rapid immune response, should an infection occur (42). Salivary secretory immunoglobulin A (SIgA) is the predominant antimicrobial protein responsible for antiviral and antibacterial protection (76) and is specifically thought to be involved in defence against infections in the upper respiratory tract (77). Salivary IgA is produced by B cells in the mucosal tissues surrounding the mouth before being translocated into the saliva across the epithelial cell. Traditionally, SIgA has been the most commonly measured salivary AMP in athletic populations. More recently however, there has been increased interested in other salivary AMPs that may contribute towards mucosal immune function. There is a wide range of salivary AMPs that contribute to the formation of a protective barrier against bacteria and pathogens. Two of the most abundant AMPs present in the mucosal secretions of the upper respiratory tract are salivary lysozyme (SLys) and salivary lactoferrin (SLac). The small collection of studies that have investigated the impact of exercise on salivary AMPs have produced mixed results. A study by Koutedakis and colleagues was the first to observe reduced salivary flow rate and SLys concentrations following intense exercise in elite swimmers (78). Since these early findings, other studies have reported increased secretion rates of SIgA and SLys following high intensity exercise (Allgrove et al 2006), no change in SLys secretion rates following endurance exercise (41,79) and no change in SIgA concentrations following endurance exercise (80) or exercise to exhaustion (81). Clearly the variation in exercise modality could lead to the discrepancies between findings; furthermore the relative exercise stress on each participant is likely to result in an individual response. The mechanisms by which exercise could affect the concentration and secretion of AMPs both acutely and chronically include: increased secretion of neuropeptides, secretion of AMPs induced by proinflammatory cytokines, secretion of AMPs from neutrophils or damaged

epithelial cells or simply hyperventilation during exercise resulting in drying of the respiratory tract (42). Furthermore, it has also been proposed that stress hormones, known to increase in concentrations during exercise, may inhibit the production, mobilization and secretion of salivary SIgA (82) and Lys (83).

1.5 The Overtraining Syndrome

1.5.1 Overtraining

Periods of increased training volume and/or intensity are regular occurrences throughout an athletic training programme. These periods of increased training load can result from heavy competition schedules, returns from injury or illness or simply from pre-planned strategies to enhance performance and prevent plateaus. The practice of periodising training programmes in this manner enables athletes to peak at pre-determined time points across an athletic season and staleness (84). Overload training encourages positive adaptation and is essential for performance enhancement. In a well-periodised training programme, overload training will be followed by a period of recovery, allowing for a super-compensatory effect of training on performance. However, if the fine balance between training and recovery is disturbed, OR can occur and in some cases, lead to development of OTS (85).

1.5.2 Defining Training Stress

Research in the area of overtraining is confounded by a lack of clear consensus on the diagnosis of OR and OTS. Furthermore, the ethical implications of inflicting overtraining regimens on study participants make it a difficult area to study. Nevertheless, numerous studies have tried to identify the changes that occur when there is a training stress imbalance, either through inducing short term OR or by studying athletes with an existing diagnosis of OTS. Before discussing some of most recent developments in the overtraining literature, it is important to understand the definitions of OR and OTS and to acknowledge the prevalence of such occurrences.

It is generally agreed that the OTS is defined as a long-term decrease in event-specific athletic performance (86-88) with an increase in mood disturbance (88-91) whereby full

restoration of performance can take several months. Importantly, the decrease in performance occurs despite increased or maintained training load (92). Overreaching can also be defined by similar physiological and psychological disturbances; however performance capacity is typically restored within a few days to several weeks. Overreaching was originally defined by Falsetti in 1983 as a form of training used by coaches to promote a fitness peak prior to a major competition, resulting in a short-term period of underperformance but ultimately leading to a supercompensation of performance capacity (93). It is understood that OR and OTS are related along a continuum, with OR developing into OTS if a period of recovery is not sufficient and/or provided at the correct time (90,91,93).

1.5.3 Prevalence

It is difficult to know the exact prevalence of the OTS in athletic populations due to the differences in diagnosis criteria and the reliance on self-report data. Despite this, numerous studies have estimated its occurrence and reported ranges from 10-20% (94-96) based on samples from a single season, up to around 60% (95,97) when assessed across the duration of an athletic career. The rates of occurrence appear to be sport-specific, with distance runners experiencing higher prevalence of OTS (~60%) compared with swimmers and wrestlers (10-21%) (98). Furthermore, elite athletes are suggested to be at a greater risk of OTS than non-elites, likely due to the greater training volumes performed by the elite (97). Women have also been reported to experience higher prevalence of OTS than males (99). Conversely, in a study of 257 elite British athletes across a range of sports, only 15% were classified as overtrained (100). Furthermore, the authors observed no differences between "aerobic" (endurance) and "anaerobic" (sprint/power) disciplines and reported higher prevalence in male athletes (17%) compared with their female counterparts (11%). Considering the wide range of athletes experiencing OTS at some point over their career, there is clearly a need for a better understanding of the causes, diagnosis and implications of overtraining.

1.5.4 Aetiology

Short-term OR and the subsequent development of the OTS is suggested to be related along a continuum, starting with a disturbance, an adaptation and eventually a maladaptation of the hypothalamic-pituitary-adrenal (HPA) axis (85,90). Disruption of the HPA axis has been suggested to lead to a neuroendocrine imbalance and a resultant change in stress hormone

response to exercise (91). Hormonal changes are often reported during periods of intensified training, specifically those involved or affected by hypothalamic control and/or subsequent glucocorticoid secretion; however, there is no clear consensus on the exact changes that occur during the development of OTS. Meeusen and colleagues have provided an excellent collection of studies and reviews presenting data on OR and OTS and have suggested that resting hormonal levels alone may not provide sufficient evidence for OTS. Rather, it has been suggested that the change in hormonal response to exercise may provided the greatest insight into the function of the HPA axis (85,91,101).

Despite the ever accumulating data in and around the area of overtraining, the practicalities and relevance of proposed markers to monitor training stress in the field are yet to be investigated and validated. At present, the most basic and commonly used methods of identifying OR and OTS in the field are a reduction in exercise performance with or without related psychological disturbances.

The aim of one of the studies in this thesis was to monitor a wide range of physiological, metabolic, immunological and psychological markers of training load during a short term period of intensified training. The novelty of the research reside in the markers that were measured, the design of the study and in the highly-trained nature of the study participants. Furthermore, an additional aim was to assess the impact of a nutritional intervention that provides high (HCHO) versus moderate (CON) carbohydrate immediately before, during and immediately after each training session. In addition, participants in the HCHO were provided with a protein recovery beverage immediately post-training whereas CON received a placebo (cellulose) tablet. With this information, we hoped to gain an understanding of some of the short-term physiological changes that occur in elite athletes undergoing 'training camp' type periods of intensified training.

1.6 Scope of Thesis

This thesis presents a series of studies which broadly investigated the impact of nutritional challenges to human health and athletic performance. Chapter 2 introduces the concept of fluid balance and presents a study which investigated the impact of caffeine on hydration status. A wide range of hydration assessment techniques were implemented, including the

gold standard measurement of total body water, to gain an understanding the impact of moderate coffee consumption on fluid balance in regular coffee consumers. Chapter 3 continues to investigate hydration; however, the focus moves to the impact of hydration status on health, exercise performance and immune function. A specific interest was in how prolonged endurance exercise in a state of moderate hypohydration modulates innate immune function. Changes in salivary flow rate were monitored and the concentration and secretion rates of numerous salivary antimicrobial proteins were assessed. The aim was to gain insight into the impact of hydration status on mucosal immunity, both during exercise and in the subsequent recovery period. Chapters 4 and 5 present data from a large training study which involved investigation into the impact of training stress on a wide range of markers of health and performance. This study aimed to determine whether a training-specific nutrition strategy would have any impact on the selected measurements. Changes within a population of highly trained athletes undergoing a short-term period of intensified training were assessed. By monitoring a selection of variables whilst tracking changes in performance and mood state, the aim was to identify potential novel markers of overreaching. Chapter 4 presents the hormonal and immunological data collected throughout the period of intensified training. Chapter 5 presents data collected using actigraphy to gain an insight into the sleep behaviours of well-trained athletes and the implications of intensified training on sleep quality. Chapter 6 discusses the results of the aforementioned studies and provides and overview of the main conclusions, practical implications and future research interests.

1.7 References

(1) Krogh A, Lindhard J. The relative value of fat and carbohydrate as sources of muscular energy. Biochem J 1920;14:290-363.

(2) Levine SA, Gordon B, Derick CL. Some changes in chemical constituents of blood following a marathon race. JAMA 1924;82:1778-1779.

(3) Bergstrom J, Hultman E. The effect of exercise on muscle glycogen and electrolytes in normals. Scand J Clin Lab Invest 1966;18(1):16-20.

(4) Bergstrom J, Hultman E. A study of glycogen metabolism during exercise in man. Scand J Clin Invest 1967;19:218-228.

(5) Burke L, Deakin V. Preparation for Competition. In: Burke L, editor. Clinical Sports Nutrition. Fourth Edition ed. Australia: McGraw-Hill Australia Pty Ltd; 2010. p. 304-329.

(6) Snyder AC, Kuipers H, Cheng B, Servais R, Fransen E. Overtraining following intensified training with normal muscle glycogen. Med Sci Sports Exerc 1995 Jul;27(7):1063-1070.

(7) Hargreaves M. Metabolic responses to carbohydrate ingestion: effects on exercise performance. In: Lamb DR, Murray R, editors. Perspectives in exercise science and sports medicine. Vol 12: The metabolic basis of performance in sport and exercise: Carmel: Cooper Publishing Group; 1999. p. 93-119.

(8) Jeukendrup AE. Carbohydrate feeding during exercise. Eur J Sport Sci 2008;8(2):77-86.

(9) Jeukendrup A. A step towards personalized sports nutrition: carbohydrate intake during exercise. Sports Med 2014 May;44 Suppl 1:S25-33.

(10) American Dietetics Association, Dietitians of Canada. Nutrition and Athletic Performance, Joint Position Statement. Medicine and Science in Sport and Exercise 2009.

(11) Maughan RJ, Shirreffs SM. IOC Consensus Conference on Nutrition in Sport, 25–27 October 2010, International Olympic Committee, Lausanne, Switzerland Journal of Sports Sciences 2011;29(S1):S3-S4.

(12) Halson S, Lancaster G, Achten J, Gleeson M, Jeukendrup A. Effects of carbohydrate supplementation on performance and carbohydrate oxidation after intensified cycling training. J Appl Physiol 2004 OCT;97(4):1245-1253.

(13) Achten J, Halson SL, Moseley L, Rayson MP, Casey A, Jeukendrup AE. Higher dietary carbohydrate content during intensified running training results in better maintenance of performance and mood state. J Appl Physiol 2004 Apr;96(4):1331-1340.

(14) Gleeson M, Bishop N, Walsh NP. Exercise Immunology. London: Routledge; 2013.

(15) Bartlett JD, Hawley JA, Morton JP. Carbohydrate availability and exercise training adaptation: Too much of a good thing? Eur J Sport Sci 2014 Jun 19:1-10.

(16) Yeo WK, Paton CD, Garnham AP, Burke LM, Carey AL, Hawley JA. Skeletal muscle adaptation and performance responses to once a day versus twice every second day endurance training regimens. J Appl Physiol (1985) 2008 Nov;105(5):1462-1470.

(17) Hulston CJ, Venables MC, Mann CH, Martin C, Philp A, Baar K, et al. Training with low muscle glycogen enhances fat metabolism in well-trained cyclists. Med Sci Sports Exerc 2010 Nov;42(11):2046-2055.

(18) Witard OC, Turner JE, Jackman SR, Kies AK, Jeukendrup AE, Bosch JA, et al. High dietary protein restores overreaching induced impairments in leukocyte trafficking and reduces the incidence of upper respiratory tract infection in elite cyclists. Brain Behav Immun 2014 Jul;39:211-219.

(19) Maughan RJ. Hydration, morbidity, and mortality in vulnerable populations. Nutr Rev 2012 Nov;70 Suppl 2:S152-5.

(20) Armstrong LE. Challenges of linking chronic dehydration and fluid consumption to health outcomes. Nutr Rev 2012 Nov;70 Suppl 2:S121-7.

(21) Maughan R, Shirreffs S. Exercise in the heat: challenges and opportunities. J Sports Sci 2004 Oct;22(10):917-927.

(22) EFSA Panel on Dietetic Products, Nutrition, and Allergies. Scientific Opinion on Dietary Reference Values for water. EFSA Journal 2010;8(3):1459.

(23) Jéquier E, Constant F. Water as an essential nutrient: the physiological basis of hydration. EJCN 2010;64:115-123.

(24) IoM (Institute of Medicine). Dietary Reference Intakes for Water, Potassium, Sodium, Chloride and Sulfate. Washington DC: National Academies Press; 2004.

(25) American College of Sports Medicine, Sawka MN, Burke LM, Eichner ER, Maughan RJ, Montain SJ, et al. American College of Sports Medicine position stand. Exercise and fluid replacement. Med Sci Sports Exerc 2007 Feb;39(2):377-390.

(26) Garth AK, Burke LM. What do athletes drink during competitive sporting activities? Sports Med 2013 Jul;43(7):539-564.

(27) Coyle EF. Fluid and fuel intake during exercise. J Sports Sci 2004 Jan;22(1):39-55.

(28) Noakes TD. Changes in body mass alone explain almost all of the variance in the serum sodium concentrations during prolonged exercise. Has commercial influence impeded scientific endeavour? Br J Sports Med 2011 May;45(6):475-477.

(29) Kleiner SM. Water: an essential but overlooked nutrient. J Am Diet Assoc 1999 Feb;99(2):200-206.

(30) WaterAid. Hydration Tips. WaterAid ;2012(11/09).

(31) Bird E, T., Parker B, D., Kim H, S., Coffield S, K. Caffeine Ingestion and Lower Urinary Tract Symptoms in Healthy Volunteers. Neurourology and Urodynamics 2005;24:611-615.

(32) Wemple RD, Lamb DR, McKeever KH. Caffeine vs caffeine-free sports drinks: effects on urine production at rest and during prolonged exercise. Int J Sports Med 1997 Jan;18(1):40-46.

(33) Neuhauser-Berthold B,S., Verwied SC, Luhrmann PM. Coffee consumption and total body water homeostasis as measured by fluid balance and bioelectrical impedance analysis. Ann Nutr Metab 1997;41:29-36.

(34) Grandjean A, Reimers K, Bannick K, Haven M. The effect of caffeinated, non-caffeinated, caloric and non-caloric beverages on hydration. Journal of the American College of Nutrition 2000;19(5):591-600.

(35) Del Coso J, Munoz G, Munoz-Guerra J. Prevalence of caffeine use in elite athletes following its removal from the World Anti-Doping Agency list of banned substances. Appl Physiol Nutr Metab 2011 Aug;36(4):555-561.

(36) Desbrow B, Leveritt M. Awareness and use of caffeine by athletes competing at the 2005 Ironman Triathlon World Championships. Int J Sport Nutr Exerc Metab 2006 Oct;16(5):545-558.

(37) Adams WM, Ferraro EM, Huggins RA, Casa DJ. The Influence of Body Mass Loss on Changes in Heart Rate during Exercise in the Heat: A Systematic Review. J Strength Cond Res 2014 Apr 14.

(38) Sawka MN, Coyle EF. Influence of body water and blood volume on thermoregulation and exercise performance in the heat. Exerc Sport Sci Rev 1999;27:167-218.

(39) Maresh CM, Whittlesey MJ, Armstrong LE, Yamamoto LM, Judelson DA, Fish KE, et al. Effect of hydration state on testosterone and cortisol responses to training-intensity exercise in collegiate runners. Int J Sports Med 2006 Oct;27(10):765-770.

(40) Fortes MB, Diment BC, Di Felice U, Walsh NP. Dehydration decreases saliva antimicrobial proteins important for mucosal immunity. Appl Physiol Nutr Metab 2012 Oct;37(5):850-859.

(41) Gill SK, Teixeira AM, Rama L, Rosado F, Hankey J, Scheer V, et al. Salivary antimicrobial protein responses during multistage ultramarathon competition conducted in hot environmental conditions. Appl Physiol Nutr Metab 2013 Sep;38(9):977-987.

(42) West NP, Pyne DB, Renshaw G, Cripps AW. Antimicrobial peptides and proteins, exercise and innate mucosal immunity. FEMS Immunol Med Microbiol 2006 Dec;48(3):293-304.

(43) Samuels C. Sleep, recovery, and performance: the new frontier in high-performance athletics. Neurol Clin 2008 Feb;26(1):169-80; ix-x.

(44) Myllymaki T, Kyrolainen H, Savolainen K, Hokka L, Jakonen R, Juuti T, et al. Effects of vigorous late-night exercise on sleep quality and cardiac autonomic activity. J Sleep Res 2011 Mar;20(1 Pt 2):146-153.

(45) Davenne D. Sleep of athletes – problems and possible solutions. Biological Rhythm Research 2009;40(1):45-52.

(46) Stickgold R, Walker MP. Sleep-dependent memory consolidation and reconsolidation. Sleep Med 2007 Jun;8(4):331-343.

(47) Weitzman ED. Circadian rhythms and episodic hormone secretion in man. Annu Rev Med 1976;27:225-243.

(48) Pilcher JJ, Huffcutt AI. Effects of sleep deprivation on performance: a meta-analysis. Sleep 1996 May;19(4):318-326.

(49) Reilly T, Edwards B. Altered sleep-wake cycles and physical performance in athletes. Physiol Behav 2007 Feb 28;90(2-3):274-284.

(50) Berger RJ, Phillips NH. Comparative aspects of energy metabolism, body temperature and sleep. Acta Physiol Scand Suppl 1988;574:21-27.

(51) Adam K. Sleep as a restorative process and a theory to explain why. Prog Brain Res 1980;53:289-305.

(52) Adam K, Oswald I. Sleep is for tissue restoration. J R Coll Physicians Lond 1977 Jul;11(4):376-388.

(53) Sheth S, Brito R, Mukherjea D, Rybak LP, Ramkumar V. Adenosine receptors: expression, function and regulation. Int J Mol Sci 2014 Jan 28;15(2):2024-2052.

(54) Dworak M, Diel P, Voss S, Hollmann W, Struder HK. Intense exercise increases adenosine concentrations in rat brain: implications for a homeostatic sleep drive. Neuroscience 2007 Dec 19;150(4):789-795.

(55) Murphy PJ, Campbell SS. Nighttime drop in body temperature: a physiological trigger for sleep onset? Sleep 1997 Jul;20(7):505-511.

(56) Krauchi K, Cajochen C, Werth E, Wirz-Justice A. Warm feet promote the rapid onset of sleep. Nature 1999 Sep 2;401(6748):36-37.

(57) Shapiro CM, Allan M, Driver H, Mitchell D. Thermal load alters sleep. Biol Psychiatry 1989 Nov;26(7):736-740.

(58) American Academy of Sleep Medicine. International Classification of Sleep Disorders, Revised: Diagnostic and Coding Manual 2001.

(59) Petruzzello SJ, Landers DM, Hatfield BD, Kubitz KA, Salazar W. A meta-analysis on the anxiety-reducing effects of acute and chronic exercise. Outcomes and mechanisms. Sports Med 1991 Mar;11(3):143-182.

(60) Kobayashi T, Yoshida H, Ishikawa T, Arakawa K. Effects of the late evening exercise on sleep onset process. Sleep Res Online 1999;2 (suppl. 1):233.

(61) Driver HS, Taylor SR. Exercise and sleep. Sleep Med Rev 2000 Aug;4(4):387-402.

(62) Souissi M, Chtourou H, Zrane A, Cheikh RB, Dogui M, Tabka S, et al. Effect of time-of-day of aerobic maximal exercise on the sleep quality of trained subjects. Biological Rhythm Research 2011;43(3):323-330.

(63) Taylor SR, Rogers GG, Driver HS. Effects of training volume on sleep, psychological, and selected physiological profiles of elite female swimmers. Med Sci Sports Exerc 1997 May;29(5):688-693.

(64) Fietze I, Strauch J, Holzhausen M, Glos M, Theobald C, Lehnkering H, et al. Sleep quality in professional ballet dancers. Chronobiol Int 2009 Aug;26(6):1249-1262.

(65) Hausswirth C, Louis J, Aubry A, Bonnet G, Duffield R, LE Meur Y. Evidence of disturbed sleep and increased illness in overreached endurance athletes. Med Sci Sports Exerc 2014 May;46(5):1036-1045.

(66) Schulman JL, Reisman JM. An objective measure of hyperactivity. Am J Ment Defic 1959 Nov;64:455-456.

(67) Stanley N. Actigraphy in human psychopharmacology: a review. Hum Psychopharmacol 2003 Jan;18(1):39-49.

(68) Sadeh A. The role and validity of actigraphy in sleep medicine: an update. Sleep Med Rev 2011 Aug;15(4):259-267.

(69) Morgenthaler T, Alessi C, Friedman L, Owens J, Kapur V, Boehlecke B, et al. Practice parameters for the use of actigraphy in the assessment of sleep and sleep disorders: an update for 2007. Sleep 2007 Apr;30(4):519-529.

(70) Nieman DC. Exercise, infection, and immunity. Int J Sports Med 1994 Oct;15 Suppl 3:S131-41.

(71) Nieman DC. Exercise, upper respiratory tract infection, and the immune system. Med Sci Sports Exerc 1994 Feb;26(2):128-139.

(72) Mackinnon LT. Immunoglobulin, antibody and exercise. Exerc Immunol Rev 1996;2:1-34.

(73) Ekblom B, Ekblom O, Malm C. Infectious episodes before and after a marathon race. Scand J Med Sci Sports 2006 Aug;16(4):287-293.

(74) Neville V, Gleeson M, Folland JP. Salivary IgA as a risk factor for upper respiratory infections in elite professional athletes. Med Sci Sports Exerc 2008 Jul;40(7):1228-1236.

(75) Malm C. Susceptibility to infections in elite athletes: the S-curve. Scand J Med Sci Sports 2006 Feb;16(1):4-6.

(76) Oliver SJ, Laing SJ, Wilson S, Bilzon JL, Walters R, Walsh NP. Salivary immunoglobulin A response at rest and after exercise following a 48 h period of fluid and/or energy restriction. Br J Nutr 2007 Jun;97(6):1109-1116.

(77) Gleeson M, Bishop NC. URI in athletes: are mucosal immunity and cytokine responses key risk factors? Exerc Sport Sci Rev 2013 Jul;41(3):148-153.

(78) Koutedakis Y, Sabin E, Perera S. Modulation of salivary lysozyme by training in elite male swimmers. Journal of Sports Sciences 1996;14(90).

(79) Gillum TL, Kuennen M, Gourley C, Schneider S, Dokladny K, Moseley P. Salivary antimicrobial protein response to prolonged running. Biol Sport 2013 Mar;30(1):3-8.

(80) Li TL, Gleeson M. The effects of carbohydrate supplementation during repeated bouts of prolonged exercise on saliva flow rate and immunoglobulin A. J Sports Sci 2005 Jul;23(7):713-722.

(81) Blannin AK, Robson PJ, Walsh NP, Clark AM, Glennon L, Gleeson M. The effect of exercising to exhaustion at different intensities on saliva immunoglobulin A, protein and electrolyte secretion. Int J Sports Med 1998 Nov;19(8):547-552.

(82) Hucklebridge F, Clow A, Evans P. The relationship between salivary secretory immunoglobulin A and cortisol: neuroendocrine response to awakening and the diurnal cycle. Int J Psychophysiol 1998 Dec;31(1):69-76.

(83) Perera S, Uddin M, Hayes JA. Salivary lysozyme: a noninvasive marker for the study of the effects of stress of natural immunity. Int J Behav Med 1997;4(2):170-178.

(84) Rhea MR, Alderman BL. A meta-analysis of periodized versus nonperiodized strength and power training programs. Res Q Exerc Sport 2004 Dec;75(4):413-422.

(85) Meeusen R, Piacentini MF, Busschaert B, Buyse L, De Schutter G, Stray-Gundersen J. Hormonal responses in athletes: the use of a two bout exercise protocol to detect subtle differences in (over)training status. Eur J Appl Physiol 2004 Mar;91(2-3):140-146.

(86) Foster C. Monitoring training in athletes with reference to overtraining syndrome. Med Sci Sports Exerc 1998 Jul;30(7):1164-1168.

(87) Armstrong LE, VanHeest JL. The unknown mechanism of the overtraining syndrome: clues from depression and psychoneuroimmunology. Sports Med 2002;32(3):185-209.

(88) Kuipers H, Keizer HA. Overtraining in elite athletes. Review and directions for the future. Sports Med 1988 Aug;6(2):79-92.

(89) Urhausen A, Gabriel HH, Weiler B, Kindermann W. Ergometric and psychological findings during overtraining: a long-term follow-up study in endurance athletes. Int J Sports Med 1998 Feb;19(2):114-120.

(90) Halson SL, Lancaster GI, Achten J, Gleeson M, Jeukendrup AE. Effects of carbohydrate supplementation on performance and carbohydrate oxidation after intensified cycling training. J Appl Physiol (1985) 2004 Oct;97(4):1245-1253.

(91) Meeusen R, Duclos M, Foster C, Fry A, Gleeson M, Nieman D, et al. Prevention, diagnosis, and treatment of the overtraining syndrome: joint consensus statement of the European College of Sport Science and the American College of Sports Medicine. Med Sci Sports Exerc 2013 Jan;45(1):186-205.

(92) Fry RW, Morton AR, Keast D. Overtraining in athletes. An update. Sports Med 1991 Jul;12(1):32-65.

(93) Falsetti HL. Overtraining in athletes - a round table. Physician and Sportsmedicine 1983;11:93-110.

(94) Hooper SL, Mackinnon LT, Hanrahan S. Mood states as an indication of staleness and recovery. Int J Sport Psychol 1997;28:1-12.

(95) Morgan WP, Brown DR, Raglin JS, O'Connor PJ, Ellickson KA. Psychological monitoring of overtraining and staleness. Br J Sports Med 1987 Sep;21(3):107-114.

(96) O'Connor PJ, Morgan WP, Raglin JS, Barksdale CM, Kalin NH. Mood state and salivary cortisol levels following overtraining in female swimmers. Psychoneuroendocrinology 1989;14(4):303-310.

(97) Morgan WP, O'Connor PJ, Sparling PB, Pate RR. Psychological characterization of the elite female distance runner. Int J Sports Med 1987 Nov;8 Suppl 2:124-131.

(98) Matos NF, Winsley RJ, Williams CA. Prevalence of nonfunctional overreaching/overtraining in young English athletes. Med Sci Sports Exerc 2011 Jul;43(7):1287-1294.

(99) Winsley R, Matos N. Overtraining and elite young athletes. Med Sport Sci 2011;56:97-105.

(100) Koutedakis Y, Sharp NC. Seasonal variations of injury and overtraining in elite athletes. Clin J Sport Med 1998 Jan;8(1):18-21.

(101) Meeusen R, Duclos M, Gleeson M, Rietjens G, Steinacker J, Urhausen A. Prevention, diagnosis and treatment of the Overtraining Syndrome - ECSS Position Statement 'Task Force'. European Journal of Sport Science 2006 MAR;6(1):1-14.

(102) The Fairtrade Foundation. Fairtrade and Coffee Report. 2012.

(103) Peters EM, Bateman ED. Ultramarathon running and upper respiratory tract infections. An epidemiological survey. S Afr Med J 1983 Oct 1;64(15):582-584.

(104) Nieman DC, Johansen LM, Lee JW, Arabatzis K. Infectious episodes in runners before and after the Los Angeles Marathon. J Sports Med Phys Fitness 1990;30:316-28.

CHAPTER 2: NO EVIDENCE OF DEHYDRATION WITH MODERATE DAILY COFFEE INTAKE: A COUNTERBALANCED CROSS-OVER STUDY IN A FREE-LIVING POPULATION

Sophie C. Killer [1,2], Andrew K. Blannin[1] and Asker E. Jeukendrup[1,2]

[1]School of Sport and Exercise Sciences, University of Birmingham, Birmingham, West Midlands. UK.

[2]Current Address: School of Sport, Exercise and Health Sciences, Loughborough University, Leicestershire, East Midlands. UK.

Conceived and designed the experiments: SCK AEJ.
Performed the experiments: SCK.
Analyzed the data: SCK AEJ AKB.
Contributed reagents/materials/analysis tools: SCK AEJ AKB.
Wrote the paper: SCK.
Statistical analysis: SCK AKB AEJ.
Editing of final paper: AKB AEJ

2.1 Abstract

It is often suggested that coffee causes dehydration and its consumption should be avoided or significantly reduced to maintain fluid balance. The aim of this study was to directly compare the effects of coffee consumption against water ingestion across a range of validated hydration assessment techniques. In a counterbalanced cross-over design, 50 male coffee drinkers (habitually consuming 3-6 cups per day) participated in two trials, each lasting three consecutive days. In addition to controlled physical activity, food and fluid intake, participants consumed either 4 x 200 mL of coffee containing 4 mg/kg caffeine (C) or water (W). Total body water (TBW) was calculated pre- and post-trial via ingestion of Deuterium Oxide. Urinary and haematological hydration markers were recorded daily in addition to nude body mass measurement (BM). Plasma was analysed for caffeine to confirm compliance. There were no significant changes in TBW from beginning to end of either trial and no differences between trials (51.5 ± 1.4 vs. 51.4 ± 1.3 kg, for C and W respectively). No differences were observed between trials across any haematological markers or in 24 h urine volume (2409 ± 660 vs. 2428 ± 669 mL, for C and W respectively), USG, osmolality or creatinine. Mean urinary Na^+ excretion was higher in C than W (p= 0.02). No significant differences in BM were found between conditions, although a small progressive daily fall was observed within both trials (0.4 ± 0.5 kg; p< 0.05). Our data show that there were no significant differences across a wide range of haematological and urinary markers of hydration status between trials. These data suggest that coffee, when consumed in moderation by caffeine habituated males provides similar hydrating qualities to water.

Key Words: Coffee, Caffeine, Hydration, Fluid Balance, Total Body Water

2.2 Introduction

Maintenance of fluid balance is essential to sustain human life. Water intake balances fluid losses to achieve adequate hydration of bodily tissues. Although there are widespread guidelines in scientific literature and media for achieving optimal hydration status and about the effects that various caffeinated beverages may have on fluid balance, there is no clear consensus about how much fluid an individual should consume (1). One study found total daily fluid intake observed in healthy adults varied from 0.416-4.316 L/day (2). The current EFSA dietary references values for water intakes for male adults is 2.5 L/day (3). However, published guidelines range from 1.5 L/day (4) to 3.7 L/day (5) for adult males. It has been suggested that caffeinated beverages should not be included in daily fluid requirement guidelines (6) and that a glass of water should be consumed with every cup of coffee or tea to ensure hydration is maintained (7).

Caffeine (1, 3, 7-trimethylxanthine) is a naturally occurring methylxanthine which can be found in coffee, tea and chocolate. Caffeine acts as an adenosine receptor antagonist to reduce fractional sodium reabsorption in both the proximal tubule and distal nephron. When consumed in large doses ($\geq$500 mg), caffeine elicits a diuretic effect (8-10). The diuretic potential of caffeine in humans has been researched for many years, with the first scientific report published over 80 y ago (11). The authors of these early findings suggested that whilst caffeine causes acute diuresis, regular caffeine consumption may lead to a tolerance developing against its diuretic effect. It has since been suggested that caffeine withdrawal of as little as 4 days is sufficient for tolerance to be lost (12). Following the work of Eddy & Downs [11], there has been a range of studies that have investigated the effects of caffeine on hydration status (Table 2.1). These studies report observations across a range of caffeine forms and doses on various markers of hydration status in either caffeine-habituated or caffeine-naive populations (individuals who do not habitually consume caffeine, or those who have abstained from caffeine consumption for $\geq$ 4 days). Although the data are somewhat varied, the general trend is that higher doses of caffeine in caffeine-naive individuals will elicit an acute increase in urine volume, yet a low to moderate dose of caffeine does not induce a diuretic effect (13-17).

Table 2.1: Effect of Caffeine Consumption on Urine Production

Authors	Caffeine Dose *(mg)*	Caffeine Source	Test population	Diuretic Effect
(8)	4 mg/d (200-700 mg)	Caffeine tablet	Habituated caffeine users	Yes –during first day only
(9)	490-680 (8.7 mg/kg bw)	Caffeine powder added to carbohydrate electrolyte drink Vs carbohydrate electrolyte drink	Caffeine naïve (Habitual coffee drinkers – 4 day pre-trial deprivation)	Yes
(10)	642	Caffeinated coffee	Caffeine naïve (Habitual coffee drinkers – 5 day pre-trial deprivation)	Yes
(14)	452	Caffeine tablet	Habitual caffeine users – 98 ± 17 mg/day	No
(17)	360	Caffeine tablet	NA	Yes
(15)	300	Caffeine tablet	Habitual coffee drinkers – 8 h pre-trial deprivation	No
(25)	250	Caffeine beverage	Caffeine naïve (Non-coffee drinkers – 3 week caffeine deprived)	Yes
(31)	250	Caffeine tablet	Caffeine naïve (1 week caffeine deprived)	Yes –during first hour only
(32)	240	Caffeine beverage	Caffeine habituated users	Yes
(14)	226	Caffeine capsules	Habitual caffeine users – 98 ± 17 mg/day	No
(16)	168 and 252	Caffeinated tea	Caffeine users – 12 h deprived	No
(13)	114-253	Caffeinated carbonated cola, Caffeinated carbonated, non-caloric cola, Instant coffee	Caffeine habituated (61-464 mg/day)	No
(33)	45, 90 and 180	Caffeine tablet	NA	No

Coffee is comprised of many bioactive compounds in addition to caffeine. These active compounds may interact with each other and therefore coffee consumption cannot be directly compared to caffeine consumption in its purest form (1,3,7-trimethyl xanthine) (18). Interestingly, only two studies have specifically investigated the effects of caffeine in the form of coffee on hydration status. One study investigated the effects of six cups of coffee (624 mg caffeine) on urine excretion following a five day caffeine deprivation period (10). Over the 24 h period, authors report a 2.7 % decrease in total body water and a 41 % increase in urine excretion, with a subsequent 66 % and 28 % increase in urinary sodium and potassium excretion, respectively. Due to the study design, which only included caffeine habituated participants who abstained from caffeine for 5 days prior to testing, the results of the study should be interpreted with caution before applying to habitual moderate-intake coffee drinkers. Another study investigated the effects of consuming equal amounts of water, caffeinated cola and caffeinated coffee (3.1 ± 0.4 mg/kg caffeine/day) against water with a mixture of caffeinated colas (1.4 ± 0.2 mg/kg caffeine/day) or non-caffeinated beverages (13). The authors found no effects of coffee consumption compared with non-caffeinated beverages across a range of hydration markers in caffeine-habituated participants. Whilst the authors concluded that the advice to exclude caffeinated beverages from daily fluid requirement was not supported by their findings, the study did not measure total body water. Total body water (TBW) estimations using the doubly labelled water dilution technique is considered the gold standard method for assessing body water fluctuations over time (14). One recent study investigated the effects of caffeine on TBW using deuterium oxide (19). Thirty participants classified as low caffeine users (<100 mg/day) consumed caffeine (5 mg/kg/day) or placebo tablets for 4 consecutive days. Although these participants could not be classified as 'caffeine-habituated', no changes in TBW measured on day 1 and 4 were found between the caffeine or placebo group. The authors suggested that a moderate dose of caffeine does not alter TBW in healthy men. To date, no studies have investigated the effects of moderate coffee consumption on TBW in caffeine-habituated adults using the doubly labelled water dilution technique.

