



Title: The use of acute responses of endocrine and immune biomarkers to highlight overreaching

Name Diogo Luis Campos Vaz Leal

This is a digitised version of a dissertation submitted to the University of Bedfordshire.

It is available to view only.

This item is subject to copyright.



The Use of Acute Responses of Endocrine and Immune  
Biomarkers to Highlight Overreaching

Diogo Luís Campos Vaz Leal

Ph.D.

2017

University of Bedfordshire





The Use of Acute Responses of Endocrine and Immune Biomarkers to  
Highlight Overreaching

by

Diogo Luís Campos Vaz Leal

A Thesis submitted to the University of Bedfordshire, in fulfilment of the requirements  
for the degree of *Doctor of Philosophy*

September 2017

## Academic Thesis: Declaration of Authorship

I, Diogo Luís Campos Vaz Leal, declare that this Thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

THE USE OF ACUTE RESPONSES OF ENDOCRINE AND IMMUNE BIOMARKERS TO HIGHLIGHT OVERREACHING

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this Thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have cited the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this Thesis is entirely my own work;
5. I have acknowledged all main sources of help;
6. Where the Thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
7. None of this work has been published before submission.

Name of candidate: Diogo Luís Campos Vaz Leal

Signature:



Date: 08/09/2107

# THE USE OF ACUTE RESPONSES OF ENDOCRINE AND IMMUNE BIOMARKERS TO HIGHLIGHT OVERREACHING

D. V. LEAL

## **Abstract**

The action of overtraining may lead to the different states of overreaching or the overtraining syndrome (OTS). Chronic maladaptation in endocrine and immune mechanisms, and performance decrements occur with the incidence of these states. Circulating cortisol and testosterone have been proposed as endocrine markers of overreaching/OTS. Salivary measurements of these hormones have been used as a non-invasive surrogate for circulating levels. Chapter 4 (study 1) on this Thesis examined the influence of consuming water 10 min, 5 min and 1 min before providing a saliva sample in diluting saliva and consequently providing invalid salivary cortisol and testosterone concentration levels. No trial effect was found. However, exercise-induced salivary cortisol and testosterone significantly elevated in response to the 10 min and 5 min trials only, with lower absolute-changes observed in the 1 min trial. No differences were found in the resting samples. It was suggested that consuming water up to 5 min before providing a saliva sample does not appear to influence the hormone concentrations at rest and during exercise. However, the recommended guidelines for saliva collection have been followed in the subsequent studies. Chapter 5 (study 2) examined the reproducibility of salivary cortisol and testosterone responses to a 30-min cycle named as the 55/80. This test has been proposed as a suitable indicator of hormonal alterations associated with overreaching/OTS. Reproducibility of salivary cortisol and testosterone to the 55/80 was confirmed by determining intra-individual coefficients of variation ( $CV_i$ ). However, the 55/80 is a cycle test and therefore, may not be appropriate for runners. Chapter 6 (study 3) focused on designing a 30-min, running bout (i.e. the  $RPE_{\text{treadmill}}$ ) to reproduce the effects of the 55/80. The  $RPE_{\text{treadmill}}$  is a self-paced test and therefore, will not require a  $\dot{V}O_{2max}$  test to determine exercise intensities. An acute elevation of plasma and salivary testosterone, but not cortisol was

observed in response to the  $RPE_{\text{treadmill}}$ . These responses have been shown to be reproducible. The data from Chapter 6 suggest that the  $RPE_{\text{treadmill}}$  may be a suitable tool to indicate hormonal alterations associated with overreaching/OTS. This led to the design of study 4 (Chapter 7). Plasma and salivary cortisol and testosterone responses were examined before and after a 12-day intensified-training period. Immunity markers (specifically salivary immunoglobulin A (SIgA), leucocyte subset proliferation and phagocytic activity) were examined before and after training. Plasma and salivary cortisol were unaffected by acute exercise and training. However, testosterone elevated to the  $RPE_{\text{treadmill}}$  Pre-Training, and these responses were reduced Post-Training. Total leucocytes and mucosal immunity were unaffected by exercise and training. However, increased upper respiratory tract infection symptoms were found Post-Training. Baseline phagocytic function was 47% lower Post-Training. This Thesis suggests that testosterone may be a more reliable exercise-stress marker. Moreover, the  $RPE_{\text{treadmill}}$  may be a suitable tool to highlight alterations in testosterone when in an overreached state in an attempt to avoid the incidence of OTS, and that this tool may be practically applied in the field of exercise science. Additionally, this Thesis shows that a 12-day intensified-training period induced a marked decrease in phagocytic responses, and therefore using the  $RPE_{\text{treadmill}}$  to highlight overreaching may be important to also prevent further impairments in immunity status.

## Dedication

To the greatest granddad that has ever lived! You will always be my T tata!

29/11/1929 – 06/03/2017



## Acknowledgements

The past 42 months have been a journey full of surprises, stories, laughs, friends, but also some tears, headaches and sometimes even disappointments. However, I am sure I will keep each second in my memory for the rest of my life and this is mainly due to the persons who have been with me all along.

Firstly, I would like to thank Dr John Hough. John, you have been the supervisor that any PhD student could ever dream of. My journey would not have been the same if you were not in it. There is nothing I can do to reciprocate the tireless hours you spent reading my work, making me think for myself, supporting my decisions and lifting me up when I was giving up, but thanking you a million times.

This has been an intense journey, but the friends who were with me at the start and the ones I made during the climbing helped me get to the end. To the ones who were and will always be there - Francisca, Zé Nuno, Chica e Diogo – my most sincere thank you for the talks, the laughs, the games, the trips, the many moments together even when in different countries. To Diana and Ricardo for the many special moments that we have had in the past three years. To Ben Maylor, Josh Foster, Jeff Aldous and Callum Mould who I met from the start and accompanied this whole journey and made every day a better day. In addition, I would like to thank Dr Andrew Mitchell who trusted my capability to share my knowledge with our University's students.

I must also thank Mr Mick Callanan and the The Connolly Foundation, for trusting me and my work and for the funding that allowed me to complete the last study of my PhD. To Dr Glen Davison and Ellie Hynes, I am grateful for the help you provided with some of the analysis from my last PhD study.

I would also like to acknowledge Dr Anna Furmanski and Dr Ariane Standing for the many hours spent in the labs for me and with me. Anna and Ariane, your help and good mood was precious, and I would not have done it without your support. And if a huge part of my PhD was spent in the labs, these days would not have been as enjoyable if it were not for Callum, Roisin and Warwick. Thanks for the smiles and for all the effort you put into keeping our labs at Bedford to the high standard we sure have. I would like to thank the students Will Craggs and Michael Harrison who were a huge help during the process of data collection in some of the studies presented in this Thesis. Without your help it would have been even harder to get these studies done. But if these studies were completed, it was due to the many participants who were involved in them, and for that reason I am grateful for their time and effort.

Most importantly, a very special, deep and sincere thank you to my family! To my mum and dad, Ana e Luís, for the 27 years spent doing their best to raise two good men. One of them, is my beloved brother, Dinis who I miss more than anything in this world! The world has become a better place 16 years ago, simply because you were born. To my godfather Álvaro for the inspiration and the many hours teaching me the laws of gravity (who cares if I was only 2 years old?) and to my aunt São to make my godfather a better, happier man. To the best grandparents someone could ever have. Vóvó, you are the most special woman (and the best cook) I will ever meet. Tátá, you were always one of my role models, my idol, the person I respected the most, the man I wanted to be. Thank you for all the great memories you have given me. You were not supposed to leave us this early. I will always remember the last words you said to me! Thank you all for all the love you have always given me. I am sorry for the time I took from us and I promise I will make it up!

Finally, I must thank my girlfriend, my partner in crime, my biggest support in the past few years, my best friend. Catinhas, I could not have done it without you. Thank you for all the love, the patience, the support, the motivation, the 'Good morning!' from every morning. Thanks for making me believe in myself, even when I thought there was nothing more I could do. For that, I will be eternally grateful!

## **Publications**

**Leal, D.;** Taylor, L. and Hough, J. (2016) Exercise-induced plasma steroid hormone responses in men: the development of a new tool to highlight overreaching-related hormonal alterations. BASES Conference 2016 – Programme and Abstracts. *Journal of Sports Sciences*, 34, i-s85.

## **Presentations**

**Leal, D.;** Standing, A.; Davison, G.; Furmanski, A. L. and Hough, J. (2017) Endocrine and immune impairments in men following a 12-day period of intensified training. *13<sup>th</sup> Symposium of the International Society of Exercise and Immunology (ISEI), Quinta das Lágrimas, Coimbra, Portugal. July, 11<sup>th</sup> – 14<sup>th</sup>.*

**Leal, D.** (2017) Exercise-induced hormonal imbalances and immune adaptations when in a healthy state compared to an overreached state – a prevention method. *Lecture to staff and MSc students, School of Sport, Polytechnic Institute of Viana do Castelo, Melgaço, Portugal. January, 17<sup>th</sup>.*

**Leal, D.;** Taylor, L. and Hough, J. (2016) Exercise-induced plasma steroid hormone responses in men: the development of a new tool to highlight overreaching-related hormonal alterations. *British Association of Sport and Exercise Sciences (BASES) Conference 2016, East Midlands Conference Centre, Nottingham, UK. November, 29<sup>th</sup> – 30<sup>th</sup>.*

**Leal, D.;** Taylor, L. and Hough, J. (2016) Exercise-induced plasma steroid hormone responses: the development of a new tool (short-duration running bout) to highlight hormonal alterations during overreaching to reduce the incidence of the overtraining syndrome. *Institute of*

*Sport & Physical Activity Research (ISPAR) Conference 2016, University of Bedfordshire, Bedford, UK. June, 28<sup>th</sup>.*

**Leal, D. (2016)** The truth about the overtraining syndrome: the use of exercise-induced hormonal alterations to prevent the incidence of symptoms of maladaptation. *Presentation/lecture to staff and 2<sup>nd</sup> year MSc students of the University Institute of Maia, University Institute of Maia, Maia, Portugal. March, 30<sup>th</sup>.*

**Leal, D.;** Taylor, L. and Hough, J. (2016) Exercise-induced plasma steroid hormone responses: the development of a new tool (short-duration running bout) to highlight hormonal alterations during overreaching to reduce the incidence of the overtraining syndrome. *British Association of Sport and Exercise Sciences (BASES) Student Conference 2016, University of Bangor, Bangor, UK. March, 22<sup>nd</sup> - 23<sup>rd</sup>.*

# List of Contents

<b>Abstract</b>	<b>iii</b>
<b>Dedication</b>	<b>v</b>
<b>Acknowledgements</b>	<b>vi</b>
<b>Publications</b>	<b>viii</b>
<b>Presentations</b>	<b>viii</b>
<b>List of Figures</b>	<b>xvii</b>
<b>List of Tables</b>	<b>xx</b>
<b>List of Abbreviations</b>	<b>xxi</b>
<b>Chapter 1: Introduction and Aims</b>	<b>1</b>
1.1 Introduction	2
<b>Chapter 2: Review of the Literature and Research Aims</b>	<b>8</b>
2.1 The General Adaptation Syndrome	9
2.2 Periodisation of Training and The Overload Principle	10
2.3 Hormonal Markers of Overreaching/OTS	13
2.3.1 Hormonal Classification, Synthesis and Secretion	14
2.3.2 Hormone Action on Their Target Cells	16
2.3.3 Patterns of Hormone Secretion	18
2.3.4 Hypothalamic-Pituitary-Adrenal (HPA) Axis	21
2.3.5 Cortisol	23
a) Acute Cortisol Responses to Exercise	25
b) Exercise-Induced Cortisol Responses After Intensified Training	28
2.3.6 The Hypothalamic-Pituitary-Gonadal (HPG) Axis	30
2.3.7 Testosterone	31
a) Acute Testosterone Responses to Exercise	32
b) Exercise-Induced Testosterone Responses After Intensified Training	35

2.3.8	Plasma and Salivary Measurement of Hormones	37
2.4	The Immune System	38
2.4.1	The Components of the Immune System	38
2.5	The Role of Leucocytes in Immunology	40
2.5.1	Granulocytes	40
2.5.2	Monocytes	41
2.5.3	Lymphocytes	41
2.6	The Basic Pattern of Immune Recognition: Innate and Adaptive Immunity	42
2.6.1	Innate Immune System	43
a)	Mucosal Epithelial	45
b)	Phagocytes	45
2.6.2	Adaptive Immune System	45
2.7	Acute Effects of Exercise in Immunity	48
2.8	Overtraining and Susceptibility to Infection	49
2.8.1	Immune and Inflammatory Responses to Overreaching/OTS	53
2.9	Aims and Structure of the Thesis	55
<b>Chapter 3:</b>	<b>General Methods</b>	<b>57</b>
3.1	Ethical Approval	58
3.2	Maximal Oxygen Uptake ( $\dot{V}O_{2max}$ ) Assessment	58
3.2.1	Maximal Oxygen Uptake and Maximal Workload Test on a Cycle Ergometer	59
3.2.2	Maximal Oxygen Uptake on a Motorised Treadmill	60
3.3	10-km Time Trial Performance Test	62
3.4	Urine Collection and Analysis of Osmolality	62
3.5	General Procedures for Hormone Analysis	63
3.6	Saliva Collection, Handling and Analysis	64
3.6.1	Saliva Collection and Handling	64
3.6.2	Enzyme-linked Immunosorbent Assays (ELISA)	65
a)	Salivary Cortisol	65
b)	Salivary Testosterone	66

c) Salivary Immunoglobulin A	66
3.7 Blood Collection, Handling and Analysis	68
3.7.1 Blood Collection and Handling	68
3.7.2 Haematology	69
3.7.3 Leucocyte Isolation	70
a) Peripheral Blood Mononuclear Cells (PBMCs) and Polymorphonuclear (PMN) granulocytes	70
b) Total Leucocytes from Whole Blood	72
3.7.4 Cell Counting using a Handheld Cell Counter	72
3.7.5 Polymorphonuclear Leucocytes Phagocytic Activity Assay	73
3.7.6 Leucocyte Subsets Activation and Acquisition	74
3.7.7 Flow Cytometry (FACS) Analysis	76
a) Phagocytosis assay	76
b) T-lymphocyte analysis	78
c) Granulocyte Analysis	80
3.7.8 Enzyme-linked Immunosorbent Assays	81
a) Plasma Cortisol	81
b) Plasma Testosterone	81
3.8 Questionnaires and Diaries	81
3.8.1 Recovery-Stress Questionnaire for Athletes (RESTQ-76 Sport)	81
3.8.2 Upper Respiratory Illness (URS) Questionnaire	82
3.8.3 Food Diary	83
3.8.4 Training Diary	83
3.9 Measurement of Training Load	84
3.10 Statistical Analysis	84
<b>Chapter 4: The Effects of Water Consumption on the Salivary Cortisol and Testosterone Responses to a Continuous, 30-Min, High-Intensity Cycle Bout</b>	<b>85</b>
4.1 Introduction	86
4.2 Methods	88

