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DOCTOR OF PHILOSOPHY

The Impact Of Extreme Physical Exertion On Salivary Anti-Microbial Protein Responses, Circulatory Endotoxin Concentrations And Cytokine Profile Do Probiotics Have A Role To Play?

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Award date:
2016

Awarding institution:
Coventry University

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PhD

06/2016

Coventry University

THE IMPACT OF EXTREME PHYSICAL EXERTION ON SALIVARY ANTI-MICROBIAL PROTEIN RESPONSES, CIRCULATORY ENDOTOXIN CONCENTRATIONS AND CYTOKINE PROFILE: DO PROBIOTICS HAVE A ROLE TO PLAY?

By

Samantha Kirsty Gill

A thesis submitted in partial fulfilment of the University's requirements for the Degree of Doctor of Philosophy.

06/2016

My contribution to the studies presented in this thesis includes: protocol development and design with supervisory input, research team coordination and all aspects of data collection, processing and storage, data analysis with and without supervisory input, and writing/preparation of manuscripts for journal submission with supervisory input.

Summary

Extreme physical exertion is commonly associated with acute physiological changes in immune variables known to disturb host defences. Likely induced by the production of stress hormones (e.g., cortisol), partaking in ultra-endurance events with accompanying physiological stressors ((e.g., environmental extremes, sleep deprivation and compromised hydration and (or) nutritional status)) may amplify stress hormone responses and compromise immune status to a greater extent. To date, research investigating the impact of extreme physical exertion (e.g., ultra-marathon events) on physiological variables is extremely limited. More recently, the potential use of probiotics with known immunomodulatory effects may be considered an appropriate nutritional strategy to improve host defences and minimise and (or) prevent sub-clinical or clinically significant outcomes in active populations.

With this in mind, the purpose of this thesis was to investigate the effects of: 1) a multi-stage ultra-marathon (total distance: 230 km) in hot ambient conditions (32-40°C), and a 24 h continuous ultra-marathon (total distance range: 122-208 km) in temperate ambient conditions (0-20°C) on salivary anti-microbial protein (S-AMP) and stress hormone response, and self-reported incidence of upper respiratory symptoms (URS); 2) a multi-stage ultra-marathon in hot ambient conditions, and a 24 h continuous ultra-marathon in temperate ambient conditions on circulatory endotoxin concentration, cytokine profile, and self-reported incidence of gastrointestinal (GI) symptoms; and 3), acute high dose supplementation of *Lactobacillus casei* (*L.casei*) on S-AMP responses, circulatory endotoxin concentration and cytokine profile in response to exertional-heat stress (EHS).

A multi-stage ultra-marathon in hot ambient conditions (**Chapter 4**) decreased post-stage salivary IgA (S-IgA) responses. Salivary alpha-amylase (S-α-amylase) and salivary lysozyme (S-lysozyme) responses increased and (or) remained unchanged post-stage throughout. Salivary cortisol (S-cortisol) responses fluctuated throughout the multi-stage ultra-marathon competition. URS were minimally reported (*n*=1) during and in the one month period following the ultra-marathon.

A 24 h continuous ultra-marathon in temperate ambient conditions (**Chapter 5**) decreased S-IgA and S-lysozyme responses post-competition. S-α-amylase and S-cortisol responses increased post-competition. No URS were reported during and in the one month following the ultra-marathon.

The implications of the results in **Chapter 4** and **Chapter 5** demonstrated perturbations to oral-respiratory mucosal immune responses during extreme physical exertion; however, this did not result in URS. Therefore, it would be prudent to minimise accompanying physiological stressors during periods of extreme physical exertion. The results in **Chapter 4** and **Chapter 5** cannot be generalised to other times of the year. Appropriate education (e.g., hydration maintenance, non-infectious episode management, medical management of established respiratory illness) and information (i.e., pollen and pollution counts at the location of the event or competition) may help prevent unwanted URS manifestations and performance decrements. However, limitations of the multi-stage ultra-marathon study (**Chapter 4**) include the failure to measure other S-AMPs with other anti-bacterial and anti-viral properties and the inability to assess and accurately differentiate between infectious *vs.* non-infectious episodes. Limitations of the 24 h continuous ultra-marathon study (**Chapter 5**) include the failure to determine the time-course of recovery of S-AMPs (i.e., the length of

time mucosal immune status is depressed). Notably, both studies also took place during the summer months (**Chapter 4:** second week of July, **Chapter 5:** first week of September) when infectious episodes (e.g., verified *Epstein-Barr virus* reactivation, *Rhinovirus* and *Influenza* infections) are fewer compared to winter months. Thus, the prevalence of URSI reported is in part, likely dependent on the time of year the event or competition takes place.

A multi-stage ultra-marathon in hot ambient conditions (**Chapter 6**) increased resting and post-stage circulatory gram-negative bacterial endotoxin concentration. Increases in pro-inflammatory cytokines post-stage (i.e., interleukin-1 beta (IL-1β), tumour necrosis factor-alpha (TNF-α), and interferon-gamma (IFN-γ)) were counteracted by a compensatory anti-inflammatory cytokine response (i.e., interleukin-10 (IL-10) and interleukin-1 receptor antagonist (IL-1ra)). GI symptoms were commonly reported (58% reported at least one severe GI symptom) during the multi-stage ultra-marathon.

A 24 h continuous ultra-marathon in temperate ambient conditions (**Chapter 7**) increased post-competition circulatory gram-negative bacterial endotoxin concentration. Increases in pro-inflammatory cytokines (i.e., interleukin-6 (IL-6) IL-1β, TNF-α)) post-competition were counteracted by a compensatory anti-inflammatory cytokine response (i.e., IL-10). GI symptoms were commonly reported (75% reported at least one severe GI symptom) during the ultra-marathon.

The implications of the results in **Chapter 6** and **Chapter 7** suggest that whilst in well-trained individuals where the exertional-heat stress is well tolerated, clinically significant episodes ((e.g., exertional-heat illness, systemic inflammatory response syndrome (SIRS), sepsis and autoimmune conditions)) may be offset, individuals who are inadequately trained

may pose a greater risk for development of clinically significant episodes. Notably, exertional-heat illness continues to be a military problem during training and operations whereby the hospitalization rate of heat stroke has markedly increased (i.e., a five-fold increase; 1.8 per 100,000 in 1980 to 14.5 per 100,000 in 2001) (Carter *et al.* 2005). Additionally, delayed elevation of inflammatory variables ((e.g., C-reactive protein (CRP) and cytokine profile)) after competition may play a role in the aetiology of undefined underperformance and chronic fatigue syndromes. Appropriate education and competition preparation (e.g., the need to be well-trained to complete the required distance, heat acclimation protocols, hydration maintenance, cooling strategies) may help prevent clinically significant episodes occurring in 'high-risk' and (or) illness prone individuals. However, limitations of the multi-stage ultra-marathon study (**Chapter 6**) include the failure to measure other variables (e.g., anti-endotoxin antibodies or endotoxin neutralising capacity) and measurements (e.g., core body temperature; T_{core}). Limitations of the 24 h continuous ultra-marathon study (**Chapter 7**) include the failure to determine the time-course of recovery of CRP and cytokine profile (i.e., the length of time inflammatory variables remain elevated).

These observational studies applied exercise models of an extreme nature (**Chapter 4** to **Chapter 7**) unlike the majority of previous research. Additionally, measuring a number of time-related immune variables and tracking over a five day period (**Chapter 4** and **Chapter 6**) is currently absent. Whilst these studies attempted to identify the key immune variables that may lead to potential sub-clinical and clinically significant outcomes, a lack of adequate research control did not allow for definitive conclusions to be made about the effects of individual and combined physiological stressors on the immune variables measured. For example, the degree and duration to which an individual is exposed to a single or combination of stressors is dependent on a number of factors (e.g., environmental conditions

during the ultra-marathons) Therefore, determining which individual or combination of stressors is responsible for the perturbations in immune variables observed, whether a cumulative effect of stressor exposure is present or whether a particular stressor (s) exerts a greater influence over others is limited.

Seven consecutive days probiotic supplementation containing *L.casei* (x 10^{11} colony forming units (CFU)/day)) did not influence S-IgA, S-α-amylase or S-lysozyme responses at rest after EHS, and during the recovery period compared with a placebo (**Chapter 8**). Probiotic supplementation did not prevent or attenuate EHS induced endotoxaemia and cytokinaemia; nor is it more positively favourable over a placebo (**Chapter 9**).

The implications of the results in **Chapter 8** and **Chapter 9** suggest that whilst in healthy individuals probiotic supplementation is not justified, further investigation into 'high-risk' and (or) illness prone individuals (i.e., those who commonly experience URS or GI symptoms) may be warranted. Limitations of the probiotic-EHS (**Chapter 8** and **Chapter 9**) include the failure for further exploration of the mechanistic responses of probiotics. For example, additional measurements such as intestinal permeability tests (e.g., urinary excretion ratio of lactulose to rhamnose). Whilst these laboratory-controlled studies (**Chapter 8** and **Chapter 9)** provide insight into the impact of a specific probiotic strategy as observed in athletic populations on key immune variables, a larger sample size including both males and females would be considered more representative of the endurance running population and would have allowed for further sub-group analysis (e.g., hydration status); whilst a longer probiotic supplementation period in accordance with previous clinical models is an area of further research.

Declaration

This work has not been previously accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.

Signed……………………………………………………..(candidate)

Date…………………………………………………………..

Statement One

This thesis is the product of my own investigation, except where otherwise stated. Other sources are acknowledged giving references.

Signed……………………………………………………..(candidate)

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Statement Two

I hereby consent for my thesis, if accepted to be available for photocopy and for interlibrary loan, and for the title and summary to be made available to outside organisations.

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Date…………………………………………………………..

Acknowledgements

First and formost, I appreciatively thank my Director of Studies Dr. Ricardo Costa, for his invaluable support and continued guidance throughout my Ph.D research programme. His commitment to excellence and high ethical and academic standards has enabled me to develop and progress my research knowledge and skills; whilst his personable, enthusiastic and positive nature created an enjoyable and pleasant research experience. For the incredible opportunities he has given me over the past five years, I express my deepest gratitide. I would also like to greatfully acknowledge and thank my Ph.D supervisors, Dr. Doug Thake and Dr. Mike Price for their support, encouragement and guidance during my Ph.D research programme. Additionally, an enormous thank you and acknowledgement must go to the members of Coventry University Sport and Exercise Nutritional Support and Research Team for their support during the process of sample and data collection, and to the Al Andalus Trail Race and Glenmore24 race directors and staff for their support and assistance during various aspects of study implementation. A special thanks to Dr. Paula Robson-Ansley, Dr. Ana Maria Teixeira, Dr. Luis Rama, Fatima Rosado, Dean Allerton, and Krystal Hemming for their technical support in sample analysis and assistance in the laboratory. Thank you to all the undergraduate and postgraduate students who were involved in various aspects of data collection, and to all the research participants for their effort and enthusiasm during the study protocols. Finally, I would like to give my upmost thanks to my family and friends for their endless support and love over the last five years.

Publications

The following list of publications arose from the material presented in this thesis. I also gratefully acknowledge the input and involvement from other named authors for each publication.

Full papers

Gill, S. K., Teixeira, A. M., Rosado, F., Cox, M., and Costa, R. J. S. (2015) 'High-Dose Probiotic Supplementation Containing *Lactobacillus casei* for 7 Days Does Not Enhance Salivary Antimicrobial Protein Responses to Exertional-Heat Stress Compared With Placebo'. *International Journal of Sport Nutrition and Exercise Metabolism* 26 (2), 150-160

Gill, S. K., Allerton D. M., Ansley-Robson, P., Hemmings, K., Cox, M., and Costa, R. J. S., (2015) 'Does Acute High Dose Probiotic Supplementation Containing *Lactobacillus casei* Attenuate Exertional-Heat Stress Induced Endotoxaemia and Cytokinaemia?' In press.

Gill, S. K., Teixeira, A., Rama, L., Rosado, F., Hankey, J., Scheer, V., Hemmings, K., Ansley-Robson, P., and Costa, R. J. S. (2015) 'Circulatory Endotoxin Concentration and Cytokine Profile in Response to Exertional-Heat Stress During a Multi-Stage Ultra-Marathon Competition'. *Exercise Immunology Review* 21, 114-128

Gill, S. K., Hankey, J., Wright, A., Marczak, S., Hemming, K., Allerton, D., Ansley-Robson, P., and Costa, R. J. S. (2015) 'The Impact of a 24-hour Ultra-Marathon on Circulatory Endotoxin and Cytokine Profile'. *International Journal of Sports Medicine* 36 (8), 688-695

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Costa, R. J. S., Teixeira, A., Rama, L., Swancott, A. J., Hardy, L. D., Lee, B., Camoes- Costa, V., Gill, S., Waterman, J. P., Freeth, E. C., Barrett, E., Hankey, J., Marczak, S., Valero-Burgos, E., Scheer, V., Murray, A., and Thake, C. D. (2013) 'Water and Sodium Intake Habits and Status Of Ultra-Endurance Runners During a Multi- Stage Ultra-Marathon Conducted in a Hot Ambient Environment: An Observational Field Based Study'. *Nutrition Journal* 12, 13.

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Gill, S. K., Teixeira, A., Rosado, F., Hankey, J., Scheer, V., Robson-Ansley, P., and Costa, R. J. S. (2013) 'Salivary Antimicrobial Protein Responses During Multi-Stage Ultra-Marathon Competition Conducted in Hot Environmental Conditions'. *Applied Physiology, Nutrition, and Metabolism* 38 (9), 977-987

Abstracts

Hankey, J., Gill, S., Costa᾽ R. J. S., Gregson᾽ W., Whyte, G., and George, K. (2014). The impact of a 37 km foot race in hot ambient conditions upon cardiac function. *European College of Sports Science conference proceedings*. held 2-5 July at VU University Amsterdam, Amsterdam.

Costa, R. J. S., Gill, S. K., Robson-Ansley, P., Hankey, J., Wright, A., Marczak, S., Hussain, H., Hemmings, K., and Murray, A. (2013). The Impact of a 24-hour Continuous Running Competition Conducted in a Thermoneutral Environment on Circulatory Endotoxin Concentration and Cytokine Profile. *International Society of Exercise and Immunology Symposium.* held 9-12 September 2013 at Newcastle Town Hall, Australia.

Costa, R.J.S., Gill, S.K., Teixeira, A., Rama, L., Rosado, F., Hankey, J., Hussain, H., Hemmings, K., and Scheer, V. (2013). Circulatory Endotoxin Concentration and Cytokine Profile During Multi-Stage Ultra-Marathon Competition Conducted in a Hot Ambient Environment. *International Society of Exercise and Immunology Symposium.* held 9-12 September 2013 at Newcastle Town Hall, Australia.

Hankey, J., Gill, S.K., Costa, R. J. S., Gregson, W., Whyte, G., and George, K. (2013). The impact of a 230 km multi-stage ultra-marathon in hot ambient conditions upon cardiac function. *British Association of Sport and Exercise Sciences Annual Conference.* held 3-5 September 2013 University of Central Lancashire, Preston, UK.

Gill, S.K., Teixiera, A., Rama, L., Morse, T., Cresswell, S., Godson, N., Scheer, V., Valero, E., and Costa, R.J.S. (2012). Haematological changes in recreational endurance runners during a multi-stage ultra-marathon conducted in hot ambient conditions. *European College of Sport Science conference proceedings*. held 4-7 July 2012 at Oud Sint-Jan Congress Centre, Bruges, Belgium.

Gill, S.K., Teixiera, A., Rama, L., Barrett, E., Freeth, E., Waterman, J., Marczak, S., Hankey, J., Scheer, V., Valero, E., and Costa, R.J.S. (2012). Oral-respiratory mucosal immune responses during a multi-stage ultra-marathon competition in hot ambient conditions. *European College of Sport Science conference proceedings*. held 4-7 July 2012 at Oud Sint-Jan Congress Centre, Bruges, Belgium.

Barrett, E., Freeth, E., Waterman, J., Gill, S.K., Hankey, J., Marczak, S., and Costa, R.J.S. (2012). Exercise-induced changes to hydration status of runners competing in a multi-stage ultra-marathon set in a hot environment: An observational study. *International Convention on Science, Education and Medicine in Sport*. held 9-24 July 2012 at Scottish Exhibition and Conference Centre, Glasgow, Scotland.

Freeth, E., Barrett, E., Waterman, J., Gill, S.K., Hankey, J., Marczak, S., and Costa, R.J.S. (2012). Do runners competing in a multi-stage ultra-marathon meet post-exercise carbohydrate and protein guidelines? An observational study. *International Convention on Science, Education and Medicine in Sport*. held 9-24 July 2012 at Scottish Exhibition and Conference Centre, Glasgow, Scotland.

Costa, R., Gill, S.K., Swancott, A., Hardy, L., Lee, B., Camões-Costa, V., Barrett, E., Freeth, E., Waterman, J., Marczak, S., Hankey, J., Scheer, V., Valero, E., Murray, A., and Thake, D. (2012). Sleep habits and recovery quality of ultra-endurance runners during a multi-stage ultra-marathon competition in the heat. *International Convention on Science, Education and Medicine in Sport*. held 9-24 July 2012 at Scottish Exhibition and Conference Centre, Glasgow, Scotland.

Gill, S.K., Barrett, E., Freeth, E., Marczak, S., Hankey, J., Wright, A., Toner, E., Wardle, K., Britton, R., Murray, A., and Costa, R. J. S. (2012). Hydration status of ultra-endurance runners during a 24 hour ultra-endurance running competition conducted in the Scottish Highlands. *International Convention on Science, Education and Medicine in Sport*. held 9-24 July 2012 at Scottish Exhibition and Conference Centre, Glasgow, Scotland. **Winner of the Human Kinetics Research Student Poster Presentation Award.**

Costa, R.J.S., and Gill, S.K. (2011). Does hydration play a role in oral-respiratory mucosal immunity during multi-stage ultra-marathon conducted in a hot ambient environment: The results. *2nd European Hydration Institute Meeting*. held October 2011, Madrid, Spain.

Gill, S.K., and Costa, R.J.S. (2010). Does hydration play a role in oral-respiratory mucosal immunity during multi-stage ultra-marathon conducted in a hot ambient environment: The experimental design. *1st European Hydration Institute Meeting*. held June 2011, London, UK.

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Thesis Format

The general introduction (**Chapter 1**) and literature review (**Chapter 2**) provides a summary of previous research, identified research gaps, and provides the rationale and justification for the aims of the current research. A description of the general methods proceeds (**Chapter 3**), which explains the common procedures, techniques and analyses performed in the subsequent studies. The thesis consists of six independent studies. The first study investigated the effects of a multi-stage ultra-marathon competition in hot ambient conditions (32-40°C) on S-AMP responses, stress hormone response, and incidence of URS (**Chapter 4**). The second study investigated the effects of a 24 h continuous ultra-marathon in temperate ambient conditions (0-20°C) on S-AMP responses, stress hormone response, and incidence of URS (**Chapter 5**). The third study investigated the effects of a multi-stage ultra-marathon competition in hot ambient conditions (32-40°C) on circulatory endotoxin concentration, cytokine profile, and GI symptoms (**Chapter 6**). The fourth study investigated the effects of a 24 h continuous ultra-marathon in temperate ambient conditions (0-20°C) on circulatory endotoxin concentration, cytokine profile, and GI symptoms (**Chapter 7**). The final two studies investigated the influence of acute high dose probiotic (*L.casei*) supplementation on S-AMP responses (**Chapter 8**); and circulatory endotoxin concentration and cytokine profile (**Chapter 9**) in response to EHS. A general discussion (**Chapter 10**) contains a summary and critical appraisal of the main findings of the research programme, including limitations and directions for future research. An additional methodological study investigating the influence of saliva flow rate (SFR) within a range of specified collection times, and after mouth rinsing with differing water temperatures can be found in the Appendix (**Appendix A**). Throughout this thesis, abbreviations are defined at first use. For clarity, a list of abbreviations, table

references and figures legends appear prior to **Chapter 1**. Due to the relatedness between thesis chapters, bold type is used when cross-referencing occurs.

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List of Abbreviations

AQUA	Allergy Questionnaire for Athletes
T_{amb}	ambient temperature
ANOVA	analysis of variance
AMPs	anti-microbial proteins
APC's	antigen presenting cells
bpm	beats per minute
BM	body mass
BALT	bronchus/tracheal-associated lymphoid tissue
CV	coefficient of variation
CFU	colony forming units
CMIS	common mucosal immune system
CON	control
T_{core}	core body temperature
CRP	C-reactive protein
Da	dalton
ºC	degrees centigrade
EU	endotoxin units
ELISA	enzyme-linked immunosorbent assay
EDTA	ethylenediaminetetraacetic acid
EH	euhydrated
EHS	exertional-heat stress
FITC	flurescein isothiocyanate
GI	gastrointestinal
g	gram
GALT	gut-associated lymphoid tissue
HR	heart rate
h	hours
HH	hypohydrated
HPA	hypothalamic-pituitary adrenal axis
I-BABP	ileal bile acid binding protein
Igs	immunoglobulins

IgA	immunoglobulin A
IFN-γ	interferon-gamma
IL-1β	interleukin-1 beta
IL-1ra	interleukin-1 receptor antagonist
IL-6	interleukin-6
IL-8	interleukin-8
IL-10	interleukin-10
IU	international units
I-FABP	intestinal fatty acid binding protein
J	joining
kCal	kilocalorie
kg	kilogram
km	kilometre
L.casei	Lactobacillus casei
LAL	limulus amebocyte lysate
LPS	lipopolysaccharide
l	litre
LRT	lower respiratory tract
mRNA	messenger ribonucleic acid
MJ	megajoule
μg	microgram
μl	microliter
mg	milligram
min	minute
ml	millilitre
mOsmol	milliosmol
MALT	mucosa-associated lymphoid tissue
NK	natural killer
ng	nanogram
nmol	nanomole
nm	nanometre
NALT	nasopharynx-associated lymphoid tissue
NF-κB	nuclear factor-kappa B
OD	optical density

Osmol	osmole
PAMPs	pathogen-associated molecular patterns
PPRs	pattern-recognition receptors
PPARγ	peroxisome proliferator-activated receptor gamma
pg	picogram
PLA	placebo
P_{Osmol}	plasma osmolality
Pv	plasma volume
PIgR	polymeric immunoglobulin receptor
K3EDTA	potassium ethylenediaminetetraacetic acid
PRO	probiotic
RPE	rating of perceived exhaustion
RH	relative humidity
RES	reticuloendothelial system
rpm	revolutions per minute
S-α-amylase	salivary alpha amylase
S-cortisol	salivary cortisol
SFR	saliva flow rate
S-IgA	salivary immunoglobulin A
S-lactoferrin	salivary lactoferrin
S-lysozyme	salivary lysozyme
SC	secretory component
SCFAs	short-chain fatty acids
SD	standard deviation
SEM	standard error of the mean
SAM	sympatheticoadrenal-medullary axis
SIRS	systemic inflammatory response syndrome
TCR	thermal comfort rating
Th1	T-helper1
Th2	T-helper2
TLRs	toll-like receptors
tSC	transmembrane secretory component
TNF-α	tumour necrosis factor-alpha
T_{re}	rectal temperature

T_{tymp}	tympanic temperature
U	unit
UPS	underperformance syndrome
URT	upper respiratory tract
URTI	upper respiratory tract infections
URSI	upper respiratory symptoms and infections
URS	upper respiratory symptoms
VO_{2max}	maximal oxygen uptake

CHAPTER ONE

General Introduction

Consisting of primary lymphoid organs (e.g., bone marrow and thymus) and secondary lymphoid organs (e.g., lymph nodes and Peyer's patches), linked by complex networks (e.g. lymphatic and circulatory systems) of circulating immune cells (i.e., leukocytes) and protein molecules ((e.g., antibodies, anti-microbial proteins (AMPs), cytokines and complement)); the immune system is a dynamic multi-faceted surveillance system strategically distributed throughout the body, with the ability to monitor, identify and differentiate between self-antigens and foreign antigens in order to maintain protection, defence and homeostasis.

An integral part of the immune system is the mucosa-associated lymphoid tissue (MALT), a highly specialised mono-layered structure estimated $400m^2$ juxtaposed to mucosal surfaces (Montilla *et al.* 2004) and further sub-divided according to anatomical site. The MALT loosely comprises of gut-associated lymphoid tissue (GALT), nasopharynx-associated lymphoid tissue ((NALT; upper respiratory tract (URT)), and bronchus/tracheal-associated lymphoid tissue ((BALT; lower respiratory tract (LRT)) (Cesta 2006). The overlapping links and cross-talk communication between different mucosal sites is often termed the common mucosal immune system (CMIS).

These mucosal surfaces comprise of highly specialised epithelial cells (e.g., enterocytes, Paneth and goblet cells) and their tight junctions, interspersed with structured lymphoid tissues (e.g., tonsils and adenoids in the BALT and NALT, and Peyer's patches in the GALT) and specialised microfold cells, beneath of which is heavily populated by intra-epithelial lymphocytes and antigen presenting cells (APC's). Continually exposed to the external

antigenic environment and the primary site for exogenous pathogen invasion whereby most bacterial and viral infections are most likely to gain entry and initiate an immune response, the innate physical and mechanical barriers (e.g., skin, mucous membranes, and ciliary function), secretions (e.g., saliva and tears) which contain anti-microbial proteins (e.g., mucins and lysozyme), cellular ((e.g., natural killer (NK)) cells, macrophages, neutrophils. and dendritic cells)) and acquired biochemical defences ((e.g., immunoglobulins (Igs)) of the MALT work synergistically to provide host protection and are considered the first line of defence against the colonisation of pathogens at the mucosal surface. For example, S-AMPs contain anti-bacterial and anti-viral properties that inhibit bacterial colonisation and neutralise viruses at the oral-respiratory mucosal epithelial surface (Brandtzaeg *et al.* 1999, McNabb and Tomasi 1981, Norderhaug *et al.* 1999, Tenovuo 1998, West *et al.* 2006), whilst the mucosal epithelium lining the GI tract is colonised by a plethora of microorganisms which serve a number of important metabolic, immunological and structural functions.

The normal immunological response is tightly controlled, regulated and maintained within homeostatic boundaries. The degree and direction ((e.g., innate, humeral-mediated and (or) cell-mediated)) of the normal immunological response is dependent upon the type of pathogen present (e.g., intracellular pathogens and extracellular pathogens), individual health status (e.g., tissue damage, injury, autoimmune disorders), circadian variations and (or) neuroendocrine control (Dimitriou, Sharp, and Doherty 2002, Dinges *et al.* 1995, Shepard and Shek 1997, Shephard, Castellani, and Shek 1998). In particular, stimulation of the hypothalamic-pituitary adrenal (HPA) and sympatheticoadrenal-medullary (SAM) axes and subsequent production of glucocorticoid hormones and catecholamines are the two main delineated pathways through which changes in immune function occur (Padgett and Glaser 2003). These pathways modulate innate (e.g., macrophages and neutrophils) and adaptive

(e.g., T- and B-lymphocyte cells) immune cell activity (e.g., cellular maturation, differentiation, proliferation and trafficking, alterations in antibody production and cytokine secretion) through bi-directional communication between the immune system and neuroendocrine system due to receptors (e.g., glucocorticoid and adrenergic) for neurotransmitters, neuropeptides and hormones located on leukocytes and lymphoid tissue (Felten *et al.* 1998, Madden 2003, Sternberg 2006).

Various physiological stressors have been shown to stimulate neuroendocrine responses (namely cortisol and catecholamines) and influence host defences, resulting in either a depressed or enhanced immune status depending on the degree of the stress stimulus (Costa *et al.* 2010, Gleeson 2006, Nieman 1997, Shephard, Castellani, and Shek 1998, Walsh and Whitham 2006). For example, whilst transient, acute stress is associated with immuno-enhancing effects (Dhabhar and McEwen 1997, Dhabhar 2002, Dhabhar 2008); paradoxically, it is frequently reported that prolonged or extreme stress resulting in amplification and (or) chronic activation of the HPA and SAM axes, suppresses innate and adaptive immune function (Gleeson *et al.* 1995, Nieman 1997) potentially increasing the susceptibility to illness and (or) infection. Notably, the nature of field-based studies allows for limited interpretation of which individual stressor or combination of stressors impact upon immune variables, and to what degree.

Whilst the hypothalamic temperature set-point remains stable with heat exposure during prolonged physical exertion, heat-stress occurs when difficulty with heat dissipation and subsequent overheating initiate body temperature increases (Shephard 1998). Indeed, prolonged physical exertion (with and without heat-stress) is associated with compromised

oral-respiratory mucosal immune status (e.g., depressions in S-AMPs) and an increased risk of URSI (e.g., verified *Epstein-Barr virus* reactivation, *Rhinovirus* and *Influenza* infections, allergen or damage induced localised inflammation) (Gleeson 2006, Gleeson and Pyne 2000, Peters and Bateman 1983, Matthews *et al.* 2002). Additionally, prolonged physical exertion (with and without heat-stress) is associated with disturbances to intestinal epithelial integrity (ter Steege and Kolkman 2012, Rehrer and Meijer 1991). Such disturbances have been linked to an endotoxin-induced cytokine-mediated inflammatory response which has been implicated in the aetiology of heat-related illness (e.g., heat stroke) (Lim and Mackinnon 2006, Opal 2010), and in the manifestation of GI symptoms (Jeukendrup *et al.* 2000, Lambert 2008, Øktedalen *et al* 1992, Peters *et al.* 1999, van Leeuwen *et al.* 1994). As a result, it is plausible that participating in extreme physical exertion (with or without heat stress) may initiate amplified or cumulative perturbations to oral-respiratory mucosal immunity and (or) to intestinal epithelial integrity, and promote sub-clinical or clinically significant outcomes in active populations (i.e., moderately and highly-trained individuals).

Several published reviews have reported depressed oral-respiratory mucosal immune status (Gleeson 2000, Gleeson and Pyne 2000, Walsh *et al.* 2011a) and disturbances to intestinal epithelial integrity (Lambert 2009, Lim and Mackinnon 2006), proportional to the degree of the exercise-stress. More recently, probiotic supplementation has been suggested as a possible nutritional strategy in active populations to counteract the depressions in oral-respiratory mucosal immunity and disturbances to intestinal epithelial integrity commonly observed following participation in prolonged physical exertion and (or) intensified training periods (Gleeson *et al.* 2011a, West *et al.* 2009). To date, the impact of extreme physical exertion (particularly running exercise), which promotes the greatest physiological responses, is scarce (Murray and Costa 2012). Whilst, the efficacy of probiotic supplementation in

counteracting depressions in oral-respiratory mucosal immunity and disturbances to intestinal epithelial integrity (i.e., increases in intestinal permeability and a subsequent endotoxin-induced cytokine-mediated inflammatory response) commonly reported after exercise-stress has not yet been thoroughly investigated.

With this in mind, the focus of this thesis was to firstly assess oral-respiratory mucosal immune status (S-IgA, S-α-amylase, S-lysozyme), stress hormone response (S-cortisol) and incidence of URS; in addition to intestinal epithelial integrity (assessed by circulatory endotoxin concentration), cytokine profile and incidence of GI symptoms in response to extreme physical exertion (with and without heat-stress) with accompanying physiological stressors and; secondly, to identify whether probiotic supplementation may prevent unwanted perturbations to oral-respiratory mucosal immune status and intestinal epithelial integrity during exposure to EHS.

CHAPTER TWO

Literature Review

Extreme, sustained activities (e.g., ultra-endurance, adventure and exploration events, military training and expeditionary operations) with or without heat-stress are commonly associated with accompanying physiological stressors which may firstly; initiate amplified and (or) cumulative perturbations to oral-respiratory mucosal immune status, leading to subsequent depressions in S-AMPs and an increased risk of sub-clinical or clinically significant outcomes (e.g., URSI) and secondly; may initiate amplified and (or) cumulative perturbations to intestinal epithelial integrity, promoting an over-exaggerated endotoxin-induced cytokine-mediated inflammatory response and an increased risk of sub-clinical or clinically significant outcomes (e.g., heat-related illness and GI symptoms) during the period after stress stimuli (Martinez-Lopez *et al.* 1993, Neville, Gleeson, and Folland 2008, Nieman 1997, Shephard, Castellani, and Shek 1998). Moreover, active populations participating in such extreme activities are potentially at high-risk for pathogen transfer and contracting illness and (or) infection (Gleeson *et al.* 2000). Indeed, unfamiliar environments (e.g., foreign locations) may harbour and expose an individual to pathogens (e.g., airborne and food) unrecognised by the immune system. Such outcomes may further jeopardise immune status and increase vulnerability to illness and (or) infection in the field (Martinez-Lopez *et al.* 1993, Neville, Gleeson, and Folland 2008, Nieman 1997, Shephard, Castellani, and Shek 1998). Additionally, exposure to unavoidable cross-contamination factors such as close contact and (or) sharing eating and drinking vessels with the surrounding population, and (or) unhygienic food and (or) drink preparation facilities may expose an individual to unrecognisable pathogens prior to and (or) after stress stimuli.

The following literature review will address the current evidence behind prolonged physical exertion (with and without heat-stress) with accompanying physiological stressors on selected immune responses, and the potential role of probiotic supplementation in counteracting perturbations to oral-respiratory mucosal immunity and in attenuating endotoxaemia. In **Section 2.1.1**, oral-respiratory mucosal immune (S-IgA, S-α-amylase, S-lysozyme) and stress hormone (S-cortisol) responses measured within **Chapter 4**, **Chapter 5** and **Chapter 8** will be reviewed. In **Section 2.1.2**, circulatory endotoxin concentration and cytokine profile measured within **Chapter 6**, **Chapter 7** and **Chapter 9** will be reviewed. Following this, **Section 2.2.1** to **2.2.2** and **Section 2.3.1** to **2.3.2** will independently review the impact of prolonged physical exertion and EHS, respectively, on oral-respiratory mucosal immune and stress hormone responses; and on circulatory endotoxin concentration and cytokine profile. **Section 2.4.1** to **2.4.3** will proceed and review the influence of probiotic supplementation on oral-respiratory mucosal immunity and intestinal epithelial integrity during physical exertion.

2.1.1 Oral-Respiratory Mucosal Immunity

The anti-bacterial and anti-viral properties of mucosal secretions, in particular S-IgA, protect oral-respiratory mucosal surfaces against foreign pathogens and provide an important first line defensive barrier (Woof and Kerr 2006). Given that pathogen colonisation is typically initiated at the mucosal surface with a high prevalence of all oral-respiratory infections originating here (~90-95%) (Bosch *et al*. 2002), the importance of the mucosal epithelium providing a protective barrier between the internal and external environment cannot be underestimated, particularly during periods of increased physiological stress when oral-respiratory mucosal immune status may be compromised (Bosch *et al*. 2003a, Bosch *et al*. 2003b, Gleeson and Pyne 2000, Neville, Gleeson, and Folland 2008). Since S-AMP responses will vary in accordance to SFR (Chicharro *et al*. 1998), a continuous saliva flow provides a mechanical washing effect, ensuring a constant supply of anti-bacterial and anti-viral constituents (e.g., S-IgA, S-α-amylase, S-lysozyme, S-lactoferrin and defensins) at the oral-respiratory mucosal surface (Tenovuo 1998) that inhibit bacterial colonisation to the mucosal epithelium, and neutralise, inactivate and prevent viral replication (Brandtzaeg *et al.* 1999, McNabb and Tomasi 1981, Norderhaug *et al.* 1999, Tenovuo 1998, West *et al.* 2006).

The primary mechanism by which S-IgA exerts its protective action is immune exclusion, rendering pathogens ineffective locally at the oral-respiratory mucosal surface without systemic immune activation. S-IgA inhibits pathogen colonisation (adherence) to the mucosal epithelium and invasion of the oral-respiratory pathway, in addition to neutralising and preventing replication of intra-epithelial viruses (Brandtzaeg *et al.* 1999, Mazanec *et al.* 1993, Woof and Mestecky 2005). To date, S-IgA is the only immune parameter that has consistently shown a positive correlation with URSI in active populations (Bishop and

Gleeson 2009, Gleeson 2000, Neville, Gleeson, and Folland 2008, Nieman *et al.* 2006a, Nieman 1997, Nieman 1994) whereby transient depressions in S-IgA are associated with an increased incidence of URSI; possibly due to compromised oral-respiratory mucosal immunity providing the opportunity for external pathogens to gain a hold on mucosal surfaces (Walsh *et al.* 2011a). URSI reports appear to be particularly common during prolonged physical exertion, intensified training periods and (or) heightened pathogen exposure (e.g., travelling and residing in foreign locations) (Bishop and Gleeson 2009, Gleeson and Pyne 2000, Libicz *et al.* 2006, Neville, Gleeson, and Folland 2008). For example, 12.9% of runners reported infectious episodes during the week following a marathon race, whilst 33.3% of runners reported infectious episodes during the two weeks after a 56 km running competition (Nieman *et al.* 1990, Peters and Bateman 1983). However, infectious episodes were assessed through self-report thus questioning the reliability of bacterial or viral infection presence. Equally, for runners participating in competitive events, URS *per se* (whether infectious or non-infectious) may lead to competing at a sub-optimal level or complete withdrawal from the event.

Immunoglobulin A (IgA) is originally synthesised and secreted from plasma cells residing in the mucosa and sub-mucosa. Whilst the long-term (days) regulation of S-IgA secretion is through modification of S-IgA synthesis, the short-term (minutes) regulation of S-IgA secretion is through transcytosis (mobilisation) stimulated by sympathetic nervous system activity (Goodrich and McGee 1998).

Figure 2.1 Salivary secretory IgA synthesis and translocation.

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Created by thesis author, based on Bishop and Gleeson (2009)

In vitro, the linking of the J-chain to the IgA has been shown to be essential for the initial complexing and stabilisation between the polymeric IgA and the tSC (Brandtzaeg and Prydz 1984). Importantly, only polymeric IgA have high affinity with the tSC which optimises the transepithelial transport of S-IgA (Johansen, Braathen, and Brandtzaeg 2000). The acquisition of the J-chain increases the effectiveness of antigen-binding sites and binding of bacteria and viruses, in addition to inducing minimal systemic immune activation (e.g., the

complement pathway), thereby allowing S-IgA to function in a non-inflammatory manner. Moreover, the glycosylated tSC protects the S-IgA against proteolytic degradation after secretion on the oral-respiratory mucosal surface (Woof and Mestecky 2005). Taking into account that considerably low concentrations of S-IgA have been observed in tSC deficient mice (Shimada *et al.* 1999), the physiological importance of the tSC cannot be underestimated. Moreover, both tSC and free secretory component (SC) exhibit several innate immune properties (e.g., bacterial neutralisation, inhibiting adherence of some gram-negative bacteria to the mucosal epithelium) (Corthesy 2010).

More recently, other constituent S-AMPs have been shown to exhibit protective properties in the oral respiratory pathway, working synergistically and in combination with S-IgA to support oral respiratory mucosal immunity, and provide an important first line defensive barrier against external pathogen invasion at oral-respiratory mucosal surfaces (Fábián *et al.* 2012, Woof and Kerr 2006). Indeed, S-α-amylase which is derived from the major salivary glands (i.e., parotid, submandibular and sublingual) in differing amounts, has been shown to assist in the prevention of bacterial attachment to mucosal surfaces, and when coupled with mucin 1, helps bacterial clearance from saliva. Additionally, S-α-amylase binds with high affinity to several oral streptococci species (Scannapieco *et al.* 1993). S-α-amylase secretion is influenced by exercise-induced sympathetic nervous system activation, the degree of which appears to be dependent on exercise intensity (Li and Gleeson 2004). For example, a five-fold increase in S-α-amylase activity was observed after a high intensity 60 min cycle exercise bout consisting of twenty 1 min periods at 100% maximal oxygen uptake (VO_{2max}), each separated by 2 min recovery at 30% VO_{2max} (Walsh *et al.* 1999). S-lysozyme, another constituent S-AMP derived from different sources (i.e., major and minor salivary glands, gingival crevicular fluid) has been shown to hydrolyse the glycosidic linkages present in

bacterial outer membranes, activate autolysins and initiate degradation. S-lysozyme can also bind bacteria and aggregate to facilitate clearance from the oral respiratory pathway (McNabb and Tomasi 1981, Tenovuo 1998, West *et al.* 2006). S-lysozyme also appears to be dependent upon exercise intensity, whereby a 55% increase in S-lysozyme concentration was observed after a graded exercise test to exhaustion (West *et al.* 2010). Extensive research has focused primarily on S-IgA as the predominant marker of oral-respiratory mucosal immunity during physical exertion, whereas other S-AMPs such as S-α-amylase and S-lysozyme have been investigated to a lesser degree and have been potentially underestimated as protective factors. Given the innate anti-bacterial and anti-viral properties of these S-AMPs and the lack of research investigating the potential impact of these individually or in combination with S-IgA, assessing the collective impact of these S-AMPs will provide a deeper understanding into how the oral-respiratory mucosal immune system responds during prolonged physiological stress (e.g., extreme physical exertion). To date, previous studies have shown variations in S-AMP responses in response to exercise. For example, 2 h of running at 75% VO$_{2max}$ in thermoneutral conditions (20°C) resulted in a 42% decrease in S-IgA concentration during recovery, but increases in S-α-amylase and S-lysozyme concentrations (81% and 109%, respectively) that remained elevated throughout recovery (Costa *et al.* 2012). Moreover, transient increases in S-IgA secretion rate (50%) were observed after an incremental exercise test to exhaustion (22.3 ± 0.8 min), while no change was observed at 75% VO$_{2max}$ of the same duration. Whereas, increases in S-lysozyme (160%) and S-α-amylase secretion rate (60%) were observed after both the exhaustion and 75% VO$_{2max}$ trials (Allgrove *et al.* 2008). These results suggest that other S-AMPs with similar protective properties as S-IgA, may counteract the depressions in S-IgA commonly observed after physical exertion. A more accurate interpretation of oral-respiratory mucosal immune status during prolonged physiological stress (i.e., depressions or counteractions between S-AMPs)

would potentially provide a more accurate indicator of URSI risk and subsequently allow for a more tailored intervention approach depending on whether the anticipatory outcome was infectious or non-infectious episode management.

In relation to oral-respiratory mucosal immunity, SFR is physiologically regulated by the autonomic nervous system with salivary glands innervated by both parasympathetic and sympathetic nerve endings. Hence, activation of neuroendocrine responses and subsequent increases in stress hormone release induced by stress stimuli have the potential to modulate oral-respiratory mucosal immunity through alterations in SFR (e.g., reduced volume of viscous saliva with increased osmolality and total protein concentration) and subsequent constituent S-AMP secretions (Bosch *et al.* 2003a, Chicharro *et al.* 1998, Gleeson and Pyne 2000, Teeuw *et al.* 2004). For example, partaking in prolonged physical exertion is generally associated with disturbances to a consistent saliva flow (i.e., decreases in SFR) (Bishop *et al.* 2000, Blannin *et al.* 1998, Walsh *et al.* 1999). Commonly used in clinical research settings, S-cortisol is considered an appropriate indicator of the stress response (activation of the HPA axis) to exercise. Firstly, concentrations are unaffected by changes in SFR; secondly, S-cortisol concentrations do closely reflect, and are highly correlated with plasma free cortisol concentrations, the biologically active component (Umeda *et al.* 1981); and thirdly, S-cortisol sample collection is considered methodologically advantageous (e.g., non-invasive). Indeed, stress hormones are considered to be immunosuppressive and are linked to the immune perturbations reported during the period after stress stimuli (Gleeson and Pyne 2000, Nieman 1997, Pedersen and Hoffman-Goetz 2000, Pedersen *et al.* 1997, Pedersen and Toft 2000, Shephard, Castellani, and Shek 1998, Walsh and Whitham 2006). Subsequently, S-cortisol was used to indicate acute changes in the stress response in **Chapter 4**, **Chapter 5** and **Chapter 8**.

While perturbations to oral respiratory mucosal immune status are transient with the majority of published research reporting S-IgA returning to pre-exercise within 1 h of exercise cessation (Blannin *et al.* 1998, Ljungberg *et al.* 1997, McDowell *et al.* 1992), S-IgA recovery can be hindered (≤72 h) (Gleeson and Pyne 2000, Nieman 2000, Nieman 1997, Nieman *et al.* 1990, Peters and Bateman 1983) compared with regular, moderate physical exertion whereby increased S-IgA responses have been reported (Akimoto *et al.* 2003, Allgrove *et al.* 2008, Bishop and Gleeson 2009, Blannin *et al.* 1998, Gleeson, Pyne, and Callister 2004, Klentrou *et al.* 2002, Libicz *et al.* 2006, McDowell *et al.* 1991).

In summary, several constituent S-AMPs bathe the oral-respiratory pathway, offering protective immune properties that inhibit pathogen colonisation. Indeed, dissimilarities in responses of different S-AMPs ((e.g., decreases in S-IgA concomitant with increases in S-α-amylase and (or) S-lysozyme)) have been reported after stress stimuli. Hence, a more comprehensive indicator of oral-respiratory mucosal immune status may be provided when S-AMPs are assessed collectively, thus potentially providing a more accurate indicator of URSI risk. **Chapter 4** and **Chapter 5** aimed to assess the influence of extreme physical exertion on S-AMP responses during a multi-stage ultra-marathon and a 24 hour continuous ultra-marathon, respectively.

2.1.2 Circulatory Endotoxin Concentration and Cytokine Profile

Comprising of enterocytes, Paneth and goblet cells, the epithelial lining along the GI tract plays a crucial role in preventing translocation of harmful microorganisms, some of which present pathogenic properties, across the intestinal epithelium and entering the portal and

systemic circulation (Ganz 2003, Lambert 2008, Lim and Mackinnon 2006, Tuma and Hubbard 2003, Ulluwishewa *et al.* 2011). In addition, the multi-protein, semi-permeable tight junctions that close the gap between adjacent enterocytes continuously regulate the transcellular and paracellular absorption of substances (i.e., fluid and macromolecules) across the intestinal epithelium.

Gram-negative bacterial endotoxins are high-molecular weight lipopolysaccharides (LPS) complexes (>100,000 dalton (Da)) with known pathogenic properties, and the major component of the outer membranes of the cell walls in all gram-negative bacteria. Endogenous bacterial endotoxins are non-toxic if they remain within the GI tract; although small quantities can routinely leak through the intestinal epithelium *via* the tight junctions and enter the portal circulation during daily function, representing a normal physiological state when cell lysis occurs (Jacob *et al.* 1977, Marshall 1998, Nolan 1981). Indeed, bacterial endotoxin translocation has been substantiated in human studies, whereby translocation occurrence as examined by bacterial analysis of intestinal serosa and mesenteric lymph nodes was identified in 10.3% of general surgical patients (*n*= 267); although this was noticeably reduced to 5% when patients with distal intestinal obstruction and inflammatory bowel disease were excluded (Sedman *et al*. 1994). While O'Boyle *et al.* (1998) cultured mesenteric lymph nodes, serosal scrapings and peripheral blood in surgical patients (*n*= 448) undergoing laparotomy and found bacterial translocation in 15.4% (*n*= 69) of all patients. Equally, these results were verified in a clinical population, whilst the methods used to vertify presence of endotoxin may have unestimated the true occurrence of endotoxin translocation. In comparison to exercise models, intestinal permeability is commonly determined through circulatory endotoxin concentrations or ingestion of sugar probes.

The liver promotes detoxification and clearance of transient bacterial endotoxins derived from the GI tract (*via* the portal vein) or from the systemic circulation (*via* the hepatic artery). Bacterial endotoxins are neutralised, degraded and removed from circulation through both innate and adaptive mechanisms such as the reticuloendothelial system (RES) (primarily hepatic Kupffer cells) (Marshall 1998), enzymes (e.g., phosphatases and hydrolases) (Poelstra *et al.* 1997), leukocytes (e.g., monocytes, neutrophils, basophils and mast cells), and lipoproteins (e.g., high-density lipoprotein, low-density lipoprotein, apolipoprotein) (Emancipator, Csako, and Elin 1992, Flegel *et al.* 1993); although anti-endotoxin antibodies (e.g., IgM, IgG and IgA class) (Camus *et al.* 1998) create the most prevalent and effective defence against bacterial endotoxins by inhibiting their biological effects.

However, disturbances to intestinal epithelial integrity (e.g., exercise-induced trauma and injury) can cause deterioration of the protective epithelial lining, leading to subsequent increases in intestinal permeability (i.e., widening of the epithelial tight junction spaces), which by definition, is the non-mediated translocation of large molecular weight molecules (>150 Da) such as bacterial endotoxins (alongside other noxious substances such as bacteria, bacterial by-products, bile, food antigens, foreign particles and hydrolytic enzymes) across the intestinal epithelium into the portal circulation (Lambert 2009, Marshall 1998, Pals *et al.* 1997). The liver has a limited bacterial endotoxin-removal capacity and when the threshold for bacterial endotoxin flux is met, whereby anti-endotoxin mechanisms are overwhelmed and the rate of bacterial endotoxin translocation from the GI tract prevails over clearance, bacterial endotoxin translocation into the systemic circulation ensues, termed endotoxaemia (Camus *et al.* 1998, Camus *et al.* 1997, Lim and Mackinnon 2006); which is considered the initial phase of heat stroke aetiology, mirroring the pathophysiological mechanisms of sepsis (Lim and Mackinnon 2006). Notably, varying degrees of circulatory endotoxin

concentrations (5 pg·ml to 294 pg·ml) have been observed following marathon, ultra-marathon and triathlon events (Bosenberg *et al.* 1988, Brock-Utne *et al.* 1988, Camus *et al.* 1997, Jeukendrup *et al.* 2000). The variations observed between and within previous field-based studies are likely due to alterations in intestinal permeability induced by the varying degrees and durations of individual or combined stressors such as the competition/event protocols (e.g., duration), the ambient temperature (T_{amb}) and the population group (e.g., training status) and number.

Endotoxaemia is a potent activator of leukocytes which in turn, stimulate the secretion of pro-inflammatory cytokines, namely TNF-α, IL-1β and IL-6 (Bouchama *et al.* 1991, Camus *et al.* 1998, Pedersen 2000, van Deventer *et al.* 1990), counterbalanced by the secretion of anti-inflammatory cytokines such as IL-10 and IL-1ra (Ostrowski *et al.* 1999, Pedersen 2000, Petersen and Pedersen 2005). The initiation of a cytokine-mediated inflammatory response has been shown to modify tight junction function (e.g., changes in protein composition and structure) and enhance intestinal permeability; in particular, TNF-α and IFN-γ (Capaldo and Nusrat 2009, Gitter *et al.* 2000, Madara and Stafford 1989, McKay and Singh 1997, Mullin and Snock 1990, Rodriguez *et al.* 1995, Youakim and Ahdieh 1999). To date, experimental models have shown that intravenous injection of endotoxin in rodents (5 µg·kg, 25 µg·kg and 1 mg·kg) signals a responsive cytokine cascade (TNF-α, IL-1β and IL-6) 10 to 30-fold above baseline (Givalois *et al.* 1994). Administration of endotoxin at 2 ng·kg in healthy human subjects resulted in peak circulating endotoxin concentrations ranging from 7 to 13 ng·l, followed by marked increases in TNF-α and IL-6, reaching peak concentrations after 60 to 90 min (68 to 1374 ng·l) and 120 to 150 min (72 to 2820 U·ml), respectively (van Deventer *et al.* 1990), whilst administration at 4 ng·kg in healthy human subjects instigated increases in TNF-α after 90-180 min, reaching peak concentrations of 240 ± 70 pg·ml (Michie *et al.*

1988). Although these studies provide insight into the mechanistic action of endotoxin, the use of animal models limits generalisability. Equally, the small sample sizes used in human models ($n= 6$) limits application to real-life settings and does not accurately reflect the general population, and more specifically active populations. Notably, administration of endotoxin *via* this method does not accurately reflect the mechanisms of intestinal permeability (e.g., leaky gut syndrome).

Synthesised and secreted primarily by leukocytes of the innate and adaptive immune system, cytokines are soluble immunoregulatory protein and glycoprotein molecules with pleiotropic properties, serving as chemical messengers that stimulate maturation, differentiation, and proliferation of leukocytes and facilitate cell-to-cell communication in a localised (autocrine, paracrine) or systemic (endocrine) manner and do so, at very low concentrations (Suzuki *et al.* 2002). *In vivo*, cytokines rarely, if ever, act alone. Instead, multiple cytokines which may have synergistic or antagonistic properties and (or) induce the synthesis of other cytokines, exert their biological effects by binding to highly specific receptors on responding target cells which initiates an intracellular signalling cascade, altered gene expression and cellular changes (e.g., up-regulation, down-regulation, stimulation or inhibition of cellular processes). Cytokines are broadly categorised into pro-inflammatory cytokines (e.g., TNF-α, IFN-γ and the interleukins; IL-1β, IL-8, IL-12, IL-18) and anti-inflammatory cytokines (e.g., the interleukins; IL-4, IL-6, IL-10, IL-11, IL-13, IL-1ra), which act to counterbalance and restrict the degree of the inflammatory response (Lim and Mackinnon 2006, Peake *et al.* 2015). For example, TNF-α and IL-1β concentrations have been reported to increase 2.3-fold and 2.1-fold respectively, immediately after a competitive marathon race; whilst IL-10 increased 27-fold and IL-1ra peaked 39-fold 1 h after the race. IL-6, which promotes an anti-inflammatory environment by inducing cortisol, IL-1ra, IL-10, and inhibiting TNF-α, also increased 128-

fold (Bethin, Vogt, and Muglia 2000, Ostrowski *et al.* 1999, Steensberg *et al.* 2003, Stouthard *et al.* 1995). Similarly, IL-10 (109%) and IL-1ra (212%) increased markedly after a marathon race, remaining elevated 1.5 h post-exercise, while significant (but of very low magnitude) increases were observed in TNF-α and IL-1β (Nieman *et al.* 2001); though, considerable individual variation in the magnitude of cytokine responses after exercise is reported (Peake *et al.* 2015).