It is estimated that 1.6 billion cups of coffee are consumed worldwide every day (20), thus it is of interest to know whether coffee contributes to daily fluid requirement, or whether it causes low-level chronic dehydration. In the present study, our aim was to directly compare the effects of a moderate intake of coffee in caffeine-habituated adults against equal amounts

of water across a wide range of hydration markers, including the gold standard TBW measure.

2.3 Methods

Ethics Statement

All participants were informed of the purposes of the study and the risks associated with the procedures. Written informed consent was obtained from all participants before the study commenced. The study was approved by the Science, Technology, Engineering and Mathematics (STEM) Ethical Review Committee at the University of Birmingham, UK.

Participants

Fifty-two healthy non-smoking males aged 18-46 y were accepted to participate in the study following the screening of over 100 volunteers. Inclusion criteria required participants to be weight stable, pass a general health questionnaire, be free from medications containing caffeine or those that might influence weight or fluid-electrolyte balance, live and work in an environment of ambient temperature with no significant temperature or humidity fluctuations, consume a diet with no extreme food, beverage or dietary supplement intakes and be free from chronic illnesses. Females were excluded from the study due to possible disruptions to fluid balance by the menstrual cycle. Participants were moderate coffee drinkers consuming 3-6 cups per day (300-600 mg/day caffeine) assessed by a 3-day weighed food diary. Two participants could not complete the study due to individual circumstances preventing them from visiting the laboratory.

Study Design

Participants reported to the School of Sport and Exercise Sciences, Human Performance Laboratory at the University of Birmingham on two occasions prior to testing. Trials ran from Tuesdays to Fridays during the months of February to December. On their first visit, participants were instructed how to complete a 3-day weighed diet diary and were provided with a set of digital scales (Electronic Scales; Salter Arc) to weigh their food and fluid intake accurately to 0.1 g. On their second visit, participants returned their completed diet diary and

baseline body weight was recorded. Each participant completed two treatments each lasting four consecutive days. Each trial was separated by a 10 day wash out period during which time participants were instructed to consume their normal diet and daily caffeine intake. See figure 2.1 for a schematic overview.

Treatment

Each trial was undertaken in a counter balanced cross-over design and participants were randomly allocated to a treatment group. The coffee trial (C) involved participants consuming four mugs (200 mL) of black coffee per day (Nescafé Original) equating to a caffeine intake of 4 mg/kg BM. The water control trial (W) involved participants consuming four mugs of water (200 mL) per day. Participants were required to abstain from alcohol and all physical activity 24 h prior to and for the duration of each trial, with the exception of walking for transport.

Diet was controlled and provided to participants throughout each testing period, including the control days. The same diet was replicated and provided for each participant's second trial. Food and fluid intake recorded in the 3-day weighed food diary was analysed for macronutrients, sodium and potassium and food fluid content using nutrition analysis software (Nutritionist Pro, Axxya Systems). Diets were designed and prescribed on an individual basis to replicate mean energy and fluid intakes from the food diary. Diets were a standard weight-maintaining composition of 50%:35%:15% for carbohydrate, fat and protein respectively. A compliance booklet was completed by participants on the morning of each trial day to ensure all food and beverages from the previous day were consumed at the correct times and that no unplanned exertions, fluid losses or nutritional variations had occurred. Participants were instructed to complete the Bristol Stool Chart each morning for indications of disruption to fluid balance (21). On the morning of each trial, participants reported to the laboratory at a standardised time between 07:00-09:00. Participants were ≥10 h fasted and had not consumed any fluids since 21:30 the previous evening. Participants produced a first morning void (MV) (trial days 1-3) and a separate 24 h urine collection (trial days 2-3). Nude body weight was recorded and a venous blood sample collected. Meals and snacks were consumed at standardised times (± 30 min); breakfast at 07:30–09:00 (immediately post testing), morning snack at 10:30, lunch at 13:30, afternoon snack at 16:30 and evening meal at 19:30.

Figure 2.1: Schematic Overview of Study Design.
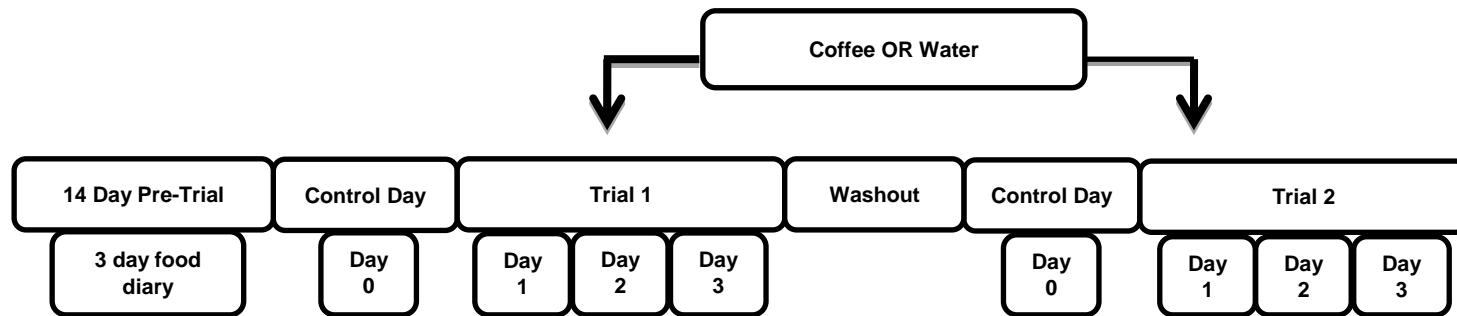
Beverages

As there is no clear consensus on how much fluid an individual should consume, yet fluid intake was required to be standardised for each participant, daily fluid intake was calculated based on mean individual fluid intakes recorded over the 3-day diet dairy. Daily fluid intake was provided with bottled water and divided into six equal bottles, measured to the nearest gram using digital scales. Participants consumed water at pre-determined time points; between 07:30-09:00 (post testing), 10:30, 13:30, 16:30, 19:30 and 21:30. Test beverages were consumed at predetermined time points; immediately after testing (07:30-09:00), 10:30, 13:30 and 16:30. Each participant was provided with a mug marked at 200 mL. During trial C, participants received four pre-weighed containers of Nescafé Original coffee to provide 4 mg/kg BM of caffeine per day (2.3 ± 0.4 g Nescafé Original per cup) and were instructed to make the beverage with boiled tap water to the 200 mL marker in the mug provided. During trial W, participants were instructed to consume 200 mL tap water in the same mug. Total test beverage intake was 800 mL/day.

2.4 Measures and Analysis

Haematology

Fasted blood samples (approximately 15 mL) were drawn from a superficial vein (21G Venisystem short butterfly, NU Care), typically from the median cubital vein by a trained phlebotomist following a 5 min resting period by participants in the supine position. Fasted blood samples were collected on the mornings of test days 1-3. Blood for serum and plasma analysis were collected directly into two separate tubes; one with clotting agent for analysis of serum sodium (Na^+), potassium (K^+), osmolality, creatinine, blood urea nitrogen (BUN) and deuterium oxide (D_2O) for total body water (TBW) calculation and one with K_2EDTA for analysis of haematocrit, total plasma protein (TPP) and caffeine. Haematocrit was analysed using a haematocrit centrifuge (Micro Haematocrit Centrifuge, Hawksley & Sons Ltd.) and ruler (Micro Haematocrit Tube Reader, Hawksley & Sons Ltd.). All other samples were centrifuged at 3500 RPM for 15 min at 4°C. Plasma and serum were frozen at -20°C for later analysis. All samples were analysed in duplicate, with the exception of D_2O. Serum osmolality was determined via freeze point depression via the Advanced Osmometer Model

765 (Advanced Instruments Inc Norwood MA). TPP, serum creatinine and BUN were analysed using the iLab 650 (Instrumentation Laboratory, UK). Plasma caffeine was analysed for participant compliance using a reversed High Performance Liquid Chromatography (HPLC) – UV method, following the protocol described elsewhere (Appendix A) (City Hospital, Sandwell and West Birmingham Hospitals NHS Trust). Na^+ and K^+ were analysed using Ion Specific Electrodes on the iLab 600 (Midland Pathology Services). Samples were loaded into cuvettes and place into the iLab without pre-preparation and analysed automatically.

Total Body Water and Body Mass

Nude body mass (BM) was recorded each morning (trial days 1-3) following the participants' first morning void. Participants were fasted and had not consumed any water since the previous evening.

Labelled isotope D_2O, was provided for each participant to drink to enable the calculation of TBW (99.9 atom % D, Aldrich Chemistry, Sigma-Aldrich). TBW was calculated on days 1 and 3 of each trial to ensure participants began the study in a state of euhydration and to assess any disruptions to fluid balance over the duration of the each trial.
Participants were provided with 0.1 g/kg BM D_2O, measured to the nearest 0.001 g on day 0 (control day) and trial day 2. Participants were instructed to consume the D_2O between 20:30-21:30 with their evening water allowance. No additional fluids were permitted following D_2O ingestion until after the fasted blood sample was collected the next morning (trial days 1 and 3) approximately 10-12 h later. Participants were instructed to continue to collect all urine losses. An additional blood sample was taken on the evening of day 2 (between 17:00-18:00) prior to the consumption of their second dose of D_2O to establish new blood deuterium enrichment baseline.

Serum D_2O enrichment was analysed using the Gas-Bench II (Thermo Electron, Bremen, Germany) – isotope mass spectrometry (Finnigan, Delta XP, Bremen, Germany) following the protocol described elsewhere (23). Briefly, 200 µL of plasma was added to a vacutainer (Labco, High Wycombe, England) with a platinum catalyst (Thermo Electron, Bremen, Germany). The vacutainer was flushed by an automated autosampler-assisted flushing procedure, using 2 % H_2 in Helium gas for 5 min. Following this, a 40 min equilibrium period occurred whereby the hydrogen isotopes in the aqueous solution exchanged with hydrogen

ions in the headspace. A sample of the headspace gas was then injected into the Isotope Ratio Mass Spectrometer (IRMS) (Thermo Electron, Bremen, Germany). A mean of the four middle measurements was taken as the measure for each sample. The isotopic enrichment was expressed as $\delta^0/_{00}$ against the international water standard Vienna Standard Mean Ocean Water (V-SMOW). The coefficient of variation of the measurement was 0.027 %. Results of the isotope ratio analysis were reported relative to the working reference gas versus V-SMOW and as atom percentage excess (APE).

The delta between sample and reference gas is defined as: *Delta D = [(Ratio of Sample - Ratio of Reference)/ (Ratio of Reference)] x 1000*. The delta deuterium values for the pre-dose (δpre) and post-dose samples (δpos) were determined. The deuterium dose was diluted with tap water. The deuterium content of tap water (δtap) and the dose (δdose) was measured. TBW in moles could then be calculated from the dilution of the heavy water isotope using the equation: *TBW (moles) = A/ (18.02a) × [δdose - δtap/ (δpost−δpre)]*. A is the amount of dose (g) administered to participants and a is amount of dose (g) diluted for analysis. To convert TBW to kilograms, the following equation was applied: *TBW (kg) = TBW (moles) x 18.02/ 1000 g*. It is known that some deuterium binds to acidic amino acids of proteins or other non-exchangeable sites and it has been experimentally determined that deuterium oxide overestimates TBW by 4% (24). Therefore, to correct for the non-exchange of deuterium in the body, the TBW measurement was divided by 1.04.

Urine Analysis

A total of five urine samples were collected during each trial; two 24 h collections (24 h) and three morning voids (V). V was collected separately each day and analysed for urine specific gravity (USG) using a hand held refractometer (Pocket Refractometer, PAL-105. Atago, Japan) and volume using digital scales (measured to 0.01 g) (Sartorius, AG Germany), applying the formula to correct for USG ($V = (M_{\text{bottle plus urine collection}} − M_{\text{empty bottle}}) / (USG \times \rho_{H2O})$): V is volume (mL), M are masses (g) and $\rho_{H2O} = 1$ g/mL is density of water).

Twenty four hour urine collections were analysed for USG and total volume using the methodology outlined above. In addition, 24 h collections were further analysed for osmolality via freeze point depression using the Advanced Osmometer Model 765 (Advanced Instruments Inc. Norwood MA), creatinine on the iLab 650 (Instrumentation Laboratory UK) and sodium (Na^+) and potassium (K^+) on the iLab 600 (Midlands Pathology Services Ltd.).

The MV volume was added to the 24 h collection to give total 24 h volume data (V24). Following the initial measures of urine volume and USG, urine samples were stored at -20°C for later analysis.

The numbers of urine samples reported throughout the paper are: Volume (*n=50*), USG (*n=50*), urine osmolality (*n=46*), urine creatinine (*n=48*), urine Na$^+$ (*n=46*) and urine K$^+$ (*n=42*). The numbers of blood samples reported throughout the paper are: haematocrit (*n=48*), serum osmolality (*n=49*), total plasma protein (*n=48*), serum creatinine (*n=46*), serum sodium (*n=45*) and serum potassium (*n=45*). Sample sizes less than 50 are the result of missing samples or technical error.

Statistical Analysis

All data were analysed using statistical software (SPSS. 18 for Windows). Two-way repeated measures ANOVA with pairwise comparisons post hoc were applied to each data set to look for significant main effects. Delta values were calculated and Student's t-tests were used to analyse changes overtime in each condition. The level of significance was set at $p<0.05$.

2.5 Results

Participant Characteristics

Fifty of the fifty two participants recruited for this study fully completed the two trials. The characteristics of the study population are presented in table 2.2.

Dietary Intakes

The habitual caffeine intake questionnaire and three day weighed diet diaries ensured participants were habitual moderate coffee drinkers with estimated mean intakes of between 300-600 mg caffeine per day from coffee. Any participant whose caffeine intake from coffee fell outside of this range was not included in the study.

During trial C, participants consumed 4 mg/kg day caffeine provided in the form of Nescafé Original, divided into four equal servings of 200 mL. During trial W, participants consumed 200 mL tap water on four occasions each day. Total test beverage intake was 800 mL/day

during both conditions. Mean caffeine consumption during trial C was 308 mg, and ranged from 204.4-453.0 mg caffeine.

Table 2.2: Participant Characteristics and Data from 3-day (pre-trial) Weighed Dietary Intakes

Characteristic	Mean ± SD	Range
Age (y)	28.1 ± 7.3	18-46
Height (cm)	181.1 ± 6.3	169.0-192.0
Weight (kg)	77.0 ± 12.1	51.1-133.6
Total Energy Intake (Kcal)	2400 ± 464	1402-3398
Dietary carbohydrate (%)	53.5 ± 7.0	33.2-72.4
Dietary Protein (%)	16.4 ± 4.8	10.3-35.3
Dietary Fat (%)	29.8 ± 5.9	14.1-43.0
Total Water Intake	2081 ± 842	1083-3614
Total Coffee Intake (mL)	979 ± 301	625-1522

Compliance booklets suggest that all participants consumed the foods and fluids that they were provided with. In addition, no participants partook in any physical activity, with the exception of walking for transport, 24 h prior to and throughout the duration of each trial. The Bristol Stool Chart monitored any unusual faecal losses. No participants reported unexpected fluid losses from diarrhoea or vomiting during either trial.

Serum caffeine was measured on day two of each trial to check for participant compliance. Results of caffeine analysis, performed by high performance liquid chromatography showed that, as expected, serum caffeine was significantly higher in the coffee trial than the water trial (1.40 ± 1.07 vs. 0.33 ± 0.46 mg/L; $p < 0.01$). These findings support participant compliance to the diet and test beverage during each trial.

Dietary macronutrient intake was calculated individually for each participant, based on energy from their food diary. Mean energy intake during the trials was 2425 ± 413 Kcal. Mean water consumption during the trials was 1953 ± 642 mL.

Body Mass Variables

Figure 2.2 illustrates the results of TBW estimates, based on deuterium oxide analysis performed by gas chromatography mass spectrometry. Fluctuations in TBW were not significantly different between the two conditions ($p = 0.90$). There were no significant changes in TBW from beginning to end of either trial ($p > 0.05$) suggesting that participants maintained a stable fluid balance throughout the study. This is further confirmed by statistical analysis showing no significant effect of trial day on either condition ($p = 0.43$).

Figure 2.3 illustrates daily body mass measurements. Mean body mass of participants during experimental trials was 76.97 ± 12.15 kg. Mean body mass did not differ between the two conditions ($p = 0.45$), however a small but progressive daily fall in BM occurred within both conditions ($p < 0.05$). Mean decrease in BM from day 1 to day 3 across both trials was 0.39 ± 0.5 kg.

Figure 2.2: Mean Total Body Water Estimates from Day 1-Day 3. *n*=25
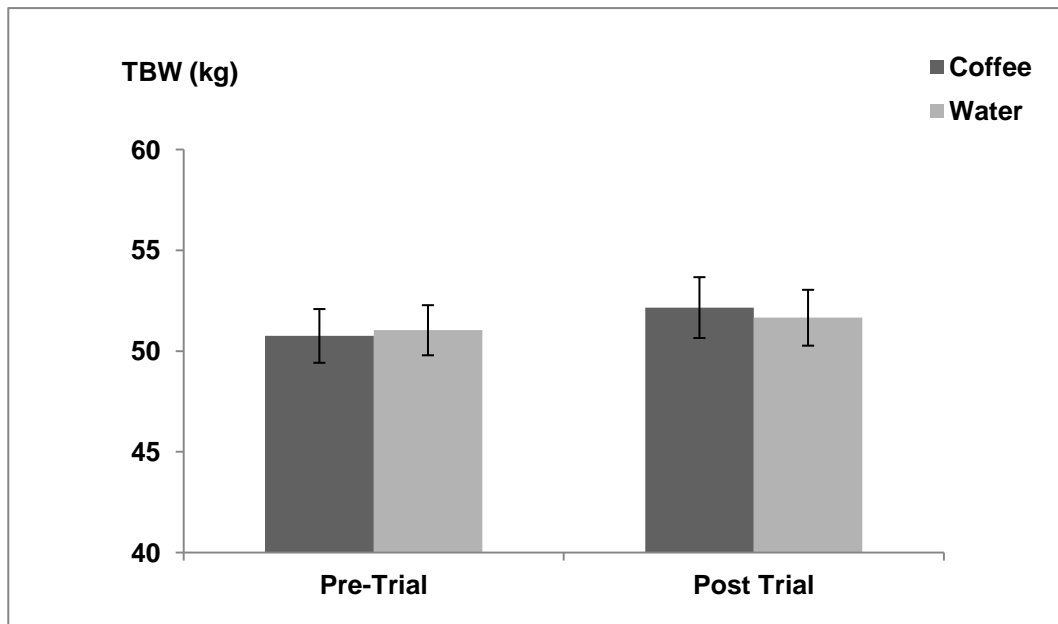
Figure 2.3: Mean Body Mass.* Significant difference between days. *n*=50
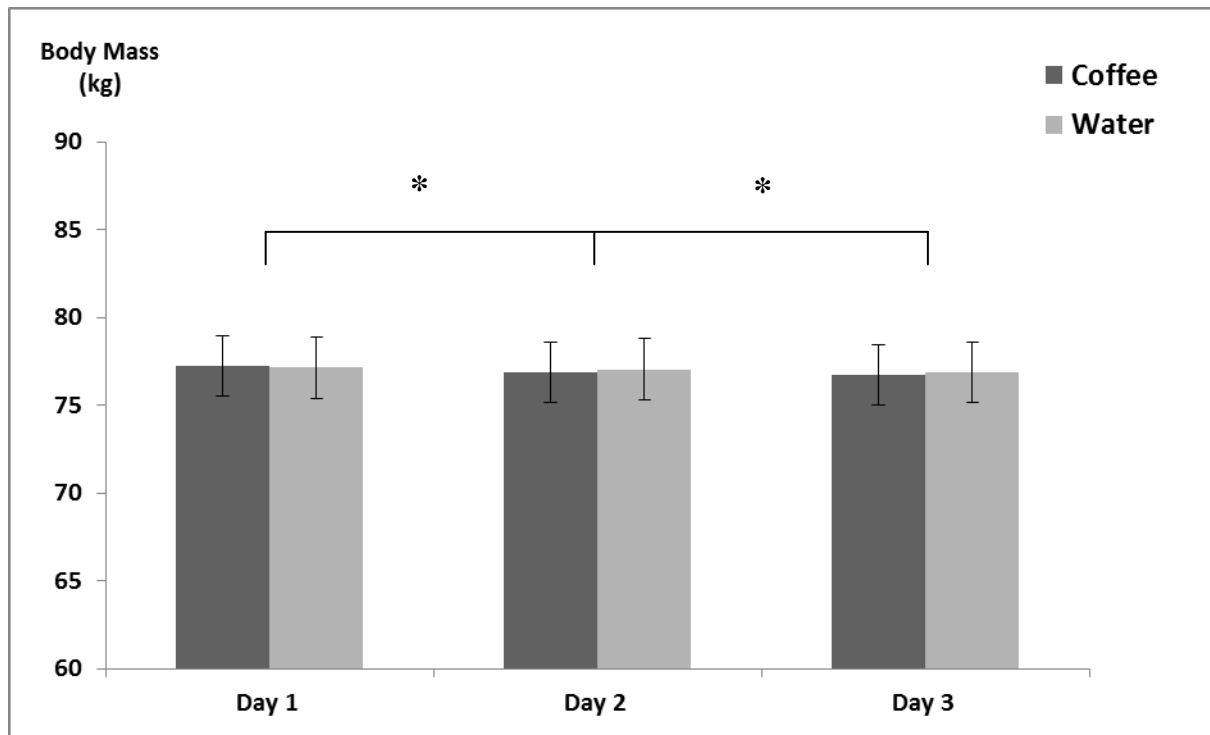
Urinary Markers

Means and standard deviations of 24 h urinary measures recorded on trial days 1 and 2 are presented in table 2.3. Urine void volume and USG collected on trial days 1-3 are presented in table 2.4. Twenty four hour urine volume, USG, urine osmolality or urine creatinine did not differ between conditions ($p > 0.05$). Urinary Na^+ was not different between trials days, however mean Na^+ excretion was significantly higher on both days in the coffee trial than the water trial ($p = 0.02$). K^+ concentration was significantly higher in C than W on

day one only. K^+ concentration were significantly higher on day 2 in both conditions ($p = 0.02$), but no between-condition difference was found.

Neither urine void volume nor urine void USG were different between conditions; $p = 0.86$ and $p = 0.95$, respectively (1.4).

Haematology

Means and standard deviations of haematological measures recorded on trial days 1, 2 and 3 are presented in table 2.5. Haematological markers did not differ between conditions across all measures: serum osmolality, haematocrit, total plasma protein, serum sodium, serum potassium ($p < 0.05$). Student's t-test analysis showed no significant differences between conditions in the delta change from day 1 to day 3 for all haematological measures.

Renal function was normal throughout each trial as assessed by urine creatinine (2.3), serum creatinine and BUN (Table 2.5). Neither urine or serum creatinine differed between conditions or time points ($p > 0.05$).

Table 2.3: Twenty-four hour Urine Collection

Condition/ Day	Urine Volume ± SD (mL/24h)	USG ± SD	Urine Osmolality ± SD (mOsm/kg)	Urine Creatinine ± SD (mg/24h)	Urine Na excretion ± SD (total mmol/24h)	Urine K excretion ± SD (total mmol/24h)
W1	2521 ± 744	1.009 ± 0.003	208.2 ± 88.2	624.8 ± 183.4	43.6 ± 15.5	32.3 ± 14.2
W2	2335 ± 826	1.009 ± 0.003	202.1 ± 89.8	696.3 ± 288.1	43.4 ± 16.5	36.4 ± 14.0†
C1	2593 ± 833	1.009 ± 0.003	217.7 ± 103.2	657.7 ± 243.8	48.7 ± 15.5*	36.6 ± 14.5*
C2	2226 ± 842	1.009 ± 0.004	243.7 ± 142.7	667.4 ± 271.1	47.0 ± 17.0*	40.3 ± 16.8†

Values are means ± SD. * Coffee (C) significantly higher than water (W) on respective days. † Day 2 significantly higher than day 1.

Table 2.4: Morning Urine Void Volume and USG

Condition/Day	Urine Void Volume (mL)	Urine Void USG
W1	349.2 ± 177.0	1.019 ± 0.005
W2	361.9 ± 168.0	1.017 ± 0.006
W3	339.8 ± 143.6	1.018 ± 0.006
C1	344.6 ± 183.6	1.017 ± 0.005
C2	360.1 ± 171.8	1.018 ± 0.005
C3	338.3 ± 157.6	1.018 ± 0.005

Values are means ± SD.

Table 2.5: Haematological data collected over each Three Day Trial.

Condition /Day	Haematocrit (%)	Serum Osmolality (mOsm/kg)	TPP (g/L)	Serum Creatinine (mmol/L)	Serum Na (mmol/L)	Serum K (mmol/L)	Blood Urea Nitrogen (mmol/L)
W1	43.9 ± 2.0	284 ± 23	73.9 ± 8.1	98.9 ± 11.7	141 ± 4	4.1 ± 0.3	4.6 ± 0.9
W2	44.0 ± 2.1	287 ± 11	73.7 ±6.8	95.9 ± 12.4	141 ± 3	4.2 ± 0.2	4.8 ± 1.0
W3	44.0 ± 2.1	285 ± 3	74.2 ± 9.2	95.2 ± 11.2	141 ± 4	4.2 ± 0.2	4.7 ± 0.8
C1	43.9 ± 2.3	286 ± 7	73.9 ± 11.0	97.7 ± 13.9	141 ± 3	4.2 ± 0.3	4.7 ± 1.2
C2	44.6 ± 2.4	288 ± 8	75.8 ± 9.4	97.5 ± 11.6	141 ± 3	4.1 ± 0.2	4.8 ± 1.0
C3	43.9 ± 2.2	286 ± 3	74.7 ± 9.4	97.1 ± 11.7	141 ± 3	4.2 ± 0.2	4.9 ± 1.3

Values are means ± SD.

2.6 Discussion

Individual daily fluid requirements, intakes and beverage preferences vary extensively within and across populations. Despite the lack of a consensus for how much fluid an individual should consume and the effects of various beverages on fluid balance, there are widespread guidelines for optimal hydration that are considered common knowledge (13). Healthy adults are often advised to avoid caffeinated beverages due to the potential negative impact they may have on hydration status (7). These opinions and recommendations are based upon a relatively small collection of caffeine studies that have been publicised in both scientific and lay literature and in the media over the past few decades (10,17,25). Interestingly however, there are a lack of data that has specifically investigated moderate doses of coffee in free living healthy adults. Thus, the question of whether moderate coffee consumption can contribute to daily fluid requirement or causes dehydration remains unanswered.

To our knowledge, this is the first study to directly compare the chronic effects of coffee ingestion with water using a wide range of hydration assessment techniques. We hypothesised that when ingested in moderation, coffee would contribute to daily fluid requirement and would not result in progressive dehydration over the course of 72 h. Our data shows no significant differences in the hydrating properties of coffee or water across a wide range of hydration assessment indices. No significant differences were observed between conditions in any of the haematological markers. No differences in blood urea nitrogen or serum creatinine suggest renal function was normal throughout both trials. Analysis of urinary data showed no significant differences between conditions in 24 h urine volume, urine void volume, USG or urine osmolality. Small daily fluctuations in TBW were observed during both trials; however this did not reach significance in either condition. A very recent study investigated the effects of caffeine provided in capsules (5 mg/kg/day) on the TBW of 30 male participants classified as 'low-caffeine users' (<100 mg/day) (19). No differences in TBW were observed between the caffeine and placebo control group. Our data confirms the author's conclusions that a moderate consumption of caffeine does not disrupt TBW in caffeine habituated males.

Urinary sodium was significantly higher in the coffee trial than the water trial on both days. The increased sodium excretion in the coffee trial supports findings of previous studies that have observed that both theophylline and caffeine enhance sodium excretion at the proximal

and distal renal tubules (26-28). The increase in sodium excretion is due to methyxanthine-induced natriuresis caused by inhibition of salt transport along the proximal convoluted tubule (29). While sodium excretion is an important determinant of urine production, it is not the only driver of urine volume. Some of the water in urine is derived from 'osmotically free' water (30), particularly when producing a relatively large volume of dilute urine as in the current study. Baseline urinary potassium was significantly elevated on day one of the coffee trial compared to day one of the water trial. The potassium content in a cup of instant coffee (200 mL and ~2 g coffee) is approximately 80 mg (30), thus participants consumed ~320 mg additional potassium during the coffee trial than during the water trial. No differences were observed between the conditions on day two, which may suggest adaptive renal handling.

Interestingly, although no changes were observed in TBW, the data showed a small fall in body mass of 190 ± 120 g/day in both conditions (0.2 % BM). Clinical dehydration is reported to be a body mass loss of between 1-3 %, therefore whilst the 0.2 % BM decrease observed in this study did reach statistical significance; participants were not near the level of clinical dehydration. These findings are similar to the results of Grandjean et al who assessed hydration status in 18 healthy adults consuming a mean caffeine intake of between 1.40-3.13 mg/kg BM (13). The authors reported a mean loss of 0.30 ± 0.39 % BM across all test conditions. Authors suggested that the loss was due to normal divergence or that their method of determining treatment volumes caused a small level of dehydration to occur. One strength of our study was that the individual fluid intake during the trials was based on three-day diet diaries instead of a fixed volume for everyone. Furthermore, if participants felt they were not allocated a sufficient volume of water at any point during the first trial or indeed if they had too much water, they were permitted to return to the laboratory to have their fluid allocation amended. The adjusted fluid intake was recorded and repeated during the second trial. The small losses in body mass observed in this study are likely to be multifactorial. As suggested by Grandjean et al (13), it is also possible that part of the mass loss observed in this study was due to natural divergence or that participants were not provided with sufficient water during the trials. The urinary data in the current study shows participants were producing relatively large amounts of dilute urine (urine osmolality < serum osmolality), which suggests dehydration is unlikely to be the cause of the fall in body mass. One other possible cause of the body mass loss could be due to unmeasured faecal losses. Participants completed a compliance booklet each day which included questions regarding faecal losses and included the validated Bristol Stool Chart. Data collected from this booklet would highlight any

unusual stool production, but as samples were not weighed it is not possible to know how much volume was excreted. No unusual faecal losses were reported by any of the participants.

This study is limited by the nature of its design. To achieve optimal results, a metabolic ward would have provided the most control of the environment and of the participants; however in an attempt to understand the effects of coffee consumption in a 'free-living' setting, some control will always be lost. Furthermore, it may have been beneficial to continue the 24 h urine collection on the third day however this was not possible due to time constraints and demands on the participants. It may have been interesting to include a decaffeinated coffee condition as this would have identified any differences specifically caused by caffeine in coffee and not any of the other bioactive components, however as we found minimal differences between coffee and water we believe that it is unlikely that we would have found any significant differences if we had included a decaffeinated coffee condition.

With acknowledgement of the study's limitations, results suggest that coffee did not result in dehydration when provided in a moderate dose of 4 mg/kg BW caffeine in four cups per day. Thus, these data suggest that coffee, when consumed in moderation by caffeine habituated males contributes to daily fluid requirement and does not pose a detrimental effect to fluid balance. The advice provided in the public health domain regarding coffee intake and hydration status should therefore be updated to reflect these findings.

2.7 Acknowledgements

2.8 References

(1) Perrier E, Vergne S, Klein A, Poupin M, Rondeau P, Le Bellego L, et al. Hydration biomarkers in free-living adults with different levels of habitual fluid consumption. Br J Nutr 2013 May;109(9):1678-1687.

(2) Bellisle F, Thornton SN, Hebel P, Tahiri M. A study of fluid intake from beverages in a sample. EJCN 2010;64:350-355.

(3) EFSA Panel on Dietetic Products, Nutrition, and Allergies. Scientific Opinion on Dietary Reference Values for water. EFSA Journal 2010;8(3):1459.

(4) Jéquier E, Constant F. Water as an essential nutrient: the physiological basis of hydrationWater as an essential nutrient. EJCN 2010;64:115-123.

(5) IoM (Institue of Medicine). Dietary Reference Intakes for Water, Potassium, Sodium, Chloride and Sulfate. Washington DC: National Academies Press; 2004.

(6) Kleiner SM. Water: an essential but overlooked nutrient. J Am Diet Assoc 1999 Feb;99(2):200-206.

(7) WaterAid. Hydration Tips. WaterAid ;2012(11/09).

(8) Bird E, T., Parker B, D., Kim H, S., Coffield S, K. Cafeine Ingestion and Lower Urinary Tract Symptoms in Healthy Volunteers. Neurourology and Urodynamics 2005;24:611-615.

(9) Wemple RD, Lamb DR, McKeever KH. Caffeine vs caffeine-free sports drinks: effects on urine production at rest and during prolonged exercise. Int J Sports Med 1997 Jan;18(1):40-46.

(10) Neuhauser-Berthold B,S., Verwied SC, Luhrmann PM. Coffee consumption and total body water homeostasis as measured by fluid balance and bioelectrical impedance analysis. Ann Nutr Metab 1997;41:29-36.

(11) Eddy N, Downs A. Tolerance and cross-tolerance in the human subject to the diuretic effect of caffeine, theobromine, and thoephylline. J Pharmacol Exper Ther 1928;33:167-174.

(12) Fisher SM, McMurray RG, Berry M, Mar MH, Forsythe WA. Influence of caffeine on exercise performance in habitual caffeine users. Int J Sports Med 1986 Oct;7(5):276-280.

(13) Grandjean A, Reimers K, Bannick K, Haven M. The effect of caffeinated, non-caffeinated, caloric and non-caloric beverages on hydration. Journal of the American College of Nutrition 2000;19(5):591-600.