4.2.1	Procedures	88
4.2.2	Preliminary Measurements and Main Experimental Trials	89
4.2.3	Saliva Collection and Handling	92
4.2.4	Statistical Analysis	92
4.3	Results	93
4.3.1	Hydration Status	93
4.3.2	Total Weight Loss	93
4.3.3	Recovery-Stress Questionnaire	93
4.3.4	Physiological Responses to Exercise	94
4.3.5	Hormonal Responses During CTL	94
	a) Salivary Cortisol	94
	b) Salivary Testosterone	95
4.3.6	Hormonal Responses to Exercise	95
	a) Salivary Cortisol	95
	b) Salivary Testosterone	96
4.4	Discussion	98
 <b>Chapter 5: The Reproducibility of the Salivary Cortisol and Testosterone Responses to the 55/80 Bout</b>		<b>104</b>
5.1	Introduction	105
5.2	Methods	108
5.2.1	Procedures	108
5.2.2	Analytical Procedures	110
5.2.3	Statistical Analysis	110
5.3	Results	111
5.3.1	Hydration Status	111
5.3.2	Recovery-Stress Questionnaire	111
5.3.3	Physiological Responses to Exercise	112
5.3.4	Hormonal Responses During CTL	112
	a) Salivary Cortisol	112



b) Salivary Testosterone	112
5.3.5 Hormonal Responses to Exercise	112
a) Salivary Cortisol	112
b) Salivary Testosterone	114
5.4 Discussion	116
<b>Chapter 6: Reproducibility of Exercise-Induced Plasma Cortisol and Testosterone Responses: The Development of a New Tool to Highlight Hormonal Alterations During Overreaching</b>	<b>122</b>
6.1 Introduction	123
6.2 Methods	126
6.2.1 Procedures	126
6.2.2 Main Experimental Trials	127
6.2.3 Analytical Procedures	128
6.2.4 Statistical Analysis	128
6.3 Results	129
6.3.1 Hydration Status	129
6.3.2 Recovery-Stress Questionnaire	129
6.3.3 Physiological Responses to Exercise	130
6.3.4 Hormonal Responses During CTL	130
a) Plasma Cortisol and Testosterone	130
b) Salivary Cortisol and Testosterone	130
6.3.5 Hormonal Responses to Exercise	131
a) Plasma Cortisol	131
b) Salivary Cortisol	131
c) Plasma Testosterone	133
d) Salivary Testosterone	133
6.4 Discussion	136

<b>Chapter 7: Effects of a 12-Day Intensified-Training Period on the Endocrine and Immune Systems: Using a Novel Short-Duration Running Test to Prevent the Incidence of NFOR/OTS</b>	<b>142</b>
7.1 Introduction	143
7.2 Methods	147
7.2.1 Procedures	147
7.2.2 Maximal Oxygen Uptake Assessment	149
7.2.3 Analytical Procedures	149
7.2.4 Statistical Analysis	150
7.3 Results	152
7.3.1 Hydration Status	152
7.3.2 Recovery-Stress Questionnaire	152
7.3.3 Upper Respiratory Symptoms (URS)	154
7.3.4 Physiological Responses to Exercise	155
7.3.5 10-km Time Trial Performance Test	156
7.3.6 Hormonal Responses to Exercise	157
a) Plasma Cortisol	157
b) Salivary Cortisol	157
c) Plasma Testosterone	159
d) Salivary Testosterone	159
7.3.7 Salivary Immunoglobulin A (SIgA)	161
7.3.8 Total Leucocytes and Polymorphonuclear Leucocyte	162
7.3.9 Leucocyte Phenotyping	163
7.3.10 Phagocytic Function	165
7.4 Discussion	166
<b>Chapter 8: General Discussion</b>	<b>176</b>
<b>References</b>	<b>183</b>
<b>Appendices</b>	<b>218</b>

Appendix 1: Physical activity readiness questionnaire (PAR-Q)	219
Appendix 2: Blood screening analysis form	220
Appendix 3: Health-related daily routine questionnaire	221
Appendix 4: Recover-stress questionnaire for athletes (RESTQ-76)	222
Appendix 5: Jackson score upper respiratory tract illness questionnaire	230
Appendix 6: Food intake diary	231
Appendix 7: Training diary	232

## List of Figures

<b>Figure 2.1</b> The stages of the supercompensation cycle (adapted from Koutedakis <i>et al.</i> (2006, p. 6)).	11
<b>Figure 2.2</b> Schematic representation of the practice of overreaching and OTS. (Taken from Budgett (1998)).	12
<b>Figure 2.3</b> Synthesis and secretion of peptide (A) and steroid (B) hormones.	15
<b>Figure 2.4</b> The action of steroid hormones in the cell. Taken from Hinson <i>et al.</i> (2010).	17
<b>Figure 2.5</b> Circadian rhythm of plasma cortisol (A), salivary cortisol (B), plasma testosterone (C) and salivary testosterone (D). Reproduced from Debono <i>et al.</i> (2009) (A), Smyth <i>et al.</i> (1997) (B), Walton <i>et al.</i> (2007) (C) and Kraemer <i>et al.</i> (2001) (D).	20
<b>Figure 2.6</b> Human adrenal glands. Adrenal cortical and medullary tissue are presented as stippled and black, respectively. Extra-adrenal sites sometimes found are also shown. Taken from Ganong (1991).	22
<b>Figure 2.7</b> The synthesis pathway of cortisol.	24
<b>Figure 2.8</b> Negative feedback control of the secretion of cortisol. The dashed and solid arrows indicate inhibitory and stimulatory effects, respectively.	25
<b>Figure 2.9</b> Negative feedback loop of the HPG axis. The dashed and solid arrows indicate inhibitory and stimulatory effects, respectively.	31
<b>Figure 2.10</b> The synthesis pathway of testosterone.	32
<b>Figure 2.11</b> The main components of the adaptive immune system. Adapted from Gleeson and Bosch (2013, p. 38).	47
<b>Figure 2.12</b> Relationship between training volume and incidence of URS: the J-shaped curve model. (Adapted from Gleeson and Robson-Ansley in Gleeson (editor) 2006, p. 116).	51
<b>Figure 2.13</b> The S-shaped curve model to describe the relationship between training volume and respiratory illness (Adapted from Malm (2006)).	52
<b>Figure 3.1</b> Graph depicting the relationship between $\dot{V}O_2$ and submaximal running speed to calculate $v\dot{V}O_{2\max}$ (adapted from Jones <i>et al.</i> , (2009)).	61

<b>Figure 3.2</b> Purification of polymorphonuclear leucocytes (PMN) using Polymorphprep™. ....	<b>71</b>
<b>Figure 3.3</b> Example of data screens from Scepter™; A) initial data screen following sample analysis; B) setting lower gate in cell diameter screen; C) setting upper gate in cell diameter screen; D) corrected cell count following gate adjustment. ....	<b>73</b>
<b>Figure 3.4</b> Polymorphonuclear leucocytes (PMNs) phagocytic activity flow cytometric analysis. A) Unstimulated PMNs; B) Dehydroethidium-stained PMNs; C) Dehydroethidium-stained and stimulated with opsonized zymosan. ....	<b>77</b>
<b>Figure 3.5</b> Lymphocyte flow cytometric gating and analysis. ....	<b>79</b>
<b>Figure 3.6</b> Granulocytes flow cytometric gating and analysis. ....	<b>80</b>
<b>Figure 4.1</b> Schematics of main experimental trial. ....	<b>91</b>
<b>Figure 4.2</b> RESTQ-76 Sport scores measured in all trials. ....	<b>94</b>
<b>Figure 4.3</b> A) Salivary cortisol responses during 10MIN, 5MIN, 1MIN and CTL. B) Absolute changes in salivary cortisol from Pre to Peak Post-Exercise. ....	<b>96</b>
<b>Figure 4.4</b> A) Salivary testosterone responses during 10MIN, 5MIN, 1MIN and CTL. B) Absolute changes in salivary testosterone from Pre to Peak Post-Exercise. ....	<b>97</b>
<b>Figure 5.1</b> RESTQ-76 Sport scores measured in all trials. ....	<b>111</b>
<b>Figure 5.2</b> Salivary cortisol responses to the 55/80 at Pre-, Post- and 30 min Post-Exercise. ....	<b>113</b>
<b>Figure 5.3</b> Individual average absolute change in salivary cortisol (nmol·L <sup>-1</sup> ) from Pre- to Post-Exercise across all trials. ....	<b>113</b>
<b>Figure 5.4</b> Salivary testosterone responses to the 55/80 at Pre-, Post- and 30 min Post-Exercise. ....	<b>114</b>
<b>Figure 5.5</b> Individual average absolute change in salivary testosterone (pmol·L <sup>-1</sup> ) from Pre- to Post-Exercise across all trials. ....	<b>115</b>
<b>Figure 6.1</b> Schematic presentation of the RPE <sub>treadmill</sub> running bout. ....	<b>128</b>
<b>Figure 6.2</b> RESTQ-76 Sport scores measured in all trials. ....	<b>129</b>
<b>Figure 6.3</b> Plasma and salivary cortisol responses to exercise and CTL. ....	<b>132</b>
<b>Figure 6.4</b> Individual average absolute changes in plasma and salivary cortisol from Pre- to Peak Post-Exercise. ....	<b>132</b>
<b>Figure 6.5</b> Plasma and salivary testosterone responses to exercise and CTL. ....	<b>134</b>

<b>Figure 6.6</b> Individual absolute changes in plasma and salivary testosterone from Pre- to Peak Post-Exercise.....	<b>134</b>
<b>Figure 7.1</b> Schematic presentation of the study design.....	<b>148</b>
<b>Figure 7.2</b> Schematic presentation of each main experimental trial. ....	<b>149</b>
<b>Figure 7.3</b> RESTQ-76 Sport scores before and after a 12-day period of intensified training. .	<b>153</b>
<b>Figure 7.4</b> RESTQ-76 Sport scores in all subscales. ....	<b>153</b>
<b>Figure 7.5</b> Average upper respiratory symptoms (URS) scores at Pre- and Post-Training.....	<b>154</b>
<b>Figure 7.6</b> Sum of individual upper respiratory symptoms (URS) scores at Pre- and Post-Training.....	<b>154</b>
<b>Figure 7.7</b> Heart rate responses during the RPE <sub>treadmill</sub> at Pre- and Post-Training. ....	<b>155</b>
<b>Figure 7.8</b> Average speed at each stage during the RPE <sub>treadmill</sub> at Pre- and Post-Training.....	<b>156</b>
<b>Figure 7.9</b> Time to completion in 10-km time-trial test.....	<b>156</b>
<b>Figure 7.10</b> Plasma and salivary cortisol at Pre- and Post-Training. ....	<b>158</b>
<b>Figure 7.11</b> Absolute change from Pre- to Post-Exercise in plasma and salivary cortisol at Pre- and Post-Training.....	<b>158</b>
<b>Figure 7.12</b> Plasma and salivary testosterone at Pre- and Post-Training.....	<b>160</b>
<b>Figure 7.13</b> Absolute change from Pre- to Post-Exercise in plasma and salivary testosterone at Pre- and Post-Training. ....	<b>160</b>
<b>Figure 7.14</b> SIgA responses at Pre- and Post-Training. ....	<b>161</b>
<b>Figure 7.15</b> SIgA secretion rate over time at Pre- and Post-Training. ....	<b>161</b>
<b>Figure 7.16</b> Total whole blood leucocyte counts. ....	<b>162</b>
<b>Figure 7.17</b> Total polymorphonuclear leucocyte (PMN) counts.....	<b>162</b>
<b>Figure 7.18</b> Phagocytic activity of zymosan-stimulated PMNs at Pre- and Post-Training.....	<b>165</b>

## List of Tables

<b>Table 2.1</b> Acute cortisol responses to continuous exercise. ....	<b>27</b>
<b>Table 2.2</b> Cortisol concentrations following a period of intensified training. ....	<b>29</b>
<b>Table 2.3</b> Acute testosterone responses to continuous exercise. ....	<b>34</b>
<b>Table 2.4</b> Testosterone concentrations following a period of intensified training. ....	<b>36</b>
<b>Table 2.5</b> Main components of the immune system. Taken from Gleeson (2006, p. 16). ....	<b>39</b>
<b>Table 2.6</b> Modules of the innate immune system. Taken from Medzhitov (2007). ....	<b>44</b>
<b>Table 4.1:</b> Participants' anthropometric and physiological characteristics at baseline. ....	<b>88</b>
<b>Table 5.1</b> Participants' anthropometric and physiological characteristics at baseline. ....	<b>108</b>
<b>Table 5.2</b> CV <sub>i</sub> and ICC values for the physiological and hormonal variables. ....	<b>115</b>
<b>Table 6.1</b> Participants' anthropometric and physiological characteristics at baseline. ....	<b>126</b>
<b>Table 6.2</b> CV <sub>i</sub> and ICC values for speed and heart rate to both running bouts. ....	<b>130</b>
<b>Table 6.3</b> CV <sub>i</sub> , ICC and ES values for the hormone responses to the RPE <sub>treadmill</sub> bouts. ....	<b>135</b>
<b>Table 7.1</b> Participants' physiological and anthropometric characteristics at baseline. ....	<b>147</b>
<b>Table 7.2</b> Haematological, total and differential leucocyte counts and leucocyte phenotyping at Pre- and Post-Training. ....	<b>164</b>