Periods of increased physiological stress augments the release of glucocorticoids hormones from the adrenal cortex, namely cortisol, which has marked anti-inflammatory and immunosuppressive properties. Indeed, stress hormones appear to promote a shift from T-helper1 (Th1) lymphocyte cells producing pro-inflammatory cytokines (e.g., TNF-α and IFN-γ) to T-helper2 (Th2) lymphocyte cell producing anti-inflammatory cytokines (e.g., IL-4, IL-10 and IL-13) (Elenkov 2004, Elenkov and Chrousos 1999), in addition to inhibiting inflammatory signalling pathways such as nuclear factor-kappa B (NF-κB) and subsequent pro-inflammatory secretion (D'Acquisto, May, and Ghosh 2002, Hu *et al.* 2003). However, periods of sustained physiological stress may induce HPA axis fatigue, glucocorticoid receptor resistance (i.e., diminished sensitivity of leukocytes to glucocorticoids) and activation of the inflammatory signalling pathways (e.g., NF-κB) resulting in the up-regulation of pro-inflammatory cytokines (Cohen *et al.* 2012, Webster, Tonelli, and Sternberg 2002). As a result, it is plausible that altered cytokine responses (i.e., continued increased pro-inflammatory cytokines leading to an over-exaggerated cytokine-mediated inflammatory response) may occur in high-risk situations (e.g., extreme physical exertion such as ultra-endurance competitions and events) in high-risk individuals (e.g., recreational runners). Notably, previous observations suggest the time course for full recovery of altered cytokine profile after ultra-endurance exercise (e.g., long-distance triathlon, ultra-marathon running)

are considerably delayed (Gomez-Merino *et al.* 2006, Neubauer, Konig, and Wagner 2008) and may play a role in the aetiology of undefined underperformance and chronic fatigue syndromes (Morris *et al.* 2014, Robson 2003). The continuous demand of competition, the opportunity for inadequate rest between exercise bouts (i.e., demanding training schedules) may promote the clinical manifestations of a systemic inflammatory response, which often reflect that of sepsis may ensue and, if left untreated, can lead to organ dysfunction and multi-organ failure.

At the same time, cytokine-induced (namely IL-6) increases in acute phase proteins such as hepatic derived CRP serve to recognise and assist in the removal of pathogens and (or) damaged cells by functioning as an opsonin (i.e., coating bacterial surfaces for recognition by phagocytes) and complement activator (i.e., amplifies inflammatory responses and phagocytosis). CRP can rapidly increase up to 1000-fold during a systemic inflammatory response peaking 24 to 48 h after initial exposure to a stress stimulus (Pepys and Hirschfield 2003, Semple 2006). Increases in CRP of nearly 300% were observed 24 h in athletes (*n*= 18) after a 160 km triathlon (Taylor *et al.* 1987), whilst a 122% increase in CRP was observed in runners (*n*= 55) 4 h after a marathon race (Siegel *et al.* 2001).

In summary, loss of intestinal epithelial integrity can promote increases in intestinal permeability and an endotoxin-induced cytokine-mediated inflammatory response, which may lead to an over-exaggerated response if the stress stimuli is of an extreme nature; for example, sustained activities with accompanying physiological stressors such as ultra-endurance competition. **Chapter 6** aimed to track the intestinal permeability of endotoxins and cytokine profile during multi-stage ultra-marathon competition, whilst **Chapter 7** aimed

to explore the intestinal permeability of endotoxins and cytokine profile during a 24 hour continuous ultra-marathon.

2.2.1 Prolonged Physical Exertion and Salivary Anti-Microbial Protein Responses

The first reported research investigating the effects of exercise on mucosal immune parameters was in cross-country skiers, whereby significantly lower resting S-IgA concentrations were observed in elite skiers compared with age-matched controls before competition, with further reductions observed after 2-3 h of intensive ski competition (Tomasi *et al.* 1982). It was speculated that following prolonged physical exertion, temporary antibody deficiency at the oral-respiratory mucosal surface may lead to an increased susceptibility of self-reported bacterial and viral infections. The potential link between exercise and infection was originally reported by Peters and Bateman (1983) who conducted a survey on the incidence of upper respiratory tract infections (URTI) two weeks after a 56 km running competition. The authors found that incidence of URTI was significantly greater in runners (33%; *n*= 141) compared with matched controls (15%; *n*= 124). Subsequent studies have since reported similar findings following endurance events (Nieman *et al.* 1990, Nieman *et al.* 2003, Peters 1990) with increased URTI associated with the degree of exercise stress (Nieman 1994). For example, a greater running mileage was correlated with an increased incidence of URTI (Heath *et al.* 1991).

In particular, it has been documented that running exercise, compared with other exercise modes, results in greater perturbations to oral respiratory mucosal immunity, contributing to increased reports of URS (Matthew *et al.* 2002, Peters 1990, Peters and Bateman 1983), possibly due to the increased ventilatory load inducing airway damage and (or) exposure to

airbourne pathogens. This is of clinical relevance, taking into account that URSI are a common medical issue amongst active populations (Cox *et al.* 2008, Reid *et al.* 2004, Robson-Ansley *et al.* 2012, Spence *et al.* 2007). Notably, individuals with selective S-IgA deficiency tend to report recurrent sinopulmonary infections (Fried and Bonilla 2009), whilst very low saliva rates and oral dryness (xerostomia) is associated with an increased risk of oral complications and an increased incidence of URTI such as pharyngitis (Fox *et al.* 2004, Fox *et al.* 1985). Whilst there is no established 'cut-off' value in predetermining URTI risk, an absolute S-IgA concentration and S-IgA secretion rate of 40 mg·l^{-1} and 40 µg·min^{-1}, respectively is associated with an increased risk of acquiring an URTI (Fahlman and Engels 2005, Gleeson *et al.* 1999).

Over the past thirty years, several reviews have discussed the effects of exercise on mucosal immunity (Bishop and Gleeson 2009, Gleeson 2000, Gleeson and Pyne 2000, Mackinnon and Hooper 1994, Walsh *et al.* 2011a). These reviews have reported similar findings, whereby depressions in S-IgA (i.e., concentration and secretion rate) have been observed immediately after various types of exercise (e.g., sprint, interval and endurance) and during the recovery period across many different sports, especially in highly trained athletes (Gleeson *et al.* 1995, Mackinnon and Hooper 1994, Mackinnon, Ginn, and Seymour 1993, Tomasi *et al.* 1982). Indeed, the degree S-IgA depression is invariably related to the volume and intensity of the exercise-stress. For example, significant depressions in S-IgA responses is more consistently reported after participation in prolonged (e.g., a marathon) and extreme (e.g., a triathlon and ultra-marathon) physical exertion (Gleeson and Pyne 2000, Libicz *et al.* 2006, Mackinnon and Hooper 1994, Nehlsen-Cannarella *et al.* 2000, Nieman *et al.* 2006a, Nieman *et al.* 2003 Steerenberg *et al.* 1997). Moreover, it has been suggested that cumulative depressions in S-IgA may occur following intensified training periods (Gleeson *et al.* 1995, Mackinnon and

Hooper 1994, Tharp and Barnes 1990). For example, pre- and post-session S-IgA levels decreased significantly in competitive swimmers during heavy training sessions compared to light or moderate training sessions over a three month training period (Tharp and Barnes 1990). Whilst, competitive runners running for 90 min at 75% VO_{2max} on three consecutive days had significantly lower S-IgA secretion rates after each exercise bout (20 to 50%), in addition to significantly lower S-IgA secretion rates on the second and third day compared with the first day (Mackinnon and Hooper 1994). As such, repetitive bouts of intense exercise (e.g., multi-stage ultra-endurance endurance events) without sufficient recovery may compromise oral respiratory mucosal immune status to a greater extent if chronic depressions occur and S-IgA returning to baseline levels is delayed or impaired. In contrast, moderate, physical exertion does not appear to induce significant alterations in S-IgA responses (Gleeson and Pyne 2000, Housh *et al.* 1991, Mackinnon and Hooper 1994, McDowell *et al.* 1991). However, inconsistencies in S-IgA responses during the recovery period following physical exertion are evident with decreases in S-IgA concentration (Costa *et al.* 2012, Costa *et al.* 2009, Gleeson *et al.* 1999, Gleeson *et al.* 2000), increases in S-IgA concentration (Blannin *et al.* 1998, Laing *et al.* 2005) and no change in S-IgA concentration (Bishop *et al.* 2000, Walsh *et al.* 1999). Similarly, decreases in S-IgA secretion rate (Laing *et al.* 2005, Mackinnon and Hooper 1994, Walsh *et al.* 2002), increases in S-IgA secretion rate (Blannin *et al.* 1998), and no change in S-IgA secretion rate (Costa *et al.* 2012, Costa *et al.* 2009, Walsh *et al.* 1999) has been previously observed. Indeed, decreases in S-IgA concentration and secretion rate have been observed after exhaustive, high-intensity and submaximal running exercise (Mackinnon and Hooper 1994, McDowell *et al.* 1992), whereas increases in S-IgA concentration have been reported after marathon competition (Ljungberg *et al.* 1997) and after two 90 min cycling bouts at 60% VO_{2max} (Li and Gleeson 2004). In addition, increases in S-IgA concentration and secretion rate have been observed after an incremental

running test to exhaustion (Allgrove *et al.* 2008) and after longer duration cycle ergometer exercise at 55% VO_{2max} for 3 h (Blannin *et al.* 1998). On the contrary, no change in S-IgA concentration was observed after running exercise bouts for durations of 15 to 45 min at 50 to 80% VO_{2max} (McDowell *et al.* 1991). Equally, no change in S-IgA concentration or secretion rate was observed after a 60 min intermittent exhaustive cycle ergometer exercise (Walsh *et al.* 1999), nor in the last 2 min of a cycling bout at 60% VO_{2max} for 2 h (Bishop *et al.* 2000). Whilst noticeable inconsistencies are apparent between previous exercise studies, the influence of extreme physical exertion on S-IgA in addition to other S-AMPs during a multi-stage ultra-marathon and 24 hour continuous ultra-marathon has yet to be explored.

To date, whilst previous research has suggested that the exercise-induced depressions in S-IgA likely accounts for the increase prevalence of URSI (Neville, Gleeson, and Folland 2008), investigation into exercise-induced perturbations of other S-AMPs (e.g., S-α-amylase, S-lysozyme and S-lactoferrin) has generally been negligible (Walsh *et al.* 2011a); and it is largely unknown whether changes in these S-AMPs alter susceptibility to URSI (West *et al.* 2006). Interestingly, a counteractive effect appears to be present, whereby a decrease or no change in one S-AMP (e.g., S-IgA) have been observed alongside simultaneous increases in other S-AMPs (e.g., S-α-amylase and S-lysozyme) (Allgrove *et al.* 2008, Costa *et al.* 2012). Indeed, a prospective observational study that investigated acute (graded exercise test) and chronic (five month period) changes in S-AMPs in a cohort of elite rowers (*n*= 11) observed increases in S-lysozyme and S-lactoferrin concentrations (55% and 50%, respectively) after a graded exercise test to exhaustion, whilst over a five month period, no significant changes in S-lysozyme concentrations were evident compared with sedentary controls. However, significantly lower S-lactoferrin concentrations (60%) were observed at baseline and mid-point of the season in elite rowers compared with sedentary controls, although no changes

were observed between groups at the end of the five month period (West *et al.* 2010). Despite the frequent association between prolonged or extreme physical exertion and depressed S-IgA responses and the diverse responses of other S-AMPs (e.g., S-α-amylase, S-lysozyme and S-lactoferrin), which do not necessarily follow the same pattern as S-IgA following physical exertion (Allgrove *et al.* 2008, Costa *et al.* 2012, West *et al.* 2010), few studies have examined the effects of S-AMP responses during ultra-endurance events. Moreover, to date, previous laboratory-controlled studies have implemented exercise protocols lasting ≤2 h (Allgrove *et al.* 2008, Costa *et al.* 2012, West *et al.* 2010) which are considerably shorter in duration compared to ultra-endurance exercise which typically lasts >4 h. Therefore, whilst exercise protocols of shorter duration that entail an overall lower level of physiological stress may result in immune perturbations to a lesser extent (i.e., increases and decreases in S-AMP responses), it could be speculated that the extreme degree of the exercise-stress associated with competitive ultra-endurance events may instead, prompt marked depressions in other S-AMPs (in addition to S-IgA), thus reducing the overall supply of protective factors at the the oral respiratory mucosal surface (Tenovuo 1998). For this reason, individuals partaking in such events may be considered a high-risk population group for compromised oral-respiratory mucosal immune status and contracting sub-clinical or clinically significant outcomes following extreme physical exertion (Gleeson *et al.* 2000).

Whilst substantial biological variation in S-IgA between individuals is reported (Neville, Gleeson, and Folland 2008), the inconsistencies in S-IgA responses between studies are likely in part, due to variations in the experimental design and methodological approaches of saliva collection and S-IgA measurement, making direct comparability between studies difficult. For example, timing of the saliva sample (diurnal variation), the exercise protocol implemented (i.e., mode, intensity and duration), saliva sample storage process,

characteristics of participants (e.g., fitness level and psychological state), expression of S-IgA (e.g., absolute concentration, secretion rate, ratio to total protein or osmolality), analytical methods used to determine S-IgA concentrations (e.g., technique, calibration, laboratory conditions and reproducibility of assay), saliva collection method (e.g., unstimulated, stimulated, spitting, swabbing and suction) and the nature or degree of saliva flow stimulus (e.g., mechanical, taste and fasting *vs*. non-fasting) (Bishop and Gleeson 2009, Gleeson and Pyne 2000, Hucklebridge, Clow, and Evans 1998, Woof and Kerr 2006).

Unstimulated saliva collection (i.e., passive drool technique) appears to be the most reliable and justified method of determining real-time S-AMPs; whereas stimulated saliva collection has been shown to alter the volume and composition of saliva resulting in an apparent decrease (dilution effect) or increase in secretion rate of salivary biomarkers (i.e., chewing stimulates epithelial cell transcytosis of S-IgA) (Proctor and Carpenter 2001). Furthermore, the major and minor salivary glands reflect variation in glandular structure and thus contribute to salivary secretions in differing quantities. For this reason, whole saliva is currently the preferred method of saliva collection.

Indeed, a major source of variation in S-IgA (and other S-AMP) responses is an alteration in SFR, which in itself is influenced by multiple confounding factors (e.g., hydration status, age, hereditary influences, drugs/medication, diurnal variation, and (or) oral-facial movement) (Bosch *et al.* 1996, Fortes *et al.* 2012, Kaufman and Lamster 2002, Li and Gleeson 2004, Naumova *et al.* 2012, Walsh *et al.* 2004a, Walsh *et al.* 2004b), although not always accounted for in previous research studies. Both unstimulated and stimulated saliva collection among individuals is highly variable. This large intra- and inter-individual variation in SFR reported between studies has consequently prevented the generation of a standardised value

for SFR (Brunstrom, Macrae, and Roberts 1997, Dawes, O'Connor, and Aspen 2000). As a range, normal values for unstimulated saliva flow have been identified at >100 µl·min (average ~300 µl·min), increasing to ≥200 µl·min (maximum of 7000 µl·min) for stimulated (Chicharro *et al.* 1998, Edgar 1990, Humphrey and Williamson 2001).

Another factor that appears to influence SFR is the temperature of the mouth rinse used prior to saliva collection. Previous research investigating varying oral temperature stimuli has observed enhanced SFR with cooler (0˚C and 8˚C; 700 µl·min) and reduced SFR with warmer (37˚C; 540 µl·min) mouth rinse fluid temperatures (Dawes, Connor, and Aspen 2000). Similar results were reported by Brunstrom, Macrae and Roberts (1997), whereby stimuli at 3°C enhanced saliva production compared with 13˚C, 23˚C and 33˚C; whilst Pangborn, Chrisp, and Bertolero (1970) observed large variations in parotid saliva flow responses among individuals, and greater SFR at extreme mouth rinse water temperatures (0°C and 55°C) compared with water temperatures of 22°C and 37°C. These observations suggest that varying procedures used to cleanse the mouth before a saliva sample collection may affect SFR, possibly due to temperature sensitive muscarinic or purinergic receptor activation involved in saliva secretion (Mukaibo *et al.* 2013).

S-IgA and other S-AMP responses are commonly reported as an absolute concentration value, which reflects the current presence of a particular salivary variable per given volume on the oral respiratory mucosal surface. Reporting concentration does not, however, take into account potential changes in saliva flow induced by external factors (e. g., exercise, hydration status and extreme environmental conditions) that may promote a concentrating or diluting effect. Therefore, additionally reporting S-AMP responses as a secretion rate provides a correctional factor for externally-induced changes in saliva flow (Costa *et al.* 2012, Fortes *et*

al. 2012). This is considered a more appropriate method of reporting alterations in S-IgA synthesis and translocation during periods of prolonged physical exertion (Blannin *et al.* 1998, Bishop and Gleeson 2009). For example, S-IgA secretion rate but not S-IgA concentration were reduced in triathletes (*n*= 42) after a triathlon race (Steerenberg *et al.* 1997). Therefore, reporting SFR, S-IgA concentration and secretion rate is possibly the most appropriate way of presenting overall S-IgA responses. With this in mind, **Chapter 4**, **Chapter 5** and **Chapter 8** reported SFR, S-IgA concentration and secretion rate.

A limited number of studies have undertaken pathology testing to differentiate between infectious and non-infectious causes of URS episodes in athletes. These studies concluded that approximately 5% and 30-40% of URS are of bacterial and viral origin respectively, with infections caused by the common respiratory pathogens affecting the general population (e.g., *Rhinovirus* and *Influenza* infections) (Cox *et al.* 2008, Spence *et al.* 2007). On the other hand, Spence *et al.* (2007) reported infectious episodes were higher in athletes (29%; 6 of 21 cases) compared to sedentary controls (22%; 2 of 9 cases). In other words, non-infectious episodes were higher in controls (78%; 7 of 9 cases) compared with athletes (71%; 15 of 21 cases). This study also took place over the summer and autumn months when the risk of contracting infectious respiratory illness is likely to be lower.

More recently, it has been suggested that self-reported URTI are not always due to infectious aetiology. It is likely that non-infectious clinical conditions (e.g., asthma, allergy, irritation, autoimmune disorders, drying of the airways, bronchial hyperresponsiveness) and (or) unknown aetiologies such as drying of the airways associated with an increased ventilation rate during exercise and (or) airway inflammation induced by mechanical damage to the epithelium (Bermon 2007, Cox *et al.* 2010a, Helenius, Lumme, and Haahtela 2005, Robson-

Ansley *et al.* 2012, Verges *et al.* 2005) that mimic infectious URS, may account for 30-40% of presenting episodes. For example, of the 47% of runners presenting URS after participation in the London Marathon, 40% were diagnosed with allergy as defined by the validated Allergy Questionnaire for Athletes (AQUA) and elevated specific IgE (Robson-Ansley *et al.* 2012). Whilst, the use of a topical anti-inflammatory agent (Difflam throat spray) for one week before and two weeks after a half-marathon resulted in a 29% difference in symptom severity between the treatment group and placebo (Cox *et al.* 2010a). Collectively, these results suggest that a significant proportion of illness symptoms experienced in athletes may be non-infectious or inflammatory in nature, and not caused through pathogen aetiology. Notably, earlier studies investigating the link between exercise, S-IgA and URTI are primarily based on subjective, self-reported recall measures rather than verification through medical diagnosis. Therefore, caution is needed when interpreting results from earlier studies.

In summary, S-IgA is the most widely researched biomarker of oral-respiratory mucosal immune status within the area of sport and exercise immunology, with depressions in S-IgA implicated as a risk factor for URSI in active populations. Salivary biomarkers are affected by a number of external factors, which likely accounts for the differences in salivary variables reported between previous studies. Given that greater depressions in S-AMPs, in particular S-IgA, are observed after prolonged physical exertion, it is plausible that participation in extreme physical exertion may exacerbate these depressions further, leading to the development of sub-clinical or clinically significant outcomes. In addition to the assessment of salivary variables, **Chapter 4** and **Chapter 5** aimed to determine the incidence of URS after a multi-stage ultra-marathon and a 24 hour continuous ultra-marathon, respectively.

2.2.2 Prolonged Physical Exertion, Circulatory Endotoxin Concentration and Cytokine Profile

An adequate splanchnic blood supply is essential in the maintenance of intestinal epithelial integrity. Prolonged physical exertion, particularly running exercise, appears to perturb intestinal epithelial integrity through redistributing blood flow to the working muscles and peripheral circulation (i.e., aiding thermoregulation), inevitably leading to splanchnic ischemia-hypoperfusion and tissue hypoxia (ter Steege and Kolkman 2012, van Wijck *et al.* 2012). A recent review by Steege and Kolkman (2012) reported ≤80% reductions in splanchnic blood flow with higher intensity and (or) prolonged physical exertion which may have particular relevance for active populations partaking in competitive endurance and ultra-endurance competitions and events. Equally, upon cessation of exercise and restoration of splanchnic blood supply, intestinal epithelial integrity can be further exacerbated by reperfusion injury (i.e., inflammation and oxidative stress) (van Wijck *et al.* 2012). Indeed, van Wijck *et al.* (2011) observed hypoperfusion-induced injury to the small intestinal epithelium as measured by plasma intestinal fatty acid binding protein (I-FABP) and ileal bile acid binding protein (I-BABP) which are considered sensitive markers of enterocyte damage, which was correlated with mild increases in intestinal permeability after healthy subjects cycled for 60 min at 70% of maximum workload capacity. Additionally, alterations to intestinal motility and direct mechanical trauma and (or) injury (i.e., increased repetitive jarring associated with prolonged periods of running) and (or) acceleration/deceleration forces during running has been shown to induce a greater intestinal burden which can further promote irritation to the intestinal mucosa, epithelial damage and (or) dysfunction, thus playing a significant role in GI pathology and symptomatic manifestations (Rehrer *et al.* 1992, Rehrer and Meijer 1991, Riddoch and Trinick 1988, Worobetz and Gerrard 1985). For

example, a previous laboratory-controlled study showed that running induces over double the degree of vibration in the GI region compared with cycling (Rehrer and Meijer 1991). It could be speculated that the repetitive jarring and the subsequent 'churning effect' induced by running, liquefies the contents of the large intestine initiating lower GI symptoms such as diarrhoea and abdominal cramps. Indeed, Peters *et al.* (1999) conducted a self-reported GI symptomology questionnaire amongst runners ($n= 199$), cyclists ($n= 197$) and triathletes ($n= 210$) and found that runners experienced more lower GI symptoms (71%) than cyclists (64%), whilst triathletes experienced more lower GI symptoms during running (79%) than cycling (45%). These results were reflected in an earlier survey of marathon runners ($n=707$) that showed long-distance running was associated with a high incidence of GI symptoms, particularly lower GI; for example, bowel movements and diarrhoea were frequently reported (34.9% and 19.2%, respectively) (Keeffe *et al.* 1984). Equally, these studies used retrospective self-reported measures to determine GI symptoms with no control groups for comparative purposes. Furthermore, rapid exercise-induced fluid shifts and (or) imbalances (e.g., compromised hydration status) may cause further irritation to the intestinal mucosa, exacerbating GI symptoms. For example, 80% of those runners who lost >4% body mass (BM) after completing a marathon race experienced GI symptoms (Rehrer *et al.* 1989), although other potential confounding factors were not accounted for (e.g., increases in body temperature, training status of runners). Equally, 50% of runners participating in a 160 km race experienced GI symptoms, although this was unrelated to fluid intake (Glace, Murphy, and McHugh 2002). In addition to the assessement of circulatory endotoxin concentrations and cytokine profile, **Chapter 6** and **Chapter 7** aimed to determine the incidence of GI symptoms after a multi-stage ultra-marathon and a 24 hour continuous ultra-marathon, respectively.

In humans, non-invasive non-metabolisable markers of intestinal permeability such as oral ingestion and urinary excretion of sucrose, lactulose, 51Cr-labelled ethylenediaminetetraacetic (EDTA) acid or the ratio of lactulose to rhamnose or manitol have been found to be elevated following exercise (Øktedalen *et al.* 1992, Pals *et al.* 1997). Indeed, a previous laboratory-controlled study observed significantly greater increases in the mean urinary excretion ratio of lactulose to rhamnose ($0.107 \pm 0.021\%$) after 60 min of treadmill running at 80% VO_{2max} in 22°C compared with treadmill running at rest, 40 and 60% VO_{2max} (0.048 ± 0.009, 0.056 ± 0.005 and $0.064 \pm 0.010\%$, respectively) (Pals *et al.* 1997). However, a more widely used method for assessing intestinal permeability following exercise in field and laboratory settings is plasma endotoxin and anti-endotoxin antibody concentrations. This method is considered practically and logistically advantageous, offering minimal disruption to those individuals partaking in intensified training periods and (or) competitive endurance and ultra-endurance competitions and events. To date, a number of studies have reported variable increases in circulatory endotoxin concentrations following exercise. An early study by Brock-Utne *et al.* (1988) reported increases in plasma LPS concentrations over 100 pg·ml in 81% of runners and decreases in plasma anti-endotoxin IgG concentrations after completion of a marathon totalling 89.4 km. Similarly, mean plasma LPS concentrations increased from 81 to 294 pg·ml after an ultra-distance triathlon, whilst mean plasma anti-endotoxin IgG concentrations decreased (67.63 to 38.99 µg·ml) (Bosenberg *et al.* 1988). These observations were reflected in a later study by Camus *et al.* (1997) who observed milder increases (7 out of 18 runners) in plasma endotoxin ranging from 5 to 13 pg·ml, with one runner having a significantly higher concentration of 72 pg·ml within the first hour of recovery after participation in a marathon. Whilst marked decreases in plasma anti-endotoxin IgG and IgM concentrations that declined to minimal values in the majority of recreational athletes were reported following a triathlon (Camus *et al.* 1998). Equally, Jeukendrup *et al.*

(2000) observed mild increases in plasma endotoxin concentrations (5 to 15 pg·ml) immediately after competition (68% of triathletes), 2 h post-competition (19% of triathletes) and 16 h post-competition (79% of triathletes), possibly attributed to continued intestinal leakage of bacterial endotoxin into the circulation after exercise. Notably, no change in plasma anti-endotoxin IgG concentrations were observed immediately after competition, although a general decline was observed 2 h post-competition, which significantly declined 16 h post-competition. Finally, mildly elevated plasma LPS concentrations (31.6%; 1.9 ± 1.9 pg·ml to 2.5 ± 1.9 pg·ml) were observed in runners after a 21 km half-marathon in warm and humid ambient conditions ((approximately 26°C to 27°C and 82 to 84% relative humidity (RH)) (Ng *et al.* 2008). However, none of the above studies included a control group for comparative purposes. Whilst the discrepancies between and within study results may in part, be attributed to individual variation and the normal fluctuating nature of plasma endotoxin concentrations and (or) analytical methods used to determine the presence of endotoxin, higher circulating concentrations of endotoxin and anti-endotoxin antibodies have been observed in untrained compared with trained individuals (Jeukendrup *et al.* 2000, Selkirk *et al*. 2008). This gained adaptation in trained individuals is likely attributed to repetitive endotoxin challenge and subsequent 'self-immunisation' (Bosenberg *et al.* 1988, Brock-Utne *et al.* 1988).

Via immune mechanisms (e.g., production of cytokines and activation of complement), exaggerated circulatory endotoxin concentrations can induce various detrimental physiological effects to the host including SIRS; a condition known as a whole body inflammatory state. In extreme cases, over-exaggerated immune activation (i.e., innate immune cell proliferation and function, and cytokine responses) and increased pro-coagulant factors results in tissue hypoperfusion, intravascular coagulation, endothelial damage,

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haemodynamic perturbations with subsequent hypotension, with the end-point being septic shock (Hodgin and Moss 2008, van Leeuwen *et al.* 1994). For example, administration of endotoxin at 2 ng·kg in healthy human subjects induced activation of the coagulation pathway as evidenced by increases in prothrombin fragments and thrombin-antithrombin 111 complexes, alongside transient increases in body temperature (oral), pulse rate and decreases in blood pressure (van Deventer *et al.* 1990).

In the mid-late 1990's, several published studies investigating the effects of exercise on cytokine responses began to appear (Nagaraju *et al.* 1998, Northoff, Weinstock, and Berg 1994, Ostrowski *et al.* 1999, Ostrowski *et al.* 1998). Today, it is known that physical exertion induces elevated plasma levels of multiple pro-, anti- and immunomodulatory cytokines, in particular muscle derived IL-6, which is the earliest and most prominent (i.e., significantly higher concentrations than any other cytokine) cytokine produced upon initiation of exercise (Bruunsgaard *et al.* 1997, Castell *et al.* 1997, Drenth *et al*. 1995, Febbraio and Pedersen 2002, Nehlsen-Cannarella *et al.* 1997, Northoff and Berg 1991, Pedersen, Steensberg, and Schjerlin 2001, Pedersen *et al.* 1998, Rohde *et al* 1997, Ullum *et al.* 1994). For example, IL-6 concentrations increased up to 100-fold after a marathon race (Ostrowski *et al.* 1999, Ostrowski *et al.* 1998). The early research consistently reporting a marked increase in IL-6 following physical exertion instigated further research into exercise-induced cytokine responses (Drenth *et al.* 1995, Sprenger *et al.* 1992, Ullum *et al*. 1994). Indeed, a number of subsequent studies have since reported increases in circulatory concentrations of both pro-inflammatory (e.g., IL-1β, TNF-α, IL-6) and anti-inflammatory (e.g., IL-10, IL-6, IL-1ra) cytokines after physical exertion (Gleeson *et al.* 2011b, Ostrowski *et al.* 1999, Ostrowski *et al.* 1998, Pedersen 2000, Petersen and Pedersen 2005).

The considerable variation in the magnitude of cytokine responses reported in the current literature is likely due to the difference in laboratory-based (exercise mode; e.g., eccentric *vs.* concentric, intensity, duration and T_{amb}) or field-based (e.g., endurance, ultra-endurance exercise and T_{amb}) protocols (Nieman *et al.* 2012) and (or) the specificity and sensitivity of assays used in earlier studies. Other mechanisms that likely contribute to cytokine release include elevated circulatory endotoxin concentrations, in addition to hyperthermia, energy imbalance and muscle injury and (or) trauma associated with the exercise-stress (Nieman *et al.* 2014, Pedersen 2000, van de Vyver and Myburgh 2014, Welc and Clanton 2013).

It is well established that moderate physical exertion elicits favourable changes in cytokine profile; such as suppression of low-grade inflammation and enhanced anti-inflammatory cytokine responses (Peterson and Pedersen 2005, Walsh *et al.* 2011a). Indeed, the classical cytokine response to moderate exercise in thermoneutral conditions (body temperature increase <1°C) results in raised circulatory IL-1ra, IL-10, and muscle-derived IL-6 concentrations; while pro-inflammatory cytokine IL-1β and TNF-α responses are generally minimal (Walsh *et al.* 2011a). For example, 2 h of running at 75% VO_{2max} in 20°C ambient conditions resulted in an approximate 150%, 570% and 1490% increase in IL-1ra, IL-10 and IL-6, respectively; while no changes in IL-1β and TNF-α were observed (Costa *et al.* 2011). On the contrary, prolonged physical exertion results in raised circulatory pro-inflammatory TNF-α and IL-1β counteracted by raised circulatory anti-inflammatory IL-1ra, IL-10, and IL-6 concentrations (Camus *et al.* 1998, Camus *et al.* 1997, Ostrowski *et al.* 1999, Ostrowski *et al.* 1998, Suzuki *et al.* 2002, Walsh *et al.* 2011a) in part, induced by the augmented increases in circulatory stress hormones with known anti-inflammatory properties. Moreover, prolonged physical exertion is associated with enhanced bacterial endotoxin translocation which may exacerbate the cytokine-mediated systemic inflammatory response, eliciting a

cytokine profile similar to that of an acute infectious episode (e.g., sepsis, trauma and fever) (Bosenberg *et al.* 1988, Fehrenbach and Schneider 2006, Jeukendrup *et al.* 2000, Selkirk *et al.* 2008). Therefore, whilst down-regulation of pro-inflammatory cytokines and up-regulation of anti-inflammatory cytokines initiated by moderate exercise is likely to re-establish homeostasis; extreme physical exertion (e.g., single- and multi-stage ultra-endurance endurance events) may lead to an over-exaggerated endotoxin-induced cytokine-mediated inflammatory response and SIRS (Webster, Tonelli, and Sternberg 2002) which has previously been implicated in the aetiology of heat stroke and septic shock (Lim and Mackinnon 2006, Opal 2010).

From a practical perspective, single- and multi-stage ultra-endurance events have dramatically increased in popularity over the past decade (Knoth *et al.* 2012), yet research into the physiological demands and immune responses to such an extreme sport is limited (Murray and Costa 2012). Therefore, to date, the short and long-term effects of such ultra-endurance exposure on intestinal epithelial integrity and immune responses have not yet been established. Furthermore, single- and multi-stage ultra-endurance events which exhibit prolonged exposure to other multiple physiological stressors may also have the potential to impact upon intestinal epithelial integrity and cytokine responses. For example, a pronounced cytokine (i.e., IL-6 and somnogenic cytokines IL-1β, TNF-α, and IFNγ) profile is commonly seen after acute periods of sleep deprivation (Dinges *et al.* 1995, Rogers *et al.* 2001) and compromised energy status (Nieman *et al.* 1998, Robson 2003). For example, inadequate carbohydrate during exercise is associated with higher stress hormone (e.g., cortisol) and cytokine responses (e.g., IL-6) which may put an individual at risk of cortisol-induced immunosuppression.

In summary, endotoxaemia and responsive cytokinaemia have consistently been observed after prolonged physical exertion. It is plausible that active populations participating in extreme physical exertion (e.g., ultra-endurance competition) are potentially a high-risk population group for pronounced acute and long-term GI and immune dysfunction, leading to the development of sub-clinical or clinically significant outcomes; including GI symptoms, GI diseases, chronic systemic inflammation, autoimmune diseases, chronic fatigue and underperformance syndrome.

2.3.1 Exertional-Heat Stress and Salivary Anti-Microbial Protein Responses

Given that hot ambient conditions (i.e., in the absence of exercise-stress) induce the classic stress hormone response (e.g., increases in cortisol and catecholamines) and partaking in physical exertion in temperate ambient conditions (i.e., in the absence of heat-stress) provokes a greater stress hormone response, the combination of exercise plus heat-stress is considered a strong stimulus for activation of the HPA and SAM axis, especially if the exercise is sustained for prolonged periods and (or) includes repetitive exercise bouts (Brenner *et al.* 1998, Galbo *et al.* 1979, Laing *et al.* 2005). Previous studies investigating the independent effects of exercise plus heat-stress on stress hormone concentrations have shown differential responses compared with exercising in temperate ambient conditions. Brenner *et al.* (1997) observed significant increases in plasma cortisol concentrations in response to exercise (two 30 min cycle ergometer exercise at 50% VO_{2max} separated by a 45 min recovery interval) in the heat only (40°C and 30% RH), compared with exercise in temperate ambient conditions (23°C and 30% RH). Notably, the repetition of exercise in the heat did not significantly alter plasma cortisol, although plasma epinephrine and norepinephrine were significantly elevated after exercise in the heat. Similarly, a 60 min swimming exercise at a

speed requiring 68% VO_{2max} in 21°C, 27°C and 33°C water, increased rectal temperature (T_{re}) in 27°C and 33°C only (0.7 ± 0.1°C and 1.3 ± 0.2°C, respectively), whilst plasma cortisol (27°C and 33°C water only) and noradrenaline (33°C water only) concentrations increased in the warmer water temperatures (Galbo *et al.* 1979). More recently, Laing *et al.* (2005) reported greater increases in plasma cortisol after a 2 h cycling bout at 62% VO_{2max} in hot ambient conditions (30.3°C and 76% RH) compared with temperate ambient conditions (20.4°C and 60% RH). Whilst robust methods were applied these laboratory-controlled studies provide insight into stress hormone responses at differing temperatures, the application to real-life ultra-endurance exercise models is limited.

In relation to oral-respiratory mucosal immunity, salivary variables are influenced by the SAM axis, whereby increases in sympathetic nervous system activity and subsequent vasoconstriction of the blood vessels supplying the salivary glands has been shown to inhibit SFR; the primary mechanism most commonly proposed to explain depressions in salivary variables (e.g., smaller volume of viscous saliva and subsequent reductions in S-AMP secretion rates). Previously, Li and Gleeson (2005) suggested that there may be a threshold level of sympathetic nervous system activation to constrict salivary glandular vessels, which may in part, explain the inconsistencies between studies investigating exercise and alterations in SFR. Additionally, sympathetic (and parasympathetic to a lesser extent) nervous system activity has been shown to increase the movement of S-IgA into saliva (i.e., increased S-IgA secretion rate), which can be maintained over an extended period of time (>2 h) (Carpenter *et al.* 1998, Carpenter *et al.* 2000). Equally, in the absence of nerve stimulation, S-IgA was shown to gradually accumulate within a glandular compartment (Carpenter *et al.* 1998). These results suggest that IgA is continuously being synthesised, of which secretion increases rapidly during periods of demand (e.g., increases in exercise-induced sympathetic nervous

system activity); whilst during periods of less demand, excess IgA may be drained into the lymphatic vessels. However, such extensively stimulated glands resulted in reduced levels of S-IgA (i.e., 77% of S-IgA levels in unstimulated control glands), suggesting that excessive stimulation may decrease S-IgA secretion rate (Bishop and Gleeson 2009), Indeed, a trend for decreased S-IgA secretion was observed with consecutive, increasing doses of isoprenaline (beta-adrenoceptor agonist) and methacholine (cholinergic agonist) (Proctor *et al.* 2003). Therefore, sympathetic nervous system activation during short duration, high intensity exercise may increase transcytosis of S-IgA into saliva; whereas, longer duration, lower intensity exercise may deplete the IgA pool available for transcytosis (i.e., excessive stimulation). For that reason, the different modes of exercise protocols implemented in previously published studies and (or) the interaction between different stimulatory mechanisms and their receptors may in part, explain the discrepancies between studies investigating the S-IgA response to physical exertion.

Similarly, the HPA axis and subsequent increases in cortisol concentrations are thought to play a significant role in inhibiting S-IgA transcytosis (Hucklebridge, Clow, and Evans 1998). Indeed, Wira *et al.* (1990) observed decreased S-IgA concentrations in rodents following glucocorticoid (dexamethasone) administration, whilst glucocorticoid administration *in vivo* has been shown to rapidly inhibit transepithelial transport (i.e., translocation) of S-IgA, possibly reflective of suppression in B-lymphocyte cell maturation, differentiation and proliferation, thus accounting for the decreases in S-IgA concentration and secretion rate consistently reported following prolonged physical exertion (Sabbadini and Berczi 1995, Saxon *et al.* 1978). Notably, whilst glucocorticoid administration has also been reported to dampen Ig responses (i.e., decreases in Ig synthesis) (Saxon *et al.* 1978), it is more likely that the acute changes (minutes to hours) observed in S-IgA following prolonged

physical exertion is due to changes in the transepithelial transport mechanism, as opposed to B-lymphocyte cell Ig synthesis and production, which requires many hours to days (Hucklebridge, Clow, and Evans 1998). Other possible mechanisms attributable to the frequently reported depressions in S-IgA is through alterations in tSC production (Woof and Mestecky 2005), the contribution of different salivary glands and different stimuli activating specific glands which may play a role in altered responses of S-AMPs (Noble 2000); or indirectly through SAM axis-induced alterations in SFR and composition (Carpenter *et al.* 2000, Carpenter *et al.* 1998, Chicharro *et al.* 1998, Teeuw *et al.* 2004, Tenovuo 1998). Indeed, sympathetic activation alters saliva composition and elicits a high protein content secretion; therefore EHS may promote a greater secretion of S-α-amylase and S-lysozyme into the saliva (Bishop *et al.* 2000; Chatterton *et al.* 1996). For example, sympathetic stimulation in anesthetised rodents substantially increased amylase and total protein secretion 430- and 490-fold, respectively, compared with S-IgA (2.7-fold) (Carpenter *et al.* 2000).

Exercising in a hypohydrated state is associated with an amplified stress hormone response, compared to a blunted stress hormone response in a euhydrated state. Previously, significantly elevated plasma cortisol have been observed after a 2 h cycling bout at 65% VO_{2max} with no fluid intake (670 ± 63 nmol·l) compared with fluid intake (592 ± 62 nmol·l) before and during exercise (Bishop *et al.* 2004); highlighting the importance of maintaining euhydration in the attenuation of stress hormone responses which may subsequently alter salivary variables. Equally, compromised hydration status *per se* and subsequent salivary gland hypofunction is another mechanism proposed to explain the depressions in salivary variables following physical exertion, particularly when performed in hot ambient conditions. Indeed, the main constituent of saliva is water (97-99.5%) and the level of salivary output is dependent on the amount of water movement from circulation (i.e., plasma) across the

salivary acinar cells; thus a copious blood flow to the salivary glands ensures an adequate saliva flow. Water movement from the plasma through the salivary acinar cells into the salivary ducts to form primary saliva, is driven osmotically in response to trans-acinar sodium gradients. However, progressive dehydration increases the extracellular sodium concentration (and plasma osmolality; P_{Osmol}). As such, a greater sodium concentration would have to be generated across the salivary acinar cell in order to drive fluid into the acinar lumen, possibly accounting for the reduced volume of viscous saliva and changes in saliva composition (Ship and Fischer 1997). Previously, Ship and Fischer (1997) reported an approximate 90% decrease in SFR compared with baseline after a 24 h period without food and fluid. More recently, laboratory-controlled studies have shown that a 48 h period of fluid restriction resulting in 3.2% BM loss was associated with a 64% decrease in SFR and a non-significant decrease in S-IgA secretion rate (19%). However, fluid and energy restriction combined resulting in 3.6% BM loss was associated with a 54% decrease in SFR and a significant decrease in S-IgA secretion rate (39%). Notably, the alterations in salivary variables were independent of alterations in cortisol, which did not change throughout the 48 h period (Oliver *et al.* 2007). This was further reflected in a study by Allgrove *et al.* (2008) who observed increases in S-IgA secretion rate (50%) after a short duration incremental exercise test to exhaustion and no change in S-IgA secretion rate after 50% or 75% VO_{2max} trials, despite no change in S-cortisol on all three trials. Collectively, these results suggest that irrespective of stress hormone responses, S-IgA regulation ((e.g., transcytosis and (or) synthesis)) can be impaired *via* other mechanisms (e.g., hydration status); as such, fluid availability *per se* to offset fluid losses appears to play a significant role in the maintenance of salivary variables (Oliver *et al.* 2007, Walsh *et al.* 2004a). Given that salivary variables normalised upon rehydration, these results support the notion that maintaining a euhydrated state during periods when hydration status is likely to be compromised (e.g., endurance and

ultra-endurance events) is an effective strategy in preventing decreases in SFR and subsequent alterations in S-AMP secretions after prolonged physical exertion (Oliver *et al.* 2008, Walsh *et al.* 2004a, Walsh *et al.* 2004b). In **Chapter 4**, **Chapter 5** and **Chapter 8**, baseline hydration status of participants was assessed *via* BM and $P_{Osmol.}$

Further studies have shown that exercise-heat induced dehydration resulting >3% BM loss resulted in a 67% decrease in SFR and accompanying decreases in S-α-amylase and lysozyme secretion rates (44% and 46%, respectively), although S-IgA secretion rate remained unchanged (Fortes *et al.* 2012). However, irrespective of ambient conditions, when adequate provision of fluids is available during periods of prolonged physical exertion, salivary variables may be perturbed to a lesser extent. For example, no differences in SFR, S-IgA concentration or secretion rate were observed in response to two separate cycling bouts of 2 h at 62% VO_{2max} performed in a hot environment (30.3°C and 76% RH) compared with a thermoneutral environment (20.4°C and 60% RH) when water was provided *ad libitum* (Laing *et al.* 2005); suggesting that regular fluid intake during EHS assists in the maintenance of SFR and subsequent S-AMP secretions. It has also been proposed recently that maintaining euhydration may be an important strategy for keeping upper respiratory mucosal passages moist, attenuating the potential surface irritation and inflammation associated with URS and exercise-induced asthma (Anderson and Kipperlen 2008, Anderson and Daviskas 2000).

In summary, amplified increases in stress hormone release and compromised hydration status are known key factors influencing SFR. Whilst extreme physical exertion in hot ambient conditions may provoke greater perturbations in salivary variables; to date, the influence of extreme physical exertion in the heat on S-AMPs is limited, whilst no research exists

examining the effects of S-AMP responses during repetitive bouts of extreme physical exertion in the heat.

2.3.2 Exertional-Heat Stress, Circulatory Endotoxin Concentration and Cytokine Profile

Exercise heat-induced thermal alterations can exacerbate GI tract functionality whereby the thermal effect of direct heat (hyperthermia) on epithelial cells is associated with increases in intestinal permeability in animal models (Hall *et al.* 2001, Oliver *et al.* 2012, Shapiro *et al.* 1986). For example, high levels of hyperthermia (41.5-42.0°C) in sedated rats promoted increases in intestinal permeability as measured by Flurescein isothiocyanate (FITC)-dextran molecule (4000 Da), as well as hyperthermia-induced epithelial damage ((e.g., sloughing (shedding) of the epithelium, cellular swelling and vacuolization)) as assessed by histological analysis (Lambert *et al.* 2002). Such results suggest that the intestinal epithelium is responsive to changes in temperature. This was further reflected in a recent laboratory-controlled study whereby an average increase of 54% in LPS after a 60 min run at 70% VO_{2max} in hot ambient conditions (33°C and 50% RH) was observed, while no significant changes in LPS were observed in temperate ambient conditions (22°C and 62% RH); suggesting that exercising in hot ambient conditions may exacerbate intestinal epithelial integrity and promote translocation of larger quantities of bacterial endotoxins into the portal and systemic circulation (Yeh, Law, and Lim 2013). Such findings are supported by *in vitro* studies observing increases in tight junction permeability with exposure to temperatures of 37°C to 41°C (Dokladny, Moseley, and Ma 2006). Several field-based studies have consistently shown increases in plasma endotoxin concentrations after endurance events (Brock-Utne *et al.* 1988, Bosenberg *et al.* 1988, Camus *et al.* 1998, Camus *et al.* 1997,

Jeukendrup *et al.* 2000, Ng *et al.* 2008), although environmental conditions (e.g., T_{amb} and RH) in the majority of studies were not reported. It is therefore difficult to establish whether the increases in endotoxin translocation were in part, due to environmental stressors or other influencing factors.

However, given that a comparably high T_{core} (>40°C) has been observed in both patients with heatstroke and in healthy active populations (Maron, Wagner, and Horvath 1977, Shapiro and Seidman 1990), hyperthermia-induced epithelial damage is implied as a pre-requisite, facilitating but not triggering the pathological manifestations of heat stroke by promoting endotoxin translocation (Lim and Mackinnon 2006). Indeed, a 100-fold range difference in plasma endotoxin neutralising capacity between individuals has been observed (Warren *et al.* 1985), suggesting that the onset of heatstroke may be dependent on an individual's endotoxin clearance capacity (i.e., threshold for endotoxin leakage). Such results were reflected in a laboratory-controlled study investigating that effects of heat-stress induced endotoxaemia in trained and untrained subjects before and after walking at 4.5 km·h (2% elevation) in a climatic chamber (40°C and 30% RH) wearing protective clothing until exhaustion. Untrained subjects had significantly greater increases in plasma endotoxin concentrations at exhaustion (14.5 pg·ml) compared with trained subjects (8.08 pg·ml), and a lower tolerance for thermal strain ($T_{re:}$ 39.1°C ± 0.1°C and 39.7 ± 0.1°C in untrained and trained, respectively) (Selkirk *et al.* 2008).

Prolonged physical exertion is an independent contributing factor of compromised intestinal epithelial integrity through exercise-induced splanchnic ischemia-hypoperfusion and tissue hypoxia (ter Steege *et al.* 2012). The addition of heat-stress (e.g., ambient conditions >30°C), which exacerbates an already challenging situation for active populations, is likely to lead to

enhanced thermoregulatory strain, increased body water losses (e.g., sweating loses exceeding fluid intake) with accompanying hypovolaemia, and promotion of a progressive dehydrated state which collectively has the potential to further promote splanchnic hypoperfusion and disrupt intestinal epithelial integrity further (Lambert 2009, Wendt, van Loon, and Lichtenbelt 2007, van Wijck *et al.* 2011). For example, a study by Lambert *et al.* (2008) found that 60 min treadmill running at 70% VO_{2max} without fluid intake resulting in 1.5% BM loss significantly increased gastroduodenal and intestinal permeability as assessed by urinary sucrose excretion and the lactulose to rhamnose excretion ratio, compared to resting conditions. Additionally, epidemiological data on heat illness hospitalisations (*n*= 5246) for the U.S Army from 1980 to 2002 found that dehydration was associated with 17% of the heat stroke cases documented (Carter *et al.* 2005), suggesting that the inability to maintain fluid balance during periods of EHS can modify an individual sensitivity and (or) accelerate pathological conditions such as exertional-heat stroke.

To date, a number of studies have observed augmented increases in stress hormone (Brenner *et al.* 1998, Galbo *et al.* 1979, Laing *et al.* 2005) and cytokine (Cosio-Lima *et al.* 2011, Peake *et al.* 2008, Rhind *et al.* 2004, Satarifard *et al.* 2012, Starkie *et al.* 2005) responses during physical exertion in hot ambient conditions compared with more temperate ambient conditions. Indeed, the impact of changes in T_{re} induced by EHS and the influence on stress hormone and cytokine responses was investigated by Rhind *et al.* (2004) who found that a 40 min cycle at 65% VO_{2max} in hot water immersion (39°C) provoked an amplified increase in plasma cortisol (80%), epinephrine (>500%), norepinephrine (>350%) and cytokine responses (e.g., >90%, >400%, 150% and 150% in TNF-α, IL-6, IL-12 and IL-1ra, respectively) compared with cold water immersion (18°C). Interestingly, cold immersion attenuated the increase in stress hormone responses and inhibited increases in cytokine

responses, which in part, may explain the pathophysiology of exertional-heat illness in active populations exercising in hot ambient conditions.

Whilst the classical cytokine response to moderate, physical exertion in thermoneutral conditions results in raised circulatory concentrations of anti-inflammatory cytokines and minimal changes in pro-inflammatory cytokines (Costa *et al.* 2014, Walsh *et al.* 2011a), prolonged physical exertion promotes augmented increases in circulatory concentrations of pro-inflammatory cytokines counteracted by anti-inflammatory cytokines (Camus *et al.* 1997, Camus *et al.* 1998, Nieman *et al.* 2001, Ostrowski *et al.* 1999, Ostrowski *et al.* 1998, Suzuki *et al.* 2002, Walsh *et al.* 2011a). Previous laboratory-controlled studies have consistently observed greater cytokine responses (e.g., IL-6, TNF-α, IL-1ra, and IL-10) in hot (32-39°C) conditions, compared with more temperate (15-22°C) conditions (Cosio-Lima *et al.* 2011, Peake *et al.* 2008, Rhind *et al.* 2004, Satarifard *et al.* 2012, Starkie *et al.* 2005); although the degree of impact from intestinal originated bacterial endotoxins was not confirmed. Such observations indicate that heat-stress appears to play a role in the degree of systemic cytokinaemia observed after physical exertion. Moreover, systemic endotoxin and cytokine responses have also been associated with symptomatic manifestations of GI symptoms (Jeukendrup *et al.* 2000, Lambert 2008, van Leeuwen *et al.* 1994) commonly associated with prolonged exposure to physical exertion and EHS (Øktedalen *et al.* 1992, Peters *et al.* 1999, Pfeiffer *et al.* 2012, Rehrer and Meijer 1991).

During EHS, a persistent endotoxin-induced cytokine-mediated inflammatory response appears to be a key feature in the aetiology of exertional-heat illnesses (i.e., exertional-heat stroke) with fatalities being acknowledged and resulting SIRS. Indeed, episodes of clinical significance with fatal outcomes have been previously reported (i.e., heat stroke and systemic

inflammatory response syndrome) in military, occupation and sporting populations (Armstrong *et al.* 2007, Hunt, Parker, and Stewart 2013, Nag, Nag, and Ashtekar 2007, Rav-Acha *et al.* 2004, van Leeuwen *et al.* 1994, Xiang *et al.* 2014). For example, from 1992-2002, diagnosed cases of heat stroke (*n*= 134) were reported in military personnel during infantry training in hot ambient conditions. Of these, mortality incidence of heat stroke (*n*= 6) was reported to be due septic shock, in which SIRS is a key feature (O'Connor *et al.* 2010, Rav-Acha *et al.* 2004). A persistent endotoxin-induced cytokine-mediated inflammatory response may be further exacerbated by repetitive bouts of extreme physical exertion (e.g., multi-stage ultra-marathon events) with insufficient recovery. This is associated with unexplained underperformance syndrome, possibly caused by excessive cytokine release instigating a chronic systemic inflammatory state and 'cytokine sickness' (Robson 2003), chronic fatigue syndrome and (or) accumulative perturbations in immune function.

From a practical perspective, competing in multi-stage ultra-endurance competition exposes active populations to consecutive days of EHS. This population may thus be pre-disposed to sub-clinical (e.g., GI symptoms) and clinical (e.g., exertional-heat illnesses and sepsis) manifestations potentially originating from loss of intestinal epithelial integrity. Indeed, mild endotoxaemia, cytokinaemia, and GI symptoms have been reported after marathon (Camus *et al.* 1997) and Ironman triathlon (Jeukendrup *et al.* 2000) events, which were also associated with decrements in overall performance (Pfeiffer *et al.* 2012).

To date, exercise immunology research in ultra-endurance sports is limited (Murray and Costa 2012), with no research exploring and tracking intestinal permeability of endotoxins and cytokine profile during multi-stage ultra-marathon competition. Besides the consecutive days of EHS, such events are also accompanied by multiple physiological stressors that have

previously been acknowledged as predisposing factors in the aetiology of fatal incidence of heat stroke and SIRS (Armstrong *et al.* 2007, O'Connor *et al.* 2010, Rav-Acha *et al.* 2004, Walsh *et al.* 2011b). These include inadequate recovery opportunities, sleep deprivation, and acute periods of compromised hydration and (or) nutritional status (Costa *et al.* 2013a, Costa *et al.* 2013b). Moreover, the predominant characteristics of those individuals generally observed (e.g., recreationally active population, not acclimatised to ambient conditions, training status suboptimal for degree of physical exertion required, high body fat, high motivation, and situation of compromised immune status) are also reported to be aetiological predisposing factors (Walsh *et al.* 2011b).