(14) Armstrong L, Pumerantz A, Roti M, Judelson D, Watson G, Dias J, et al. Fluid, electrolyte, and renal indices of hydration during 11 days of controlled caffeine consumption. International Journal of Sport Nutrition and Exercise Metabolism 2005;15:252-265.

(15) Dorfman LJ, Jarvik ME. Comparative stimulant and diuretic actions of caffeine and theobromine in man. Clinical pharmacology and therapeutics 1970;11(6):869-872.

(16) Ruxton CH, Hart VA. Black tea is not significantly different from water in the maintenance of normal hydration in human subjects: results from a randomised controlled trial. Br J Nutr 2011 Aug;106(4):588-595.

(17) Passmore AP, Kondowe GB, Johnston GD. Renal and cardiovascular effects of caffeine: a dose-response study. Clin Sci (Lond) 1987;72(6):749-756.

(18) Graham TE, Hibbert E, Sathasivam P. Metabolic and exercise endurance effects of coffee and caffeine ingestion. J Appl Physiol 1998 Sep;85(3):883-889.

(19) Silva A, Judice P, Matias C, Santos D, Magalhaes J, St-onge M, et al. Total body water and its compartments are not affected by ingesting a moderate dose of caffeine in healthy young adult males. Appl Physiol Nutr Metab 2013;38(6):626-632.

(20) The Fairtrade Foundation. Fairtrade and Coffee Report. 2012.

(21) Lewis SJ, Heaton KW. Stool form scale as a useful guide to intestinal transit time. Scand J Gastroenterol 1997 Sep;32(9):920-924.

(22) Hodgson AB, Randell RK, Jeukendrup AE. The metabolic and performance effects of caffeine compared to coffee during endurance exercise. PLoS One 2013;8(4):e59561.

(23) Currell K, Urch J, Cerri E, Jentjens RL, Blannin AK, Jeukendrup AE. Plasma deuterium oxide accumulation following ingestion of different carbohydrate beverages. Appl Physiol Nutr Metab 2008 Dec;33(6):1067-1072.

(24) Metabolic Solutions Inc. Measurement of Total Body Water, Extracellular and Intracellular Water 2009.

(25) Robertson D, Wade D, Workman R, Woosley RL, Oates JA. Tolerance to the humoral and hemodynamic effects of caffeine in man. J Clin Invest 1981 Apr;67(4):1111-1117.

(26) Dodd SL, Herb RA, Powers SK. Caffeine and exercise performance. An update. Sports Med 1993 Jan;15(1):14-23.

(27) Fredholm BB. Cardiovascular and renal actions of methylxanthines. Prog Clin Biol Res 1984;158:303-330.

(28) Osswald H, Schnermann J. Methylxanthines and the kidney. Handb Exp Pharmacol 2011;(200):391-412. doi(200):391-412.

(29) Lote CJ. Principles of Renal Physiology. New York: Springer; 2012.

(30) Debry G. Coffee and Health. Montrouge (France): John Libbey Eurotext; 1994.

(31) Nussberger J, Mooser V, Maridor G, Juillerat L, Waeber B, Brunner HR. Caffeine-induced diuresis and atrial natriuretic peptides. J Cardiovasc Pharmacol 1990 May;15(5):685-691.

(32) Riesenhuber A, Boehm M, Posch M, Aufricht C. Diuretic potential of energy drinks. Amino Acids 2006 Jul;31(1):81-83.

(33) Passmore AP, Kondowe GB, Johnston GD. Renal and cardiovascular effects of caffeine: a doseresponse study. Clin Sci (Lond) 1987;72:749-756.

CHAPTER 3: THE INFLUENCE OF HYDRATION STATUS DURING PROLONGED ENDURANCE EXERCISE ON SALIVARY ANTIMICROBIAL PROTEINS

Sophie C. Killer, Ida S Svendsen and Michael Gleeson

School of Sport, Exercise and Health Sciences, Loughborough University, Leicestershire, East Midlands. UK.

Conceived and designed the experiments: SCK, MG
Performed the experiments: SCK, ISS
Analyzed the data: SCK
Contributed reagents/materials/analysis tools: SCK, MG
Wrote the paper: SCK.
Statistical analysis: SCK
Editing of final paper: SCK MG

3.1 Abstract

Several antimicrobial proteins (AMPs) in saliva including secretory immunoglobulin A (SIgA), lysozyme (SLys) and lactoferrin (SLac) are important in host defence against oral and respiratory infections. The aim of this study was to investigate the effects of hydration status on saliva AMP responses to prolonged endurance exercise. Using a randomized design, 10 healthy male participants (age 23 ± 4 y, body mass 73.8 ± 7.4 kg, $\dot{V}O_{2max}$ 56.8 ± 6.5 ml/kg/min) completed 2 h cycling at 60% $\dot{V}O_{2max}$ in states of euhydration (EH) or moderate dehydration (DH) induced by 24 h fluid restriction. Timed unstimulated saliva samples were collected before, during and immediately post-exercise and each hour for 3 h recovery. Baseline body mass was determined over 3 consecutive days prior to the first trial. Fluid restriction resulted in a 1.5 ± 0.5% loss of body mass from baseline and a 4.3 ± 0.7% loss immediately post-exercise. Pre-exercise urine osmolality was significantly elevated in DH compared to EH (958 ± 134 vs 721 ± 237 mOsm/kg; p=0.03). Heart rate and perceived exertion were significantly higher throughout exercise in DH compared to EH. Saliva flow rate was significantly reduced in DH compared to EH. Baseline SIgA secretion rates were not different between conditions; however exercise induced a significant increase in SIgA concentration in DH (161 ± 134 to 309 ± 271 mg/L; p<0.05) but not EH and remained significantly elevated throughout 3 h recovery. There was a significant increase in SLac secretion rate from pre- to post-exercise in both conditions (1.2 ± 1.2 to 3.2 ± 3.0 µg/mL and 1.7 ± 1.7 to 4.2 ± 1.8 µg/mL for DH and EH, respectively) which remained elevated in DH but returned to baseline by 1 h post-exercise in EH. Overall, SLac concentrations were significantly higher in DH than EH (p=0.02). Pre-exercise SLys concentration was significantly lower in DH compared to EH (1.2 ± 1.6 vs 5.5 ± 6.7 ng/mL; p<0.05). Exercise elicited a significant increase in SLys secretion rate in both conditions. Post-exercise SLys concentrations remained significantly elevated from baseline in DH whereas concentrations in EH returned to baseline by 1 h post-exercise. Exercise in DH caused a reduction in saliva flow rate yet induced greater secretion rates of SLac and higher concentrations of SIgA and SLys. Thus, DH does not impair saliva AMP responses to endurance exercise.

Key Words: Hydration Status, Mucosal Immunity, Endurance Exercise, Salivary Antimicrobial Proteins

3.2 Introduction

Mucosal secretions play an important role in immunity as the first line of defence against potential pathogens invading the oral cavity and upper respiratory tract (1). Salivary secretory IgA (SIgA) has been the most commonly studied marker of mucosal immunity and its deficiency has been correlated to a high incidence of infections (2-5). More recently the importance of other antimicrobial proteins (AMPs) in saliva has gained greater recognition. There are a diverse range of AMPs in saliva, many of which act to form a protective barrier against bacteria and pathogens. Two of the most abundant AMPs present in the mucosal secretions of the upper respiratory tract are salivary lysozyme (SLys) and salivary lactoferrin (SLac). The presence of AMPs such as SLys and SLac in salivary secretions without prior exposure to infectious agents is indicative of their integral role in the innate immune system (6).

Immune resilience is an important factor in the success of an elite athlete (7). Prolonged and intense periods of exercise can cause transient perturbations in many cellular and hormonal immune factors (8). A small collection of studies have investigated the effects of endurance exercise on salivary AMPs. Koutedakis et al (9) was the first to report significant reductions in salivary flow rate (FR) and SLys concentrations following exercise in elite swimmers. Other studies have reported equivocal results; short-duration, high intensity exercise lead to increased secretion rates of AMPs such as SIgA and SLys in active men (10) and prolonged endurance exercise either decreased SIgA with no change in SLys secretion rates (11,12) or resulted in no change in SIgA concentrations (13-15). The variation in the findings of these studies may be due to the differences in exercise intensity, duration and relative stress on the participant. Furthermore, there are numerous possible mechanisms by which exercise could affect the concentration and secretion of AMPs both acutely and chronically including; increased secretion of neuropeptides, secretion of AMPs induced by proinflammatory cytokines, secretion of AMPs from neutrophils or damaged epithelial cells or simply hyperventilation during exercise resulting in drying of the respiratory tract (for detailed review see 16). In addition, circulating stress hormones such as cortisol have been implicated in the inhibition of salivary IgA (17) and Lys (18) production, mobilization and secretion.

Saliva secretion is regulated by the autonomic nervous system and humans typically produce ~1500 mL saliva per day (8). However, fluid balance studies have observed significant reductions in salivary FR when in a state of dehydration (11,19). Fortes and colleagues reported significant reductions in salivary FR and secretion of AMPs at a modest dehydration of -3% body mass loss (BM) induced by fluid restriction (19). Dehydration of 1.3-2.4% BM resulting from prolonged endurance exercise has also been shown to reduce salivary FR (11). In addition to a reduction in salivary FR the authors reported a significant decrease in SIgA secretion rates but no change in SLys. During exercise, the concentration of the saliva, and its FR, are thought to be influenced by sympathetic nervous activity and the hypothalamic-pituitary-axis (HPA axis) (10). It has been proposed that increases in protein content are influenced by noradrenalin release via sympathetic nervous stimulation, whereas FR is considered to respond primarily to parasympathetic stimulation (20).

As a result of well-established guidelines, athletes are regularly advised to drink sufficient fluids before, during and after exercise to limit dehydration to <2% loss in BM (21,22). However studies suggest that commencing exercise in a mild state of dehydration is not uncommon across athletic populations (23-25). Furthermore, it has been shown that many athletes fail to consume sufficient fluids during exercise to offset fluid loses, resulting in levels of dehydration in excess of 2% BM loss (26,27). Exercising in a state of dehydration has been shown to increase cardiovascular strain (increased heart rate (HR)) (28) and to significantly increase plasma cortisol levels (29) compared to euhydration. Thus the potential for a disturbed immune response when exercising in a state of dehydration is significantly augmented.

It is apparent that prolonged exercise can effect secretion of salivary AMPs however it is not known how the hydration status of an athlete may affect these changes. The aims of this study were to investigate the effects of dehydration during prolonged endurance cycling on exercise performance and salivary markers of immune function.

3.3 Methods

Participants

Ten recreational male cyclists were recruited to participate in this study. Participant characteristics are presented in Table 3.1. Inclusion criteria required participants to be weight stable, involved in regular exercise, complete a general health questionnaire without reporting any chronic or underlying illness or disease, free from medications that may disrupt fluid-electrolyte balance and to have not suffered any symptoms of an upper respiratory tract infection during the four weeks prior to the study. Females were not included in the study due to potential disruptions in fluid balance resulting from the menstrual cycle. All participants were informed of the purposes of the study and the risks associated with the procedures. Written informed consent was obtained from each participant and a health questionnaire was completed before the study commenced. The study was approved by the Loughborough University ethical advisory committee.

Table 3.1: Participant Characteristics

	Age (y)	**Height (cm)**	**Body Mass (kg)**	$\dot{V}O_{2max}$ **ml/kg/min**
Mean ± SD	21 ± 1 y	178.0 ± 7.9 cm	72.4 ± 8.0	58.2 ± 6.2

Experimental Design

Participants underwent two experimental trials in a randomised, counterbalanced-cross over design, consisting of 2 h cycling on an electronically braked ergometer at 60% $\dot{V}O_{2max}$ in either a euhydrated (EH) or dehydrated state (DH). Saliva samples were collected pre-exercise, throughout the exercise session and each hour for 3 h post-exercise. Urine samples were collected pre- and immediately post-exercise and again at 3 h post-exercise. Trials were separated by a minimum of 6 days.

Pre-trial Tests & Familiarisation

Prior to the exercise trials, participants completed an incremental cycle exercise test to volitional exhaustion on an electronically braked cycle ergometer (Lode Excalibur Sport, Groningen, Netherlands) in order to determine $\dot{V}O_{2max}$. Briefly, following a 10 min warm up

at 70 W, participants began the exercise test at 95 W, undergoing 35 W increments every 3 min. Breath samples were collected from participants into Douglas bags during the final minute of each stage and a rating of perceived exertion (RPE) was recorded (40). Heart rate (HR) was measured throughout the test using short-range telemetry (Polar RS800CX, Kempele, Finland). A paramagnetic oxygen analyser (Servomex 1420B, Crowborough, UK) and infrared carbon dioxide analyser (Servomex 1415B) were used in combination with a dry gas meter (Harvard Apparatus, Edenbridge, UK) for determination of $\dot{V}_E$, $\dot{V}O_2$ and $\dot{V}CO_2$. The work rate in Watts corresponding to 60% $\dot{V}O_{2max}$ was then calculated from the $\dot{V}O_2$–work rate relationship using a linear equation. Following a 15 min rest period, during which time participants were familiarised with the saliva collection protocols, participants then completed a 20 min familiarisation at 60% $\dot{V}O_{2max}$ representing the workload of the subsequent trials. Expired gas samples were collected during each trial at 5, 15 and 20 min to ensure the calculated work rate elicited the desired relative intensity. In addition to the pre-trial $\dot{V}O_{2max}$ test, participants were required to visit the laboratory on the final three mornings prior to their first trial to establish fasted baseline body mass. On each occasion nude body mass was recorded and the average of these data was used to estimate baseline BM at euhydration.

Exercise Trials

Participants reported to the laboratory between 07:00-09:00 for each exercise trial having completed an overnight fast ($\geq$10 h). Each participant performed both trials at the same time of day to reduce inter-trial effects of diurnal variations in cortisol (30,31). Participants were requested to complete a 24 h weighed food diary prior to their first trial and to avoid consumption of foods with a high fluid content, such as yoghurts, soups and fruit. Participants were then instructed to replicate their nutritional intake 24 h prior to their second trial. Participants were also requested to abstain from any physical activity and alcohol consumption for 48 h prior to each trial to help prevent disturbances in fluid balance. Prior to commencing exercise trials, participants provided a urine void, nude BM measurement and saliva sample for analysis. Participants cycled for 120 min at 60% $\dot{V}O_{2max}$ on a stationary cycle ergometer (Lode Excalibur Sport, Groningen, Netherlands) in a laboratory environment maintained at 21.0 ± 1.0°C. Saliva samples, expired gas and RPE were collected at 15, 45, 75 and 105 min of exercise. Heart rate was recorded continually during each exercise trial with

short range telemetry (Polar RS800CX, Kempele, Finland). Post-exercise saliva and urine samples were collected following exercise cessation and BM was measured before participants were provided with 500 mL water. Participants remained in the laboratory for 180 min, sitting quietly, to enable hourly saliva sampling and a final urine sample to be collected.

Treatment

To induce DH, participant's fluid intakes were restricted to 500 mL water during the 24 h period pre- trial. Furthermore, no water was provided prior to or during the 2 hour cycling protocol. On completion of the exercise trial, following saliva and urine sampling, participants were provided with 500 mL water. During EH, participants were encouraged to consume their normal fluid intakes during the 24 h lead into the trial. In addition, participants were provided with 500 mL water before commencing the exercise trial and given an additional 250 mL water every 20 min during exercise. The exercise protocol was identical for both trials.

3.4 Measures and Analysis

Unstimulated saliva samples were collected from all participants at each time point. Participants were instructed to be in the seated position, leaning forward with their head tilted forward. Participants were asked to swallow to empty their mouth of any residual saliva before the timed sample collection began. Saliva collections lasted 3 min, during which time participants were requested to minimise orofacial movement and passively dribble into a pre-weighed vial. Samples were weighed to estimate saliva volume and micro-centrifuged to remove cells and insoluble matter before storing at -20°C. The saliva flow rate (ml/min) was determined by dividing the volume of saliva by the collection time. Subsequently, saliva samples were analysed for secretory immunoglobulin A (SIgA) using an ELISA kit (Salimetrics, Philadelphia, USA) and both lactoferrin and lysozyme were analysed using commercially available ELISA kits (Calbiochem, USA and Biomedical Technologies, USA, respectively). Secretion rates for each of the salivary AMPs were calculated as the multiple of the saliva FR and the antimicrobial protein concentration. All saliva assays were carried out

in duplicate. The intra-assay CV for SIgA, lactoferrin and lysozyme were 1.8%, 8.1% and 5.3%, respectively. Urine osmolality was analysed via freezing-point depression using a single sample osmometer (Osomamat 030, Gonotec, Berlin, Germany). Venous blood samples were collected immediately prior to exercise, immediately post-exercise (<3 min) and at 2 h post-exercise. After clotting, whole blood was centrifuged at 3500 rpm for 10 min at 4°C and serum was immediately stored at -20°C before later analysis of cortisol with an enzyme-linked immunosorbent assay (DRG Instruments, Cortisol ELISA EIA − 1887; intra assay CV: ±5.6%).

Statistical Analysis

All data were analysed using statistical software (IBM SPSS Statistics 21) and are presented as Mean ± Standard Deviation. Changes in hydration and saliva markers and plasma cortisol were analysed using a 2-way repeated measures analysis of variance (ANOVA). A Bonferroni adjustment was included into the analysis to correct for multiple comparisons. Data sets that were found to be significantly non-normal (Lys and SLac) were log transformed prior to analysis. Between trial differences in performance parameters (mean HR, $\dot{V}O_2$ and RPE) were determined using an independent samples t-test. The level of significance was set at $p<0.05$.

3.5 Results

Hydration Variables

Table 3.2 provides an overview of the hydration variables during each trial. Twenty-four hour fluid restriction resulted in a 1.5 ± 0.5% loss of body mass from baseline and a 4.3 ± 0.7% loss immediately post-exercise. No differences in BM were reported between pre-exercise body mass and baseline BM (72.5 kg ± 8.0 vs. 72.4 kg ± 8.0, respectively) in EH. Urine osmolality was significantly higher at pre-and post-exercise in DH compared to EH (p<0.05). Haematocrit was non-significantly higher at baseline in DH compared to EH (46.9 ± 3.4 vs 46.0 ± 3.4%, for DH and EH, respectively). Haematocrit was significantly elevated by exercise in both conditions (46.5 ± 0.9 to 48.8 ± 1.0%; p<0.01) and remained elevated at 2 h

post-exercise (47.7 ± 1.0%; p<0.01; figure 3.1). No difference in baseline salivary FR was observed between conditions. However, immediately post-exercise and for the remainder of the trial, FR was significantly higher in EH than DH (p<0.05; figure 3.3).

Performance Data

Average power output during trials was 175 ± 22 W, resulting in an exercise $\dot{V}O_2$ of 60 ± 4% $\dot{V}O_{2max}$ with no difference between trials. Mean HR and RPE were significantly higher throughout exercise in DH compared to EH (157 ± 13 vs. 151 ± 11 bpm; p<0.01, and 14.1 ± 2.2 vs. 13.0 ± 1.8 RPE; p<0.05).

Haematology

Figure 3.1 presents plasma cortisol concentrations pre-exercise, immediately post- and 2 h post-exercise. Exercise elicited a significant rise in plasma cortisol levels in both conditions (632 ± 26 to 771 ± 33 nmol/L; p=0.01). At 2 h post-exercise, cortisol concentrations had fallen and were significantly lower than pre-exercise levels (p=0.03).

Salivary Analysis

Salivary AMP concentrations are presented in table 3.3. There was a significant increase in SLac concentrations with exercise (p<0.01) which returned to baseline by 1 h post-exercise in EH, but remained elevated in DH for the entirety of the post-exercise recovery period. Overall, SLac concentrations were higher in DH than EH (p=0.02). Lys concentrations were significantly lower at baseline (pre-exercise) in DH compared to EH (p<0.05). Exercise resulted in an increase in Lys concentration (p<0.01), which remained elevated above baseline during DH, but returned to baseline in EH by 1 h post-exercise. Baseline SIgA concentrations were not statistically different between conditions. Following exercise SIgA concentrations were higher in DH than EH; overall SIgA concentrations were significantly high in DH than EH (p<0.01).

No differences were observed between pre-exercise salivary FR, however FR was significantly lower during DH than EH at all time points during and post-exercise (p<0.05; figure 3.3). SLac secretion rate increased significantly following exercise (p=0.01; figure 3.4A). No differences between EH and DH were observed. Exercise elicited a significant

increase in SLys secretion rate in both conditions; with a tendency to be higher in EH than DH (p=0.06; figure 3.4B). SLys secretion rate fell significantly after exercise in EH (p=0.02) but remained elevated in DH at the end of the trial. SIgA secretion rates were not acutely effected by exercise, however at 3 h post-exercise SIgA secretion rates were significantly higher in DH than EH (p<0.01; figure 3.4C).

Table 3.2: Overview of Hydration Variables

	Body Mass (kg)		**Urine Osmolality (mOsm/kg)**		**Haematocrit (%)**	
	EH	DH	EH	DH	EH	DH
Pre-Exercise	72.5 ± 8.0	71.3 ± 8.0	721 ± 237	958 ± 134*	46.0 ± 3.4	46.9 ± 2.4
Post- Exercise	71.6 ±7.7	69.4 ± 7.8	584 ± 252	918 ± 137*	48.0 ±3.7[‡]	49.7 ± 3.4[‡]

Data are means ± SD. * Indicates significant condition effect. [‡] Indicates significant change from baseline (pre-exercise).

Figure 3.1: Haematocrit Concentrations
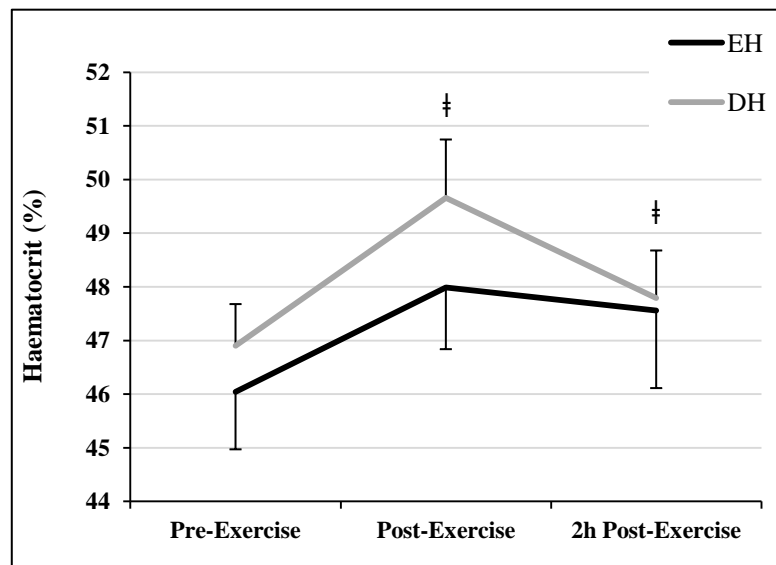
Figure 3.2: Plasma Cortisol Concentrations
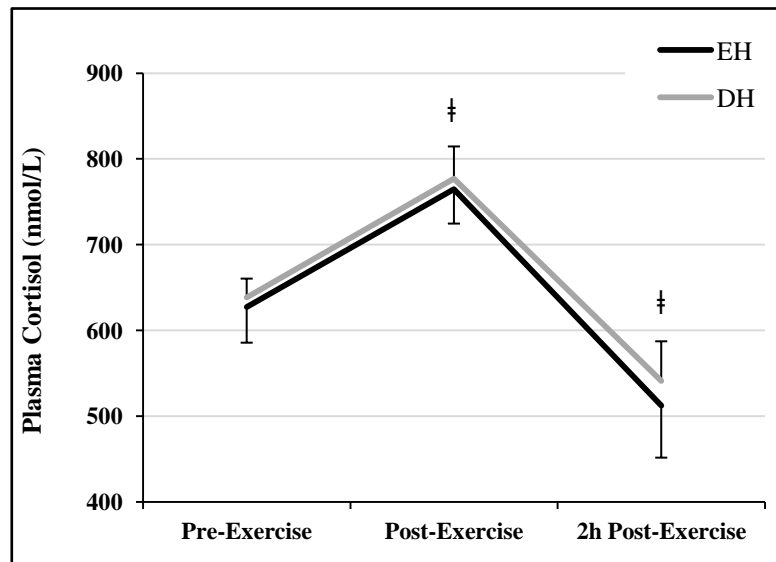
Figures 3.1-3.2: Haematocrit and Plasma cortisol concentrations at pre-exercise, immediately post-exercise and at 2 h post exercise. Data are means ± SE. [‡] Indicates significant change from baseline. No differences between conditions were observed.

Table 3.3: Salivary AMP Concentrations

Mean ± SD	SLac (mg/L)		SLys (mg/L)		SIgA (mg/L)	
	EH	DH	EH	DH	EH	DH
Pre-Exercise	5.0 ± 5.5	3.5 ± 2.8	5.5 ± 6.7	1.6 ± 2.0*	104.9 ± 84.4	160.6 ± 134.3
Post-Exercise	11.9 ± 7.0‡	15.0 ± 12.7‡	11.1 ± 4.8‡	9.5 ± 9.1‡	136.9 ± 98.3	309.3 ± 271.2*
1 h Post	4.8 ± 3.1	13.6 ± 9.3*‡	2.4 ± 1.9	9.1 ± 5.3*	73.9 ± 32.5	241.6 ± 214.7*
2 h Post	7.9 ± 8.6	18.7 ± 21.0‡	2.1 ± 2.8	9.5 ± 7.4*	87.9 ± 56.2	239.9 ± 140.7*
3 h Post	5.4 ± 4.5	15.3 ± 12.3*‡	3.1 ± 3.5	8.9 ± 6.7	74.6 ± 41.6	246.6 ± 146.6*

Data are means ± SD. * Indicates significant condition effect. ‡ Indicates significant change from baseline (pre-exercise)

Figures: 3.3 and 3.4A-C
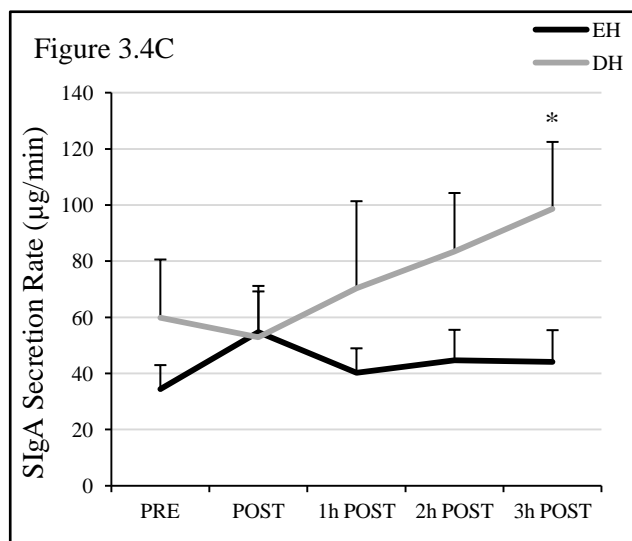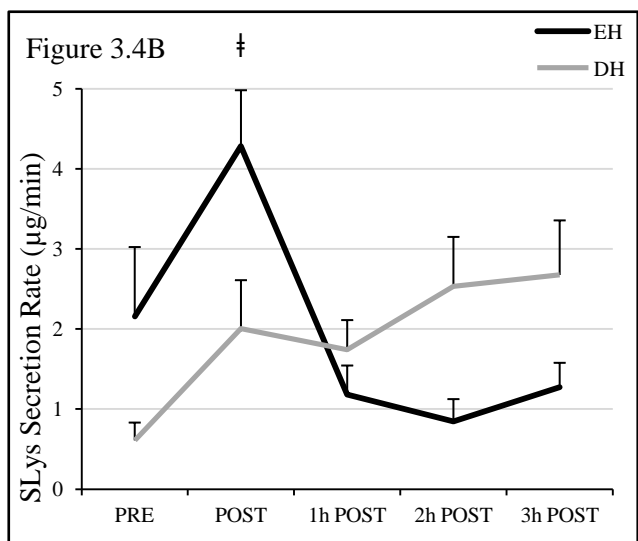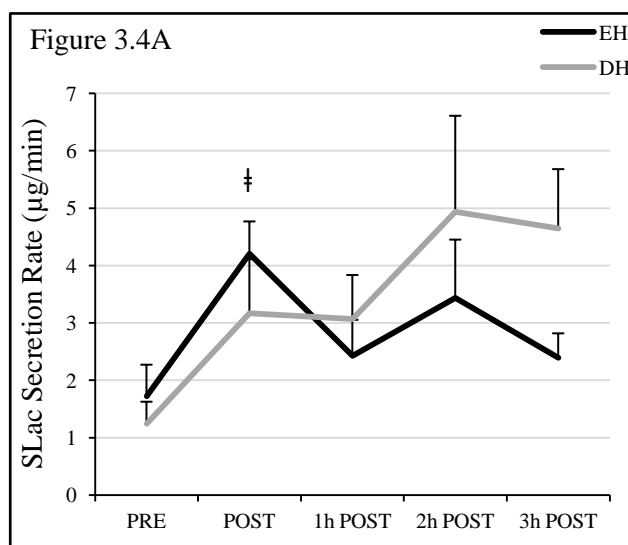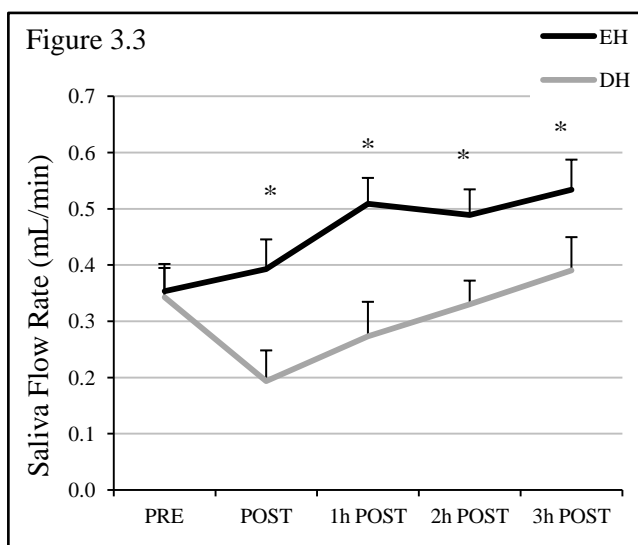
Figures 3.3 and 3.4A-C: Salivary flow rate and AMP secretion rates pre- and post-exercise for Euhydrated (EH) and Dehydrated (DH) trials. Data are means ± SE.* Indicates significant difference between conditions. ‡ Indicates significant change from baseline.

3.6 Discussion

The aim of the present study were to investigate the effects of dehydration during prolonged endurance cycling on exercise performance and salivary markers of immune function. With the knowledge that many athletes commence exercise with pre-existing fluid imbalances (32), we were interested in the effects of prior dehydration (induced by fluid restriction) on exercise performance and mucosal immunity during exercise and short term recovery. The main findings of this study were that exercise in a mildly dehydrated state caused a reduction in salivary FR and resulted in transient changes in salivary AMPs during and immediately post-exercise that had mostly returned to baseline by 3 h of recovery.

A small collection of studies have reported the effects of a single bout of exercise on AMPs involved in host defence. Allgrove and colleagues (10) investigated salivary AMPs in acute exercise (22 min) at 50% and 70% $\dot{V}O_{2max}$ or during an incremental test to exhaustion. The incremental test to exhaustion resulted in temporal increases in the secretion rates of SIgA and SLac, with exercise at 70% $\dot{V}O_{2max}$ only resulting in a post-exercise increase in SLys secretion rate and no effects at 50% $\dot{V}O_{2max}$. The authors concluded that sympathetic stimulation during high intensity exercise was sufficient to increase SIgA and SLys transport, despite the short-duration of the exercise period. No studies have investigated the effects of endurance exercise (>90 min) on AMPs, with the exception of two field studies which analysed SIgA and SLys (and SLac (12)) during ultra-marathon racing and reported equivocal results. The authors of the multi-stage ultra-marathon observed exercise-induced body mass losses over the duration of the race and postulate that hydration status may play a role in protecting the upper respiratory trace when exercising (11).

Current hydration guidelines advise athletes to limit body mass losses during exercise to no greater than 2% in order to prevent deleterious effects of dehydration on exercise performance and health (21,22). Dehydration has been shown to increase cardiovascular strain, core temperature, perceived exertion response (33) and a mean change in HR of 3 bpm for every 1% change in BM loss has recently been reported (28), ultimately inhibiting exercise performance. (34). In line with these findings, we observed significantly higher mean HR and RPE at the same work rate in DH compared with EH. Twenty-four hour fluid restriction in this study resulted in a mean body mass loss of 1.5%, (0.3-1.9 kg loss). Following exercise in DH, mean body mass loss was 3.9% (2.1-4.2 kg loss). Baseline urine

osmolality was 972 mOsmol/kg (777-1113 mOsmol/kg) in DH compared to 687 mOsmol/kg (353-1092 mOsmol/kg) in EH and baseline haematocrit was 47% (43-50%) and 46% (43-50%) for DH and EH, respectively. These data highlight the large individual variability in sweat rates and fluid homeostasis between individuals undergoing the same level of fluid restriction.

Given the known effects of fluid intake (35) and sweat rates (36,37) on salivary FR, the potential for exercising in a dehydrated state to interfere with the salivary AMP activity is high. Despite this, the effects of dehydration on salivary AMPs during exercise are relatively understudied. To the author's knowledge, only one study has investigated the effects of dehydration and exercise on salivary AMPs. Fortes and colleagues investigated the effects of dehydration caused by exercise in the heat, with subsequent overnight fluid restriction on markers of immune function (19). The authors reported a significant reduction in salivary FR immediately following exercise in the dehydration trial, which remained suppressed until rehydration was permitted the following morning. In addition, they observed an increase in SIgA concentration, with no change in secretion rate and a decrease in SLys secretion rates with no change in concentration. Other studies that have investigated the effects of exercise on SLys, independent of hydration status, have reported decreased SLys concentrations (9), increased secretion rates (10) and no change in secretion rates (11,12) following a range of exercise sessions. We did not observe any differences in salivary FR at baseline between EH and DH, despite 24 h fluid restriction. However, during exercise, salivary FR decreased significantly in DH, with no change in EH, and remained significantly lower than baseline until 2 h post-exercise. SIgA secretion rates were not acutely affected by exercise; however by 3 h post-exercise SIgA secretion rates were significantly higher in DH than EH. We also observed significantly higher concentrations of SIgA and SLac in DH than EH throughout the trial, likely the result of a concentrating effect from the reduced salivary FR. SLac secretion rates increased with exercise, with no differences between conditions. Participants in both trials presented a significant increase in SLys concentrations which returned to baseline within 1 h post-exercise in EH but remained elevated for the duration of the trial in DH. Furthermore, we observed a transient increase in SLys secretion rate despite no change in salivary FR during exercise in EH. Despite a reduction in salivary FR in DH, we observed an increase in SLys secretion rates that remained elevated for the duration of the trial. Due to the difference in salivary FR between conditions, it is important to consider secretion rates when looking at AMP immune responses, and not absolute concentrations. Increases in SIgA

secretion rates in DH (above that reported in EH) and elevated SLys secretion rates above baseline at 3 h post exercise in DH suggest that DH exacerbated the immune response to endurance exercise. Whilst we did not observe any significant differences in cortisol concentrations between conditions, it may be possible that a heightened stress response in DH (evidenced by increased HR and RPE during exercise) resulted in a great immune response. Furthermore, it has been suggested that high-intensity exercise may increase the mobilization of SIgA into the saliva via sympathetic nervous activity (10), therefore it could be speculated that the increased exercise strain in DH effectively increased the relative intensity of the exercise and caused a greater mobilization of SIgA.