## List of Abbreviations

**55/80** A continuous 30-min cycle consisting of alternating blocks of 1 min at 55%

$\dot{W}_{\max}$  and 4 min at 80%  $\dot{W}_{\max}$

<b><math>\alpha</math></b>	Alpha
<b><math>\gamma</math></b>	Gamma
<b><math>\delta</math></b>	Delta
<b><math>\mu\text{L}</math></b>	Microliters
<b><math>\mu\text{m}</math></b>	Micrometers
<b><math>^{\circ}\text{C}</math></b>	Degree celsius
<b>%</b>	Percentage
<b>~</b>	Approximately
<b>&lt;</b>	Less than
<b>&gt;</b>	Greater than
<b><math>\pm</math></b>	Plus-minus
<b>ACTH</b>	Adrenocorticotrophic hormone
<b>AMP</b>	Adenosine monophosphate
<b>ANOVA</b>	Analysis of variance
<b>APC</b>	Allophycocyanin conjugate
<b>ATP</b>	Adenosine triphosphate
<b>cAMP</b>	Cyclic adenosine monophosphate
<b>CD</b>	Cluster of differentiation
<b>CD4<sup>+</sup></b>	T-helper cell
<b>CD8<sup>+</sup></b>	T-cytotoxic cell
<b>cm</b>	Centimetres
<b>CO<sub>2</sub></b>	Carbon dioxide
<b>CRH</b>	Corticotropin-releasing hormone
<b>CTL</b>	Control resting trial
<b>CV<sub>i</sub></b>	Intra-individual coefficient of variation



<b>DNA</b>	Deoxyribonucleic acid
<b>ELISA</b>	Enzyme-linked immunosorbent assay
<b>ES</b>	Cohen's effect size
<b>FITC</b>	Fluorescein isothiocyanate conjugate
<b>FOR</b>	Functional overreaching
<b>FSH</b>	Follicle-stimulating hormone
<b>G</b>	Gauge
<b>g</b>	Grams
<b><i>g</i></b>	Gravitational force
<b>GAS</b>	General adaptation syndrome
<b>GnRH</b>	Gonadotropin-releasing hormone
<b>h</b>	Hour
<b>H<sub>2</sub>O</b>	Water
<b>HPA</b>	Hypothalamic-pituitary-adrenal
<b>HPG</b>	Hypothalamic-pituitary-gonadal
<b>HR</b>	Heart rate
<b>HR<sub>max</sub></b>	Maximum heart rate
<b>HR<sub>rest</sub></b>	Resting heart rate
<b>ICC</b>	Intraclass correlation coefficient
<b>Ig</b>	Immunoglobulin
<b>IL</b>	Interleukin
<b>L</b>	Litre
<b>LH</b>	Luteinising hormone
<b>K<sub>3</sub>EDTA</b>	Tri-potassium ethylenediaminetetraacetic acid
<b>kcal</b>	Kilocalories
<b>kg</b>	Kilograms
<b>kJ</b>	Kilojoules
<b>km</b>	Kilometer
<b>mL</b>	Mililiter

<b>max</b>	Maximum/maximal
<b>MHC</b>	Major histocompatibility complex
<b>min</b>	Minute
<b>nm</b>	Nanometers
<b>nmol</b>	Nanomoles
<b>mRNA</b>	Messenger ribonucleic acid
<b>NFOR</b>	Non-functional overreaching
<b>NK</b>	Natural killer
<b>OTS</b>	The overtraining syndrome
<b>pL</b>	Picoliters
<b>pmol</b>	Picomoles
<b>PAMPs</b>	Pathogen-associated molecular patterns
<b>PCV</b>	Packed cell volume
<b>PBMC</b>	Peripheral blood mononuclear cell
<b>PBS</b>	Phosphate buffered saline
<b>PE</b>	Phycoerythrin
<b>PerCP-CY5.5</b>	Peridin-chlorophyll-protein complex: cyanine 5.5 conjugate
<b>PKA</b>	Protein kinase A
<b>PMN</b>	Polymorphonuclear leucocyte
<b>PMP™</b>	Polymorphprep™
<b>PRRs</b>	Pattern-recognition receptors
<b>RBC</b>	Red blood cell
<b>RPE</b>	Rating of perceived exertion
<b>RPE<sub>treadmill</sub></b>	A continuous 30-min treadmill-run consisting of alternating blocks of 1 min at 11 and 4 min at 15 on the Borg scale
<b>rpm</b>	Revolutions per minute
<b>RNA</b>	Ribonucleic acid
<b>S</b>	Secretory
<b>s</b>	Second

<b>SD</b>	Standard deviation
<b>SHBG</b>	Sex hormone-binding globulin
<b>StAR</b>	Steroidogenic acute regulatory protein
<b>TLR</b>	Toll-like receptors
<b>TNF</b>	Tumour necrosis factor
<b>URS</b>	Upper respiratory symptoms
<b>URTI</b>	Upper respiratory tract infections
$\dot{V}O_2$	Volume of oxygen utilized
$\dot{V}CO_2$	Volume of carbon dioxide produced
$\dot{V}O_{2max}$	Maximal oxygen uptake
$\dot{W}$	Workload
$\dot{W}_{max}$	Maximum workload
<b>W</b>	Watt

## **CHAPTER 1**

### **Introduction**

## 1.1 Introduction

To improve physical performance an athlete must increase the intensity and/or volume of exercise training (Costill *et al.*, 1991, Häkkinen *et al.*, 1987, Laursen and Jenkins, 2002, Lehmann *et al.*, 1992a, Mujika *et al.*, 1995, Sperlich *et al.*, 2010). In addition to this, there is a requirement for appropriate recovery to be completed during this elevation in training load in order to elicit increases in physical performance (Meeusen *et al.*, 2013). If appropriate recovery is not present after prolonged exposure to excessive exercise loads, then signs of overreaching may be developed within a period as short as 7 days (Halsen *et al.*, 2002). These signs may include acute performance decrements (Le Meur *et al.*, 2014, Meeusen *et al.*, 2004), mood and sleep disturbances (Morgan *et al.*, 1987), an increased rate of infection (Gleeson, 2006a, Nieman, 1997) and a continuous fatigued state (Meeusen *et al.*, 2010). If overreaching is recognised early and appropriate recovery is completed, athletes may fully recover and experience a supercompensatory increase in performance. This phenomenon is referred to as Functional Overreaching (FOR) (Dupuy *et al.*, 2010, Halsen and Jeukendrup, 2004, Meeusen *et al.*, 2013). It normally takes days to weeks for athletes to fully recover from FOR. However, if the recovery duration is not sufficient and the body is continually exposed to stress then Non-Functional Overreaching (NFOR) may occur. It takes weeks to months for full recovery to occur from this state. If NFOR is not recognised/diagnosed and the training/recovery imbalance continues an individual may develop the Overtraining Syndrome (OTS), which may take months to years for recovery to occur with some cases reporting no recovery at all (Dupuy *et al.*, 2010, Meeusen *et al.*, 2013, Urhausen and Kindermann, 2002). Signs of overreaching happen in both athletes from individual (37%) and team (17%) sports (Matos *et al.*, 2011), with the overall incidence of NFOR/OTS in an elite athlete's career ranging from ~35% (Kenttä *et al.*, 2001, Raglin *et al.*, 2000), to 67% in elite distance athletes (Morgan *et al.*, 1987), for example. Despite the prevalence of overreaching/OTS symptoms during an athlete's career, little progress has been made on uncovering objective biomarkers that

focus on identifying the onset of overreaching/OTS (Armstrong and VanHeest, 2002, Wyatt *et al.*, 2013).

Endocrine and immune markers of NFOR and OTS have been proposed but have been found to be inconclusive as useful biomarkers of these states, with research reporting increases (Grandys *et al.*, 2009, McConnel and Sinning, 1984), decreases (Hoogeveen and Zonderland, 1996, Kageta *et al.*, 2015, Wheeler *et al.*, 1991) and no changes (Miloski *et al.*, 2015, Mujika *et al.*, 1996, Vesterinen *et al.*, 2016) in the resting concentrations of certain hormones when comparing before and after a period of intensified training. Two proposed endocrine biomarkers, measured at rest, of the overreaching phenomena are circulating cortisol and testosterone due to their ability to indicate the body's catabolic/anabolic state, respectively. As resting levels of endocrine biomarkers of overreaching may not be informative, it has been proposed that an examination of the exercise-induced responses of particular hormones may be a useful approach to highlight and overreached state. A blunted plasma cortisol and adrenocorticotrophic hormone (ACTH) to a double cycle to fatigue protocol has been reported to occur following a 10-day training camp (Meeusen *et al.*, 2004). This absolute change in the exercise-induced blunted response was in the magnitude of 73% to the second bout to exhaustion (13.2 to 3.6 ngL<sup>-1</sup>). Moreover, this cycling protocol was reported to be sensitive enough to distinguish between NFOR athletes and those diagnosed with OTS, with NFOR athletes showing a considerably greater acute response of ACTH to the second cycle bout to fatigue (Meeusen *et al.*, 2010).

Despite the useful findings from Meeusen *et al.* (2010), it is reasonable to suggest that the exercise tool utilised in this study may not be of an ideal duration (the duration of 2 x cycles to fatigue and a 4h resting recovery) for individuals presenting with symptoms of overreaching/OTS. It was for this reason that Hough *et al.* (2011) developed a shorter (30 min), high-intensity cycle test that is capable of elevating salivary cortisol (by ~120% from

pre-exercise) and testosterone (by ~35% from pre-exercise) when in a normal-trained state. This high-intensity cycle bout was referred to as the 55/80. The 55/80 is a continuous 30 min cycling bout formed of alternate blocks of 1 min at 55% maximal work rate ( $\dot{W}_{\max}$ ) and 4 min at 80%  $\dot{W}_{\max}$ . Hough *et al.* (2013) and Hough *et al.* (2015) further identified that salivary cortisol and testosterone responses to the 55/80 are blunted following short periods of intensified training in physically active males and in a male elite triathlete population. Overall this suggests that the use of the exercise-induced responses of particular hormones (cortisol and testosterone more specifically) may be a useful method to highlight an overreached state (FOR or NFOR). In addition, salivary cortisol and testosterone measurements are widely utilized in exercise science as a non-invasive surrogate for blood sampling (Ahn *et al.*, 2007, Hayes *et al.*, 2015a, Hayes *et al.*, 2016, Hough *et al.*, 2011, Maso *et al.*, 2004). Salivary cortisol was reported for the first time in the early 1960's (Greaves and West, 1963), whereas salivary testosterone was first detected in 1976 (Landman *et al.*, 1976). Current guidelines recommend that water consumption should be ceased at least 10 minutes before collecting a saliva sample. However, to this author's knowledge there are no studies that examine the effect water consumption has on the exercise-induced salivary hormones concentrations. Therefore, it is of interest to examine if consuming water shortly before providing a saliva sample has an influence in the salivary cortisol and testosterone concentration levels in saliva.

Despite the interesting findings from Hough *et al.* (2013) and Hough *et al.* (2015), the reproducibility of the salivary cortisol and testosterone responses to the 55/80 has not been examined yet. Moreover, although the 55/80 may be a useful short-duration exercise tool to highlight a hormonal dysfunction following intensified training periods, this exercise test is completed on a cycle ergometer. It must be recognised not all athletes are familiar to or comfortable with cycling and therefore, the design of a similar running test may be beneficial to increase the population that can be tested for NFOR/OTS.

Continuously overloading the body by intensifying the training sessions has been reported to suppress the immune system (Lancaster *et al.*, 2003, Nieman, 2000, Pedersen *et al.*, 1996), eventually increasing the risk of common infections and upper respiratory symptoms (URS) (Heath *et al.*, 1992, Nieman, 1994b). As a link to the hormones referred to earlier in this Introduction, it is known that cortisol has an influence in the regulation of the exercise-induced, acute inflammatory response and it has been reported to be associated with a raise in neutrophil numbers and suppression of lymphocytes such as natural killer (NK) cells, and CD3<sup>+</sup> CD4<sup>+</sup> CD8<sup>+</sup> and CD3<sup>+</sup> CD4<sup>-</sup> CD8<sup>-</sup> T-cells following 2 x 45 min competitive football games (Malm *et al.*, 2004). Furthermore, the magnitude and function of circulating cells of the innate immune system, such as neutrophils and NK-cells, and the acquired immune system (e.g. T and B lymphocytes) may be altered by acute and chronic exposure to exercise (Walsh *et al.*, 2011b). Blood lymphocyte numbers [e.g. NK-cells, CD8<sup>+</sup> T-cells, CD4<sup>+</sup> T-cells, and Gamma Delta ( $\gamma\delta$ ) T-lymphocytes] have been shown to exponentially increase following an acute bout of exercise (Anane *et al.*, 2009, Mohr *et al.*, 2016). Anane *et al.* (2009) were the first to report that  $\gamma\delta$  T-lymphocytes are stress-responsive cells that are mobilized during physical (exercise) and psychological stress, therefore it will be of interest to analyse these cells proliferation in athletes suffering symptoms consistent with overreaching. It has been previously described that athletes suffering from NFOR/OTS have uncommonly low blood leucocyte counts and that depletion of the bone marrow's reserves of mature neutrophils may occur following repeated bouts of prolonged intensified-exercise training over a period of weeks or months (Gleeson and Robson-Ansley, 2006). Additionally, changes in neutrophil function [e.g. reactive oxygen species production (ROS) and phagocytic activity (PA)] have also been shown to be a useful marker when predicting the prevalence of overreaching/OTS (Yaegaki *et al.*, 2008). The authors reported a significant exercise-induced increase in leucocyte/neutrophil ratio in female athletes and an unexpected ~23% decrease in ROS without any compensation (~15% increase in PA) when comparing before to after a 7-day training camp. Therefore, regularly examining neutrophil functions





(Mackinnon and Hooper, 1994, Tiollier *et al.*, 2005) (Mackinnon and Hooper, 1994, Tiollier *et al.*, 2005) (Mackinnon and Hooper, 1994, Tiollier *et al.*, 2005) (Mackinnon and Hooper, 1994, Tiollier *et al.*, 2005) (Mackinnon and Hooper, 1994, Tiollier *et al.*, 2005) (Mackinnon and Hooper, 1994, Tiollier *et al.*, 2005) have reported a decrease in SIgA concentrations over the course of a 6-month swimming season, with overreached swimmers showing lower SIgA concentrations than well-trained, healthy swimmers. These contrasting results may be explained by the different training programmes, the fitness level of participants, and even the method used to express IgA data (Bishop and Gleeson, 2009). However, the fluctuations in SIgA in response to exercise and its association with upper respiratory symptoms (URS) in athletes may be a good indicator of mucosal immunity in athletes suspected to be overreached.