In summary, previous findings support the notion that prolonged and (or) extreme physical exertion in hot ambient conditions poses a greater threat to intestinal epithelial integrity, leading to increases in intestinal permeability, an endotoxin-induced cytokine-mediated inflammatory response and subsequent sub-clinical or clinically significant episodes with fatal outcomes being reported. Notably, extreme physical exertion such as competitive ultra-endurance events meet all of the above requirements for SIRS (Suzuki *et al.* 2000, Suzuki *et al.* 1999), while chronic altered cytokine responses may promote undefined underperformance and chronic fatigue syndromes.

2.4.1 Probiotics and Intestinal Integrity

The GI tract is colonised by a plethora of microorganisms (~1000 species) which have the potential to promote either beneficial or harmful effects on GI integrity, function, and overall health (Gareau, Sherman, and Walker 2010, Holzapfel *et al.* 1998, Murguía-Peniche *et al.* 2013). Colonic microflora concentration and composition varies along the GI tract, remaining

relatively sparse in the stomach and duodenum (e.g., x 10^1 to x 10^3), gradually increasing to x 10^4 to x 10^7 in the jejunum and ileum, and achieving concentrations of up to 10^{12} cells per gram (dry weight) (35-50% of the volume of colonic contents) in the large intestine (O'Hara and Shanahan 2006). Colonic microflora interact with the intestinal epithelium, stimulating immunomodulatory effects. These interactions represent a functionally paradoxical relationship whereby some defence mechanisms (e.g., mucus membranes, secretion of AMPs such as IgA, lysozyme, proteases and defensins) aim to prevent penetration of pathogens and inflammation, whilst others (e.g., structured lymphoid sites such as Peyer's patches and lymphoid follicles) aim to sample bacteria in the intestinal lumen to initiate adaptive immune responses (Sansonetti 2004). Indeed, enterocytes bear pattern-recognition receptors (PPRs) such as the membrane-associated Toll-like receptors (TLRs) which interact with pathogen-associated molecular patterns (PAMPs) present on colonic microflora (and pathogens) (Akira, Takeda, and Kaisho 2001). In the majority of cases, ligand recognition by PPRs induce the production of pro-inflammatory cytokines. For example, the major ligand for endotoxin is TLR-4; this interaction activates enterocytes, triggering an intracellular cellular cascade and release the transcription NF-κB leading to the subsequent transcription and expression of localised pro-inflammatory cytokines which is appropriately downregulated by anti-inflammatory cytokines in order to prevent chronic inflammation of the intestinal epithelium (Walker 2008).

All individuals have the predominant genera of colonic microflora (e.g., *Bacteroides, Eubacterium, Bifidobacterium* and *Peptostreptococcus* species) detectable in faeces. However, there is considerable inter-individual variation in the species composition and diversity, which can fluctuate under particular circumstances (e.g., antibiotic therapy and psychosocial stress). (Eckburg *et al.* 2005, Moore and Moore 1995). The colonic microflora

serves to maintain homeostasis of the mucosal epithelium and modulate innate and adaptive immune responses *via* a number of specific metabolic, immunological and structural functions. Indeed, colonic microflora can *de novo* synthesise vitamins (e.g., vitamin K and most B vitamins) (Conly *et al.* 1994, Hill 1997, LeBlanc *et al.* 2013) and promote micronutrient absorption (e.g., calcium, magnesium, and iron) (Miyazawa *et al.* 1996, Younes *et al.* 2001). The major metabolic function of colonic microflora is the fermentation of substrates (i.e., non-digestible dietary carbohydrates; e.g., resistant starches, cellulose, pectins and gums) to generate readily absorbed short-chain fatty acids (SCFAs); in particular butyrate, acetate and propionate. SCFAs provides the major energy source for colonic microbial growth and intestinal epithelial cells (i.e., proliferation and differentiation; Frankel *et al*. 1994), in addition to involvement in cellular processes such as modulation of inflammation and oxidative stress (e.g., exerting anti-carcinogenic and anti-inflammatory activity *via* inhibition of pro-inflammatory cytokines such as IFN-γ) (Hamer *et al.* 2008, Inan *et al.* 2000, Lührs *et al.* 2002, Segain *et al.* 2000, Vinolo *et al.* 2011a, Vinolo *et al.* 2011b), and enhancement of intestinal epithelial integrity *via* regulating the assembly of tight junction proteins (Peng *et al.* 2009).

Furthermore, complex interactions between colonic microflora and the intestinal epithelium which harbours MALT and intra-epithelial lymphocytes appears to promote mucosal immune modelling and competency. For example, undeveloped mucosal immunity (e.g., hypoplastic Peyer's patches containing few germinal centres and reduced numbers of plasma cells) in germ-free animal models has previously been observed (Cash and Hooper 2005, Macpherson and Harris 2004), suggesting that colonic microflora is necessary for optimal immune functioning and the driving force behind adaptive immune development in the GI tract. Finally, colonic microflora plays a structural role by creating a defensive barrier at the

surface of the intestinal epithelium. For example, exogenous microorganisms with pathogenic properties may have difficulty colonising and becoming established within the GI tract since they have to directly compete with the colonic microflora for space, nutrients and attachment sites (Canny and McCormick 2008, Kamada *et al.* 2013).

Probiotics are defined as viable non-pathogenic microorganisms that, when administered in adequate amounts, confer a health benefit to the host (WHO/FAO 2002), of which the most commonly studied and traditionally used probiotic species in fermented dairy products belong to the *Lactobacillus* and *Bifidobacterium* anaerobic species. These species are known to colonise the GI tract in the majority of humans. The major functional effects of probiotics firstly refer to their ability to alter the composition of the colonic microflora (e.g., altering the ratio of pathogenic to non-pathogenic bacteria by increasing numbers and (or) activity of non-pathogenic bacteria) and promote re-establishment of balanced microflora and homeostasis; for example, *Bifidobacterium* has been shown to inhibit the activity of gram-positive and gram-negative bacteria (Gibson and Wang 1994). Secondly, to interfere with and prevent pathogen adherence (i.e. displacing pathogens), colonisation and overgrowth by competitively adhering to epithelial cells (i.e., binding to and occupying adhesion receptor sites) (Altenhoefer *et al.* 2004). Thirdly, to stimulate selected aspects of innate and adaptive immune function (e.g., pro- and anti-inflammatory cytokine production, IgA and mucin secretion) (Otte and Podolsky 2004, Salminen, Isolauri, and Salminen 1996, Qiao *et al.* 2002); for example, *Bifidobacterium* and *Lactobacillus* have been shown to initiate production of AMPs which express adhesive properties (Servin 2004), in addition to other metabolic molecules (e.g., SCFAs); and fourthly, to enhance tight junction stability (Otte and Podolsky 2004, Seth *et al.* 2008). Additionally, novel mechanisms of action by which probiotics may exert anti-inflammatory effects is *via* activation of peroxisome proliferator-

activated receptor gamma (PPARγ), a nuclear receptor that is expressed largely in the colon that regulates intestinal inflammation (i.e., interferes with inflammatory signalling pathways) through inhibition of NF-κβ. For example, studies *in vitro* have reported up-regulation of PPARγ expression ((messenger ribonucleic acid (mRNA) and protein levels)) and supressed pro-inflammatory cytokine responses (e.g., TNF-α) with *L.casei* and *L.GG Saccharomyces boulardii* probiotic strains (Eun *et al.* 2007, Isolauri *et al.* 2001, Majamaa and Isolauri 1997). Collectively, these functional effects of probiotics may assist in the maintenance of intestinal epithelial integrity, preventing increases in intestinal permeability, translocation of bacterial endotoxins and a resultant cytokine-mediated inflammatory response. The use of probiotics in reducing intestinal permeability and endotoxin leakage has been substantiated in animal (Isolauri *et al.* 1993) and human studies (Salimen *et al.* 1996), subsequently leading to probiotic research within the last decade diversifying into the area of sport and exercise immunology (Clancy *et al.* 2006, Cox *et al.* 2010b, Kekkonen *et al.* 2007, Shing *et al.* 2013); although, to date, the efficacy of probiotic supplementation in general healthy population groups is limited, including active populations. Therefore, **Chapter 8** and **Chapter 9** aimed to determine the influence of probiotic supplementation on selected immune variables in an active population group.

Research has attempted to address the mechanistic action of probiotics with a number of studies reporting improvements in intestinal epithelial integrity, immunological parameters and (or) clinical outcomes *in vitro*, *in vivo* and in a limited number of human studies (Kekkonen *et al.* 2007, Liu and Rhoads 2013, Mack *et al.* 2003, Mattar *et al.* 2002, Möndel *et al.* 2009, Schlee *et al.* 2008, Wehkamp *et al.* 2004). However, valid conclusions about the health benefits of probiotics have yet to be made due to the inconsistencies in reported results; possibly due to the variations in the experimental design and methodological

approaches employed. For example, the use of different probiotic strains (e.g., bioavailability and level of activity), probiotic dose provided, criteria used to define an illness episode (i.e., URSI or GI episode) duration of treatment (i.e., supplementation) period, route of administration (e.g., yogurt, beverage, liquid form, freeze-dried tablet form and mucosal spray), differences in patient characteristics (e.g., severity of the clinical condition, present microflora composition, current immunological state and age) and subjective data interpretation. Recent meta-analysis and systematic reviews of randomised controlled trials have yielded inconsistent results (Hempel *et al.* 2012, Hoveyda *et al.* 2009, McFarland and Dublin 2008), potentially questioning the clinical relevance of probiotics. Additionally, major concerns exist around the effectiveness of commercially available probiotics since the effectiveness of a particular probiotic strain is dependent upon whether it is resistant and viable to industrial handling, processing and preparation methods. For example, liquid-form fermented dairy products containing probiotics have a relatively short shelf life in their active form and bacterial damage may occur through the pasteurization or centrifugation process and (or) the use of additives and preservatives (Marteau 2001). Whilst the process of freeze-drying powdered probiotics (e.g., tablet form) may cause bacterial damage, resulting in poor colonisation and survival in the GI tract and (or) upon water absorption whereby bacteria may become activated, then cease (Sekhon and Jairath 2010). Furthermore, previous research has indicated that commercially available probiotics in the UK may lack the number of microbiota otherwise stated (Hamilton-Miller, Shah, and Smith 1996), possibly due to the practicalities surrounding handling, processing, preparing, storage and transportation of the probiotic product. Finally, given that the GI tract provides a multifaceted and hostile environment to many microorganisms, it could be questioned as to whether ingested probiotics manage to survive GI transit and colonise the GI tract.

Given that probiotics have been shown to modulate various innate and adaptive aspects of immune function (Delcenserie *et al.* 2008, Isolauri *et al.* 2001), it is theoretically plausible that ingestion of large volumes of probiotics may create excessive stimulation, aggravation and (or) inflammation of the mucosal immune system in the GI tract leading to systemic infections and detrimental metabolic activities (Marteau 2001). Moreover, clinical studies using probiotics have reported substantial inter-individual variation whereby some individuals have had favourable outcomes whilst others have not (responders *vs.* non-responders) (Reid *et al.* 2010). The limited understanding of the mechanistic action of probiotics is a major disadvantage when attempting to address whether a particular probiotic strain is likely to induce immuno-enhancing or immuno-suppressing effects which may be considered protective or harmful (e.g., pathogenic, toxic) to the host. It is possible that under certain conditions and (or) in certain individuals, probiotics may fail to maintain intestinal epithelial integrity and promote translocation of large molecular weight molecules such as endotoxins or induce infections themselves (Sanders *et al.* 2010). For example, in 'high-risk' individuals (e.g., untrained, illness-prone, history of GI-related issues) that participate in extreme exercise in extreme environmental conditions, amplified disturbances to intestinal epithelial integrity may increase the risk of clinically significant episodes including sepsis.

The general consensus is that probiotics are safe in healthy population groups (Boyle, Robins-Browne, and Tang 2006) and side effects (if any) tend to be mild, with large doses failing to exhibit any toxicity (Ahmed *et al.* 2007, Holzapfel *et al.* 1998, Larsen *et al.* 2006, Sekhon and Jairath 2010). To date, research investigating the dose-response of probiotic supplementation (typical dosages: x 10^{10} CFU·100ml^{-1}·day^{-1}) and the 'ideal' dose to induce a definitive pharmaceutical effect on S-AMP responses is absent. Adverse probiotic effects are rarely reported in studies, although identification and interpretation may prove difficult if

weak experimental designs lacking rigour and power (e.g., small participant numbers) are implemented (Dunne *et al.* 2001).

In summary, whilst limited studies have shown that probiotic supplementation may provide some immunological benefit, the mechanisms of action of probiotics are yet to be fully elucidated, whilst their efficacy in attenuating and (or) preventing illness requires further exploration. The potential use of probiotics in clinical health conditions (e.g., irritable bowel syndrome) has since generated interest in other sub-groups (e.g., active populations). However, very few studies in active populations have investigated probiotic supplementation on immunological parameters and (or) clinical illness symptoms; thus overall results have yielded insufficient evidence to provide recommendations in active populations. **Chapter 8** and **Chapter 9** aimed to explore the use of probiotic supplementation as a potential nutritional strategy in active population groups.

2.4.2 Probiotics, Salivary Anti-Microbial Protein Responses and Physical Exertion

With the considerable personal and economic investment in training and competition, it is crucial for active populations to maintain host defence, thus minimising the risk of clinically significant outcomes (e.g., URSI) that may negatively affect exercise performance. Although considered medically harmless in sedentary populations, sub-clinical or clinically significant outcomes in athletes may result in competing at a sub-optimal level or missing the event completely (West *et al.* 2009). Therefore, the immunostimulating properties of probiotics may induce a small worthwhile effect on host defence and possibly a favourable option amongst active populations to minimise perturbations to oral respiratory mucosal immunity. Indeed, it has been suggested that probiotics may modulate immune function beyond the GI

tract, exerting a desirable influence at other mucosal sites (e.g., URT) and prevent common cold or *Influenza* symptoms. For example, a systematic review of randomised controlled trials showed that probiotics may be a more favourable option over placebo in attenuating the incidence, severity and duration of acute URTI in healthy population groups (Berggren *et al.* 2011, Hao *et al.* 2011), and in sub-groups of the population, including the elderly and children (Leyer *et al.* 2009, Makino *et al.* 2010). Additionally, it has been suggested that probiotics may exert their therapeutic effects in the oral respiratory pathway through direct contact, interaction and adherence to the mucosal epithelium (Stamatova and Meurman 2009). Although athletes are not considered immunocompromised, the transient depressions in salivary variables following physical exertion may justify their use as a nutritional adjunct pre and (or) during periods of intensified training and (or) competitive events (Gleeson *et al.* 2011a, West *et al.* 2009). For example, practical barriers to consistently meeting nutritional and (or) hydration requirements during prolonged physical exertion commonly exist (Costa *et al.* 2013a, Costa *et al.* 2013b). Consequently, probiotics may have particular relevance for active populations partaking in prolonged physical exertion, especially when performed in hot ambient conditions when oral-respiratory mucosal immune status is more likely to be compromised compared with exertional stress in temperate conditions.

Currently, a limited number of studies have investigated the influence of probiotic supplementation amongst highly trained athletes on oral-respiratory mucosal immunity with conflicting results. Recently, significantly higher S-IgA concentrations were observed in active individuals during and after four months of winter training after consuming a fermented milk drink containing *L. casei Shirota* (two 65ml bottles containing 6.5×10^9 CFU each). In this study, participants were randomised to probiotic or placebo. Whilst no differences in S-IgA concentration were observed at baseline between probiotic and placebo,

S-IgA concentration was significantly higher at eight and sixteen weeks in the probiotic group compared with placebo. This was further reflected in a lower mean number of URTI symptom weeks in the probiotic group (1.9 ± 1.5) compared to placebo (3.5 ± 2.0), and a lower mean number of URTI episodes in the probiotic group (1.2 ± 1.0) compared to placebo (2.1 ± 1.2) (Gleeson *et al.* 2011a). However, S-IgA secretion rates were not reported. Given that reporting concentration values alone do not take into account potential changes in saliva flow rate, these results are limited in their interpretation. Conversely, a study involving elite male distance runners (*n*= 20) over a four month period of winter training observed no significant changes in S-IgA concentration after ingestion of a freeze-dried probiotic powder capsule containing 1.2 x 10^{10} CFU·day. Still, less than half the number of URT and LRT episodes were reported in the probiotic group (*n*= 4) compared with placebo (*n*= 9) and the number of total illness days was significantly less (30 illness days *vs.* 72 illness days in probiotic and placebo, respectively), although the mean URT and LRT episode duration did not differ between groups (Cox *et al.* 2010b). Furthermore, three weeks of military training followed by a five day combat course did not induce changes in S-IgA concentration between probiotic (a fermented milk drink containing *L. casei* strain DN-114 001) and placebo at any of the four saliva sampling time points; although S-IgA concentration was significantly lower after the five day combat course in placebo, whereas S-IgA concentrations did not change over time throughout the study period in the probiotic group. However, medical examinations assessing URT and LRT infections confirmed that no differences were observed in those subjects who presented at least one infection during the study period (46%; *n*=11 *vs.* *n*=13; 57% in probiotic and placebo, respectively). Moreover, no differences were observed in RTI incidence (0.8 ± 0.2 *vs.* 0.6 ± 0.1 in probiotic and placebo, respectively), nor in the duration of symptoms (5.5 ± 1.6 *vs.* 6.1 ± 1.7 in probiotic and placebo, respectively) between the groups. Interestingly, analysis of the clinical symptomology revealed that during the trial

period, the majority of participants in the probiotic group experienced rhinopharyngitis (70%), whereas diagnosed symptoms in the placebo group were more evenly distributed (e.g., 40% rhinopharyngitis, 20% bronchitis and 17% tonsillitis) These findings suggested that infections were predominantly confined to the nasopharyngeal area and that probiotic supplementation may have prevented the spread of infection beyond the respiratory tract (Tiollier *et al.* 2007). Similarly, S-IgA secretion rate was not reported.

A further randomised, double-blind intervention study investigating probiotic supplementation (2 x 65ml milk drink containing a total of 4.0 x 10^{10} of *Lactobacillus rhamnosus* GG (LGG) or 2 capsules containing a total of 1 x 10^{10} LGG)) during a three month training period prior to a marathon race reported no differences in number of healthy days (79.0 *vs.* 73.4 days in probiotic and placebo, respectively). Additionally, no differences were observed in the number of participants with a self-reported URTI episode (46% *vs.* 37% in probiotic and placebo, respectively), in the duration of those individuals who reported an URTI (6.3 days *vs.* 7.9 days in probiotic and placebo, respectively), nor in the two week follow-up period after the marathon between groups (Kekkonen *et al.* 2007). On the contrary, powered-form single-strain probiotic supplementation (*Bifidobacterium animalis* subsp. *lactis* Bl-04, 2.0×10^9 CFU·day) for one-hundred and fifty days in healthy active individuals was associated with a significant reduction (27%) in URTI episode risk, and a non-significant reduction (19%) with powdered-form multi-strain probiotic supplementation (*Lactobacillus acidophilus* NCFM and *Bifidobacterium animalis subsp. lactis* Bi-07, 5×10^9 CFU·day) compared with placebo (West *et al.* 2014). Moreover, multi-strain probiotic supplementation (*L. gasseri* PA 16/8, *B. longum* SP 07/3, *B. bifidum* MF 20/5) over a three month training period significantly decreased the mean duration of self-reported common cold episodes, although no significant differences were observed in the incidence of episodes (158 and 153

episodes in the probiotic and placebo group, respectively) (de Vrese *et al.* 2005). Finally, a randomised controlled trial involving eleven weeks of probiotic supplementation (minimum of 1×10^9 *Lactobacillus fermentum*) in competitive cyclists observed no differences in S-IgA, S-lysozyme or S-lactoferrin concentrations between probiotic and placebo (West *et al.* 2011). The authors reported large within- and between-subject variability in the AMPs measured and ambiguity of clinical outcomes (self-reported URTI) (West *et al.* 2011). Notably, with the more recent development of a 'non-infectious' hypothesis, it could be speculated that the previous positive impacts of probiotic supplementation may target inflammation suppression rather than prevention and (or) attenuation of pathogen infection *via* anti-inflammatory mechanisms such as the upregulation of anti-inflammatory cytokines, IgA and mucin production.

Collectively, results suggest that probiotic supplementation in active populations may induce favourable changes in S-IgA. However, all probiotic studies, with the exception of one, have solely reported S-IgA responses in the assessment of oral-respiratory mucosal immune status. Given the protective properties of other S-AMPs, probiotic studies to date are currently limited in their interpretation of the exerted influence on oral-respiratory mucosal immune status. Moreover, the majority of studies establish the existence of an URTI through self-reported measures, without determining the causality of the URTI (e.g., pathogen presence through medical diagnosis). This is considered important to identify for the tailoring of a treatment plan which differs depending on whether the URTI is infectious or non-infectious. Additionally, S-IgA secretion rate has not been reported in any of the aforementioned studies, which is surprising given the exercise-induced changes on SFR. To date, research investigating the influence of probiotic supplementation on S-AMP responses during EHS when oral-respiratory mucosal immune status is likely to be compromised, is absent.

Although S-IgA does not appear to be affected by exercise (≤2 h) in environmental extremes in laboratory-controlled studies (Housh *et al.* 1991, Laing *et al.* 2005, Walsh *et al.* 2002), extreme physical exertion (e.g., single- or multi-stage ultra marathon events) in addition to heat-stress with accompanying physiological stressors is likely to perturb oral-respiratory mucosal immune status to a greater extent.

Given that probiotics are ingested orally and inevitably come into direct contact with oral mucosal surfaces, another mechanism in which probiotics may induce health-promoting effects is directly through modification of the oral microbial ecology. Analogically to the GI tract, it is plausible that adherence to mucosal surfaces, competition for adhesion receptors and (or) modification of the pellicle may favourably impact upon enhancing host defence. To date, studies have shown that probiotics may affect the oral ecology (e.g., preventing adherence of other microorganisms, modifying protein composition of the salivary pellicle) through temporary colonisation (Haukioja *et al.* 2008). Equally, colonisation potential and binding efficacy to the oral mucosa appears to be strain-specific (Haukioja *et al.* 2006), whilst unaltered S-IgA concentrations have been reported after three weeks of probiotic supplementation (Kekkonen *et al.* 2007, Paineau *et al.* 2008).

In summary, previous research suggests that probiotic supplementation may play a role in attenuating and (or) preventing URSI during periods of compromised oral-respiratory mucosal immune status. Given that prolonged physical exertion is associated with significant depressions in S-IgA and an increased susceptibility to URSI; and probiotics have the potential to stimulate aspects of the immune system beyond the GI tract, probiotic supplementation in the weeks leading up to competitive endurance and ultra-endurance events may be considered an appropriate nutritional strategy in active populations to

counteract the depressions in oral-respiratory mucosal immune status. However, to date, the efficacy of probiotics on salivary immune variables in active populations is extremely limited.

2.4.3 Probiotics, Endotoxaemia and Physical Exertion

Despite unsubstantiated claims, commercially available probiotics (e.g., *L.casei*) products dominate the global gut-health market (Cummings *et al.* 2004), increasing in popularity amongst active populations; with reported focus on prevention-management of GI related symptoms (e.g., traveller's diarrhoea) (Sazawal *et al.* 2006, Tillett and Loosemore 2009). It has been suggested that probiotics may play a beneficial role in the maintenance of gut-barrier function in active populations, potentially counteracting the disturbances to intestinal epithelial integrity and preventing or attenuating the manifestation of GI symptoms.

Probiotics have been shown to exert protective effects and contribute to intestinal epithelial integrity through a number of different mechanisms. Firstly, by inhibiting pathogenic activity through production of anti-microbial substances and preventing pathogenic colonisation and translocation of the intestinal epithelium through competitive adherence for cell surface receptors. Indeed, studies *in vitro* and *in vivo* have shown up-regulation of mucin and β-defensin gene expression in intestinal epithelial cells, augmenting mucus and β-defensin secretion (Mack *et al.* 2003, Mattar *et al.* 2002, Möndel *et al.* 2009, Otte and Podolsky 2004, Schlee *et al.* 2008, Wehkamp *et al.* 2004) and production of substances such as SCFAs, hydrogen peroxide, nitric oxide and bacteriocins (Atassi and Servin 2010, Chenoll *et al.* 2010, Corr, Hill, and Gahan 2009). Secondly, through stimulation of innate and acquired immune responses (e.g., secretion of pro-inflammatory cytokines from intestinal epithelial

cells ((e.g., IL-8), anti-inflammatory cytokines (e.g., IL-10) from dendritic cells and S-IgA (Hart *et al.* 2004, Liu and Rhoads 2013, Vizoso Pinto *et al.* 2007); and thirdly, through regulation and stabilisation of the intestinal epithelial cell barrier (i.e., modulating homeostasis). For example, secretion of probiotic proteins (e.g., p75 and p40) with apparent anti-inflammatory effects which activate Akt (i.e., protein kinase B_1). Akt protects intestinal epithelial cells from apoptosis (Otte and Podolsky 2004, Seth *et al.* 2008).

A systematic review of randomised controlled trials on the impact of probiotics on GI health has demonstrated favourable effects of probiotic bacteria (e.g., *L.casei*) on markers of intestinal epithelial integrity; albeit within inflammatory gut diseases (Moayyedi *et al.* 2010). In animal models, multi- and single-strain probiotic supplementation has been shown to be effective in maintaining intestinal epithelial integrity in animals with sepsis, colitis and alcohol-induced intestinal integrity and liver injury (Ewaschuk *et al*. 2007, Mennigen *et al.* 2009, Wang *et al*. 2012). While in human models, single-strain probiotics have also been shown to induce favourable changes on tight junction and intestinal epithelium integrity (Anderson *et al.* 2010, Resta-Lenert and Barrett 2006, Ukena *et al.* 2007, Qin *et al.* 2009). For example, Karczewski *et al.* (2010) observed increases in the expression of tight junction proteins (zonula occludens and occludin) after probiotic administration (*Lactobaccilus plantarum*) through an intraduodenal feeding catheter. Indeed, a recent randomised controlled trial investigated the influence of a multi-strain probiotic supplementation (*Bifidobacterium bifidum* W23, *Bifidobacterium lactis* W51, *Enterococcus faecium* W54, *Lactobacillus acidophilis* W22, *Lactobacillus brevis* W63, *Lactococcus lactis* W58) containing 10^{10} CFU·day on tight junction competency and intestinal permeability in endurance trained males (*n*= 23) by measuring faecal concentrations of zonulin (a surrogate marker of impaired intestinal barrier function; Fasano 2011) at baseline and at fourteen weeks. Results from this

study showed that zonulin, a protein synthesised by intestinal epithelial cells that modulates the permeability of intercellular tight junctions whereby increased concentrations indicate increased intestinal permeability, significantly decreased in faeces after fourteen weeks in the probiotic group (>20%; p= 0.019) compared with placebo and; although failing to reach significance (p= 0.054), reduced concentrations of TNF-α compared to placebo after fourteen weeks. The authors concluded that probiotic supplementation can initiate improvements in intestinal epithelial integrity and reduce intestinal permeability (Lamprecht *et al.* 2012). However, this study focused solely on zonulin. The inclusion of a wider range of biochemical and stool markers would have allowed for a deeper mechanistic insight, in addition to a more comprehensive overview of intestinal permeability. Moreover, Christensen *et al.* (2006) established that as dosage of probiotic supplementation (e.g., *Bifidobacterium animalis* ssp. *lactis* x 10^{10} *vs.* x 10^{11} CFU·day^{-1}) increases, the proportion of bacteria specific positive subjects also increases (i.e., 40% *vs.* 90%, respectively). A further randomised controlled trial involving eleven weeks of probiotic supplementation (minimum of 1×10^9 *Lactobacillus fermentum*) in competitive cyclists reported attenuated exercise-induced changes in pro- and anti-inflammatory cytokines (IL-1ra, IL-6, IL-8, IL-10, IFN-γ and TNF-α) when adjusted for mean training load, although no significant differences in resting cytokine concentrations from pre- to post-supplementation were observed between groups, with no associations with illness symptoms. Notably, the number and duration of mild GI symptoms were 2-fold greater in the probiotic group, possibly reflecting the introduction of additional bacterial species and subsequent adaptive changes in the GI tract; although severity of GI symptoms was substantially lower at higher training loads in males in the probiotic group (West *et al.* 2011).

To knowledge, only one study has investigated the influence of probiotics on circulatory LPS concentrations and cytokine profile following exercise. In contrast to results reported by Lamprecht *et al.* (2012), Shing *et al.* (2013) demonstrated that despite 4 weeks of multi-strain probiotic (*Lactobaccilus, Bifidobacterium, Streptococcus*) supplementation (i.e., modest dose of 4.5^{10} CFU·day^{-1}), no significant differences in pre- and post-exercise (incremental run to fatigue at 80% ventilatory threshold in hot ambient conditions; 35°C and 40% RH) circulatory plasma LPS concentrations, or the ratio of urinary lactulose to rhamnose were observed between probiotic and placebo in male runners (*n*= 10). Similarly, no significant differences in cytokine profile (IL-6, TNF-α, IL-1ra and IL-10) were observed between groups (Shing *et al.* 2013). These outcomes were however in response to short duration, high intensity exercise, with relatively short heat exposure time (<40 min at 35˚C), and modest rises in T_{core} (38.1˚C). Whilst perturbations to intestinal epithelial integrity are more likely to be amplified with longer duration exercise and the duration of the exercise implemented in this study does not reflect endurance populations, **Chapter 9** aimed to determine the influence of probiotic supplementation during a 2 h bout of EHS.

Notably, in an *in vitro* human colonic microbiota model, daily administration of some specific probiotics strains belonging to the *Lactobacillus* and *Bifidobacterium* species for fourteen days have been shown to reduce concentrations of gut-derived LPS which was positively and significantly correlated with cytokine production (e.g., TNF-α and IL-1β) (Rodes *et al.* 2013). These results suggest that the pro-inflammatory cytokine tone can be attenuated if intestinal epithelial integrity can be maintained through probiotic use. Indeed, it has been suggested that attenuating cytokine responses such as IL-6 during exercise may reduce susceptibility to unexplained underperformance syndrome (Robson 2003). Moreover, given the potential for an endotoxin-induced cytokine-mediated inflammatory response during prolonged physical exertion, especially when performed in hot ambient conditions,

probiotic supplementation in the weeks leading up to endurance and ultra-endurance events may be justified. Despite this, no research exists examining the effects of probiotics during prolonged physical exertion in the heat (Brock-Utne *et al.* 1988, Bosenberg *et al.* 1988, Camus *et al.* 1998, Camus *et al.* 1997, Jeukendrup *et al.* 2000, Ng *et al.* 2009). It is plausible that using probiotics as a preventative measure to counteract disturbances to intestinal epithelial integrity may also attenuate or prevent symptomatic manifestations of GI symptoms in active populations, although this has yet to be substantiated.

To date, a limited number of studies have assessed the efficacy of probiotic supplementation on GI symptoms amongst active population groups, with inconclusive results. For example, probiotic supplementation (2 x 65ml milk drink containing a total of 4.0 x 10^{10} of *Lactobacillus rhamnosus* GG (LGG) or 2 capsules containing a total of 1 x 10^{10} LGG)) during a three month training period prior to a marathon race was shown to reduce the duration of GI symptoms by 33% compared with placebo, and by 57% in the two week follow-up period after the marathon compared with placebo. However, no differences in the frequency of GI symptom episodes were observed during the training period nor in the two week follow-up period after the marathon between groups (Kekkonen *et al.* 2007).

In summary, previous research has suggested that probiotic supplementation may enhance gut-barrier function. However, to date, the efficacy of probiotics in counteracting disturbances to intestinal epithelial integrity (e.g., increases in intestinal permeability and a subsequent endotoxin-induced cytokine-mediated inflammatory response) in active populations is extremely limited. Whilst the impact of probiotic supplementation during prolonged physical exertion, especially in hot ambient conditions when intestinal epithelium integrity is likely to be compromised to a greater extent, has yet to be substantiated.

2.5.1 Thesis Aims

The presented literature suggests that participating in prolonged or extreme physical exertion with or without heat-stress, but with accompanying physiological stressors has the potential to perturb oral-respiratory mucosal immunity, potentially increasing the risk of URSI. Additionally, prolonged or extreme physical exertion with or without heat-stress is associated with disturbances to intestinal epithelial integrity and a systemic endotoxin-induced cytokine-mediated inflammatory response which is a key feature in the aetiology of exertional-heat illnesses. While chronic altered cytokine responses may play a role in the aetiology of undefined underperformance and chronic fatigue syndromes (Morris *et al.* 2014, Robson 2003). It is plausible that participating in extreme physical exertion (e.g., single- or multi-stage ultra-marathon events) with or without heat-stress, but with accompanying physiological stressors, may amplify and (or) instigate cumulative perturbations to oral respiratory mucosal immunity and (or) intestinal epithelial integrity. Furthermore, the presented literature suggests that probiotics may have a role to play in preventing perturbations to oral respiratory mucosal immunity, support in the maintenance of intestinal epithelial integrity and attenuate and (or) prevent sub-clinical or clinically significant episodes in active population groups. Although research investigating the potential use of probiotic supplementation during prolonged physical exertion is extremely limited, the presence of other key factors (e.g., amplified circulatory stress hormone release and compromised hydration status) associated with prolonged physical exertion plus heat-stress may further justify the use of probiotics in these higher-risk situations.

With this information in mind, the broad aims of the studies contained within this thesis were to investigate: 1. the effects of: a multi-stage ultra-marathon in hot ambient conditions, and a

24 h continuous over-night ultra-marathon in temperate ambient conditions on S-AMP responses, stress hormone response and incidence of URS; 2. the effects of a multi-stage ultra-marathon in hot ambient conditions, and a 24 h continuous over-night ultra-marathon in temperate ambient conditions on circulatory endotoxin concentration, cytokine profile and incidence of GI symptoms; and finally, 3. acute high dose supplementation of *L. casei* on S-AMP responses, circulatory endotoxin concentration and cytokine profile in response to EHS.

It was hypothesised that: 1. a multi-stage ultra-marathon performed in hot ambient conditions would depress salivary variables, leading to an increased reports of URS in ultra-endurance runners (UER); 2. a 24 h continuous overnight ultra-marathon performed in temperate ambient conditions would depress salivary variables, leading to an increased reports of URS in UER; 3. a multi-stage ultra-marathon performed in hot ambient conditions would initiate progressive increases in endotoxaemia, mirrored by a cytokinaemic response which correlated with GI symptoms in UER; 4. a 24 h continuous overnight ultra-marathon performed in temperate ambient conditions would initiate pronounced increases in endotoxaemia, mirrored by a cytokinaemic response which correlated with GI symptoms in UER; and finally, 5. enhanced S-AMP responses would be seen after one week of supplementation of *L.casei*, after EHS, and during the recovery period compared with placebo; additionally, decreases in circulatory endotoxin concentrations and cytokine profile would be seen after EHS compared with placebo.

CHAPTER THREE

General Methods

3.1 Ethical Approval

Prior to the commencement of each study, approval was received from the Local Ethics Committee (Coventry University) that conformed to the 2008 Helsinki Declaration for Human Research Ethics. All participants were made fully aware of the nature and purpose of each study, and the risks and benefits were fully explained, both verbally and in a written format *via* participant information forms (**Appendix B**). Participants gave written informed consent (**Appendix B**) freely and voluntarily; and completed a health screen questionnaire (**Appendix C**) prior to the commencement of each study. Each participant was made fully aware that they were able to withdraw from any study at any time, and without reason. Each study had individual inclusion and exclusion criteria. Participants were only eligible to initiate and complete the study if they met and fulfilled this criteria.

3.2 Preliminary Measures

Height was measured by a wall-mounted stadiometer. BM was determined using calibrated electronic scales (BF510, Omron Healthcare, Ukyo-ku, Kyoto, Japan) placed on a hard levelled surface. Waist and hip circumferences were measured using a standard clinical tape measure by trained researchers, in accordance with International Standards for ISAK international standards for anthropometric assessment (ISAK 2001). BM and circumference measures were used when conducting multi-frequency bioelectrical impedance analysis (Quadscan 4000, Bodystat, Douglas, Isle of Man, UK), which was used to estimate body composition.

3.3 Dietary and Hydration Analysis

Total energy and water intake through foods and fluids were determined and analysed through Dietplan6 dietary analysis software (v6.60, Forestfield Software, Horsham, West Sussex, UK) by a trained dietetic researcher. To improve the validity of the dietary analysis, all the nutritional information gathered from food-beverage packages during the interview procedure was entered into the dietary analysis software program. In addition, to improve the reliability of the dietary analysis, all the completed dietary interview logs were blindly analysed by a 2nd trained dietetic researcher. The coefficient of variation (CV) for the energy, macronutrients, and water variables analysed are individually reported within each study chapter.

Exercise-induced BM change (pre- to post-exercise BM difference) was determined from pre- and post-stage BM values (**Chapters 4** to **9**). Pre- and post-exercise P_{Osmol} was determined from 50 µl lithium heparin plasma samples in duplicate by freezepoint osmometry (Osmomat 030, Gonotec, Germany), as previously recommended (Seifarth *et al.* 2004) (**Chapters 4** to **9**). For **Chapters 4** to **7**, P_{Osmol} was determined once samples had been frozen and thawed, as described in **Section 3.6**, whilst for **Chapters 8** and **9**, P_{Osmol} was determined immediately after sample collection.

3.4 Tympanic and Rectal Temperature Measurements

To determine tympanic temperature (T_{tymp}) (**Chapter 6** and **Chapter 7**) participants were asked to position their head in the Frankfort plane and avoid head movement until T_{tymp} measurement was completed. A disposable thermometer tip cover was placed on the sensor

(Braun Thermoscan, Kronberg, Germany); the right auricle was then gently pulled up and back before the sensor was insertion into the right external auditory canal for five seconds, without touching the tympanic membrane.

To monitor body temperature during EHS (**Chapter 9**), participants inserted a thermocouple 12 cm beyond the external anal sphincter (T_{re}); CorTemp Core Body Temperature Sensor, Florida, USA).

3.5 Saliva Sample Collection and Analysis

Prior to saliva collection, participants were asked to thoroughly rinse the mouth with plain water (15–25°C) and swallow in order to fully empty contents of the mouth. Participants were then asked to lean forward and passively drool into the universal tube with minimal orofacial movements. Unstimulated whole saliva samples were collected by a dribbling method into pre-weighed 30 ml universal tubes (HR 120-EC, A & D instruments, Tokyo, Japan). Saliva volume was measured by weighing the universal tube immediately after collection to the nearest milligram. By assuming saliva density to be 1.00 g·ml (Cole and Eastoe 1988), SFR in µl·min was determined by dividing the volume of saliva by the collection time. Aliquots of saliva were pipetted into Eppendorf tubes and stored frozen initially at -20°C prior to transferring to -80°C storage after completion of the experimental procedure (**Chapter 4** and **Chapter 5**). Aliquots of saliva were pipetted into Eppendorf tubes and stored frozen at -80°C storage after completion of the EHS protocol (**Chapter 8**).

After thawing and gentle mixing by vortex, 50 µl of the saliva sample was used to determine the saliva osmolality (S_{Osmol}) in duplicate by freeze point osmometry (Osmomat 030)

(Chapter 4). After thawing and centrifuging at 5000g for 2 min, S-IgA (Salimetrics Europe, Suffolk, UK), S-lysozyme (Bioquote, York, UK), and S-cortisol (Salimetrics Europe) concentrations were determined by enzyme linked immunosorbent assay (ELISA), and the S-α-amylase concentration was determined by an enzyme reaction assay kit (Salimetrics Europe). To account for stress-induced changes in SFR, the secretion rates for S-AMPs (and the appearance rate for S-cortisol) were calculated by multiplying the SFR by the concentration.

S-IgA

S-IgA concentrations (Salimetrics Europe, Suffolk, UK) were determined by ELISA using an indirect competitive immunoassay as per manufacturer's instructions. Using a 1:5 dilution, 25 µl of sample was diluted in 100 µl of S-IgA diluent. 10 µl of standards, controls and samples were added to tubes containing 4ml of S-IgA diluent. 50 µl of diluted antibody-enzyme conjugate was then added to the tubes containing 4 ml of S-IgA diluent. All tubes were gently inverted and incubated for 90 min at room temperature (20°C). 50 µl from each tube was pipetted in duplicate, into each well on the microtitre plate. The plate was covered and incubated at room temperature with continuous mixing at 500 rpm for 90 min. After plate washing, 50 µl of TMB substrate solution was added to each well and placed on a microtitre plate shaker for 5 min at 500 rpm and incubated in the dark at room temperature for an additional 40 min. 50 µl of stop solution was then added to each well and placed on the microtitre plate shaker for 3 min at 500 rpm, followed by reading at optical density (OD) 450 nm. Concentration was calculated by plotting the absorbance against standards in a linear regression curve. The CV's for S-IgA were <3.4% for studies included in this thesis (**Chapter 4, Chapter 5** and **Chapter 8**).

S-lysozyme

S-lysozyme concentrations (Bioquote, York, UK) were determined by a sandwich ELISA assay as per manufacturer's instructions. Using a 1:4000 dilution, 10 µl of sample was diluted in 390 µl (step 1), and 10 µl in 990 µl (step 2) with Phosphate-saline buffer into tubes. 50 µl of standards, controls and samples were pipetted in duplicate, into each well on the microtitre plate. The plate was covered and incubated at room temperature for 60 min. After plate washing, 100 µl of the lysozyme antiserum was added to each well. Once more, the plate was covered and incubated at room temperature for 60 min. After plate washing, 100 µl of the diluted Donkey anti-Goat IgG Peroxidase was added to each well. Again, the plate was covered and incubated at room temperature for 60 min. After plate washing, 100 µl of substrate mix (TMB solution and Hydrogen Peroxide solution) was added to each well and incubated at room temperature for 15 min. 100 µl of Stop solution was added to each well, followed by reading at 450 nm. Concentration was calculated by plotting the absorbance against standards in a linear regression curve. The CV's for S-lysozyme were <3.6% for studies included in this thesis (**Chapter 4, Chapter 5** and **Chapter 8**).

S-α-amylase

S-α-amylase concentrations (Salimetrics Europe, Suffolk, UK) were determined by enzyme reaction assay kit as per manufacturer's instructions. Using a 1:10 dilution, 10 µl of sample was diluted in 90 µl of α-amylase diluent with a final dilution of 1:200. 8 µl of controls and samples were pipetted in duplicate, into each well on the microtitre plate. 320 µl of pre-heated (37°C) α-amylase substrate solution was added to each well, followed by reading at exactly 1 and 3 min. The 1 min readings are subtracted from the 3 min readings and multiplied by the conversion factor. The CV's for S-α-amylase were <4.2% for studies included in this thesis (**Chapter 4, Chapter 5** and **Chapter 8**).

S-cortisol

S-cortisol concentrations (Salimetrics Europe, Suffolk, UK) were determined by ELISA using an indirect competitive immunoassay as per manufacturer's instructions. 25 µl of standards, controls and samples were pipetted in duplicate, into each well on the microtitre plate. A 1:1600 dilution of the conjugate was made by adding 15 µl of the conjugate to a tube containing 24 ml of assay diluent, followed by gentle inverting. 200 µl of the conjugate were pipetted into each well and the plate was placed onto a microtitre plate shaker at 500 rpm for 5 min. After 4 plate washes, 200 µl of TMB solution was added to each well. The plate was placed back onto the microtitre plate shaker at 500 rpm for 5 min, and incubated in the dark at room temperature for an additional 25 min. 50 µl of Stop solution was added to each well, placed on the microtitre plate shaker at 500 rpm for 3 min, followed by reading at 450 nm. Concentration was calculated by plotting the OD against standards in a linear regression curve. The CV's for S-cortisol were <4.1% for studies included in this thesis (**Chapter 4, Chapter 5** and **Chapter 8**).

All salivary variables analysed were individually run on the same day, with standards and controls on each plate, and each participant assayed on the same plate.

3.6 Blood Sample Collection and Analysis

Whole blood samples were collected by venepuncture without venostasis from an antecubital vein using a 21G butterfly syringe into one lithium heparin (6 ml, 1.5 IU·ml heparin; Becton Dickinson, Oxford, UK) and one K3EDTA (6 ml, 1.6 mg·ml of K3EDTA; Becton Dickinson, Oxford, UK) vacutainer tube. Blood samples were immediately centrifuged at 3000 rpm for 10 min. Plasma was aliquoted into Eppendorf tubes and stored frozen initially at -20°C prior

to transferring to -80°C storage after completion of the experimental procedure (**Chapter 6** and **Chapter 7**). Plasma was aliquoted into Eppendorf tubes and stored frozen at -80°C storage after completion of the EHS protocol (**Chapter 9**).

Whole blood haemoglobin concentration and haematocrit were used to estimate changes in plasma volume (Pv) relative to pre-Stage 1 (**Chapter 6**), pre-competition (**Chapter 7**) and to baseline (**Chapter 9**). Haemoglobin concentration and hematocrit content of K3EDTA blood samples (100 µl) were determined using an automated cell counter (Coulter ACT Diff, Beckham Coulter, USA) immediately after sample collection (**Chapter 6**). Whilst, haemoglobin concentration was determined using a haemoglobin analyser (HemoCue Hb 201+ Analyzer, California, USA) and hematocrit was determined by capillary method in triplicate using lithium heparin blood samples and a microhematocrit reader immediately after sample collection (**Chapter 7** and **Chapter 9**). All blood parameters were corrected for changes in Pv (Dill and Costill 1974).

Endotoxin

Gram-negative bacterial endotoxin concentration (HIT302, Hycult Biotech, Uden, Netherlands) was determined by limulus amebocyte lysate (LAL) chromogenic endpoint assay using K3EDTA plasma as per manufacturer's instructions. 20 µl of sample was diluted in 380 µl of endotoxin-free water, and then incubated at 75°C for 10 min. Once at room temperature, 50 µl of standards, blank, positive control, and samples were added to plate wells in duplicate. To enhance assay validity, background plate reading without LAL reagent was performed at OD 405nm. 50 µl LAL reagent was then added. Plate was covered and incubated at 22ºC for 30 min, followed by reading at OD 405 nm. Concentration was calculated by plotting the absorbance against standards in a linear regression curve and

eliminating background error. The CV's for endotoxin were ≤5.5% for studies included in this thesis (**Chapter 6, Chapter 7** and **Chapter 9**).

Cytokine Profile

Circulatory concentrations of IL-6, TNF-α, IL-1β, IFN-γ, IL-1ra and IL-10 (Invitrogen, Carlsbad, US) were determined by ELISA using K_3EDTA plasma as per manufactures instructions (**Chapter 6**). Prior to analysis, wells on the microtitre plate were coated with 100 µl of diluted coating antibody (10 µl of coating antibody and 9.99 µl of coating buffer). Plates were covered and incubated for 12-18 h at 4°C. After plate washing, 300 µl of Assay Buffer was added to each well. The microtitre plate was incubated at room temperature for 60 min. 100 µl of diluted standards and samples were pipetted in duplicate, into each well on the microtitre plate, followed by 50 µl of detection antibody. The plate was placed onto a microtitre plate shaker at 700 rpm and incubated for 120 min. After 5 plate washes, 100 µl of the streptavidin-HRP solution was added to each well. The plate was placed back onto the microtitre plate shaker at 700 rpm and incubated for 30 min. After 5 washes, 100 µl of TMB substrate was added to each well, placed onto the microtitre plate shaker at 700 rpm and incubated for 30 min, followed by 100 µl of Stop Solution and reading at 450 nm. Concentration was calculated by plotting the absorbance against standards in a linear regression curve.

Circulatory concentrations of IL-6, TNF-α, IL-1β, IFN-γ, IL-8 and IL-10 (human pro-inflammatory 7-plex ultra-sensitive kit (K15008C-1), MesoDiscovery, Gaithersburg, MD, USA) were determined by a sandwich ELISA assay using K_3EDTA plasma as per manufactures instructions (**Chapter 7** and **Chapter 9**). 25 µl of diluent was added to each well. The plate was covered, placed onto a microtitre plate shaker (700 rpm) and incubated at

room temperature for 30 min. 25 µl of standards and samples were pipetted in duplicate, into each well on the MSD plate. The plate was covered, placed onto the microtitre plate shaker (700rpm) and incubated at room temperature for 120 min. After 3 plate washes, 25 µl of the Detection Antibody Solution was added to each well. Once more, the plate was covered, placed onto the microtitre plate shaker (700rpm) and incubated at room temperature for 120 min. After 3 plate washes, 150 µl of Read Buffer T was added to each well, followed by analysis using a SECTOR imager (SECTOR imager 2400, MesoScale Discovery, Rockville, MD, USA). The CV's for cytokines were ≤5.5% **(Chapter 6)** and ≤14.7% (**Chapter 7** and **Chapter 9**) for studies included in this thesis.

C-reactive protein

Circulatory concentrations of CRP (eBioscience, Hatfield, UK) were determined by ELISA as per manufacturer's instructions. Using a 1:4000 dilution, 10 µl of sample was diluted in 390 µl (step 1), and 10 µl in 990 µl (step 2) with wash buffer into tubes. 100 µl of standards and samples were pipetted in duplicate, into each well on the microtitre plate. The plate was covered and incubated at room temperature for 120 min. After 5 plate washes, 100 µl of diluted HRP-conjugate was added to each well. Once more, the plate was covered and incubated at room temperature for 60 min. After 5 plate washes, 100 µl of the TMB substrate solution was added to each well and left for 5-10 min, followed by reading at 450 nm. Concentration was calculated by plotting the absorbance against standards in a linear regression curve. The CV's for CRP were ≤5.5% **(Chapter 6)** and ≤14.7% (**Chapter 7** and **Chapter 9**) for studies included in this thesis.

All plasma variables analysed were individually run on the same day, with standards and controls on each plate, and each participant assayed on the same plate.

3.7 Assessment of Upper Respiratory Symptoms and Gastrointestinal Symptoms

The assessment of illness symptoms in **Chapter 4** and **Chapter 5** (URS) and in **Chapter 6** and **Chapter 7** (GI symptoms) was included to explore whether alterations in physiological variables (e.g., S-AMP concentration, circulatory endotoxin concentrations and cytokine profile) resulted in the manifestation of clinically significant outcomes.

The Wisconsin Upper Respiratory Symptom Survey (WURSS) was used to assess illness symptoms during and daily for four weeks after the competition in **Chapter 4** and **Chapter 5**. In the absence of a medical diagnosis, the WURSS is considered a useful, validated tool for measuring symptoms (Barrett *et al.* 2009, Spence *et al.* 2007).

A standardised questionnaire assessing GI symptoms during endurance events (Pfeiffer *et al.* 2009, Pfeiffer *et al.* 2012) was adapted for use in **Chapter 6** and **Chapter 7** to measure GI symptoms and severity.

3.8 Statistical Analysis

The primary outcomes of this thesis were SFR, S-IgA, S-α-amylase, S-lysozyme concentration and secretion rate, S-cortisol concentration and appearance rate, circulatory endotoxin concentration, circulatory cytokine profile (IL-6, TNF-α, IL-1β, IFN-γ, IL-8, IL-1ra and IL-10) and circulatory CRP concentration. Given the variation in methodological design and experimental protocols between presented studies in this thesis, statistical analysis is individually reported within each study chapter. The secondary outcomes of this thesis

were self-reported illness symptoms, specifically URS and GI symptoms. Illness symptoms were assessed as described in **Section 3.7**.

For **Chapter 4** and **Chapter 6**, 23 out of 69 (33%) and 19 out of 69 (28%) UER entering multi-stage ultra-marathon volunteered to participate in the study, respectively. For **Chapter 5** and **Chapter 7**, 25 out of 48 (52%) and 17 out of 48 (35%) UER entering the 24 h ultra-marathon volunteered to participate in the study. Previous field-based studies exploring the effects S-AMPs and URSI have used sample sizes between 14 and 38 participants (Gleeson *et al.* 1999, Neville *et al.* 2008, Pacque *et al.* 2007, Peters *et al.* 2010), whilst previous field-based studies exploring the effects of circulatory endotoxin concentrations and cytokine profile have used sample sizes between 18 and 29 participants (Bosenberg *et al.* 1988, Camus *et al.* 1998, Jeukendrup *et al.* 2000, Nieman *et al.* 2006).

For **Chapter 8** and **Chapter 9**, $n=$ 8 endurance runners volunteered to participate in the study. Previous data examining the effects of heat-stress and nutritional interventions on physiological variables have used sample sizes between 6 and 10 participants (Costa *et al.* 2014, Costa *et al.* 2009, Shing *et al.* 2013).

CHAPTER FOUR

Salivary Anti-Microbial Protein Responses During Multi-stage Ultra-Marathon Competition Conducted in Hot Ambient Conditions.

4.1 Summary: Prolonged physical exertion is commonly reported to depress oral-respiratory immune status and increase the incidence of URS. This novel investigation aimed to determine the S-AMP responses and hydration status of UER (n= 23) and a control group (CON, n= 12) during a 230 km multi-stage ultra-marathon conducted in hot ambient conditions (32–40°C). BM was measured and unstimulated saliva and venous blood samples were taken before and after each stage of the ultra-marathon. *Ad libitum* fluid intake was permitted throughout each race day. URS were monitored during and until four weeks after race completion. Samples were analysed for S-IgA, S-lysozyme, S-α-amylase, and S-cortisol, as well as for P_{Osmol} and S_{Osmol}. Mean exercise-induced BM loss over the five stages ranged from 1.3% to 2.4%. Overall mean pre- and post-stage P_{Osmol} measurements in the UER were 279 ± 14 mOsmol·kg^{-1} and 293 ± 15 mOsmol·kg^{-1}, respectively. Decreases in SFR (overall change 22%) and post-stage increases in S_{Osmol} (36%) were observed in the UER during the ultra-marathon. Reduced S-IgA (32%) (p <0.001 *vs.* pre-stage S-IgA), enhanced S-α-amylase (187%) (p <0.001 *vs.* pre-stage S-α-amylase), and no change in S-lysozyme secretion rates were observed in the UER throughout the ultra-marathon. Only 1 UER reported URS during and one month after competition. Observed depressions in S-IgA secretion rates were offset by favourable increases in S-α-amylase and unchanged S-lysozyme responses in the majority of runners during the competition. Ensuring euhydration throughout a multi-stage ultra-marathon competition in the heat may play a role in protecting the URT.

4.2 Introduction

Reported episodes of URS are often attributed to the commonly observed transient decreases in S-IgA responses (Costa *et al.* 2009, Gleeson *et al.* 2000, Neville *et al*. 2008). Such a response has been suggested as creating an "open window", whereby viruses and (or) bacteria may gain a hold on oral-respiratory mucosal surfaces (Nieman 1994).