Exercising in a state of dehydration (5% and 7% BM loss) has previously been shown to increase resting and post-exercise cortisol concentrations compared to euhydration (29,38). However, moderate dehydration in this study did not influence resting cortisol, or differentially effect post-exercise response compared to euhydration in this study. These data support the findings of Mitchell et al 2002, who reported no effect of hydration status (~1 % BM loss versus EH) on cortisol concentrations when exercising in an ambient environment (39). It is interesting that we observed higher RPE and HR in DH compared to EU without seeing differences in cortisol. This may be explained by relatively high baseline concentrations due to the diurnal pattern of cortisol and the fact the testing was carried out early in the morning which may have somewhat masked an influence of hydration. Alternatively, the level of dehydration may not have been sufficiently severe to trigger a stress response. Elevated levels of cortisol are indicative of stress on the body; with stressors (physiological or psychological) leading to activation of the hypothalamic-pituitary-adrenal axis resulting in secretion of adrenocorticotrophic hormone (ACTH) from the anterior pituitary gland and subsequent release of the adrenal glucocorticoid, cortisol. Glucocorticoids are potent anti-inflammatory and immunosuppressant agents, thus it is feasible to postulate that exercise-related changes in cortisol concentrations, with or without the impact of dehydration, would impact salivary AMP activity. Subsequently, stress hormones such as cortisol have been reported to inhibit the production, mobilization and secretion of SIgA (17) and SLys (18). However, in a field study whereby a low level of dehydration occurred in participants, no correlation was observed between cortisol concentrations and mucosal immunity (SIgA, SLac and SLys) (12). Similarly, we did not observe any differences in cortisol concentrations between conditions and the change in cortisol from pre to post-exercise was not correlated with pre to post changes in AMPs.

In conclusion, we observed transient changes in salivary AMPs during and immediately post-exercise that had mostly returned to baseline by 3 h of recovery. The responses may have been greater if participants were exercising in the heat and/ or if the level of dehydration was greater, however in the view of the author, exercising in ambient temperature at low levels of dehydration is a common occurrence in athletic populations and therefore requires further investigation. It would appear that the moderate levels of dehydration achieved in this study do not impair salivary AMP responses to endurance exercise; however more research is required to fully understand the implications of a range of fluid deficits on innate mucosal immunity.

3.7 Acknowledgments

3.7 References

(1) Gleeson M, Pyne DB. Special feature for the Olympics: effects of exercise on the immune system: exercise effects on mucosal immunity. Immunol Cell Biol 2000 Oct;78(5):536-544.

(2) Fahlman MM, Engels HJ. Mucosal IgA and URTI in American college football players: a year longitudinal study. Med Sci Sports Exerc 2005 Mar;37(3):374-380.

(3) Neville V, Gleeson M, Folland JP. Salivary IgA as a risk factor for upper respiratory infections in elite professional athletes. Med Sci Sports Exerc 2008 Jul;40(7):1228-1236.

(4) Gleeson M. Mucosal immunity and respiratory illness in elite athletes. Int J Sports Med 2000;21(Suppl 1):S33-S43.

(5) Gleeson M, Bishop NC. URI in athletes: are mucosal immunity and cytokine responses key risk factors? Exerc Sport Sci Rev 2013 Jul;41(3):148-153.

(6) West NP, Pyne DB, Renshaw G, Cripps AW. Antimicrobial peptides and proteins, exercise and innate mucosal immunity. FEMS Immunol Med Microbiol 2006 Dec;48(3):293-304.

(7) Malm C. Exercise immunology: the current state of man and mouse. Sports Med 2004;34(9):555-566.

(8) Gleeson M, Bishop N, Walsh NP. Exercise Immunology. London: Routledge; 2013.

(9) Koutedakis Y, Sabin E, Perera S. Modulation of salivary lysozyme by training in elite male swimmers. Journal of Sports Sciences 1996;14(90).

(10) Allgrove JE, Gomes E, Hough J, Gleeson M. Effects of exercise intensity on salivary antimicrobial proteins and markers of stress in active men. J Sports Sci 2008 Apr;26(6):653-661.

(11) Gill SK, Teixeira AM, Rama L, Rosado F, Hankey J, Scheer V, et al. Salivary antimicrobial protein responses during multistage ultramarathon competition conducted in hot environmental conditions. Appl Physiol Nutr Metab 2013 Sep;38(9):977-987.

(12) Gillum TL, Kuennen M, Gourley C, Schneider S, Dokladny K, Moseley P. Salivary antimicrobial protein response to prolonged running. Biol Sport 2013 Mar;30(1):3-8.

(13) Davison G, Allgrove J, Gleeson M. Salivary antimicrobial peptides (LL-37 and alpha-defensins HNP1-3), antimicrobial and IgA responses to prolonged exercise. Eur J Appl Physiol 2009 May;106(2):277-284.

(14) Blannin AK, Robson PJ, Walsh NP, Clark AM, Glennon L, Gleeson M. The effect of exercising to exhaustion at different intensities on saliva immunoglobulin A, protein and electrolyte secretion. Int J Sports Med 1998 Nov;19(8):547-552.

(15) Li TL, Gleeson M. The effects of carbohydrate supplementation during repeated bouts of prolonged exercise on saliva flow rate and immunoglobulin A. J Sports Sci 2005 Jul;23(7):713-722.

(16) West NP, Pyne DB, Renshaw G, Cripps AW. Antimicrobial peptides and proteins, exercise and innate mucosal immunity. FEMS Immunol Med Microbiol 2006 Dec;48(3):293-304.

(17) Hucklebridge F, Clow A, Evans P. The relationship between salivary secretory immunoglobulin A and cortisol: neuroendocrine response to awakening and the diurnal cycle. Int J Psychophysiol 1998 Dec;31(1):69-76.

(18) Perera S, Uddin M, Hayes JA. Salivary lysozyme: a noninvasive marker for the study of the effects of stress of natural immunity. Int J Behav Med 1997;4(2):170-178.

(19) Fortes MB, Diment BC, Di Felice U, Walsh NP. Dehydration decreases saliva antimicrobial proteins important for mucosal immunity. Appl Physiol Nutr Metab 2012 Oct;37(5):850-859.

(20) Chicharro JL, Lucia A, Perez M, Vaquero AF, Urena R. Saliva composition and exercise. Sports Med 1998 Jul;26(1):17-27.

(21) American Dietetics Association, Dietitians of Canada. Nutrition and Athletic Performance, Joint Position Statement. Medicine and Science in Sport and Exercise 2009.

(22) Maughan RJ, Shirreffs SM. IOC Consensus Conference on Nutrition in Sport, 25–27 October 2010, International Olympic Committee, Lausanne, Switzerland Journal of Sports Sciences 2011;29(S1):S3-S4.

(23) Garth AK, Burke LM. What do athletes drink during competitive sporting activities? Sports Med 2013 Jul;43(7):539-564.

(24) Volpe SL, Poule KA, Bland EG. Estimation of prepractice hydration status of National Collegiate Athletic Association Division I athletes. J Athl Train 2009 Nov-Dec;44(6):624-629.

(25) Maughan RJ, Shirreffs SM, Merson SJ, Horswill CA. Fluid and electrolyte balance in elite male football (soccer) players training in a cool environment. J Sports Sci 2005 Jan;23(1):73-79.

(26) Gore CJ, Bourdon PC, Woolford SM, Pederson DG. Involuntary dehydration during cricket. Int J Sports Med 1993 Oct;14(7):387-395.

(27) Kurdak SS, Shirreffs SM, Maughan RJ, Ozgunen KT, Zeren C, Korkmaz S, et al. Hydration and sweating responses to hot-weather football competition. Scand J Med Sci Sports 2010 Oct;20 Suppl 3:133-139.

(28) Adams WM, Ferraro EM, Huggins RA, Casa DJ. The Influence of Body Mass Loss on Changes in Heart Rate During Exercise in the Heat: A Systematic Review. J Strength Cond Res 2014 Apr 14.

(29) Maresh CM, Whittlesey MJ, Armstrong LE, Yamamoto LM, Judelson DA, Fish KE, et al. Effect of hydration state on testosterone and cortisol responses to training-intensity exercise in collegiate runners. Int J Sports Med 2006 Oct;27(10):765-770.

(30) Petrovsky N, McNair P, Harrison LC. Diurnal rhythms of pro-inflammatory cytokines: regulation by plasma cortisol and therapeutic implications. Cytokine 1998 Apr;10(4):307-312.

(31) Kanaley JA, Weltman JY, Pieper KS, Weltman A, Hartman ML. Cortisol and growth hormone responses to exercise at different times of day. J Clin Endocrinol Metab 2001 Jun;86(6):2881-2889.

(32) American College of Sports Medicine, Sawka MN, Burke LM, Eichner ER, Maughan RJ, Montain SJ, et al. American College of Sports Medicine position stand. Exercise and fluid replacement. Med Sci Sports Exerc 2007 Feb;39(2):377-390.

(33) Sawka MN, Coyle EF. Influence of body water and blood volume on thermoregulation and exercise performance in the heat. Exerc Sport Sci Rev 1999;27:167-218.

(34) Institute of Medicine. Water: Dietary Reference Intakes for Water, Sodium, Chloride, Potassium and Sulfate. Washington D.C: National Academy Press; 2005.

(35) Bishop NC, Walker GJ, Scanlon GA, Richards S, Rogers E. Salivary IgA responses to prolonged intensive exercise following caffeine ingestion. Med Sci Sports Exerc 2006 Mar;38(3):513-519.

(36) Oliver SJ, Laing SJ, Wilson S, Bilzon JL, Walters R, Walsh NP. Salivary immunoglobulin A response at rest and after exercise following a 48 h period of fluid and/or energy restriction. Br J Nutr 2007 Jun;97(6):1109-1116.

(37) Walsh NP, Montague JC, Callow N, Rowlands AV. Saliva flow rate, total protein concentration and osmolality as potential markers of whole body hydration status during progressive acute dehydration in humans. Arch Oral Biol 2004 Feb;49(2):149-154.

(38) Francesconi RP, Sawka MN, Pandolf KB, Hubbard RW, Young AJ, Muza S. Plasma hormonal responses at graded hypohydration levels during exercise-heat stress. J Appl Physiol (1985) 1985 Dec;59(6):1855-1860.

(39) Mitchell JB, Dugas JP, McFarlin BK, Nelson MJ. Effect of exercise, heat stress, and hydration on immune cell number and function. Med Sci Sports Exerc 2002 Dec;34(12):1941-1950.

(40) Borg GA. Psychophysical bases of perceived exertion. Med Sci Sports Exerc 1982;14(5):377-381.

CHAPTER 4: THE IMPACT OF 9 DAYS OF INTENSIFIED TRAINING WITH A HIGH CARBOHYDRATE FEEDING STRATEGY ON HORMONAL BALANCE AND IMMUNE FUNCTION IN TRAINED CYCLISTS

Sophie C Killer[1], Ida S Svendsen[1], Rebecca K Randell[2], James M Carter[2], Asker E Jeukendrup[1,2] and Michael Gleeson[1]

[1]School of Sport, Exercise and Health Sciences, Loughborough University, Leicestershire, East Midlands. UK.

[2] Gatorade Sports Science Institute, Loughborough.. LE11 3TU. UK

4.1 Abstract

Short periods of intensified training (IT) are a regular feature in a cyclist's training programme. The aim of this study was to monitor changes in performance, mood state, plasma hormones and mucosal immune function during IT in well-trained cyclists and observe the effects of a carbohydrate nutritional intervention. Thirteen trained male cyclists (age: 25 ± 6 y, $\dot{V}O_{2max}$: 72 ± 5 ml/kg/min) participated in two 9-day periods of IT while undergoing a high (HCHO) or moderate (CON) carbohydrate nutritional intervention before, during and after training sessions. Performance was assessed throughout IT with maximal oxygen uptake ($\dot{V}O_{2max}$) and 1 h time trial (TT). Mood state questionnaires were completed daily to monitor total mood disturbance (TMD). Venous blood samples and unstimulated saliva samples were collected before and immediately after each TT for analysis. Weekly training volume and intensity increased significantly during IT (9.3 ± 2.4 h to 23.5 ± 3.4 h/week for volume and 2.6 ± 2.5 to 6.5 ± 4.0 h/week for intensity). Total energy intake and dietary CHO were significantly higher in HCHO than CON. TMD was elevated during IT and was significantly higher in CON than HCHO ($p<0.05$). Performance in the $\dot{V}O_{2max}$ exercise protocol fell significantly with IT in both conditions ($p<0.05$). No significant changes in TT performance were observed over IT, or between conditions. However, absolute mean power during TT decreased non-significantly in both conditions (-1.2 and -7.2 W for HCHO and CON, respectively). Pre-exercise cortisol and BDNF tended to be lower after IT in both conditions ($p=0.07$). Post-exercise cortisol, ACTH, BDNF and prolactin concentrations decreased with IT ($p<0.05$), with no difference between conditions. Vitamin D status was positively correlated with ability to maintain TT performance during IT. Transient changes in salivary antimicrobial proteins were observed throughout IT. A relatively short period of IT resulted in significant disturbances in mood state and minor performance decrements. Hormonal changes present signs of chronic stress, including decreased post-exercise prolactin, BDNF, cortisol and ACTH suggesting these cyclists may have exhibited early signs of overreaching.

Key Words: Intensified Training, Overreaching, Carbohydrate, Hormones, Mucosal Immunity, Performance.

4.2 Introduction

Short-term bouts of intensified training occur throughout well periodised training programmes. This practice enables athletes to peak at pre-determined time points across an athletic season and to avoid performance plateaus (1). Overload training is required for performance enhancement and when the balance between training and recovery is optimised, a heavily demanding training programme can have remarkable performance benefits. However, if the fine balance between training and recovery is disturbed, overreaching (OR) can occur and in some cases, lead to development of the overtraining syndrome (OTS) (2). Short-term OR and the OTS have been suggested to be related along a continuum (3) with a disturbance, an adaptation and eventually a maladaptation of the hypothalamic-pituitary-adrenal axis (HPA) (2) leading to a neuroendocrine imbalance and an abnormal hormonal stress response (4). The HPA axis is one of the main pathways activated in response to stressors that controls the immune system. Activation of the HPA axis results in secretion of adrenocorticotrophic hormone (ACTH) from the anterior pituitary gland and subsequent release of the adrenal glucocorticoid cortisol and other neuroendocrine factors. HPA activation occurs as a result of stressors such as intense or prolonged physical exercise and psychological stress (5). Numerous studies have investigated the effects of exercise training on stress hormones, dating back to 1985 (6) yet results have been inconclusive. It is likely that the variation in study design and diagnoses of both OR and OTS has led to equivocal results.

A growing body of evidence indicates that regular physical activity induces neurobiological adaptations that result in improved health and cognitive function (7). Endurance exercise, and recently resistance exercise (8), have been shown to increase skeletal muscle brain derived neurotrophic factor (BDNF) expression and protein content, in addition to acute increases in circulating BDNF concentrations (8-14). At present, it is unknown how a short-term intensified endurance training programme may affect circulating BDNF concentrations. It is also unknown how serum prolactin concentration, an anterior pituitary hormone involved in neuroendocrine function and hypothalamic pituitary capacity, may be affected by short term intensified cycling training. A small collection of studies have previously investigated prolactin in relation to overtraining, finding suppressed post-exercise prolactin levels (2,4,15) and increased prolactin response to a neuroendocrine challenge (16) in overtrained athletes. A range of suggested mechanisms have been proposed: increased dopaminergic action due to

excessive exercise, inhibition of prolactin secretion resulting from noradrenalin action via adrenoreceptors (17), or decreased serotonergic drive due to an acute stressor (as seen in rats) (18). Circulating concentrations of the gonadal steroid testosterone have also been reported to be lower during periods of intensified exercise training (19) and it may therefore be of interest to observe changes in prolactin responses during short-term intensified training alongside a range of additional hormones (including stress and fertility hormones) and immune markers, to help provide an insight into the physiological and biochemical changes occurring in athletes undergoing these practices.

Production of salivary secretory immunoglobulin A (SIgA) is the major effector function of the immune system providing the first line of defence against pathogens (20). While SIgA is largely unaffected by acute bouts of moderate exercise, prolonged endurance exercise can lead to a suppression in its secretion (21-24) and thus increasing susceptibility to upper respiratory tract infections (URTIs) (25). The mechanisms behind this suppression are suggested to be related to the sympathetic nervous system and changes in HPA axis activity resulting in the inhibition of IgA synthesis and/or transcytosis (20). The effects of intensified training periods on other salivary antimicrobial proteins (AMPs) have been less well researched; however, AMPs such as lysozyme (SLys) and lactoferrin (SLac) are known to be present at mucosal surfaces and to contribute to the first line of defence against pathogens along with SIgA. Little is known about how prolonged intensified training may impact secretion of these AMPs.

One proposed mechanism behind the development of OTS is the glycogen depletion hypothesis. Since the early findings from Bergstrom and Hultman (26), it has been understood that prolonged exercise leads to muscle glycogen depletion. Low muscle glycogen levels are associated with exercise-induced fatigue (peripheral and central) (27) and studies have found high carbohydrate intakes during periods of intensified training help to maintain performance (3,28). It is also known that low nutrient availability (specifically carbohydrate) can negatively impact immune function via direct (reduced immune cell energy metabolism and synthesis of antibody, cytokine and acute phase proteins) and indirect (stimulation of stress hormone secretion) mechanisms (25). Furthermore, consumption of carbohydrate during endurance exercise has been shown to attenuate the rise in stress hormones such as cortisol and adrenaline. However, whilst sufficient carbohydrate to prevent glycogen depletion during heavy training regimens may aid performance and support

immune function, it has been reported that this does not prevent the development of short term OR (3,28,29).

Current guidelines for carbohydrate intakes state that 30-60 g (30) or 0.7 g/kg carbohydrate (31) should be consumed per hour to support endurance exercise lasting longer than an hour and to reduce the likelihood of injury or illness (30). Recently studies have shown that higher intakes of mixed carbohydrates (90 g/h) may have even greater performance effects (32) and it has been suggested that these higher intakes may be more appropriate for exercise lasting 2.5 h and longer. Furthermore, high carbohydrate diets (6-10 g/kg/day) are generally recommended for athletes undergoing heavy training regimens (30,31). High carbohydrate intakes (8.5-9.4 g/kg/day) during intensified training have been shown to result in reduced mood state disturbances and performance decrements compared to lower intakes (5.4-6.4 g/kg/day) (3,28). Interestingly, one study found that high dietary protein has also been found to reduce the decline in both mood state and performance resulting from short term intensified training in endurance trained cyclists (3 g/kg/day vs 1.5 g/kg/day) (33).

Some evidence suggests that dietary carbohydrate intakes may fall short of the recommendation in a large percentage of athletes undergoing such training (34). Lower intakes may be the result of an inability to access the foods and fluids required during training due to the restrictions or implications of the sport, concerns about total energy intakes and body composition, post-exercise appetite suppression or simply due to an inability to tolerate such large intakes during exercise due to gastrointestinal complaints (35). The aims of this study were to investigate the effects of 9 days of intensified training on hormonal balance and immune function (both at rest and in response to exercise) in addition to monitoring mood state and exercise performance. Furthermore, we aimed to investigate the potential impact of high carbohydrate availability during exercise (in line with current ACSM recommendations) on the aforementioned variables. With this information, it was hoped that a better understanding of some of the short-term physiological changes that occur in elite athletes undergoing 'training camp' type periods of intensified training could be gained.

4.3 Methods

Participants

Fifteen trained cyclists were accepted to participate in this study. Participants were included if they had a competitive cycling background of at least 3 y, were currently cycling at least three times per week for a minimum of 2 h/day and reported a $\dot{V}O_{2max}$ of ≥ 65 mL/kg/min. Participants were excluded from the study if they were currently suffering from any cycling-related injuries or had experienced any symptoms of an upper respiratory tract infection (URTI) in the four weeks prior to the study. Two participants were unable to complete the study due to personal circumstances. All participants were informed of the purposes of the study and the risks associated with the procedures. Written informed consent was obtained from each participant and a health questionnaire was completed before the study commenced. The study was approved by the Loughborough University ethical advisory committee.

Characteristics of the 13 participants who completed the study are reported in Table 4.1.

Experimental Design

Participants underwent two 9-day periods of intensified training (IT) while ingesting one of two nutritional interventions in a randomised counterbalanced, double-blind cross-over model. During the high carbohydrate intervention (HCHO), participants consumed carbohydrate beverages at intakes consistent with current ACSM guidelines (30) before and during exercise, followed by a high carbohydrate and protein recovery beverage in the immediate recovery period (<10 min post-training). During the control condition (CON), participants were provided with lower carbohydrate concentration beverages before, during and after training. No supplemental protein was provided in the control condition. Prior to their first trial, participants underwent a two week pre-trial period to provide protocol familiarisation and allow baseline measures to be taken. A 10 day washout period separated the two trials. Participants were monitored for 2 weeks following their last trial to ensure full recovery. See Figure 4.1 for a schematic overview.

Table 4.1: Participant Characteristics

	Age (y)	Height (cm)	Body Mass (kg)	Body Fat (%)	$\dot{V}O_{2max}$ mL/kg/min
Mean ±SD	25.0 ± 5.8	178.3 ± 3.9	69.7 ± 6.3	13.4 ± 4.1	72.2 ± 4.9

Figure 4.1: Schematic Overview of Study Design

IT	1	2	3	4	5	6	7	8	9	10
am	**MAX**	**TT**	🚲	🚲	🚲	**TT**	🚲	🚲	**MAX**	**TT**
pm	🚲	🚲	🚲	🚲	🚲	🚲	🚲	🚲	🚲	

🚲 Training Session   **MAX** $\dot{V}O_{2max}$ Test   **TT** Time Trial Performance Test

Pre-trial Tests and Familiarisation

Participants visited the laboratory for pre-trial exercise testing to confirm suitability to participate. During their first visit, participants underwent an incremental cycle test to exhaustion on an electronically braked ergometer (Lode Excalibur Sport, Groningen, Netherlands) to determine suitability for the study. Expiratory gases were collected continually throughout the test and breath-by-breath analysis was performed automatically with a Moxus metabolic systems analyser (AEI Technologies Inc., Naperville, IL, USA). Heart rate (HR) was recorded continually using short range telemetry (Polar RS800CX, Polar, Kempele, Finland) and ratings of perceived exertion (RPE) were recorded from participants during the final minute of each stage. If participants achieved a $\dot{V}O_{2max}$ of ≥ 65 ml/kg/min and were suitable according to all other inclusion criteria, they were included in the study. Following this, each suitable participant completed a 2 week pre-trial training diary, including mood-state questionnaires and a 3-day weighed diet diary. Participants were provided with HR monitors and SRM power meters (SRM Shimano DA7900 PowerMeter) and requested to train according to their normal programmes during this phase. During this time, participants underwent a full body composition assessment and baseline blood screening for vitamin D and iron status (Spire Healthcare Ltd). Body composition was determined by DXA at the Gatorade Sports Science Laboratory at Loughborough University. No participants exhibited symptoms of overreaching during their pre-trial phase according to the guidelines set by Meeusen and colleagues (4). Following the pre-trial phase, participants began 9 days of IT.

Intensified Training

Individual IT programmes were based on participants' pre-trial training diaries and discussed with each individual athlete. Training volume and intensity (time spent in the highest 3 HR zones) was increased 2.5-fold from baseline. HR zones were calculated from maximum HR achieved during the $\dot{V}O_{2max}$ test. Five zones were devised, based on previous research within our group (3,36), and expressed as a percentage of maximum HR; Zone 1: <69%, Zone 2: 69% - 81%, Zone 3: 82% - 87%, Zone 4: 88% - 94%, Zone 5: >94%. HR zones were used to define the intensity of the training sessions. Power zones were also applied to interval sessions. Power zones were calculated as a percentage of Watt max attained by participants in the first $\dot{V}O_{2max}$ of each trial and used as a guideline during training sessions, in addition to

HR zones. It was predicted IT would result in a reduction of participants' ability to achieve HR zones; therefore, power zones were put in place as an additional measure to encourage participants to achieve the highest workload possible. An example of a typical training programme can be seen in Appendix B. SRM power meters (SRM Shimano DA7900 PowerMeter) were fitted to each participant's bicycle to enable the monitoring of power output during each session. Participants were also provided with a HR monitor to use specifically for training (Suunto ANT, Amer Sports Corporation, Finland). Participants were responsible for uploading data files (power and HR) after each training session via secure online training software (Training Peaks Ltd.). This enabled the researchers to monitor sessions that were not performed in the laboratory.

Mood State

The Profile of Mood State shortened version (POMS-24) and the Daily Analysis of Lifestyle Demands on Athletes (DALDA) were completed daily, first thing after waking and before visiting the laboratory or partaking in a training sessions. In addition, the full 65 item POMS questionnaire (POMS-65) was completed on days 1, 3, 6 and 9. Global mood state (GMS) was calculated from POMS questionnaires by summing the five negative measures of affect (tension, depression, anger, fatigue and confusion) and subtracting scores for vigour. Total mood disturbance (TMD) was monitored throughout IT by changes in GMS. The DALDA questionnaire identifies the sources (part A) and symptoms (part B) of stress for athletes. Changes in TMD were determined from DALDA B scores by analysing changes in the number of 'a' (worse than normal) and 'c' (better than normal) scores.

Nutritional Intervention and Assessment of Dietary Intake

Participants and lead investigators were blind to the intervention order. During IT, participants ingested either high (HCHO) or control (CON) nutritional beverage before, during and after each training session. Table 4.2 outlines the nutritional composition of the HCHO and CON. Participants were instructed to consume their normal diet throughout each training period and keep their diets as similar as possible between the two conditions. In addition, participants completed a weighed diet diary each day through the entirety of each trial and at select time points during the pre-trial and washout periods. Evening meals consumed before the performance tests were replicated as closely as possible each time,

across both conditions. Caffeine was not permitted for 12 h before performance testing. Participants consumed a pre-exercise beverage within 15 min of starting each training session. Pre-exercise beverages were taste matched carbohydrate solutions (118 mL); containing 24 g or 2 g carbohydrate for HCHO and CON, respectively. During exercise, participants consumed 1 L of a taste matched carbohydrate-electrolyte solution per hour of exercise; containing 60 g (6 m/v %) or 20 g (2 m/v %) carbohydrate for HCHO and CON conditions, respectively. Following exercise, participants were provided with recovery nutrition; both trials received a carbohydrate-electrolyte solution (30 g vs 10 g carbohydrate for HCHO and CON, respectively) in addition to a recovery product; a beverage during the HCHO condition and a placebo tablet in CON. The HCHO recovery beverage contained 14 g CHO and 17 g protein. The CON placebo tablet contained only 1 g cellulose. Participants were informed that the variation in the nutritional intervention was in the recovery nutrition formulation, either tablet or beverage. When questioned after the study, no participants reported noticing any differences between carbohydrate beverages or that the intervention involved the manipulation of carbohydrate ingestion.

Table 4.2: Composition of Nutritional Interventions

	HCHO			**CON**		
	PRE	During/h	POST	PRE	During/h	POST
kcal	98	250	250	12	100	50
CHO (g)	24	60	44	2	20	10
PRO (g)	0	0	17	0	0	0

Table 4.2: Pre-exercise (PRE), per hour of exercise (During/h) and post-exercise (POST) caloric intakes (Kcal), carbohydrate intakes (CHO) and protein intakes (PRO) for high carbohydrate (HCHO) and control (CON) conditions. Data are absolute values.

Performance Testing – Time Trial

Participants arrived at the laboratory at the same time for each TT (between 06:30-08:30) following an overnight fast of $\geq$ 8 h. Blood and saliva samples were collected from participants pre-TT (after resting for 5 min) and immediately post-TT (<5 min). Blood samples (20 mL) were drawn from a superficial vein by a trained phlebotomist from participants in the seated position. Unstimulated saliva samples were collected during a 3 min collection period from participants in the seated position.

Following a 10-min warm-up at a self-selected intensity, participants performed an all-out 1 h TT on their own bikes on a turbo trainer (CycleOps Flow) in a quiet laboratory environment. Heart rate was recorded continually during each TT with short range telemetry (Suunto). Participants were blinded from their power, cadence and HR during the TT and were only provided with a stop clock. Changes in TT performance were calculated from mean power (watts). Participants were permitted to drink water *ad libitum* during TT. Post-TT blood and saliva samples were collected within 5 min of exercise cessation.

Performance Testing – $\dot{V}O_{2max}$

Participants arrived at the laboratory at the same time for each $\dot{V}O_{2max}$ test (between 06:30-08:00), following an overnight fast of $\geq$ 8 h. Blood samples (approximately 10 mL) were drawn from a superficial vein by a trained phlebotomist from participants in the seated position. Following this, participants completed an incremental cycle test to exhaustion on an electronically braked ergometer (Lode Excalibur Sport, Groningen, Netherlands). Results were used to determine maximal $\dot{V}O_2$, HR and power output (W_{max}). No warm-up time was permitted prior to testing; however, the test began at 60 W and power increased by 35 W every 3 min until volitional exhaustion. Expiratory gases were collected continually throughout the test and each breath was analysed automatically with a Moxus metabolic systems analyser (AEI Technologies Inc., Naperville, IL, USA). Heart rate was recorded continually using short range telemetry (Polar RS800CX, Polar, Kemple, Finland) and ratings of perceived exertion were recorded from participants during the final minute of each stage.

W_{max} was calculated with the following equation:

$$W_{max} = W_{final} + (t/T) \cdot W_{inc}$$

W_{final} is the power output (W) of the final completed stage, *t* is the time achieved in the final uncompleted stage (s), T is the duration of each stage (180 s) and W_{inc} is the workload increment (35 W).

Haematology and Circulating Hormone Analysis

Venous blood samples were collected at rest prior to each TT and immediately after the cessation of exercise (≤5 min). Blood was collected into chilled EDTA vacutainers and analysed for haematological variables and plasma metabolomic profiling. K_3EDTA blood was used for immediate haematological analysis (including haemoglobin, haematocrit and total and differential leukocyte counts) using an automated cell-counter (A^c.T™5diff haematology analyser, Beckman Coulter, High Wycombe, UK) and the remaining blood centrifuged at 3500 rpm for 10 min at 4˚C and the plasma was immediately stored at -80˚C. The intra-assay coefficient of variation (CV) for all measured blood variables was less than 3.0%. In addition, venous blood was collected into serum clotting vacutainers and analysed for brain-derived neurotrophic factor (BDNF), cortisol (CORT), testosterone (TEST), prolactin (PRL), luteinising hormone (LH) and follicle stimulating hormone (FSH). After clotting, whole blood was centrifuged at 3500 rpm for 10 min at 4°C and serum was immediately stored at -20°C. Serum LH, FSH, PRL and TEST were analysed with a chemiluminescent immunoassay (RANDOX, Fertility Hormone Array – EV3610) (mean intra assay CV was ±5.6%). Serum CORT, ACTH and BDNF were analysed with an enzyme-linked immunosorbent assay (DRG Instruments, Cortisol ELISA EIA – 1887; intra assay CV: ±5.6%, Biomercia ACTH ELISA 7023; intra assay CV: ±4.5% and ChemiKine™ BDNF ELISA CTY306; intra assay CV: ±3.7%). Serum vitamin D (25-Hydroxyvitamin D) and ferritin were analysed using electrochemiluminescence immunoassays (Elecsys Vitamin D Total; intra assay CV: ±<5% and Elecsys Ferritin; intra assay CV: ±<3%; Spire Healthcare Ltd.). The vitamin D analysis methodology used in the study has been shown to correlate well with the gold standard liquid chromatography/mass spectrometry method; Pearson r=0.877.

Salivary Analyses

Unstimulated saliva samples were collected from participants before and immediately after the 1 h TT (≤5 min). Participants were familiarised with the saliva collection procedure prior

to their first trial. Saliva collections were made with participants in the seated position, leaning forward with their head tilted forward. Participants were asked to swallow to empty their mouth of any residual saliva before the timed sample collection began. Saliva collections lasted 3 min, during which time participants were requested to minimise orofacial movement and passively dribble into a pre-weighed vial. Samples were weighed to estimate saliva volume and micro-centrifuged to remove cells and insoluble matter before storing at -20°C. The saliva volume collected was estimated by weighing and saliva flow rate (mL/min) was determined by dividing the volume of saliva by the collection time. Saliva samples were analysed for SIgA using an ELISA kit (Salimetrics, Philadelphia, USA) and both SLac and SLys were analysed using commercially available ELISA kits (Calbiochem, USA and Biomedical Technologies, USA, respectively). Secretion rates for each of the salivary AMPs were calculated as the multiple of the saliva flow rate and the antimicrobial protein concentration. All saliva assays were carried out in duplicate. The intra-assay CV for SIgA, SLac and SLys was 1.8%, 8.1% and 5.3%, respectively.

Statistical Analysis

All data were analysed using statistical software (IBM SPSS Statistics 21) and are presented as Mean ± Standard Deviation. Changes in performance, blood markers and mood state parameters were analysed using a 2-way repeated measures analysis of variance (ANOVA). A Bonferroni adjustment was included into the analysis to correct for multiple comparisons. Data that were found to be significantly non-normal were log transformed prior to analysis. Between trial and pre- to post-IT changes in performance parameters were determined using an independent samples t-test. Pearson's correlation was used to bivariate correlations between vitamin D status and performance outcomes. The level of significance was set at $p < 0.05$.