The following chapter will focus on the relevant background evidence that led to the design and development of the experimental chapters presented in this Thesis.

## **CHAPTER 2.**

# **Review of the Literature and Research Aims**

## 2.1 The General Adaptation Syndrome

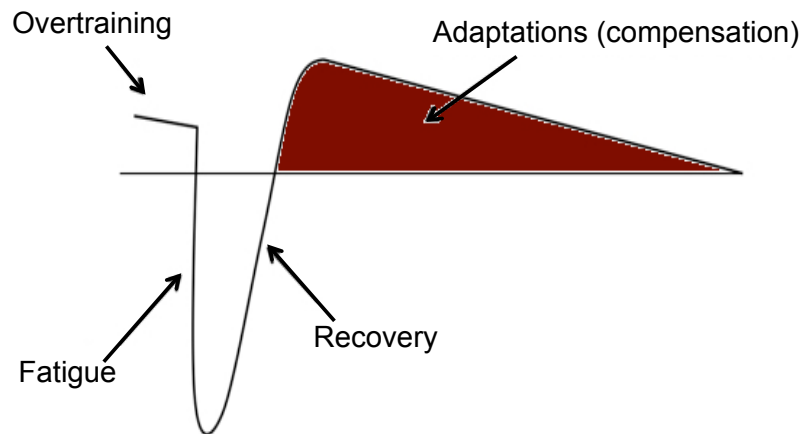
The General Adaptation Syndrome (GAS) describes the biological process of an organism's adaptive response to a new condition. This adaptation is developed in three stages (Selye, 1936). The first stage, known as the 'general alarm reaction' begins when the body identifies the onset and source of the biological stress resulting in the decline of its physical and mental capacity. As the biological stressor persists, a decline in the body's capability to cope with external stressors falls below baseline levels (Selye, 1946). In the second stage the organism activates a series of recovery mechanisms after the imposed imbalance, aiming to restore homeostasis. This stage is referred to as the 'stage of resistance'. At this point, depending on the treatment and duration of stimulus, the organism may build up such resistance that in the later part of the second stage a state of full recovery and/or adaptation may occur. However, depending on the severity of the damaging agent, the organism may potentially lose its resistance and succumb to the stress and present with symptoms resembling those experienced in the first stage. This latter manifestation may occur in the so called 'exhaustion phase', which is the third and final stage of the syndrome.

Extrapolating GAS to what would be expected to happen in response to continuous physical stress (i.e. exercise training) the first stage would be considered to occur when the individuals overload their body beyond their capability and start fatiguing. This would lead to an inability to maintain a given exercise intensity over a certain period of time (Fitts, 1994), may be considered as the 'general alarm reaction' from the organism highlighting a decrease in its capacities following a stressful situation. The human body will attempt to recover from the exposure to the physical stress ('stage of resistance') and may adapt to improve its capability to cope with this physical stress exposure. The 'exhaustion phase' may then be experienced if the exposure to physical stress persists

and the human body is not allowed sufficient recovery, precluding the development of a mechanism of adaptation.

### **2.2 Periodisation of Training and The Overload Principle**

To achieve positive physiological adaptations in line with the GAS, athletes and coaches must plan their training programmes appropriately. Periodisation is the planned variation of a training programme in an attempt to lead the athlete to their optimal physical performance. Periodisation will normally involve three main phases: the preparation, the competition and the transition phases (Koutedakis *et al.*, 2006). The preparation stage is the longest of the processes, involving the development and improvement of all technical and tactical aspects required for the competition. It is fully focused on improving the overall performance of the athletes, to prepare them for the forthcoming phase. The competition phase will vary in duration and complexity depending on the number of competition dates. During this stage, there is a large focus on improving and maintaining fitness levels (Argus *et al.*, 2009, Argus *et al.*, 2012, Baker, 2001). Periodisation planning typically ends after this phase, with the implementation of lower-intensity training sessions and longer periods of recovery, referred to as the transition phase (Bompa, 1996). In summary, if a periodisation period is completed successfully it will lead an athlete into a physical state that may lead to an optimal physical performance. This adaptation is achieved by an appropriate balance of stress and recovery, which is illustrated in Figure 2.1.

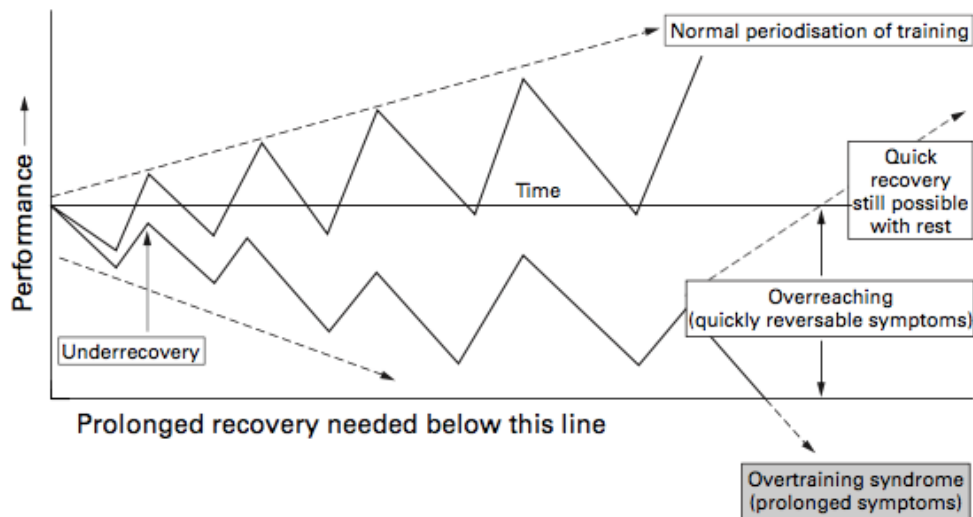


**Figure 2.1** The stages of the supercompensation cycle (adapted from Koutedakis *et al.* (2006, p. 6)).

This figure presents the supercompensation cycle that links intensified-training load and recovery as the bases for a positive physical adaptation (Bompa and Haff, 2009). Similarly to GAS, the first stage begins when athletes overtrain by increasing volume, intensity and/or frequency of exercise training. Overtraining is likely to increase fatigue levels, leading to the onset of the ‘Fatigue’ stage. This stage should lead to acute declines in exercise performance (Morton, 1997). Following the ‘Fatigue’ stage the recovery stage begins, aiming an adaptation to higher levels of physical performance (Koutedakis *et al.*, 2006) (Figure 2.1).

A disrupted balance between the accumulation or increase in training load (either by increasing volume and/or intensity) and the recovery from it may trigger acute or chronic states of fatigue with concomitant decreases in physical performance. If the repetition of training cycles is not accompanied by sufficient recovery this may lead the athletes to overreach. If overreaching is recognised early and appropriate recovery is completed, a supercompensatory response in exercise performance may be achieved. This state is referred to as FOR (Dupuy *et al.*, 2010, Halson and Jeukendrup, 2004, Meeusen *et al.*, 2013). However, if the necessary recovery is insufficient and the athlete continues the exposure to stress, then NFOR may occur. If NFOR is not diagnosed and the

training/recovery imbalance continues further, then OTS may be developed (Meeusen *et al.*, 2013). The difference between these stages is simply the duration required for full recovery to occur. Recovery from FOR may occur within days to weeks, whereas several weeks to months may be required for recovery from NFOR to occur. Recovery from OTS may take years, and more severe cases have reported no recovery at all (Dupuy *et al.*, 2010, Meeusen *et al.*, 2013, Urhausen and Kindermann, 2002). The figure below schematically clarifies the concepts of overreaching – FOR when quick recovery is still possible, or NFOR where recovery is still possible - and the more serious states of OTS.



**Figure 2.2** Schematic representation of the practice of overreaching and OTS. (Taken from Budgett (1998)).

The most common symptoms of maladaptation associated with NFOR/OTS are chronic declines in athletic performance associated with increased fatigue levels (Coutts *et al.*, 2007, Halson and Jeukendrup, 2004, Le Meur *et al.*, 2014, Meeusen *et al.*, 2004, Urhausen *et al.*, 1995, Urhausen *et al.*, 1998b), mood and sleep disturbances (Fry *et al.*, 1991, Hollander *et al.*, 1995, Kenttä *et al.*, 2001, Morgan *et al.*, 1987, Raglin, 1993, Raglin *et al.*, 2000), prolonged fatigue and weakness (Hough *et al.*, 2013, Meehan *et al.*, 2004,

Meeusen *et al.*, 2010, Urhausen *et al.*, 1995), and an elevated incidence of infections (Bishop and Gleeson, 2009, Fahlman and Engels, 2005, Gleeson, 2006b, Gleeson *et al.*, 2012, Neville *et al.*, 2008, Nieman, 1997, 2009, Robson *et al.*, 1999b, Tiollier *et al.*, 2005). Even with this said, these signs and symptoms are not always present in a NFOR or OTS athlete and therefore these states are often diagnosed retrospectively.

### 2.3 Hormonal Markers of Overreaching/OTS

Two proposed endocrine biomarkers of the overreaching phenomena are plasma/serum cortisol and testosterone, which can indicate the body's catabolic/anabolic state, due to their roles in protein degradation/synthesis, respectively.

Measuring resting concentrations of plasma/serum cortisol and testosterone has been proposed as a possible method to help diagnose NFOR and OTS (Hooper *et al.*, 1993, Hooper *et al.*, 1995, Mackinnon *et al.*, 1997a, Snyder *et al.*, 1995, Urhausen *et al.*, 1998a). However, on detailed examination of this research it is clear that the findings are inconclusive with increased, decreased and unaltered responses of the resting concentrations of these hormones being shown when individuals are suffering from overreaching or OTS. For example, decreased resting serum cortisol (33% decrease from pre-race values) and testosterone (40% decrease from pre-race values) concentrations in elite cyclists have been reported over the course of a 3-week professional cycle race (Lucia *et al.*, 2001). Contrastingly, an increase of 17% in circulating testosterone and 26% in free testosterone from baseline measures were found in young, previously untrained men following a 5-week endurance training programme (4 sessions per week at 90% maximal oxygen uptake ( $\dot{V}O_{2max}$ )), with no alteration in circulating cortisol (Grandys *et al.*, 2009). Finally, Hoogeveen and Zonderland (1996) reported a ~15% decrease in resting



testosterone and a ~39% increase in resting cortisol concentrations in elite cyclists over the course of 4 months of intensified training.

These conflicting findings are likely due to the many different training stresses experienced by the study participants (i.e. the training protocols to induce overreaching), the timing of day of the sample collection, the controls or the absence of particularly important controls (e.g. hydration status) when measuring these hormones.

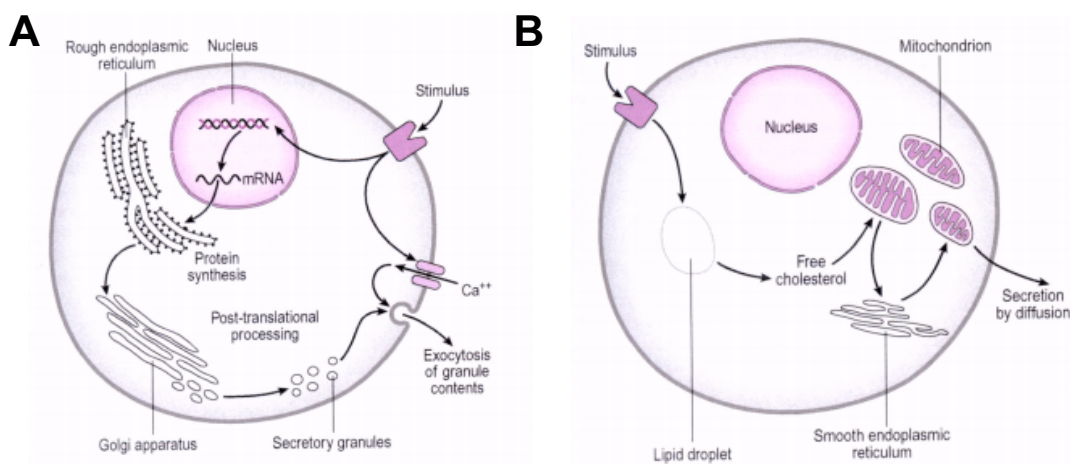
### **2.3.1 Hormonal Classification, Synthesis and Secretion**

The endocrine system connects to all other body systems and controls its metabolic and physiological functions (Barrett *et al.*, 2005). It incorporates the ductless glands which are responsible for synthesising hormones and releasing them directly into the circulatory system (Martin, 1976). These glands are located in different areas of the human body and, depending on their function, they can secrete more than one type of hormone.

Hormones are chemical messengers classified as signalling molecules that act through receptors to cause action in their target cells, thus regulating the metabolic, physiological and reproductive mechanisms of the body (Hinson *et al.*, 2010, Martin, 1976). The chemical structure of a hormone directly influences the way in which hormones are stored, secreted, transported through the bloodstream and their mechanism of action. There are three main categories of hormones that differ on the basis of their chemical structure (Hinson *et al.*, 2010). The most numerous in the human body are the peptide hormones, formed of amino acid chains connected via peptide bonds. The second group of hormones is the steroid hormones, which originate from cholesterol and have a common fundamental structure. The third major group of hormones comprises those that are derived from amino acids (amine hormones). This Thesis focuses on steroid hormones

with a theoretic focus on their peptide precursors. Steroid hormones may be divided into more specific subcategories. This Thesis will specifically focus on androgens (male sex hormones) and glucocorticoids. The glucocorticoids play a role in glucose homeostasis (Olefsky and Kimmerling, 1975).