Because of the nature of multi-stage ultra-marathon events, UER are a high-risk population group for contracting sub-clinical or clinically significant illnesses and (or) infections during, and in the month after, competition (Gleeson *et al.* 2000). The presence of URS *per se* may have both clinical and performance significance for UER participating in a multi-stage ultra-endurance competition, because symptom aggravation and discomfort leading to negative effects on nutrition and hydration status (e.g., loss of appetite, food–fluid disinterest, and (or) pain when eating or drinking) may ultimately result in withdrawal from competition and (or) in medical intervention.

With this in mind, the aims of the current study were to determine the S-AMP responses and hydration status of UER during a multi-stage ultra-marathon competition in hot ambient conditions, in which *ad libitum* fluid intake was permitted throughout each race day. Additionally, URS during the race and daily for four weeks after race completion were monitored. It was hypothesized that the water intake habits of UER would not be sufficient to consistently maintain baseline euhydration levels throughout the competition, and subsequently, depressed salivary variables (i.e., SFR and S-AMP responses) would be evident throughout the competition, leading to increased reports of URS.

4.3 Methods

Setting. The study was conducted during the 2011 Al Andalus Ultimate Trail (www.alandalus-ut.com), held during the 2nd week of July, in the region of Loja, Spain. The multi-stage ultra-marathon was conducted over five stages (five days) totalling a distance of 230 km (Stage 1: 37 km, Stage 2: 48 km, Stage 3: 38 km, Stage 4: 69 km, and Stage 5: 38 km, respectively), and performed on a variety of terrains; predominantly off-road trails and paths, but also included steep and narrow mountain passes, and occasional road. Mean running intensity was (mean ± SD) 7.2 ± 0.9 METs (SenseWear 7.0, BodyMedia Inc., Pittsburgh, PA, USA). Sleeping arrangements from Stages 1 to 5 included a combination of outdoor tent and village sports hall accommodation (mean sleep duration per night was 7 h 54 min ± 0 h 37 min) (Pittsburgh Sleep Diary) (Monk *et al.* 1994). Moreover, the run course was routed over an altitude ranging between 473 to 1443 m above sea level (Garmin International, Olathe, Kansas, US). Average daytime maximum T_{amb} during the running stages ranged between 32-40°C, with maximum daytime RH also ranging between 32-40% (Garmin International, Olathe, Kansas, US).

Participants. After ethical approval, a convenience sampling observational cohort was studied, whereby 23 out of 69 UER who entered this ultra-marathon competition volunteered to participate in the study [UER (Male *n*= 16, Female *n*= 7): age 41 ± 9 y, height 1.75 ± 0.06 m, BM 70.0 ± 9.0 kg, body fat mass 16 ± 4%]. For comparative purposes, 12 matched individuals who accompanied the UER along the ultra-marathon course, but did not compete (absence of exercise stress), volunteered to participate in the study as part of the CON group [(Male *n*= 5, Female *n*= 7): age 35 ± 13 y, height 1.67 ± 0.09 m, BM 69.9 ± 16.2 kg, body fat mass 21 ± 6%]. All participants arrived at location ≤48 h prior to the start of Stage 1. Only

32% of participants resided in countries with hot ambient conditions similar to those of the race location ($\geq 30°C$ T_{amb}) at the time of competition; whilst the remaining 68% of participants resided in countries with cold or thermoneutral ambient conditions ($\leq 20°C$). No participant reported any incidence of illness and/or infection in the twelve weeks leading up to the ultra-marathon.

Study design and data collection. Following participant recruitment and informed consent, preliminary measures were taken to determine participant characteristics (**Section 3.2**). The current ultra-marathon was semi self-sufficient, whereby participants (including CON) planned and provided their own foods and fluids (except plain water) along the five days of competition. Participants' equipment and sustenance was transported to each stage section by the race organisation. Only plain water was provided by the race organisers *ad libitum* during the rest phase throughout competition. Additionally, aid stations along the running phase of competition were situated approximately 10 km apart, and only provided plain water, fruit (oranges and watermelon), and electrolyte supplementation (Elete electrolyte add-in, Mineral Resources International**,** South Ogden, Utah, US). Participants were advised to adhere to their programmed habitual dietary practices throughout the entire competition.

Each day, for five consecutive days, running stages commenced at either 08:00 or 09:00 h. Within the hour prior to the start of each running stage, pre-stage measurements were determined and samples collected. BM measurements were taken, as previously described (**Section 3.2**)**.** Participants were then required to sit in an upright position for 10 min before whole blood and 2 min saliva samples were collected, as previously described (**Section 3.5 and 3.6)**. All measurement techniques and samples were consistently conducted and collected in a large partitioned research field tent (four sections, 3 m x 3 m) or sports hall

facility. BM was re-measured in those participants who needed to urinate prior to the stage start. Immediately post-stage and before any foods or fluids could be consumed, BM was measured, followed by blood and saliva sampling, as previously described (**Section 3.5 and 3.6**). For consistency, the order, positioning and technique of measurements and sampling were similar pre- and post-stage for all stages, and were taken by the same trained researcher throughout.

At the end of each competition day (20:00 to 22:00 h) on Stages 1 to 4, trained dietetic researchers conducted a standardised structured interview (dietary recall interview technique) on participants to ascertain total daily food and fluid ingestion. To avoid inter-observer variations, each trained researcher conducted the interview on the same participant throughout the entire ultra-marathon. Participants also completed an URS log (Wisconsin Upper Respiratory Symptom Survey, St. Madison, WI, USA) during this period and each day for four weeks following race completion.

Dietary analysis and hydration status. Dietary analysis and hydration status were determined as previously described in **Section 3.3.** The mean CV for energy, macronutrient, and water variables analysed was 1.3%, 2.3%, and 0.7%, respectively. The CV for P_{Osmol} was 3.5%.

Saliva sample analysis. Unstimulated whole saliva samples were analysed as previously described in **Section 3.5.** In CON, salivary variables were determined on pre-Stages 1, 3 and 5 only.

A CON group was included to allow for comparability between resting values (UER *vs.* CON) at various time points along the multi-stage ultra-marathon (Stage 1, Stage 3 and Stage

5). Since the CON group did not compete (absence of exercise stress) and the focus of **Chapter 4** and **Chapter 6** was not to determine the time effect of salivary or plasma variables in CON, respectively; collecting samples post-stages of competition was not justified.

Data analysis. Data in text (overall mean value unless otherwise specified) and tables are presented as mean ± standard deviation (SD). For clarity, the data in figures are presented as mean ± SEM, unless otherwise specified. Because of pronounced diurnal variation in cortisol responses (Shephard and Shek 1997), statistical analysis was performed between stages for pre- and post-stage time points only. A one-way analysis of variance (ANOVA) was applied to determine differences in variables between stages, whereas a two-way ANOVA was applied to determine differences between groups (UER *vs.* CON), and between pre- and post-stage values within stages (SPSS v.20, Illinois, US). Significant main effects were analysed using a post hoc Tukey's honestly significant difference test. All data were checked for normal distribution by calculating skewness and kurtosis co-efficients. When the data violated the assumption of normality, they were log transformed, and the transformed data were used in the analysis. For comparative purposes, an independent sample t-test with non-parametric verification was used to assess differences in post-stage salivary anti-microbial protein responses between euhydrated (EH) runners (mean ≤1.5% exercise-induced BM loss) and hypohydrated (HH) runners (mean >3.0% exercise-induced BM loss). The acceptance level of significance was set at $p < 0.05$.

4.4 Results

Energy, macronutrient, and water ingestion. Total daily energy intake did not vary significantly among stages in UER (15.0 ± 3.3 MJ·day^{-1}; 12% protein, 61% carbohydrates, 27% fat) and CON (11.6 ± 4.8 MJ·day^{-1}; 10% protein, 67% carbohydrates, 23% fat). Compared with CON, the total daily energy intake was higher in UER on Stages 1 to 3 ($p < 0.001$). Total daily water intake through foods and fluids did not vary significantly among stages in UER (7383 ± 1578 ml·day^{-1}) and CON (3301 ± 163 ml·day^{-1}). Compared with CON, the total daily water intake through foods and fluids was higher in UER on all stages ($p < 0.001$). Total and rate of water intake through foods and fluids during running did not differ among stages in UER (4030 ± 1307 ml and 731 ± 226 ml·h^{-1}, respectively).

Body mass. No significant changes in pre-stage BM in UER (pre-Stage 1, 71.4 ± 9.2 kg to pre-Stage 5, 70.7 ± 9.0 kg) and CON (pre-Stage 1, 68.9 ± 16.2 kg to pre-Stage 5, 69.1 ± 16.9 kg) were observed during the ultra-marathon. However, as expected, BM loss did occur from pre- to post-stage ($p < 0.001$) in UER on all stages, with no significant differences among stages (**Figure 4.1**).

Figure 4.1: Exercise induced body mass loss of ultra-endurance runners participating in a 230 km multi-stage ultra-marathon competition conducted in a hot ambient environment. Data are presented as mean and range (*n*= 23).
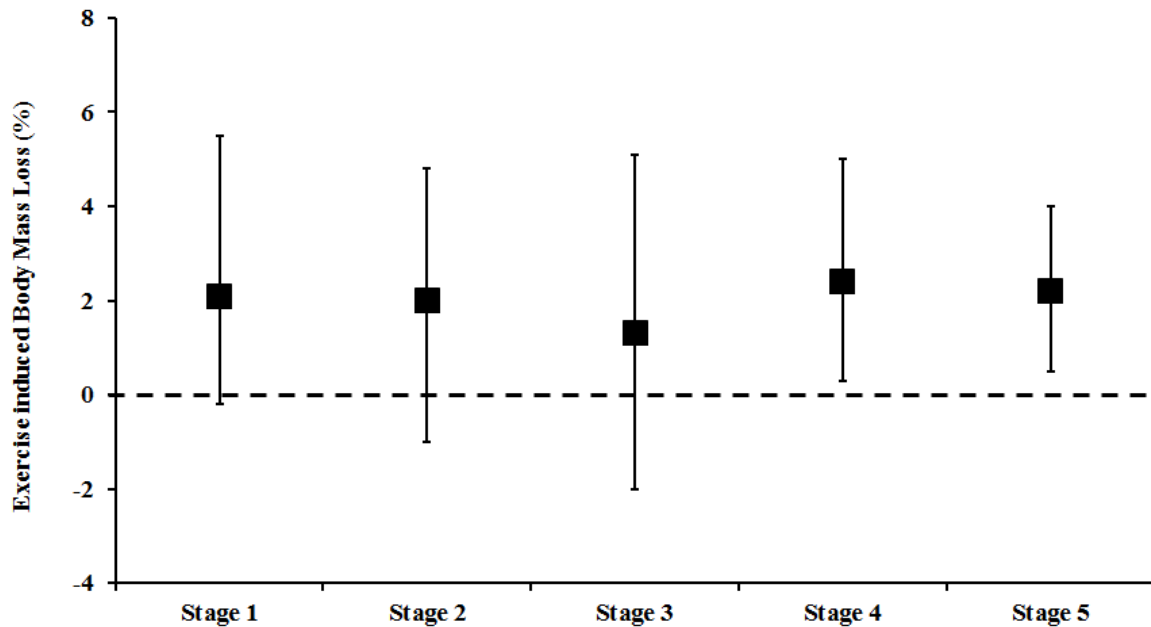
Plasma osmolality. Pre-stage P_{Osmol} was significantly higher on Stage 2 compared with Stage 4 only in UER (*p*= 0.045) (**Figure 4.2**) but did not differ among stages in CON (pre-stage, 278 ± 12 mOsmol·kg^{-1}). Pre-stage P_{Osmol} did not differ between UER and CON within stages throughout the ultra-marathon. Post-stage P_{Osmol} did not differ among stages in UER. Post-stage P_{Osmol} increased from pre-stage P_{Osmol} in UER (*p* <0.001) on Stages 3 to 5 (**Figure 4.2**).

Figure 4.2: Changes in plasma osmolality of ultra-endurance runners participating in a 230 km multi-stage ultra-marathon competition conducted in a hot ambient environment. Data are presented as mean and range ($n= 23$). a, Normal clinical reference range (280–300 mOsmol·kg^{-1}) (Fischbach and Dunning 2004; Thomas *et al.* 2008). †, $p< 0.01$ *vs.* pre-stage; §, $p< 0.01$ *vs.* pre-Stage 2.
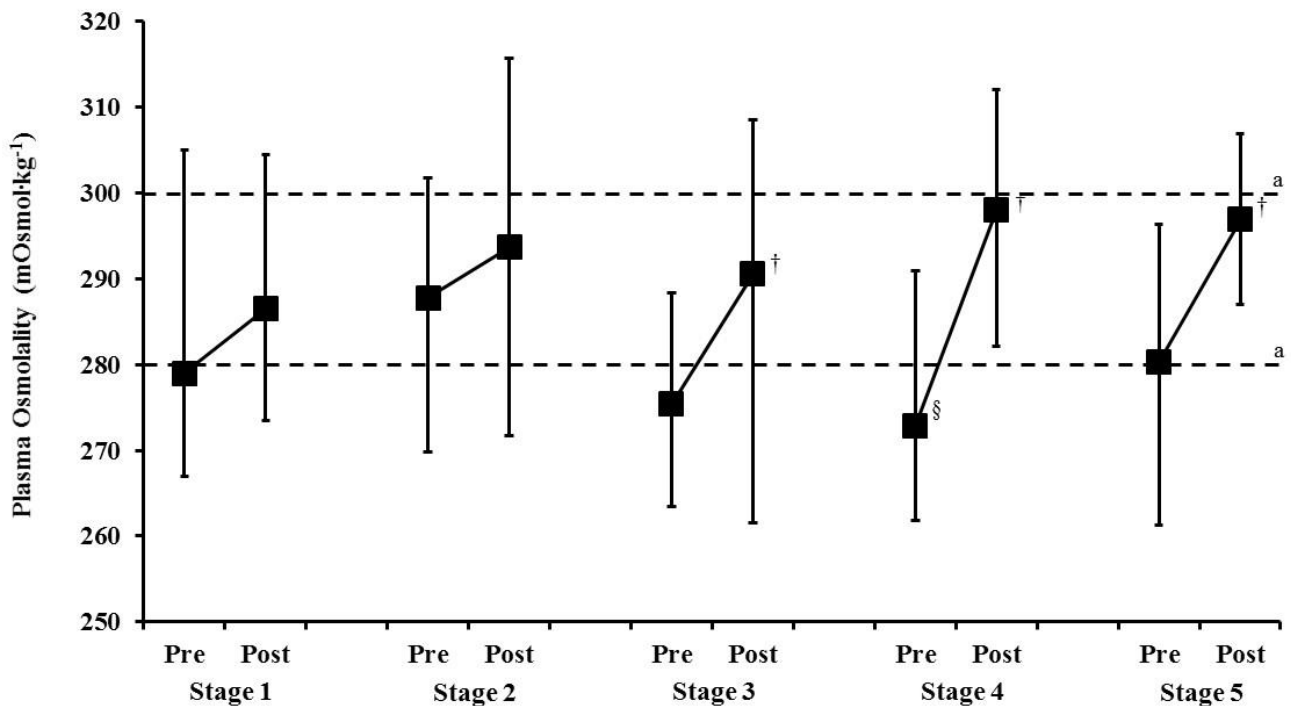
Saliva flow rate and saliva osmolality. The pre- and post-stage SFR did not significantly differ among stages in UER and CON (**Table 4.1**). Compared with pre-stage values, decreases in the SFR ($p= 0.002$) occurred after Stages 1 to 4 (37%, 34%, 20%, and 31%, respectively). Before Stage 3, the SFR was higher in UER than in CON ($p< 0.001$). An 18% lower post-stage SFR was evident in HH runners compared with EH runners ($p= 0.095$). S$_{Osmol}$ was higher ($p< 0.001$) before Stage 5 (53%) compared with before Stage 1 in UER, but did not change significantly in CON (**Table 4.1**). No significant changes in post-stage S$_{Osmol}$ were observed in UER throughout the ultra-marathon. Compared with pre-stage values, increases in S$_{Osmol}$ ($p< 0.001$) occurred after Stages 1 to 4 (44%, 34%, 20%, and 36%,

respectively). No differences in S_{Osmol} were observed between UER and CON throughout the ultra-marathon. A 26% higher post-stage S_{Osmol} was evident in HH runners compared with EH runners, but this failed to reach significance ($p= 0.140$).

Table 4.1: Changes in saliva flow rate and osmolality of ultra-endurance runners participating in a 230 km multi-stage ultra-marathon competition conducted in a hot ambient environment.

	Stage 1		Stage 2		Stage 3		Stage 4		Stage 5	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Saliva flow rate (μl·min^{-1})										
UER	651 ± 579	$412 \pm 249^*$	523 ± 330	$343 \pm 292^\dagger$	$535 \pm 319^\ddagger$	$430 \pm 291^*$	601 ± 350	$415 \pm 247^\dagger$	414 ± 318	473 ± 331
CON	458 ± 198				353 ± 153				365 ± 297	
Saliva osmolality (mOsmol·kg^{-1})										
UER	45 ± 12	$65 \pm 36^*$	47 ± 19	$63 \pm 24^*$	49 ± 15	$59 \pm 15^*$	50 ± 11	$68 \pm 25^*$	$69 \pm 28^\S$	67 ± 31
CON	44 ± 12				53 ± 15				57 ± 28	

Mean $\pm$ SD: UER ($n = 23$) and control (CON, $n = 12$). $^*p < 0.05$ *vs.* pre-stage. $^\dagger p < 0.01$ *vs.* pre-stage. $^\ddagger p < 0.05$ *vs.* CON. $^\S p < 0.01$ *vs.* pre-Stage 1.

S-IgA responses. The pre- and post-stage S-IgA concentration did not alter significantly throughout the ultra-marathon in UER and CON (**Table 4.2; Figure 4.3A**). Similarly, no significant pre- to post-stage changes in S-IgA concentration were observed throughout the ultra-marathon in UER. Compared with CON, the UER S-IgA concentration was lower at baseline (before Stage 1) ($p< 0.001$). The pre- and post-stage S-IgA secretion rate did not alter significantly throughout the ultra-marathon in UER and CON **(Table 4.2; Figure 4.3B)**. Compared with pre-stage values, decreases in the S-IgA secretion rate ($p= 0.001$) occurred after Stages 2 to 5 (35%, 36%, 23%, and 33%, respectively) in UER. No significant differences in the S-IgA secretion rate were observed between UER and CON throughout the ultra-marathon. The post-stage S-IgA concentration ($p= 0.026$) and secretion rate ($p= 0.05$) were 37% and 48% lower, respectively, in HH runners compared with EH runners.

Figure 4.3: Changes in salivary IgA concentration (A) and secretion rate (B) of ultra-endurance runners participating in a 230 km multi-stage ultra-marathon competition conducted in a hot ambient environment. Data are presented as mean (squares; *n*= 23) and individual UER responses (circles).
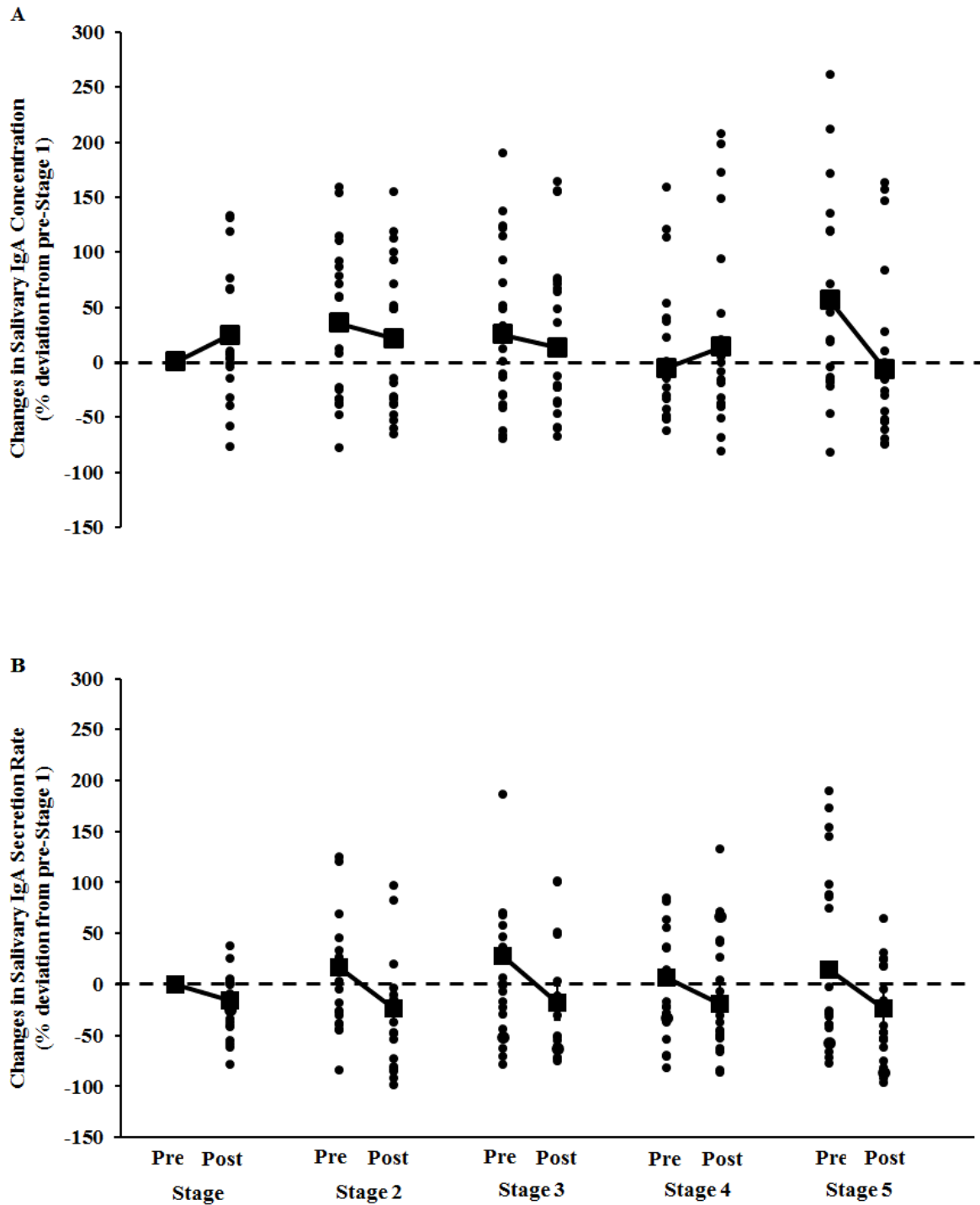
S-α-amylase responses. The pre- and post-stage S-α-amylase concentration did not alter significantly throughout the ultramarathon in UER and CON (**Table 4.2; Figure 4.4A**). Compared with pre-stage values, increases in the S-α-amylase concentration ($p < 0.001$) occurred on all stages (194%, 573%, 257%, 87%, and 28%, respectively) in UER. The S-α-amylase concentration was significantly lower in UER compared with CON before Stage 3 ($p = 0.002$). The pre- and post-stage S-α-amylase secretion rate did not change significantly throughout the ultra-marathon in UER and CON (**Table 4.2; Figure 4.4B**). Compared with pre-stage values, increases in the S-α-amylase secretion rate ($p < 0.001$) occurred on Stages 1, 2, 3, and 5 (106%, 380%, 195%, and 68%, respectively) in UER. No significant differences in the S-α-amylase secretion rate were observed between UER and CON throughout the ultra-marathon. No differences in the post-stage S-α-amylase concentration and secretion rate were observed between HH and EH runners.

Figure 4.4: Changes in salivary α-amylase concentration (A) and secretion rate (B) of ultra-endurance runners participating in a 230 km multi-stage ultra-marathon competition conducted in a hot ambient environment. Data are presented as mean (squares; *n*= 23) and individual UER responses (circles).
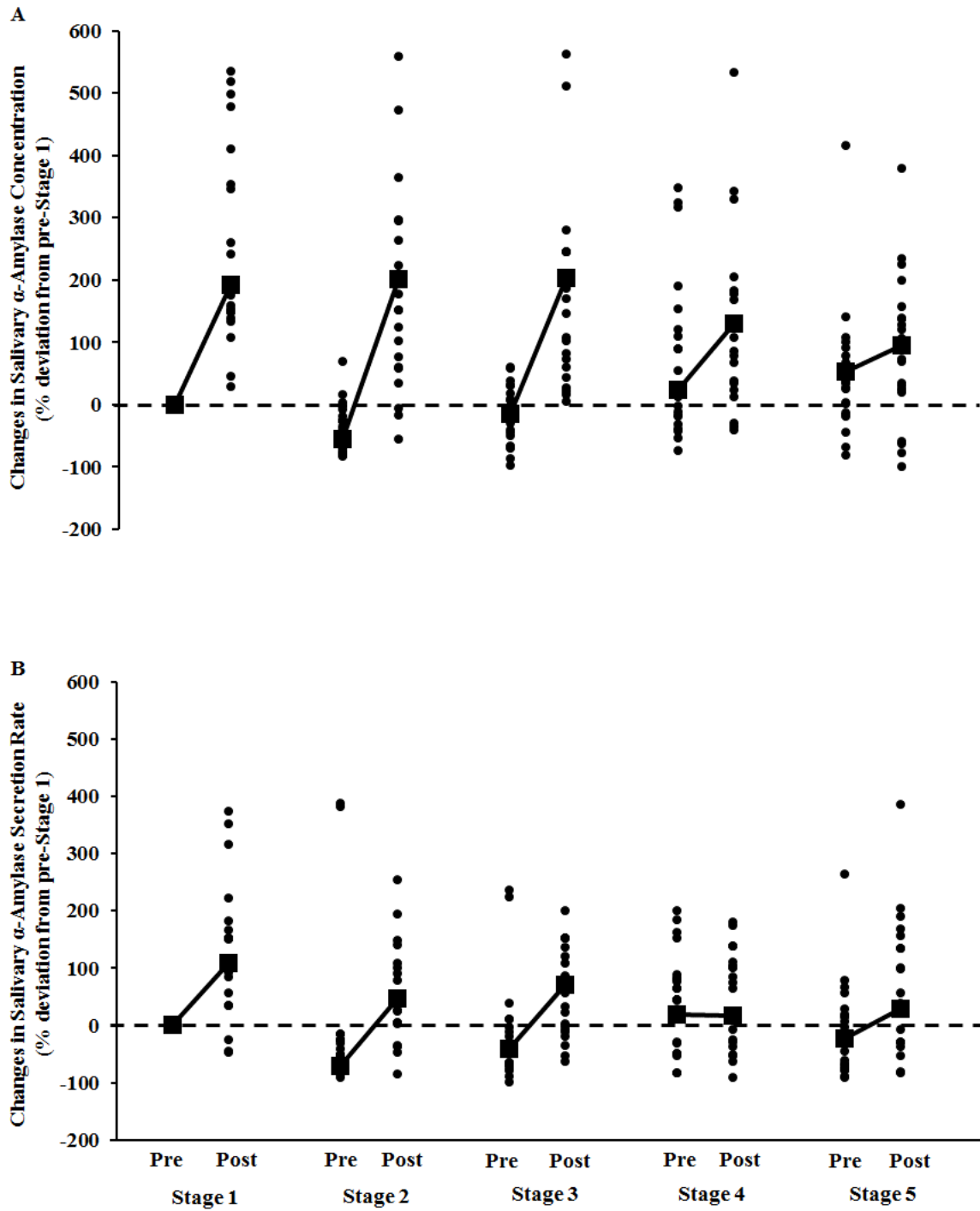
S-lysozyme responses. The pre- and post-stage S-lysozyme concentration did not alter significantly throughout the ultra-marathon in UER and CON (**Table 4.2; Figure 4.5A).** Compared with pre-stage values, increases in the S-lysozyme concentration ($p= 0.002$) occurred on Stages 1 to 4 (33%, 180%, 60%, and 100%, respectively) in UER. No significant differences in the S-lysozyme concentration were observed between UER and CON throughout the ultra-marathon. The pre- and post-stage S-lysozyme secretion rate did not alter significantly throughout the ultra-marathon in UER and CON (**Table 4.2; Figure 4.5B**). Similarly, no significant changes in the pre- to post-stage S-lysozyme secretion rate were observed in UER during the ultra-marathon competition. No significant differences in the S-lysozyme secretion rate were observed between UER and CON throughout the ultra-marathon. The post-stage S-lysozyme concentration ($p= 0.004$) was 70% lower in HH runners compared with EH runners. A 56% lower post-stage S-lysozyme secretion rate was evident in HH runners compared with EH runners ($p= 0.068$).

Figure 4.5: Changes in salivary lysozyme concentration (A) and secretion rate (B) of ultra-endurance runners participating in a 230 km multi-stage ultra-marathon competition conducted in a hot ambient environment. Data are presented as mean (squares; *n*= 23) and individual UER responses (circles).
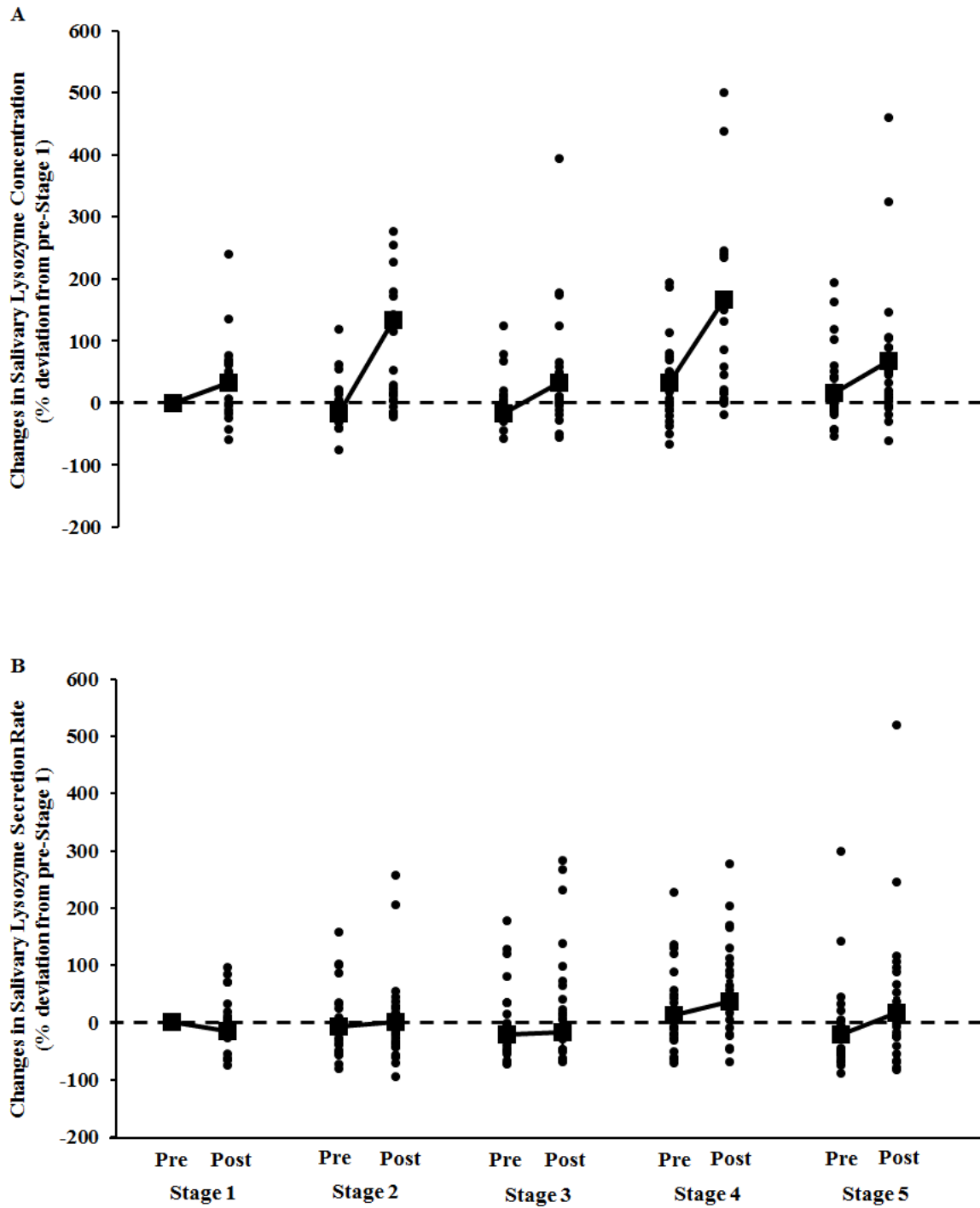
Table 4.2: Salivary anti-microbial protein responses of ultra-endurance runners participating in a 230 km multi-stage ultra-marathon competition conducted in a hot ambient environment.

	Stage 1		Stage 2		Stage 3		Stage 4		Stage 5	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Salivary IgA concentration ($\mu g \cdot ml^{-1}$)										
UER	$203 \pm 115^{\ddagger}$	253 ± 150	274 ± 183	246 ± 167	253 ± 173	229 ± 146	192 ± 123	230 ± 162	316 ± 184	190 ± 124
CON	364 ± 118				447 ± 124				487 ± 166	
Salivary IgA secretion rate ($\mu g \cdot min^{-1}$)										
UER	105 ± 64	88 ± 57	123 ± 87	$80 \pm 77^{*}$	134 ± 129	$86 \pm 78^{*}$	111 ± 89	$85 \pm 59^{*}$	120 ± 104	$80 \pm 78^{*}$
CON	158 ± 61				157 ± 84				176 ± 93	
Salivary α-amylase concentration ($U \cdot ml^{-1}$)										
UER	49 ± 58	$144 \pm 120^{\dagger}$	22 ± 27	$148 \pm 126^{\dagger}$	$42 \pm 59^{\ddagger}$	$149 \pm 117^{*}$	60 ± 49	$113 \pm 102^{*}$	75 ± 89	$96 \pm 82^{*}$
CON	56 ± 19				58 ± 10				39 ± 51	
Salivary α-amylase secretion rate ($U \cdot min^{-1}$)										
UER	33 ± 48	$68 \pm 58^{*}$	10 ± 11	$48 \pm 55^{\dagger}$	19 ± 30	$56 \pm 54^{\dagger}$	39 ± 43	39 ± 33	25 ± 31	$42 \pm 37^{*}$
CON	26 ± 8				32 ± 10				31 ± 43	

Salivary lysozyme concentration (µg·ml^{-1})

UER	5.7 ± 5.2	$7.9 \pm 7.6^{*}$	5.4 ± 4.2	$13.8 \pm 18.8^{*}$	5.4 ± 4.5	$7.9 \pm 7.2^{*}$	8.1 ± 8.6	$16.5 \pm 18.6^{\dagger}$	7.4 ± 7.8	10.4 ± 10.6
CON	3.4 ± 1.8				4.4 ± 1.7				8.9 ± 4.7	

Salivary lysozyme secretion rate (µg·min^{-1})

UER	3.5 ± 4.0	3.0 ± 2.5	3.3 ± 3.4	3.6 ± 5.0	2.8 ± 2.7	3.0 ± 2.9	4.0 ± 3.5	4.9 ± 4.6	2.8 ± 2.6	4.1 ± 4.0
CON	1.3 ± 0.5				1.7 ± 0.6				4.2 ± 4.3	

Mean ± SD: UER ($n = 23$) and control (CON, $n = 12$). $^{*}p < 0.05$ *vs.* pre-stage. $^{\dagger}p < 0.01$ *vs.* pre-stage. $^{\ddagger}p < 0.05$ *vs.* CON.

S-cortisol responses. The pre-stage S-cortisol concentration did not alter significantly throughout the ultra-marathon competition in UER and CON (**Table 4.3**). The post-stage S-cortisol concentration was lower (54%) on Stage 3 compared with Stage 1 in UER ($p= 0.006$). The S-cortisol concentration was higher ($p< 0.001$) before Stages 1 and 3 in UER compared with CON. The pre-stage S-cortisol appearance rate did not alter significantly throughout the ultra-marathon competition in UER and CON (**Table 4.3**). The post-stage S-cortisol appearance rate was lower on Stages 2 and 3 (36% and 50%, respectively) compared with Stage 1 in UER ($p= 0.006$). The S-cortisol appearance rate was higher ($p< 0.001$) before Stages 1 and 3 in UER compared with CON.

Upper respiratory symptoms. Only 1 runner reported URS during and four weeks after the ultra-marathon competition. The initial symptoms were reported on Stage 3 and continued, varying in severity, until seven days after the ultra-marathon competition. Over the five stages, the runner presented a mean exercise-induced BM loss of 3.2%, a pre-stage mean P_{Osmol} of 288 mOsmol·kg^{-1}, and a post-stage mean P_{Osmol} of 305 mOsmol·kg^{-1}. The runner also presented a pre- to post-stage decrease in SFR (25%), a decrease in S-IgA (21%), no change in S-lysozyme, and an increase in S-amylase secretion rates (30%). No URS were reported by CON.

Table 4.3: Salivary cortisol responses of ultra-endurance runners participating in a 230 km multi-stage ultra-marathon competition conducted in a hot ambient environment.

	Stage 1		Stage 2		Stage 3		Stage 4		Stage 5	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Salivary cortisol concentration ($\mu g \cdot ml^{-1}$)										
UER	$66 \pm 28^{\parallel}$	63 ± 37	65 ± 26	48 ± 28	$59 \pm 24^{\parallel}$	$29 \pm 17^{\P}$	60 ± 29	51 ± 31	51 ± 33	42 ± 27
CON	30 ± 16				25 ± 11				36 ± 18	
Salivary cortisol appearance rate ($\mu g \cdot min^{-1}$)										
UER	$39 \pm 29^{\dagger}$	22 ± 18	34 ± 26	$14 \pm 15^{\P}$	$31 \pm 25^{\dagger}$	$11 \pm 8^{\P}$	38 ± 27	23 ± 19	27 ± 25	20 ± 21
CON	15 ± 7				12 ± 6				9 ± 8	

Mean ± SEM: UER ($n = 23$) and control (CON, $n = 12$). $^{\parallel}p < 0.01$ *vs.* CON. $^{\P}p < 0.01$ *vs.* post-Stage 1. $^{\dagger}p < 0.05$ *vs.* CON.

4.5 Discussion

The novel outcomes of this field-based investigation suggest that the UER were able to maintain oral-respiratory mucosal surface immune integrity, possibly through their effective hydration strategies. Moreover, a counteractive effect appears to be present in the majority of runners, whereby the observed post-stage decreases in S-IgA responses were counterbalanced by increases in S-α-amylase and S-lysozyme responses. The observed maintenance of oral-respiratory mucosal immune integrity may have reduced the likelihood of URS.

The overall average total water intake in the runners through foods and fluids was 7.4 $l \cdot day^{-1}$, whereas the overall average rate of water intake during running was 731 $ml \cdot h^{-1}$, resulting in an average exercise-induced BM loss of 2.0% and a post-stage P_{Osmol} of 293 $mOsmol \cdot kg^{-1}$. Because exercise-induced BM loss and post-stage P_{Osmol} indicative of hypohydration were observed in only 33% of UER (Fischbach and Dunning 2004, Sawka *et al.* 2007, Thomas *et al.* 2008) and because they returned to baseline values prior to the onset of the next day's stage, the fluid intake habits of the majority of UER appear to have been sufficient to maintain euhydration throughout the current ultra-marathon. Taking into account the influence of hydration status on SFR (Oliver *et al.* 2008, Walsh *et al.* 2004a, Walsh *et al.* 2004b), the maintenance of euhydration may have contributed to the prevention of substantial depressions in the SFR.

In the current study, the pre- to post-stage reduction in SFR ranged from 20-37%. In relation to S-AMP responses, a consistent post-stage decrease in the S-IgA secretion rate was observed (32%), whereas S-α-amylase and S-lysozyme increased or remained unchanged. Notably, any observed reductions in post-stage S-IgA responses (91% of UER showed

reductions in post-stage S-IgA secretion rates) were compensated for by increases in S-α-amylase (91% of UER showed increases in post-stage S-α-amylase secretion rates) and S-lysozyme (57% of UER showed increases in post-stage S-lysozyme secretion rates) responses during the competition. Of the 43% of UER that showed decreased post-stage S-lysozyme secretion rates, 88% demonstrated increased post-stage S-α-amylase secretion rates. Given that saliva secretions are under neuroendocrine control (Carpenter *et al.* 2000, Carpenter *et al.* 1998, Chicharro *et al.* 1998; Teeuw *et al.* 2004, Tenovuo 1998), the increased circulating stress hormone concentrations associated with exercise-heat stress may have influenced the small fluctuations in S-AMPs observed during the ultra-marathon.

The novel findings in the current study indicated that a low prevalence (*n*= 1) of URS during and up to four weeks after a 230 km multi-stage ultra-marathon competition are not in accordance with the findings of symptomology reports of other field-based, prolonged running studies that showed a high incidence of URT illness symptoms (Heath *et al.* 1991, Linde 1987, Nieman *et al.* 1990, Peters 1990, Peters and Bateman 1983). Additionally, the multi-stage ultra-marathon took place during the summer months and therefore the risk of contracting an illness of viral aetiology (e.g., the common cold) is less likely compared to if the multi-stage ultra-marathon took place during the winter months.

In regards to limitations, the sample of UER recruited to take part in the study was not randomised and therefore the potential for self-selection bias was not eliminated. This can potentially lead to a sample not being representative of the population studied, which in this case are UER. Additionally, the control group had different characteristics to the sampled cohort of UER, thus applying a statistical correction method during analysis would have allowed adjustments for differences between UER and the control group.

In conclusion, S-AMP responses appear to be maintained throughout multi-stage ultra-marathon competition irrespective of exposure to environmental extremes and other accompanying physiological stressors known to perturb immune function and hydration. Regardless of neuroendocrine regulation, maintaining euhydration through the maintenance of the saliva flow rate may play a fundamental role in protecting the oral respiratory mucosal surface, thereby ensuring an adequate presence of S-AMPs and preventing oral-respiratory surface drying and associated inflammatory responses. The observed unfavourable reductions in S-IgA responses were offset by favourable increases in the S-α-amylase and S-lysozyme responses. This is further reflected by the low prevalence of URS reported during and up to four weeks after the competition.

CHAPTER FIVE

Salivary Anti-Microbial Protein Responses During a 24 h Ultra-Marathon in Temperate Ambient Conditions.

Gill, S. K., Teixeira, A. M., Rosado, F., Hankey, J., Wright, A., Marczak, S., Murray, A., and Costa, R. J. (2014) 'The Impact of a 24-h Ultra-Marathon on Salivary Antimicrobial Protein Responses'. *International Journal of Sports Medicine* 35 (11), 966-971

5.1 Summary: Depressed oral-respiratory mucosal immunity and increased incidence of URS are commonly reported after bouts of prolonged physical exertion. The current study observed the impact of a 24 h continuous overnight ultra-marathon competition (distance range: 122-208 km; T_{amb} range: 0-20°C) on S-AMP responses and incidence of URS. BM, unstimulated saliva and venous blood samples were taken from UER (n=25) and CON (n=17), before and immediately after competition. URS were assessed during and until four weeks after event completion. Samples were analysed for S-IgA, S-lysozyme, S-α-amylase, and S-cortisol in addition to P_{Osmol}. Decreased SFR (overall mean change 36%; $p < 0.001$ vs. pre-competition), S-IgA (overall mean change 33%; $p < 0.001$ vs. pre-competition) and S-lysozyme (overall mean change 41%; $p = 0.015$ vs. pre-competition) secretion rates, and increased S-α-amylase secretion rates (overall mean change 92%; $p < 0.001$ vs. pre-competition) and S-cortisol responses (overall mean change 71%; $p < 0.001$ vs. pre-competition) were observed post-competition in UER; with no changes being observed in CON. No incidences of URS were reported by participants. A 24 h continuous overnight ultra-marathon resulted in the depression of some S-AMP responses, but no incidences of URS were evident during or following competition. S-AMP synergism, effective management of non-infectious episodes, maintaining euhydration, and (or) favourable environmental influences could have accounted for the low prevalence of URS.

5.2 Introduction

Taking into account the extreme nature of ultra-endurance events, oral-respiratory mucosal immune status may be compromised to a greater extent compared with shorter bouts of endurance exercise reporting high incidence of URS (Linde 1987, Nieman *et al.* 1990). To date, the impact of a 24 h continuous ultra-marathon on oral-respiratory mucosal immunity has not been determined. The unique characteristics of ultra-endurance running events expose participants to a combination of physiological stressors known to perturb oral-respiratory immune status, through neuroendocrine (i.e., stress hormones) regulated salivary activity and hydration status. Both of these mechanisms have been shown to contribute towards depressed SFR and S-AMP responses (Chicharro *et al.* 1998, Costa *et al.* 2010, Fortes *et al.* 2012, Oliver *et al.* 2007, Teeuw *et al.* 2004, Tenovuo 1998). Even though previous laboratory-controlled studies have reported modest alterations in SFR and S-IgA responses at rest or in response to physical exertion after an acute period of sleep deprivation with and without energy-nutrient restriction (Costa *et al.* 2010, Costa *et al.* 2008, Costa *et al.* 2005, Oliver *et al.* 2007); exposure to cold T_{amb} (0°C) resulted in a 36% and 24% decrease in S-IgA concentration and secretion rate, respectively (Costa *et al.* 2010). This is on the contrary to heat exposure, in which there is general consensus that S-AMP (i.e., S-IgA) perturbations are no greater when exercising in hot T_{amb} (>30°C) compared with thermoneutral ambient conditions (Laing *et al.* 2005, Walsh and Whitham 2006). It therefore appears that both neuroendocrine (i.e., stress hormones) responses and hydration status are potentially two key factors influencing oral-respiratory mucosal immune status under conditions of physical exertion with accompanying physiological stressors.

With this in mind, the current study aimed to determine S-AMP responses of UER before and immediately after a 24 h continuous overnight ultra-marathon competition, in which participants were also exposed to a period of cold ambient conditions and energy inadequacy. Additionally, in order to assess the possible impact of S-AMPs (or other immune) responses to the ultra-marathon upon URS susceptibility, URS were recorded during the event and monitored daily until four weeks after completion of the event. It was hypothesized that depressed salivary variables (i.e., saliva flow and S-AMP responses) would be evident immediately after competition, leading to a large incidence of URS.

5.3 Methods

Setting and participants. The study was conducted during the 2011 and 2012 Glenmore24 Trail Race (www.glenmore24.com), held during the first week of September, in the Cairngorms National Park, Scottish Highlands, UK (T_{amb} range: 0-20°C, RH range: 54-82%). The 24 h continuous ultra-marathon was conducted on a 6 km looped-course on a variety of terrains; including off-road trails, paths, and grassland. Distance covered by participants ranged between 122-208 km at an estimated average intensity of 7.2 ± 1.2 METs (SenseWear 7.0, Bodymedia, PA, Pittsburgh, USA), and in an altitude averaging 342 m above sea level. After ethical approval, a convenience sampling observational cohort was studied; whereby 25 out of 48 UER entering the event volunteered to participate in the study (male n= 19, female n= 6: age 39 ± 7 y, height 177 ± 8 cm, BM 78 ± 11 kg). Additionally, 17 individuals who did not compete (absence of exercise stress), volunteered to participate in the study as part of the CON group (male n= 6, female n= 11: age 32 ± 11 y, height 170 ± 10 cm, BM 69 ± 13 kg), for comparative purposes. All participants reported no illness and (or) infection in the twelve weeks leading up to the ultra-marathon.

Study design and data collection. Within the hour prior to commencement of competition (11:00-12:00 h), BM measurements were taken, as previously described **(Section 3.2)**. Participants were then required to sit in an upright position for 10 min before whole blood and 2 min saliva samples were collected, as previously described **(Section 3.5 and Section 3.6)**, for all participants (UER and CON). BM was re-measured in those participants who needed to urinate prior to the start of competition. Immediately after competition (12:00 h the following day) and before any foods or fluids could be consumed, BM was measured, followed immediately by blood and saliva sampling, identical to pre-competition procedures.

All measurements and samples were collected for all participants (UER and CON) within 1 h after completing the ultra-marathon (12:00-13:00 h). Also, within 1 h following competition, trained dietetic researchers conducted a standardised structured interview on participants to ascertain total foods and fluids ingested during the ultra-marathon. Participants were also educated and instructed to complete a validated URS log (Wisconsin Upper Respiratory Symptom Survey, St. Madison, WI, USA) for the ultra-marathon period and daily until four weeks after event completion.

Dietary analysis and hydration status. Dietary analysis and hydration status were determined as previously described in **Section 3.3.** Energy expenditure was measured by a triaxial accelerometer, which also included measurements of heat flux, skin temperature and galvanic skin responses (SenseWear 7.0, Bodymedia, PA, Pittsburgh, USA). Pre- and post-competition P_{Osmol} was determined as previously described in **Section 3.3.** The mean CV for energy, macronutrient, and water variables analysed was 0.8%, 1.4%, and 0.5%, respectively. The CV for P_{Osmol} was 3.5%.

Saliva sample analysis. Unstimulated whole saliva samples were analysed for S-IgA, S-α-amylase, S-lysozyme, and S-cortisol as previously described in **Section 3.5.**

Data analysis. Data in text are presented as mean ± SD. For clarity, data in figures are presented as mean ± SEM. Due to the commonly large individual variation in salivary variables (S-AMP responses) (Gleeson and Pyne 2000, Walsh *et al.* 2011a), data are presented as mean and 95% confidence interval (CI) for mean (lower and upper boundary). Data were processed and analysed in SPSS for Windows (SPSS v.17.0.2, Illinois, US). Diagnostic checks (Shapiro-Wilks test of normality and Levene's homogeneity of variance

test) were performed prior to analysis. Where data violated the assumption of normality, data were log transformed, and the transformed data was analysed using parametric statistics, verified against non-parametric equivalents where appropriate. Paired-sample t-tests were applied to determine pre- to post-competition variable differences; while independent-sample t-tests were applied for group (UER *vs.* CON) and sub-group comparisons (pre- to post-competition variable changes for running distance (<160 km *vs.* ≥160 km)) (Pacque *et al.* 2007, Peters and Bateman 1983) and hydration status (EH (≤1.5% exercise-induced BM loss) *vs.* HH (>3.0% exercise-induced BM loss)) (Fortes *et al.* 2012). The acceptance level of significance was set at p< 0.05. Descriptive statistics were used to explore URS (percentage of participants experiencing URS in accordance with the Wisconsin Upper Respiratory Symptom Survey).

5.4 Results

Energy balance and hydration status. Total energy intake and energy expenditure over the 24 h period was 20 ± 11 MJ and 55 ± 11 MJ for UER, and 12 ± 1 MJ and 14 ± 5 MJ for CON, respectively. Total fluid ingestion over the 24 h period was 9.1 ± 4.0 l for UER, and 2.1 ± 0.4 l for CON. While rate of fluid ingestion was 378 ± 164 ml·h^{-1} for UER. Significant BM loss occurred pre- to post-competition ($p= 0.001$) in UER (pre-competition: 78.2 ± 11.5 kg, post-competition: 77.0 ± 11.7 kg; $1.6 \pm 2.0\%$). No significant changes in P$_{Osmol}$ were observed pre- to post-competition in UER (pre-competition: 285 ± 11 mOsmol·kg^{-1}, post-competition: 287 ± 10 mOsmol·kg^{-1}) and remained within normal clinical reference range (280-300 mOsmol·kg^{-1}) (Thomas *et al.* 2008). Compared with CON, P$_{Osmol}$ pre- and post-competition was lower in UER ($p= 0.05$ and $p< 0.001$, respectively).

Saliva flow rate and salivary anti-microbial protein responses. Decreased SFR (overall mean change: 36%; $p= 0.001$ *vs.* pre-competition) was observed post-competition in 88% of sampled UER, with no significant change in CON evident (**Figure 5.1**). No significant differences in SFR pre- to post-competition were observed for sub-group comparisons.

Figure 5.1: Saliva flow rate in ultra-endurance runners before and immediately after a 24 h continuous overnight ultra-marathon competition. Mean ± SEM: UER (closed squares; $n=$ 25) and CON (open squares; $n=$ 17). ** $p<0.01$ *vs.* pre-competition.
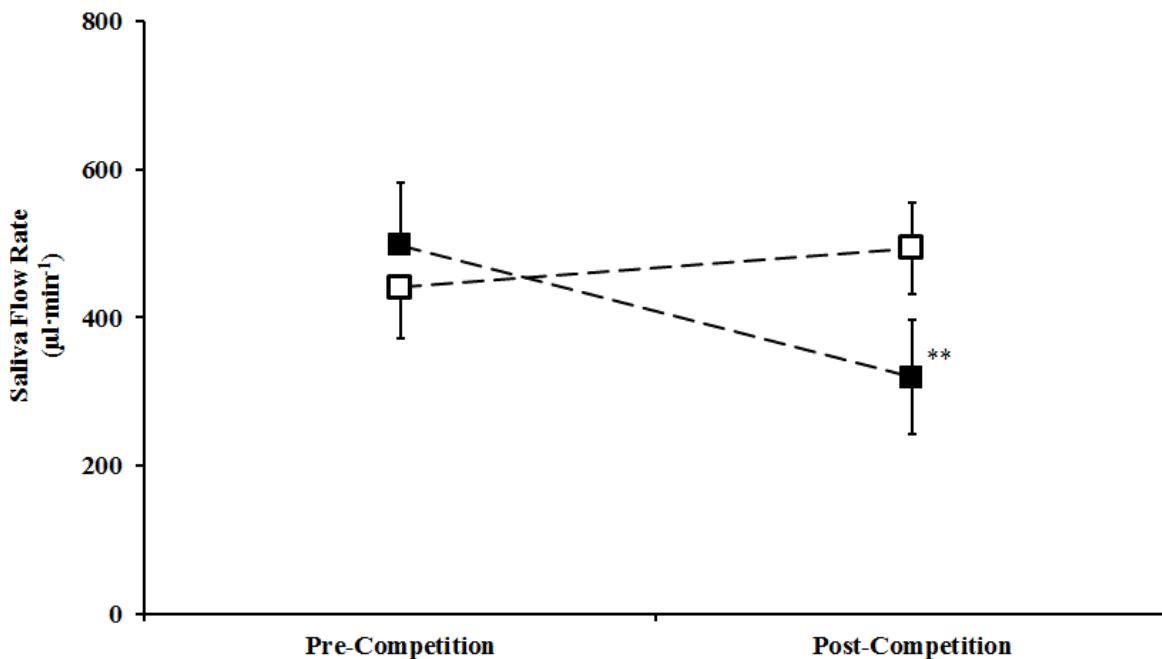
A pre- to post-competition increase in S-IgA concentration (overall mean change: 34%) was observed in UER ($p=$ 0.077; **Table 5.1**), but failed to reach significance. Decreased S-IgA secretion rate (overall mean change: 33%; $p<0.001$ *vs.* pre-competition) was observed post-competition in 84% of sampled UER. No significant differences in pre- to post-competition S-IgA concentration and secretion rate were observed in CON, nor observed for sub-group comparisons.