4.4 Results

Diet and Training

Total energy intake (EI) and dietary CHO intakes increased significantly during IT compared to baseline ($p < 0.05$). EI and CHO were significantly higher during HCHO than CON (4148 ±

766 vs 3501 $\pm$ 617 kcal and 9.9 $\pm$ 1.5 vs 7.4 $\pm$ 1.6 g/kg CHO, respectively; $p < 0.05$). Dietary protein intake was significantly higher in HCHO than CON (2.1 $\pm$ 0.5 vs 1.7 $\pm$ 0.4 g/kg protein; $p < 0.05$) and compared with pre-trial intakes (1.7 $\pm$ 0.5 g/kg protein; $p < 0.05$). Fat intake was not different between conditions, or between HCHO and baseline; however, fat intake was significantly higher in CON than at baseline (1.5 $\pm$ 0.4 vs 1.2 $\pm$ 0.3 g/kg; $p = 0.02$). See Appendix C for participant dietary intakes.

Weekly training volume increased significantly during IT compared with pre-trial baseline training (153% increase: 9.3 $\pm$ 2.4 to 23.5 $\pm$ 3.4 h/week; $p < 0.001$). Total time spent training during the 9 days of IT was 30.2 $\pm$ 4.4 h. Weekly training intensity increased significantly during IT compared with pre-trial baseline training (146% increase: 2.6 $\pm$ 2.5 to 6.5 $\pm$ 4.0 h/week spent training above 82% HRmax; $p < 0.01$). Total time spent training above 82% HRmax (zone 3 and above) during IT was 8.3 $\pm$ 5.2 h.

Performance

High intensity exercise performance ($\dot{V}O_{2max}$) fell significantly with IT in both conditions ($p < 0.05$); peak power (391 $\pm$ 37 to 375 $\pm$ 38 W; figure 4.2), maximum HR (190 $\pm$ 10 to 179 $\pm$ 8 bpm) and completion time (31:15 $\pm$ 03:12 to 30:00 $\pm$ 03:30). IT did not result in a significant reduction in absolute $\dot{V}O_{2max}$; 4.91 $\pm$ 0.48 to 4.86 $\pm$ 0.48 l/min for pre- and post-IT respectively. Declines in TT performance over IT and differences between conditions did not reach significance. However, performance (mean Watts) fell by 2.8% in CON compared to 0.3% in HCHO (-7.2 W $\pm$ 2.9 compared to -1.2 W $\pm$ 0.5 decrements for CON and HCHO, respectively; $p > 0.05$; figure 4.3). Effort, as a percentage of peak power during TT did not change over IT in HCHO (64.8 $\pm$ 3.3 to 64.7 $\pm$ 5.2%) or CON (63.3 $\pm$ 5.5 to 61.5 $\pm$ 5.9%). Mean HR tended to decrease during the TT in CON ($p = 0.06$).

Figure 4.2: Individual change in Peak Power during VO_{2max} testing following IT
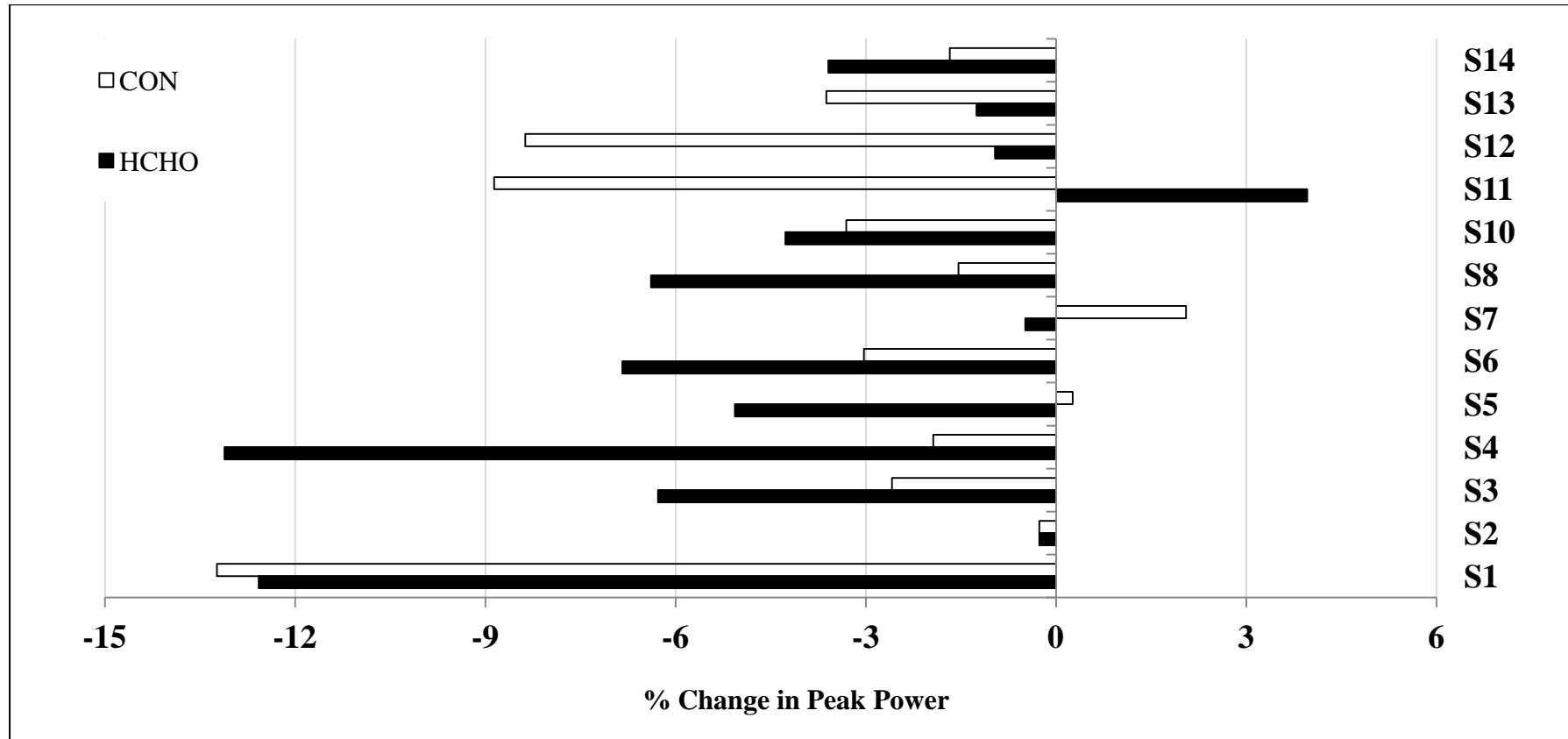
Figure 4.2: Individual performance response to IT represented as percentage change in peak power during the $\dot{V}O_{2max}$ test from pre- to post-IT.

Figure 4.3: Individual change in Mean Power during 1 h time trial following IT
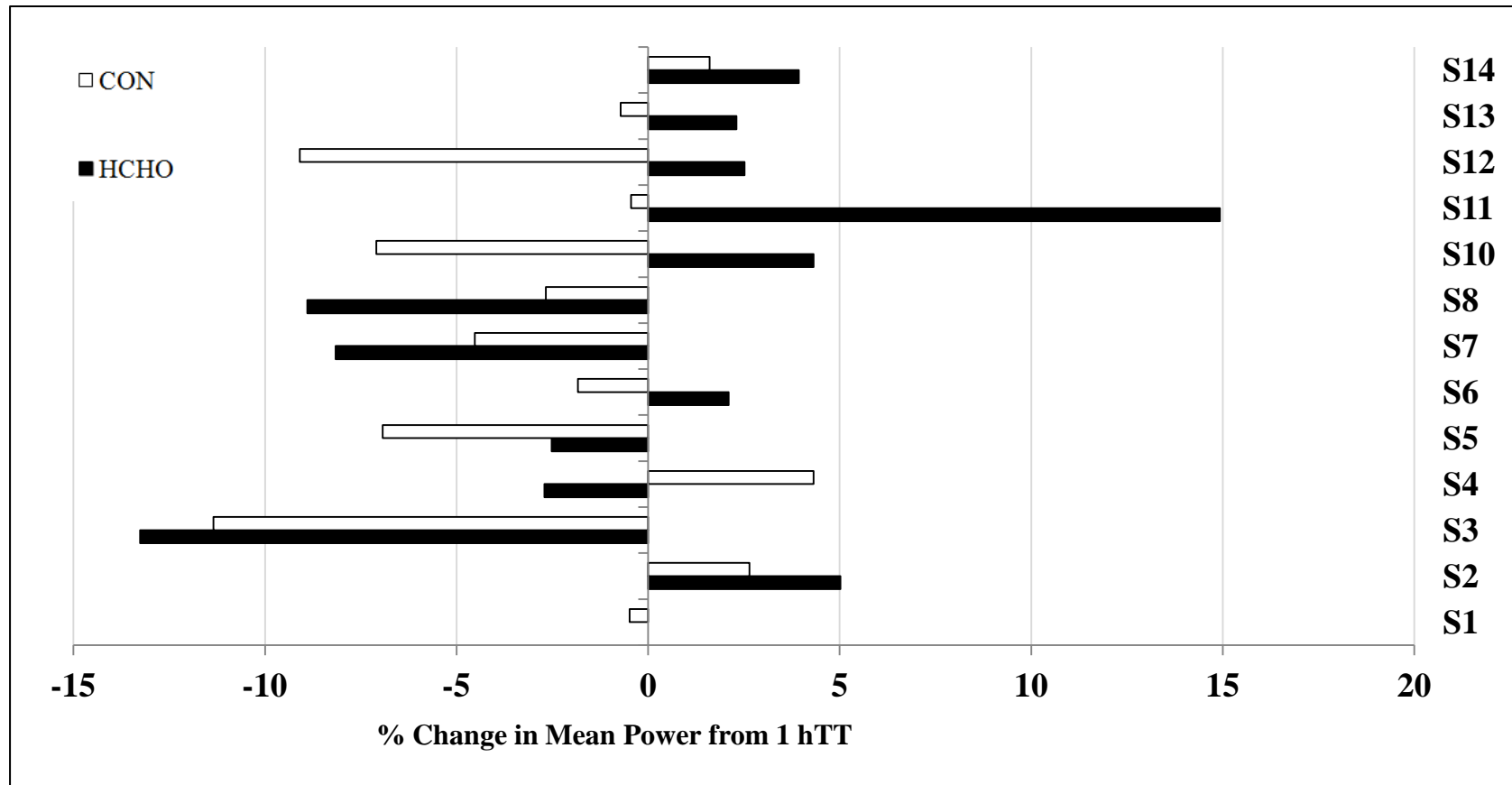
Figure 4.3: Individual performance response to IT represented as percentage change in mean power during the 1-h time trial test from TT1-TT3.

Mood State

Daily records of the POMS-24 questionnaire responses showed a continual increase in TMD throughout IT ($p < 0.01$). Overall, TMD was significantly higher in CON than HCHO throughout the period of IT ($p = 0.04$). Data from the POMS-65 questionnaire, recorded every 3 days supported findings from the shortened version, showing a significant increase in TMD with time ($p < 0.05$) and TMD was significantly higher in CON than HCHO at day 9 (figure 4.4; $p < 0.05$). Results from the DALDA questionnaire also indicated a significant overall disturbance in mood state, with an increase in section B 'a scores' (figure 4.5) and a decrease in number of 'c scores' with IT ($p < 0.05$).
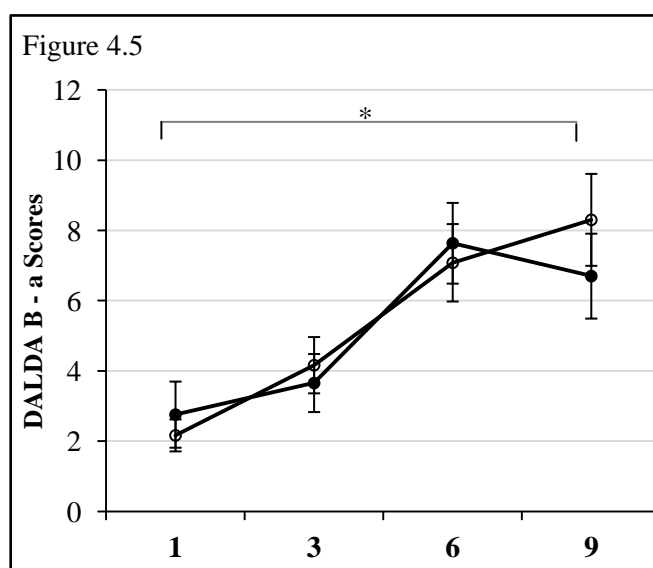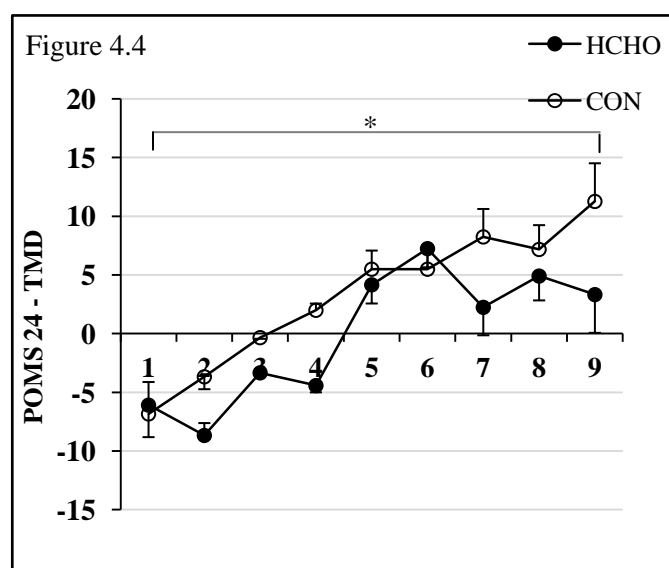
Figures 4.4 and 4.5: Daily changes in Profile of Mood States – 24 item questionnaire (POMS-24) and changes in the number of 'a' scores from the Daily Analysis of Lifestyle Demands on Athletes (DALDA) – part B from baseline over IT in the high carbohydrate (HCHO) and control (CON) trials. Black circles represent HCHO and white circles represent CON. Data are mean ± SE. * Indicates significant increase with time ($p < 0.05$).

Vitamin D Status

Six of 12 participants tested for vitamin D status reported <75 nmol/L and were classified as having insufficient levels (37). Vitamin D status was significantly correlated with better maintenance of performance (mean W) following IT, $r = 0.69$; p (one-tailed) < 0.01 (figure 4.6). No relationship between vitamin D status and changes in $\dot{V}O_{2max}$; peak power were observed, $r = -0.198$; p (one-tailed) > 0.05.

Hormonal Concentrations

Changes in key hormonal responses to exercise are presented in figure 4.7. Resting CORT concentrations tended to be lower after IT (752 ± 23 to 700 ± 42 nmol/L; $p=0.07$), with no differences between conditions. Post-exercise CORT decreased significantly with IT (805 ± 78 to 646 ± 67 nmol/L; $p<0.01$), with no differences observed between conditions. Pre-exercise ACTH tended to be lower in CON than HCHO (46.7 ± 4.8 vs 61.0 ± 6.3 pg/mL, for CON and HCHO respectively; $p=0.06$). Post-exercise ACTH fell significantly with IT (115.9 ± 20.8 to 71.2 ± 12.0 pg/mL; $p<0.01$), with no difference between conditions.

Baseline BDNF concentrations tended to decrease following IT ($p=0.08$) with no difference between conditions. Exercise resulted in a significant increase in BDNF concentration compared to pre-exercise baseline ($p<0.01$). Post-exercise BDNF concentrations were significantly lower following IT ($p<0.05$). The percentage increase in serum BDNF after TT3 was non-significantly lower in CON (12.5%) than HCHO (22.0%).

Figure 4.6: Vitamin D Status and TT Performance
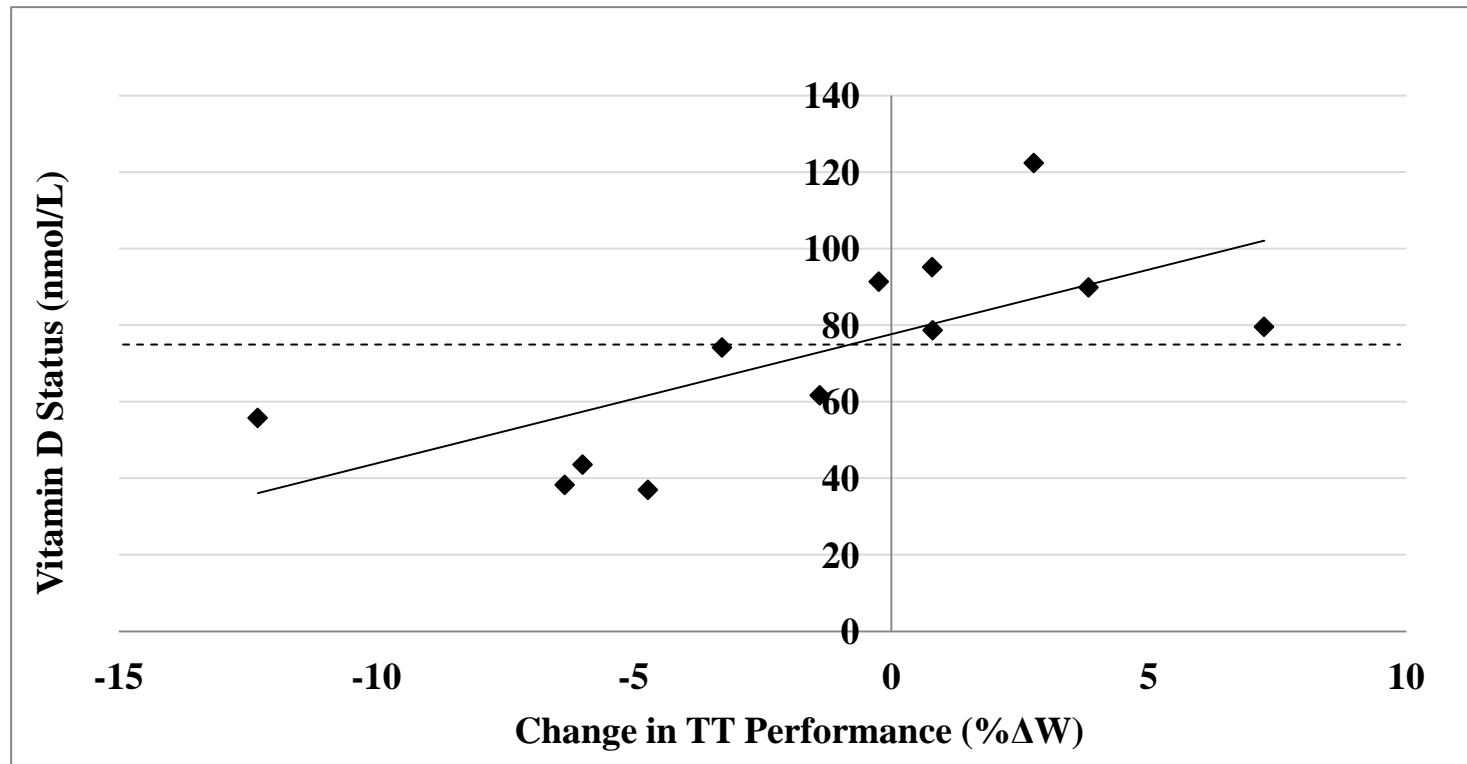
Figure 4.6: Relationship between vitamin D status and percentage change in TT performance (mean W) from TT1-TT3.Solid line represents the linear regression. Broken line marks the 75 nmol/L cut off for sufficiency levels of Vitamin D.

A significant change in the acute PRO response to exercise was observed following IT (4.6A). Post-exercise PRO concentration increased from baseline at TT1 and decreased following TT3. Post-exercise PRO concentration decreased significantly with IT, with no significant difference between conditions.

No changes in baseline serum testosterone were observed from pre-post exercise (TT), or between conditions. Post-exercise serum testosterone was significantly lower in CON than HCHO (13.2 ± 0.7 vs 14.6 ± 0.9 nmol/L; $p<0.01$). Pre-exercise cortisol/testosterone ratio tended to be higher at TT2 in CON than HCHO ($p=0.06$). Overall, post-exercise CORT/TEST ratio declined during IT (63.9 ± 8.5 to 48.2 ± 5.6 C/T ratio; $p=0.02$).

No differences were observed in pre-exercise LH concentrations over IT, or between conditions. Post-exercise LH concentrations increased significantly with IT from TT1-TT3 (2.8 ± 0.4 to 3.4 ± 0.4 mLU/mL; $p=0.02$). Post-exercise FSH did not change from TT1-TT2, but tended to increase from TT2-TT3 (3.2 ± 0.5 to 3.5 ± 0.5 mLU/mL; $p=0.06$).

Figure 4.7: Changes in Hormone Responses to 1 h Time Trial over IT
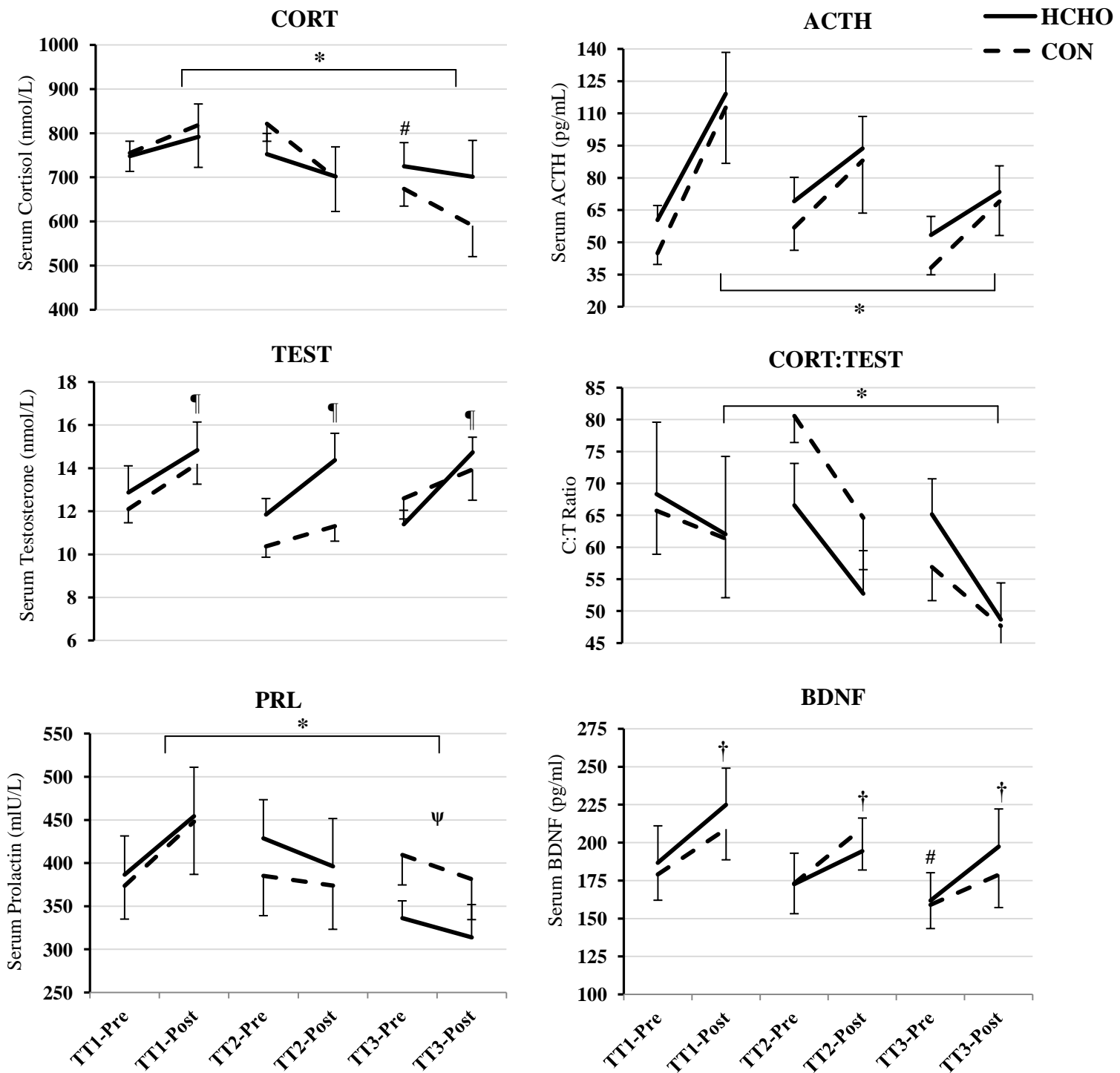
Figure 4.7: Change in hormone responses to each 1 h time trial over (TT) IT for HCHO (solid line) and CON (broken line). Data are mean ± SE. *Significant change with IT. † Significant acute change following TT. $^\Psi$ Significant change in acute response to exercise with IT. ¶ Significant difference between conditions. # Tendency to decrease with IT.

Salivary AMP Concentrations

Tables 4.3 and 4.4 report salivary AMP concentrations and secretion rates, respectively, at rest (PRE) and post-exercise (POST) with the delta values (Δ POST minus PRE) for each of the three TT performance tests.

Table 4.3: Pre-exercise (PRE) and post-exercise (POST) salivary secretory immunoglobulin A (SIgA), lactoferrin (SLac) and lysozyme (SLys) concentrations for high carbohydrate group (HCHO) and control trial (CON) for each time trial (TT) performance test.

	HCHO			CON		
SIgA (mg/L)	PRE	POST	Δ	PRE	POST	Δ
TT1	154.4 (71.9)	227.4 (66.5)	73.0 (64.9)	127.6 (54.9)	208.4 (50.6)	80.8 (93.6)
TT2	160.7 (76.6)	216.6 (118.8)	55.9 (106.5)	165.4 (72.9)	217.2 (99.9)	51.8 (96.2)
TT3	158.4 (76.2)	204.7 (33.3)	46.3 (62.1)	140.2 (66.2)	195.1 (45.3)	54.9 (77.2)
SLac (mg/L)	PRE ¶	POST ¶	Δ †	PRE	POST	Δ †
TT1	2.1 (1.5)	6.9 (2.4)	4.8 (3.1)	2.1 (1.5)	9.5 (4.6)	7.4 (4.1)
TT2	3.9 (4.7)*	9.0 (5.6)*	5.2 (2.8)	2.4 (1.8)*	13.1 (11.2)*	10.7 (11.5)
TT3	3.1 (3.1)	8.3 (4.6)	5.2 (4.3)	2.2 (1.8)	8.8 (5.7)	6.5 (4.4)
SLys (mg/L)	PRE	POST	Δ*	PRE	POST	Δ†
TT1	1.9 (0.9)	2.8 (1.2)	0.8 (0.6)	2.0 (0.8)	3.0 (1.3)	0.9 (0.9)
TT2	2.1 (0.9)	2.7 (1.1)	0.6 (0.7)	2.2 (0.9)	2.7 (1.2)	0.5 (1.0)
TT3	2.0 (0.9)	2.5 (1.0)	0.5 (0.4)*	2.1 (1.1)	2.4 (1.0)	0.3 (0.6)*

Data are mean (± SD). *Significant change with IT from TT1. †Significant change with exercise bout. ¶ Significant difference between conditions; $p < 0.05$.

Table 4.4: Pre-exercise (PRE) and post-exercise (POST) salivary secretory immunoglobulin A (SIgA), lactoferrin (SLac) and lysozyme (SLys) secretion rates for high carbohydrate (HCHO) and control (CON) treatments for each time trial (TT) performance test.

	HCHO			CON		
SIgA (µg/min)	PRE	POST	Δ	PRE	POST	Δ
TT1	73.2 (43.8)	101.2 (39.8)	28.1 (36.9)	82.6 (55.4)	90.3 (32.4)	6.9 (40.3)
TT2	84.1 (64.0)	159.8 (150.1)	21.2 (107.7)	100.5 (53.1)	104.0 (66.7)	16.4 (84.3)
TT3	112.4 (103.7)	107.6 (41.1)	-4.9 (74.8)	90.0 (55.1)	94.0 (45.3)	4.0 (40.0)
SLac (µg/min)	PRE	POST	Δ	PRE	POST	Δ
TT1	0.9 (0.6)	3.0 (1.6)	2.1 (1.6)	1.1 (0.9)	4.8 (3.6)	3.7 (2.9)
TT2	1.4 (0.9)	5.7 (5.1)	4.2 (4.9)	1.2 (1.2)	5.0 (5.4)	3.8 (5.3)
TT3	1.9 (1.4)˚	3.7 (2.0)	1.8 (1.8)	1.5 (1.5)˚	3.9 (2.4)	2.4 (2.2)
SLys (µg/min)	PRE	POST	Δ †	PRE	POST	Δ †
TT1	0.7 (0.3)	1.1 (0.7)	0.4 (0.6)	1.0 (0.5)	1.3 (0.6)	0.3 (0.3)
TT2	0.9 (0.4)	1.7 (2.0)	0.8 (1.7)	1.1 (0.5)	1.2 (0.6)	0.1 (0.6)
TT3	1.0 (0.4)	1.1 (0.6)	0.1 (0.4)˚	1.0 (0.6)	1.2 (0.7)	0.1 (0.4)˚

Data are mean (± SD). †Significant change with exercise bout; $p < 0.05$. ˚Tendency to change from TT1-TT3 ($p = 0.07$)

No differences in baseline or post-exercise SIgA concentrations or secretion rates were observed between conditions or with IT. No significant differences were seen in the response to exercise. Figure 4.8 presents the significant increase in SLac concentration from pre-post exercise, observed in both conditions ($p<0.01$). SLac concentration increased significantly from TT1-TT2 ($p<0.05$). Overall, SLac concentration was significantly higher in HCHO than CON ($p<0.05$). No change in baseline SLac secretion rates were observed following IT; 1.02 vs 1.71 mg/min for TT1 and TT3, respectively ($p>0.05$) and no differences were observed in post-exercise secretion rates after IT or between conditions.

SLys concentrations increased significantly with exercise from 0.95-2.63 mg/L in HCHO ($p<0.01$) and tended to increase in CON ($p=0.06$). There was a significant attenuation in the magnitude of increase in concentration from pre-post exercise from TT1-TT3 (figure 4.9; $p<0.05$). Exercise resulted in a significant increase in SLys secretion rates in both conditions ($p<0.05$). The magnitude of the post-exercise increase in SLys secretion rate tended to decrease over IT ($p=0.08$) from 0.38-0.12 mg/min and 0.28-0.10 mg/min for HCHO ($p=0.21$) and CON ($p=0.08$), respectively. No changes in baseline SLys secretion rate or concentration were observed during IT or between conditions.
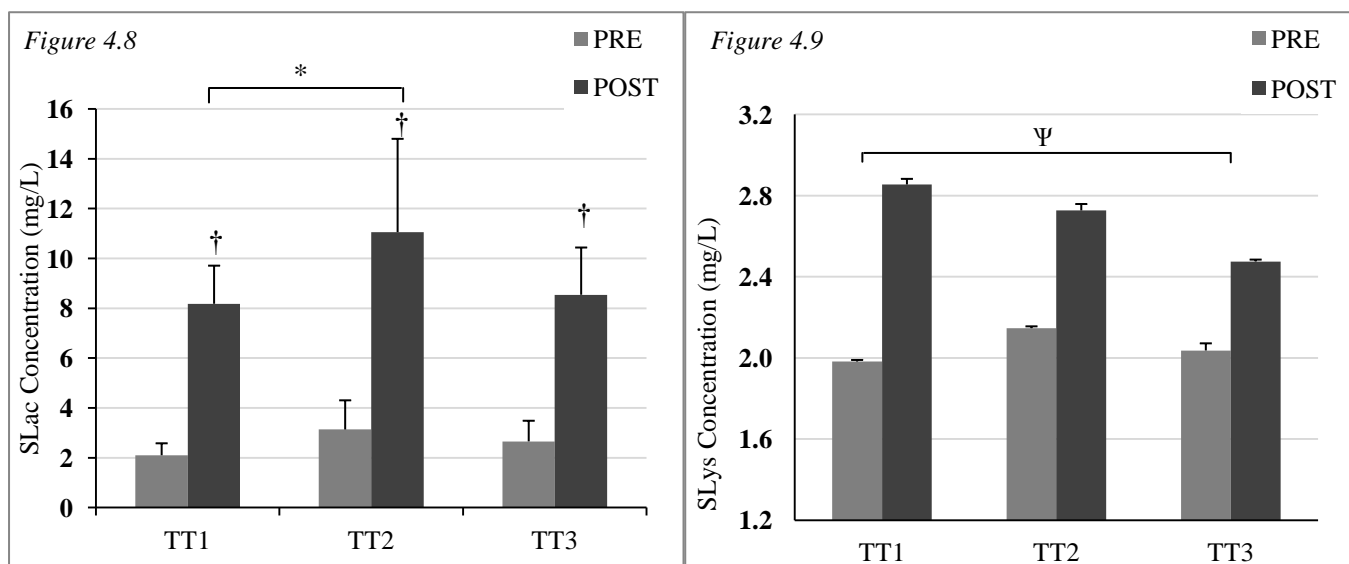
Figure 4.8-4.9: Data are mean ± SE.* Significant change with IT from TT1. Ψ Significant change in acute response to exercise bout with IT. † Significant change with exercise; $p<0.05$

4.5 Discussion

The main aims of this study were to investigate the effects of 9 days of IT on hormonal balance and mucosal immune function (both at rest and in response to exercise) in addition to monitoring mood state and exercise performance in highly trained athletes. In addition, we were interested in the potential impact of high carbohydrate availability before, during and after exercise (in line with current ACSM recommendations) on the aforementioned variables. The main findings of our study were that a relatively short period of IT resulted in large declines in mood state, significant reductions in maximal exercise performance and minor endurance performance decrements. Furthermore, significant changes in hormone regulation and markers of immune function were observed during 9 days of IT. The HCHO nutritional intervention appeared to provide a protective effect against some, but not all, of the detrimental physiological and psychological effects of IT.

Short-term periods of training at higher volumes and/or intensities are commonplace throughout an athletic training programme, enabling targeted performance peaks and preventing staleness. If the periodisation of this practice is not properly considered, short term OR (whereby performance is restored within several days to a few weeks of recovery) can develop into the OTS (38). Within ethical restraints, numerous studies have attempted to investigate the causes and effects of OR and OT, and have reported a range of different findings. In light of our data, it is not possible to confirm whether all cyclists experienced OR; however, some level of performance decrement occurred in almost all athletes. It is important to consider that whilst changes in TT performance over IT did not reach significance ($p<0.05$), many athletes and coaches would consider a 2.8% attenuation in 1 h TT performance in CON to have meaningful implications in the field, especially when compared to a 0.3% change in HCHO.