Peptide hormones are synthesized in cells in which the rough endoplasmic reticulum and Golgi apparatus are abundantly present (Hinson *et al.*, 2010). The synthesised peptide hormones are stored within granules in the endocrine cells, before being released to the bloodstream in response to a stimulus (Kelly, 1987). Peptide hormones secretion to the bloodstream occurs through a mechanism referred to as exocytosis (see Figure 2.3 A). Exocytosis is the movement of molecular materials out of cells through vesicles or secretory granules that contain the hormone ready for secretion and is triggered by an elevation in intra-cellular levels of calcium ions ( $Ca^{2+}$ ) or depolarisation of the cell. In contrast, cells responsible for secreting steroids are abundant in mitochondria and smooth endoplasmic reticulum and lipid droplets are found in the cytoplasm (Herz and Fried, 1958, Hinson *et al.*, 2010). Steroid hormones do not require any secretory mechanism and simply diffuse out of the cell membrane to the extracellular fluid (see Figure 2.3 B).



**Figure 2.3** Synthesis and secretion of peptide (A) and steroid (B) hormones. (Taken from Hinson *et al.* (2010)).

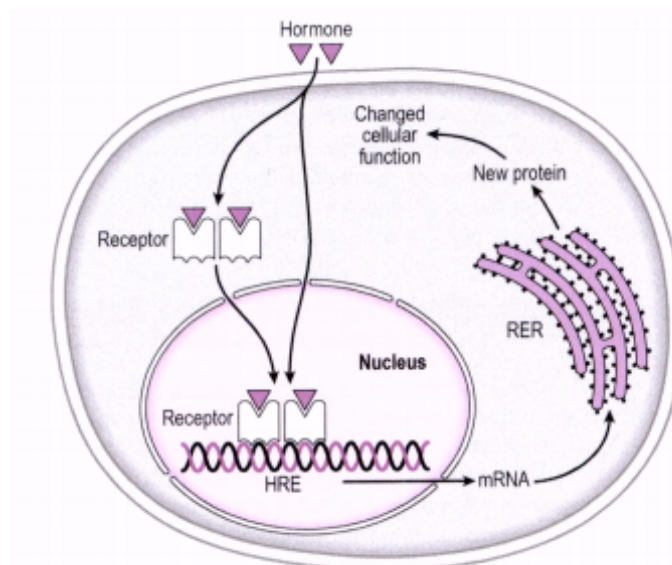
### 2.3.2 Hormone Action on Their Target Cells

Typically, hormones travel in the bloodstream in very low concentrations from the cells in which they are synthesized to reach the target cells where they will cause action. All hormones act by binding to specific sensing units, receptor proteins, on the cell membrane or in the cytoplasm of their target cells, which will in turn trigger an intracellular response and consequently activate a biological reaction. For this to occur, hormones travelling in the bloodstream to a specific tissue must first be unbound from any protein carrier (i.e. in a free state) before they can cause their action (Norman and Litwack, 1997).

The location of the receptors to which hormones will bind to depends on the chemical nature of the hormone itself. A target cell may express two types of receptors – a membrane-bound surface receptor and a soluble intracellular receptor. As peptide hormones are water-soluble they dissolve readily in plasma. However, these hormones cannot enter the target cell as they are either too large and hydrophilic and, therefore, interact with receptors on the cell membrane, forming a hormone-receptor complex (Martin, 1976). There are two main classes of cell surface receptors: the G-protein coupled receptors (GPCRs) which act through the generation of a second messenger - chemical signals that spread the initial signal sent by the hormone (i.e. the first messenger) – which in turn are linked to protein kinase activation, and those receptors that directly activate a protein kinase (Hinson *et al.*, 2010). The GPCRs are the most common within the endocrine system (Hinson *et al.*, 2010). The most abundant central diffusible intracellular second messenger is cyclic adenosine monophosphate (cAMP) (Rasmussen and Barrett, 1984). Peptide hormone-receptor complexes activate certain enzymes such as adenylyl cyclase, triggering the dephosphorylation of adenosine triphosphate (ATP) to cAMP (Hurley, 1999). An elevation in cAMP concentrations activates a family of enzymes termed protein kinase A (PKA), which in turn catalyses the

phosphorylation of a number of proteins involved in specific cell mechanisms (Walsh and Van Patten, 1994).

Steroids are mostly insoluble in plasma, due to their lipophilic character. These hormones bind to carrier proteins to be transported through the circulatory system, entering into the cells via diffusion to interact with the intracellular receptor in the cytoplasm or in the nucleus of the cell (Hinson *et al.*, 2010). Subsequently, the formed hormone-receptor complex binds to specific response elements in the promoter region of specific genes (Hinson *et al.*, 2010). This stimulates gene transcription, the first step of gene expression, where a specific DNA sequence is used as a template to generate a messenger RNA (mRNA) molecule (Mooren *et al.*, 2005). The genomic effects of steroids are mediated through particular steroid hormone receptors that act as intracellular transcription factors (Beato, 1989). This process is accomplished through use of an RNA polymerase enzyme. The obtained genetic code will then travel to the cytoplasm where proteins will be synthesized (Branden and Tooze, 1993, Maestroni, 2010). In this way, steroid hormones stimulate protein synthesis and thereby alter cellular function (see Figure 2.4).



**Figure 2.4** The action of steroid hormones in the cell. Taken from Hinson *et al.* (2010).

### 2.3.3 Patterns of Hormone Secretion

The regulation of the endocrine system involves preventing a cascade of catabolic events, and aims to maintain homeostasis (Hinson *et al.*, 2010). This regulation is coordinated by episodic secretion of regulatory hormones involved in the process. The pattern of episodic secretion of hormones is important when regulating factors such as the half-life of the hormone itself and the frequency and amplitude of secretory incidences (Hinson *et al.*, 2010).

A daily pattern is also involved in hormone secretion, and this is referred to as diurnal variation or circadian rhythm. This is any endogenous, regular biological process that follows a 24-hour (diurnal) pattern (Edgar *et al.*, 2012, Vitaterna *et al.*, 2001). The singular diurnal pattern of each hormone is mediated by the so called 'body clock' i.e. the suprachiasmatic nucleus in the hypothalamus (Hinson *et al.*, 2010, Martin, 1976).

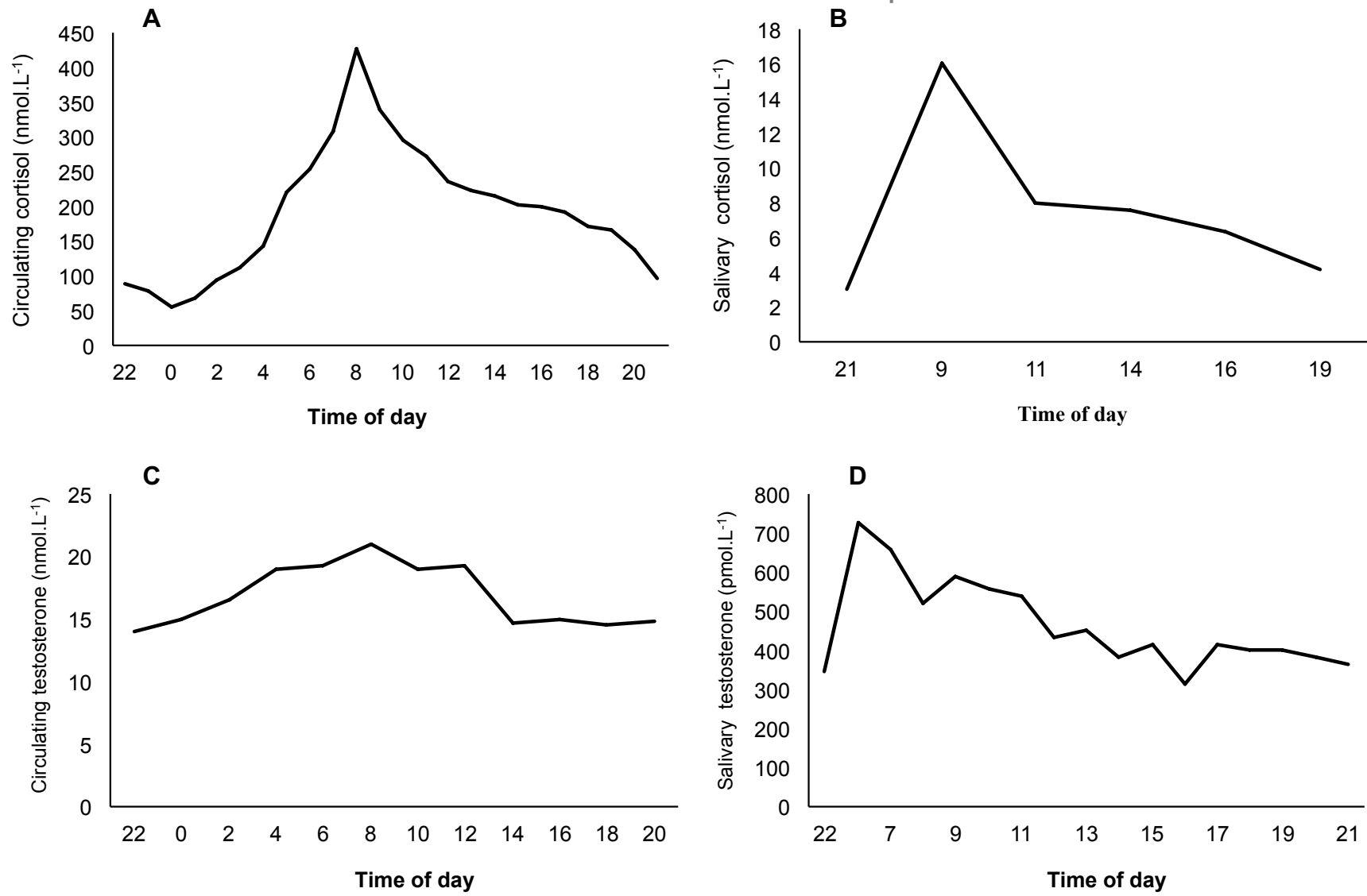
Cortisol and testosterone are two hormones that are mediated by a marked circadian rhythm (Chan and Debono, 2010, Debono *et al.*, 2009, Sinha-Hikim *et al.*, 2003, Weitzman *et al.*, 1971). This is depicted in Figure 2.5. In humans, circulating cortisol lowest secretion rates in healthy individuals typically occur during the first few hours of night-time sleep (Chan and Debono, 2010, Weitzman *et al.*, 1971). This is followed by a robust, immediate rise, with peak concentrations occurring after awakening and decreasing from then (Born *et al.*, 1999, Van Cauter, 1990, Weibel *et al.*, 1995). A similar pattern is seen in the testosterone circadian rhythm, with research reporting peak morning circulating levels and a nadir occurring in the early evening (Cooke *et al.*, 1993, Diver *et al.*, 2003, Plymate *et al.*, 1989, Walton *et al.*, 2007).

## Chapter 2: Review of the Literature and Research Aims

An age-related response is also known to occur in both circulating cortisol and testosterone. The concentration of cortisol in the circulation has been reported to increase with age, with research reporting that a progressive elevation in the levels of cortisol nocturnal nadir levels may be the main cause for an increase in mean plasma cortisol with age in both sexes (Sherman *et al.*, 1985, Van Cauter *et al.*, 1996). Contrastingly, the production of testosterone starts gradually declining with age from around early 30's onwards (Baker *et al.*, 1976, Dillon *et al.*, 2010, Ferrini and Barrett-Connor, 1998, Harman *et al.*, 2001, Rubens *et al.*, 1974, Vermeulen *et al.*, 1972).

Cortisol and testosterone can be measured in saliva and blood. Resting serum cortisol levels throughout the day in healthy individuals oscillate, with nadir concentrations of  $<50 \text{ nmol}\cdot\text{L}^{-1}$  and peak concentrations being approximately  $\sim 428 \text{ nmol}\cdot\text{L}^{-1}$  (Debono *et al.*, 2009). Moreover, resting salivary cortisol concentrations vary from  $\sim 2$  to  $\sim 14 \text{ nmol}\cdot\text{L}^{-1}$  during the day (Kudielka *et al.*, 2007). Resting plasma testosterone concentrations in healthy, young individuals range from approximately 13 to  $35 \text{ nmol}\cdot\text{L}^{-1}$  (Sinha-Hikim *et al.*, 2003, Traish *et al.*, 2011, Walton *et al.*, 2007), whereas in elderly men these are reduced ranging between approximately 5 to  $20 \text{ nmol}\cdot\text{L}^{-1}$  (Wierman, 2007). Daily average salivary testosterone concentrations have been shown to be around  $\sim 716 \pm 92 \text{ pmol}\cdot\text{L}^{-1}$  (Landman *et al.*, 1976).

Secretion and regulation of circulating cortisol and testosterone fluctuations is accomplished by a cascade of precursor hormones synthesized and released within the hypothalamic-pituitary-adrenal (HPA) and the hypothalamic-pituitary-gonadal (HPG) axes, respectively.



**Figure 2.5** Circadian rhythm of plasma cortisol (A), salivary cortisol (B), plasma testosterone (C) and salivary testosterone (D). Reproduced from Debono *et al.* (2009) (A), Smyth *et al.* (1997) (B), Walton *et al.* (2007) (C) and Kraemer *et al.* (2001) (D).

### 2.3.4 Hypothalamic-Pituitary-Adrenal (HPA) Axis

The hypothalamus, located beneath the thalamus at the base of the brain, is regulated by a wide range of neural inputs that in turn adjust its secretory functions following exposure to stress (Akana *et al.*, 1992), exercise (Duclos *et al.*, 1998, Duclos *et al.*, 1997) and time of day (Crofford *et al.*, 1997). It is directly connected to the pituitary gland by the pituitary stalk. Nuclei in the hypothalamus (i.e. areas of the hypothalamus that are anatomically or functionally distinct) detect circulating levels of certain hormones and control the release of anterior pituitary hormones by producing releasing factors, which are carried to the anterior pituitary to stimulate the release of other hormones via a specific blood portal known as the hypophysial portal (Edington and Edgerton, 1976).