Increased S-α-amylase concentration (overall mean change: 85%) was observed post-competition in UER ($p=$ 0.001 *vs.* pre-competition; **Table 5.1**). Similarly, increased S-α-amylase secretion rate (overall mean change: 92%) was observed post-competition in 90% of sampled UER ($p<0.001$ *vs.* pre-competition). No significant differences in pre- to post-

competition S-α-amylase concentration and secretion rate were observed in CON, nor observed for sub-group comparisons.

A pre- to post-competition increase in S-lysozyme concentration (overall mean change: 98%) was observed in UER ($p= 0.085$; **Table 5.1**), but failed to reach significance. Decreased S-lysozyme secretion rate (overall mean change: 41%) was observed post-competition in 73% of sampled UER ($p= 0.015$ *vs.* pre-competition). No significant differences in pre- to post-competition S-lysozyme concentration and secretion rate were observed in CON, nor observed for sub-group comparisons.

Table 5.1: Salivary antimicrobial protein responses in ultra-endurance runners before and immediately after a 24 h continuous overnight ultra-marathon competition.

	Pre-Competition	Post-Competition
Salivary IgA concentration ($\mu g \cdot ml^{-1}$)		
UER	193 (136-249)	258 (166-350)[†]
CON	202 (104-298)	204 (74-332)
Salivary IgA secretion rate ($\mu g \cdot min^{-1}$)		
UER	91 (52-130)	61 (36-85)[**]
CON	95 (44-144)	74 (30-117)
Salivary α-amylase concentration ($U \cdot ml^{-1}$)		
UER	35 (17-55)	70 (45-99)[**aa]
CON	34 (19-48)	27 (16-37)
Salivary α-amylase secretion rate ($U \cdot min^{-1}$)		
UER	17 (8-24)	33 (15-46)[**aa]
CON	12 (5-19)	11 (6-16)
Salivary lysozyme concentration ($\mu g \cdot ml^{-1}$)		
UER	4.9 (2.6-7.6)	9.7 (2.9-15.9)[‡a]
CON	4.8 (3.1-7.2)	3.2 (1.6-5.2)
Salivary lysozyme secretion rate ($\mu g \cdot min^{-1}$)		
UER	3.4 (2.0-5.0)[a]	2.0 (0.7-3.3)[**b]
CON	1.4 (0.6-2.2)	1.0 (0.4-1.5)

Mean and 95% CI (lower and upper bound): UER ($n= 25$) and CON ($n= 17$). ** $p< 0.01$ *vs.* pre-competition, † $p= 0.077$ and ‡ $p= 0.085$ *vs.* pre-competition, [a] $p< 0.05$ and [aa] $p< 0.01$ *vs.* CON, [b] $p= 0.089$ *vs.* CON.

Salivary cortisol responses. Increased S-cortisol concentration (overall mean change: 71%; $p < 0.001$ *vs.* pre-competition) was observed post-competition in UER (**Figure 5.2).** A 66% increase in pre- to post-competition S-cortisol appearance rate was observed in UER, but failed to reach significance ($p = 0.164$). No significant differences in pre- to post-competition S-cortisol concentration and appearance rate were observed in CON, nor observed for sub-group comparisons.

Figure 5.2: Salivary cortisol concentration (A) and appearance rate (B) in ultra-endurance runners before and immediately after a 24 h continuous overnight ultra-marathon competition. Mean ± SEM: UER (closed squares; *n*= 25) and CON (open squares; *n*= 17). ** *p*< 0.01 *vs.* pre-competition, [a] *p*< 0.05 and [aa] *p*< 0.01 *vs.* CON, [b] *p*= 0.088 *vs.* CON.
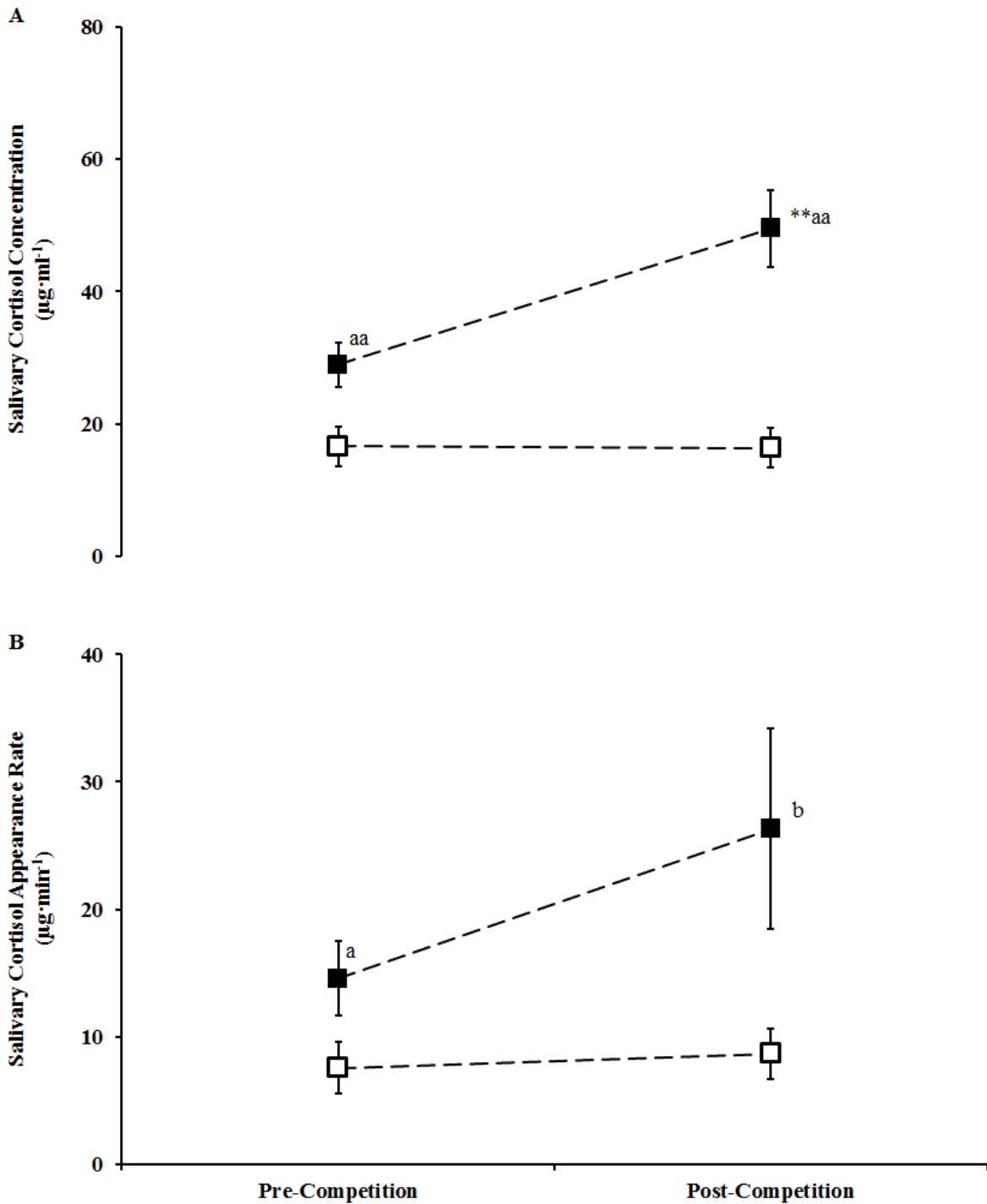
Upper respiratory symptoms. In accordance with the Wisconsin Upper Respiratory Symptom Survey, no (0%) URS *per se* were reported by UER and CON during the ultra-marathon. However, sub-symptoms were acknowledged by UER, whereby $n= 1$ UER reported fever, $n= 2$ UER reported cold-flu like symptoms, and $n= 4$ UER reported headache during the event. Additionally, no (0%) URS and sub-symptoms were reported by UER and CON in the four weeks after completion of the 24 h ultra-marathon.

5.5 Discussion

In accordance with the hypothesis, irrespective of distance covered, results show reductions in S-IgA and S-lysozyme secretion rates post-competition; whereas in contrast to the hypothesis S-α-amylase responses increased. Despite exposure to multiple physiological stressors and unfavorable reductions in some S-AMP responses, a novel finding was that no URS were reported by UER during and four weeks following the ultra-marathon.

Compared with the current study, greater depressions in S-AMP responses (i.e., S-IgA), have previously been observed after 82 km (50%) and 160 km (57%) ultra-marathons (Nieman *et al.* 2003, Pacque *et al.* 2007), although hydration status was not accounted for in these studies. Given that the majority of previous studies have solely reported S-IgA responses after exercise-stress, collectively determining S-AMPs may provide a clearer interpretation of oral-respiratory mucosal immune status (Costa *et al.* 2012). For example, the increased S-IgA, S-α-amylase, and S-lysozyme concentrations post-competition likely reflected a concentrating effect of reduced SFR (Costa *et al.* 2012, Costa *et al.* 2009). Whereas, even though reduced secretion rates of S-IgA and S-lysozyme were observed post-competition, a compensatory level of mucosal protection may have been provided (increased S-α-amylase secretion rate), irrespective of distance covered.

In the current study, amplified S-cortisol concentrations post-competition, suggesting raised HPA activation, possibly attributed to the combination of extreme physical exertion and energy inadequacy (Costa *et al.* 2005), and not necessarily sleep deprivation (Costa *et al.* 2010), likely played a significant role in the modest depressions in salivary variables observed (Allgrove *et al.* 2008, Bosch *et al.* 2003b, Costa *et al.* 2005).

Contrary to previous field-based studies, showing high incidence of URS after prolonged running (Heath *et al.* 1991, Linde 1987, Nieman *et al.* 1990, Peters and Bateman 1983), commonly attributed to the reductions in S-IgA responses (Costa *et al.* 2005, Gleeson and Pyne 2000, Neville, Gleeson, and Folland 2008), more recent ultra-marathon studies have reported minimal incidence of URS (Pacque *et al.* 2007). In agreement with these studies, despite the extreme physical exertion and exposure to multiple physiological stressors known to perturb immune function (Walsh *et al.* 2011a, Walsh and Whitham 2006), low incidences of URS were reported during and up to four weeks following competition (**Chapter 4**).

In conclusion, a 24 h continuous overnight ultra-marathon resulted in the modest depressions of some S-AMP responses, but no incidences of URS were evident during or following competition. Given that S-AMPs work synergistically to protect the URT against pathogen adhesion, replication, and invasion; the counteractive effect observed in S-AMP responses may have offered some level of oral-respiratory mucosal immune protection.

Circulatory Endotoxin Concentrations and Cytokine Profile During a Multi-Stage Ultra-Marathon Competition Conducted in Hot Ambient Conditions.

Gill, S. K., Teixeira, A., Rama, L., Rosado, F., Hankey, J., Scheer, V., Hemmings, K., Ansley-Robson, P., a
Costa, R. J. S. (2015) 'Circulatory Endotoxin Concentration and Cytokine Profile in Response to Exertional-
Heat Stress During a Multi-Stage Ultra-Marathon Competition'. *Exercise Immunology Review* 21, 114-128

6.1 Summary: Exertional-heat stress has the potential to disturb intestinal integrity, leading to enhanced permeability of enteric pathogenic microorganisms and associated clinical manifestations. The study aimed to determine circulatory endotoxin concentration and cytokine profile of UER ($n= 19$) and a control group (CON, $n= 12$) during a 230 km multi-stage ultra-marathon (mean ± SD: 27 h 38 min ± 3 h 55 min) conducted in hot ambient conditions (32-40ºC) and to explore the relationship between these responses with severe GI symptoms and perceptive thermal tolerance rating. BM and T_{tymp} were measured, and venous blood samples were taken before (pre-stage) and immediately after (post-stage) each stage of the ultra-marathon. Samples were analysed for gram-negative bacterial endotoxin, CRP, cytokine profile (IL-6, IL-1β, TNF-α, IFN-γ, IL-10, and IL-1ra), and P_{Osmol}. GI symptoms and perceptive thermal tolerance rating were also monitored throughout competition. Mean exercise-induced BM loss over the five stages ranged 1.0% to 2.5%. Pre- and post-stage P_{Osmol} in UER ranged 277-282 mOsmol·kg and 286-297 mOsmol·kg, respectively. Pre-stage concentrations of endotoxin (peak: 21% at Stage 5), CRP (889% at Stage 3), IL-6 (152% at Stage 2), IL-1β (95% at Stage 5), TNF-α (168% at Stage 5), IFN-γ (102% at Stage 5), IL-10 (1271% at Stage 3), and IL-1ra (106% at Stage 5) increased as the ultra-marathon progressed in UER; while no changes in CON were observed (except for IL-1β; 71%). Pre- to post-stage increases were observed for endotoxin (peak: 22% at Stage 3), CRP (25% at Stage 1), IL-6 (238% at Stage 1), IL-1β (64% at Stage 1), TNF-α (101% at Stage 1), IFN-γ (39% at Stage 1), IL-10 (1100% at Stage 1), and IL-1ra (207% at Stage 1) concentrations in UER. 58% of

UER reported at least one severe GI symptom (including 33% of sampled UER reporting nausea) during competition, while no GI symptoms were reported by CON. Multi-stage ultra-marathon competition in the heat resulted in a modest circulatory endotoxaemia accompanied by a pronounced pro-inflammatory cytokinaemia by post-Stage 1, both of which were sustained throughout competition at rest (pre-stage) and after stage completion. Compensatory anti-inflammatory responses and other external factors (i.e., training status, cooling strategies, heat acclimatization, nutrition and hydration) may have contributed towards limiting the extent of pro-inflammatory responses in the current scenario.

6.2 Introduction

Perturbations to intestinal epithelial integrity during EHS have been linked to increased intestinal permeability of gram-negative bacterial endotoxins, leading to endotoxaemia and responsive cytokinaemia (Camus *et al.* 1998, Camus *et al.* 1997). Endotoxin-induced cytokinaemia has previously been implicated in the aetiology of heat-related illness (Lim and Mackinnon 2006, Opal 2010). Taking into account that previous research has predominantly focused on single bouts of prolonged physical exertion with and without heat-stress, to date, it is still unclear the extent to which consecutive days of EHS may impact on circulatory endotoxin and cytokine responses along the duration of exposure.

With this in mind, the aims of the current study were to: 1) determine circulatory endotoxin concentration and cytokine profile of UER throughout a five days (five stages) multi-stage ultra-marathon competition conducted in hot ambient conditions; 2) determine the relationship between these responses with those individuals reporting severe GI symptoms, and perceptive thermal tolerance rating; and 3) determine if gender, fitness level, and hydration status influence responses. Taking into account the nature of the event, it was hypothesised that a progressive endotoxaemia would be seen along the ultra-marathon, and a cytokinaemic response would mirror the endotoxaemia. It was also hypothesised that correlations between circulatory responses with severe GI symptoms (positive) and perceptive thermal tolerance rating (negative) would be seen. Additionally, it was hypothesised that no difference in responses would be seen between genders, faster runners with higher fitness levels would show lower responses, and a state of hypohydration would result in greater responses compared with euhydration.

6.3 Methods

Setting. The study was conducted during the 2011 Al Andalus Ultimate Trail (www.alandalus-ut.com), held during the 2nd week of July, in the region of Loja, Spain. The multi-stage ultra-marathon was conducted over five stages (five days) totalling a distance of 230 km (Stage 1: 37 km, Stage 2: 48 km, Stage 3: 38 km, Stage 4: 69 km, and Stage 5: 38 km, respectively), and performed on a variety of terrains; predominantly off-road trails and paths, but also included steep and narrow mountain passes, and occasional road. Mean running intensity was (mean ± SD) 7.5 ± 0.5 METs (SenseWear 7.0, BodyMedia Inc., Pittsburgh, PA, USA). Sleeping arrangements from Stages 1 to 5 included a combination of outdoor tent and village sports hall accommodation (mean sleep duration per night was 8 h 18 min ± 1 h 22 min) (Pittsburgh Sleep Diary) (Monk *et al.* 1994). Moreover, the run course was routed over an altitude ranging between 473 to 1443 m above sea level (Garmin International, Olathe, Kansas, US). Average daytime maximum T_{amb} during the running stages ranged between 32-40°C, with maximum daytime RH also ranging between 32-40% (Garmin International, Olathe, Kansas, US).

Participants. After ethical approval, a convenience sampling observational cohort was studied, whereby 19 out of 69 UER who entered this ultra-marathon competition volunteered to participate in the study [UER (Male *n*= 13, Female *n*= 6): age 45 ± 6 y, height 1.71 ± 0.05 m, BM 69.0 ± 7.0 kg, body fat mass 17.5 ± 4%]. For comparative purposes, 12 matched individuals who accompanied the UER along the ultra-marathon course, but did not compete (absence of exercise stress), volunteered to participate in the study as part of the CON group [(Male *n*= 5, Female *n*= 7): age 35 ± 13 y, height 1.67 ± 0.09 m, BM 69.9 ± 16.2 kg, body fat mass 21 ± 6%]. All participants arrived at location ≤48 h prior to the start of Stage 1. Only *n*=

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2 of participants resided in countries with hot ambient conditions similar to those of the race location (≥30°C T_{amb}) at the time of competition; the remaining participants resided in countries with cold or thermoneutral ambient conditions (≤20°C). No participant reported any incidence of illness and (or) infection in the twelve weeks leading up to the ultra-marathon.

Oral anti-inflammatory agents. The use of non-steroidal anti-inflammatory drugs (NSAIDs) and other anti-inflammatory agents amongst UER included: paracetamol (500-1000mg), ibuprofen (400mg), cocodamol (500-1000mg), Celebrex (200mg), and fish oils (1-2 capsules). Anti-inflammatory agent use by UER ranged from n= 2 on Stage 1 to n= 6 on Stage 5. Of the total number of participants, n= 13 did not use any form of oral anti-inflammatory agents throughout the ultra-marathon.

Study design and data collection. Following participant recruitment and informed consent, preliminary measures were taken to determine participant characteristics (**Section 3.2**). The current ultra-marathon was semi self-sufficient, whereby participants (including CON) planned and provided their own foods and fluids (except plain water) along the five days of competition. Participants' equipment and sustenance was transported to each stage section by the race organisation. Only plain water was provided by the race organisers *ad libitum* during the rest phase throughout competition. Additionally, aid stations along the running phase of competition were situated approximately 10 km apart, and only provided plain water, fruit (oranges and watermelon), and electrolyte supplementation (Elete electrolyte add-in, Mineral Resources International**,** South Ogden, Utah, US). Participants were advised to adhere to their programmed habitual dietary practices throughout the entire competition.

Each day, for five consecutive days, running stages commenced at either 08:00 or 09:00 h. Within the hour prior to the start of each running stage, pre-stage measurements were determined and samples collected. BM measurements were taken, as previously described **(Section 3.2).** Participants were then required to sit in an upright position for 10 min before T_{tymp} was determined, as previously described **(Section 3.4)** and whole blood samples collected, as previously described **(Section 3.6).** All measurement techniques and samples were consistently conducted and collected in a large partitioned research field tent (four sections, 3 m x 3 m) or sports hall facility. BM was re-measured in those participants who needed to urinate prior to the stage start. Immediately post-stage and before any foods or fluids could be consumed, BM was measured, followed by blood sampling, as previously described (**Section 3.6**). For consistency, the order, positioning and technique of measurements and sampling were similar pre- and post-stage for all stages, and were taken by the same trained researcher throughout.

At the end of each competition day (20:00 to 22:00 h) on Stages 1 to 4, trained dietetic researchers conducted a standardised structured interview (dietary recall interview technique) on participants to ascertain total daily food and fluid ingestion. To avoid inter-observer variations, each trained researcher conducted the interview on the same participant throughout the entire ultra-marathon. Severe GI symptoms (Pfeiffer *et al.* 2012) and thermal tolerance rating through a Likert scale (-3 very poor to +3 very good, with 0 being a neutral response) (Hollies and Goldman 1977: 107) were also explored at this time through a research-generated symptomology tool. Exertional heat illness symptoms were verified by a qualified Sports Physician.

Dietary analysis and hydration status. Dietary analysis and hydration status were determined as previously described in **Section 3.3.** The mean CV for energy, macronutrient, and water variables analysed was 1.3%, 2.3%, and 0.7%, respectively. The CV for P_{Osmol} was 3.5%.

Blood sample analysis. Whole blood samples were analysed for circulatory concentrations of CRP, cytokines (IL-6, TNF-α, IL-1β, IFN-γ, IL-10, and IL-1ra) and gram-negative bacterial endotoxin, as previously described in **Section 3.6.** In CON, blood-borne indices were determined on pre-Stages 1, 3 and 5 only.

Data analysis. Data in text (overall mean value otherwise specified) and tables are presented as mean ± SD. Due to commonly large individual variation in immunological responses to exercise (Walsh *et al.* 2011), data in figures are presented as mean and individual participant responses. A one-way ANOVA was applied to determine differences in variables between stages. Whereas, a two-way ANOVA was applied to determine differences between groups [UER vs CON, genders (total and BM corrected values), oral anti-inflammatory agent administration, running speed (slow runners (SR, *n*= 11), who completed the entire distance of the ultra-marathon using a mixture of walking and running (overall mean speed <8 km·h), and fast runners (FR, *n*= 8), who completed the majority of the ultra-marathon distance running (overall mean speed ≥8 km·h))], and between pre- and post-stage values within stages (SPSS v.20, Illinois, US). All data were checked for normal distribution by calculating skewness and kurtosis co-efficients. Where data violated the assumption of normality, data were log transformed, and the transformed data was used in analysis. Significant main effects were analysed using post hoc Tukey's HSD tests. For comparative purposes, an independent-sample t-test with nonparametric verification was used to assess post-stage endotoxin and cytokine responses between hydration status (euhydrated (EH) runners (mean ≤1.5% exercise-induced BM loss) and hypohydrated (HH) runners (mean >3.0% exercise-induced

BM loss)). Pearson's coefficient correlation analysis was used to assess associations between endotoxin with CRP and cytokine profile. Spearman's rank correlation analysis was used to assess associations between blood-borne variables with self-reported GI symptoms and perceptive thermal tolerance rating. Individual participant ratios between IL-1β and TNF-α with IL-10 were calculated to determine the pro- to anti-inflammatory cytokines balance. The acceptance level of significance was set at $p < 0.05$.

6.4 Results

Energy, macronutrient, and water ingestion. No difference in daily energy (16.0 ± 3.0 MJ·day) and macronutrient [protein: 1.5 ± 0.4 g·kgBM·day (12% of energy intake), carbohydrate: 7.5 ± 1.6 g·kgBM·day (62% of energy intake), fat: 1.4 ± 0.5 g·kgBM·day (26% of energy intake)] intakes were seen between stages in UER and CON (11.4 ± 4.4 MJ·day; 10% protein, 67% carbohydrate, 23% fat). Total daily energy intake was higher in UER on Stage 1 and Stage 4 compared with CON ($p < 0.001$). Overall rate of carbohydrate intake during running was 28 ± 12 g·h in UER, with no difference seen between stages. No difference in total daily water intake through foods and fluids was seen between stages in UER (7.3 ± 1.7 l·day) and CON (3.3 ± 0.2 l·day). Total daily water intake through foods and fluids was higher in UER on all stages compared with CON ($p < 0.001$). Total and rate of water intake through foods and fluids during running did not differ between stages in UER (4.2 ± 1.5 l, 775 ± 248 ml·h; respectively).

Body Mass, *plasma osmolality, and plasma volume change.* Pre- and post-stage BM did not significantly alter throughout competition in UER (pre-Stage 1: 71.7 ± 9.5 kg to pre-Stage 5: 71.2 ± 9.2 kg; and post-Stage 1: 69.8 ± 8.9 kg to post-Stage 5: 69.6 ± 9.5 kg) and CON (pre-Stage 1: 67.4 ± 15.0 kg to pre-Stage 5: 67.0 ± 14.8 kg). Stage 1 (2.5%) resulted in a greater exercise-induced BM loss compared with Stages 2 to 5 in UER (2.0%, 1.0%, 2.2%, and 2.2%, respectively; $p < 0.001$). Pre-stage (range: 277 to 282 mOsmol·kg) and post-stage (range: 286 to 297 mOsmol·kg) P_{Osmol} did not differ between stages in UER. Pre-stage P_{Osmol} did not differ from CON throughout the ultra-marathon. Pre- to post-stage increases in P_{Osmol} ($p < 0.001$) were observed on all stages in UER. Relative to pre-Stage 1, resting pre-stage P_V increased significantly ($p < 0.001$) by Stage 2 (7.0 ± 1.4%) and peaked at Stage 5 (22.7 ±

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2.0%) in UER; while no significant change in P_V was observed in CON, relative to pre-Stage 1. UER presented greater P_V change at pre-Stages 3 and 5 compared with CON ($p < 0.001$).

Tympanic temperature. T_{tymp} was within normal range pre- (overall mean: $36.3 \pm 0.4°C$) and post-stage (overall mean: $37.0 \pm 0.3°C$) in UER. Pre-stage T_{tymp} gradually decreased ($p < 0.001$) in UER as the ultra-marathon progressed (pre-Stage 1: $36.5°C$ and pre-Stage 5: $36.0°C$). No change in pre-stage T_{tymp} ($36.7 \pm 0.5°C$) was observed for CON throughout the ultra-marathon. Pre- to post-stage increases ($0.7°C$; $p < 0.001$) in T_{tymp} were also observed in UER throughout the ultra-marathon. No differences in T_{tymp} were observed for sub-group comparisons (gender, running speed, and hydration status).

Circulatory gram-negative bacterial endotoxin concentration. Pre-stage circulatory endotoxin concentration gradually increased ($p < 0.001$) in UER as the ultra-marathon progressed (**Table 6.1, Figure 6.1A**), peaking at Stage 5 (21%). No change in pre-stage circulatory endotoxin concentration was observed between Stages 1, 3, and 5 for CON. Pre- to post-stage increases ($p = 0.002$) in circulatory endotoxin concentration were also observed in UER throughout the ultra-marathon (**Table 6.1, Figure 6.1B**). No differences in circulatory endotoxin concentration were observed for sub-group comparisons (oral anti-inflammatory agent administration, gender, running speed, and hydration status).

Table 6.1: Circulatory gram-negative bacterial endotoxin concentration, C-reactive protein concentrations, and plasma cytokine profile of a control group and ultra-endurance runners participating in a 230 km multi-stage ultra-marathon competition conducted in a hot ambient environment.

	Stage 1		Stage 2		Stage 3		Stage 4		Stage 5	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Gram-negative endotoxin (EU/ml)										
UER	2.8 ± 0.3	$3.2 \pm 0.8^{\S}$	3.0 ± 0.4	3.1 ± 0.5	2.9 ± 0.4	$3.5 \pm 0.8^{**}$	$3.3 \pm 0.5^{\dagger\dagger}$	$3.6 \pm 0.6^{*}$	$3.4 \pm 0.5^{\dagger\dagger}$	3.6 ± 0.9
CON	3.0 ± 0.2				3.0 ± 0.4				3.1 ± 0.5	
C-reactive protein (μg/ml)										
UER	1.1 ± 1.7	1.6 ± 2.4	$7.4 \pm 5.3^{\dagger\dagger}$	8.8 ± 5.4	$10.0 \pm 5.7^{\dagger\dagger aa}$	9.6 ± 5.9	$9.2 \pm 5.9^{\dagger\dagger}$	$10.0 \pm 6.7^{*}$	$8.8 \pm 5.6^{\dagger\dagger aa}$	$11.0 \pm 6.4^{*}$
CON	1.4 ± 0.7				1.3 ± 0.8				1.3 ± 0.8	
IL-6 (pg/ml)										
UER	8.2 ± 4.5	$27.9 \pm 23.4^{**}$	$20.8 \pm 18.5^{\dagger\dagger}$	20.7 ± 14.8	$20.7 \pm 16.8^{\dagger\dagger aa}$	$25.3 \pm 24.3^{**}$	$19.2 \pm 14.1^{\dagger\dagger}$	$21.7 \pm 12.6^{**}$	$18.2 \pm 11.6^{\dagger\dagger aa}$	$23.4 \pm 13.1^{**}$
CON	7.5 ± 2.5				5.5 ± 7.1				6.5 ± 5.7	
IL-1β (pg/ml)										
UER	0.6 ± 0.3	$1.0 \pm 0.3^{**}$	$1.1 \pm 0.4^{\dagger\dagger}$	1.1 ± 0.4	$1.2 \pm 0.4^{\dagger\dagger}$	1.2 ± 0.4	$1.1 \pm 0.3^{\dagger\dagger}$	$1.4 \pm 0.4^{**}$	$1.2 \pm 0.4^{\dagger\dagger}$	$1.4 \pm 0.4^{*}$
CON	0.7 ± 0.2				$1.2 \pm 0.2^{\dagger}$				$1.3 \pm 0.5^{\dagger\dagger}$	

TNF-α (pg/ml)

UER	3.1 ± 2.9	$6.3 \pm 5.0^{**}$	$6.1 \pm 4.5^{\dagger\dagger}$	6.6 ± 3.7	$6.9 \pm 4.4^{\dagger\dagger aa}$	$6.1 \pm 3.8^{*}$	$6.5 \pm 4.2^{\dagger\dagger}$	$8.1 \pm 4.3^{**}$	$7.1 \pm 3.8^{\dagger\dagger aa}$	8.3 ± 5.0
CON	1.3 ± 0.4				1.8 ± 0.7				2.3 ± 0.7	

IFN-γ (IU/ml)

UER	9.3 ± 5.5	$12.9 \pm 6.0^{**}$	15.2 ± 6.8	16.9 ± 5.7	$16.7 \pm 6.7^{\dagger}$	15.2 ± 5.2	$16.2 \pm 7.2^{\dagger}$	$19.9 \pm 8.3^{**}$	$18.8 \pm 10.0^{\dagger\dagger}$	$22.7 \pm 9.9^{**}$
CON	16.8 ± 5.5				14.3 ± 2.0				16.8 ± 5.1	

IL-10 (pg/ml)

UER	0.7 ± 0.6	$7.9 \pm 10.1^{**}$	$7.0 \pm 10.8^{\dagger\dagger}$	7.9 ± 9.1	$9.0 \pm 10.2^{\dagger\dagger aa}$	8.0 ± 9.4	$9.0 \pm 12.2^{\dagger\dagger}$	9.3 ± 10.1	$8.2 \pm 11.2^{\dagger\dagger aa}$	$10.9 \pm 15.0^{*}$
CON	0.6 ± 0.1				1.4 ± 0.3				1.4 ± 0.7	

IL-1ra (pg/ml)

UER	22.9 ± 8.0	$70.3 \pm 28.1^{**}$	$39.8 \pm 12.4^{\dagger\dagger}$	$61.0 \pm 39.8^{**}$	$45.5 \pm 20.6^{\dagger\dagger aa}$	$53.9 \pm 19.0^{*}$	$37.9 \pm 14.7^{\dagger\dagger}$	$56.3 \pm 30.4^{**}$	$47.1 \pm 22.4^{\dagger\dagger aa}$	$63.2 \pm 28.1^{**}$
CON	23.4 ± 7.3				36.4 ± 9.2				33.1 ± 9.3	

Mean ± SD: ultra-endurance runners (UER, n= 19) and control group (CON, n= 12). †† $p< 0.01$ and † $p< 0.05$ vs pre-Stage 1, ** $p< 0.01$ and * $p< 0.05$ vs respective pre-stage, § $p= 0.058$ vs respective pre-stage, ^{aa} $p< 0.01$ vs CON.

Figure 6.1: Individual changes in pre-stage resting (A) and pre- to post-stage (B) circulatory gram-negative endotoxin concentration of ultra-endurance runners participating in a 230 km multi-stage ultra-marathon competition conducted in a hot ambient environment. Individual ultra-endurance runner responses (●; *n*= 19).
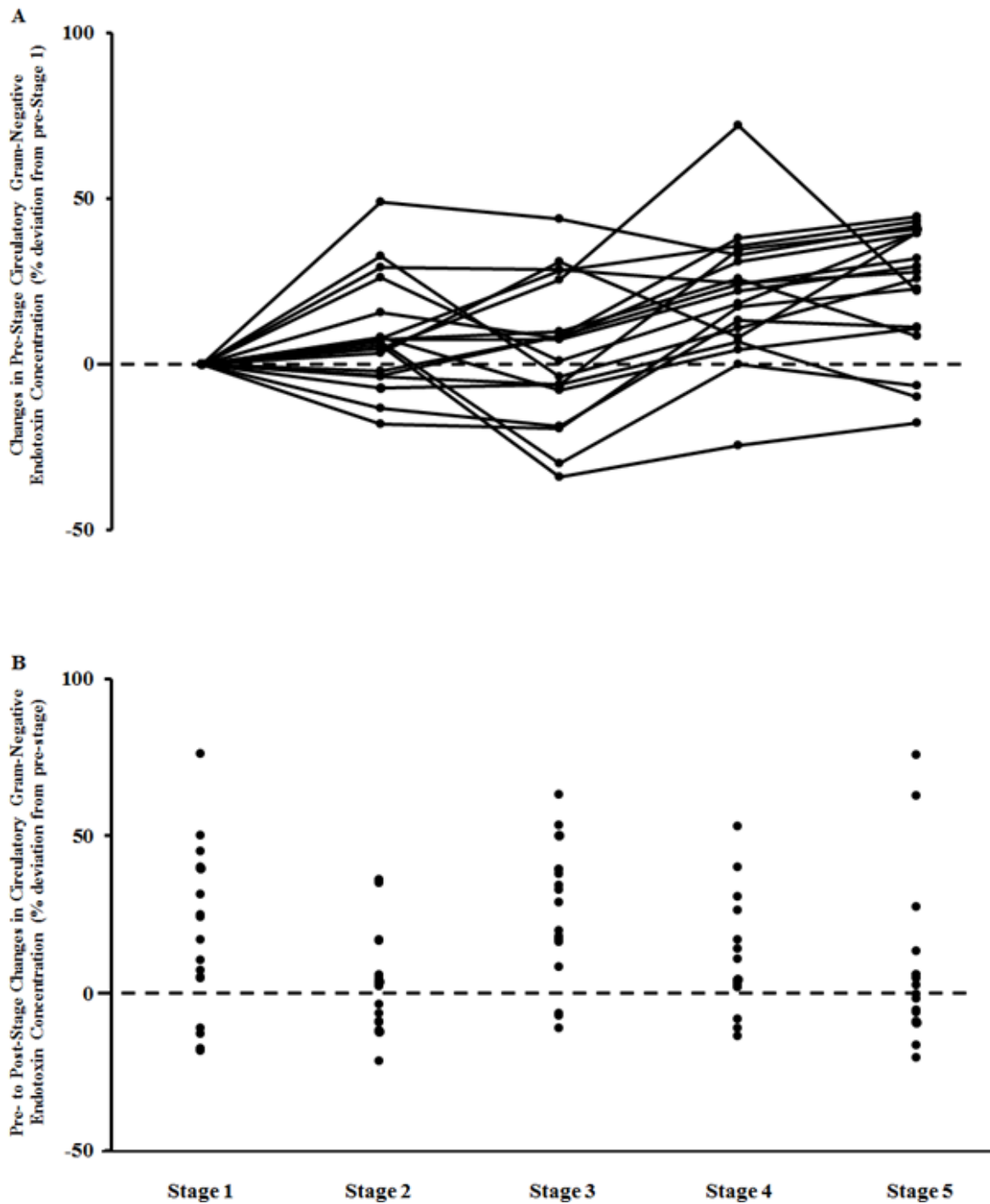
Plasma C-reactive protein concentration. Pre-stage plasma CRP concentration increased ($p<$ 0.001) by Stage 2 in UER, and remained elevated thereafter (**Table 6.1, Figure 6.2A**), peaking at Stage 3 (889%). No change in pre-stage plasma CRP concentration was observed between Stages 1, 3, and 5 for CON, and levels were lower than UER pre-Stages 3 and 5 ($p<$ 0.001). Pre- to post-stage increases ($p<$ 0.05) in plasma CRP concentration were also observed in UER throughout the ultra-marathon (**Table 6.1, Figure 6.2B**). Plasma CRP concentration was observed to be higher ($p<$ 0.001) in males (pre-stage: 8.9 ± 3.6 µg·ml, post-stage: 10.1 ± 4.0 µg·ml) compared with females (pre-stage: 4.2 ± 2.6 µg·ml, post-stage: 4.3 ± 2.5 µg·ml) throughout the ultra-marathon. This difference was also observed when corrected for BM ($p<$ 0.001). No differences in other sub-group comparisons were observed.

Figure 6.2: Individual changes in pre-stage resting (A) and pre- to post-stage (B) plasma C-reactive protein concentration of ultra-endurance runners participating in a 230 km multi-stage ultra-marathon competition conducted in a hot ambient environment. Individual ultra-endurance runner responses (●; $n= 19$). Additional individual responses outside the figure range for pre- to post-stage changes: Stage 1, $n= 1$ at 1827%; Stage 2, $n= 1$ at 429% and $n= 1$ at 620%; and Stage 4, $n= 1$ at 1192%.
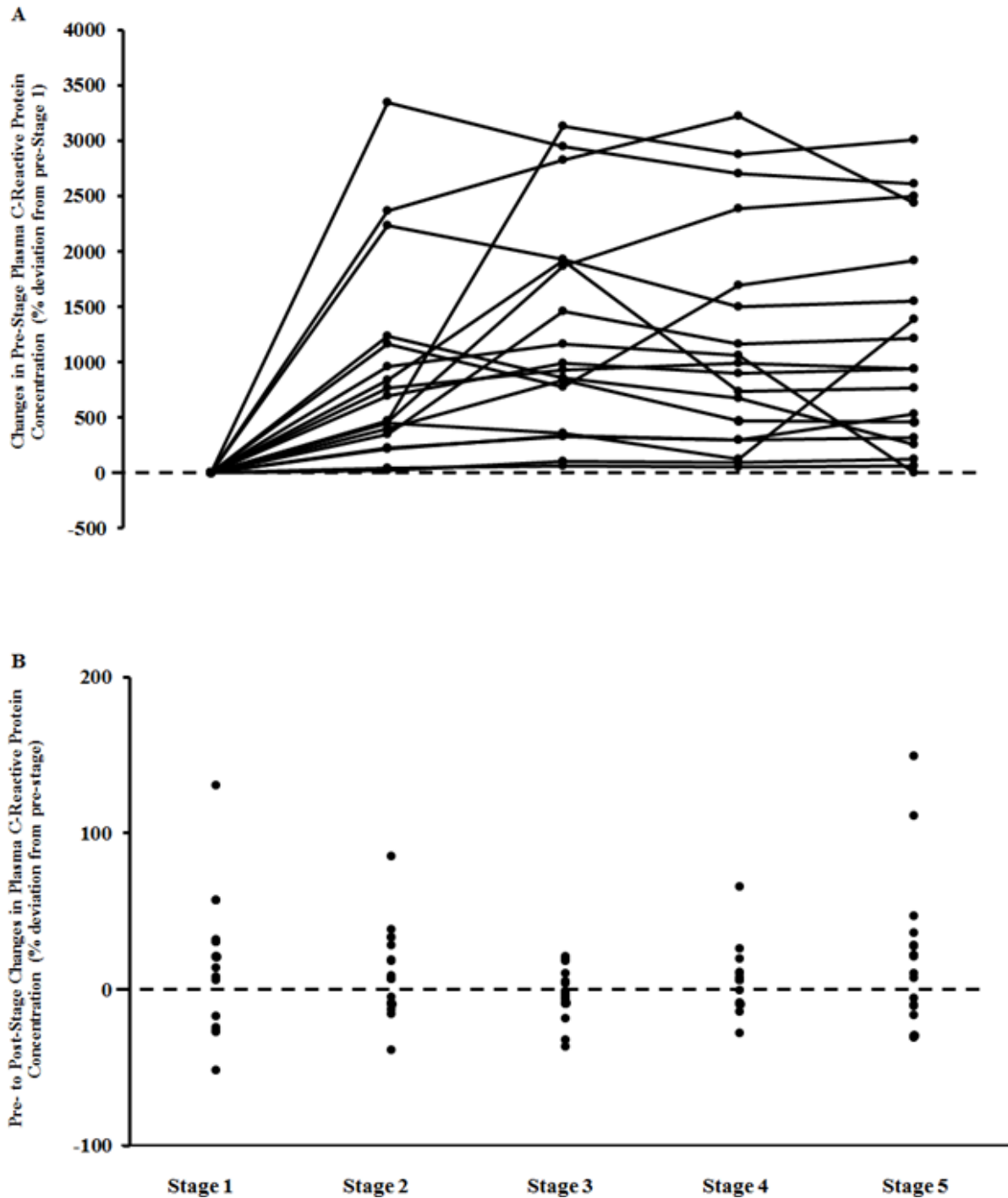
Plasma interleukin-6 concentration. Pre-stage plasma IL-6 concentration increased ($p<$ 0.001) by Stage 2 (152%) in UER, and remained elevated thereafter (**Table 6.1, Figure 6.3A**). No change in pre-stage plasma IL-6 concentration was observed between Stages 1, 3, and 5 for CON, and levels were lower than UER pre-Stages 3 and 5 ($p<$ 0.001). Pre- to post-stage increases ($p<$ 0.001) in plasma IL-6 concentration were also observed in UER (**Table 6.1, Figure 6.3B**). Post-stage plasma IL-6 concentration was observed to be higher ($p=$ 0.054) in males (26.7 ± 20.5 pg·ml) compared with females (17.6 ± 5.7 pg·ml) throughout the ultra-marathon. However, when corrected for BM no substantial difference was observed. There was also a tendency for higher ($p=$ 0.094) pre-stage plasma IL-6 concentration in SR (20.4 ± 15.2 pg·ml) compared with FR (13.3 ± 5.4 pg·ml) throughout the ultra-marathon. Additionally, dehydration status had a tendency to promote higher (42%, $p=$ 0.068) post-stage plasma IL-6 concentrations compared with a euhydrated status. No differences in other sub-group comparisons were observed.

Figure 6.3: Individual changes in pre-stage resting (A) and pre- to post-stage (B) plasma interleukin-6 concentration of ultra-endurance runners participating in a 230 km multi-stage ultra-marathon competition conducted in a hot ambient environmen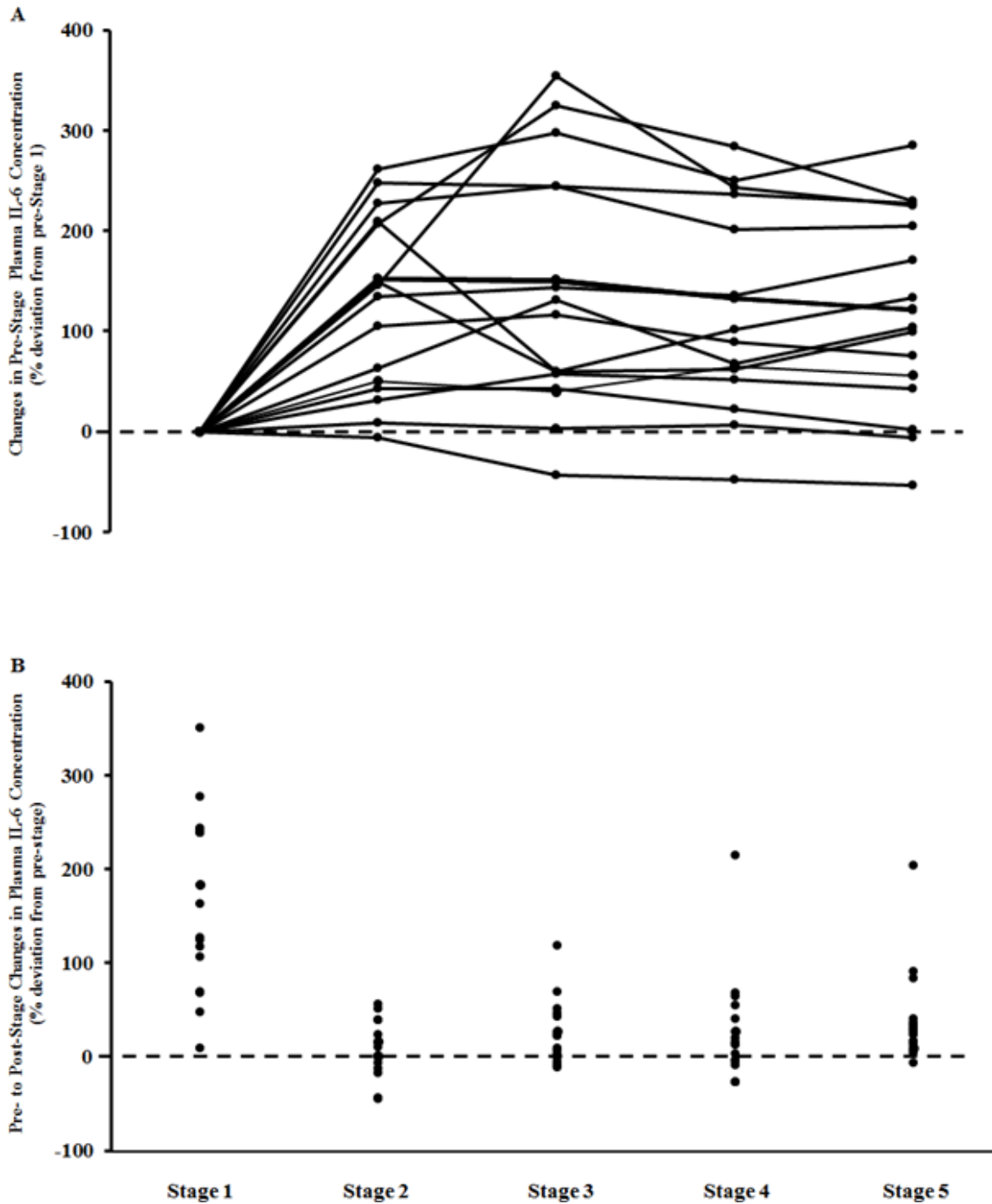t. Individual ultra-endurance runner responses (●; $n= 19$). Additional individual responses outside the figure range for pre- to post-stage changes: Stage 1, $n= 1$ at 605%, $n= 1$ at 704%, and $n= 1$ at 1205%.

Plasma interleukin-1β concentration. Pre-stage plasma IL-1β concentration increased ($p<$ 0.001) by Stage 2 in UER (**Table 6.1, Figure 6.4A**) and remained elevated thereafter, peaking at Stage 5 (95%). While unexpected increases were also observed for CON ($p<$ 0.001), whereby plasma IL-1β concentration increased by Stage 3 and remained elevated thereafter. Pre- to post-stage increases ($p=$ 0.001) in plasma IL-1β concentration were also observed in UER **(Table 6.1, Figure 6.4B**). Pre- and post-stage plasma IL-1β concentration was observed to be lower ($p=$ 0.014) in males (pre-stage: 1.0 ± 0.2 pg·ml, post-stage: 1.1 ± 0.2 pg·ml) compared with females (pre-stage: 1.2 ± 0.4 pg·ml, post-stage: 1.3 ± 0.4 pg·ml) throughout the ultra-marathon. However, when corrected for BM no substantial difference was observed. There was a tendency for higher ($p=$ 0.054) pre-stage plasma IL-1β concentration in SR (1.1 ± 0.2 pg·ml) compared with FR (0.9 ± 0.5 pg·ml) throughout the ultra-marathon. No differences in other sub-group comparisons were observed.

Figure 6.4: Individual changes in pre-stage resting (A) and pre- to post-stage (B) plasma interleukin-1β concentration of ultra-endurance runners participating in a 230 km multi-stage ultra-marathon competition conducted in a hot ambient environment. Individual ultra-endurance runner responses (●; *n*= 19).
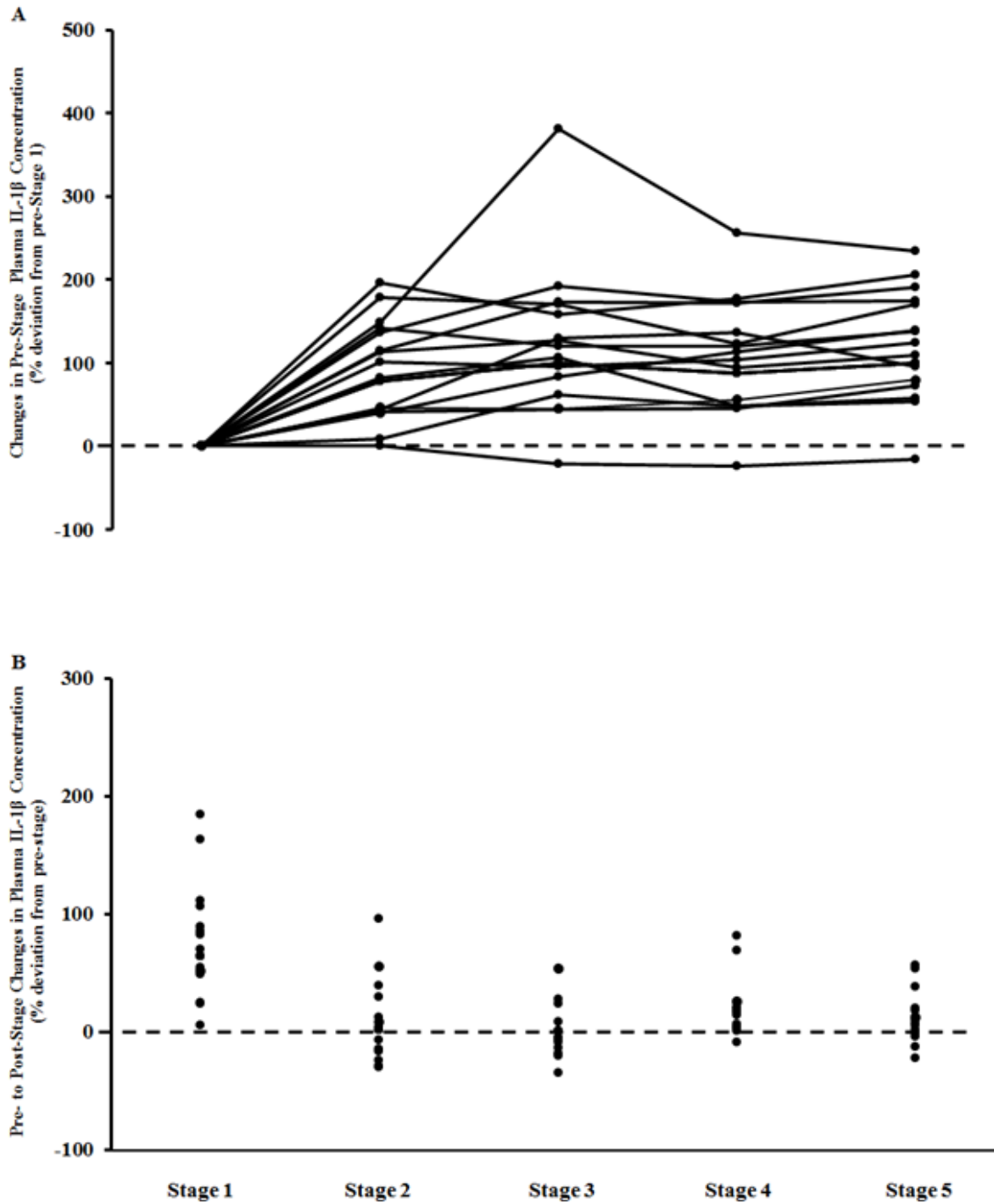
Plasma tumour necrosis factor-α concentration. Pre-stage plasma TNF-α concentration increased ($p < 0.001$) at Stage 2 in UER (**Table 6.1, Figure 6.5A**) and remained elevated thereafter, peaking at Stage 5 (168%). No change in pre-stage plasma TNF-α concentration was observed between Stages 1, 3, and 5 for CON, and levels were lower than UER pre-Stages 3 and 5 ($p < 0.001$). Pre- to post-stage increases ($p = 0.007$) in plasma TNF-α concentration were also observed in UER (**Table 6.1, Figure 6.5B**). Pre- and post-stage plasma TNF-α concentration was observed to be lower ($p < 0.001$) in males (pre-stage: 4.8 ± 1.8 pg·ml, post-stage: 6.1 ± 2.8 pg·ml) compared with females (pre-stage: 8.4 ± 6.1 pg·ml, post-stage: 9.3 ± 6.1 pg·ml) throughout the ultra-marathon. This difference was also observed when corrected for BM (pre-stage: $p < 0.001$, post-stage: $p = 0.001$). Pre-stage plasma TNF-α concentration was observed to be higher ($p = 0.016$) in SR (6.9 ± 1.8 pg·ml) compared with FR (4.7 ± 6.1 pg·ml) throughout the ultra-marathon. No differences in other sub-group comparisons were observed.