An alternative statistical method was considered when analysing these data. Inferential statistics can help provide insight into whether results from such performance tests are meaningful when translated into performance outcomes in the field. Inferential statistics can be used to make conclusions about the true value of an effect statistic derived from a sample. It has been suggested that magnitude based inferences help determine whether the true value of an outcome has any meaningful implications (70). Furthermore, calculating clinical inferences with confidence intervals and probabilities of benefit and harm has been suggested

to be used in place of null-hypothesis significance testing (70). However, the use of this alternative statistical method was not implemented within the body of research in this thesis due to the potential confusion over the use of two statistical methodologies. The use of traditional hypothesis testing with p-values is well understood by readers of scientific literature and most commonly used. Whilst this traditional method of statistical analysis may not give true insight into the meaning of the result outside of the laboratory environment; there can be no confusion over the statistical outcome. Furthermore, the use of traditional statistics in this study enable the findings to be easily compared and contrasted against previous literature, which can therefore contribute to a greater understanding in the field.

Previous research has reported significant performance decrements following similar protocols (3,28,39). The differences in our findings may be due to the training status of our participants. It appears that the highly-trained athletes in this study exhibited a level of resilience in their ability to perform endurance exercise following short-term IT, almost independently of the nutritional intervention, despite experiencing significant declines in mood state and hormonal disruptions.

It is understood that carbohydrate consumption is an important component of an athlete's diet, both in its ability to enhance physical performance (3,26,28) and to enhance recovery after exercise (31,34). Furthermore, low carbohydrate diets have been implicated with suppressed immune function (40) and increased stress hormones responses to exercise (3) in athletic populations. A small collection of studies have investigated the effects of high versus low/moderate carbohydrate diets during periods of intensified training and reported attenuation in performance decrements (3,28). Differences in our outcome measurements between conditions were not as clear as have been previously reported (3,28). The carbohydrate intervention completed in this study resulted in high (HCHO: 10 g/kg/day) and moderate (CON: 7 g/kg/day) dietary CHO intakes whereas other studies have compared high (10.0-8.5 g/kg/day) versus considerably lower (5.4-5.0 g/kg/day) doses of carbohydrate and have found more pronounced differences between conditions (28,41,42). Results from this study fall more closely in line with findings from Lamb and colleagues who found no difference in maintenance of swim intensity following 9 days of training between high (12.1 g/kg/day) and a moderate (6.5 g/kg/day) carbohydrate interventions (43). Furthermore, Sherman and colleagues observed no change in time to exhaustion following 7 days of cycling training between high (10 g/kg/day) and low (5 g/kg/day) carbohydrate groups, despite significant reductions in muscle glycogen in the low group (44). It is important to

note that neither of these studies increased the training load of participants during the testing periods, which may explain the absence of a significant change in performance. Participants in the present study displayed a level of tolerance to IT that has not previously been observed, potentially due to the high aerobic capacity of the cyclists which was approximately 9 ml/kg/min greater than the aforementioned studies (3,28).

A recent publication has also highlighted the importance of protein intake during periods of IT (45). High dietary protein (3 g/kg/day) attenuated endurance performance decrements in trained cyclists undergoing 7 of days IT compared to a moderate protein diet (1.5 g/kg/day) (45). The addition of small amount of protein to the post-exercise recovery beverage in the present study resulted in a significant increase in total dietary protein in HCHO compared to CON and may have provided an additional stimulus for immediate post-exercise recovery that was absent in CON. The ingestion of protein during recovery from endurance exercise has been shown to improve endurance capacity of subsequent exercise sessions compared to carbohydrate alone (46,47). It is therefore possible that the additional protein, in combination with the additional carbohydrate in HCHO, contributed to the small differences in endurance performance and hormone responses to exercise observed in this study. It is of further interest that Witard and colleagues reported an association between high dietary protein during IT and fewer symptoms of URTI and suggest that a high protein diet may support the immune system during periods of high intensity training (45).

It is understood that moderate intensity, regular exercise can enhance immune function above that of a sedentary individual, but that prolonged strenuous exercise or periods of increased training load can be immunosuppressive (48). Mucosal immunity is an essential first line of defence of the immune system and its popularity for measurement in athletic populations is ever increasing due to its non-invasive sampling methods and relatively straightforward analysis. Salivary IgA is perhaps the most commonly measured AMP in saliva and changes in SIgA have been reported during periods of IT and/or chronic stress (20) and suggested to be linked to HPA axis activity. Interestingly, we did not observe any significant changes in SIgA in our participants during IT, or any differences between conditions. A small collection of studies have also failed to observe differences in SIgA concentrations with carbohydrate nutritional interventions during exercise (24,49,50). However, it is unusual that we did not observe any changes over the period of IT. Both the inter- and intra-subject variability was high, which may explain the absence of a clear trend. We did, however, observe significant

increases in SLys concentration and secretion rate with exercise. Despite a paucity of data on SLys responses to exercise, it has been proposed as a potential marker of both psychological (51) and physical stress (52), making it a potentially interesting marker of the OTS. Previous research has shown transient increases in SLys with exercise (53) and suggested that its increase may be the result of changes in sympathetic nervous activity resulting in mobilisation of SLys to the saliva, providing a temporary enhancement of immune function. Interestingly, over the course of IT, we observed a significant attenuation in the post-exercise increase in SLys secretion rate. Whilst the regulation of SLys is not entirely understood, reductions in SLys concentrations have been attributed to secretion of glucocorticoids (51) and thus activation of the HPA axis. Our data report a suppression of SLys with IT. Furthermore, the increase in SLys with exercise was lower in CON compared to HCHO and a larger attenuation in this increase was seen over IT. These data suggest HCHO may have supported the mobilization of SLys to the saliva via sympathetic nervous activation. In line with these findings, SLac concentrations were significantly higher in HCHO than CON throughout IT. SLac concentrations also increased acutely with exercise. There was no change in SLac secretion rates at baseline or post-exercise following IT.

Hormonal disturbances during training, IT and under conditions of OR or OTS have been well documented in recent years. However the ability of practitioners to utilise these data in a meaningful way with their athletes is not as straight forward. Clearly, there is huge individual variability in hormonal responses to exercise, therefore a change in an individual hormone or a single threshold value during a season could never been sufficient in monitoring training load. In addition to standard measures of stress, including alterations to hypothalamic control and subsequent glucocorticoid secretion, we were interested in changes in prolactin concentrations. Prolactin is an immunoregulatory hormone that has previously been suggested to be affected by chronic stress (2,54). Meeusen and colleagues observed suppressed exercise-induced prolactin concentrations in athletes diagnosed as OT. Indeed, we observed a significant change in post-exercise response of prolactin over IT from an increased concentration (TT1) to a complete attenuation of prolactin (TT3) in just 9 days. The link between prolactin suppression and training stress has been proposed to be the result of altered central dopaminergic or serotonin activity due to accumulating fatigue (2,55). The suppression in prolactin suggests an increase in immunosuppression during IT. Furthermore, analysis of CORT and ACTH, showing attenuated post-exercise concentrations provide evidence of HPA axis disturbance. Previous research has shown elevated CORT in low

carbohydrate groups, compared with high following one-off endurance exercise (56) and following IT (3); however, no condition effects were reported in this study. Interestingly, baseline ACTH tended to be lower in CON compared with HCHO during IT, suggesting some level of reduced adrenocortical secretion or reactivity, or enhanced negative feedback inhibition of the HPA axis (3) in the CON condition. Furthermore, whilst we found no effect of IT on serum testosterone levels, we did observe significantly lower post-exercise levels in CON, compared with HCHO following IT. Lowered testosterone levels result from a decrease in steroid synthesis which is thought to be a consequence of physiological and/or psychological strain (54).

In addition to ACTH and prolactin, additional pituitary hormones were analysed. In vitro studies have reported inhibition of LH by glucocorticoids (57), however one study of professional cyclists during a 3-week multi-stage competition (Vuelta a Espana) found no changes in LH or FSH following the race (58). To the authors' knowledge, no studies have investigated the effects of short term intensified training on LH and FSH in line with the more commonly measured pituitary hormones. Whilst we did not observe changes in baseline LH or FSH or differences between conditions, we did see post-exercise increases in LH following IT, which could be associated with the attenuated CORT and ACTH responses to exercise after IT.

In addition to the measurement of neuroendocrine function, we were interested in specific neurobiological changes during short term periods of IT. Neurotrophic factors, in particular BDNF, are understood to play a role in central and peripheral energy metabolism in skeletal muscle (59). It is thought that exercise is a key stimulus for mediation of this process (60) due to its characteristics as a contraction-induced protein in the skeletal muscle (61). In a recent review, Knaepen and colleagues reported that 69% of healthy participants showed a 'mostly transient' increase in serum BDNF concentrations following acute aerobic exercise (60) and that the increase in post-exercise BDNF concentrations appears to occur in a dose response manner with exercise intensity. A number of studies have investigated the effects of aerobic training in healthy populations on baseline and post-exercise BDNF concentrations and have reported mixed findings. No training-induced effects on basal BDNF were observed by Schiffer and colleagues (71), while the three studies that adopted more intense aerobic training interventions, reported elevated basal plasma BDNF concentrations following the training period (13,62,63). Elevated pre- to post-exercise changes in BDNF have also been

observed following aerobic training (13,63,64). As BDNF is a key component of the hypothalamic pathway (65), which is understood to be disrupted as a result of periods of intense exercise training, it was of interest to observe BDNF concentrations in this study. As predicted, our results showed an acute increase in post-exercise BDNF; however, we also observed a significant attenuation in post-exercise concentrations from TT1 to TT3. In addition, we observed a tendency for decreased baseline BDNF concentrations following IT (9 of 13 participants decreased; p=0.08). It is also of interest to note that the percentage increase in BDNF concentrations at TT3 were almost double in HCHO (22%) compared to CON (12%), although this was not statistically significant. It appears that IT had a suppressive effect on BDNF concentrations, not only at baseline, but in the typical response to exercise. Low levels of BDNF have been associated with neurodegenerative diseases and depression thus BDNF could potentially be considered as a tool for monitoring training load and identifying OR or OT within athletic populations, especially considering the prevalence of significant mood disturbance in overtrained athletes.

This study not only provides a novel insight into neurobiological and neuroendocrine changes in well trained athletes undergoing short term IT, but also supports previous research in the common goal of identifying the changes that occur during strenuous and prolonged exercise in the aim to understand the aetiology of the OTS. In line with previous studies, the maintenance of mood state was favoured by HCHO, but its decline could not be prevented. All participants experienced disturbed mood state during IT. The DALDA appeared to be a less sensitive indicator of mood state when compared to the POMS-24 and 65 as was less able to identify a condition effect. Whilst only minor differences were observed in exercise performance between nutrition interventions, the authors believe this study highlights the importance of carbohydrate availability during exercise for maintenance of performance during IT, perhaps independent of absolute volume of carbohydrate per day. In addition to measuring the impact of macronutrients during IT, this is the first study to investigate the importance of micronutrient status, specifically the impact of vitamin D status on performance. It is interesting to observe the significant correlation between vitamin D status and better maintenance of TT performance following IT. It is of further interest to note that all athletes presenting a vitamin D insufficiency (<75 nmol/L; (66)) experienced a decline in performance and those that were classified as sufficient (≥75 nmol/L) experienced no change or an improvement in TT performance. This finding supports the current literature suggesting the importance of vitamin D status for athletic performance (67-69) and highlights the

importance of vitamin D status for endurance athletes undergoing demanding training programmes.

Based on the findings of this study, the authors conclude that a combination of carefully selected markers; such as prolactin, BDNF, salivary AMPs and vitamin D should be included in general athlete screening and may be useful in monitoring of training stress when used in combination with performance, mood state and the more commonly measured glucocorticoids and hormonal markers of HPA axis functionality. Each measurement is highly individual, as are the responses to training; both acute and chronic. Repeated sampling throughout a season will help identify abnormal responses to exercise training and is strongly advised for athletes prone to overtraining or those regularly undergoing periods of intensified training.

4.6 Acknowledgments

4.7 References

(1) Rhea MR, Alderman BL. A meta-analysis of periodized versus nonperiodized strength and power training programs. Res Q Exerc Sport 2004 Dec;75(4):413-422.

(2) Meeusen R, Piacentini MF, Busschaert B, Buyse L, De Schutter G, Stray-Gundersen J. Hormonal responses in athletes: the use of a two bout exercise protocol to detect subtle differences in (over)training status. Eur J Appl Physiol 2004 Mar;91(2-3):140-146.

(3) Halson S, Lancaster G, Achten J, Gleeson M, Jeukendrup A. Effects of carbohydrate supplementation on performance and carbohydrate oxidation after intensified cycling training. J Appl Physiol 2004 OCT;97(4):1245-1253.

(4) Meeusen R, Duclos M, Foster C, Fry A, Gleeson M, Nieman D, et al. Prevention, diagnosis and treatment of the overtraining syndrome: Joint consensus statement of the European College of Sport Science (ECSS) and the American College of Sports Medicine (ACSM). EJSS 2012;1-24(iFirst article).

(5) Galbo H. The hormonal response to exercise. Diabetes Metab Rev 1986;1(4):385-408.

(6) Barron JL, Noakes TD, Levy W, Smith C, Millar RP. Hypothalamic dysfunction in overtrained athletes. J Clin Endocrinol Metab 1985 Apr;60(4):803-806.

(7) Dishman RK, Berthoud HR, Booth FW, Cotman CW, Edgerton VR, Fleshner MR, et al. Neurobiology of exercise. Obesity (Silver Spring) 2006 Mar;14(3):345-356.

(8) Yarrow JF, White LJ, McCoy SC, Borst SE. Training augments resistance exercise induced elevation of circulating brain derived neurotrophic factor (BDNF). Neurosci Lett 2010 Jul 26;479(2):161-165.

(9) Ferris LT, Williams JS, Shen CL. The effect of acute exercise on serum brain-derived neurotrophic factor levels and cognitive function. Med Sci Sports Exerc 2007 Apr;39(4):728-734.

(10) Gold SM, Schulz KH, Hartmann S, Mladek M, Lang UE, Hellweg R, et al. Basal serum levels and reactivity of nerve growth factor and brain-derived neurotrophic factor to standardized acute exercise in multiple sclerosis and controls. J Neuroimmunol 2003 May;138(1-2):99-105.

(11) Matthews VB, Astrom MB, Chan MH, Bruce CR, Krabbe KS, Prelovsek O, et al. Brain-derived neurotrophic factor is produced by skeletal muscle cells in response to contraction and enhances fat oxidation via activation of AMP-activated protein kinase. Diabetologia 2009 Jul;52(7):1409-1418.

(12) Rasmussen P, Brassard P, Adser H, Pedersen MV, Leick L, Hart E, et al. Evidence for a release of brain-derived neurotrophic factor from the brain during exercise. Exp Physiol 2009 Oct;94(10):1062-1069.

(13) Seifert T, Brassard P, Wissenberg M, Rasmussen P, Nordby P, Stallknecht B, et al. Endurance training enhances BDNF release from the human brain. Am J Physiol Regul Integr Comp Physiol 2010 Feb;298(2):R372-7.

(14) Zoladz JA, Pilc A, Majerczak J, Grandys M, Zapart-Bukowska J, Duda K. Endurance training increases plasma brain-derived neurotrophic factor concentration in young healthy men. J Physiol Pharmacol 2008 Dec;59 Suppl 7:119-132.

(15) Lehmann M, Gastmann U, Petersen KG, Bachl N, Seidel A, Khalaf AN, et al. Training-overtraining: performance, and hormone levels, after a defined increase in training volume versus intensity in experienced middle- and long-distance runners. Br J Sports Med 1992 Dec;26(4):233-242.

(16) Budgett R, Hiscock N, Arida RM, Castell LM. The effects of the 5-HT2C agonist m-chlorophenylpiperazine on elite athletes with unexplained underperformance syndrome (overtraining). Br J Sports Med 2010 Mar;44(4):280-283.

(17) Colthorpe KL, Nalliah J, Anderson ST, Curlewis JD. Adrenoceptor subtype involvement in suppression of prolactin secretion by noradrenaline. J Neuroendocrinol 2000 Apr;12(4):297-302.

(18) Thorre K, Chaouloff F, Sarre S, Meeusen R, Ebinger G, Michotte Y. Differential effects of restraint stress on hippocampal 5-HT metabolism and extracellular levels of 5-HT in streptozotocin-diabetic rats. Brain Res 1997 Oct 24;772(1-2):209-216.

(19) Urhausen A, Gabriel HH, Kindermann W. Impaired pituitary hormonal response to exhaustive exercise in overtrained endurance athletes. Med Sci Sports Exerc 1998 Mar;30(3):407-414.

(20) Walsh NP, Gleeson M, Shephard RJ, Gleeson M, Woods JA, Bishop NC, et al. Position statement. Part one: Immune function and exercise. Exerc Immunol Rev 2011;17:6-63.

(21) Tomasi TB, Trudeau FB, Czerwinski D, Erredge S. Immune parameters in athletes before and after strenuous exercise. J Clin Immunol 1982 Jul;2(3):173-178.

(22) Mackinnon LT. Immunoglobulin, antibody and exercise Exerc Immunol Rev 1996;2:1-34.

(23) Steerenberg PA, van Asperen IA, van Nieuw Amerongen A, Biewenga A, Mol D, Medema GJ. Salivary levels of immunoglobulin A in triathletes. Eur J Oral Sci 1997 Aug;105(4):305-309.

(24) Nieman DC, Henson DA, Fagoaga OR, Utter AC, Vinci DM, Davis JM, et al. Change in salivary IgA following a competitive marathon race. Int J Sports Med 2002 Jan;23(1):69-75.

(25) Gleeson M, Bishop N, Oliveira M, Tauler P. Influence of training load on upper respiratory tract infection incidence and antigen-stimulated cytokine production. Scand J Med Sci Sports 2013 Aug;23(4):451-457.

(26) Bergstrom J, Hultman E. The effect of exercise on muscle glycogen and electrolytes in normals. Scand J Clin Lab Invest 1966;18(1):16-20.

(27) Snyder AC, Kuipers H, Cheng B, Servais R, Fransen E. Overtraining following intensified training with normal muscle glycogen. Med Sci Sports Exerc 1995 Jul;27(7):1063-1070.

(28) Achten J, Halson SL, Moseley L, Rayson MP, Casey A, Jeukendrup AE. Higher dietary carbohydrate content during intensified running training results in better maintenance of performance and mood state. J Appl Physiol 2004 Apr;96(4):1331-1340.

(29) Snyder AC, Kuipers H, Cheng B, Servais R, Fransen E. Overtraining following intensified training with normal muscle glycogen. Med Sci Sports Exerc 1995 Jul;27(7):1063-1070.

(30) American Dietetics Association, Dietitians of Canada. Nutrition and Athletic Performance, Joint Position Statement. Medicine and Science in Sport and Exercise 2009.

(31) Maughan RJ, Shirreffs SM. IOC Consensus Conference on Nutrition in Sport, 25–27 October 2010, International Olympic Committee, Lausanne, Switzerland Journal of Sports Sciences 2011;29(S1):S3-S4.

(32) Smith JW, Pascoe DD, Passe DH, Ruby BC, Stewart LK, Baker LB, et al. Curvilinear dose-response relationship of carbohydrate (0-120 g.h(-1)) and performance. Med Sci Sports Exerc 2013 Feb;45(2):336-341.

(33) Witard OC, Jackman SR, Kies AK, Jeukendrup AE, Tipton KD. Effect of increased dietary protein on tolerance to intensified training. Med Sci Sports Exerc 2011 Apr;43(4):598-607.

(34) Burke LM, Cox GR, Culmmings NK, Desbrow B. Guidelines for daily carbohydrate intake: do athletes achieve them? Sports Med 2001;31(4):267-299.

(35) Garth AK, Burke LM. What do athletes drink during competitive sporting activities? Sports Med 2013 Jul;43(7):539-564.

(36) Halson S, Bridge M, Meeusen R, Busschaert B, Gleeson M, Jones D, et al. Time course of performance changes and fatigue markers during intensified training in trained cyclists. J Appl Physiol 2002 SEP;93(3):947-956.

(37) Larson-Meyer E. Vitamin D supplementation in athletes. Nestle Nutr Inst Workshop Ser 2013;75:109-121.

(38) Meeusen R, Piacentini M, Busschaert B, Buyse L, De Schutter G, Stray-Gundersen J. Hormonal responses in athletes: the use of a two bout exercise protocol to detect subtle differences in (over)training status. Eur J Appl Physiol 2004 MAR;91(2-3):140-146.

(39) Rietjens GJ, Kuipers H, Adam JJ, Saris WH, van Breda E, van Hamont D, et al. Physiological, biochemical and psychological markers of strenuous training-induced fatigue. Int J Sports Med 2005 Jan-Feb;26(1):16-26.

(40) Burke LM. Fueling strategies to optimize performance: training high or training low? Scand J Med Sci Sports 2010 Oct;20 Suppl 2:48-58.

(41) Simonsen JC, Sherman WM, Lamb DR, Dernbach AR, Doyle JA, Strauss R. Dietary carbohydrate, muscle glycogen, and power output during rowing training. J Appl Physiol (1985) 1991 Apr;70(4):1500-1505.

(42) Halson SL, Lancaster GI, Achten J, Gleeson M, Jeukendrup AE. Effects of carbohydrate supplementation on performance and carbohydrate oxidation after intensified cycling training. J Appl Physiol (1985) 2004 Oct;97(4):1245-1253.

(43) Lamb DR, Rinehardt KF, Bartels RL, Sherman WM, Snook JT. Dietary carbohydrate and intensity of interval swim training. Am J Clin Nutr 1990 Dec;52(6):1058-1063.

(44) Sherman WM, Doyle JA, Lamb DR, Strauss RH. Dietary carbohydrate, muscle glycogen, and exercise performance during 7 d of training. Am J Clin Nutr 1993 Jan;57(1):27-31.

(45) Witard OC, Turner JE, Jackman SR, Kies AK, Jeukendrup AE, Bosch JA, et al. High dietary protein restores overreaching induced impairments in leukocyte trafficking and reduces the incidence of upper respiratory tract infection in elite cyclists. Brain Behav Immun 2014 Jul;39:211-219.

(46) Saunders MJ, Kane MD, Todd MK. Effects of a carbohydrate-protein beverage on cycling endurance and muscle damage. Med Sci Sports Exerc 2004 Jul;36(7):1233-1238.

(47) Niles ES, Lachowetz T, Garfi J, Sullivan W, Smith JC, Leyh BP, et al. Carbohydrate-protein drink improves time to exhaustion after recovery from endurance exercise. Journal of Exercise Physiology 2001;4(1):45.

(48) Gleeson M, Bishop N, Walsh NP. Exercise Immunology. London: Routledge; 2013.

(49) Bishop NC, Blannin AK, Armstrong E, Rickman M, Gleeson M. Carbohydrate and fluid intake affect the saliva flow rate and IgA response to cycling. Med Sci Sports Exerc 2000 Dec;32(12):2046-2051.

(50) Li TL, Gleeson M. The effects of carbohydrate supplementation during repeated bouts of prolonged exercise on saliva flow rate and immunoglobulin A. J Sports Sci 2005 Jul;23(7):713-722.

(51) Perera S, Uddin M, Hayes JA. Salivary lysozyme: a noninvasive marker for the study of the effects of stress of natural immunity. Int J Behav Med 1997;4(2):170-178.

(52) Koutedakis Y, Sabin E, Perera S. Modulation of salivary lysozyme by training in elite male swimmers. Journal of Sports Sciences 1996;14(90).

(53) Allgrove JE, Gomes E, Hough J, Gleeson M. Effects of exercise intensity on salivary antimicrobial proteins and markers of stress in active men. J Sports Sci 2008 Apr;26(6):653-661.

(54) Gomez-Merino D, Chennaoui M, Burnat P, Drogou C, Guezennec CY. Immune and hormonal changes following intense military training. Mil Med 2003 Dec;168(12):1034-1038.

(55) Collomp K, Arlettaz A, Portier H, Lecoq AM, Le Panse B, Rieth N, et al. Short-term glucocorticoid intake combined with intense training on performance and hormonal responses. Br J Sports Med 2008 Dec;42(12):983-988.

(56) Mitchell JB, Pizza FX, Paquet A, Davis BJ, Forrest MB, Braun WA. Influence of carbohydrate status on immune responses before and after endurance exercise. J Appl Physiol (1985) 1998 Jun;84(6):1917-1925.

(57) Bambino TH, Hsueh AJ. Direct inhibitory effect of glucocorticoids upon testicular luteinizing hormone receptor and steroidogenesis in vivo and in vitro. Endocrinology 1981 Jun;108(6):2142-2148.

(58) Fernandez-Garcia B, Lucia A, Hoyos J, Chicharro JL, Rodriguez-Alonso M, Bandres F, et al. The response of sexual and stress hormones of male pro-cyclists during continuous intense competition. Int J Sports Med 2002 Nov;23(8):555-560.

(59) Matthews VB, Astrom MB, Chan MH, Bruce CR, Krabbe KS, Prelovsek O, et al. Brain-derived neurotrophic factor is produced by skeletal muscle cells in response to contraction and enhances fat oxidation via activation of AMP-activated protein kinase. Diabetologia 2009 Jul;52(7):1409-1418.

(60) Knaepen K, Goekint M, Heyman EM, Meeusen R. Neuroplasticity - exercise-induced response of peripheral brain-derived neurotrophic factor: a systematic review of experimental studies in human subjects. Sports Med 2010 Sep 1;40(9):765-801.

(61) Pedersen BK, Pedersen M, Krabbe KS, Bruunsgaard H, Matthews VB, Febbraio MA. Role of exercise-induced brain-derived neurotrophic factor production in the regulation of energy homeostasis in mammals. Exp Physiol 2009 Dec;94(12):1153-1160.

(62) Baker LD, Frank LL, Foster-Schubert K, Green PS, Wilkinson CW, McTiernan A, et al. Effects of aerobic exercise on mild cognitive impairment: a controlled trial. Arch Neurol 2010 Jan;67(1):71-79.

(63) Zoladz JA, Pilc A, Majerczak J, Grandys M, Zapart-Bukowska J, Duda K. Endurance training increases plasma brain-derived neurotrophic factor concentration in young healthy men. J Physiol Pharmacol 2008 Dec;59 Suppl 7:119-132.

(64) Schulz KH, Gold SM, Witte J, Bartsch K, Lang UE, Hellweg R, et al. Impact of aerobic training on immune-endocrine parameters, neurotrophic factors, quality of life and coordinative function in multiple sclerosis. J Neurol Sci 2004 Oct 15;225(1-2):11-18.

(65) Wisse BE, Schwartz MW. The skinny on neurotrophins. Nat Neurosci 2003 Jul;6(7):655-656.

(66) Larson-Meyer E. Vitamin D supplementation in athletes. Nestle Nutr Inst Workshop Ser 2013;75:109-121.

(67) Close GL, Russell J, Cobley JN, Owens DJ, Wilson G, Gregson W, et al. 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 2013;31(4):344-353.

(68) Rejnmark L. Effects of vitamin d on muscle function and performance: a review of evidence from randomized controlled trials. Ther Adv Chronic Dis 2011 Jan;2(1):25-37.

(69) Wyon MA, Koutedakis Y, Wolman R, Nevill AM, Allen N. The influence of winter vitamin D supplementation on muscle function and injury occurrence in elite ballet dancers: a controlled study. J Sci Med Sport 2014 Jan;17(1):8-12.

(70) Hopkins W. Statistical Analysis and Data Interpretation: an Introduction. Sportscience 2013;17(ii).

(71) Schiffer T, Schulte S, Hollmann W, Bloch W, Struder HK. Effects of strength and endurance training on brain-derived neurotrophic factor and insulin-like growth factor 1 in humans.   Horm   Metab   Res   2009   Mar;41(3):250-254.

CHAPTER 5: EVIDENCE OF DISTURBED SLEEP AND MOOD STATE IN WELL-TRAINED ATHLETES DURING SHORT-TERM INTENSIFIED TRAINING WITH AND WITHOUT A HIGH CARBOHYDRATE NUTRITIONAL INTERVENTION

Sophie C Killer[1], Ida S Svendsen[1], Asker E Jeukendrup[1,2] and Michael Gleeson[1]

[1]School of Sport, Exercise and Health Sciences, Loughborough University, Leicestershire, East Midlands. UK.

[2] Gatorade Sports Science Institute, Loughborough. LE11 3TU. UK

Conceived and designed the experiments: SCK, MG, AEJ
Performed the experiments: SCK, ISS
Analyzed the data: SCK
Contributed reagents/materials/analysis tools: SCK, MG, AEJ
Wrote the paper: SCK.
Statistical analysis: SCK
Editing of final paper: SCK, MG, AEJ

This chapter includes data collected from a larger study (chapter 4). A brief re-introduction to overreaching and overtraining is present in the introduction to remind the reader of the background of this study. A small selection of data from chapter 4 has been included within this chapter to re-familiarise the reader of the methods and to help portray the context of the results. However the main focus of this chapter is on the sleep data collected and analysed with actigraphy.

5.1 Abstract

Few studies have investigated the effects of exercise training on sleep physiology in well–trained athletes. We investigated changes in sleep markers, mood state and exercise performance in well-trained cyclists undergoing short-term intensified training (IT) and observed the effects of a high carbohydrate intervention. Thirteen highly-trained male cyclists (age: 25±6y, $\dot{V}O_{2max}$: 72±5ml/kg/min) participated in two 9-day periods of IT while undergoing a high (HCHO) or moderate (CON) carbohydrate nutritional intervention before, during and after training sessions. Sleep was measured each night via wristwatch actigraphy. Mood state questionnaires were completed daily. Performance was assessed with maximal oxygen uptake ($\dot{V}O_{2max}$). Time in bed increased during IT (456±50 to 509±48min; p=0.02) however percentage sleep time fell (87.9±1.5 to 82.5±2.3%; p<0.05). Actual sleep time was significantly higher in CON than HCHO throughout IT. Sleep efficiency decreased during IT (83.1±5.3 to 77.8±8.6%; p<0.05). Mood disturbance increased during IT and was higher in CON than HCHO (p<0.05). Performance in the $\dot{V}O_{2max}$ exercise protocol fell significantly with IT. The main findings of this study were that as little as 9-days of IT in highly-trained cyclists resulted in significant and progressive declines in sleep quality. In addition, we observed significant declines in mood state and maximal exercise performance.

Key Words: Sleep, Cycling, Intensified training, Actigraphy.

5.2 Introduction

It is understood that several compounding factors, such as poor nutrition, inadequate sleep, illness and psychological stressors can augment the development of overreaching (OR). Sleep is considered to be the 'gold standard' post-exercise recovery procedure amongst athletes (1,2). During sleep, muscles are in a complete state of relaxation allowing effective myofibre restoration. This process is further enhanced by the release of growth hormone from the pituitary gland during the night which stimulates protein synthesis and has important effects on muscle and bone growth, repair and adaptation (3). When sleep quality or duration (or combinations of the two) become compromised, there can be a significant detrimental impact on human functioning and mood state (4). Sleep data from normal athletic populations is scarce, but sleep deprivation studies in athletes have reported increased errors, impaired decision making, reduced maximal power, increased fatigue (5) and an attenuation of the ability to perform maximal exercise, as both aerobic and anaerobic pathways are affected (3).

Research has shown numerous beneficial effects of sleep on exercise performance, and equally several studies have reported positive effects of exercise on sleep physiology (6,7). Regular physical activity has been shown to improve self-reported sleep compared with a sedentary lifestyle (3,8,9). It is also understood that exercise improves mood state (10,11), which can be an additional factor in improving (or disturbing) sleep (12). Acute exercise in the evenings has been suggested to have positive effects on sleep (7,13,14), especially when it is of moderate intensity (15). However, it has been proposed that high-intensity exhaustive exercise may be disruptive to sleep by causing decreased rapid eye movement (REM) sleep and increasing wakefulness (9)(9). It has also reported that elite athletes experience poorer sleep quality than age-matched sedentary individuals, but still remain within the range for healthy sleep (16). Research to date suggests that interactions between sleep and exercise do exist, but are not yet fully understood.

Relatively few studies have investigated the effects of exercise training on sleep physiology in athletic populations (17). Thus, the manner in which sleep duration and efficiency are affected by strenuous exercise and training remains unclear (18). Previous sleep research has primarily focused on physically active groups of 'good sleepers' whereby the exercise stress is low and the margin for sleep improvement is relatively small (ceiling effect) (9). A small collection of studies have reported sleeping behaviours in athletes. Taylor and colleagues

monitored sleep patterns with polysomnography in female swimmers across a competitive season. The authors reported increased movement time during sleep during the busy parts of the season (beginning and peak) compared to during a taper and concluded that some sleep disruption was present during these times. The authors also reported increases in slow wave sleep during these busy periods which suggested improved sleep quality and thereby supporting the restorative theory of sleep (19). More recently, a study used actigraphy to monitor sleeping patterns of athletes experiencing overreaching (20). The authors reported reduced sleep efficiency, duration and immobile time in athletes who were classified as being overreached following three weeks of overload training (30% increase in training volume from baseline). Another study investigated the sleep quality of professional ballerinas during the lead up to a Premier performance (18). This is a period of increased training volume in an environment that is often highly stressful. Fietze and colleagues used actigraphy to monitor sleep parameters throughout the study and observed impaired sleep efficiency and duration, increased time awake after sleep onset and reduced time in bed. It is difficult to draw parallels between this study and well-trained endurance athletes undergoing intensified training periods, although it may be possible that any period of increased training load, with or without heightened stress and anxiety, may have an impact on an athlete's sleeping behaviours. Fietze and colleagues concluded that dancers should focus more attention on relaxation and sleep-wake behaviour (i.e. napping) during high stress periods of training, in addition to their physical condition and nutrition.

The effects of nutritional interventions on various sleep parameters have been investigated over recent years. Aside from testing numerous herbal extracts, research has focussed on how ingestion of different macronutrients may impact sleep. A small number of studies have investigated the effects of different macronutrients and/or total energy consumed prior to bedtime (21-24) or overall high carbohydrate (CHO) vs. low CHO diets (25) on sleep quality. These data are limited and equivocal, likely due to varying study designs; however, based on the available information, it has been suggested that reduced energy intakes may result in poorer sleep (26). No studies to date have investigated the effects of targeted nutrition interventions during exercise on sleep parameters. The timing of nutrient intake to ensure high availability for performance and recovery is of great importance to athletes, but how this may impact sleep and thus, longer term recovery remains unknown.

Given the known restorative qualities of sleep for athletic performance, it is of great interest to understand how periods of intensified training affect sleep quality in trained athletes. It is possible that either training will elicit a level of stress that induces fatigue and the body responds with enhanced or at least sufficient sleep or that both the physiological and the psychological stresses associated with intensified training programmes will result in a reduction in sleep efficiency and/or duration. It is of further interest to understand how a training-specific high CHO nutritional intervention may impact not only performance and mood state, but subsequent sleep parameters compared with a moderate CHO alternative. Interestingly, sleep deprivation can slow glucose metabolism (reducing the rate of glucose disposal and blunting the insulin response) by as much as 30-40% (27), which has implications for glycogen resynthesis for athletes which could hinder subsequent exercise performance. Therefore, there may be important links between sleep, exercise and nutrition that are yet to be understood.