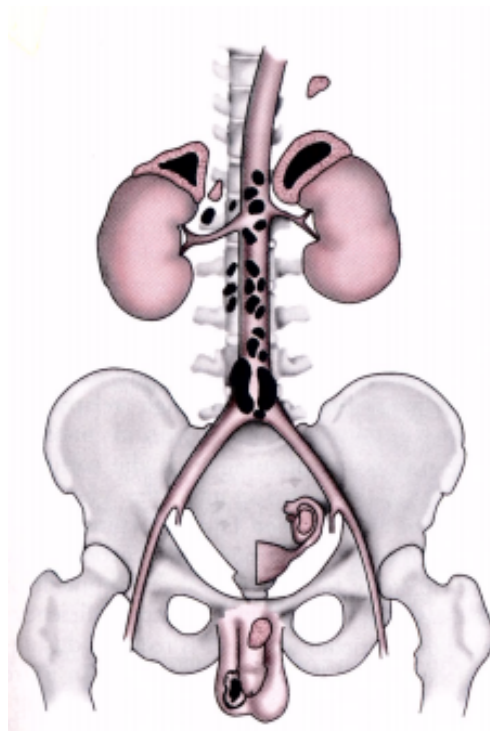
Corticotrophin-releasing hormone (CRH) is a peptide that is synthesized and released by the hypothalamus (Ganong, 1991) following activation of the sympathetic nervous system in response to a stressful situation (Vale *et al.*, 1981). It acts by traveling through the portal hypophysial vessel to the anterior pituitary gland (Ganong, 1991) to stimulate the secretion of ACTH (Vale *et al.*, 1981), therefore regulating the glucocorticoid synthesis by the adrenal cortex (Lundblad and Roberts, 1988).

The adrenal gland is controlled by a circadian rhythm, regulated via the splanchnic nerve (Jasper and Engeland, 1997). This means that there are times during the day when this gland most effectively responds to ACTH. In turn, ACTH is the key regulator of glucocorticoid secretion (Tsigos and Chrousos, 2002). ACTH is found in the circulatory system mainly bound to a glycoprotein called transcortin (~75%), but it may also be found loosely bound to plasma albumin (~15%) with the remainder of this hormone circulating unbound/free (~10%) (Norman and Litwack, 1997). Secreted ACTH travels through the bloodstream to the adrenal glands, located at the superior pole of each kidney (Ganong, 1991).



## Chapter 2: Review of the Literature and Research Aims

There are two endocrine organs in the adrenal gland, one surrounding the other: the inner adrenal medulla, which constitutes 28% of the mass of the adrenal gland, and the outer adrenal cortex. In adult mammals, the adrenal cortex itself is divided into three distinct zones that are connected between each other. The outer zona glomerulosa is made up of cell spirals that are connected to the columns of cells that form the zona fasciculata. The inner portion of the latter merges into the zona reticularis, where the cell columns interlace in a network (Ganong, 1991). Cortical and medullary tissue may sometimes be found in some extraadrenal sites (see Figure 2.6) (Williams, 1962). Under resting conditions, an increase in CRH pulses in the early hours in the morning results in subsequent bursts of ACTH and cortisol in the circulation (Horrocks *et al.*, 1990). During acute stress events, the amplitude of CRH pulsations prominently elevates, thus increasing the ACTH and, consequently cortisol secretory events (Tsigos and Chrousos, 1994). Prolonged exposure to either acute or chronic stress has been shown to reduce the sensitivity of the HPA axis to glucocorticoid (specifically cortisol) negative feedback (Akana *et al.*, 1992, Duclos *et al.*, 1998, Duclos *et al.*, 1997).



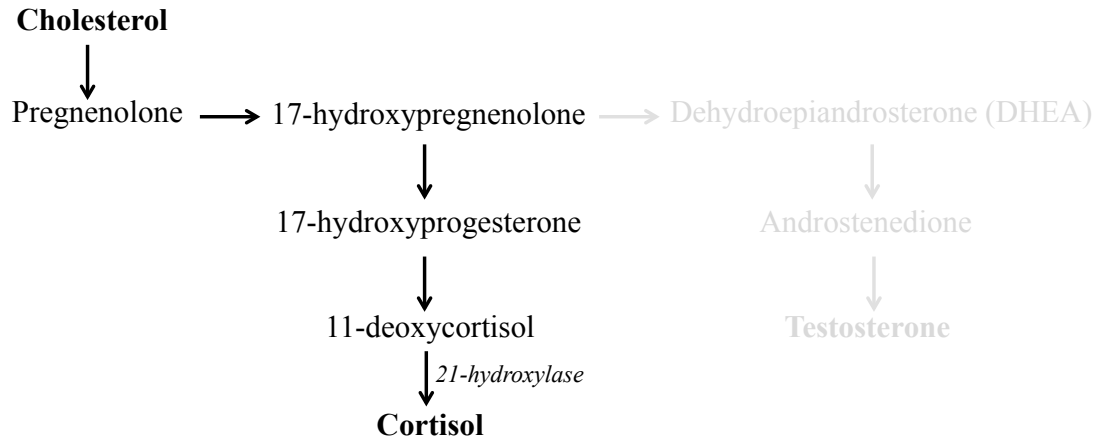
**Figure 2.6** Human adrenal glands. Adrenal cortical and medullary tissue are presented as stippled and black, respectively. Extra-adrenal sites sometimes found are also shown. Taken from Ganong (1991).

### 2.3.5 Cortisol

Cortisol is a glucocorticoid synthesized from cholesterol (Figure 2.7) and is produced in the zona fasciculata of the adrenal cortex through the activation of the HPA axis (Ganong, 1991). This steroid travels in the circulatory system bound to transcortin (~90%), albumin (~7%) or is found unbound (free) (1 to 3%). Free cortisol represents the active form of this hormone. Cortisol is released in response to both physiological and psychological stress (Hinson *et al.*, 2010). This Thesis will predominantly focus on the exercise-induced cortisol responses and how this may alter following a period of intensified exercise training stress.

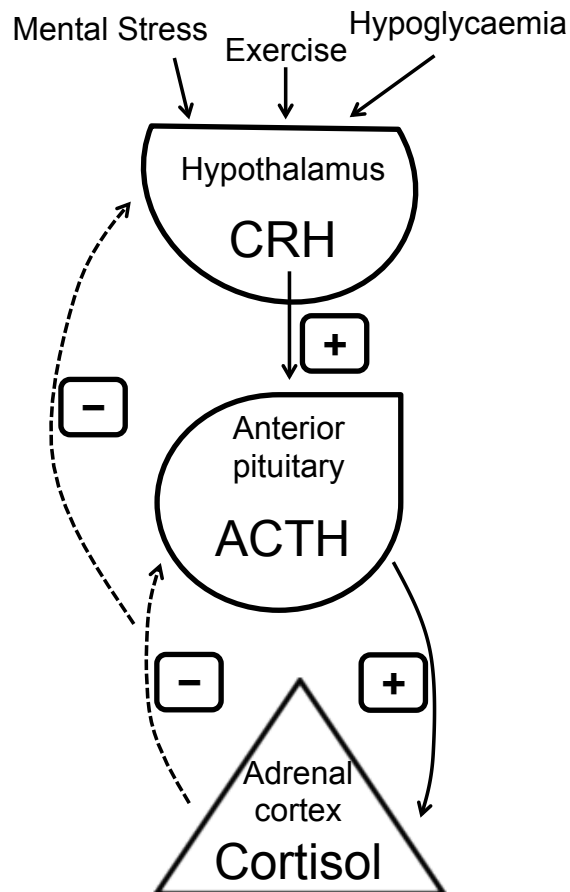
Cortisol is known to have many actions in the human body. This steroid is important in maintaining blood glucose levels and glycogen stores when in a fasting state (Friedmann *et al.*, 1967, Hinson *et al.*, 2010), and has also been shown to increase glucose production by stimulating gluconeogenesis (Khani and Tayek, 2001), and glycogenolysis and the onset of the insulin-dependent regulatory pathway (Plas and Nunez, 1976).

Furthermore, it has been reported that cortisol can lead to a reduction in leucocyte mobilisation in healthy adult individuals, specifically an eosinopenia being explained by eosinophils migrating from the circulation to lymphoid organs in response to elevated blood cortisol levels (Sabag *et al.*, 1978), and lymphocytes showing an inverse relation with cortisol levels (Miyawaki *et al.*, 1984, Thomson *et al.*, 1980). In contrast, cortisol has been proposed to sustain neutrophilia following long periods of intensified exercise (Pedersen and Ullum, 1994; Pedersen *et al.*, 1997).



**Figure 2.7** The synthesis pathway of cortisol.

Cortisol diurnal variation is controlled by a circadian signal generated by the circadian pacemaker located in the suprachiasmatic nucleus of the hypothalamus, and by the alteration in wakefulness and sleep patterns (Czeisler and Klerman, 1998). The regulation of the synthesis and secretion of cortisol is controlled by a homeostatic process called negative feedback. Thus, cortisol (the final product of the HPA axis cascade) acts to inhibit the release of CRH, which in turn downregulates the secretion of ACTH and eventually, cortisol itself (Hinson *et al.*, 2010) (Figure 2.8).



**Figure 2.8** Negative feedback control of the secretion of cortisol. The dashed and solid arrows indicate inhibitory and stimulatory effects, respectively.

**a) Acute Cortisol Responses to Exercise**

Cortisol response to exercise is influenced by factors such as duration and intensity of the exercise session, and the training status of the individual (Bloom *et al.*, 1976, Galbo *et al.*, 1977, Kuoppasalmi *et al.*, 1980, Sutton *et al.*, 1973, Tremblay *et al.*, 2004). Furthermore, although cortisol has been shown to increase in response to endurance (examples in Table 2.1) and resistance exercise (Di Blasio *et al.*, 2016, Guezennec *et al.*, 1986, Häkkinen *et al.*, 1988, Kraemer *et al.*, 1999, Leite *et al.*, 2011, McCaulley *et al.*, 2009), this Thesis will focus on the acute cortisol responses to high-intensity, short-duration (30 min) aerobic exercise.

## Chapter 2: Review of the Literature and Research Aims

It has been proposed that a threshold of exercise intensity exists to elevate cortisol concentrations. It has been shown that short-duration exercise above  $\sim 50\% \dot{V}O_{2\max}$  decreases cortisol concentrations and exercise above  $60\% \dot{V}O_{2\max}$  may induce an elevated response (Davies and Few, 1973). Furthermore, it has been reported that continuous cycling exercise for  $\sim 120-180$  min at moderate intensities of  $\sim 50\% \dot{V}O_{2\max}$  (Tabata *et al.*, 1991) and  $\sim 55\% \dot{V}O_{2\max}$  (Robson *et al.*, 1999b, Tremblay *et al.*, 2005) - induces a robust elevation in circulating cortisol. However, these responses are not immediate and more recently, several studies reported that higher exercise intensities ( $>60\% \dot{V}O_{2\max}$ ) elevate both plasma and salivary cortisol following short-duration ( $\sim 30$  min) exercise bouts (Cadore *et al.*, 2012, Hough *et al.*, 2013, Hough *et al.*, 2011, Robson *et al.*, 1999b). A summary of studies reporting the acute responses of cortisol to exercise is outlined in Table 2.1.

**Table 2.1** Acute cortisol responses to continuous exercise.  
\*Plasma cortisol; \*\*Salivary cortisol.

Authors (Publication year)	Participant population	Mean $\pm$ SD maximal oxygen uptake ( $\dot{V}O_{2max}$ ) values	Type of exercise	Duration	Direction of concentration variation	Percentage change
Verde, Thomas and Shephard (1992)	Trained distance runners	$65.3 \pm 4.9 \text{ mL} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$	Treadmill-run at 80% $\dot{V}O_{2max}$	30 min	Increase	*20%
Gray <i>et al.</i> (1993)	Trained male athletes	$64.3 \pm 3.8 \text{ mL} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$	Interval treadmill- run at max speed on $\dot{V}O_{2max}$ test	To exhaustion	Increase	*60%
Nieman <i>et al.</i> (1994)	Well-conditioned male runners	$66.0 \pm 1.9 \text{ mL} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$	Treadmill-run at 50% $\dot{V}O_{2max}$	45 min	No change	*N/A
VanBruggen <i>et al.</i> (2011)	Endurance trained males	$58.2 \pm 6.4 \text{ mL} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$	Cycle at 60% $\dot{V}O_{2max}$	30 min	No change	*N/A **N/A
VanBruggen <i>et al.</i> (2011)	Endurance trained males	$58.2 \pm 6.4 \text{ mL} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$	Cycle at 80% $\dot{V}O_{2max}$	30 min	Increase	*40% **171%
Hough <i>et al.</i> (2011)	Physically active males	$50.9 \pm 8.8 \text{ mL} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$	Cycle at ~75% $\dot{W}_{max}$	30 min	Increase	*~80% **~120%
Hough <i>et al.</i> (2013)	Recreationally active males	$52 \pm 5 \text{ mL} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$	Cycle at ~75% $\dot{W}_{max}$	30 min	Increase	**~210%
Sgrò <i>et al.</i> (2014)	Healthy males	$49 \pm 5 \text{ mL} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$	Cycle at anaerobic threshold (~50% $\dot{V}O_{2peak}$ )	30 min	Decrease	*6%
Peake <i>et al.</i> (2014)	Well-trained male cyclists and triathletes	$4.8 \pm 0.3 \text{ L} \cdot \text{min}^{-1}$	Cycle at ~65% $\dot{V}O_{2max}$	61min $\pm$ 14s	No change	*N/A

### **b) Exercise-Induced Cortisol Responses After Intensified Training**

The HPA axis is involved in the body's short- and long-term response to stress, such as the one induced by exercise. Cortisol, the final product of the HPA cascade, has a long half-life (~66 min) in the circulatory system (Weitzman *et al.*, 1971). As shown in the previous section of this Thesis, plasma and salivary cortisol concentrations acutely elevate in response to high-intensity exercise bouts. It has been hypothesized that a hormone-mediated dysregulation occurs with the incidence of OTS (Fry *et al.*, 1994a, Kraemer and Ratamess, 2005, Lehmann *et al.*, 1993, Meeusen *et al.*, 2004, Urhausen *et al.*, 1998a).

Resting concentrations of cortisol have been proposed as a possible biomarker of NFOR and OTS. However, on detailed examination of this research it is clear that the findings are inconclusive with increased (Kirwan *et al.*, 1988, Svendsen *et al.*, 2016), decreased (Lehmann *et al.*, 1993) and unaltered (Balsalobre-Fernández *et al.*, 2014, Mujika *et al.*, 1996, Verde *et al.*, 1992) concentrations shown after intensified training. This is likely due to the many differently designed training programmes experienced by the participants in these studies.