Figure 6.5: Individual changes in pre-stage resting (A) and pre- to post-stage (B) plasma tumour necrosis factor-α concentration of ultra-endurance runners participating in a 230 km multi-stage ultra-marathon competition conducted in a hot ambient environment. Individual ultra endurance runner responses (●; $n= 19$).
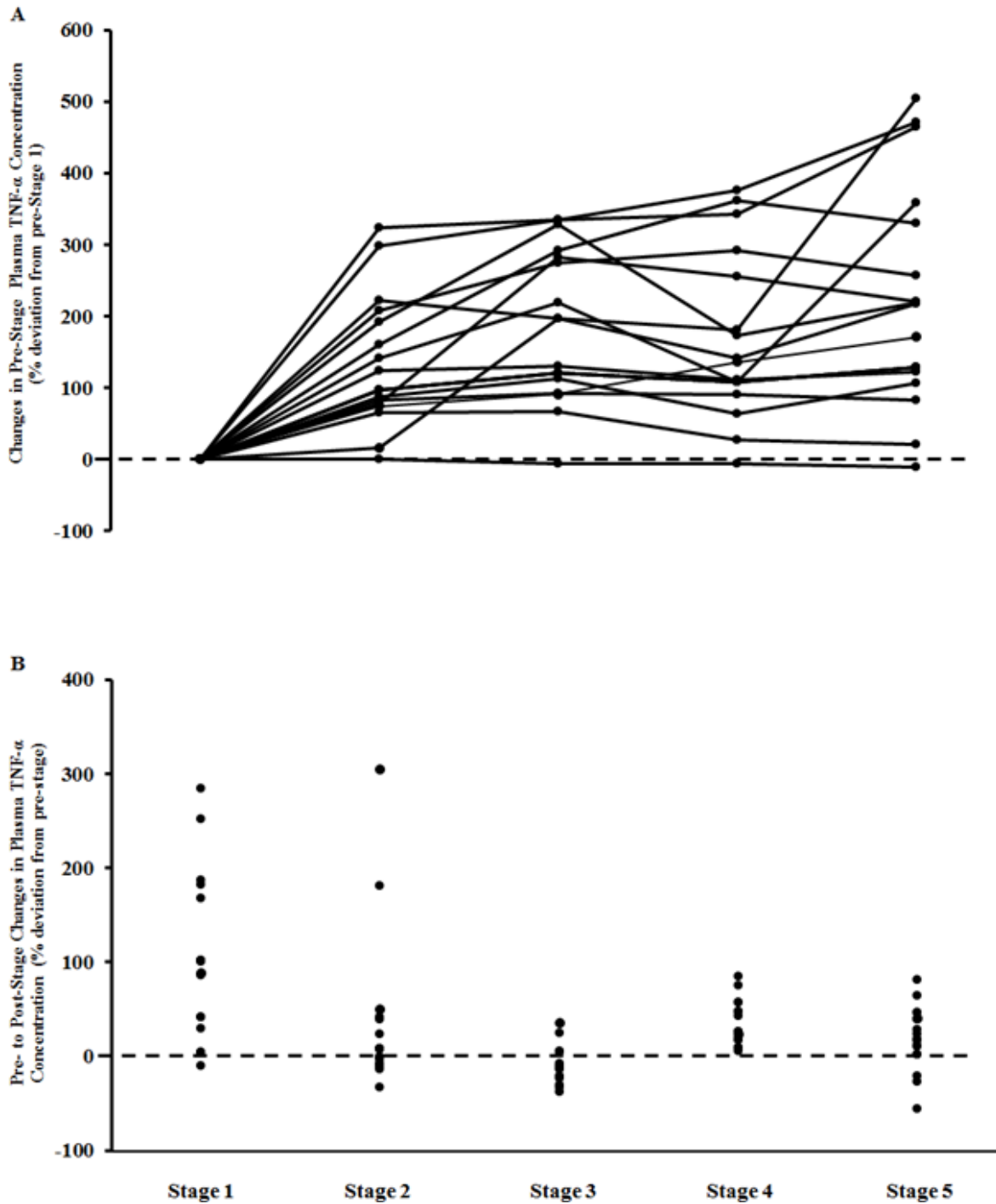
Plasma interferon-γ concentration. Pre-stage plasma IFN-γ concentration increased (*p*= 0.008) at Stage 3 in UER (**Table 6.1, Figure 6.6A**), peaking at Stage 5 (102%). No change in pre-stage plasma IFN-γ concentration was observed for CON. Pre- to post-stage increases (*p*= 0.021) in plasma IFN-γ concentration were also observed in UER (**Table 6.1, Figure 6.6B**). Pre-stage plasma IFN-γ concentration was observed to be higher (*p*= 0.016) in SR (16.7 ± 6.4 IU·ml) compared with FR (13.2 ± 9.2 IU·ml) throughout the ultra-marathon. No differences in other sub-group comparisons were observed.

Figure 6.6: Individual changes in pre-stage resting (A) and pre- to post-stage (B) plasma interferon-γ concentration of ultra-endurance runners participating in a 230 km multi-stage ultra-marathon competition conducted in a hot ambient environmen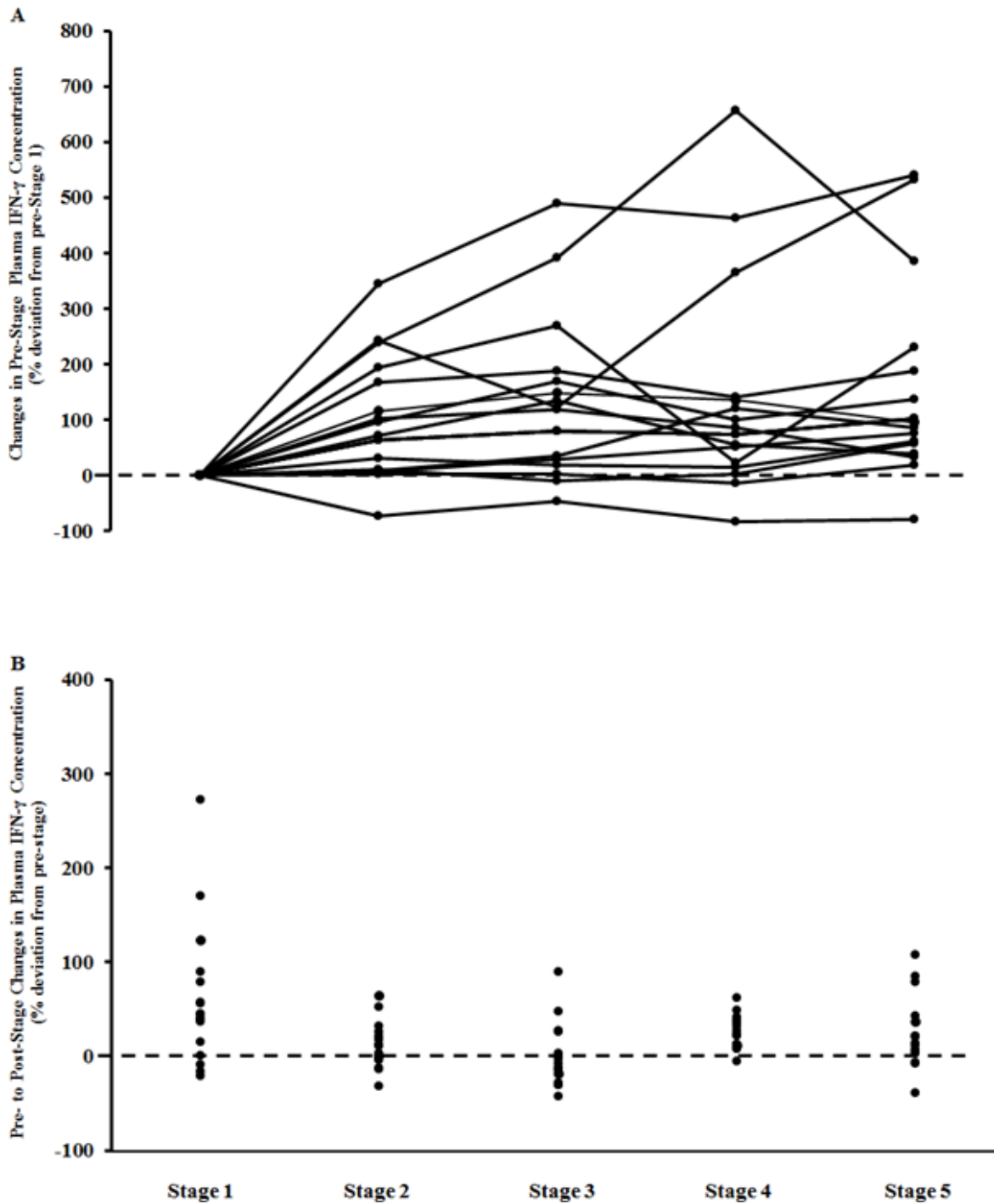t. Individual ultra-endurance runner responses (●; $n= 19$). Additional individual responses outside the figure range for pre- to post-stage changes: Stage 2, $n= 1$ at 533%; and Stage 5, $n= 1$ at 511%.

Plasma interleukin-10 concentration. Pre-stage plasma IL-10 concentration increased ($p<$ 0.001) by Stage 2 in UER, and remained elevated thereafter (**Table 6.1, Figure 6.7A**), peaking at Stage 3 (1271%). No change in pre-stage plasma IL-10 concentration was observed between Stages 1, 3, and 5 for CON, and levels were lower than UER pre-Stages 3 and 5 ($p<$ 0.001). Pre- to post-stage increases ($p=$ 0.003) in plasma IL-10 concentration were also observed in UER (**Table 6.1, Figure 6.7B**). Pre- and post-stage plasma IL-10 concentration was observed to be lower ($p<$ 0.001) in males (pre-stage: 4.1 ± 4.1 pg·ml, post-stage: 6.3 ± 7.6 pg·ml) compared with females (pre-stage: 12.6 ± 13.6 pg·ml, post-stage: 14.2 ± 14.3 pg·ml) throughout the ultra-marathon. This difference was also observed when corrected for body mass (pre-stage: $p<$ 0.001, post-stage: $p=$ 0.001). No differences in other sub-group comparisons were observed.

Figure 6.7: Individual changes in pre-stage resting (A) and pre- to post-stage (B) plasma interleukin-10 concentration of ultra-endurance runners participating in a 230 km multi-stage ultra-marathon competition conducted in a hot ambient environment. Individual ultra-endurance runner responses (●; *n*= 19). Additional individual responses outside the figure range for pre- to post-stage changes: Stage 1, *n*= 1 at 2305%, *n*= 1 at 3781%, *n*= 1 at 4761%, and *n*= 1 at 6226%; and Stage 3, *n*= 1 at 1810%.
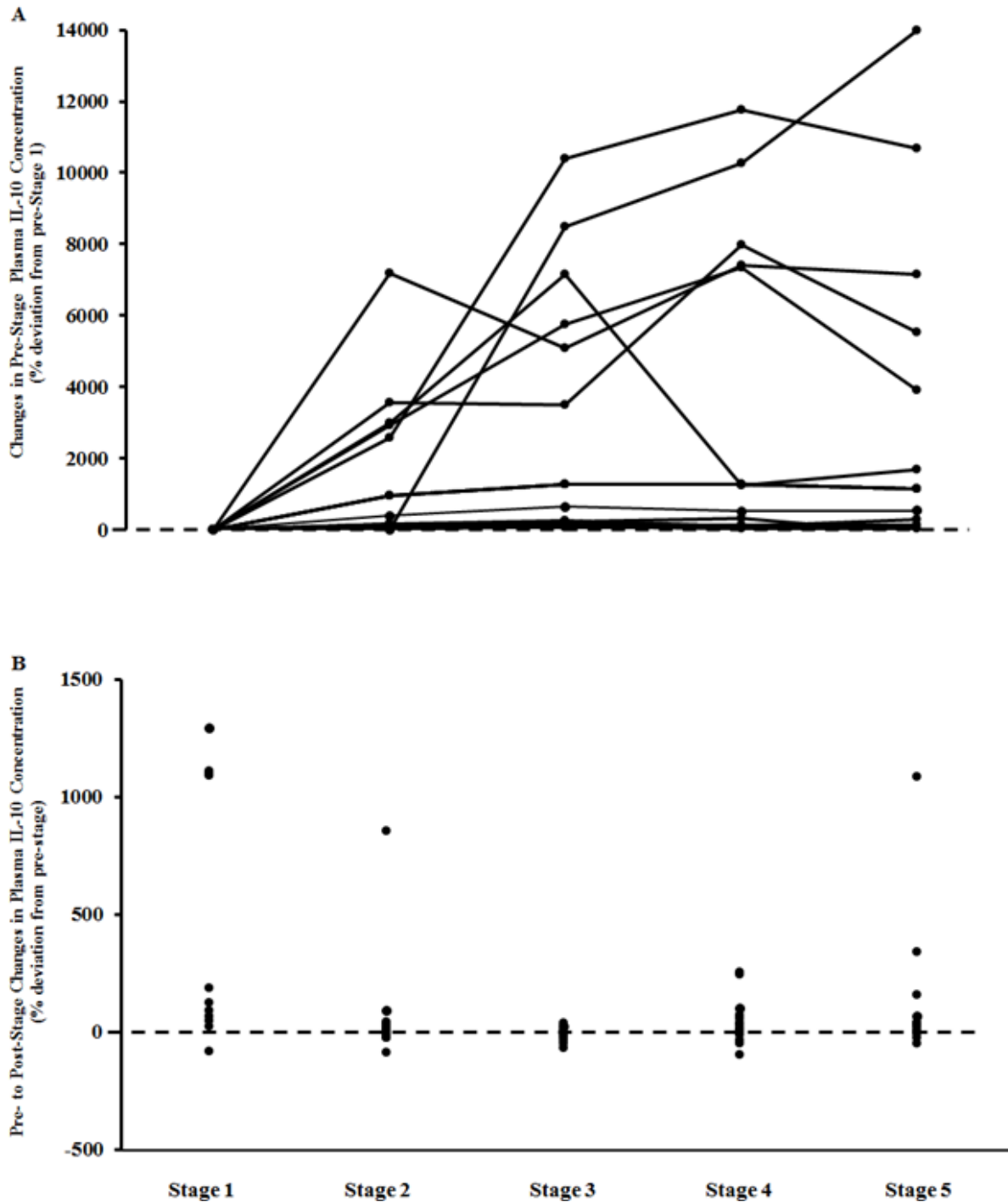
Plasma interleukin-1 receptor antagonist concentration. Pre-stage plasma IL-1ra concentration gradually increased ($p< 0.001$) in UER as the ultra-marathon progressed (**Table 6.1, Figure 6.8A**), peaking at Stage 5 (106%). No change in pre-stage plasma IL-1ra concentration was observed between Stages 1, 3, and 5 for CON, and levels were lower than UER pre-Stages 3 and 5 ($p< 0.001$). Pre- to post-stage increases ($p< 0.001$) in plasma IL-1ra concentration were also observed in UER throughout the ultra-marathon (**Table 6.1, Figure 6.8B**). No differences in other sub-group comparisons were observed.

Figure 6.8: Individual changes in pre-stage resting (A) and pre- to post-stage (B) plasma interleukin-1ra concentration of ultra-endurance runners participating in a 230 km multi-stage ultra-marathon competition conducted in a hot ambient environment. Individual ultra-endurance runner responses (●; $n= 19$). Additional individual responses outside the figure range for pre- to post-stage changes: Stage 2, $n= 1$ at 509%; and Stage 4, $n= 1$ at 696%.
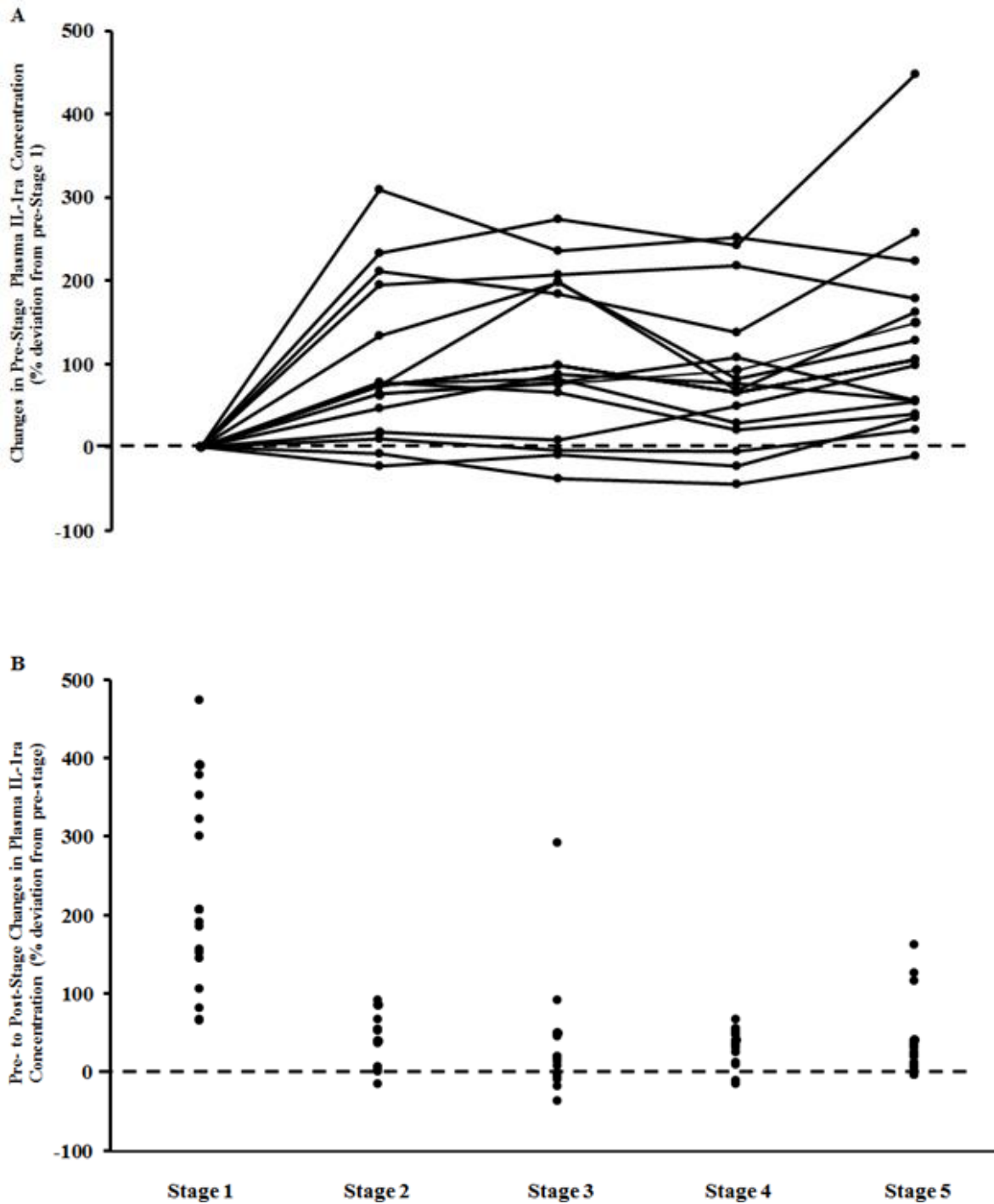
Pro-inflammatory to anti-inflammatory cytokine ratio. Pre-stage IL-1β: IL-10 and TNF-α: IL-10 ratios decreased in UER ($p= 0.041$ and $p< 0.001$, respectively) as the ultra-marathon progressed, with the lowest ratios seen at pre-Stage 4. Ratios in UER did not differ from CON pre-Stages 3 and 5. Only pre- to post-stage decreases in IL-1β: IL-10 ratio were observed in UER ($p= 0.05$), with the largest decrease occurring on Stage 1. No differences in sub-group comparisons were observed for IL-1β: IL-10 and TNF-α: IL-10 ratios.

Gastrointestinal symptoms and thermal tolerance rating. GI symptoms were a common feature amongst UER sampled for endotoxin and cytokine responses; with 58% reporting at least one severe GI symptom (including 33% of sampled UER reporting nausea) during competition, while no GI symptoms were reported by CON. No difference in the reported rates of severe GI symptoms was observed between stages in UER. Perceptive thermal tolerance rating in UER improved as the ultra-marathon progressed ($p= 0.005$), with no change in CON. Additionally, no heat related symptoms or illnesses were observed in UER and CON throughout the ultra-marathon.

Correlation analysis. Small but significant positive correlations were observed between pre-stage circulatory gram-negative bacterial endotoxin concentration with pre-stage plasma CRP ($r^2= 0.343$, $p= 0.001$), IL-6 ($r^2= 0.246$, $p= 0.019$), IL-1β ($r^2= 0.305$, $p= 0.003$), TNF-α ($r^2= 0.370$, $p< 0.001$), IFN-γ ($r^2= 0.282$, $p= 0.007$), IL-10 ($r^2= 0.309$, $p= 0.003$), and IL-1ra ($r^2= 0.268$, $p= 0.011$) concentrations; and between post-stage circulatory endotoxin concentration with post-stage plasma CRP concentration ($r^2= 0.213$, $p= 0.043$). No correlations were observed between circulatory gram-negative bacterial endotoxin concentration and plasma cytokine concentrations with severe GI symptoms (including nausea). A strong relationship between perceptive thermal tolerance rating and severe GI symptoms was observed ($r^2= -$

0.665, $p< 0.001$), whereby lower perceptive tolerance rating to heat was associated with greater reports of severe GI symptoms in UER. However, no correlations were observed between circulatory gram-negative bacterial endotoxin concentration and plasma cytokine concentrations with perceptive thermal tolerance rating.

6.5 Discussion

Findings confirm that consecutive days of EHS resulted in a modest rise in both resting and post-stage circulatory gram-negative bacterial endotoxin concentration. Despite overnight recovery between stages, results show that pyrogenic pro-inflammatory cytokines (i.e., IL-1β, TNF-α, and IFN-γ) increased in response to EHS and remained elevated at rest throughout competition. These responses however were counteracted by anti-inflammatory cytokines (IL-10 and IL-1ra) which predominated throughout competition. Although the characteristics and magnitude of cytokine responses observed were similar to that of an acute infectious episode and in accordance with the aetiology of exertional-heat illnesses (i.e., exertional-heat stroke and SIRS), no diagnosis of heat related symptoms or illnesses by a qualified Sports Physician were established in UER along the ultra-marathon. Severe GI symptoms reported by UER were generally high; but in contrast to our hypothesis, no relationship between severe GI symptoms with circulatory gram-negative endotoxin concentration and cytokine profile were observed.

In the current study, plasma CRP concentrations of UER increased by Stage 2 and remained elevated thereafter. Interestingly, on this occasion, males showed high plasma CRP concentration throughout competition compared with females, suggesting greater general inflammatory presence in males.

The current ultra-marathon resulted in modest increases in post-stage circulatory endotoxin concentrations throughout competition (i.e., 30 pg·ml average increase from pre- to post-stage, with the highest increase observed at 92 pg·ml). However, a novel finding was the gradual increases in resting levels as the ultra-marathon progressed (i.e., 60 pg·ml average

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increase from Stage 1 to 5, with 32% of runners had concentrations >100 pg·ml and the highest increase observed at 130 pg·ml). While, observed increases in resting pre-stage and pre- to post-stage plasma IL-6, IL-1β, TNFα, and IFN-γ concentrations remained elevated throughout competition; while no change in CON was observed (except for IL-1β). The cytokine profile of the current study mirrors that of an acute infectious episode. The current ultra-marathon also resulted in substantial increases in resting pre-stage and pre- to post-stage anti-inflammatory cytokines that remained elevated thereafter to a similar degree as compensatory anti-inflammatory syndrome (Adib-Conquy and Cavaillon 2009, Shubin, Monaghan, and Ayala 2011).

Perceptive thermal comfort rating improved as competition progressed in the sampled population, and likely reflect heat acclimatization as evidenced by gradual increase in P_V and reductions in T_{tymp} as the ultra-marathon progressed, with no changes in CON being observed (Costa *et al.* 2014, Costa *et al.* 2013b). In contrast to previous studies, no associations between GI symptoms with circulatory endotoxin concentration and cytokine responses were observed on this occasion. However, a strong relationship ($r^2 = -0.665$) between severe GI symptoms and perceptive thermal tolerance rating was confirmed ($p < 0.001$).

As previously described in **Chapter 4**, the limitations in the sampled cohort are stated.

In conclusion, multi-stage ultra-marathon competition in the heat resulted in a modest circulatory endotoxaemia accompanied by a pronounced pro-inflammatory cytokinaemia and compensatory anti-inflammatory responses. No incidences of exertional heat symptoms or illnesses were evident throughout competition. Even though severe GI symptoms were reported, no relationships with blood borne indices were identified. The expected exacerbated cytokine responses were possibly attenuated by the maintenance of hydration status in the

majority of runners, and thermoregulatory-induced adaptations and behaviours (e.g., heat acclimatisation and cooling strategies) adopted by participants.

Circulatory Endotoxin Concentrations and Cytokine Profile During a 24 h Ultra-Marathon in Temperate Ambient Conditions.

Gill, S. K., Hankey, J., Wright, A., Marczak, S., Hemming, K., Allerton, D., Ansley-Robson, P., and Costa, R. S. (2015) 'The Impact of a 24-hour Ultra-Marathon on Circulatory Endotoxin and Cytokine Profile'. *International Journal of Sports Medicine* 36 (8), 688-695

7.1 Summary: The study aimed to determine circulatory endotoxin concentration, cytokine profile, and GI symptoms of UER (n= 17) in response to a 24 h continuous ultra-marathon competition (total distance range: 122-208 km) conducted in temperate ambient conditions (0-20°C) in mountainous terrain. BM and body temperature were measured, and venous blood samples were taken before and immediately after competition. Samples were analysed for gram-negative bacterial endotoxin, CRP, cytokine profile**,** and P_{Osmol}. GI symptoms were also monitored throughout competition. Mean exercise-induced BM loss was (mean ± SD) 1.7 ± 1.8% in UER. Pre- and post-competition P_{Osmol} in UER was 286 ± 11 mOsmol·kg^{-1} and 286 ± 9 mOsmol·kg^{-1}, respectively. Pre- to post-competition increases ($p<$ 0.05) were observed for endotoxin (37%), CRP (2832%), IL-6 (3436%), IL-1β (332%), TNF-α (35%), IL-10 (511%), and IL-8 (239%) concentrations in UER, with no change in CON (n= 12) observed (p >0.05). GI symptoms were reported by 75% of UER, with no symptoms reported by CON. IL-10 (r= 0.535) and IL-8 (r= 0.503) were positively correlated with GI symptoms. A 24 h continuous ultra-marathon competition in temperate ambient conditions resulted in a circulatory endotoxaemia and pro-inflammatory cytokinaemia, counteracted by a compensatory anti-inflammatory response.

7.2 Introduction

Even though exercising in hot ambient conditions *per se* appear to disturb intestinal epithelial integrity and promote an endotoxin-induced cytokine-mediated inflammatory response, it is plausible that extreme ultra-endurance competition, in the absence of heat-stress, but with prolonged exposure to a multitude of other multiple physiological stressors (Costa *et al.* 2013a, Costa *et al.* 2013b), may also have the potential to impact intestinal epithelial integrity, and the subsequent cytokine-mediated inflammatory cascade in a similarly negative manner.

With this in mind, the aims of the current study were to determine circulatory endotoxin concentration and cytokine profile of UER in response to a 24 h continuous ultra-marathon competition conducted in temperate ambient conditions; and additionally to determine the relationship between these responses with GI symptoms. Taking into account the nature of the event, it was hypothesised that a pronounced circulatory endotoxaemia would be seen after the event, and a pro-inflammatory cytokinaemic response would mirror the endotoxaemia. Additionally, it was hypothesised that correlations between circulatory responses and GI symptoms would be seen.

7.3 Methods

Setting and participants. The study was conducted during the 2011 and 2012 Glenmore24 Trail Race (www.glenmore24.com), held during the first week of September, in the Cairngorms National Park, Scottish Highlands, UK (T_{amb} range: 0-20°C, RH range: 54-82%). The 24 h continuous ultra-marathon was conducted on a 6 km looped-course on a variety of terrains; including off-road trails, paths, and grassland. Distance covered by participants ranged between 122-208 km at an estimated average intensity of 7.0 ± 1.3 METs (SenseWear 7.0, Bodymedia, PA, Pittsburgh, USA), and in an altitude averaging 342 m above sea level. After ethical approval, a convenience sampling observational cohort was studied; whereby 25 out of 48 UER entering the event volunteered to participate in the study; however complete blood sampling and biomarker data were only achieved in n= 17 (male n= 14, female n= 3: age 40 ± 7 y, height 177 ± 9 cm, BM 78.1 ± 11.9 kg). Additionally, 17 individuals who did not compete (absence of exercise stress), but were present at the race location, volunteered to participate in the study as part of the CON group for comparative purposes, however complete blood sampling and biomarker data were only achieved in n= 12 (male n= 4, female n= 8: age 30 ± 12 y, height 169 ± 10 cm, BM 68 ± 13 kg). All participants reported no illness and (or) infection in the twelve weeks leading up to the ultra-marathon. Additionally, no anti-inflammatory agents of any form were consumed by all participants in the week leading up and during competition.

Study design and data collection. Within the hour prior to commencement of competition (11:00h-12:00 h), BM measurements were taken, as previously described **(Section 3.2).** Participants were then required to sit in an upright position for 10 min before T_{tymp} was determined, as previously described **(Section 3.4)** and whole blood samples collected, as

previously described **(Section 3.6)**, for all participants (UER and CON). BM was re-measured in those participants who needed to urinate prior to the start of competition. Immediately after competition (12:00 h the following day) and before any foods or fluids could be consumed, BM and T_{tymp} were measured, followed immediately by blood sampling, identical to pre-competition procedures. Also, within 1 h following competition, trained dietetic researchers conducted a standardised structured interview on participants to ascertain total foods and fluids ingested during the ultra-marathon. Severe GI symptoms (Pfeiffer *et al.* 2012) were also explored at this time through a research-generated symptomology tool by trained researchers.

Dietary analysis and hydration status. Dietary analysis and hydration status were determined as previously described in **Section 3.3.** Energy expenditure was measured by a triaxial accelerometer, which also included measurements of heat flux, skin temperature and galvanic skin responses (SenseWear 7.0, Bodymedia, PA, Pittsburgh, USA). Pre- and post-competition P_{Osmol} was determined as previously described in **Section 3.3.** The mean CV for energy, macronutrient, and water variables analysed was 0.8%, 1.4%, and 0.5%, respectively. The CV for P_{Osmol} was 3.5%.

Blood sample analysis. Whole blood samples were analysed for circulatory concentrations of CRP, cytokines (IL-6, TNF-α, IL-1β, IFN-γ, IL-10, and IL-8) and gram-negative bacterial endotoxin, as previously described in **Section 3.6.**

Data analysis. Data in text are presented as mean ± SD, otherwise specified. For clarity, data in figures are presented as mean ± SEM. Due to the commonly large individual variation in cytokine responses (Suzuki *et al.* 2002), data in tables are presented as mean and 95% CI for

mean (lower and upper boundary). Data were processed and analysed in SPSS for Windows (SPSS v.17.0.2, Illinois, US). Prior to data analysis, outlying values for all variables were detected through box-plot analysis and appropriately removed. Diagnostic checks (Shapiro-Wilks test of normality and Levene's homogeneity of variance test) were performed prior to analysis. Where data violated the assumption of normality, data were log transformed, and the transformed data was analysed using parametric statistics, verified against non-parametric equivalents where appropriate. Paired-sample t-tests were applied to determine pre- to post-competition variable differences; while independent-sample t-tests were applied for group comparisons (UER *vs.* CON, and distance covered (<160 km *vs.* ≥160 km)) (Pacque *et al.* 2007, Peters and Bateman 1983). Due to insufficient female UER sample size, statistical analysis was not viable on this occasion, Spearman's rank correlation analysis was used to assess associations between blood-borne variables with perceived GI symptoms. Individual participant ratios between IL-1β and TNF-α with IL-10 were calculated to determine the pro- to anti-inflammatory cytokine balance. Descriptive statistics were used to explore severe GI symptoms. The acceptance level of significance was set at $p < 0.05$.

7.4 Results

Energy balance and hydration status. Total energy intake and energy expenditure over the 24 h period was 21 ± 12 and 53 ± 11 MJ for UER, and 12.4 ± 1.3 and 13.6 ± 4.9 MJ for CON, respectively. Significant BM loss occurred pre- to post-competition ($p = 0.001$) in UER (pre-competition: 78.1 ± 11.9 kg, post-competition: 76.8 ± 12.0 kg, $1.7 \pm 1.8\%$). No significant difference in P_{Osmol} were observed pre- to post-competition in UER (pre-competition: 286 ± 11 mOsmol·kg^{-1}, post-competition: 286 ± 9 mOsmol·kg^{-1}) and remained within normal clinical reference range (Thomas *et al.* 2008). Compared with CON, P_{Osmol} pre- and post-competition was lower in UER ($p = 0.05$ and $p < 0.001$, respectively).

Tympanic temperature. T_{tymp} was within normal range pre- and post-stage (36.0°C to 37.5°C) in UER and CON, with no difference between pre- and post-competition T_{tymp} observed in both groups. No difference in T_{tymp} was observed between UER and CON.

Circulatory gram-negative bacterial endotoxin concentration. A pre- to post-competition increase in circulatory endotoxin concentration (overall mean change: 37%) was observed in UER ($p = 0.009$; **Figure 7.1**), with no significant change in CON evident ($p = 0.005$ *vs.* UER). No difference in circulatory endotoxin concentration was observed for distance covered.

Figure 7.1. Circulatory gram-negative bacterial endotoxin concentration of ultra-endurance runners participating in a 24 h continuous ultra-marathon. Mean ± SEM: UER (*n*= 17, ■) and CON (*n*= 12, O). ** *p*< 0.01 *vs.* pre-competition; [aa] *p*< 0.01 *vs.* CON.
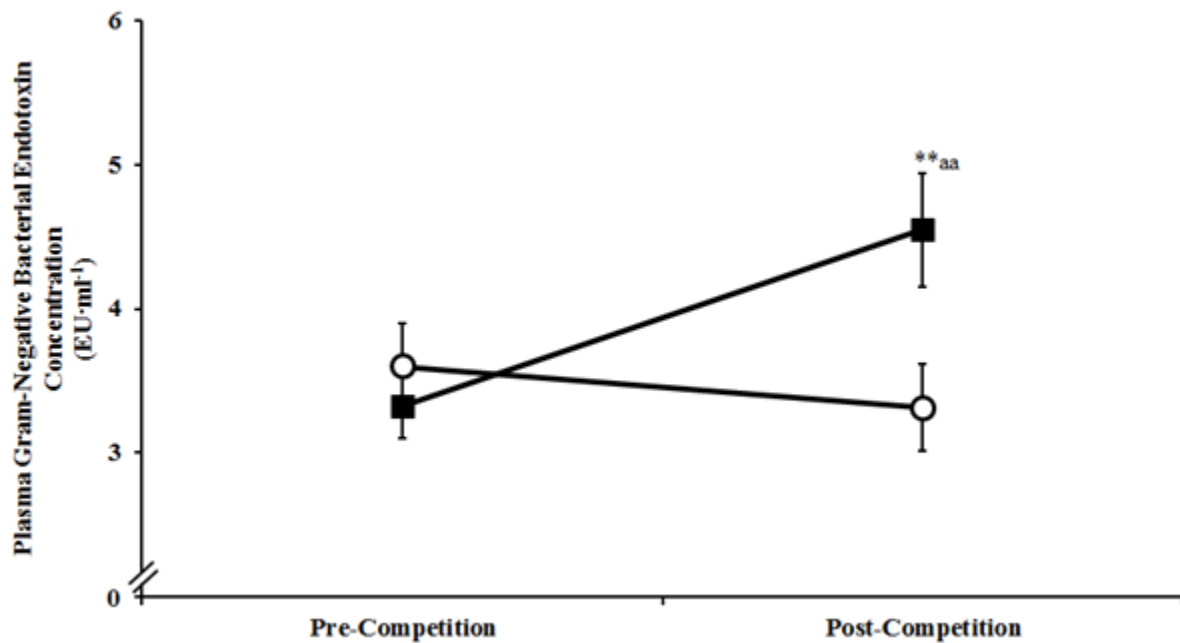
Plasma C-reactive protein concentration. A pre- to post-competition increase in plasma CRP concentration (2832%) was observed in UER (*p*< 0.001; **Figure 7.2**), with no significant change in CON evident (*p*< 0.001 *vs.* UER). No difference in plasma CRP concentration was observed for distance covered.

Figure 7.2. Plasma C-reactive protein concentration of ultra-endurance runners participating in a 24 h continuous ultra-marathon. Mean ± SEM: UER (*n*= 17, ■) and CON (*n*= 12, O). ** *p*< 0.01 *vs.* pre-competition; [aa] *p*< 0.01 *vs.* CON.
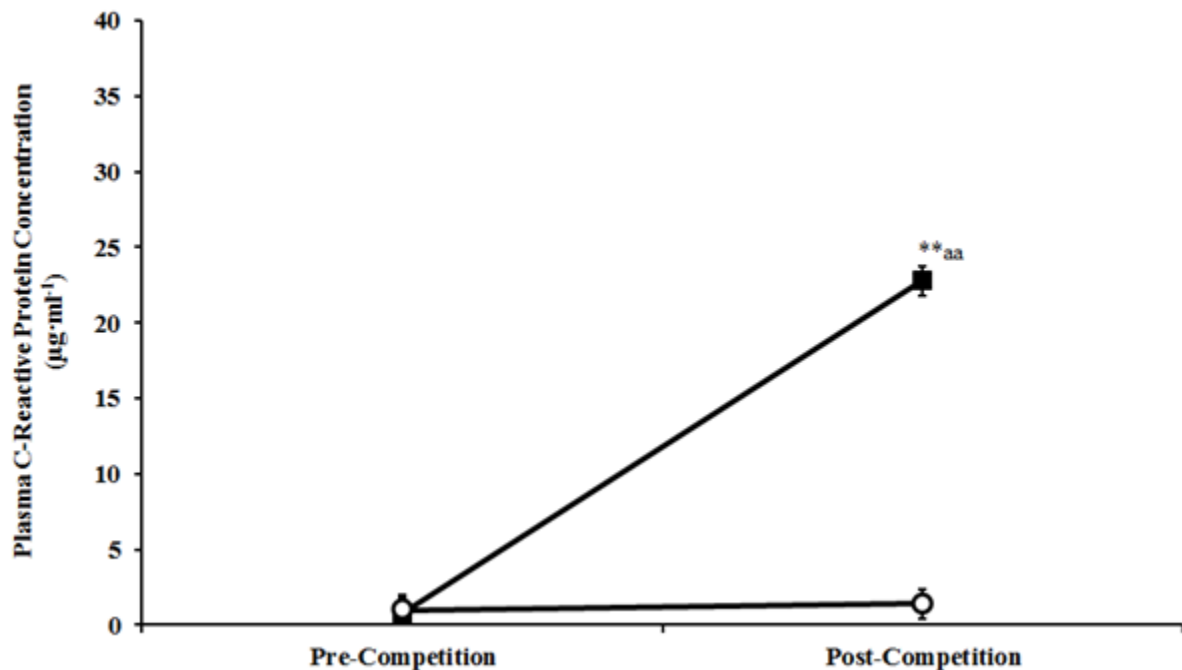
Plasma interleukin-6 concentration. A pre- to post-competition increase in plasma IL-6 concentration (3436%) was observed in UER (*p*< 0.001; **Table 7.1**), with no significant change in CON evident (*p*< 0.001 *vs.* UER). Post-competition plasma IL-6 concentration was higher (*p*= 0.018) in UER totalling a distance ≥160 km compared to UER totalling a distance <160 km (**Table 7.2**).

Plasma interleukin-1 beta concentration. A pre- to post-competition increase in plasma IL-1β concentration (332%) was observed in UER (*p*= 0.05; **Table 7.1**), with no significant change in CON evident (*p*= 0.032 *vs.* UER). No difference in plasma IL-1β concentration was observed for distance covered.

Plasma tumour necrosis factor alpha concentration. A pre- to post-competition increase in plasma TNF-α concentration (35%) was observed in UER ($p < 0.001$; **Table 7.1**), with no significant change in CON evident. No difference in plasma TNF-α concentration was observed for distance covered.

Plasma interferon gamma concentration. No significant change in pre- to post-competition plasma IFN-γ concentration was observed in UER or CON. No difference in plasma IFN-γ concentration was observed for distance covered.

Plasma interleukin-10 concentration. A pre- to post-competition increase in plasma IL-10 concentration (511%) was observed in UER ($p < 0.001$; **Table 7.1**), with no significant change in CON evident ($p < 0.001$ *vs.* UER). Post-competition plasma IL-10 concentration was higher ($p = 0.002$) in UER totalling a distance ≥160 km compared to UER totalling a distance <160 km (**Table 7.2**).

Plasma interleukin-8 concentration. A pre- to post-competition increase in plasma IL-8 concentration (239%) was observed in UER ($p < 0.001$; **Table 7.1**), with no significant change in CON evident ($p < 0.001$ *vs.* UER). A higher post-competition plasma IL-8 concentration was observed in UER totalling a distance ≥160 km compared to UER totalling a distance <160 km (**Table 7.2**), although this failed to reach significance ($p = 0.102$).

Table 7.1: Plasma cytokine profile of ultra-endurance runners before and immediately after a 24 h continuous ultra-marathon competition.

	Pre-Competition	Post-Competition
Plasma IL-6 concentration (pg·ml^{-1})		
UER	0.4 (0.3-0.5)	14.5 (9.3-19.7)[**aa]
CON	0.6 (0.2-0.9)	1.8 (0.5-3.1)
Plasma IL-1β concentration (pg·ml^{-1})		
UER	0.1 (0.0-0.3)	0.6 (0.1-1.1)[*a]
CON	0.0 (0.0-0.1)	0.0 (0.0-0.1)
Plasma TNF-α concentration (pg·ml^{-1})		
UER	2.8 (2.5-3.2)	3.8 (3.5-4.2)[**]
CON	2.6 (1.8-3.3)	3.2 (2.4-4.0)
Plasma IFN-γ concentration (pg·ml^{-1})		
UER	1.0 (0.6-1.4)	1.2 (0.3-2.2)
CON	1.1 (0.6-1.6)	1.1 (0.6-1.6)
Plasma IL-10 concentration (pg·ml^{-1})		
UER	2.1 (1.3-2.9)	12.8 (7.3-18.2)[**aa]
CON	1.6 (0.1-3.2)	1.7 (0.1-3.3)
Plasma IL-8 concentration (pg·ml^{-1})		
UER	11.4 (9.4-13.4)	38.7 (26.3-51.1)[**aa]
CON	14.0 (10.2-17.7)	14.2 (11.1-17.2)

Mean and 95% CI (lower and upper bound): UER ($n= 17$) and CON ($n= 12$). * $p< 0.05$ and ** $p< 0.01$ *vs.* pre-competition, [a] $p< 0.05$ and [aa] $p< 0.01$ *vs.* CON.

Table 7.2: Pre- and post-competition circulatory gram-negative bacterial endotoxin concentration, plasma C-reactive protein concentration, and plasma cytokine profile of ultra-endurance runners who completed ≥160 km and <160 km during a 24 h continuous ultra-marathon competition.

	Pre-Competition	Post-Competition
Circulatory gram-negative bacterial endotoxin concentration (EU·ml^{-1})		
≥160 km	3.5 (2.7-4.3)	4.5 (3.6-5.2)
<160 km	3.1 (2.5-3.7)	4.7 (3.3-6.0)
Plasma C-reactive protein concentration (µg·ml^{-1})		
≥160 km	0.5 (0.2-0.8)	22.2 (19.4-26.1)
<160 km	1.0 (0.0-2.1)	23.4 (21.1-25.8)
Plasma IL-6 concentration (pg·ml^{-1})		
≥160 km	0.5 (0.2-0.6)	19.8 (12.0-30.4)[a]
<160 km	0.4 (0.3-0.5)	8.5 (5.6-11.5)
Plasma IL-1β concentration (pg·ml^{-1})		
≥160 km	0.3 (0.0-0.7)	0.6 (0.0-1.6)
<160 km	0.0 (0.0-0.0)	0.6 (0.0-1.4)
Plasma TNF-α concentration (pg·ml^{-1})		
≥160 km	2.7 (1.8-3.4)	3.8 (3.3-4.4)
<160 km	3.0 (2.7-3.3)	3.8 (3.2-4.5)
Plasma IFN-γ concentration (pg·ml^{-1})		
≥160 km	1.0 (0.3-1.5)	1.2 (0.1-1.7)
<160 km	0.7 (0.2-1.1)	0.5 (0.2-0.7)

Plasma IL-10 concentration (pg·ml^{-1})

≥160 km	2.0 (0.8-3.1)	19.7 (10.1-27.0)[aa]
<160 km	2.2 (0.7-3.7)	4.9 (3.3-6.5)

Plasma IL-8 concentration (pg·ml^{-1})

≥160 km	11.5 (9.0-15.3)	47.7 (24.8-76.6)[§]
<160 km	11.3 (8.0-14.6)	28.5 (22.3-34.8)

Mean and 95% CI (lower and upper bound): ≥160 km (*n*= 9) and <160 km (*n*= 8). [a] *p*< 0.05 and [aa] *p*< 0.01 *vs.* <160 km, [§] *p*= 0.102 *vs.* <160 km

Pro-inflammatory to anti-inflammatory cytokine ratio. A pre- to post-competition decrease in TNF-α: IL-10 ratio (72%; *p*< 0.001) was observed in UER, with no significant change in CON evident (*p*= 0.011 *vs.* UER). While a 32% decrease in IL-1β: IL-10 ratio was also observed in UER (*p*= 0.063 *vs.* CON), but this failed to reach significance (*p*> 0.05). Post-competition TNF-α: IL-10 ratios were substantially lower (*p*= 0.008) in UER totalling a distance ≥160 km compared to UER totalling a distance <160 km.

Gastrointestinal symptoms. Severe GI symptoms were a common feature amongst the cohort sampled for endotoxin and cytokine responses; with 75% of the cohort reporting at least one severe GI symptom (including 63% reporting nausea) during competition. Greater reports of severe GI symptoms were reported by UER totalling a distance ≥160 km compared to UER totalling a distance <160 km (*p*= 0.012). Spearman's rank correlation analysis showed a positive correlation between reported rates of GI symptoms and plasma IL-10 (r= 0.535, *p*= 0.034) and IL-8 (r= 0.503, *p*= 0.047) concentrations.

7.5 Discussion

Findings confirm that in the absence of heat-stress, but with prolonged exposure to other multiple physiological stressors, resultant endotoxaemia with accompanying cytokinaemia were characteristic of an acute infectious episode and in accordance with the aetiology of SIRS (Fehrenbach and Schneider 2006, Marshall 1998, Suzuki *et al.* 2002, Suzuki *et al.* 1999). Severe GI symptoms were a common feature, with higher symptom occurrence associated with greater compensatory anti-inflammatory (i.e., IL-10) and immune activation (i.e., IL-8) responses. Additionally, post-competition IL-6, IL-10, and IL-8 responses were higher and TNF-α:IL-10 ratio was lower in those UER totalling a distance ≥160 km.

In comparison to a 230 km multi-stage ultra-marathon conducted in hot ambient conditions (32°C to 40°C) (**Chapter 6**), whereby resting plasma CRP concentration increased 889% and circulatory endotoxin concentration peaked at 21% by Stage 5, plasma CRP and circulatory endotoxin concentration in the current study were markedly more pronounced despite the absence of heat stress, increasing 2832% and 37% post-competition, respectively.

Despite temperate ambient conditions (0-20°C), the current study observed increases in pro-inflammatory cytokine responses post-competition, similar to that of an acute infectious episode; whereby IL-6, IL-1β, and TNF-α increased 3436%, 332%, and 35%, respectively. Acute pro-inflammatory response is commonly accompanied by compensatory anti-inflammatory (i.e., 511% increase in IL-10) and immune activation (i.e., 239% increase in IL-8) responses. As such, the observed decreases in TNF-α: IL-10 and IL-1β:IL-10 ratios in the current study suggest that the anti-inflammatory properties of IL-10 may have restricted the magnitude of pro-inflammatory cytokine responses induce by the extreme nature of this

ultra-marathon. Notably, those UER totalling a distance of ≥160 km had significantly greater post-competition IL-6 and IL-10 responses (overall mean: 19.8 pg·ml^{-1} and 19.7 pg·ml^{-1}, respectively) compared to those UER totalling a distance <160 km (overall mean: 8.5 pg·ml^{-1} and 4.9 pg·ml^{-1}, respectively). Additionally, post-competition TNF-α: IL-10 ratio was substantially lower in UER totalling a distance ≥160 km compared to UER totalling a distance <160 km.

In accordance with a recent multi-stage ultra-marathon study (**Chapter 6**), despite 58% of UER sampled for endotoxin and cytokine responses reporting severe GI symptoms during competition, and reporting being greater in UER totalling a distance ≥160 km compared to UER totalling a distance <160 km, no relationships between GI symptoms with circulatory endotoxin and pro-inflammatory cytokine concentrations was observed in the current study. Interestingly, an association between the reported number of severe GI symptoms and plasma concentrations of anti-inflammatory IL-10 (r= 0.535) and immune activator IL-8 (r= 0.503) was observed.

In conclusion, a 24 h continuous ultra-marathon competition conducted in temperate ambient conditions with inclusion of multiple physiological stressors (i.e., sleep deprivation and energy deficit) resulted in endotoxaemia with accompanying cytokinaemia characteristic of an acute infectious episode. GI symptoms were commonly reported amongst UER, with higher symptom occurrence associated with greater compensatory anti-inflammatory responses and immune activation suggesting that alterations to intestinal motility and mechanical trauma may have promoted intestinal mucosa and epithelial disturbance, inducing GI symptoms.

CHAPTER EIGHT

Acute Supplementation of *Lactobacillus Casei* on Salivary Anti-Microbial

Proteins in Response to Exertional-Heat Stress.

Summary 8.1: The study aimed to determine if acute high dose probiotic supplementation containing *L.casei* enhances S-AMP responses to EHS. Eight endurance trained male volunteers completed a blinded randomised cross-over design. Oral supplementation of the probiotic beverage (*L.casei* x 10^{11} CFU·day^{-1}) (PRO) or placebo (PLA) was consumed for seven consecutive days before 2 h running exercise at 60% VO_{2max} in hot ambient conditions (34.0°C and 32% RH). BM, unstimulated saliva and venous blood samples were collected one week before EHS (baseline), pre-EHS, during recovery (1 h, 2 h, and 4 h), and at 24 h. Saliva samples were analysed for S-IgA, S-α-amylase, S-lysozyme, and S-cortisol. Plasma samples were analysed for P_{Osmol}. BM and P_{Osmol} did not differ between trials. SFR remained relatively constant throughout the experimental design in PRO (overall mean ± SD: 601 ± 284 µl·min) and PLA (557 ± 296 µl·min). PRO did not induce significant changes in resting S-AMP responses compared with PLA ($p > 0.05$). Increases in S-IgA, S-α-amylase, S-lysozyme, and S-cortisol concentration were observed after EHS ($p < 0.05$). No main effects of trial or time x trial interaction were observed for S-AMP and S-cortisol responses. Acute supplementation of a probiotic beverage containing *L.casei* for seven days before EHS does not provide any further oral-respiratory mucosal immune protection, with respect to S-AMP, over a PLA. Maintenance of hydration and exertional stress *per se* appears to have a more favourable effect on S-AMP status than acute high dose probiotic supplementation.

8.2 Introduction

It has been suggested that probiotic supplementation may play a role in preventing or attenuating URSI during periods of compromised oral-respiratory mucosal status through alterations in S-AMP responses (Gill and Prasad 2008, West *et al.* 2009). For example, probiotic supplementation studies in active populations have reported mild decreases (Cox *et al.* 2010b; Gleeson *et al.* 2011a), but also no change (Kekkonen *et al.* 2007), in URSI. Considering the clinical significance of exertion-induced compromised S-AMP responses, with respect to URSI risk, the influence of probiotic supplementation on each individual S-AMP (i.e., S-IgA, S-α-amylase, and S-lysozyme) and how these respond in combination to exertion has not yet been thoroughly investigated. It is thus plausible and proposed that repetitive exposure of a substantial non-toxic bacterial load at the oral-respiratory mucosal surface over an acute period may promote enhanced S-AMP responses (e.g. up-regulation of S-AMP secretion). *L.casei* is one of the most popular commercially available probiotics worldwide and appears to modulate mucosal surfaces beyond the GI tract (Spanhaak *et al.* 1998).

A lower dose of probiotic supplementation between two weeks to six months are generally used within clinical research models (Ford *et al.* 2014, Moayyedi *et al.* 2010). Considering that previous research has established that as dosage of probiotic supplementation increases, the proportion of bacteria specific positive subjects also increases (Christensen *et al.* 2006); providing a higher dosage over a shorter duration, which mimics athlete population consumption behaviours, replicates the supplemented bacterial load of previous lower dosage longer duration research models. From a practical perspective, long-term consumption of probiotics is not generally practiced and (or) sustained in active populations; whereas acute

consumption of probiotics (e.g., beverage form) is common before event participation, during periods of intensified training, and in anticipation to international travel, with reported focus on prevention-management of URSI episodes (West *et al.* 2009; Walsh *et al.* 2011b). Indeed, high volumes of commercially available probiotic beverages consumption has been observed before multi-stage ultra-marathon competition (**Chapter 4**).

With this in mind, the study aimed to determine if oral supplementation of a commercially available probiotic beverage containing *L.casei* (x 10^{11} CFU·day^{-1}) for seven consecutive days can enhance S-AMP responses to EHS in an endurance trained population. It was hypothesised that enhanced S-AMP responses would be seen after one week of supplementation, after EHS, and during the recovery period compared with placebo.

8.3 Methods

Participants. Eight non-heat acclimatised healthy endurance trained male runners (mean ± SD: age 26 ± 6 years, nude BM 70.2 ± 8.8 kg, height 1.75 ± 0.05 m, VO_{2max} 59 ± 5 ml·kg^{-1}·min^{-1}; endurance sport competitive experience types include: triathlon, road and trail running, and ultra-endurance running) volunteered to participate in the study. All participants gave written informed consent, which received ethical approval from the local Ethics Committee that conforms with the 2008 Helsinki Declaration for Human Research Ethics. Participants confirmed that they had not consumed probiotics, in any form, within the previous three month period and consumption was prohibited during the study. **Figure 8.1** is a CONSORT Flow Diagram illustrating the process of participants through the study.