The aims of this study were to investigate the impact of 9-days of intensified training in well-trained cyclists on sleep quality, through analysis of nocturnal actigraphy, and mood state. Secondly, we aimed to understand the effects of a high vs. moderate CHO intervention on sleep parameters during intensified training. Ultimately, we aimed to gain insight into the sleeping behaviours of well training athletes undergoing a training camp style intervention.

5.3 Methods

Participants

Thirteen highly-trained cyclists (age: 25.0 ± 5.8 y, maximal oxygen uptake ($\dot{V}O_{2max}$): 72.2 ± 4.9 ml/kg/min, height: 178.3 ± 3.9 cm, body mass: 69.8 ± 6.3 kg, body fat: $13.4 \pm 4.1\%$) completed the study. Participants were included if they had a competitive cycling history of at least 3 y, were currently cycling at least three times per week for a minimum of 2 h/day and had a $\dot{V}O_{2max}$ of ≥ 65 mL/kg/min. Participants were excluded from the study if they were currently suffering from any cycling-related injuries or had experienced any symptoms of URTI in the four weeks prior to the study. Fifteen participants were recruited, two of which were unable to complete the study due to individual circumstances preventing them from training. All participants were informed of the purposes of the study and the risks associated

with the procedures. Written informed consent was obtained from each participant and a general health questionnaire was completed before the study commenced. The study was approved by the Loughborough University ethical advisory committee.

Experimental Design

In a double blind, randomised and counterbalanced, cross-over design, participants underwent two 9-day periods of intensified training (IT) while ingesting one of two nutritional interventions. During the high carbohydrate intervention (HCHO), participants consumed carbohydrate beverages at intakes consistent with current ACSM guidelines (36) before and during exercise, followed by a high carbohydrate and protein recovery beverage in the immediate recovery period (<10 min post-training). During the control condition (CON), participants were provided with lower carbohydrate concentration beverages before, during and after training. No protein was provided in the control condition. Prior to their first trial, participants completed a 2-week pre-trial period to allow baseline measurements to be taken and familiarisation with study procedures. At the beginning and end of each period of IT, participants underwent a $\dot{V}O_{2max}$ test to assess performance. A 10-day washout period separated the two trials. Sleep was quantified each night throughout the trial with wrist watch actigraphy.

Sleep Measurement

Participants were fitted with an actiwatch (MotionWatch 8, CamNtech, Cambridgeshire, UK) during their pre-trial period to obtain baseline sleep scores using wrist watch actigraphy. Baseline data was collected for 5 consecutive nights (mean). Participants continued to wear the actiwatch each night for the duration of the study. The actiwatch is a light-weight, waterproof, wrist-worn tri-axial actigraph designed to provide an empirical measurement of movement throughout the night. The actiwatch uses activity counts to apply published algorithms for measurement on actigraphy data, resulting in a reliable and valid method of sleep research (42). These data were sampled at 50 Hz and processed into 30s epochs. Sleep measurement included; time in bed (TB), actual sleep time (AST), percentage sleep time (ST%) expressed as a percentage of assumed sleep time, sleep efficiency (SE) defined as AST expressed as a percentage of TB, sleep onset latency (SOL), wake bouts (WB) defined as the number of continuous sections categorised as awake in the epoch-by-epoch wake/sleep

categorisation, mobile time (MT) expressed as a percentage of assumed sleep time, and the fragmentation index (FI) defined as the sum of the mobile time (%) and the immobile bouts <=1min (%). The FI is an indication of the degree of fragmentation of the sleep period, and can be used as an indication of sleep quality. Sleep data was collected each night and grouped into four quarters of IT for statistical analysis; sleep period 1 (nights 1-2), 2 (nights 3-4), 3 (nights 5-6) and 4 (nights 7-8). In addition, participants completed a daily sleep diary, reporting morning resting HR, time to bed and wake up time. Participants were provided with a HR monitor to measure overnight heart rate (Polar RS800CX, Polar, Kemple, Finland). Participants were instructed to fit the HR chest strap when preparing for bed to ensure a strong signal was detected by the watch. HR recordings began after turning lights out for the night. Upon waking in the morning, participants were requested to note down their resting heart rate before stopping the recording session and getting out of bed.

For methodological details on training and dietary procedures, performance testing and collection of mood state data, please refer to Chapter 4.

Statistical Analysis

All data were analysed using statistical software (IBM SPSS Statistics 21) and are presented as Mean ± Standard Deviation. Changes in performance parameters, mood state and sleep parameters were analysed using a 2-way repeated measures analysis of variance (ANOVA). A Bonferroni adjustment was included into the analysis to correct for multiple comparisons. Data that were found to be significantly non-normal were log transformed prior to analysis. Between trial and pre- to post-IT changes in performance parameters were determined using an independent samples t-test. Trial order effects were tested using an independent samples t-test to compare group means. The level of significance was set at $p < 0.05$.

5.4 Results

A summary of participants dietary intakes and both the training and performance data can be found in Chapter 4 (page 84).

Sleep Analysis

IT resulted in a significant increase in TB (456 ± 50 to 509 ± 48 min; p=0.02) (figure 5.1) with a non-significant increase in AST and resultant decrease in percentage sleep time (87.9 ± 1.5 to 82.5 ± 2.3%). Overall, AST was significantly higher in CON than HCHO throughout IT (396 ± 11 vs. 377 ± 16 min; p=0.03). No trial order effects were observed between measured sleep parameters. Ten participants successfully recorded complete sets of sleep files from both trials, therefore the number of participants included in the sleep analysis is n=10.

There was a significant progressive increase in the number of WB during the night (28.1 ± 2.8 to 35.0 ± 2.6 bouts; p=0.03) (figure 5.1) and MT (7.7 ± 0.9 to 12.7 ± 1.6 %; p<0.01) throughout IT and a resultant increase in sleep FI (p=0.02) (figure 5.1).

Figure 5.1: Overview of Sleep Measurements
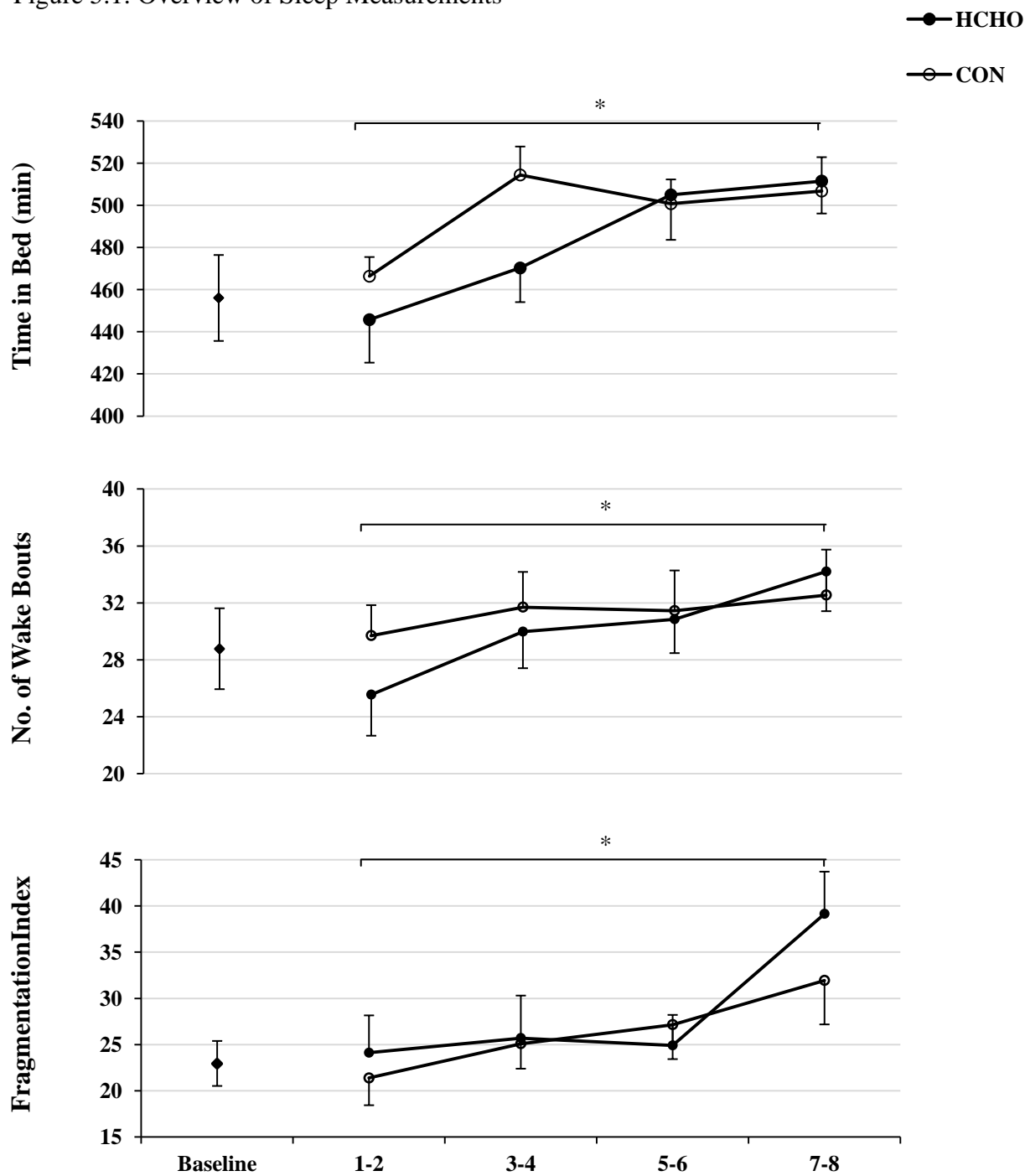
Figure 5.1: Change in sleep parameters from baseline over IT in the high carbohydrate (HCHO) and control (CON) trials. Black circles represent HCHO and white circles represent CON. Data are mean ± SE. * Indicates significant increase with time ($p < 0.05$).

SE decreased significantly with time from beginning to end of IT (83.1 ± 5.3 to 77.8 ± 8.6%; p<0.05) (figure 5.2). No changes were observed in SOL with IT, or between conditions.

Figure 5.2: Sleep Efficiency
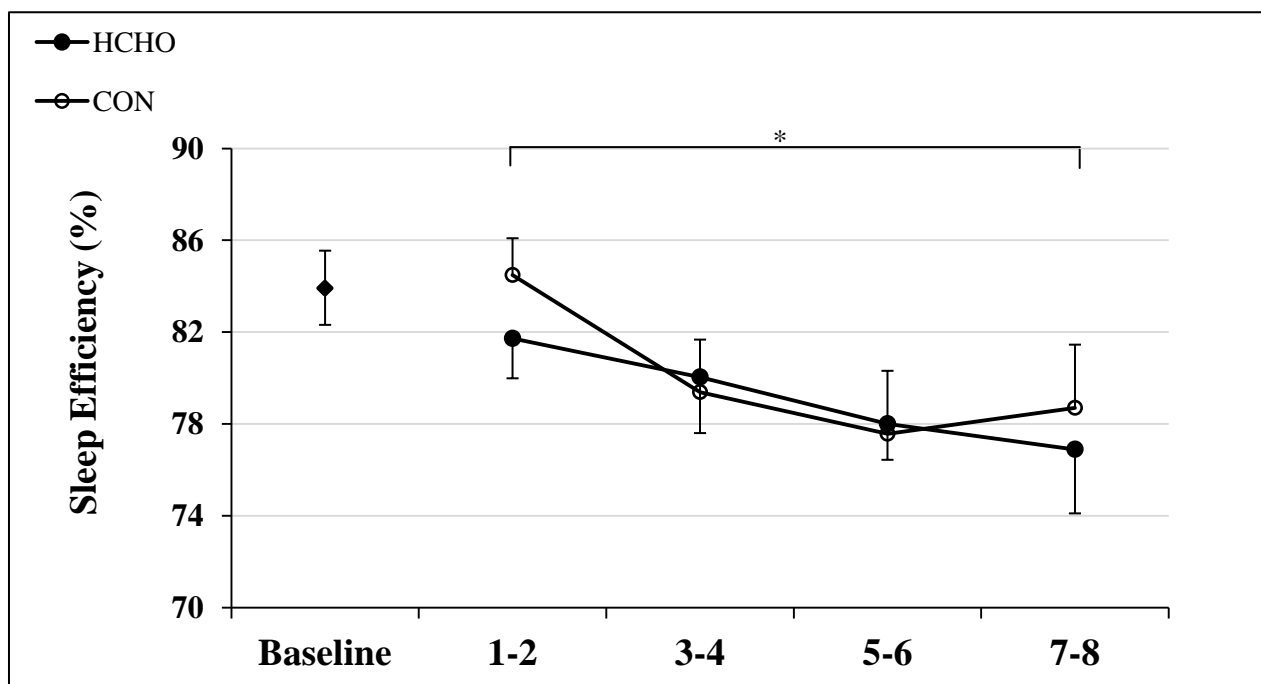
Figure 5.2: Changes in sleep efficiency from baseline over IT in the high carbohydrate (HCHO) and control (CON) trials. Black circles represent HCHO and white circles represent CON. Data are mean ± SE. * Indicates significant decrease with time (p<0.05).

Heart Rate Variables

Morning HR did not change significantly from beginning to end of IT. However, following a non-significant increase during the first quarter of IT in HCHO, HR tended to fall by the end of IT (p=0.08). Post hoc analysis shows morning HR was significantly higher during days 5-6 in CON than HCHO (p=0.01); however, no main effect for condition was observed (figure 5.3).

Figure 5.3: Morning Resting Heart Rate
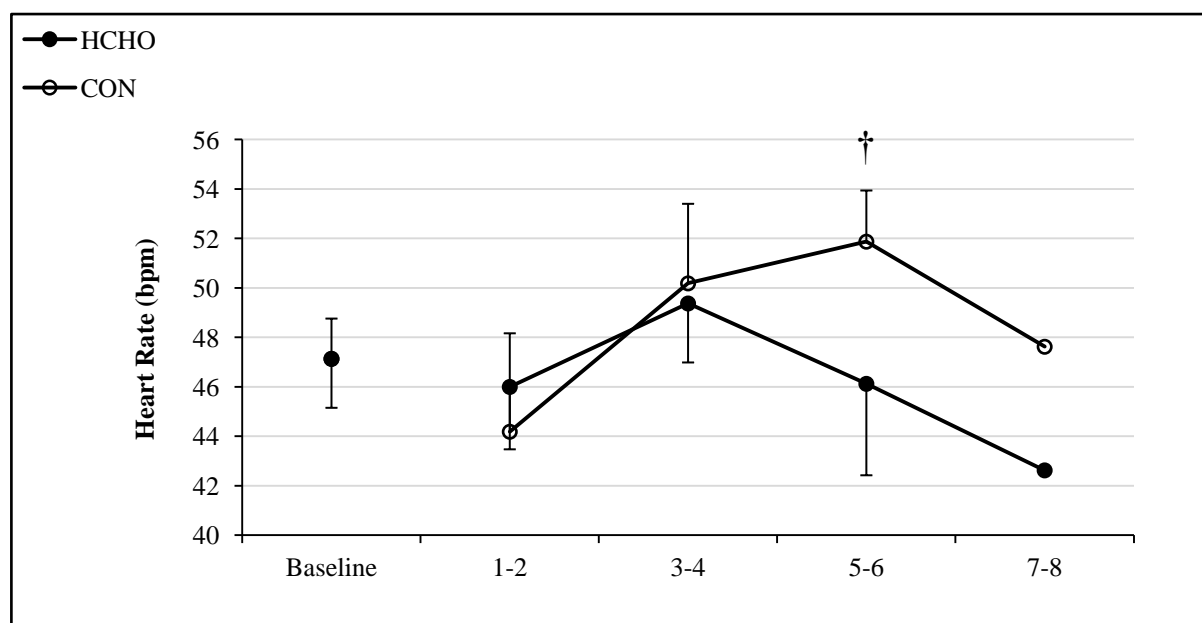
Figure 5.3: Change in resting morning heart rate (HR) from baseline over IT in the high carbohydrate (HCHO) and control (CON) trials. Trials days were grouped in two's for analysis. Black circles represent HCHO and white circles represent CON. Data are mean ± SE. †Indicates significant difference between conditions ($p < 0.05$).

Overnight HR proved difficult to record, with significant losses of signal throughout the night. Complete data sets were collected from 4 participants and therefore have not been included in the final analysis.

5.5 Discussion

The main aim of the present study was to investigate the impact of 9 days of intensified training on the subjective sleep quality of well trained cyclists. Furthermore, we aimed to determine the impact of a high CHO nutritional intervention provided immediately before, during and after each training session, compared with a moderate CHO control condition on sleep quality. The main finding of this study was that as little as 9 days of intensified training in highly-trained cyclists resulted in a significant and progressive decline in sleep quality, as assessed with actigraphy. In addition, we observed significant declines in mood state and

maximal exercise capacity during a test to exhaustion. The high CHO nutritional intervention reduced some, but not all of the detrimental effects of IT.

Sleep is an essential aspect of recovery and fatigue management (1,2)(1,2). Disturbed sleep is often reported as both a contributor (28-30) (28-30)and/or symptom of overreaching (28,29,31,32), however there is a paucity of data to support either of these claims. Whilst a full state of overreaching may not have occurred in all of the participants in this study, many reported performance decrements and declined mood states during the period of IT, thus these findings support the anecdotal evidence and suggested hypothesis that IT, leading to overreaching can result in compromised sleep quality. It is of interest that these athletes increased the time they spent in bed (TB) during IT, suggesting that they were exhibiting symptoms of fatigue; and although the increased TB did not result in an increase in AST, it did compensate for impaired sleep quality such that AST was not significantly changed during IT. Our data revealed that SE was significantly reduced during the intensified training period from 83.1 ± 5.3 to 77.8 ± 8.6 %. Furthermore, there was an increase in the number of WB throughout the night and overall a more fragmented sleep period. In addition, the cyclists experienced significant disruptions to mood state, reporting increased tension, anger, fatigue, confusion, depression and increased feelings and symptoms of stress.

These findings contradict the theory that sleep is proportional to restorative needs and instead fall in line with results from similar studies that have reported impaired SE during periods of intense training (18) or when athletes have become overreached (20). However, it is interesting to note that AST was significantly higher during IT in CON than HCHO, which may indicate an increased requirement for recovery and/or expression of fatigue. It is understood that some forms of training are known to result in detrimental psychological effects (33) and that mood disturbances increase in a progressive manner with step-wise increases in training load (34). Significant increases in POMS-24 and POMS-65 TMD were observed in both conditions as a result of IT. Furthermore, there was a significant increase in DALDA scores for symptoms of stress being 'worse than normal'. These findings support the work of previous research into the psychological impacts of intensified training, overreaching and overtraining in athletes (34-40).

To our knowledge, our study is the first to investigate the effects of intensified training with a high CHO nutritional intervention on sleep parameters. Despite intensified training having a detrimental effect across a range of sleep parameters, the only difference between conditions

observed was significantly longer AST in CON compared with HCHO (396 ± 11 vs. 377 ± 16 min). It was also observed that during the third quarter of IT (days 5-6), HR was significantly higher in CON than HCHO (52 ± 9 vs. 46 ± 7 bpm). These, together, could indicate a greater level of fatigue in the CON condition and that HCHO may have provided a level of protection against the changes in sympathetic nervous activity that are thought to be linked to the OTS.

Overall, these findings suggest that relatively short periods of intensified training, similar to a training camp, can result in significant disruptions to sleep quality and mood state. It is interesting to note that high intensity exercise performance decreased significantly following IT independent of nutritional intervention, which suggests athletes may have been exhibiting early signs of overreaching. The athletes in this study were not aware that the two trials involved varying CHO intakes, therefore it is unlikely that differences observed in mood state were a result of knowing that they were exercising with insufficient energy replacement (according to the current ACSM guidelines (41)). With sleep and passive rest providing an important form of recovery, athletes undergoing such programmes should plan ahead with their coaches to ensure sleep is optimised. Strategies to enhance sleep during these times may include improving sleep hygiene before bedtime, ensuring adequate time in bed and incorporating time to nap into the day routine.

5.6 Acknowledgments

5.7 References

(1) Myllymaki T, Kyrolainen H, Savolainen K, Hokka L, Jakonen R, Juuti T, et al. Effects of vigorous late-night exercise on sleep quality and cardiac autonomic activity. J Sleep Res 2011 Mar;20(1 Pt 2):146-153.

(2) Samuels C. Sleep, recovery, and performance: the new frontier in high-performance athletics. Neurol Clin 2008 Feb;26(1):169-80; ix-x.

(3) Davenne D. Sleep of athletes – problems and possible solutions Biological Rhythm Research 2009;40(1):45-52.

(4) Pilcher JJ, Huffcutt AI. Effects of sleep deprivation on performance: a meta-analysis. Sleep 1996 May;19(4):318-326.

(5) Reilly T, Edwards B. Altered sleep-wake cycles and physical performance in athletes. Physiol Behav 2007 Feb 28;90(2-3):274-284.

(6) Kubitz KA, Landers DM, Petruzzello SJ, Han M. The effects of acute and chronic exercise on sleep. A meta-analytic review. Sports Med 1996 Apr;21(4):277-291.

(7) Youngstedt SD, O'Connor PJ, Dishman RK. The effects of acute exercise on sleep: a quantitative synthesis. Sleep 1997 Mar;20(3):203-214.

(8) Youngstedt SD, Kline C, E. Epidemiology of exercise and sleep Sleep and Biological Rhythms 2006;4(215):221.

(9) Driver HS, Taylor SR. Exercise and sleep. Sleep Med Rev 2000 Aug;4(4):387-402.

(10) Blumenthal JA, Babyak MA, Moore KA, Craighead WE, Herman S, Khatri P, et al. Effects of exercise training on older patients with major depression. Arch Intern Med 1999 Oct 25;159(19):2349-2356.

(11) Dunn AL, Trivedi MH, Kampert JB, Clark CG, Chambliss HO. Exercise treatment for depression: efficacy and dose response. Am J Prev Med 2005 Jan;28(1):1-8.

(12) Uchida S, Shioda K, Morita Y, Kubota C, Ganeko M, Takeda N. Exercise effects on sleep physiology. Front Neurol 2012;3:48.

(13) O'Connor PJ, Breus MJ, Youngstedt SD. Exercise-induced increase in core temperature does not disrupt a behavioral measure of sleep. Physiol Behav 1998 Jun 1;64(3):213-217.

(14) Flausino NH, Da Silva Prado JM, de Queiroz SS, Tufik S, de Mello MT. Physical exercise performed before bedtime improves the sleep pattern of healthy young good sleepers. Psychophysiology 2012 Feb;49(2):186-192.

(15) Vuori I, Urponen H, Hasan J, Partinen M. Epidemiology of execise effecrs on sleep. Acta Physiol Scand Suppl 1988;574:3-7.

(16) Leeder J, Glaister M, Pizzoferro K, Dawson J, Pedlar C. Sleep duration and quality in elite athletes measured using wristwatch actigraphy. J Sports Sci 2012;30(6):541-545.

(17) Souissi M, Chtourou H, Zrane A, Cheikh RB, Dogui M, Tabka S, et al. Effect of time-of-day of aerobic maximal exercise on the sleep quality of trained subjects. Biological Rhythm Research 2011;43(3):323-330.

(18) Fietze I, Strauch J, Holzhausen M, Glos M, Theobald C, Lehnkering H, et al. Sleep quality in professional ballet dancers. Chronobiol Int 2009 Aug;26(6):1249-1262.

(19) Oswald I. Sleep. Harmondsworth: Penguin; 1970.

(20) Hausswirth C, Louis J, Aubry A, Bonnet G, Duffield R, LE Meur Y. Evidence of disturbed sleep and increased illness in overreached endurance athletes. Med Sci Sports Exerc 2014 May;46(5):1036-1045.

(21) Afaghi A, O'Connor H, Chow CM. Acute effects of the very low carbohydrate diet on sleep indices. Nutr Neurosci 2008 Aug;11(4):146-154.

(22) Porter JM, Horne JA. Bed-time food supplements and sleep: effects of different carbohydrate levels. Electroencephalogr Clin Neurophysiol 1981 Apr;51(4):426-433.

(23) Hartmann MK, Crisp AH, Evans G, Gaitonde MK, Kirkwood BR. Short-term effects of CHO, fat and protein loads on total tryptophan/tyrosine levels in plasma as related to %REM sleep. Waking Sleeping 1979 Jan;3(1):63-68.

(24) Zammit GK, Kolevzon A, Fauci M, Shindledecker R, Ackerman S. Postprandial sleep in healthy men. Sleep 1995 May;18(4):229-231.

(25) Phillips F, Chen CN, Crisp AH, Koval J, McGuinness B, Kalucy RS, et al. Isocaloric diet changes and electroencephalographic sleep. Lancet 1975 Oct 18;2(7938):723-725.

(26) Halson S. Nutritional interventions to enhance sleep. Sports Science Exchange 2013;26(116):1-5.

(27) Spiegel K, Leproult R, Van Cauter E. Impact of sleep debt on metabolic and endocrine function. Lancet 1999 Oct 23;354(9188):1435-1439.

(28) Meeusen R, Duclos M, Foster C, Fry A, Gleeson M, Nieman D, et al. Prevention, diagnosis, and treatment of the overtraining syndrome: joint consensus statement of the European College of Sport Science and the American College of Sports Medicine. Med Sci Sports Exerc 2013 Jan;45(1):186-205.

(29) Kuipers H, Keizer HA. Overtraining in elite athletes. Review and directions for the future. Sports Med 1988 Aug;6(2):79-92.

(30) Jurimae J, Maestu J, Purge P, Jurimae T. Changes in stress and recovery after heavy training in rowers. J Sci Med Sport 2004 Sep;7(3):335-339.

(31) Kentta G, Hassmen P. Overtraining and recovery. A conceptual model. Sports Med 1998 Jul;26(1):1-16.

(32) Taylor SR, Rogers GG, Driver HS. Effects of training volume on sleep, psychological, and selected physiological profiles of elite female swimmers. Med Sci Sports Exerc 1997 May;29(5):688-693.

(33) Raglin JS, Morgan WP. Development of a scale for use in monitoring training-induced distress in athletes. Int J Sports Med 1994 Feb;15(2):84-88.

(34) Morgan WP, Costill DL, Flynn MG, Raglin JS, O'Connor PJ. Mood disturbance following increased training in swimmers. Med Sci Sports Exerc 1988 Aug;20(4):408-414.

(35) Achten J, Halson SL, Moseley L, Rayson MP, Casey A, Jeukendrup AE. Higher dietary carbohydrate content during intensified running training results in better maintenance of performance and mood state. J Appl Physiol 2004 Apr;96(4):1331-1340.

(36) Halson SL, Lancaster GI, Achten J, Gleeson M, Jeukendrup AE. Effects of carbohydrate supplementation on performance and carbohydrate oxidation after intensified cycling training. J Appl Physiol (1985) 2004 Oct;97(4):1245-1253.

(37) Halson SL, Bridge MW, Meeusen R, Busschaert B, Gleeson M, Jones DA, et al. Time course of performance changes and fatigue markers during intensified training in trained cyclists. J Appl Physiol 2002;93:947-956.

(38) Morgan WP, Brown DR, Raglin JS, O'Connor PJ, Ellickson KA. Psychological monitoring of overtraining and staleness. Br J Sports Med 1987 Sep;21(3):107-114.

(39) Halson SL, Lancaster GI, Jeukendrup AE, Gleeson M. Immunological responses to overreaching in cyclists. Med Sci Sports Exerc 2003 May;35(5):854-861.

(40) Witard OC, Jackman SR, Kies AK, Jeukendrup AE, Tipton KD. Effect of increased dietary protein on tolerance to intensified training. Med Sci Sports Exerc 2011 Apr;43(4):598-607.

(41) American Dietetics Association, Dietitians of Canada. Nutrition and Athletic Performance, Joint Position Statement. Medicine and Science in Sport and Exercise 2009.

CHAPTER 6: DISCUSSION

Section A – Hydration Implications for Health and Performance

The volume of fluid required to maintain euhydration is highly variable between individuals and for this reason it is impossible to establish drinking guidelines on a 'one rule fits all' policy. A recent study of fluid intakes in an adult population reported a range from 416-4316 mL per day (40). Indeed, within our population of healthy sedentary adult males, we observed large variation in habitual daily fluid intakes (1082-3614 mL). Despite this there are a range of published guidelines on how much fluid one should consume, when it should be consumed and in what form, both with a sedentary population (41-44) and within sport (35,45-47).

6.1 Fluid Imbalances: Causes and Effects

There is some evidence that certain populations experience chronic low level hypohydration; however, the potential long term implications of this are not well understood (48). Despite this, it has been suggested that low habitual fluid intakes may be associated with a range of chronic diseases such as urolithiasis (urinary tract stone infection), constipation, asthma, cardiovascular disease, diabetic hyperglycaemia and certain cancers (48). Within athletic populations, sweating poses an additional challenge to fluid balance, with fluid losses of between 10-15 L/day having been reported in athletes training in hot environments (equal to 25-30 % of total body water) (49). Although such high sweat rates are uncommon, individual sweat rates are highly variable and are dependent on environmental conditions that are generally out of the athlete's control. Not only may athletes experience acute hypohydration following exercise, it is possible that if they do not rehydrate sufficiently between sessions that they carry chronic low-level fluid deficits. A combination of complicated factors determine the opportunities to drink during exercise, including the rules of the event, the format of play, back to back events that limit the opportunity to rehydrate, the cost/benefit calculation of time spent drinking during the event and/or power-weight ratio implications. It has been reported that successful athletes take calculated risks over their hydration strategies; weighing up to costs of drinking (time, weight etc) versus the potential benefits (46).

With caffeine regularly reported as a potential diuretic, but coffee consumption hugely popular across the globe by the general and athletic population (50,51), it is of interest to understand the impact of coffee consumption on fluid balance. In an attempt to gain a better

insight into the hydration implications of regular coffee consumption, we designed a study to investigate the effects of coffee on fluid balance in a cohort of healthy coffee drinkers. Our aim was to understand the effects of habitual coffee consumption on chronic hydration status using the most validated and comprehensive hydration assessment methods available. In the knowledge that a tolerance to caffeine can develop in habitual caffeine consumers (52), we were specifically interested in the effects of coffee consumption in a population of coffee drinkers, not in caffeine naïve individuals whereby the outcomes may have been different and the practical message for coffee drinkers would be unclear. Using a wide range of hydration assessment techniques, including doubly labelled water for total body water (TBW) estimation, we observed no significant differences in hydrating properties between coffee and water across a wide range of hydration assessment indices. Small daily fluctuations in TBW were observed during both trials; however, this did not reach statistical significance in either condition. Interestingly, these data support the findings of a recent study that also used TBW estimations to assess fluid balance following ingestion of pure anhydrous caffeine (5 mg/kg/day) and reported no differences between caffeine and placebo over a 4-day period (53). We did observe some minor differences in urinary sodium and potassium excretion between conditions. The observed increase in urinary excretion of sodium in the coffee condition supports previous studies that have reported increased sodium excretion at the proximal and distal renal tubules following consumption of caffeine and/or theophylline (54-56). Furthermore, the content of potassium in a cup of instant coffee (200 mL and ~ 2 g coffee) is reported to be ~80 mg (57) which may explain the increased urinary potassium excretion during the coffee trial. Therefore, it is possible that these differences between trials were the result of the numerous bioactive compounds in coffee that were not controlled for and provided in the water trial. Future studies should consider a 4-way analysis that investigates caffeinated coffee, decaffeinated coffee, water and caffeinated water. This form of analysis would enable a level of 'blinding' to both the participants and investigators that was not possible in this study. The data may also provide insight into the small variability in urinary electrolyte excretion observed between conditions in the present study. The limitations of a 4-way analysis would be the large time commitment imposed on the participants and the withdrawal of coffee for the duration of two trials may impact upon their developed caffeine tolerance. The main conclusion drawn from our study, in light of its limitations, is that that the consumption of coffee in moderate doses, by caffeine habituated males, contributes to the daily fluid requirement and does not pose a detrimental effect to

fluid balance. Furthermore, the advice provided in the public health domain regarding coffee intake and hydration status should therefore be updated to reflect these findings. Within 4 months of publication, this study achieved the status of being the 3rd most viewed paper of all time in the Public Library of Science One (PLOS ONE) and currently has received 51,461 views (October 2014).

6.2 Hydration implications for athletes

As previously mentioned, it is not uncommon for athletes to commence exercise in a negative fluid balance (46). This fluid deficit can arise from a combination of factors including failure to rehydrate between sessions, poor hydration strategies and/or result from weight making complications. The potential for a disturbed immune response during exercise in a state of dehydration has been highlighted within the context of this thesis. Most notably, a significant decrease in salivary flow rate can occur at relatively modest levels of hypohydration (58) and the implications of this reduction on salivary AMPs are not fully understood.

To date, salivary secretory IgA (SIgA) has been the most studied marker of mucosal immunity within athletic populations; however the importance of other AMPs has recently gained greater recognition. Salivary lysozyme (SLys) and salivary lactoferrin (SLac) are both present in the mucosal secretions of the upper respiratory tract and are understood to play an integral role in the innate immune system. We were interested to know whether exercise in a dehydrated state had any impact on salivary secretion and concentrations of a selection of under researched salivary AMPs including SLys and SLac.

To our knowledge, no laboratory controlled studies to date have investigated the effects of endurance exercise (>90 min) on salivary AMPs. In line with previous research, we observed a chronic reduction in salivary flow rate during and following exercise in the dehydrated trial. In addition, we observed transient changes in salivary AMPs during and immediately post-exercise, which had mostly returned to baseline values by 3 h post-exercise. Due to the significantly decreased salivary flow rate in the dehydration trial, it is important to assess secretion rates when looking for comparisons in AMP immune responses between conditions, and not absolute concentrations. A reduction in salivary flow rate will have an inevitable concentrating effect on the AMPs present. With mild dehydration induced by fluid restriction in the 24 h period prior to exercise we did not observe a significant difference in the plasma cortisol response to exercise between conditions and the secretion rates of SIgA, SLys and

SLac were only transiently affected. Exercise in the mildly dehydrated state caused a reduction in saliva flow rate, yet induced greater secretion rates of SLac and higher concentrations of SIgA and SLys. This suggests that prolonged exercise in a state of mild dehydration does not detrimentally affect mucosal immunity and it is unlikely that these small transient fluctuations in salivary AMPs would translate into clinical relevance.