Table 2.2 presents an overview of several studies reporting resting and exercise-induced cortisol responses following a period of intensified training.

**Table 2.2** Cortisol concentrations following a period of intensified training.  
\*Plasma cortisol; \*\*Salivary cortisol.

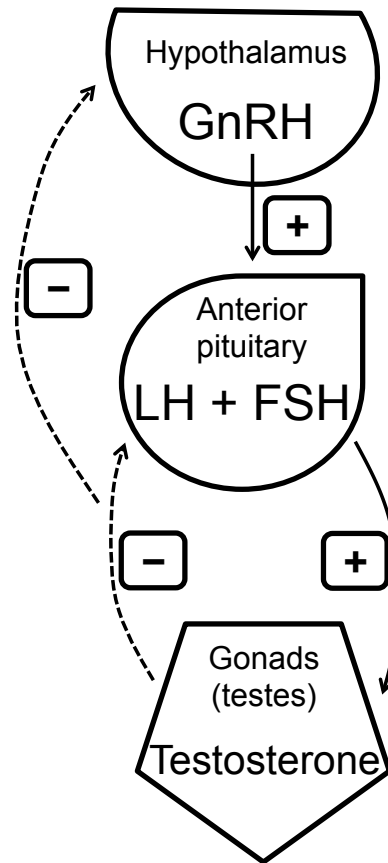
Authors (Publication year)	Participant population	Fitness level	Duration of training	Resting or exercise sample	Concentration variation	Percentage change
Kirwan <i>et al.</i> (1988)	Trained collegiate swimmers	No data	10 days	Rest	Increase	*~24%
Verde, Thomas and Shephard (1992)	Trained distance runners	65.3 ± 4.9 mL·kg <sup>-1</sup> ·min <sup>-1</sup>	3 weeks	Exercise	No change	*N/A
Verde, Thomas and Shephard (1992)	Highly trained distance runners	65.3 ± 4.9 mL·kg <sup>-1</sup> ·min <sup>-1</sup>	3 weeks	Rest	Increase	*~11%
Lehmann <i>et al.</i> (1993)	Recreationally active males	52.0 ± 5.2 mL·kg <sup>-1</sup> ·min <sup>-1</sup>	6 weeks	Rest	Decrease	*~13%
Mujika <i>et al.</i> (1996)	Elite swimmers	No data	12 weeks	Rest	No change	*N/A
Hough <i>et al.</i> (2013)	Recreationally active males	52 ± 5 mL·kg <sup>-1</sup> ·min <sup>-1</sup>	11 days	Exercise	Blunted response	**166%
Balsadore-Fernández <i>et al.</i> (2014)	Elite endurance runners	1500-m personal bests of 3:38 – 3:58 min	39 weeks	Rest	No change	**N/A
Hough <i>et al.</i> (2015)	Male elite triathletes	67.6 ± 4.5 mL·kg <sup>-1</sup> ·min <sup>-1</sup>	10 days	Exercise	No change	**N/A
Svendsen <i>et al.</i> (2016)	Trained male cyclists	72 ± 5 mL·kg <sup>-1</sup> ·min <sup>-1</sup>	8 days	Rest	Increase	*~67%



### 2.3.6 The Hypothalamic-Pituitary-Gonadal (HPG) Axis

Similarly to the HPA axis, the HPG axis is composed by the hypothalamus and the anterior pituitary gland, and terminates at the gonads (testes in males). Among other functions, the HPG axis is responsible for regulating both androgen synthesis and spermatogenesis (Hinson *et al.*, 2010, Saez *et al.*, 1989). Androgens are synthesized in the Leydig cells in the testes (Eik-Nes, 1975). In the HPG axis, gonadotropin-releasing hormone (GnRH) is secreted in an episodic fashion from the hypothalamus and travels to the anterior pituitary gland via the hypothalamus-hypophysial portal system, consequently activating the synthesis of a class of hormones called gonadotropins, specifically follicle-stimulating hormone (FSH) and luteinising hormone (LH) (Santoro *et al.*, 1988, Santoro *et al.*, 1986). The hormones FSH and LH are subsequently released in a pulsatile mode, with LH acting on the Leydig cells to stimulate testosterone production. Concomitantly, FSH acts with testosterone on Sertoli cells to stimulate spermatogenesis (Williams, 1962).

Testosterone exerts a negative feedback at the level of the hypothalamus and pituitary to control normal reproductive function (Figure 2.9) (Hinson *et al.*, 2010). A marginal concentration of the circulating testosterone (~5%) originates from the adrenal cortex (Williams, 1962).



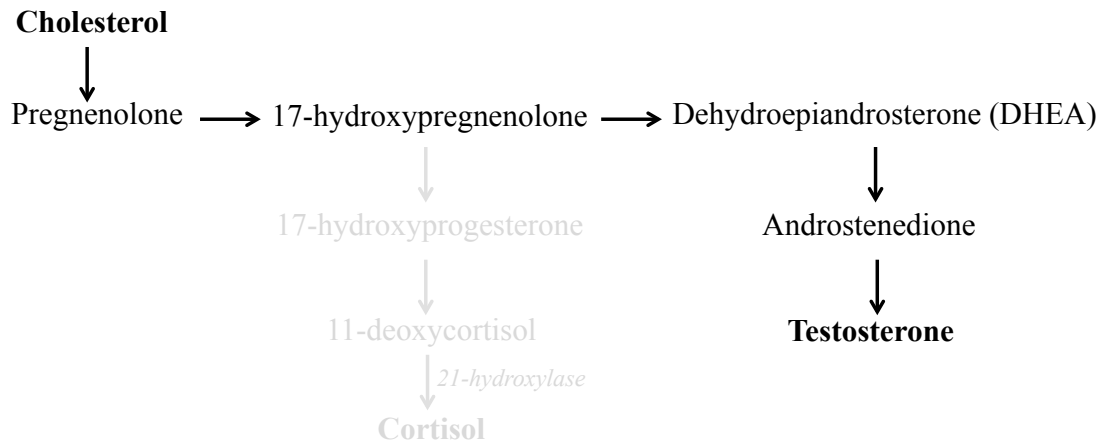
**Figure 2.9** Negative feedback loop of the HPG axis. The dashed and solid arrows indicate inhibitory and stimulatory effects, respectively.

### 2.3.7 Testosterone

As stated earlier in this Thesis, cholesterol is the basis for all adrenal steroids synthesis and therefore testosterone (Figure 2.10). Testosterone is responsible for regulating many physiological processes such as promoting secondary sexual characteristics, muscle maintenance, cognitive function and bone metabolism (Bhasin *et al.*, 1996, Evans, 2004).

Testosterone synthesis increases in response to an elevation in the stimulation of the peptide GnRH (Lin *et al.*, 1995), which will in turn activate LH. This mechanism increases the production of intracellular cAMP, which will in turn activate PKA. The steroidogenic acute regulatory (StAR) protein originated from the enzymatic function of PKA (Chen *et*

*al.*, 2005, Stocco and Clark, 1996) is fundamental in transferring free cholesterol - released from the lipid stores by activated cholesterol ester hydrolase - from cytoplasm into the inner membrane of mitochondria. This process will induce cholesterol conversion to pregnenolone (Stocco and Clark, 1996). Pregnenolone is then transported to the smooth endoplasmic reticulum for further synthesis to testosterone (Saez, 1994).



**Figure 2.10** The synthesis pathway of testosterone.

### **a) Acute Testosterone Responses to Exercise**

It has been reported that endurance-trained men present a ~20-30% lower baseline testosterone concentration than sedentary men (Hackney *et al.*, 2003), perhaps due to a training-induced adaptation at a peripheral level (i.e. testicles). This adaptation in endurance-trained males has been proposed to cause an inhibited response of testosterone from the tests to endogenous stimuli, as a result from a suppressed response of the precursor LH in these athletes (Hackney *et al.*, 1990, Wheeler *et al.*, 1984).

Circulating concentrations of testosterone are known to acutely elevate following exercise of different duration and intensity (Guglielmini *et al.*, 1984). It has been reported that a running test to fatigue at 100% of the individual ventilatory threshold (average duration being 85 min) induced a 31% elevation in plasma total testosterone, with concentration levels remaining elevated within the following 30 min (Daly *et al.*, 2005). In

addition, cycling for 30 min at an average 75%  $\dot{V}O_{2\text{peak}}$  (Hough *et al.*, 2011, 2013 & 2015) is stressful enough to induce a robust elevation of salivary testosterone levels. Furthermore, plasma testosterone has also been shown to elevate following a ~30-min incremental maximal cycle by 14% to 30% (Cumming *et al.*, 1983, Hoogeveen and Zonderland, 1996, Lehmann *et al.*, 1993) and by 33% following a 30-min submaximal cycle at the individual anaerobic threshold test (Sgrò *et al.*, 2014). When examining testosterone responses to a running bout, different responses have been reported. Testosterone concentrations were not shown to acutely elevate following a 45 min run at ~18 km.h<sup>-1</sup> in male sprinters (Kuoppasalmi *et al.*, 1980), but significantly increased after 60 min at 55%  $\dot{V}O_{2\text{max}}$  endurance-trained male individuals (Tremblay *et al.*, 2005).

A summary of studies reporting the acute responses of testosterone to exercise is outlined in Table 2.3, presented in the next page.

**Table 2.3** Acute testosterone responses to continuous exercise.  
\*Plasma testosterone; \*\*Salivary testosterone.

Authors (Publication year)	Participant population	Fitness level	Type of exercise	Duration	Direction of concentration variation	Percentage change
Guglielmini <i>et al.</i> (1984)	Middle-distance runners	90-100 km weekly	Training session (running at low to submaximal speed)	60 min	Increase	*38.2%
Cumming <i>et al.</i> (1986)	Physically active males	40 to 50 mL·kg <sup>-1</sup> ·min <sup>-1</sup>	Cycling incremental max test	~30 min	Increase	*30%
Lehmann <i>et al.</i> (1993)	Recreationally active males	52.0 ± 5.2 mL·kg <sup>-1</sup> ·min <sup>-1</sup>	Cycling incremental max test	~30 min	Increase	*23%
Hoogeveen and Zonderland (1996)	Professional male cyclists	Average workload max of 420W	Cycling incremental max test	~30-35 min	Increase	*14%
Daly <i>et al.</i> (2005)	Endurance- trained males	62.1 ± 1.1 mL·kg <sup>-1</sup> ·min <sup>-1</sup>	Running at 100% ventilatory threshold	To exhaustio n	Increase	*~31%
Tremblay <i>et al.</i> (2005)	Endurance- trained males	> 75 km weekly	Running at 55% $\dot{V}O_{2max}$	80 min	Increase	*22%
Hough <i>et al.</i> (2011)	Physically active males	50.9 ± 8.8 mL·kg <sup>-1</sup> ·min <sup>-1</sup>	Cycle at ~75% $\dot{W}_{max}$	30 min	Increase	**~35%
Hough <i>et al.</i> (2013)	Recreationally active males	52 ± 5 mL·kg <sup>-1</sup> ·min <sup>-1</sup>	Cycle at ~75% $\dot{W}_{max}$	30 min	Increase	**~53%
Sgrò <i>et al.</i> (2014)	Healthy males	49 ± 5 mL·kg <sup>-1</sup> ·min <sup>-1</sup>	Cycle at anaerobic threshold (~50% $\dot{V}O_{2peak}$	30 min	Increase	*33%

### ***b) Exercise-Induced Testosterone Responses After Intensified Training***

In the previous section of this Thesis varied acute responses of testosterone to exercise were presented and compared. As described, most studies reported significant elevations in testosterone in response to cycling bouts of various duration and intensities.

As stated earlier in this Thesis, resting concentrations of testosterone have been proposed as a possible biomarker of the overreaching phenomena. However, similarly to cortisol concentrations following intensified periods of training, resting concentrations of testosterone to a period of intensified training have also been shown to be inconclusive, with increases (Alghadir *et al.*, 2015, Grandys *et al.*, 2009, McConnel and Sinning, 1984), decreases (Hoogeveen and Zonderland, 1996, Kageta *et al.*, 2015, Wheeler *et al.*, 1991) and no changes (Miloski *et al.*, 2015, Mujika *et al.*, 1996, Vesterinen *et al.*, 2016) reported when comparing before to after the intensified training period. This may be due to the differences in the duration and intensity of the training periods (presented in Table 2.4). In addition, it may be reasonable to remember that it is still difficult to diagnose overreaching/OTS as there is still no reliable biomarker, and that these states are normally diagnosed retrospectively, and therefore whether these study participants were truly overreached cannot be answered.

As stated earlier in this Thesis, it has been proposed that the exercise-induced responses of both salivary and plasma testosterone may be a more reliable indicator of any overreaching-induced alterations in this hormone, with blunted responses occurring following a period of intensified training (Hough *et al.*, 2013, Hough *et al.*, 2015, Mäestu *et al.*, 2005).

**Table 2.4** Testosterone concentrations following a period of intensified training.  
\*Plasma testosterone; \*\*Salivary testosterone.