Figure 8.1: CONSORT Flow Diagram
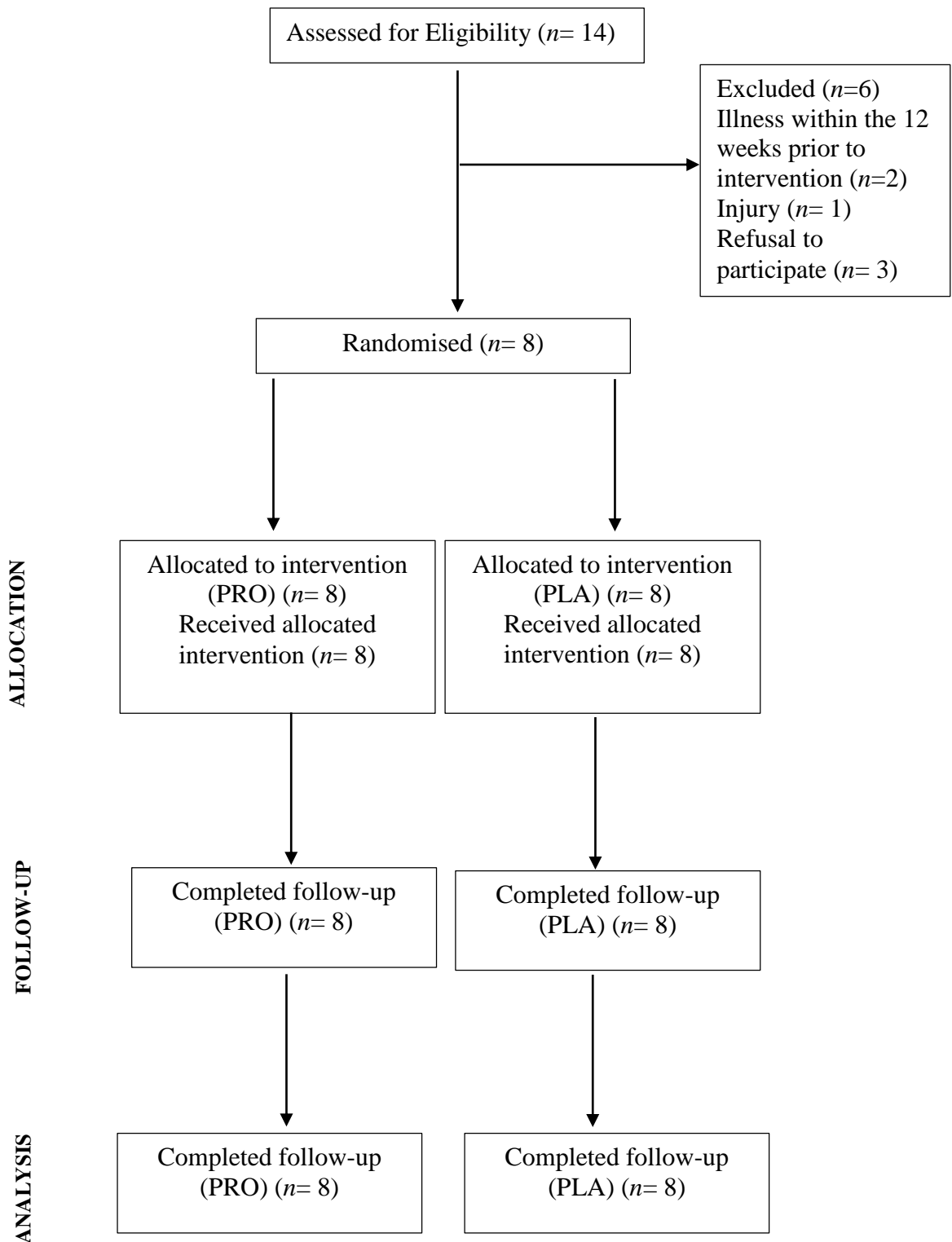
Preliminary measures. Previous research has indicated that commercially available probiotics in the UK may lack the number of microbiota otherwise stated (Hamilton-Miller *et al.* 1996). Therefore, prior to the study, preparations were cultured in duplicate (CV: 2.5%) for *Lactobacillus* on DeMan-Rogosa-Sharpe agar to estimate the level of *Lactobacillus* contained within the probiotic beverage (stated count: 1.0×10^8 CFU·ml^{-1}). The counts from these cultured plates were 1.9×10^8 CFU·ml^{-1}. The commercially available probiotic beverage containing *L.casei* used was kept confidential to comply with product anonymity ethical procedures.

Two weeks before the first experimental trial, height and nude BM were recorded. VO_{2max} (Cortex MetaMax 3B, Biophysik, Leipzig, Germany) was estimated by means of a continuous incremental exercise test to volitional exhaustion on a motorized treadmill (h/p/cosmos Mercury 4.0, Nussdorf-Traunstein, Germany), as previously reported (Costa et al., 2009). From the VO_2–work rate relationship, the treadmill speed at 60% VO_{2max} and 1% gradient was extrapolated (9.9 ± 1.1 km·h^{-1}).

Experimental procedure. One week before the EHS trial (baseline) whole blood samples were collected, as previously described (**Section 3.6**). Participants then provided a 4 min saliva sample, as previously described (**Section 3.5**). Immediately after baseline samples, in a blinded randomised and counterbalanced order, participants were provided with either PRO or PLA for consumption over the subsequent seven consecutive days. The overall PRO and PLA dose was split into two equal boluses (500 ml) that were consumed between 08:00-09:00 h and 16:00-17:00 h, respectively. The placebo formulation was similar in taste, consistency, colour and nutritional value to the probiotic formulation, but contained no *L.casei* (PRO per 100ml: 66 kCal, 14.7 g carbohydrate, 1.3 g protein, fat <0.1 g; PLA per

100ml: 70 kCal, 14.7 g carbohydrate, 1.9 g protein, fat 0.1 g). Dietary intake was assessed and analysed as previously reported (Costa *et al.* 2013a).

On two occasions following seven consecutive days of either PRO or PLA, and separated by one month washout, participants reported to the laboratory at 8:00h after consumption of a standardised breakfast (526 kCal, 118 g carbohydrate, 9 g protein, and 2 g fat) with 400 ml of water (07:00h). Within 30 min before the EHS trial, participants were asked to void before nude BM measurements. Blood and saliva samples were then collected. Participants initiated (09:00h) running exercise on a motorised treadmill for 2 h at the previously determined treadmill speed that elicited 60% VO_{2max}, dressed in athletic shorts, socks and shoes. The 2 h exercise bout was performed in an environmental chamber with ambient conditions of $34.0 \pm 0.4°C$ and $32 \pm 2\%$ RH. Heart rate (HR) measured by a short-range radiotelemetry monitor (Polar Electro, Kempele, Finland), rating of perceived exertion (RPE), thermal comfort rating (TCR), and T_{re} (CorTemp Core Body Temperature Sensor, Florida, USA) were recorded every 10 min during EHS (Costa *et al.* 2010). Participants were provided with water *ad libitum.* Immediately post-EHS, blood and saliva samples were collected, and nude BM was recorded, as previously described (**Section 3.2, Section 3.5, and Section 3.6**). Blood and saliva samples were additionally collected 1 h, 2 h, and 4 h post-EHS, and 24 h after the EHS protocol (09:00h the next morning). To reduce any seasonal heat acclimatisation, the experimental procedure was conducted in during winter (Costa *et al.* 2014).

Sample analysis. P_{Osmol} was determined as previously described (**Section 3.3**). Saliva samples were analysed, as previously described (**Section 3.5**). On this occasion, due to sample availability, S-lysozyme was determined at baseline, pre-EHS, post-EHS, 2 h and 4 h post-EHS only.

Statistical analysis. Based on the typical standard deviation of 73 µl·min^{-1}, 33 U·min^{-1} and 3 µl·min^{-1} for S-IgA, S-α-amylase, and S-lysozyme secretion rates to exertional stress, respectively (Costa *et al.* 2009, **Chapter 4** and **Chapter 5**); using standard alpha (0.05) and beta values (0.8), a sample size of *n*= 8 is estimated to provide adequate statistical precision to detect a >29% change in S-AMP responses to exertional-heat stress (Costa *et al.* 2012, Fortes *et al.* 2012). Such reductions in S-AMPs (namely, S-IgA) have been associated with increased incidence of URSI (Gleeson *et al.* 2011a, Neville *et al.* 2008). Data in the text and tables are presented as mean ± SD. For clarity, data in figures are presented as mean ± SEM. The data were examined using two-way repeated-measures ANOVA, except for nude BM loss and water intake that were examined using paired sample t-test and Wilcoxon signed-rank test where appropriate. Assumptions of homogeneity and sphericity were checked, and when appropriate adjustments to the degrees of freedom were made using the Greenhouse–Geisser correction method. Significant main effects were analysed using a post hoc Tukey's HSD test. The acceptance level of significance was set at *p*< 0.05.

8.4 Results

Energy intake and hydration status. No significant differences in total daily energy intake (mean ± SD: PRO 2329 ± 245 kCal·day^{-1}, PLA 2449 ± 364 kCal·day^{-1}; p= 0.190), carbohydrate (PRO 307 ± 72 g·day^{-1}, PLA 320 ± 77 g·day^{-1}; p= 0.304), protein (PRO 101 ± 11 g·day^{-1}, PLA 102 ± 19 g·day^{-1}; p= 0.833), fat (PRO 76 ± 9g·day^{-1}, PLA 82 ± 16 g·day^{-1}; p= 0.503), and water intake (PRO 2.6 ± 0.4 L·day^{-1}, PLA 2.8 ± 0.4L·day^{-1}; P= 0.064) during the seven-days supplementation period were observed between PRO and PLA.

No significant difference in *ad libitum* water intake during EHS was observed between PRO and PLA (1.7 ± 1.4 L and 1.9 ± 1.8 L, respectively). Exercise-induced BM loss occurred pre- to post-EHS on PRO (1.5 ± 1.5%; p= 0.030) and PLA (1.3 ± 1.4%; p= 0.029). Compared with pre-EHS values, no significant changes in immediate post-EHS and recovery P_{Osmol} were observed on PRO (pre-EHS: 296 ± 7 mOsmol·kg^{-1}, post-EHS: 302 ± 10 mOsmol·kg^{-1}, and overall recovery: 298 ± 12 mOsmol·kg^{-1}) and PLA (pre-EHS: 294 ± 6 mOsmol·kg^{-1}, post-EHS: 297 ± 11 mOsmol·kg^{-1}, and overall recovery: 297 ± 8 mOsmol·kg^{-1}). Moreover, P_{Osmol} did not differ between PRO and PLA throughout the experimental protocol.

Cardiovascular and thermoregulatory strain. No significant difference in HR (165 ± 16 bpm) and 165 ± 16 bpm), T_{re} (38.6 ± 0.8°C and 38.6 ± 0.7°C), RPE (12 ± 3 and 13 ± 3), and TCR (11 ± 1 and 11 ± 1) were observed between PRO and PLA during EHS, respectively.

Saliva flow rate. A significant main effect of time was observed (p= 0.004; **Table 8.1**), whereby a significantly higher SFR was observed 4 h post-EHS and at 24 h compared with

pre-EHS. No significant main effects of trial or trial x time interaction were observed for SFR.

Table 8.1: Saliva flow rate in response to 2 h running at 60% VO_{2max} in hot ambient conditions (exertional-heat stress) following seven consecutive days of probiotic and placebo supplementation.

	Baseline	Pre EHS	Post EHS	1 h post EHS	2 h post EHS	4 h post EHS	24 h
Saliva Flow Rate ($\mu l \cdot min^{-1}$)						†	†
PRO	472 ± 224	558 ± 280	543 ± 293	699 ± 300	659 ± 229	644 ± 360	631 ± 298
PLA	435 ± 314	501 ± 355	430 ± 285	535 ± 214	648 ± 284	717 ± 367	631 ± 255

Mean ± SD (n= 8). Main effect of time † $p<$ 0.05 *vs.* pre-EHS.

Salivary anti-microbial protein responses. PRO did not induce significant changes in resting pre-EHS S-IgA, S-α-amylase and S-lysozyme concentrations and secretion rates compared with PLA (**Table 8.2**). Significant main effects of time were observed for S-IgA (concentration $p=$ 0.004 and secretion rate $p=$ 0.005) and S-α-amylase (concentration $p=$ 0.011 and secretion rate $p=$ 0.045) responses only. Whereby, S-IgA concentration and secretion rate were significantly higher immediately post-EHS (37% and 23%, respectively), but S-IgA concentration was lower at 24 h (32%), compared with pre-EHS values; and S-α-amylase concentration and secretion rate were significantly higher immediately (267% and 186%, respectively) and 1 h post-EHS (90% and 93%, respectively) compared with pre-EHS values (**Table 8.2**). No significant main effects of trial or trial x time interaction were observed for S-IgA, S-α-amylase, and S-lysozyme concentrations and secretion rates (**Figures 8.1 to 8.3**).

Table 8.2: Salivary anti-microbial protein responses to 2 h running at 60% VO_{2max} in hot ambient conditions (exertional-heat stress) following seven consecutive days of probiotic and placebo supplementation.

	Baseline	Pre EHS	Post EHS	1 h post EHS	2 h post EHS	4 h post EHS	24 h
Salivary IgA concentration ($\mu g \cdot ml^{-1}$)			†				††
PRO	264 ± 110	396 ± 113	527 ± 317	360 ± 215	423 ± 193	360 ± 225	283 ± 147
PLA	383 ± 185	432 ± 182	606 ± 170	448 ± 105	369 ± 128	396 ± 187	282 ± 160
Salivary IgA secretion rate ($\mu g \cdot min^{-1}$)			†				
PRO	121 ± 84	229 ± 131	260 ± 183	255 ± 201	286 ± 157	203 ± 117	192 ± 126
PLA	160 ± 160	196 ± 120	264 ± 182	245 ± 125	236 ± 116	223 ± 124	165 ± 94
Salivary α-amylase concentration ($U \cdot ml^{-1}$)			††	†			
PRO	96 ± 91	139 ± 156	385 ± 302	200 ± 174	169 ± 145	134 ± 103	48 ± 41
PLA	70 ± 41	67 ± 57	371 ± 380	191 ± 169	178 ± 205	173 ± 187	117 ± 127
Salivary α-amylase secretion rate ($U \cdot min^{-1}$)			†	†			
PRO	42 ± 32	97 ± 117	238 ± 226	162 ± 183	113 ± 94	100 ± 95	32 ± 33
PLA	38 ± 39	50 ± 68	184 ± 242	122 ± 145	141 ± 165	126 ± 118	75 ± 71

Salivary lysozyme concentration ($\mu g \cdot ml^{-1}$)

PRO	4.4 ± 9.3	4.7 ± 10.4	7.4 ± 10.9	----	3.8 ± 8.7	5.1 ± 10.0	----
PLA	2.8 ± 5.5	2.2 ± 2.6	4.4 ± 7.9	----	4.3 ± 7.2	5.3 ± 10.6	----

Salivary lysozyme secretion rate ($\mu g \cdot min^{-1}$)

PRO	1.3 ± 2.1	1.9 ± 3.7	3.2 ± 5.4	----	2.4 ± 5.2	3.0 ± 5.7	----
PLA	0.9 ± 1.3	1.0 ± 1.0	2.5 ± 4.5	----	3.0 ± 5.5	2.8 ± 4.4	----

Mean $\pm$ SD (*n*= 8). Main effect of time [†] *p*< 0.05 and [††] *p*< 0.01 *vs.* pre-EHS.

Figure 8.2: Changes in salivary IgA concentration (A) and secretion rate (B) in response to 2 h running at 60% VO_{2max} in hot ambient conditions (exertional-heat stress) following seven consecutive days of probiotic and placebo supplementation. Mean ± SEM (*n*= 8): PRO (O) and PLA (■).
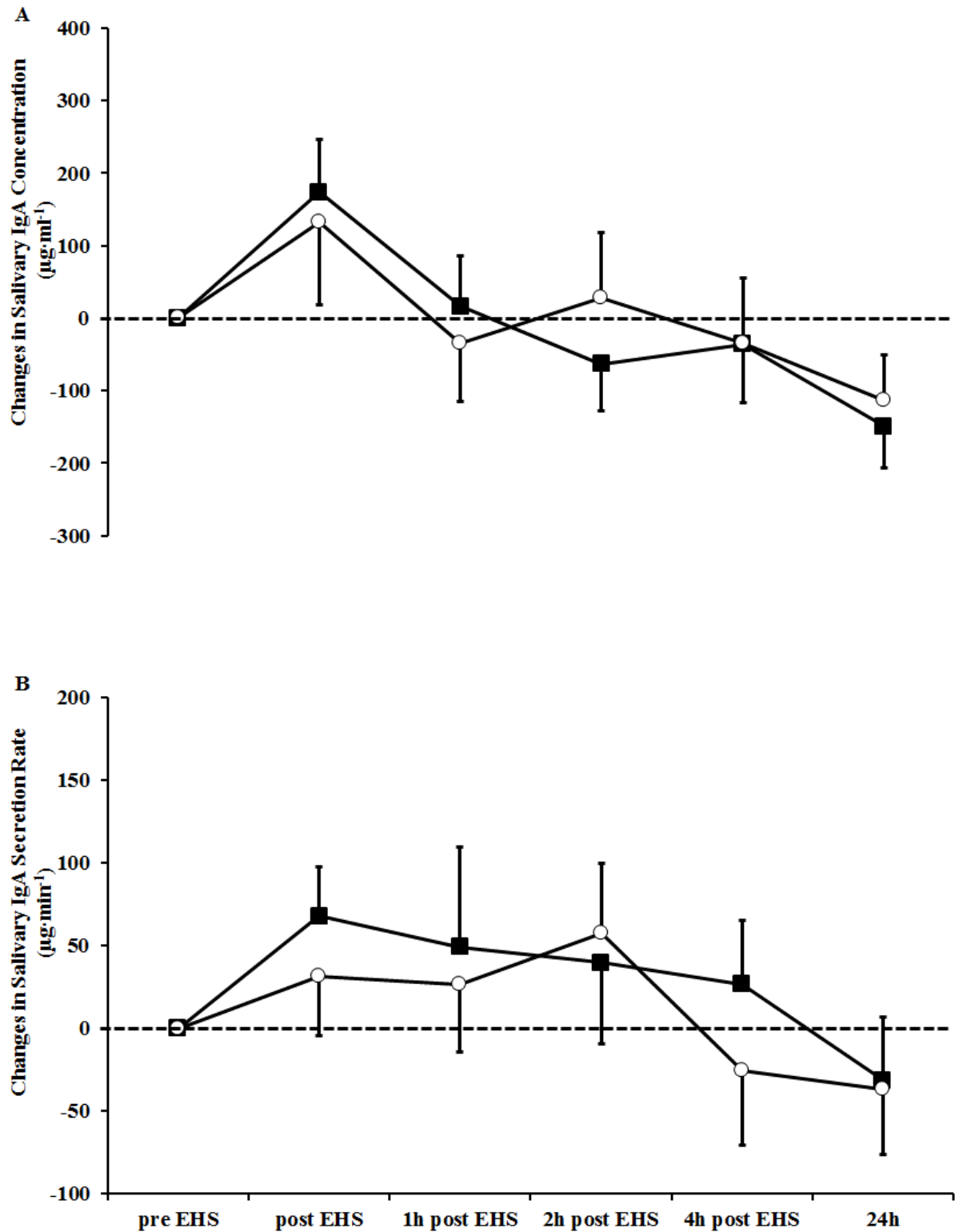
Figure 8.3: Changes in salivary α-amylase concentration (A) and secretion rate (B) in response to 2 h running at 60% VO_{2max} in hot ambient conditions (exertional-heat stress) following seven consecutive days of probiotic and placebo supplementation. Mean ± SEM (*n*= 8): PRO (O) and PLA (■).
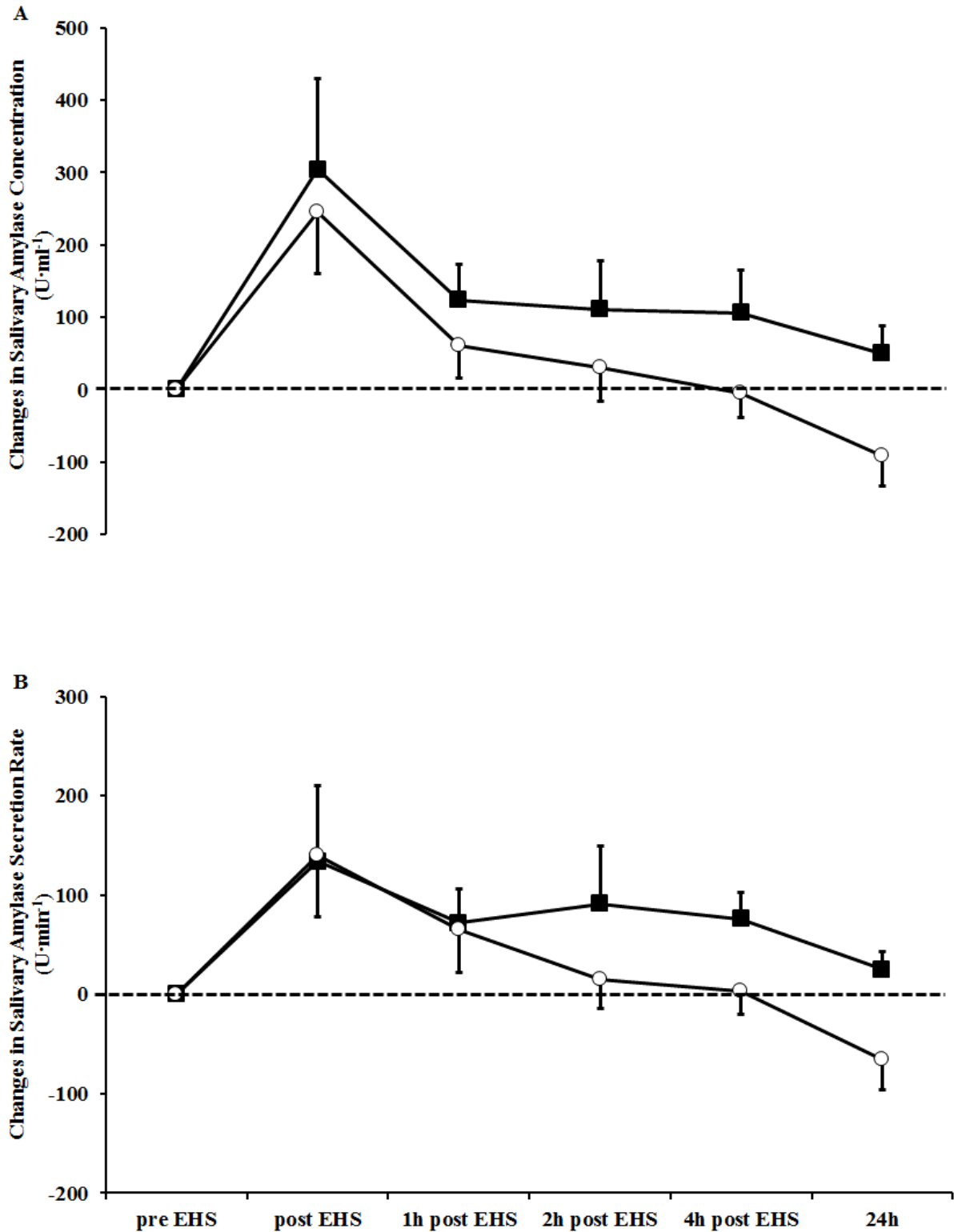
Figure 8.4: Changes in salivary lysozyme concentration (A) and secretion rate (B) in response to 2 h running at 60% VO_{2max} in hot ambient conditions (exertional-heat stress) following seven consecutive days of probiotic and placebo supplementation. Mean ± SEM (*n*= 8): PRO (O) and PLA (■).
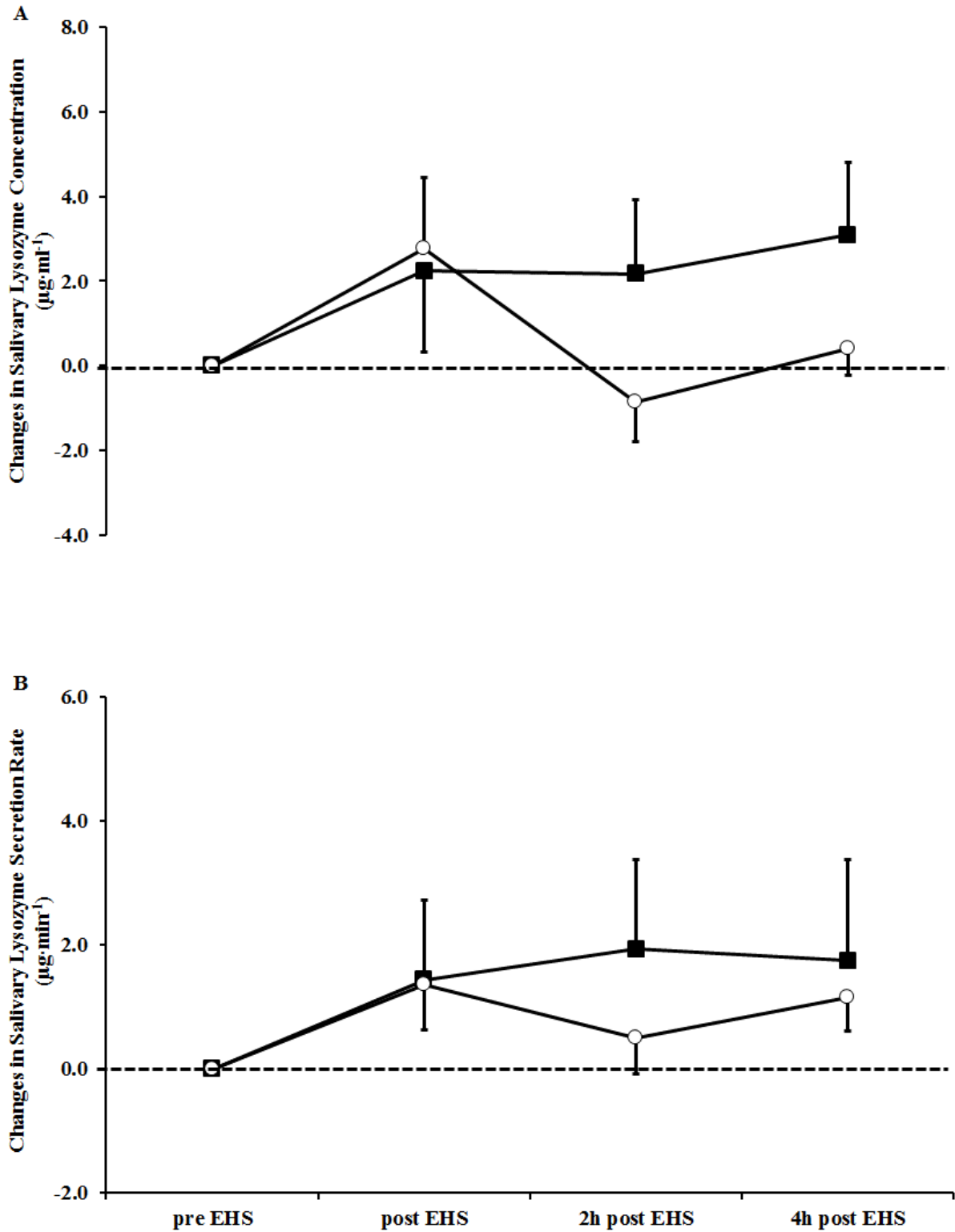
Salivary cortisol responses. PRO did not induce significant changes in resting pre-EHS S-cortisol concentration and appearance rate compared with PLA. A significant main effect of time was observed for S-cortisol concentration ($p= 0.05$), but not appearance rate ($p= 0.072$) (**Table 8.3**); whereby S-cortisol concentration was significantly higher immediately and 1 h post-EHS (84% and 47%, respectively), but lower 2 h and 4 h post-EHS (32% and 64%, respectively) compared with pre-EHS values. No main effects of trial or trial x time interaction were observed for S-cortisol concentration and appearance rate.

Table 8.3: Salivary cortisol responses to 2 h running at 60% VO_{2max} in hot ambient conditions (exertional-heat stress) following seven consecutive days of probiotic and placebo supplementation.

	Baseline	Pre EHS	Post EHS	1 h post EHS	2 h post EHS	4 h post EHS	24 h
Salivary cortisol concentration (μg·ml^{-1})			*	†	†	††	
PRO	22 ± 9	29 ± 13	55 ± 44	42 ± 22	20 ± 9	9 ± 4	35 ± 23
PLA	32 ± 15	38 ± 20	68 ± 68	56 ± 44	25 ± 15	15 ± 10	30 ± 35
Salivary cortisol appearance rate (μg·min^{-1})							
PRO	11 ± 7	16 ± 10	30 ± 23	31 ± 21	13 ± 6	5 ± 3	25 ± 22
PLA	13 ± 11	17 ± 11	34 ± 39	28 ± 20	17 ± 13	10 ± 6	13 ± 10

Mean $\pm$ SD ($n = 8$). Main effect of time † $p< 0.05$ and †† $p< 0.01$ *vs.* pre-EHS; main effect of time * $p= 0.074$ *vs.* pre-EHS.

8.5 Discussion

The current study aimed to determine if oral supplementation of a commercially available probiotic beverage containing *L.casei* (x 10^{11} CFU·day^{-1}) for seven consecutive days can enhance S-AMP responses to EHS in an endurance trained population. In contrast with our hypothesis, one week of PRO supplementation did not enhance S-AMP responses after or during recovery from EHS compared with PLA. Despite the large bacterial exposure, all resting S-AMP values were unaltered after seven days of PRO supplementation. S-AMP increased in response to EHS, with depressions in S-IgA seen after 24 h, and no difference between PRO and PLA observed. It is thus unlikely that acute high dose supplementation of a probiotic beverage containing *L.casei* prior to EHS provides any further oral-respiratory mucosal immune protection (i.e., prevention or reduction in URSI episodes), with respect to S-AMP, over a placebo.

It is plausible that repetitive probiotic administration over consecutive days may lead to enhanced S-AMP responses, especially during compromised periods (Gleeson *et al.* 2011a). On the contrary, despite the high probiotic dosage of the current study, S-IgA responses were unaltered after seven days of supplementation, and did not differ from PLA immediately post-EHS and during the recovery period. Equally, no significant changes in S-IgA responses with probiotic supplementation (L.fermentum VRI-003) was observed over four months of winter training in elite male distance runners (Cox *et al.* 2010b); whilst no difference in S-IgA responses was observed with probiotic supplements (L.casei DN-114 001) during three weeks of intensified military training followed by a five-days combat course compared with placebo (Tiollier *et al.* 2007).

In the current study, no changes in S-α-amylase and S-lysozyme responses were observed after seven days of PRO; but on both trials, S-α-amylase responses increased after EHS, which likely reflect the degree of the exertional stress *per se* (Bosch *et al.* 2003b, Rosa *et al.* 2014). Thus, the clinical relevance of probiotic use in populations exposed to EHS is unclear. It is possible that in immunocompromised and (or) illness-prone individuals partaking in exertional or EHS who commonly experience URSI may benefit from longer term low dose probiotic supplementation, given that previous research has shown mild reductions in severity and (or) duration of URSI (Gleeson *et al.* 2011a, Cox *et al.* 2010b). However, these outcomes are likely to be external to S-AMP responses.

In conclusion, the results of the current study suggest that a daily oral supplementation of a commercially available probiotic beverage containing *L.casei* for seven consecutive days does not influence S-AMP responses and subsequent oral-respiratory mucosal immune status above placebo in response to EHS in an endurance trained population. During the trial periods, maintenance of hydration status (and SFR) and the exertional stress *per se* (e.g., increases in S-α-amylase responses) appear to have a more favourable impact on S-AMP status than acute high dose probiotic supplementation.

CHAPTER NINE

Acute Supplementation of *Lactobacillus Casei* on Circulatory Endotoxin Concentrations

and Cytokine Profile in Response to Exertional-Heat Stress.

Gill, S. K., Allerton D. M., Ansley-Robson, P., Hemmings, K., Cox, M., and Costa, R. J. S., (2015) 'Does Acute High Dose Probiotic Supplementation Containing *Lactobacillus casei* Attenuate Exertional-Heat Stress Induced Endotoxaemia and Cytokinaemia?' In press.

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10.1 Background

Prolonged physical exertion with accompanying physiological stressors ((e.g., environmental extremes, sleep deprivation and compromised hydration and (or) nutritional status)) have all been individually reported to influence host defence through stimulation of neuroendocrine responses (namely cortisol and catecholamines), leading to alterations in immune responses. It is therefore plausible that participating in extreme physical exertion (e.g., activities such as ultra-endurance, adventure and exploration events, military training, expeditionary operations) with accompanying physiological stressors, particularly in unfamiliar environments harboured by pathogens unrecognisable by the immune system, may induce neuroendocrine responses to a greater extent, prompting amplified or cumulative perturbations to host defence and increasing susceptibility of unwanted clinically significant outcomes (Martinez-Lopez *et al.* 1993, Neville, Gleeson, and Folland 2008, Nieman 1997, Shephard, Castellani, and Shek 1998). Notably, due to the observational nature of the ultra-marathon studies (**Chapter 4** to **Chapter 7**) and a subsequent lack of adequate research control, determining which individual or combination of stressors responsible for the perturbations in immune variables observed is limited.

More recently, probiotic consumption in active populations as a strategy to attenuate and (or) prevent immune perturbations and unwanted clinically significant outcomes associated with the aforementioned physiological stressors, has gained considerable research interest within exercise immunology. Known for their immunostimulatory effects, it is plausible that probiotic use may attenuate and (or) prevent immune perturbations, thus reducing

susceptibility to illness and infection and promoting sporting potential and performance amongst active populations. The probiotic-EHS studies (**Chapter 8** and **Chapter 9)** was conducted using $n= 8$ and thus considered a relatively small sample size. Notably, a larger sample size would be considered more representative of the endurance running population and would have allowed for further sub-group analysis.

The broad aims of this thesis were therefore to investigate: 1. oral-respiratory mucosal immune status (S-IgA, S-α-amylase and S-lysozyme), stress hormone response (S-cortisol) and incidence of URS in response to extreme physical exertion with accompanying multi-stressors reported to disturb immune homeostasis; 2. intestinal epithelial integrity (assessed by circulatory endotoxin concentration), cytokine profile and incidence of GI symptoms in response to extreme physical exertion with accompanying multi-stressors reported to disturb immune homeostasis and finally, 3. acute high dose probiotic supplementation in the prevention of unwanted perturbations to oral-respiratory mucosal immune status and intestinal epithelial integrity in response to EHS reported to disturb immune homeostasis.

10.2 Salivary Anti-Microbial Protein Responses and Extreme Physical Exertion, With and Without Heat-Stress

The field-based investigative studies presented in **Chapter 4** and **Chapter 5** provide a novel insight into the effects of ultra-marathon competition on oral-respiratory mucosal immunity. The first study (**Chapter 4**) aimed to determine the effects of a multi-stage ultra-marathon in hot ambient conditions, whilst the second study (**Chapter 5**) aimed to determine the effects of a 24 h continuous ultra-marathon in temperate ambient conditions. Previous studies have indicated that partaking in prolonged physical exertion is associated with depressions in S-IgA and an increased susceptibility to URSI (Bishop and Gleeson 2009, Gleeson 2000, Neville, Gleeson, and Folland 2008, Nieman *et al.* 2006a, Nieman 1997, Nieman 1994) *via* mechanisms such as the degree of exercise-stress (e.g., duration), environmental extremes, increased circulatory stress hormone concentrations, and compromised hydration status and energy inadequacy (Costa *et al.* 2005, Fortes *et al.* 2012, Heath *et al.* 1991, Sabbadini and Berczi 1995, Saxon *et al.* 1978). Albeit, the influence of extreme physical exertion on S-IgA is extremely limited, whilst the influence of other S-AMPs on oral respiratory mucosal immune status have been investigated to a far lesser degree. To date, the study presented in **Chapter 4** was the first to assess and track oral-respiratory mucosal immune parameters (S-IgA, S-α-amylase, and S-lysozyme) during a multi-stage ultra-marathon in the heat; whilst the study presented in **Chapter 5** was the first to assess and explore oral-respiratory mucosal immune parameters during a 24 h continuous ultra-marathon in the absence of heat stress but with inclusion of other physiological stressors known to perturb immune function.

The field-based study conducted in **Chapter 4** demonstrated that only mild alterations in S-AMP responses were seen as a result of the ultra-marathon competition in the heat. These

mild alterations may not have been large enough to promote and (or) increase pathogen adherence and infection incidence. Additionally, the lower incidence of the common cold during the mid-summer months is likely to have contributed to the low incidence of illness symptoms reported by runners.

In the multi-stage ultra-marathon study, an average exercise-induced BM loss of 2.0% and a post-stage P_{Osmol} of 293 mOsmol·kg^{-1} was observed along the ultra-marathon course. The consistent euhydration status observed in the majority of runners ($\leq$1.5% BM loss in 67% of UER) may have prevented substantial depressions in SFR and provided protection against the drying of oral-respiratory mucosal surfaces that has been associated with URS (e.g., inflammation and exercise-induced asthma). Indeed, previous laboratory-controlled dehydration protocols (3.5% BM loss and P_{Osmol} of 297 mOsmol·kg^{-1}) have reported reductions in the SFR (67%), resulting in depressed S-AMP responses (Fortes *et al.* 2012). In the multi-stage ultra-marathon study, the pre- to post-stage reduction in the SFR ranged from 20-37%, whereas the S-AMP responses generally remained unchanged or showed increased responses (**Table 4.2**). These results are in accordance with the findings of previous laboratory-based trials reporting 20-28% reductions in SFR, 1.9-2.4% exercise-induced BM loss, and S-AMP responses generally remaining unchanged or increased during the recovery period after 2 h of strenuous running (Costa *et al.* 2012, Costa *et al.* 2009). If larger numbers of UER had produced greater degrees of post-stage hypohydration (e.g., >3.0%), it is speculated that greater overall reductions in the SFR and larger overall perturbations to the S-AMP responses may have been observed.

It is likely that the changes in S-AMP responses observed (i.e., decreases in S-IgA secretion rate and increases or unchanged S-α-amylase and S-lysozyme) were attributable to

mechanistic differences in the storage and secretion (endocytosis *vs.* transcytosis) of S-AMPs (Bishop and Gleeson 2009) and (or) neuroendocrine influences. For instance, S-α-amylase and S-lysozyme are synthesized and stored within secretory granules, unlike S-IgA, which is produced by B-lymphocyte cells and transported across epithelial cells to the mucosal surface by PIgR activity (Bishop and Gleeson 2009). Because sympathetic activation alters saliva composition and elicits a high protein content secretion, exercise heat-induced stimulation may promote a greater secretion of S-α-amylase and S-lysozyme into the saliva (Bishop *et al.* 2000, Chatterton *et al.* 1996). Alternatively, the contribution of different salivary glands and different stimuli activating specific glands may have also played a role in the observed altered responses of the S-AMPs (Noble 2000). Moreover, saliva secretions are under neuroendocrine control (Carpenter *et al.* 2000, Carpenter *et al.* 1998, Chicharro *et al.* 1998; Teeuw *et al.* 2004, Tenovuo 1998). Therefore, HPA activation and an increase in cortisol concentrations (**Table 4.3**) causing inhibitory effects on the transepithelial transport of IgA, rather than IgA synthesis (Hucklebridge, Clow, and Evans 1998), in conjunction with reductions in the SFR and altered saliva composition (Carpenter *et al.* 2000, Carpenter *et al.* 1998, Chicharro *et al.* 1998, Teeuw *et al.* 2004, Tenovuo 1998), may in part, explain the pre- to post-stage reductions in the SFR on Stages 1-4 and the S-IgA secretion rates on Stages 2-5 in UER, as well as the large discrepancies in pre-stage S-IgA values between UER and CON, although this failed to reach significance (**Table 4.1**).

In the multi-stage ultra-marathon study, transient fluctuations were observed in all salivary immune markers throughout the ultra-marathon competition, with the post-stage changes being the most noticeable. Any observed reductions in post-stage S-IgA responses were compensated for by increases in S-α-amylase and S-lysozyme responses during the competition. These results are similar to those of previous laboratory-controlled studies

(Allgrove *et al.* 2008; Costa *et al.* 2012); they highlight the importance of collectively assessing oral-respiratory immune markers when determining oral-respiratory mucosal immune status in active populations and suggest no evidence of depressed oral-respiratory immune status in the majority of runners during a multi-stage ultra-marathon.

Taking into account that S-AMPs act synergistically to protect the oral respiratory pathway from pathogen invasion, and more specifically, that S-α-amylase and S-lysozyme exhibit similar protective properties similar as S-IgA, the previously proposed "open window hypothesis", whereby viruses and (or) bacteria may gain a hold on oral-respiratory mucosal surfaces, leading to illness and (or) infection (Nieman 1994), often attributed to the commonly observed transient decreases in S-IgA responses (Costa *et al.* 2009, Gleeson *et al.* 2000, Neville *et al.* 2008), may be misleading. Given the low prevalence (*n*= 1) of URS during and up to four weeks after competition, future studies should investigate alterations in S-AMPs, other than S-IgA, in relation to illness and (or) infection incidence during the common cold season.

Often, illness symptoms are assessed through self-report because of the practical issues associated with medical diagnosis in a field-based setting. It is recognized that the illness symptoms reported by runners may simply reflect other conditions that mimic URS (Cox *et al.* 2010a; Cox *et al.* 2008, Robson-Ansley *et al.* 2012), and for this reason, it is difficult to distinguish causation (Spence *et al.* 2007). More recently, a 'non-infectious' hypothesis was developed, in which inflammatory factors (e.g., irritants, pollen, pollution, dust, other allergens) manifest as infectious URS (Spence *et al.* 2007, Robson-Ansley *et al.* 2012, Walsh *et al.* 2011a).This may explain, in part, these seemingly higher incidence of symptoms frequently reported after endurance exercise (Gleeson 2000, Gleeson and Pyne 2000). In

view of the extreme nature of multi-stage ultra-marathon competition, the illness symptom reports in the runners were extremely low. The effective management of allergy–respiratory illness symptoms (e.g., asthma) through the appropriate use of medication around competition; forecasts of low to moderate pollen counts across the region during the time of competition; low levels of environmental air pollution in the course vicinity; and (or) maintaining hydration status, thus preventing the drying of mucosal surfaces and ensuring the permanent presence of S-AMPs at the oral-respiratory mucosal surface (Anderson and Kipperlen 2008, Cox *et al.* 2010a, Cox *et al.* 2008, Robson-Ansley *et al.* 2012, Tenovuo 1998) are all factors that may have contributed to the low incidence of URS.

However, a potential limitation of the multi-stage ultra-marathon study is the failure to measure other S-AMPs (e.g., lactoferrin) with anti-bacterial and anti-viral properties, which act synergistically with those reported and play a significant role in maintaining oral-respiratory mucosal immune status (Walsh *et al.* 2011a, West *et al.* 2006), giving a better global overview of salivary immune responses to consecutive days of EHS. Additionally, the extreme nature of the event and the invasive techniques used to determine blood borne hydration status at frequent points during the ultra-marathon created a potential barrier to obtaining full data sets from all the recruited participants ($n = 37$).

From a practical viewpoint, the findings from the multi-stage ultra-marathon study suggest that appropriate education (e.g., hydration maintenance, non-infectious episode management, medical management of established respiratory illness) and information (i.e., pollen and pollution counts at the competition location) may help prevent significant clinical and performance decrements from occurring in UER during multi-stage ultra-marathon competitions conducted in hot ambient environments.

The field-based study presented in **Chapter 5** demonstrated reductions in some S-AMP secretion rates post-competition (i.e., S-IgA and S-lysozyme), and increases in others (i.e., S-α-amylase). Despite the 24 h continuous exercise-stress, in which participants were exposed to a period of cold ambient conditions and energy inadequacy, no URS were reported by UER during and 4 weeks following the ultra-marathon.

Given that S-α-amylase exhibits similar protective properties as other S-AMPs (Walsh *et al.* 2011a, West *et al.* 2006), in the 24 h continuous ultra-marathon study, its enhanced response may have offset any potential negative impact of reduced S-IgA and S-lysozyme responses (**Table 5.1**). Similar increases in S-α-amylase responses have been observed during recovery from 2 h running at 75% VO_{2max} in controlled laboratory trials (Costa *et al.* 2012, Costa *et al.* 2009) and a 230 km multi-stage ultra-marathon competition (**Chapter 4**); although both studies also showed increased S-lysozyme responses, dissimilar to that of the 24 h continuous ultra-marathon study.

The degree of S-α-amylase response has previously been associated with stress stimulus *per se* (Bosch *et al.* 2003b, Filaire *et al.* 2010, Filaire *et al.* 2009, Rosa *et al.* 2014). It is therefore not surprising that large increases were observed as a result of 24 h of extreme duration, physical exertion. Interestingly, the distance covered by UER did not influence the degree of S-α-amylase response on this occasion. Taking this into account, stress induced increases in S-α-amylase response may actually provide a protective mechanism to oral-respiratory immunity, when other protective components are compromised. Even though there exists evidence of S-AMPs responding to pathogenic microbial colonization (i.e., adherence and coadherence activity) on mucosal microflora (Bosch *et al.* 2003b) under stress conditions, it remains unclear the degree to which each relevant S-AMP contributes individually towards

oral-respiratory immunity, and the degree of compensatory and collaborative mucosal immune activity under stress conditions. Future investigations could potentially aim at developing an oral-respiratory mucosal immune index which determines URTI risk based upon collective S-AMP values (e.g., S-IgA, S-α-amylase, S-lysozyme, S-lactoferrin) and acknowledges deviations in individual S-AMP activity and interactions with each other in response to stress stimulus.

Given the extreme nature of ultra-endurance events, UER partaking in the 24 h continuous ultra-marathon study were free from illness in the twelve weeks before the ultra-marathon, thereby excluding mildly immunocompromised and (or) illness-prone individuals, whose training load may have been affected and their ability to complete the 24 h continuous ultra-marathon markedly reduced. Illness-infection symptoms reported by runners, in field-based experimental models, are frequently assessed through self-reports due to the practical issues associated with medical diagnosis. Since verification of infectious upper respiratory episodes are rarely confirmed, the majority of studies reporting high incidences of URS are more likely to reflect non-infectious and (or) inflammatory factors that mimics infectious URS (Cox *et al.* 2010a, Cox *et al.* 2008, Robson-Ansley *et al.* 2012), generally induced by environmental influences (e.g., irritants, pollen, pollution, dust, and (or) other allergens). In the 24 h continuous ultra-marathon study, forecasts of low to moderate pollen counts across the region during the time of competition, reduced risk of opportunistic infections due to the isolated competition location and low population aggregation, low levels of environmental air pollution in the course vicinity, seasonal variations in levels of Vitamin D that are highest during the late summer, and (or) optimal prevention and management strategies of established respiratory illness, are all factors that may have contributed to the low incidence of infectious and non-infectious URS observed (Anderson and Kipperlen 2008, Anderson and

Daviskas 2000, Cox *et al.* 2010a, Cox *et al.* 2008, Robson-Ansley *et al.* 2012, Tenovuo 1998).

In the 24 h continuous ultra-marathon study, amplified cortisol concentrations were observed post-competition **(Figure 5.2).** Such hormone derived S-AMP responses are potentially irrespective of hydration status (Fortes *et al.* 2012, Oliver *et al.* 2007). For example, euhydration was seen in the majority of UER (≤2% BM loss in 63% of UER, P_{Osmol} <300 mOsmol·kg^{-1} in all UER), with only 29% of UER having a ≥3% BM loss post-competition indicative of a hypohydrated state (Sawka *et al.* 2007), shown to be necessary to substantially impact upon S-AMP responses (Fortes *et al.* 2012). Interestingly, SFR and S-AMP responses in the 24 h continuous ultra-marathon study did not differ according to hydration status (≤1.5% *vs.* ≥3.0% BM loss). Despite modest reductions in SFR observed in the hypohydrated group (40%), reductions were considerably less than previous dehydration protocols reporting 62-67% decreases in SFR with BM losses ≥3.0% (Oliver *et al.* 2007, Walsh *et al.* 2004a). Moreover, favourable environmental conditions (i.e., high RH) during competition may have played a role in preventing airway drying, and subsequently attenuating mucosal surface irritation, inflammation, and symptom presentation and (or) aggravation (Anderson and Kipperlen 2008, Anderson and Daviskas 2000, Robson-Ansley *et al.* 2012).

Reflecting on the current findings which acknowledges no reports of URS in response to extreme physical exertion, effective management strategies of non-infectious respiratory illnesses (i.e., allergies and asthma) and providing environmental information before ultra-endurance events (i.e., RH, pollen and pollution counts at competition location) may help to prevent and manage sub-clinical and (or) clinical manifestations of URS from occurring amongst UER before, during, and after ultra-marathon events through adequate preparation

strategies (i.e., administration of pre-event medications based on environmental information, carrying allergy medications, and (or) ensuring sufficient fluids to optimise hydration). In addition, such preventative and management strategies can also be applied to those populations exposed to similar physiological strains (e.g., military personnel, adventure-expedition crews (i.e., medical, health, and media), miners, and (or) park rangers) who regularly undertake occupational-related activities in extreme environmental conditions for prolonged periods of time.

A particular strength of the 24 h continuous ultra-marathon study was the assessment of multiple salivary immune biomarkers, as reported previously (Costa *et al.* 2012); but measurements of other salivary proteins (e.g., lactoferrin and (or) α-defensin), with known antibacterial-viral properties, would have complemented the current findings. Due to practical constraints, S-AMP measurements were limited to pre- and immediately post-competition; therefore the time-course of S-AMP recovery was not determined on this occasion. In accordance with previous studies, it is likely that perturbations observed in S-AMPs would have returned to similar baseline levels within 4 h into recovery, and fully recovered within 24 h (Costa *et al.* 2013a, Costa *et al.* 2012).

From a practical viewpoint, effective management of non-infectious episodes, favourable environmental conditions, maintaining euhydration, and the isolated location of the ultra-marathon course vicinity may have accounted for the low prevalence of URS in UER on this occasion.

10.3 Circulatory Endotoxin, Cytokine Profile and Extreme Physical Exertion With and Without Heat-Stress

The field-based investigative studies presented in **Chapter 6** and **Chapter 7** present a new aspect into the effects of ultra-marathon competition on intestinal epithelial integrity (assessed by circulatory endotoxin concentration). The third study (**Chapter 6**) aimed to determine circulatory endotoxin concentration and cytokine profile of UER throughout a multi-stage ultra-marathon competition conducted in hot ambient conditions; whilst the second study (**Chapter 7**) aimed to determine the effects of a 24 h continuous ultra-marathon in temperate ambient conditions. Previous studies have indicated that partaking in prolonged physical exertion is associated with disruption to intestinal epithelial integrity and increases in intestinal permeability, leading to endotoxaemia and responsive cytokinaemia (Camus *et al.* 1998, Camus *et al.* 1997, Lambert 2009, van Wijck *et al.* 2012) *via* mechanisms such as the degree of exercise-stress, environmental extremes (and subsequent hyperthermia), splanchnic ischemia-hypoperfusion and tissue hypoxia, direct mechanical trauma and compromised hydration status (Lambert *et al.* 2008, Rehrer and Meijer 1991, ter Steege and Kolkman 2012, van Wijck *et al*. 2012). In a military setting, exertional-heat illness continues to be a problem during training and operations with epidemiological data showing marked increases in the hospitalization rate of heat stroke from 1980 to 2001 (Carter *et al.* 2005). To date, the study presented in **Chapter 6** was the first to explore and track intestinal epithelial permeability of endotoxins and cytokine profile over consecutive days of exercise-heat stress and to determine the relationship between these responses with severe GI symptoms and perceptive thermal tolerance rating; whilst the study presented in **Chapter 7** was the first to investigate intestinal epithelial permeability and cytokine profile during a 24 h continuous ultra-marathon in the absence of heat-stress, but with exposure to other multi-stressors known

to perturb intestinal epithelial integrity, to determine whether a similar rendotoxaemic and cytokinaemic response would be observed. Additionally, to determine the relationship between these responses with GI symptoms.

The field-based study presented in **Chapter 6** showed modest circulatory endotoxaemia accompanied by a pronounced pro-inflammatory cytokinaemia which was counteracted by a compensatory anti-inflammatory cytokine response. Circulatory endotoxin concentration, pro- and anti-inflammatory responses were sustained throughout competition at rest and post-stage. Strength of the findings is supported by the control group showing no change in circulatory endotoxin concentration and cytokine profile (except IL-1β) throughout the ultra-marathon period.

Even though UER presented no heat-related medical issues during the multi-stage ultra-marathon study, the endotoxin and cytokine responses observed provide a novel and valuable insight into a potentially high-risk situations, whereby individuals with predisposition and multiple risk factors for deranged pro-inflammatory and compensatory anti-inflammatory responses are likely to develop consequential clinically significant issues. For example, the degree of cytokinaemia observed has been linked to the aetiology of heat stroke, septic shock, autoimmune disease, GI disease, and chronic fatigue (Caradonna *et al.* 2000, Lim and Mackinnon 2006, Morris *et al.* 2014, Opal 2010, Robson 2003).

Plasma CRP concentration, which is normally low and undetectable in the circulation of healthy populations, is an acute phase reactant that dramatically rises in the presence of inflammation (e.g., induced by trauma, bacterial infection, and (or) inflammatory responses). In the multi-stage ultra-marathon study, plasma CRP concentrations of UER increased in

response to EHS (increasing by Stage 2) and remained elevated throughout competition; while no change in CON was observed. These responses are similar to that observed during a six-days (total distance: 468 km) endurance mountain bike event (Robson-Ansley *et al.* 2009), whereby plasma concentrations were significantly elevated at rest for the duration of the event. However, the level of rise in plasma CRP concentration was less pronounced in comparison to levels (1.9 to 18.4 mg·l) reported in ultra-endurance athletes after six-days of track based running race totalling 622 km (Fallon 2001), and after the 246 km Spartathlon ultra-marathon race (0.65 to 97.3 mg·l) (Margeli *et al.* 2005). Taking into account the responsive nature of CRP to general inflammation, the wide variations in plasma concentrations observed in the current and previous ultra-endurance studies likely reflect multi-influential stimulating factors, such as intestinal originated bacterial endotoxin leakage into circulation ($r^2 = 0.343$, *p*= 0.001) and soft tissue damage (e.g., exertional rhabdomyolysis) (Clarkson 2007). It is possible that persistent elevations in CRP at rest in UER may contribute to progressive perceptions of fatigue and subsequent impaired performance over the given time course (Neubauer, Konig, and Wagner 2008, Robson 2003).

Interestingly, males showed high plasma CRP concentration throughout competition compared with females. The reason for this observation is unclear; it is however likely to be attributed to muscle originated responses (Steensberg *et al.* 2000), taking into account greater plasma IL-6 concentrations concomitant with lower IL-1β and TNF-α responses observed in males compared with females. Due to practical limitations in monitoring parameters after competition (i.e., ultra-endurance athletes returning to country of origin after cessation of the ultra-marathon), the multi-stage ultra-marathon study was not able to determine the recovery time course of CRP. However, such responses have been shown to remain elevated above pre-exercise values for a considerable period of time (i.e., up to nineteen-days after an

Ironman triathlon event) (Neubauer, Konig, and Wagner 2008), suggesting time course for full recovery of altered inflammatory status is considerably delayed.