6.3 Concluding Comments

To conclude, fluid balance is in a constant flux, with fluid intake balancing fluid losses to ensure euhydration is maintained. Our data suggest that the human body is able to adapt to potential diuretic challenges and maintain a level of hydration status that ensures we remain in fluid balance. However, under some conditions, such as fluid restriction and/or exercise in the heat, fluid balance may be compromised and without sufficient rehydration there may be health and performance implications for athletes and the general population. Commencing exercise in a state of modest dehydration resulted in acute differences in salivary flow rate and minor fluctuations in saliva AMP secretion rates. It is currently unclear whether these findings have any clinical relevance therefore future studies should consider both acute and chronic assessment of mucosal immune function across a range of fluid deficits and in particular states of more severe dehydration sufficient to increase activation of the HPA axis.

Section B – Intensified Training: Novel Insights and Future Directions for Understanding Training Load

Training harder, longer and more frequently was once considered the recipe for the success of an athlete. Indeed, many athletes and coaches will still pursue this philosophy. However, within the past few decades the science of recovery and training periodisation has provided invaluable insight into the physical, psychological and biochemical basis of elite performance (1). Currently, a search on 'exercise training' in PubMed will return over 62,000 publications, detailing and reviewing training practices, outlining responses to training interventions, describing the impact of nutrition and supplementation and defining the health and performance implications. Training periodisation involves a complex balance of overload training and tapering, by adjusting the volume and/or intensity of the sessions (2). Overload training can lead to acute physical and psychological disturbances that ultimately lead to attenuation in exercise performance (3). However with sufficient recovery these temporary stresses resolve and a super-compensation of physical performance usually occurs (4,5). A long-term imbalance between training and recovery may manifest into prolonged performance decrements, psychological disturbances and physical and biochemical disruptions. The stages of overtraining have been proposed to occur along a continuum of severity from overreaching (OR), with mild performance decrements that are recovered relatively quickly (several days to a few weeks) to the overtraining syndrome (OTS) whereby serious performance decrements prevail in the long term (months), with or without psychological and biochemical implications. For a recent and thorough review of the overtraining syndrome see Meeusen et al (6).

6.4 Markers for monitoring training load

By definition, overtraining is diagnosed as a reduction in performance and an increase in mood disturbance; therefore at the most basic level these two factors can be used to monitor training stress. A negative change in sport-specific performance, in the absence of a decrease in training load, is one of the easiest markers used to monitor OR in the field. Furthermore, mood state is commonly monitored by athlete support teams, either objectively (with validated questionnaires) or subjectively by people close to the athlete. Whilst these factors may provide early indications for athletes and coaches, it is important to understand why

these changes occur and the extent of the physiological effects on the athlete. A wide range of studies have been conducted over recent years, each analysing various aspects of OT, including the metabolic, biochemical, hormonal and immune responses, physiological effects on sleep, psychology of life stressors and environmental impacts on training. Below is a summary of some of the most relevant findings within the scope of this thesis.

Performance

Performance testing is a key component in the identification of OTS. It has been suggested that athletes suffering from underperformance due to OR or OTS are often able to begin training sessions or races at a normal pace but are not able to sustain the performance for the duration of the exercise period (6). Time to exhaustion tests have been commonly reported in the scientific literature (7,8); however, it is argued that this test is not sufficiently sport-specific. We adopted a time trial performance test in our study as one of our aims was that the study would have an applied focus that could be related to practical outcomes in the field. Aside from its sport-specific nature, the time trial also offers a level of standardisation that cannot be ascertained from a time to exhaustion protocol. A 1 h time trial enabled direct comparisons of markers taken from standardised time points.

Unlike other studies that have adopted similar training protocols (9,10) we did not observe significant changes in time trial performance following IT, though some level of performance decrement occurred in almost all athletes. These data make diagnosis of OR extremely difficult. However, a strength of our study is the ability to quantify exact training load via the use of power meters and heart rate monitors in each session. Independent of whether our athletes were officially OR or not, we have a collected a vast amount of data from highly-trained athletes that provides a novel insight into the physiological, metabolic and immunological impact of such training. To this author's knowledge, data of this type has not been previously collected within such a highly trained population of athletes.

In addition to monitoring endurance performance, we were interested in the athlete's ability to sustain short-term high intensity exercise performance following IT. Interestingly, despite no change in absolute $\dot{V}O_{2max}$, we observed a significant reduction in time to complete the test, peak power and maximum heart rate. This suggests that the athletes were unable to sustain high intensity exercise performance, despite its relatively short duration, and were therefore exhibiting signs of acute fatigue.

Mood State

Disturbed mood state is commonly reported in relation to OR and OTS. Whether mood disturbance is a contributor to or result of under-performance is not entirely understood. Firstly, we were interested in whether we would observe changes in mood state during 9-days of IT according to the standard POMS-65 mood state questionnaire and secondly, whether it was possible to correlate these changes with shorter, less time consuming questionnaires such as the POMS-24 and DALDA. We observed disturbed mood states in all of our participants during IT, validated by each of our three mood state questionnaires. The DALDA questionnaire was least sensitive and was unable to clearly distinguish differences between our nutritional interventions, unlike both versions of the POMS. In this study, the POMS-24 proved to be a simple, useful and valid tool for monitoring changes in mood state on a daily basis.

Hormones/Endocrine function

A multitude of hormones have been analysed in relation to the OTS in recent years. This discussion will not list the merits of each hormone (for review see (6)), but will focus on the most recent developments and future directions.

There is a strong consensus within the overtraining literature that OR and OTS occur along a continuum; starting with a disturbance, an adaptation and an eventual maladaptation of the HPA axis (6,7,11,12). The HPA axis is one of the main pathways that becomes activated under stressful conditions, such as exercise, resulting in a stress hormone response (increased ACTH secretion and subsequent release of cortisol). Original research linking OTS with hypothalamic function dates back to 1985 (13). In a study by Barron and colleagues to assess hypothalamic-pituitary function, healthy trained athletes and a small number (n=4) of athletes diagnosed as suffering from OTS underwent an insulin-induced hypoglycaemic challenge (in the rested state) to stimulate the release of ACTH, growth hormone and prolactin. Plasma cortisol, ACTH, growth hormone and prolactin responses to insulin-induced hypoglycaemia were much lower in OTS athletes when compared with the trained athletes, indicating hypothalamic dysfunction.

To date, numerous hormones have been measured in overtrained athletes in an attempt to identify these so-called 'abnormalities' of the HPA axis. Accumulating data suggests that

resting hormonal levels may not provide sufficient evidence for OTS. Research has shown that resting hormone measurements are highly variable and individual and therefore difficult to interpret (9). Indeed, we observed large individual variability in baseline stress hormone concentrations between participants which may, in part, explain our reported 'tendencies' instead of significant observations (e.g. for cortisol and ACTH). Meeusen and colleagues were among the first to highlight and test the importance of measuring hormonal responses to exercise (14). It is understood that hormonal fluctuations during exercise influence post-exercise hormonal responses. This can impact the athlete's ability to recover and perform subsequent exercise sessions. In a cleverly designed study, Meeusen and colleagues employed a two-bout maximal exercise testing protocol to look at neuroendocrine dysfunction in athletes diagnosed with OTS (14). The authors reported suppression of ACTH response to the second exercise test in the OTS athletes, something that has been previously observed after a single test (13,15). Whilst the authors believe the use of a two-bout test protocol is more beneficial for detecting OR, they accept that a single-exercise test is also an appropriate tool to use. We did not utilise a two-bout exercise protocol in our study, however the training protocol was designed as such that each participant trained on two occasions each day (for a minimum or 90 min per session) for 9 days, thus minimising the recovery window. Using a time trial test as our performance marker, we observed significant changes in stress hormone response to exercise from pre- to post-IT. In line with previous reports in OR athletes, ACTH and cortisol concentrations were significantly suppressed following exercise after IT, indicating HPA axis disturbance.

It is not only important to observe individual hormone responses to exercise sessions, the concurrent measurement and analysis of multiple related hormones must be also performed. The interactions of various hormones contribute to a better understanding of endocrine function. An interesting observation in our study was the change in prolactin response to exercise. Post-exercise prolactin concentration increased from baseline at TT1 (before IT) and decreased from baseline following TT3 (following IT). Overall, post-exercise prolactin concentrations decreased significantly with IT suggesting chronic fatigue and potential immunosuppression (16,17). In line with suggestions made by Barron and colleagues, if measured appropriately, prolactin should be involved in the monitoring and/or diagnosis of OT (13).

An additional marker that could provide insight into an athlete's training status is BDNF. It has been reported that repeated exposure to stress may alter the responsiveness to subsequent

stressors leading to an altered neurotransmitter and receptor response (6). BDNF is understood to be a key component of the hypothalamic pathway (18) and numerous studies have reported transient increases in circulating BDNF concentrations following exercise (19-21). BDNF is an important mediator in brain neuroplasticity, particularly in survival, differentiation and neuronal growth and is thought to influence numerous brain functions, such as learning and memory (22). Low levels of BDNF have been associated with psychiatric disorders, including major depressive disorder (23) and it has been suggested that exercise-induced increases in circulatory BDNF concentrations might be neuroprotective and important in the prevention of neurodegenerative diseases (22). To our knowledge, this is the first study to measure BDNF concentrations in highly trained athletes undergoing short-term IT. Our results indicate that IT had a suppressive effect on BDNF concentrations. We observed a decrease in baseline BDNF concentrations in 9 of 13 participants (p=0.08) and a significant attenuation in post-exercise concentrations following IT. Given that clinical research has highlighted the negative implications of low circulating BDNF concentrations, including psychiatric disorders, it is interesting to note that decreased levels of BDNF occur in participants who at the same time were experiencing significant mood disturbances. Excess glucocorticoids have been associated with reduced levels of BDNF (24); however, the mechanisms behind the relationship have not been well researched. A recent study investigated the interactions between BDNF and glucocorticoids in the regulation of corticotrophin-releasing hormone (CRH) in the hypothalamus (25). In this study, the researchers generated mice that were defective in glucocorticoid receptor (GR) signalling in the parventricular nucleus (similar to a condition in certain human pathologies). This adaptation resulted in increased CRH expression, up-regulation of hypothalamic levels of BDNF and disinhibition of the HPA axis. The authors report that BDNF concentrations (regulated by cAMP response-element binding protein (CREB) and its coactivator protein (CRTC2)) regulate the ability of glucocorticoids to influence expression of CRH (25). A common observation following rigorous exercise is an increase glucocorticoid 'stress-hormone' secretion. Our participants underwent a highly strenuous period of IT and we observed attenuated levels of BDNF. However, we also observed an attenuation in stress hormone response by the end of IT, a common observation in athletes that become overreached. There is strong evidence for interactions between BDNF, glucocorticoids and training stress; however, more research is required to gain a full understanding of the mechanisms and implications.

A further interest in BDNF concentrations and the OTS is its central involvement in cellular energy metabolism and peripheral roles in skeletal muscle metabolism (26,27). Studies in mice have found BDNF to be involved in energy expenditure through activation of the sympathetic nervous system (28). BDNF has also been suggested to exert a satiating effect (29). Human genetics studies have shown that BDNF gene mutations (resulting in high levels of BDNF) may account for certain types of obesity and low circulating levels of BDNF have been found to correlate with eating disorders including anorexia nervosa and bulimia nervosa (29). Within the OTS complex, the role of BDNF in cellular and skeletal energy metabolism is particularly interesting and much more work is required in this field.

Sleep Physiology

Few studies have investigated the effects of exercise training on sleep physiology in athletes. However, with sleep considered as the 'gold standard' post-exercise recovery procedure among athletic populations, it is of great interest to understand the sleeping patterns of trained athletes. We used wrist watch actigraphy (activity-based monitoring) to assess sleep in our participants in the lead up to and during two periods of intensified training. Modern day actigraphy allows objective measures of sleep to be collected over extended periods of time with little inconvenience to the participant (30). In light of its limitations, as discussed in the general introduction, actigraphy has been recommended as an acceptable method of estimating sleep patterns in normal, healthy adult populations (31). The importance of sleep for physical and mental recovery from exercise is well understood and inadequate sleep is thought to lead to impaired cognitive function and inability to concentrate (6). A small collection of studies have monitored sleep in athletes using actigraphy and one study in particular has tracked sleep patterns in athletes diagnosed as OR (32). Participants in this study underwent a 3-week intensified raining period whereby training load was increased by 30%. Nine of 18 participants exhibited significant declines in time to complete an incremental cycle test to exhaustion ($\dot{V}O_{2max}$) and were therefore classified as OR. The authors reported reduced sleep quality (duration, efficiency and immobile time) in the OR group compared with a those who were not classified as OR. Whilst this study has interesting implications, it is limited by its design. It is unlikely that a 30% increase in training load would actually induce OR over a 3-week period. Indeed, in our study we observed significant declines in our $\dot{V}O_{2max}$ test following 9 days of IT (with a 150% increase in training load), but did not observe such consistent observations in a 1 h TT, therefore it cannot be confirmed

whether these athletes were truly OR. Nevertheless, we observed significant and progressive declines in sleep quality (decreased sleep efficiency and increased fragmentation) during 9 days of IT.

It has been proposed that non-restorative sleep can occur during times when sleep duration is sufficient, but high sleep fragmentation results in poor quality sleep. It is likely that our participants experienced some level of non-restorative sleep as we saw no change in actual sleep time and an increase in fragmentation. It has been proposed that athletes who suffer from non-restorative sleep may be tired from training and may not be achieving optimal restoration from sleep (33). In the longer term, this lack of full recovery could potentially contribute to the development of the OTS.

These novel findings suggest that athletes undergoing relatively short training camps should allow extra time for napping to try to compensate for nocturnal sleep loss. Coaches and support staff should be aware of the potential implications of disturbed sleep on recovery and performance and should design the training programme accordingly, for example by avoiding training very late in the evening or very early in the morning to prevent additional sleep disruption.

Nutrition

High carbohydrate diets are commonplace within athletic populations, particularly in sports with an endurance component. High carbohydrate diets during periods of intensified training have been shown to help maintain performance and attenuate declines in mood state (8,10). In our study (Chapters 4 and 5), we were interested not only in the impact of a high vs moderate carbohydrate diet, but specifically in how carbohydrate availability immediately before, during and immediately after exercise may impact our results. Current ACSM and IOC guidelines advise athletes to consume 30-60 g or 0.7 g/kg carbohydrate per hour to support endurance exercise lasting longer than 60 min (34,35). Therefore, we were interested in how these recommendations might impact short-term periods of intensified training, similar to that of a typical training camp. We provided 60 g/h carbohydrate in our high carbohydrate group and 20 g/h in our control group. In addition, we provided either 24 or 2 g of carbohydrate within the 30 min prior to each training session and 44 or 10 g in the immediate post-exercise recovery period, for HCHO and CON respectively. Furthermore, the addition of the post-exercise protein in the HCHO contributed to an overall significantly

higher dietary protein compared with CON. Despite the differences in nutrient intakes between conditions, we did not observe the significant differences across our analyses that we had hypothesised. However, whilst we did not observe differences between groups in performance of the $\dot{V}O_{2max}$ tests, we did observe small differences between time trial performance (-2.8% vs -0.3% change in performance, for CON and HCHO, respectively) that may be meaningful in the field. We also observed small differences in immune response to exercise; including attenuated SLys secretion rate response to exercise in CON and lower concentrations of SLac throughout IT in CON compared with HCHO. Our data contributes to a growing body of research that proposes SLys as a potential marker for psychological (36) and physiological stress (37). Reductions in SLys concentrations are thought to be associated with secretion of glucocorticoids (36) and therefore activation of the HPA-axis. It may be that with further research, SLys could be used as a marker for OTS. Within the context of this study, it was interesting to observe potential interactions of salivary AMPs with our nutrition intervention. Our data suggest HCHO may have supported the mobilization of SLys to the saliva via sympathetic nervous activation.

The nutritional intervention resulted in minor effects on hormonal responses during IT. Pre-exercise ACTH tended to be lower in CON than HCHO. ACTH suppression in CON may suggest reduced adrenocortical secretion or reactivity, or enhanced negative feedback inhibition of the HPA axis (8) however this did not translate into a measureable difference in cortisol concentrations and no difference in prolactin concentrations were observed between conditions. We also observed significantly lower post-exercise testosterone concentrations in CON compared with HCHO following IT, which is thought to result from a decrease in steroid synthesis due to physiological and/or psychological strain (17).

Following IT, we observed attenuated BDNF increases in response to exercise. Interestingly this attenuation was more pronounced in the CON (12% increase) compared with HCHO (22% increase). The condition effect was not statistically significant, potentially due to lack of power, but also because of the time course of BDNF response to exercise. We collected blood samples immediately post-exercise (within 5 min of exercise cessation); however in a future study it may be interesting to study the acute response with repeated blood sampling over a number of hours post-exercise.

It is likely that we would have observed more significant differences between our measures if participants had been provided with the nutritional intervention during the 1 h performance

test. It is also an interesting thought from a practical perspective as it would be extremely rare for an athlete to undergo a time trial in a fasted state and without consuming any sports foods (carbohydrate, caffeine etc). However, due to the importance of standardising the results and avoiding short-term effects of the nutritional intervention that may mask some of the underlying markers of interest, no nutrition was provided.

One of the key nutritional messages from this study is that the current ACSM and IOC carbohydrate recommendations for endurance exercise helped prevent some, but not all signs of chronic fatigue and early markers of OR during 9 days of IT. Interestingly, the lower carbohydrate dose in our CON group enabled better maintenance of performance than has previously been observed in training studies which provided ~2 g/kg/day carbohydrate less than in their control/low carbohydrate groups (7 g/kg/day vs ~5 g/kg/day (8,38,39). It would seem that the availability of carbohydrate during exercise, independent of absolute amount, was able to help maintain exercise performance over the course of IT.

6.5 Practical Messages and Application in the Field

- In order to determine changes in performance, an athlete must have undergone a performance test prior to developing OR or OTS. This is not often the case in elite sports, especially team sports, whereby the non-specificity of the performance test makes the data collection appear to be pointless. However, the benefit of a standardised performance test, whether it is sport-specific or not, is in the ability to accurately monitor performance over the course of a season, which otherwise may not be possible.
- Daily monitoring of mood state prior to and during periods of intensified training is a beneficial tool in monitoring training stress. The POMS-24 is able to detect changes in mood state as effectively as the longer version and has the benefit of being concise and not overly time consuming.
- Periods of intensified training and/or training camps should be carefully designed with recovery and sleep in mind. Coaches and support staff should be aware of the potential implications of disturbed sleep on recovery and performance and should design the training programme accordingly, for example by avoid training very late in the evening or very early in the morning to prevent additional sleep disruption.

- Salivary AMPs, specifically SLys, may prove to be a useful non-invasive tool for monitoring OR in the field.

- BDNF is potentially a very important marker in monitoring training load and with further research could be used as a marker for OTS.

- Current ACSM and IOC guidelines for carbohydrate intakes during exercise; both at the upper (60 g/h) and lower (30 g/h) ends appear to be appropriate for short-term periods of IT. Such carbohydrate intakes may help prevent performance decrements resulting from OR and may support immune function, mood state and sleep quality during IT.

6.6 References

(1) Mujika I, Padilla S, Pyne D, Busso T. Physiological changes associated with the pre-event taper in athletes. Sports Med 2004;34(13):891-927.

(2) Bosquet L, Montpetit J, Arvisais D, Mujika I. Effects of tapering on performance: a meta-analysis. Med Sci Sports Exerc 2007 Aug;39(8):1358-1365.

(3) Mujika I, Padilla S. Scientific bases for precompetition tapering strategies. Med Sci Sports Exerc 2003 Jul;35(7):1182-1187.

(4) Busso T. Variable dose-response relationship between exercise training and performance. Med Sci Sports Exerc 2003 Jul;35(7):1188-1195.

(5) Banister EW, Carter JB, Zarkadas PC. Training theory and taper: validation in triathlon athletes. Eur J Appl Physiol Occup Physiol 1999 Jan;79(2):182-191.

(6) Meeusen R, Duclos M, Foster C, Fry A, Gleeson M, Nieman D, et al. Prevention, diagnosis, and treatment of the overtraining syndrome: joint consensus statement of the European College of Sport Science and the American College of Sports Medicine. Med Sci Sports Exerc 2013 Jan;45(1):186-205.

(7) Urhausen A, Gabriel HH, Weiler B, Kindermann W. Ergometric and psychological findings during overtraining: a long-term follow-up study in endurance athletes. Int J Sports Med 1998 Feb;19(2):114-120.

(8) Halson SL, Lancaster GI, Achten J, Gleeson M, Jeukendrup AE. Effects of carbohydrate supplementation on performance and carbohydrate oxidation after intensified cycling training. J Appl Physiol (1985) 2004 Oct;97(4):1245-1253.

(9) Halson S, Lancaster G, Achten J, Gleeson M, Jeukendrup A. Effects of carbohydrate supplementation on performance and carbohydrate oxidation after intensified cycling training. J Appl Physiol 2004 OCT;97(4):1245-1253.

(10) Achten J, Halson SL, Moseley L, Rayson MP, Casey A, Jeukendrup AE. Higher dietary carbohydrate content during intensified running training results in better maintenance of performance and mood state. J Appl Physiol (1985) 2004 Apr;96(4):1331-1340.

(11) Lehmann M, Foster C, Keul J. Overtraining in endurance athletes: a brief review. Med Sci Sports Exerc 1993 Jul;25(7):854-862.

(12) Urhausen A, Gabriel H, Kindermann W. Blood hormones as markers of training stress and overtraining. Sports Med 1995 Oct;20(4):251-276.

(13) Barron JL, Noakes TD, Levy W, Smith C, Millar RP. Hypothalamic dysfunction in overtrained athletes. J Clin Endocrinol Metab 1985 Apr;60(4):803-806.

(14) Meeusen R, Piacentini M, Busschaert B, Buyse L, De Schutter G, Stray-Gundersen J. Hormonal responses in athletes: the use of a two bout exercise protocol to detect subtle differences in (over)training status. Eur J Appl Physiol 2004 MAR;91(2-3):140-146.

(15) Urhausen A, Gabriel HH, Kindermann W. Impaired pituitary hormonal response to exhaustive exercise in overtrained endurance athletes. Med Sci Sports Exerc 1998 Mar;30(3):407-414.

(16) Meeusen R, Piacentini MF, Busschaert B, Buyse L, De Schutter G, Stray-Gundersen J. Hormonal responses in athletes: the use of a two bout exercise protocol to detect subtle differences in (over)training status. Eur J Appl Physiol 2004 Mar;91(2-3):140-146.

(17) Gomez-Merino D, Chennaoui M, Burnat P, Drogou C, Guezennec CY. Immune and hormonal changes following intense military training. Mil Med 2003 Dec;168(12):1034-1038.

(18) Wisse BE, Schwartz MW. The skinny on neurotrophins. Nat Neurosci 2003 Jul;6(7):655-656.

(19) Seifert T, Brassard P, Wissenberg M, Rasmussen P, Nordby P, Stallknecht B, et al. Endurance training enhances BDNF release from the human brain. Am J Physiol Regul Integr Comp Physiol 2010 Feb;298(2):R372-7.

(20) Nofuji Y, Suwa M, Moriyama Y, Nakano H, Ichimiya A, Nishichi R, et al. Decreased serum brain-derived neurotrophic factor in trained men. Neurosci Lett 2008 May 23;437(1):29-32.

(21) Vaynman S, Ying Z, Gomez-Pinilla F. Hippocampal BDNF mediates the efficacy of exercise on synaptic plasticity and cognition. Eur J Neurosci 2004 Nov;20(10):2580-2590.

(22) Coelho FG, Gobbi S, Andreatto CA, Corazza DI, Pedroso RV, Santos-Galduroz RF. Physical exercise modulates peripheral levels of brain-derived neurotrophic factor (BDNF): A systematic review of experimental studies in the elderly. Archives of Gerontology and Geriatrics 2012.

(23) Gustafsson G, Lira CM, Johansson J, Wisen A, Wohlfart B, Ekman R, et al. The acute response of plasma brain-derived neurotrophic factor as a result of exercise in major depressive disorder. Psychiatry Res 2009 Oct 30;169(3):244-248.

(24) McEwen BS. Physiology and neurobiology of stress and adaptation: central role of the brain. Physiol Rev 2007 Jul;87(3):873-904.

(25) Jeanneteau FD, Lambert WM, Ismaili N, Bath KG, Lee FS, Garabedian MJ, et al. BDNF and glucocorticoids regulate corticotrophin-releasing hormone (CRH) homeostasis in the hypothalamus. Proc Natl Acad Sci U S A 2012 Jan 24;109(4):1305-1310.

(26) Knaepen K, Goekint M, Heyman EM, Meeusen R. Neuroplasticity - exercise-induced response of peripheral brain-derived neurotrophic factor: a systematic review of experimental studies in human subjects. Sports Med 2010 Sep 1;40(9):765-801.

(27) Matthews VB, Astrom MB, Chan MH, Bruce CR, Krabbe KS, Prelovsek O, et al. Brain-derived neurotrophic factor is produced by skeletal muscle cells in response to contraction and enhances fat oxidation via activation of AMP-activated protein kinase. Diabetologia 2009 Jul;52(7):1409-1418.

(28) Tsuchida A, Nonomura T, Ono-Kishino M, Nakagawa T, Taiji M, Noguchi H. Acute effects of brain-derived neurotrophic factor on energy expenditure in obese diabetic mice. Int J Obes Relat Metab Disord 2001 Sep;25(9):1286-1293.

(29) Lebrun B, Bariohay B, Moyse E, Jean A. Brain-derived neurotrophic factor (BDNF) and food intake regulation: a minireview. Auton Neurosci 2006 Jun 30;126-127:30-38.

(30) Sadeh A, Acebo C. The role of actigraphy in sleep medicine. Sleep Med Rev 2002 Apr;6(2):113-124.

(31) Morgenthaler T, Alessi C, Friedman L, Owens J, Kapur V, Boehlecke B, et al. Practice parameters for the use of actigraphy in the assessment of sleep and sleep disorders: an update for 2007. Sleep 2007 Apr;30(4):519-529.

(32) Hausswirth C, Louis J, Aubry A, Bonnet G, Duffield R, LE Meur Y. Evidence of disturbed sleep and increased illness in overreached endurance athletes. Med Sci Sports Exerc 2014 May;46(5):1036-1045.

(33) Samuels C. Sleep, recovery, and performance: the new frontier in high-performance athletics. Neurol Clin 2008 Feb;26(1):169-80; ix-x.

(34) American College of Sports Medicine, Sawka MN, Burke LM, Eichner ER, Maughan RJ, Montain SJ, et al. American College of Sports Medicine position stand. Exercise and fluid replacement. Med Sci Sports Exerc 2007 Feb;39(2):377-390.

(35) Maughan RJ, Shirreffs SM. IOC Consensus Conference on Nutrition in Sport, 25–27 October 2010, International Olympic Committee, Lausanne, Switzerland Journal of Sports Sciences 2011;29(S1):S3-S4.

(36) Perera S, Uddin M, Hayes JA. Salivary lysozyme: a noninvasive marker for the study of the effects of stress of natural immunity. Int J Behav Med 1997;4(2):170-178.

(37) Koutedakis Y, Sabin E, Perera S. Modulation of salivary lysozyme by training in elite male swimmers. Journal of Sports Sciences 1996;14(90).

(38) Achten J, Halson SL, Moseley L, Rayson MP, Casey A, Jeukendrup AE. Higher dietary carbohydrate content during intensified running training results in better maintenance of performance and mood state. J Appl Physiol 2004 Apr;96(4):1331-1340.

(39) Simonsen JC, Sherman WM, Lamb DR, Dernbach AR, Doyle JA, Strauss R. Dietary carbohydrate, muscle glycogen, and power output during rowing training. J Appl Physiol (1985) 1991 Apr;70(4):1500-1505.

(40) Bellisle F, Thornton SN, Hebel P, Tahiri M. A study of fluid intake from beverages in a sample. EJCN 2010;64:350-355.

(41) EFSA Panel on Dietetic Products, Nutrition, and Allergies. Scientific Opinion on Dietary Reference Values for water. EFSA Journal 2010;8(3):1459.

(42) Jéquier E, Constant F. Water as an essential nutrient: the physiological basis of hydrationWater as an essential nutrient. EJCN 2010;64:115-123.

(43) Kleiner SM. Water: an essential but overlooked nutrient. J Am Diet Assoc 1999 Feb;99(2):200-206.

(44) WaterAid. Hydration Tips. WaterAid ;2012(11/09).

(45) American College of Sports Medicine, Sawka MN, Burke LM, Eichner ER, Maughan RJ, Montain SJ, et al. American College of Sports Medicine position stand. Exercise and fluid replacement. Med Sci Sports Exerc 2007 Feb;39(2):377-390.

(46) Garth AK, Burke LM. What do athletes drink during competitive sporting activities? Sports Med 2013 Jul;43(7):539-564.

(47) Noakes TD. Changes in body mass alone explain almost all of the variance in the serum sodium concentrations during prolonged exercise. Has commercial influence impeded scientific endeavour? Br J Sports Med 2011 May;45(6):475-477.

(48) Maughan RJ. Hydration, morbidity, and mortality in vulnerable populations. Nutr Rev 2012 Nov;70 Suppl 2:S152-5.

(49) Maughan R, Shirreffs S. Exercise in the heat: challenges and opportunities. J Sports Sci 2004 Oct;22(10):917-927.

(50) Del Coso J, Munoz G, Munoz-Guerra J. Prevalence of caffeine use in elite athletes following its removal from the World Anti-Doping Agency list of banned substances. Appl Physiol Nutr Metab 2011 Aug;36(4):555-561.

(51) Desbrow B, Leveritt M. Awareness and use of caffeine by athletes competing at the 2005 Ironman Triathlon World Championships. Int J Sport Nutr Exerc Metab 2006 Oct;16(5):545-558.

(52) Eddy N, Downs A. Tolerance and cross-tolerance in the human subject to the diuretic effect of caffeine, theobromine, and thoephylline. J Pharmacol Exper Ther 1928;33:167-174.

(53) Silva A, Judice P, Matias C, Santos D, Magalhaes J, St-onge M, et al. Total body water and its compartments are not affected by ingesting a moderate dose of caffeine in healthy young adult males. Appl Physiol Nutr Metab 2013;38(6):626-632.

(54) Dodd SL, Herb RA, Powers SK. Caffeine and exercise performance. An update. Sports Med 1993 Jan;15(1):14-23.

(55) Fredholm BB. Cardiovascular and renal actions of methylxanthines. Prog Clin Biol Res 1984;158:303-330.

(56) Osswald H, Schnermann J. Methylxanthines and the kidney. Handb Exp Pharmacol 2011;(200):391-412. doi(200):391-412.

(57) Debry G. Coffee and Health. Montrouge (France): John Libbey Eurotext; 1994.

(58) Gill SK, Teixeira AM, Rama L, Rosado F, Hankey J, Scheer V, et al. Salivary antimicrobial protein responses during multistage ultramarathon competition conducted in hot environmental conditions. Appl Physiol Nutr Metab 2013 Sep;38(9):977-987.

Appendix A: Reverse Phase HPLC Methodology for Caffeine Analysis

Sample Analysis Notes:

Caffeine sample preparation:

1. 200 µL of plasma added to 100 µL internal standard (Proxyphylline, Sigma, United Kingdom)
2. Mix thoroughly using vortex (30 s)
3. Add 500 µL of acetic acid.
4. Inject supernatant (5 µL) onto the Phenomenex Prodigy 150 x 4.60mm 5 µ Octadecyl Silane (ODS) column using the auto sampler
5. Add two QCs (High and Low) with each batch of samples.

The sample will be detected at a UV of 273 nm.

Use the one-point calibrator to quantify caffeine concentration.

NB: One point calibration has been validated against 9 point calibration curve.

Appendix B: Program for 9-day intensified training period:

Name: xxx Date: xxx

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Last Day
AM	VO2max	1hr TT	2 h @ 272W Must inc. 3 sets of (5 x 30s on/ 30s off) **MAX EFFORT** *Total session 2h*	2h @ 272W Must inc. 6 x 10 min @ 341W (3-5 min rec) *Total session 2h*	2h @ 272W Must inc. 5 x 12 min @ 341W (3-5 min rec) *Total session 2h*	1hr TT	2h @ 272W Must inc. 3 sets of (5 x 30 sec on/ 30 sec off) **MAX EFFORT** *Total session 2h*	2 h @ 272W Must inc. 5 x 12 min @ 341W (3-5 min rec) *Total session 2h*	VO2max	1hr TT
PM	LAB SESSION 10 x 4 min @ >386W (1 min rec) 5 x 1 min **MAX EFFORT** (1 min rec) *Total session 2h*	LAB SESSION 20 min @272W 7x1min - 5x30s - 7x1min **MAX EFFORT** (1min/30s rec) 35 min @272W 2 x 2 min (4 min rec) *Total session 2h*	2 h @ 210W Must inc. 4 x 5 min @>386W + 5x 30sec MAX EFFORT *Total session 2h*	LAB SESSION 20min @ 272W 7x1min - 5x30s - 7x1min **MAX EFFORT** (1min/30s rec) 35min @ 272W 2 x 2 min (4 min rec) *Total session 2h*	LAB SESSION 10 x 4 min @ >386W (1 min rec) 5 x 1 min **MAX EFFORT** (1 min rec) *Total session 2h*	2 h @272W Must inc. 4 x 5 min @>386W + 6x 30sec **MAX EFFORT** *Total session 2h*	2 h @ 210W Must inc. 5 x 4 min @>386W + 6x 30sec **MAX EFFORT** *Total session 2h*	LAB SESSION 20min @ 272W 7x1min - 5x30s - 7x1min **MAX EFFORT** (1min/30s rec) 35min @ 272W 2 x 2 min (4 min rec) *Total session 2h*	LAB SESSION 10 x 4 min @>386W (1 min rec) 5 x 1min **MAX EFFORT** (1 min rec) *Total session 2h*	

Appendix C: Participant Dietary Intakes.

	Pre-Trial		HCHO		CON	
	Mean	SD	Mean	SD	Mean	SD
Energy (Kcal/d)	2910.7	590.0	4148.0*¶	766.4	3501.5*¶	616.6
CHO (g/d)	429	81.2	678.7¶	105.3	505.4¶	106.8
PRO (g/d)	114.5	31.5	143.9¶	31.2	117.0¶	25.9
Fat (g/d)	81.9	20.5	92.0	26.6	102.7*	24.6

Data are mean ± SD. * Significant increase from Pre-Trial. ¶ Significant difference between HCHO and CON.