In comparison with previous endurance and ultra-endurance studies observing mild (e.g., marathon, 160 km ultra-marathon, and Ironman distance triathlon: 5 to 15 pg·ml) (Camus *et al.* 1997, Jeukendrup *et al.* 2000) and substantial (e.g., 89.4 km ultra-marathon whereby 81% of runners had concentrations >100 pg·ml and an ultra-distance triathlon reporting 81 to 294 pg·ml) (Bosenberg *et al.* 1988, Brock-Utne *et al.* 1988) increases in circulatory endotoxin concentrations, the current ultra-marathon resulted in modest increases in post-stage circulatory endotoxin concentrations throughout competition (i.e., 30 pg·ml average increase from pre- to post-stage, with the highest increase observed at 92 pg·ml). However, a novel finding was the gradual increase in resting levels as the ultra-marathon progressed (i.e., 60 pg·ml average increase from Stage 1 to 5, with 32% of runners had concentrations >100 pg·ml and the highest increase observed at 130 pg·ml), possibly attributed to a delayed and sustained intestinal leakage upon exercise cessations, which is accompanied by a redistribution of blood flow into the splanchnic region (van Wijck *et al.* 2012). The cumulative affect observed as the ultra-marathon progressed suggests a reduced tolerance for EHS induced endotoxin leakage, subsequent to anti-endotoxin antibodies not restoring to their optimal level on consecutive occasions (Leon and Helwig 2010). For example, depressed anti-endotoxin antibodies have been reported after a marathon race, which remained below pre-exercise values for 24 h (Camus *et al.* 1997). Moreover, a 100-fold range difference in endotoxin neutralizing capacity in plasma has been observed between individuals (Warren *et al.* 1985), likely associated with training adaptations (Bosenberg *et al.* 1988). Indeed, higher circulating concentrations of endotoxin and anti-endotoxin antibodies have been observed in untrained compared with trained individuals (Jeukendrup *et al.* 2000,

Selkirk *et al.* 2008). The proposed gained adaptation to endotoxin tolerance in trained individuals is likely attributed to repetitive endotoxin challenge resulting from exercise-stress inducing endotoxin intestinal leakage and subsequent 'self-immunisation' (Bosenberg *et al.* 1988, Brock-Utne *et al.* 1988). Therefore during the current ultra-marathon, it is possible that the experience level of UER and frequent endotoxin exposure induced as part of their competition preparation may have initiated training adaptations favouring an attenuated circulatory endotoxin peak along competition (i.e., UER developing adaptations that enhance resistance and resilience to enteric pathogenic endotoxin exposure); such plausibility, however, warrants further investigation. Favourable adaptations would reduce the risk of developing clinically significant issues associated with endotoxaemia and subsequent cytokinaemia during consecutive days of EHS with or without additional stressors. Conversely, inadequate training and not being physically prepared for such an extreme event would potentially increase the risk.

Even though no differences in endotoxin was seen between running speeds, pre-stage plasma IL-1β, TNFα, and IFN-γ concentrations were substantially higher in SR throughout the ultra-marathon compared with FR; potentially suggesting greater intestinally originated endotoxin exposure above capable clearance capacity in less trained UER. This explanation however also warrants further investigation (e.g., role of intestinal originated endotoxin in training adaptions-immune competence), and may provide valuable findings into the role of endotoxin leakage in physiological adaptations to exercise stress, especially in environmental extremes. Moreover, it has also been suggested that plasma endotoxin concentrations may reach equilibrium during endurance exercise, whereby endotoxin influx from the GI tract into circulation matches endotoxin clearance by anti-endotoxin antibodies (Camus *et al.* 1998,

Selkirk *et al.* 2008); which may in part explain why only modest fluctuations in circulatory endotoxin concentrations were observed.

The increases in resting pre-stage and pre- to post-stage plasma IL-6, IL-1β, TNFα, and IFN-γ concentrations remained elevated throughout competition; mirroring that of an acute infectious episode and is similar to pro-inflammatory cytokine responses seen after endotoxin (e.g., LPS) infusion in both animal (Givalois *et al.* 1994) and human (van Deventer *et al.* 1990) models. These results are in accordance with previous endurance based (e.g., marathon) experimental designs observing modest increases in IL-6, IL-1β, and TNFα; which were also accompanied by a compensatory anti-inflammatory response (i.e., an increase in plasma IL-10 and IL-1ra concentrations) (Nieman *et al.* 2006b, Ostrowski *et al.* 1999, Suzuki *et al.* 2003). It is possible that the anti-inflammatory properties of IL-10, with adjunct IL-1ra in the multi-stage ultra-marathon study may have restricted the magnitude of pro-inflammatory cytokine production along competition as reflected by the IL-1β:IL-10 and TNF-α:IL-10 ratios. Interestingly, no differences in pro- and anti-inflammatory cytokine responses were observed between UER that ingested and did not ingest oral anti-inflammatory agents. This observation may suggest that exposure to EHS induced by the event far outweighs any impact of inconsistent use of low dose anti-inflammatory medication on cytokine responses, and questions the efficacy of such inconsistent ingestion of anti-inflammatory pharmaceutical agents within medical management of UER during extreme events.

In well trained individuals where the EHS may be better tolerated (Costa *et al.* 2014), anti-inflammatory responses predominated, off-setting potential clinically significant episodes associated with cytokinaemia (Fehrenbach and Schneider 2006, Goldhammer *et al.* 2005,

Nielsen and Pederson 2007, Okita *et al.* 2004, Pischon *et al.* 2003, Suzuki *et al.* 2006). It is however concerning that inadequately trained individuals may not present such competent anti-inflammatory responses, and may be a prime risk population for developing heat illness from immune aetiology (i.e., exertional-heat stroke, SIRS) (Leon and Helwig 2010, O'Connor *et al.* 2010, Rav-Acha *et al.* 2004). Indeed, SR presented higher resting pro-inflammatory cytokine profile compared with FR. It also needs to be taken into consideration that SR were on the course routes for a greater amount of time than FR; and thus SR may have been exposed to greater volumes of EHS and a time-dependant effect on cytokine production during recovery may produce delayed anti-inflammatory responses in SR.

The recovery time course of cytokine responses after the ultra-marathon was not determined on this occasion due to practical limitations; however previous ultra-endurance studies (e.g., long-distance triathlon and ultra-marathon running) have observed variations in cytokine responses during the recovery period. For example, IL-6 and TNF-α returned to baseline by 24 h after a 50 km ultra-marathon (Mastaloudis *et al.* 2004); whilst IL-6 returned to baseline values 16 h, with no significant changes observed in TNF-α, after a long-distance triathlon (Jeukendrup *et al.* 2000). Furthermore, on cessation of two endurance events of similar duration (long-distance triathlon, 100 km run), IL-6, IL-10, and IL-1ra peaked after competition, returning to baseline values seven days after the events (Gomez-Merino *et al.* 2006). Whereas after a long distance triathlon, IL-6 remained elevated on day one (345%) and day five (79%); while IL-10 was elevated on day one (37%), declining by 4% below pre-competition concentrations on day five (Neubauer, Konig, and Wagner 2008). These observations suggest the time course for full recovery of altered cytokine profile in response to extreme events are considerably delayed, and may play a role in the aetiology of undefined underperformance and chronic fatigue syndromes (Morris *et al.* 2014, Robson 2003). The

potential role of extreme event induced immune perturbations initiating autoimmune disease in individuals with predisposition warrants attention since chronic elevations in cytokine responses are reported in many autoimmune conditions (e.g., systemic lupus erythematosus, fibromyalgia, myalgicencephalomyelitis, idiopathic inflammatory myopathies, arthritic conditions, and inflammatory bowel diseases) (Caradonna *et al.* 2000, Morris *et al.* 2014, Thomas 2013, Woolley *et al.* 2005).

Despite amplified cytokine responses similar to that of an acute infectious episode and in accordance with the aetiology of heat-related illnesses, none of the current $n= 19$ UER were diagnosed with heat related symptoms or illnesses. Previously, only $n= 1$ UER competing in the five-days 2010 Al Andalus Ultra-Trail race suffered heat-related problems (Scheer and Murray 2011), reported to be due to UER experience (e.g., training status), the hot ambient conditions, and the nature of the race course (e.g., limited shade availability).

In the multi-stage ultra-marathon study, perceptive thermal comfort rating improved as the competition progressed. Interestingly, the two UER that originated from countries with hot ambient conditions at the time of competition showed similar circulatory endotoxin and cytokine responses to the main cohort, with substantial increases in Pv indicative of heat acclimatization still being observed in these UER (pre-Stage 1 to pre-Stage 5: UER 1= 30.4% and UER 2 = 24.9%); suggesting exertional stress is an essential key feature of heat adaptions (Costa *et al.* 2014). In view of the unique and challenging characteristics of ultra-marathon competitions (i.e., prolonged physical exertion, sleep deprivation, environmental extremes, acute periods of under-nutrition and hypohydration) and associated factors (i.e., training status, inadequate rest, tolerated injury and trauma) having the potential to disturb intestinal epithelial integrity and promote cytokine-mediated inflammatory responses, the maintenance

of hydration status in the majority of runners, thermoregulatory-induced adaptations, and cooling behaviours throughout competition may have contributed to improved heat tolerance despite prolonged exposure to EHS (Armstrong *et al.* 2007, Costa *et al.* 2014, Costa et al 2013b, ter Steege and Kolkman 2012).

The systemic endotoxin and cytokine responses seen in the multi-stage ultra-marathon study have previously been associated with symptomatic manifestations of GI symptoms (Jeukendrup *et al.* 2000, Lambert 2009, Lambert 2008, van Leeuwen *et al.* 1994), commonly associated with prolonged exposure to EHS (Øktedalen *et al.* 1992, Peters *et al.* 1999, Pfeiffer *et al.* 2012, Rehrer *et al.* 1992). For example, GI symptoms, such as nausea and vomiting, have been observed in endurance athletes presenting endotoxaemia after an Ironman triathlon event (Jeukendrup *et al.* 2000).

In the multi-stage ultra-marathon study, no assocations between GI symptoms with circulatory endotoxin and cytokine concentrations were observed. However, a strong relationship ($r^2 = -0.665$) between severe GI symptoms and thermal tolerance was confirmed ($p < 0.001$). These results suggest that severe GI symptoms likely originate from heat stress during exercise, potentially through splanchnic hypoperfusion and hypoxia (i.e., exercising in the heat creating greater redistribution of blood flow away from the splanchnic area) (ter Steege and Kolkman 2012, van Wijck *et al.* 2012, van Wijck *et al.* 2011) Such physiological changes in splanchnic blood flow, which have symptomatic outcomes, likely lead to disturbances in intestinal mucosal and epithelial integrity that enhances local enteric endotoxin leakage and subsequent cytokinaemia; and not necessarily that endotoxaemia and cytokinaemia induce GI symptoms.

In regards to future directions, it is still unknown how the degree of exertional stress, with or without environmental extremes and between different exercise modes, impacts on overall intestinal epithelial integrity. Additionally, does the nutritional and hydration status before exertional stress, and the changes that occurs to status during physical exertion, influence the degree of GI disturbance? Conducting a set of laboratory-controlled experiments assessing varying T_{amb}, exercise intensities, durations and modes whilst assessing intestinal epithelial integrity measures (van Wijck *et al.* 2012) would contribute substantially to the current knowledge base and provide a foundation to investigate potential strategies to overcome GI complications associated with EHS. For example, dietary strategies during physical exertion, development of gut training protocols, functional foods, heat acclimation protocols, external pre-cooling (e.g., cold water bath or cooling vest) and (or) during physical exertion internal cooling (e.g., cold beverages) are proposed strategies that may attenuate EHS induced GI perturbations. Indeed, due to gut plasticity, there is potential for the GI tract to adapt to a challenge load ('training the gut') (Jeukendrup and McLaughlin 2011). Whereas, previous investigations have demonstrated favourable effect of prebiotic oligosaccharides (e.g., inulin and oligofructose) and probiotic bacteria (e.g., Lactobacillus casei and Bifidobacterium) on markers of GI integrity; albeit within inflammatory diseases of the gut (Sator 2004). Knowledge into the impact of such biotics on gut integrity during EHS is, however, scarce. Anecdotal evidence during the multi-stage ultra-marathon study highlighted that UER who consistently consumed commercial probiotic product in the week leading up to the ultra-marathon presented no incidence of GI symptoms; suggesting further controlled investigation is needed to confirm any beneficial effects of biotics on intestinal epithelial integrity in response to EHS.

Multi-stage ultra-marathon competition in the heat resulted in a modest circulatory endotoxaemia accompanied by a pronounced pro-inflammatory cytokinaemia and compensatory anti-inflammatory responses, both of which were sustained throughout competition at rest (pre-stage) and after stage completion. The findings from the multi-stage ultra-marathon study suggest that appropriate informed training (e.g., physically trained to complete the required distance in environmental extremes) and competition preparation (e.g., effective and evidence-based heat acclimation protocols, hydration maintenance and (or) cooling strategies) may help prevent significant exertional-heat related subclinical and clinical manifestations from occurring in high-risk UER competing in extreme events.

The field-based study presented in **Chapter 7** demonstrated that in the absence of heat-stress, but with inclusion of other multi-stressors, endotoxaemia with accompanying pro-inflammatory cytokinaemia ensued, counteracted by a compensatory anti-inflammatory response.

Despite temperate ambient conditions, the markedly more pronounced increases in plasma CRP **(Figure 7.2)** and circulatory endotoxin concentration **(Figure 7.1)** in the 24 h continuous ultra-marathon study (2832% and 37% post-competition, respectively), compared to that of **Chapter 6** (889% and 21%, respectively) suggest that amplified CRP concentrations likely reflect other multi-factorial influences, such as the degree of the exertional stress, sleep deprivation, energy deficit, and bacterial endotoxin presence in circulation simultaneously. Whereas, the endotoxin leakage was likely induced by disturbances to intestinal epithelial integrity (i.e., splanchnic hypoperfusion, ischemia, and mechanical trauma), associated with the physical exertion volume; despite endotoxin leakage being more commonly studied amongst EHS protocols (Bosenberg *et al.* 1988, Brock-Utne *et*

al. 1988). Due to practical limitations in monitoring parameters after competition (i.e., participant follow-up at race location during the acute and prolonged recovery period), the 24 h continuous ultra-marathon study was not able to determine the recovery time course of CRP. However, such responses have been shown to remain elevated above pre-exercise values for a considerable period of time (i.e., up to nineteen days after an Ironman triathlon event) (Neubauer, Konig, and Wagner 2008).

The pro-inflammatory cytokinaemic response observed post-competition are similar to previous field-based studies that have observed cytokinaemia during EHS (Bosenberg *et al.* 1988, Brock-Utne *et al.* 1988, Jeukendrup *et al.* 2000, Suzuki *et al.* 2003). For example, increases in IL-6 (152%), IL-1β (95%), TNF-α (168%), and IFN-γ (102%) were consistently observed post-stage during a 230 km multi-stage ultra-marathon conducted in hot ambient conditions (**Chapter 6**). Likewise, the increased pro-inflammatory response was accompanied by a compensatory anti-inflammatory response **(Table 7.1).**

Results from the 24 h continuous ultra-marathon study suggest that in well-trained individuals, where the exertional stress is well tolerated, compensatory anti-inflammatory responses may off-set potential clinically significant episodes associated with profound and prolonged cytokine profile disturbance (Fehrenbach and Schneider 2006, Goldhammer *et al.* 2005, Nielsen and Pederson 2007, Suzuki *et al.* 2006); thus acting as a safeguard. Indeed, disturbances to cytokine profile have been linked to the aetiology of GI and autoimmune diseases (Caradonna *et al.* 2000, Elenkov and Chrousos 2002); while the fatigue inducing properties of certain cytokines, especially IL-6, have recently been confirmed and established (Morris *et al.* 2014, Robson-Ansley, Blannin, and Gleeson 2007, Robson-Ansley *et al.* 2009, Robson-Ansley *et al.* 2004). Previous research has shown that illness-prone individuals have

an altered cytokine response (i.e., pro- to anti-inflammatory balance) after a standardised bout of treadmill running compared with healthy runners who have a more proportionally regulated cytokine response (Cox *et al.* 2007). Therefore, it is plausible that in illness-prone individuals partaking in prolonged physical exertion who experience impaired cytokine regulation (i.e., over-exaggerated cytokine-mediated inflammatory response) not adequately counteracted by anti-inflammatory responses, the resulting cytokine imbalance may potentially contributing to pathophysiology (Fehrenbach and Schneider 2006). Investigation into the role of challenging compensatory anti-inflammatory responses through internal (e.g., endotoxin challenge) and external (e.g., physical strain) mechanisms is warranted, and may act to strengthen tolerance to exertional exposure with or without additional stressors (e.g., heat stress, sleep deprivation, and (or) compromised nutrition) in illness-prone individuals.

As previously reported, due to practical limitations, the 24 h continuous ultra-marathon study was not able to determine the recovery time course of the cytokine profile. Previous ultra-endurance studies (e.g., long-distance triathlon and ultra-marathon running) have however observed variations in cytokine recovery. For example, IL-6 and TNF-α returned to baseline by 24 h after a 50 km ultra-marathon (Mastaloudis *et al.* 2004); whilst IL-6 returned to baseline values after 16 h, with no significant change observed in TNF-α after a long-distance triathlon (Jeukendrup *et al.* 2000). Furthermore, on cessation of two endurance events of similar duration (i.e., long-distance triathlon and 100 km running event), IL-6, IL-10, and IL-1ra peaked after competition, returning to baseline values seven days after the events (Gomez-Merino *et al.* 2006). Whereas, after a long distance triathlon, IL-6 remained elevated on day one (345%) and day five (79%); while IL-10 was elevated on day one (37%), declining by 4% below pre-competition concentrations on day five (Neubauer, Konig, and Wagner 2008).

Circulatory endotoxin and pro-inflammatory cytokine responses seen in the 24 h continuous ultra-marathon study have previously been associated with symptomatic manifestations of GI distress (Jeukendrup *et al.* 2000, Lambert 2009, Lambert 2008, van Leeuwen *et al.* 1994). In the 24 h continuous ultra-marathon study, the association between the reported number of severe GI symptoms and plasma concentrations of anti-inflammatory IL-10 and immune activator IL-8 suggest that alterations to intestinal motility and mechanical trauma (i.e., repetitive jarring) associated with the prolonged running period may have promoted intestinal epithelial disturbance and potential damage (Rehrer *et al.* 1992, Rehrer and Meijer 1991), inducing GI symptoms and stimulating immune responses into protection and repair; and not necessarily that endotoxaemia and cytokinaemia causes symptoms.

Further research is required to clarify the impact of over-exaggerated cytokine-mediated inflammatory responses, similar to those observed in the 24 h continuous ultra-marathon study, on the potential long-term health implications in 'high-risk' illness-prone individuals.

10.4 Probiotics, Salivary Anti-Microbial Protein Responses, Circulatory Endotoxin, Cytokine Profile and Exertional-Heat Stress

The laboratory-controlled experimental trials presented in **Chapters 8** and **9** are the first to determine the effects of acute high dose probiotic supplementation on multiple markers of oral-respiratory mucosal immunity (**Chapter 8**), intestinal epithelial integrity (assessed by circulatory endotoxin concentration), and cytokine profile (**Chapter 9**) during exposure to EHS. All participants confirmed through self-report that the full amount of probiotic (and placebo) were consumed over the seven day supplementation period. Participants were instructed to split the doses into two equal boluses (500ml between 08:00 h-09:00 h and 500ml between 08:00 h-09:00h). This was to increase overall compliance and to make the total daily dose (1000 ml) more manageable to consume. A higher dosage of probiotic supplementation over a shorter duration was chosen as this mimics athlete population consumption behaviours. Additionally, there is currently limited probiotic research in the area of exercise immunology and to date, whilst probiotics have been shown to induce immunomodulatory effects, research investigating the dose-response effect of probiotic supplementation (typical dosages: x 10^{10} CFU·100ml^{-1}·day^{-1}) and the 'ideal' dose to induce a definitive pharmaceutical effect on S-AMP responses and in the management of endotoxaemia and responsive cytokinaemia is absent. Therefore, to date, it is possible that the disparities between previous research results simply reflect the concentration and (or) the volume of probiotic provided to participant and theoretically, it is plausible that a substantially larger probiotic dose may induce a greater oral-respiratory mucosal immune response (e.g., up regulation of S-IgA) and (or) attenuate disturbances to intestinal epithelium integrity and a subsequent endotoxin-induced cytokine-mediated inflammatory response. Given that exercising in the heat compared with exertional stress in temperate ambient

conditions is associated with greater perturbations to oral-respiratory mucosal immunity (*via* mechanisms such as compromised hydration status depressing SFR and amplified stress hormone release) and intestinal epithelial integrity (*via* mechanisms such as hyperthermia, splanchnic ischemia-hypoperfusion and tissue hypoxia, direct mechanical trauma and compromised hydration status), the studies in **Chapter 8** and **9** present a novel aspect into the effects of probiotic supplementation prior to and upon exposure to EHS. To date, the study presented in **Chapter 8** was the first to investigate the effects of an acute high dose probiotic on multiple markers of oral-respiratory mucosal immunity (i.e., S-IgA, S-α-amylase, and S-lysozyme) prior to and upon exposure to EHS; whilst the study presented in **Chapter 9** was the first to investigate the effects of an acute high dose probiotic on intestinal epithelial integrity (i.e., circulatory endotoxin concentration) and cytokine profile prior to and upon exposure to EHS.

The laboratory-controlled study presented in **Chapter 8** demonstrated that daily high dose probiotic supplementation did not enhance resting S-AMP values after seven days, nor did supplementation enhance S-AMP responses after or during recovery from EHS compared with placebo.

To date, research into the mechanisms of action by which probiotics exerts an effect on oral-respiratory mucosal immunity is limited. Previously, it has been suggested that strain-specific probiotic supplementation can enhance mucosal immunity through stimulation of lymphoid tissue (e.g., Peyer's patches) in the GI tract resulting in localised effects (i.e., up-regulation of S-IgA) at other mucosal sites (i.e., URT) (Glück and Gebbers 2003). Alternatively, it is possible that colonisation and probiotic interaction at the oral mucosal epithelium may affect the oral ecology, inducing modulation of innate and adaptive immune responses, enhancing

production of anti-microbial substances and epithelial barrier function (Stamatova and Meurman 2009). On the contrary, despite the high probiotic dose in the probiotic-EHS study, S-IgA responses were unaltered after seven days of supplementation, and did not differ from PLA immediately post-EHS and during the recovery period **(Figure 8.1).**

Taking into account the impact of hydration status on SFR (Walsh *et al.* 2004a), it is possible that the maintenance in S-IgA responses in the probiotic-EHS study are partly due to the maintenance of euhydration observed during and after EHS, which may have contributed towards attenuating substantial depressions in SFR and thus maintaining S-AMP responses. This response is irrespective of the increases in S-cortisol immediately post-EHS in both trials **(Table 8.3)**, which have been associated with inhibitory effects on the transepithelial transport of S-IgA and reductions in SFR (Chicharro *et al.* 1998, Teeuw *et al.* 2004). For example, the overall mean exercise-induced BM loss for the PRO and PLA trial was 1.5% and 1.3%, respectively; while P_{Osmol} remained within clinical reference ranges (280-300 mOsmol·kg^{-1}) throughout recovery (PRO: 298 mOsmol·kg^{-1} and PLA: 297 mOsmol·kg^{-1}). These results are consistent with a previous studies showing greater S-AMP responses in euhydration compared with dehydration (Fortes *et al.* 2012, **Chapter 4**), suggesting hydration status play a more important role in oral-respiratory mucosal immunity than probiotics, and may account for the discrepant outcomes observed in previous research (i.e., hydration status not reported, controlled, or accounted for in the majority of previous research).

Studies investigating the influence of probiotic supplementation on oral-respiratory mucosal immune status have predominantly focused on S-IgA responses. It is however conceivable that the reported immunostimulatory properties of probiotics may induce changes in other salivary immune parameters. Therefore collectively determining S-IgA concomitant with

other S-AMP may provide a clearer interpretation of oral-respiratory mucosal immune status. Indeed, whilst reductions in S-IgA responses are commonly reported after prolonged physical exertion, adjunct increases in S-α-amylase and S-lysozyme responses have recently been reported (Costa *et al.* 2012, Costa *et al.* 2009). In the probiotic-EHS study, no changes in S-α-amylase and S-lysozyme responses were observed after seven days of daily high dose probiotic supplementation, whilst the increases in S-α-amylase responses after EHS is likely a reflection of the degree of the exertional stress *per se* (Bosch *et al.* 2003b, Rosa *et al.* 2014). Moreover, considering all participants in the probiotic-EHS study showed higher S-AMP profiles (i.e., either S-IgA, S-α-amylase, or S-lysozyme relative to pre-EHS values) on PLA compared with PRO, the current sample size provides sufficient power to reject the hypothesis and clearly show that acute high dose probiotic supplementation does not enhance S-AMP responses over placebo. However, considering PRO showed consistently lower S-AMP responses during recovery from EHS compared with PLA (i.e., relative to pre-EHS values), but which failed to reach significance on this occasion, the limited sample size in this instance did not confirm a clear negative effect of acute high dose probiotic supplementation.

Given that S-α-amylase and S-lysozyme exhibits similar protective properties as S-IgA, it is suggested that acute stress induced increases in these S-AMP may actually provide a protective mechanism to oral-respiratory mucosal immunity. For example, increases in S-α-amylase and S-lysozyme responses have been consistently observed after each stage of a 230 km multi-stage ultra-marathon and after a 24 h continuous ultra-marathon resulting in low occurrence of URSI in both events, despite depressions in S-IgA responses (**Chapter 4** and **Chapter 5**). It is also important to highlight the large individual variation in responses observed in the probiotic-EHS study, especially in relation to S-α-amylase and S-lysozyme. This suggests that acute high dose probiotic supplementation leading up to EHS induces a

'responder' and 'non-responder' outcome, which may actually result in a compromised S-AMP status (e.g., dampened exercise-induced increases in S-α-amylase in adjunct with depressed S-IgA responses) in individuals that are sensitive to an oral probiotic exposure, compared with avoiding exposure altogether.

While the mechanisms by which probiotic supplementation may possibly enhance immune function remains controversial, further research is required to determine whether probiotic supplementation can translate into immunocompromised active populations and (or) those who commonly present clinically significant symptoms as a possible form of 'health insurance' when other nutritional measures are unable to be maintained.

The laboratory-controlled study presented in **Chapter 9** demonstrated that daily high dose probiotic supplementation did not reduce concentrations of circulatory endotoxin and inflammatory cytokines after EHS or during the recovery period on the probiotic trial compared with placebo. Although mild in nature, the data actually shows circulatory endotoxin concentration and inflammatory cytokine responses were generally higher post-EHS and during recovery from EHS on PRO compared with PLA. However, from an overview standpoint, it appears that an acute high dosage of oral supplementation of a commercially available probiotic beverage containing *L.casei* induces inconsequential effects on intestinal endotoxin translocation and cytokine responses after EHS.

The results of the probiotic-EHS study showing modest increases in endotoxaemia are comparable to some studies (Camus *et al.* 1997, Jeukendrup *et al.* 2000), but not all (**Chapter 4**). Discrepancies in such results compared to that of the probiotic-EHS study are likely due to the duration and (or) repetitive nature of the exertional stress, coupled with insufficient

recovery between exercise bouts and presence of other stressors (e.g., hydration status) known to compromise intestinal epithelial integrity and promote endotoxin translocation (Lambert *et al.* 2008). Indeed, body temperature and hydration status appear to be contributing factors towards the degree of EHS induced endotoxaemia (Lambert *et al.* 2008, Selkirk *et al.* 2008). For example, in contrast with previous laboratory-controlled studies that have observed increases in circulatory endotoxin concentrations (3.8 $pg \cdot ml^{-1}$ at baseline to 16.5 $pg \cdot ml^{-1}$) in trained subjects, concomitant with considerable elevations in T_{core} at exhaustion (36.9°C at baseline to 39.7°C) (Selkirk *et al.* 2008), the current experimental design instigated relatively mild T_{core} elevations in comparison (PRO: 38.6°C and PLA: 38.6°C), which may in part, account for the milder endotoxaemia observed. Moreover, participants in the probiotic-EHS study were provided with fluid *ad libitum*, potentially lessening the negative impact of dehydration during EHS on intestinal epithelial integrity. It could be speculated that if T_{core} was higher (i.e., >39.0°C) and exercise-induced dehydration greater, a higher endotoxaemia may have been observed. Indeed, those participants in the probiotic-EHS study who had an exercise-induced BM loss of >2% and a P_{Osmol} >300 $mOsmol \cdot kg^{-1}$ showed higher average circulatory endotoxin concentrations (e.g., up to 40 $pg \cdot ml^{-1}$). These results are reflected in another laboratory controlled study that observed significant increases in intestinal permeability after 60 min of treadmill running at 70% VO_{2max} without fluid intake resulting in 1.5% BM loss (Lambert *et al.* 2008); suggesting that increases body water losses may exacerbate perturbations to intestinal epithelial integrity.

In contrast to previous studies showing enhancement of gut-barrier function with probiotic administration (Ohland and MacNaughton 2010, Rodes *et al.* 2013), the current data show that, although mild in nature, circulatory endotoxin concentrations were higher on PRO during recovery in all participants compared with PLA **(Figure 9.4)**. Although the

mechanisms for this outcome cannot be fully explained and warrant further exploration, it is clear that the circulatory endotoxin flux in the post-EHS period was quenched by competent anti-endotoxin antibody responses in both trials, likely associated with training adaptations (Bosenberg *et al.* 1988, Selkirk *et al.* 2008).

In the probiotic-EHS study, the increases in IL-6, TNF-α, IL-10, and IL-8 on PRO and PLA observed **(Table 9.1)** are in accordance with previous field-based studies (Bosenberg *et al.* 1988, Brock-Utne *et al.* 1988). Indeed, increases in IL-6 (152%), TNF-α (168%) and IL-10 (1271%) were observed post-stage during a 230 km multi-stage ultra-marathon conducted in hot ambient conditions (**Chapter 6**). Such results, including those of the probiotic-EHS study, suggest that the compensatory anti-inflammatory response of IL-10 may have restricted the magnitude and counteracted any further exacerbation of pro-inflammatory cytokine responses. Notably, in relation to pre-EHS values, the cytokineamic response was generally higher in the PRO trial, which is not surprising given the greater circulatory endotoxin concentrations observed after exposure to EHS.

Results also suggest that if individuals are well-trained (i.e., adaptations to exercise and environmental stress) and prepared (e.g., hydration maintenance) for EHS, these characteristics *per se* may help dampen EHS induced endotoxaemia, and responsive cytokinaemia. Despite the probiotic-EHS study not primarily focusing on intestinal epithelial mechanistic responses, future research could potentially establish the degree to which varying heat stress models during exercise, with or without a state of dehydration, impacts on intestinal permeability through the lactulose-rhamnose sugars test in adjunct to circulatory endotoxin responses (van Wijck *et al.* 2012).

Furthermore, whilst long-term probiotic supplementation is not generally practiced amongst active populations, further adequately controlled studies are required to assess whether longer term probiotic use has a greater influence on changes in immune variables. The probiotic intervention period ranges between two weeks to six months in clinical research models to promote optimal colonisation in the GI tract. Despite the differences in intestinal epithelial surface area, probiotic dosage in human trials are based on animal models. Furthermore, dose-response studies are limited. It is unknown whether different probiotic strains (single or in combination) and species may induce differing immunological effects dependent upon the disease state. Consequently, it is extremely difficult to extrapolate the inconsistent results of previous studies.

Similar to the current findings, Shing *et al.* (2013) reported that whilst pre- and post-exercise LPS concentrations were lower following four weeks of probiotic supplementation, there was no significant difference between the probiotic and placebo group, nor were differences evident in cytokine responses. At present, acute high dose probiotic beverage consumption prior to EHS is not justified, and it would be advisable to withhold intake before EHS due to the potential unknown and (or) underlying hidden outcomes. For example, increases in intestinal permeability, endotoxin translocation and cytokine responses which may accelerate symptomatic outcomes and the aetiology of heat stroke.

Conclusions

The major conclusions from this thesis are:

1. S-AMP responses appear to be maintained throughout multi-stage ultra-marathon competition. The observed unfavourable reductions in S-IgA responses were offset by favourable increases in S-α-amylase and S-lysozyme responses. A low prevalence (n=1) of URS were reported during and up to 4 weeks after the competition.

2. Modest depressions in some S-AMP responses (S-IgA and S-lysozyme) were counteracted by increases in others (S-α-amylase) after a 24 h continuous overnight ultra-marathon. No incidences of URS were evident during or following competition.

   To date, the influence of extreme exercise models in the investigation of oral-respiratory mucosal immune status, in addition to the tracking of S-MAP status over time is extremely limited; whilst reporting multiple salivary immune variables allowed for a more comprehensive overview of oral-respiratory mucosal immune status. Equally, the inclusion of additional salivary immune variables such as S-lactoferrin, determining the time-course of recovery and conducting the research protocol during the winter months when the risk of an infectious episode is greater would have provided further insight into the impact of extreme physical exertion on immune variables. Furthermore, the lack of research control limits data interpretation.

3. Modest circulatory endotoxaemia accompanied by a pronounced pro-inflammatory cytokinaemia and compensatory anti-inflammatory responses were observed throughout multi-stage ultra-marathon competition. No incidences of exertional-heat

symptoms or illnesses were evident throughout competition. Even though severe GI symptoms were reported, no relationships with blood borne indices were identified.

4. Circulatory endotoxaemia with accompanying cytokinaemic characteristics of an acute infectious episode were observed after a 24 h continuous overnight ultra-marathon. GI symptoms were commonly reported amongst UER, with higher symptom occurrence associated with greater compensatory anti-inflammatory responses (IL-10).

To date, the influence of extreme exercise models in the investigation of intestinal epithelial integrity (as assessed by circulatory endotoxin concentrations) and cytokine profile, in addition to the tracking of endotoxin and cytokine responses over time is extremely limited. Equally, the inclusion of additional variables (e.g., anti-endotoxin antibodies), measurements (e.g., T_{core}) and determining the time-course of recovery of cytokine profile would have provided further insight into the impact of extreme physical exertion on immune variables. The lack of research control (e.g., sporadic use of NSAIDs) limits data interpretation.

5. Acute supplementation of the probiotic *L. casei* for a period of one week does not enhance S-AMP responses after or during recovery from EHS compared with placebo.

6. Acute supplementation of the probiotic *L. casei* for a period of one week does not prevent or attenuate EHS induced circulatory endotoxaemia or cytokinaemia; nor is it more positively favourable over a placebo.

To date, laboratory controlled studies exist investigating the influence of probiotic supplementation on oral-respiratory mucosal immune status and intestinal epithelial integrity (as assessed by circulatory endotoxin concentrations) and cytokine profile during prolonged EHS is absent. Equally, a larger sample size would be considered more representative of the endurance running population, whilst a longer probiotic supplementation period to promote optimal colonisation and in accordance with previous clinical models is an area of further research.

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Coventry University: Sports & Exercise Science Applied Research Group

Oral-Respiratory Mucosal Immune, Circulatory Endotoxin and Cytokine Responses, Assessment of Nutrition & Hydration Status, and Illness & Infection Rates During Multi-Stage Ultra-Marathon Competition in Hot Ambient Temperatures.

2011 Al Andalus Ultra Trail

Participant Information & Informed Consent Form

Thank you for agreeing to take part in this research study. This form explains what you will be asked to do. If you have any questions about this research please ask one of the researcher team.

Research Investigators: Samantha Gill and Dr. Ricardo Costa.

The aims of the research are:
1. Determine oral-respiratory mucosal immune responses during a multi-stage ultra-marathon competition in a hot ambient environment.
2. Determine circulatory endotoxin concentrations and pro and anti-inflammatory cytokine responses during a multi-stage ultra-marathon competition in a hot ambient environment.
3. Assess the rates and severity of upper respiratory and gastrointestinal symptoms during and the month following a multi-stage ultra-marathon competition in a hot ambient environment.
4. Assess nutrition and hydration status of ultra-endurance runners during a multi-stage ultra-marathon competition in a hot ambient environment.

What will I have to do?
You are only required to follow YOUR NORMAL COMPETITION STRATEGIES. In addition:
- Have your body mass taken before and after each stage.
- Provide one urine, saliva and blood sample before and after each stage.
- Let the research team conduct a ~10min interview each evening between 6-10pm.

What will I get from this?
1. You will gain an insight into the scientific aspects of the study and you may find it interesting to know what we are trying to achieve or how various measurements are recorded.

2. You will gain an insight into how your immune system responses to an ultra-marathon competition in the heat. From this information the research team and provide some advice on strategies to avoid unwanted immune profiles associated with the ultra-marathon competition.

3. After the ultra-marathon, you will be provided with individual feedback on the measurements made during the research. Advice will be given to help you improve your dietary habits for subsequent ultra-marathon competition.

By signing this form you agree to take part in the study. However, please note that you are free to stop taking part at any time.

Foreseeable risks or discomforts?

Blood sampling: Only qualified phlebotomy trained researchers with experience at performing this procedure will collect blood samples. To ensure you are completely comfortable with giving blood samples, we will familiarise you with the blood sample collection procedure. The amount of blood collected during each blood sampling will be (18 ml), which will not impact upon your normal physical functioning during strenuous exercise in a hot ambient environment.

No risks are associated with urine sampling, or saliva sampling that will be collected via an unstimulated passive dribble method.

We will require ~10mins of your time every evening to conduct an interview of the days dietary activities and recovery. We have a very sympathetic and qualified team, which will make the interview a pleasant experience.

What will happen to your data?

Confidentially will be maintained throughout data collection. All data is annonymised as soon as it is collected and will be stored electronically using participant codes so that individuals cannot be identified. Any data from your participation in the study will be used by the research team. It may also be disseminated externally, however your name or identity will remain anonymous.

I confirm that I have read the participant information. The nature, demands and risks of the project have been explained to me. Any additional enquiries about the project have been answered satisfactorily by the researchers.

I knowingly assume the risks involved and understand that I may withdraw my consent and discontinue participation at any time without penalty and without having to give any reason.

I hereby give my consent to participate in this research project.

Participant's signature: _____Date _____

Participant's email: _____

Researcher's signature: _____Date _____

For any further questions or specific research information please feel free to contact any of the research team.

Samantha Gill:gills19@coventry.ac.uk Dr Ricardo Costa:ricardo.costa@coventry.ac.uk

Illness reported four weeks prior to participation: Yes   No

(If yes, please complete the WURSS questionnaire)

Coventry University: Sports & Exercise Science Applied Research Group

Oral-Respiratory Mucosal Immune, Circulatory Endotoxin and Cytokine Responses, Assessment of Nutrition & Hydration Status, Energy Expenditure, and Illness & Infection Rates During a 24 hour Ultra-Endurance Running Competition Conducted in the Scottish Highlands

2011 & 2012 Glenmore 24h

Participant Information & Informed Consent Form

Thank you for agreeing to take part in this research study. This form explains what you will be asked to do. If you have any questions about this research please ask one of the researcher team.

Research Investigators: Samantha Gill, Dr. Ricardo Costa, Dr. Andrew Murray & Mike Adams

The aims of the research are:
5. Determine oral-respiratory mucosal immune responses during a 24 hour ultra-endurance running competition.
6. Determine circulatory endotoxin concentrations and pro and anti-inflammatory cytokine responses during a 24 hour ultra-endurance running competition.
7. Assess the rates and severity of upper respiratory and gastrointestinal symptoms during and the month following a 24 hour ultra-endurance running competition.
8. Assess nutrition and hydration status of ultra-endurance runners during a 24 hour ultra-endurance running competition.
9. Determine accurate energy expenditure during a 24 hour ultra-endurance running competition.

What will I have to do?
You are only required to follow YOUR NORMAL COMPETITION STRATEGIES. In addition:
- Have your body mass taken before and after the 24 hour race.
- Provide one urine, saliva and blood sample before and after the 24 hour race.
- Wear an armband during the 24 hour race, which will measure your energy expenditure.
- Let the research team conduct a ~10 minute interview after the 24 hour race.

What will I get from this?
4. You will gain an insight into the scientific aspects of the study and you may find it interesting to know what we are trying to achieve or how various measurements are recorded.

5. You will gain an insight into how your immune system responses to an ultra-endurance competition. From this information the research team and provide some advice on strategies to avoid unwanted immune profiles associated with the ultra-endurance competition.

6. After the 24 hour race, you will be provided with individual feedback on the measurements made during the research. Advice will be given to help you improve your dietary habits for subsequent ultra-endurance performance.

By signing this form you agree to take part in the study. However, please note that you are free to stop taking part at any time.

Foreseeable risks or discomforts?
Blood sampling: Only qualified phlebotomy trained researchers with experience at performing this procedure will collect blood samples. To ensure you are completely comfortable with giving blood samples, we will familiarise you with the blood sample collection procedure. The amount of blood collected during each blood sampling will be (6 ml), which will not impact upon your normal physical functioning during strenuous exercise.

No risks are associated with urine sampling, or saliva sampling that will be collected via an unstimulated passive dribble method.

We will require ~10 minutes of your time at the end of the 24 hour races to collect the armband and conduct an interview of the 24 hour's dietary activities and symptoms experienced. We have a very sympathetic and qualified team, which will make the interview a pleasant experience.

What will happen to your data?
Confidentially will be maintained throughout data collection. All data is annonymised as soon as it is collected and will be stored electronically using participant codes so that individuals cannot be identified. Any data from your participation in the study will be used by the research team. It may also be disseminated externally, however your name or identity will remain anonymous.

I confirm that I have read the participant information. The nature, demands and risks of the project have been explained to me. Any additional enquiries about the project have been answered satisfactorily by the researchers.

I knowingly assume the risks involved and understand that I may withdraw my consent and discontinue participation at any time without penalty and without having to give any reason.

I hereby give my consent to participate in this research project.

Participant's signature: _____Date _____

Participant's email: _____

Researcher's signature: _____Date _____

For any further questions or specific research information please feel free to contact any of the research team.

Samantha Gill:gills19@coventry.ac.uk Dr Ricardo Costa:ricardo.costa@coventry.ac.uk

Illness reported four weeks prior to participation: Yes   No

(If yes, please complete the WURSS questionnaire)

Coventry University: Sports & Exercise Science Applied Research Group

Oral-respiratory mucosal immune responses, and circulatory endotoxin concentration and cytokine responses to exercise-heat stress: Does probiotic supplementation play a role in mucosal health during exercise-heat stress.

Participant Information & Informed Consent Form

Thank you for agreeing to take part in this research study. This form explains what you will be asked to do. If you have any questions about this research please ask one of the researcher team.

Research Title: Oral-respiratory mucosal immune responses, and circulatory endotoxin concentration and cytokine responses to exercise-heat stress: Does probiotic supplementation play a role in mucosal health during exercise-heat stress.

Research Investigators: Samantha Gill; gills19@coventry.ac.uk
Dr. Ricardo Costa; ricardo.costa@coventry.ac.uk

Invitation to take part
You have been invited to take part in a research investigation. Before you do so, it is important for you to understand why the research is being conducted and what you will be required to do once you agree to be involved. Please read the following information carefully. You should ask us if there is anything that is not clear or if you would like more information.

Do I have to take part?
This is entirely your decision. If you decide to take part you will be given an information sheet to keep and be asked to sign a consent form. **If you decide to take part you are still free to withdraw at any time, without giving a reason.** If you decide not to participate or withdraw during the study, your decision will not affect your relationship with the Health and Life Sciences faculty, or any of the investigators involved in the study.

Background
Current research suggests that prolonged, strenuous exercise in the heat is associated with disturbances to mucosal immunity, potentially increasing the risk of illness and infection. The general consensus is that probiotics are safe in healthy population groups and side effects (if any) tend to be mild with high doses failing to exhibit any toxicity. To date, only a handful of studies have investigated probiotic supplementation specifically in athletes with results demonstrating some immune modulating effects. However, there is insufficient evidence to set recommendations for the athletic population and limitations apply to a number of studies. Currently, the use and effectiveness of probiotic supplementation in athletes to reduce immune disturbances and prevent exercise-heat stress occurrence and severity is currently unknown and warrants further investigation. In summary, the aims of this study are to examine the effects of probiotic supplementation on oral-respiratory mucosal immune status and circulatory endotoxin and cytokine profile during exercise-heat stress.

The aims of the research are:

10. Determine oral-respiratory mucosal immune and stress responses during a 120 minute exercise bout in two treatment groups in a hot environment.
11. Determine circulatory endotoxin concentrations and cytokine profile during a 120 minute exercise bout in two treatment groups in a hot environment.
12. Assess the rates and severity of upper respiratory symptoms and gastrointestinal symptoms during each treatment group.
13. Assess nutritional status during the weeks of supplementation.

What will I have to do?

Prior to completing a trial you will be expected to:

- Complete an initial assessment through performance of a maximal aerobic test (10-15 minutes) on an electric treadmill. This test will be used to measure your aerobic fitness (maximal oxygen uptake). Briefly, the treadmill speed and gradient will be increased every 3 minutes until you reach volitional exhaustion. This test will require you to run at your maximal aerobic capacity for approximately 1 minute. We will require you to wear a face mask during this fitness test so that we can make measurements on your expired air; the facemask is not uncomfortable and will not impede your breathing.

Completing a trial

1. 120 minute exercise bout, 60% VO_{2max} in a hot environment (30-35ºC) with placebo.
2. 120 minute exercise bout, 60% VO_{2max} in a hot environment (30-35ºC) with probiotic supplementation.

Whilst completing the experimental trials you will be expected to:

- On 2 separate occasions perform a 2h run at 60% of your maximal exercise capacity on an electric treadmill.
- To have blood samples taken from the antecubital vein by a trained researcher. These will occur one week prior to the exercise trial, immediately before the exercise trial, immediately after the exercise trial and 1h, 2h, 4h and 24h after the exercise trial. This totals 14 blood samples for the course of the study (7 blood samples per exercise trial). To provide 126ml blood for each exercise trial.
- To provide a 4 minute timed saliva sample one week prior to the exercise trial, immediately before the exercise trial, immediately after the exercise trial and 1h, 2h, 4h and 24h after the exercise trial via a dribble method that will be explained to you. This will total 14 saliva samples for the course of the study (7 saliva samples per exercise trial).
- To provide one urine sample one week prior to the exercise trial, immediately before the exercise trial, immediately after the exercise trial and 1h, 2h, 4h and 24h after the exercise trial. This will total 14 urine samples for the course of the study (7 urine samples per exercise trial).
- Consume 1000ml yoghurt drink per day (AM and PM) during the study (a total of 7 days for each exercise trial). On two of the treatment groups, the yogurt drink will contain probiotics. On the other two treatment groups, the yogurt drink will contain no probiotics. However, you will be unaware which one you are consuming throughout the study.
- Wear a rectal probe during exercise. The rectal probe is a thin flexible cable that you insert approximately 10cm past your anal sphincter for the measurement of body temperature.

- Wear a heart rate monitor during exercise.
- Complete a food and fluid diary & training log during the weeks of supplementation.
- Complete an illness log assessing upper respiratory and gastrointestinal symptoms during the weeks of supplementation.
- Complete a compliance log for the yogurt drink consumed during the weeks of supplementation.

If you decide to take part in this study, there will be a number of constraints placed upon your normal everyday life and activities.
- You will be asked to refrain from consuming any other types of probiotics (i.e. drinking yogurts, capsules, tablet forms).
- You will be asked to refrain from consuming any dietary supplements one month prior to and during the study.

Advantages of taking part
By taking part in this study you will contribute to our knowledge about the influence of probiotics on immune function. Currently, there are insufficient therapeutic treatment options to enhance defence mechanisms, particularly in an athletic population group. Indeed, immune function appears to be lowered after heavy exercise, possibly accounting for the increased illness and infection incidence at this time; as such, any information about how to improve host defence after exercise would be most welcome. This information will be useful to sports scientists and research outcomes will have considerable relevance, not only catering for recreational and elite endurance athletes routinely performing strenuous exercise for extended durations in the heat, but additionally, can be largely applied across a wide range of other sports (teams, international competition) and professional activities (military personnel, expedition crews (medical, health or media), and/or park rangers) regularly exposed to hot environmental conditions.

You will gain an insight into the scientific aspects of the study and you may find it interesting to know what we are trying to achieve or how various measurements are recorded.

You will gain an insight into how your immune system responds to a strenuous bout of exercise in a hot and thermo-neutral environment and the influence of probiotic supplementation on immune responses and symptoms. From this information, the research team can provide some advice on strategies to avoid unwanted immune profiles associated with strenuous exercise in the heat.

You will receive comprehensive feedback on the results and measurements made during the research, with full explanations; for your fitness level, immune system status and advice on strategies to avoid unwanted immune profiles associated with strenuous exercise in the heat.

Disadvantages of taking part
The disadvantages of taking part in this study, which you will probably be most concerned about are: blood sampling, physical exertion, and time commitment.

1.Blood sampling
Only qualified phlebotomy trained researchers with experience at performing this procedure will collect blood samples. To ensure you are completely comfortable with giving blood samples, we will familiarise you with the blood sample collection procedure during the initial assessment. We will insert a small syringe into the forearm vein on several occasions for the

multiple blood collections before and during the recovery period. The amount of blood collected during each experimental exercise trial (126ml) will not impact upon normal physiological functioning.

2.Physical exertion
The physical components of this study include; the incremental aerobic fitness test, and 2 2h exercise bouts at 60% of maximal effort. The incremental aerobic fitness test will only require you to run at your maximal capacity for approximately 1 minute. You most probably feel that the same type of exhaustion and fatigue for longer periods during your normal training and competition habits. The 2 2h exercise bouts at 60% of maximal effort may promote post-exercise fatigue. However, they are of a similar volume and intensity of effort to that experienced during your training and competitions. As such, the 2h runs can also be seen as useful intensive and prolonged training sessions. You will be closely supervised during the exercise trials and by monitoring your body temperature; we will stop the trial should your body temperature rise too quickly.

3.Time commitment
To complete all aspects of the study we will require you to visit the laboratory on 7 occasions for a total of approximately 10 hours.

As the study involves prolonged, strenuous exercise in extreme conditions, all safety measures will be discussed with you prior to any testing. You will also be asked to complete a heath/medical history questionnaire prior to any testing. It is important that we closely monitor your body temperature during exercise to ensure you do not get too hot; we will withdraw you from the exercise trial if this happens.

What will happen to your data?
Confidentially will be maintained throughout data collection. All information collected during the study will be coded and treated confidentially. All data is annonymised as soon as it is collected and will be stored electronically using participant codes so that individuals cannot be identified. Any data from your participation in the study will be used by the research team. It may also be disseminated externally, however your name or identity will remain anonymous.

I confirm that I have read the participant information. The nature, demands and risks of the project have been explained to me. Any additional enquiries about the project have been answered satisfactorily by the researchers.

I knowingly assume the risks involved and understand that I may withdraw my consent and discontinue participation at any time without penalty and without having to give any reason.

You will excluded if you are a smoker or if you are on specific types of medication, supplements or take probiotics on a regular basis. Asthmatics and Diabetes patients are also prohibited. You should not take part if you have a medically diagnosed cardiac condition, are currently on any cardiac related medication (e.g. beta-blockers) or have a physical injury that may prevent you from completing the task.

If you have or recently had (within one month) an infection, illness, injury, and/or taking medication you will be excluded from taking part in this study.

If you experience any illness/infection during the course of the study, testing will be terminated.

IN TOTAL, THE STUDY WILL REQUIRE YOU TO GIVE UP ~ 10 HOURS OF YOUR TIME.

Please tick boxes

1	I confirm that I have read and understand the Participant Information Sheet dated................. for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.	
2	I understand that my participation is voluntary and that I am free to withdraw at any time without giving a reason, without my medical care or legal rights affected.	
3	I understand that my participation is voluntary and that I am free to withdraw at any time without giving a reason. If I do decide to withdraw I understand that it will have no influence on the outcome of my period of study or members of staff within Health and Life Sciences.	
4	I understand that I may register any complaint I might have about this experiment with the Head of Sport Science, and that I will be offered the opportunity of providing feedback on the experiment using the standard report forms.	
5	I agree to take part in the above study.	

Name of participant _____

Participant's signature: _____Date _____ _____

Participant's email: _____

Name of Researcher _____

Researcher's signature: _____Date _____ _____

For any further questions or specific research information please feel free to contact any of the research team.

Sam Gill: gills19@coventry.ac.uk Dr Ricardo Costa: ricardo.costa@coventry.ac.uk

Appendix C: Health Screen Questionnaire

Name of Participant _____

Date of Birth _____

General Physical Fitness

How often do you take regular physical exercise?
- Less than once a week
- Once a week
- Two to three times a week

How long have you been exercising at this frequency?
- Less than 1 month
- 1-6 months
- More than 6 months

Is your current body weight:
- Normal range
- Overweight
- Underweight

Smoking habits (circle all that apply)
- Never smoked
- Gave up more than 1 month ago
- Total years smoked for …………
- Smoke/used to smoke less than 20 cigarettes per day
- Smoke/used to smoke more than 20 cigarettes per day

General Health

Do you suffer or have you ever suffered from the conditions below? (give details if yes)
- Heart disease and/or circulatory problems
- Diabetes
- High blood pressure
- High cholesterol
- Asthma or any other type of lung disease
- Kidney disease
- Clotting disorders
- Anaemia or other blood disorders
- Any other long term medical disorder

Details

_____

_____

Do you regularly take:
- Any prescribed medicines
- Any over the counter medicines
- Any other drugs
- Any supplements

Details

_____

_____

Have you ever had past injuries that might be affected by the tests planned for today?

_____

_____

Is there any other information that might affect your safety/health in carrying out these tests?

_____

_____

Your Health Today

Have you had any of the following health problems in the last few days:
- Coughs/colds
- Headaches
- Shortness of breath
- Muscle/joint pain
- Any other health problems

_____

_____

Do you currently have any of the following symptoms:
- Sore throat or blocked nose
- Shortness of breath
- Headache and/or dizziness
- Nausea
- Pain in muscles/tendons/bones

- Any other feelings/pains that you do not normally have

_____

_____

Are you pregnant?

_____

Is there any other information that might affect your safety/health in carrying out the tests today?

_____

_____

I confirm that I have given details of any information that may affect my suitability to participate as a subject today.

I have also completed a consent form for this experiment which stated the tests to be carried out and any adverse effects.

Signature _____ **Date** _____
Participant

Signature _____ **Date** _____
Experimenter