Authors (Publication year)	Participant population	Fitness level	Duration of training	Resting or exercise sample	Concentration variation	Percentage change
Lehmann <i>et al.</i> (1993)	Recreationally active males	52.0 ± 5.2 mL·kg <sup>-1</sup> ·min <sup>-1</sup>	6 weeks	Rest	No change	*N/A
Mujika <i>et al.</i> (1996)	Elite swimmers	No data	12 weeks	Rest	No change	*N/A
Flynn <i>et al.</i> (1994)	Long-distance runners	65.2 ± 1.3 mL·kg <sup>-1</sup> ·min <sup>-1</sup>	6 weeks	Rest	No change	*N/A
Hoogeveen and Zonderland (1996)	Professional male cyclists	Average workload max of 420W	4 months	Exercise	Increase	*N/A
Hoogeveen and Zonderland (1996)	Professional male cyclists	Average workload max of 420W	4 months	Rest	Decrease	*15%
Urhausen <i>et al.</i> (1998a)	Male cyclists and triathletes	61.2 ± 1.8 mL·kg <sup>-1</sup> ·min <sup>-1</sup>	19 months	Rest	No change	*N/A
Hough <i>et al.</i> (2013)	Recreationally active males	52 ± 5 mL·kg <sup>-1</sup> ·min <sup>-1</sup>	11 days	Exercise	Blunted response	**21%
Hough <i>et al.</i> (2015)	Male elite triathletes	67.6 ± 4.5 mL·kg <sup>-1</sup> ·min <sup>-1</sup>	10 days	Exercise	Blunted response	**44%
Alghadir <i>et al.</i> (2015)	Healthy males	No data	4 weeks	Rest	Increase	**55%

### 2.3.8 Plasma and Salivary Measurement of Hormones

Determination of cortisol and testosterone in saliva is a common non-invasive method in exercise-related endocrine research (Ahn *et al.*, 2007, Hayes *et al.*, 2015a, Hayes *et al.*, 2016, Hough *et al.*, 2011, Maso *et al.*, 2004). Due to the good permeability of the acini (secretory cells) in the saliva glands to steroid hormones (Aps and Martens, 2005, Vining *et al.*, 1983), cortisol and testosterone concentrations are not influenced by the rate of production of saliva (Chicharro *et al.*, 1998, Lewis, 2006, Vining *et al.*, 1983).

Salivary cortisol has been shown to be a good surrogate for circulating free cortisol (Perogamvros *et al.*, 2010), and therefore has been proposed as a tool for monitoring exercise-related hormone and immune markers (Papacosta and Nassis, 2011). Salivary and circulatory cortisol have been shown to have a moderate to strong positive correlation both in healthy, active men (Pearson's  $r = 0.86$ ,  $p < 0.001$ ,  $n = 50$ ) (Port, 1991), and in endurance-trained male individuals (Pearson's  $r = 0.73$ ,  $p < 0.0001$ ,  $n = 12$ ) (VanBruggen *et al.*, 2011). Furthermore, similar responses were shown when correlating plasma and salivary cortisol concentrations to the 55/80 cycle bout developed by Hough *et al.* (2011) (Pearson's  $r = 0.78$ ,  $p = 0.012$ ,  $n = 10$ ). Similarly to cortisol, salivary testosterone allegedly reflects the unbound hormone fraction, as the glycoprotein sex hormone-binding globulin (SHBG) has been reported not to enter saliva. However, Selby, Lobb and Jeffcoate (1988) have observed the presence of SHBG in saliva. Although salivary and plasma testosterone have been shown to strongly correlate in 37 healthy male individuals with Pearson's  $r = 0.71$ ,  $p < 0.001$  (Khan-Dawood *et al.*, 1984) and Pearson's  $r = 0.97$ ,  $p < 0.001$  in 45 healthy male and female individuals (Vittekk *et al.*, 1985), a strong correlation is not always observed for this hormone (Granger *et al.*, 2004), with research reporting poor levels of agreement between serum and salivary total ( $r = 0.271$ ,  $p = 0.180$ ) and free ( $r = 0.257$ ,  $p = 0.205$ ) testosterone concentrations to exercise in aging male individuals (Hayes *et al.*, 2015b). In addition, Shirtcliff *et al.* (2002) has also reported a sex difference in the



serum-salivary testosterone responses, with a significant correlation in serum-salivary free testosterone being observed in males ( $r = 0.67$ ,  $p < 0.01$ ), but not in females ( $r = 0.37$ ,  $p > 0.05$ ).

### **2.4 The Immune System**

The concepts of immunology have long been derived primarily from the study of the ability to resist to infection. Immunity refers to all the physiological mechanisms that provide the human body with the capacity to recognize foreign materials and to neutralize, eliminate or metabolize them (Bellanti, 1978b). Therefore, the function of the immune system relies not only on defending the body from infectious agents (Roitt, 1977), but also on mediating biological functions such as homeostasis and surveillance (Ward, 1978). Exercise is known to influence the immune system mechanisms and functions, either positively or harmfully (Walsh *et al.*, 2011b), which will be discussed in more detailed further in this Thesis.

#### **2.4.1 The Components of the Immune System**

The immune system is composed of cellular and soluble elements, as seen in Table 2.5 below. Cellular elements are synthesized in the bone marrow from stem cells, which can differentiate into erythrocytes (red blood cells needed for oxygen transport), megakaryocytes (precursors of platelets, needed for blood clotting) and leucocytes (white blood cells with distinct functions in immune defence). This chapter will focus on leucocytes and its importance in immune function.

Around 60-70% of circulating leucocytes are granulocytes, which are polymorphonuclear leucocytes (PMNs) due to its multi-lobulated nucleus, 10-15% are monocytes and the remainder 20-25% are lymphocytes (Gleeson, 2006b).

Granulocytes are further subdivided in neutrophils (>90%), eosinophils (2-5%) and basophils (0-2%). All granulocytes have cytoplasmic granules with specific biologically active substances involved in inflammatory and allergic processes, being involved mainly in phagocytic mechanisms (Barrett *et al.*, 2010). This will be detailed further in section 2.5.1. Monocytes are phagocytic mononuclear cells and the largest type of leucocytes, which migrate into tissue and develop into macrophages after a few hours in the circulation (Nichols *et al.*, 1971, Pederson, 2005). Lymphocytes are mononuclear cells responsible for the adaptive immune response. Several lymphocyte subsets, such as B cells (bursa-derived), T cells (thymus-derived) and natural killer (NK) cells may be identified by the use of monoclonal antibodies with fluorescent properties that aid in differentiating cell surface markers, known as clusters of differentiation (CD) (Gleeson, 2006b).

**Table 2.5** Main components of the immune system. Taken from Gleeson (2006, p. 16).  
\*CD = Clusters of Differentiation or Cluster Designators

Innate Components	Adaptive components
<p><u>Cellular:</u></p> <ul style="list-style-type: none"> <li>Natural killer cells (*CD16<sup>+</sup>, CD56<sup>+</sup>)</li> <li>Phagocytes (neutrophils, eosinophils, basophils, monocytes, macrophages)</li> </ul> <p><u>Soluble:</u></p> <ul style="list-style-type: none"> <li>Cytokines (interleukins (IL), interferons (IFN), colony-stimulating factors (CSF), tumour-necrosis factors (TNF))</li> <li>Lysozymes</li> <li>Complement</li> <li>Acute-phase proteins</li> </ul>	<p><u>Cellular:</u></p> <ul style="list-style-type: none"> <li>T-cells (CD3<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup>)</li> <li>B-cells (CD19<sup>+</sup>, CD20<sup>+</sup>, CD22<sup>+</sup>)</li> </ul> <p><u>Soluble:</u></p> <ul style="list-style-type: none"> <li>Immunoglobulins (Ig): IgA, IgD, IgE, IgG, IgM</li> </ul>

## 2.5 The Role of Leucocytes in Immunology

Most immune effector cells circulate in the bloodstream as leucocytes. These include granulocytes, monocytes and lymphocytes. Presence of these cells in the tissues after extravascular migration amplify the immune responses, alongside tissue macrophages and mast cells (Barrett *et al.*, 2010). These cells, when operating in synchrony, powerfully protect the body against tumours and any type of infections.

### 2.5.1 Granulocytes

The average half-life of circulating neutrophils (the major granulocyte in circulation) is around 6 hours, hence the production of approximately 100 billion neutrophils per day (Barrett *et al.*, 2010). Neutrophils act in the tissues, particularly if stimulated to do so by an infection or inflammatory proteins. Shortly after injury to the tissues (including inflammation), neutrophils start clustering along endothelial cells in the injured area, threading their way out of the vessels and building the first line of defence against invading microorganisms, engulfing and destroying potentially dangerous agents, in a process known as phagocytosis (Bellanti, 1978a, Ward, 1978).

Similarly to neutrophils, the eosinophils also have a short half-life in the circulatory system (~30 min) and go through a similar process of binding to proteins that attach to the walls of vessels, entering the tissue by diapedesis (the process of passing through the walls of the capillaries between endothelial cells) (Barrett *et al.*, 2005). Like neutrophils, eosinophils also synthesize inflammatory proteins, such as cytokines and chemokines (Cassatella, 1995). These cells are capable of destroying foreign organisms, release histamine, and have anti-parasitic and anti-allergic properties (Rosenberg and Domachowske, 2001). Eosinophils make up 2-5% of total leucocytes in healthy individuals (Ward, 1978).

Basophils also enter tissues and secrete cytokines, and release histamine and several other inflammatory mediators following activation from specific antigens when bound to cell-fixed IgE molecules (MacGlashan *et al.*, 1999).

### 2.5.2 Monocytes

Monocytes are mononuclear phagocytes that circulate in the bloodstream for around 72 hours, after which they enter the tissues and differentiate further into macrophages (Barrett *et al.*, 2005, Ward, 1978). The release of specific cytokines from T lymphocytes will activate macrophages (Jovanovic *et al.*, 1998), which in turn migrate, engulf and kill bacteria through mechanisms similar to those happening in neutrophils (Barrett *et al.*, 2010).

### 2.5.3 Lymphocytes

This type of cells has a key role in maintaining immunity, albeit only about 2% of the total lymphocytes are found in peripheral blood. It has been estimated that in humans, each day around  $3.5 \times 10^{10}$  lymphocytes enter the bloodstream through the thoracic duct alone (Barrett *et al.*, 2005, Dhabhar *et al.*, 1994).

Some lymphocytes originate in the bone marrow and are transported through the circulation before entering the tissues. The cells that enter the thymus (thymocytes, T cells) may either be modified or pass through the organ to be eliminated (Herscowitz, 1978). Some lymphocytes may also differentiate in the gut-associated lymphoid tissues and fetal liver of mammals (B-cells) (Herscowitz, 1978). However, most lymphocytes are formed in the lymph nodes, thymus and spleen from precursor cells (Barrett *et al.*, 2005).

## 2.6 The Basic Pattern of Immune Recognition: Innate and Adaptive Immunity

The human immune system has an innate and an adaptive component, which main goal is to repel the body's own cells that have become senescent or abnormal (Barrett *et al.*, 2005). The main difference between these two types of host-defense mechanisms is the receptor types used in pathogen recognition. The innate immune system consists of functionally distinctive elements (modules) that protect the body against pathogens by sensing the latter through pattern-recognition receptors (PRRs), which in turn activate antimicrobial activity and stimulate the immune response (Janeway, 1989, Medzhitov, 2007). This immune response will involve the existence of a pathogenic 'cell-eating' process (phagocytosis), which may occur as part of an inflammatory response. This inflammatory response is normally associated with a cascade of cellular and systemic mechanisms aiming to restore homeostasis under adverse environmental influences (Bellanti, 1978b).

Contrastingly, the adaptive component triggers the activation of innate effector mechanisms through specific foreign substances, referred to as antigens. Antigens are a class of foreign molecules that bind to specific immune receptors to induce an immune response (Alberts *et al.*, 2002). The final outcome of the reaction occurring following recognition and disposal of the foreign microorganisms by the human body is highly determined upon the substance's molecular properties. These include its size, structure, chemical nature and magnitude (Ward, 1978).

### 2.6.1 Innate Immune System

The innate immune system constitutes the major defence mechanism against an invading microorganism by immediately stabilising a pathogen attempting to infect the human body. This is accomplished through its surface barriers (such as biological acids, commensal bacteria, skin, lungs, etc.), soluble factors, phagocytes and natural killer (NK) cells (Gleeson, 2006b). The cellular receptors of the innate immune system have a wide specificity and may bind to an extensive number of molecules that share a mutual structural pattern. These cellular receptors target specific pathogenic molecular structures, referred to as pathogen-associated molecular patterns (PAMPs), which may also be found in the gut as symbiotic bacteria (Medzhitov, 2001). There are a number of PRR classes that differ in function, one of these being the Toll-like receptors (TLRs) (Takeda *et al.*, 2003). A relevant characteristic of pattern recognition is that PRRs do not have the capability to distinguish pathogenic from symbiotic (non-pathogenic) microorganisms, due to the fact that the receptors' ligands are not unique to the pathogens, but rather shared by entire classes of bacteria (Medzhitov, 2007). However, TLRs may function as sensors of microbial infection, being critically important in initiating inflammatory and immune defence reactions (Rakoff-Nahoum *et al.*, 2004). TLRs are able to identify conserved molecular products of microorganisms such as lipopolysaccharide (LPS) (Beutler and Rietschel, 2003), and activate macrophages found in tissues to produce pro-inflammatory cytokines (Medzhitov, 2007).

In summary, the innate immune system is composed by a group of subsystems (also called modules) that present distinct functions in host defence mechanisms (Table 2.6). The main modules that this Thesis focuses on are the mucosal epithelia (specifically the secretion of SIgA) and the phagocytes (i.e. polymorphonuclear leucocyte concentrations and phagocytic function).

**Table 2.6** Modules of the innate immune system. Taken from Medzhitov (2007).

<b>Innate host-defence module</b>	<b>Primary sensors (PRRs)</b>	<b>Prototypical responses</b>
Mucosal epithelia	TLRs and NOD proteins	<ul style="list-style-type: none"> <li>• Production of antimicrobial peptides</li> <li>• Production of mucins</li> </ul>
Phagocytes	TLRs, dectins and NOD proteins	<ul style="list-style-type: none"> <li>• Production of antimicrobial proteins</li> <li>• Production of cytokines: IL-1<math>\beta</math>, IL-6 and TNF</li> </ul>
Acute-phase proteins and complement system	Collectins, pentaxins and ficolins	<ul style="list-style-type: none"> <li>• Lysis or opsonisation of pathogens</li> <li>• Chemotactic attraction of leucocytes</li> </ul>
Inflammasomes	NALPs and NAIPs	<ul style="list-style-type: none"> <li>• Production of IL-1 family members</li> <li>• Apoptosis of infected host cells</li> </ul>
NK cells	ND	<ul style="list-style-type: none"> <li>• Apoptosis of infected host cells</li> </ul>
Type-I-IFN-induced antiviral proteins	RIG-I, MDA5, DAI and TLRs	<ul style="list-style-type: none"> <li>• Induction of an antiviral state</li> <li>• Apoptosis of infected host cells</li> </ul>
Eosinophils and basophils	ND	<ul style="list-style-type: none"> <li>• Contractions of smooth muscle</li> <li>• Production of mucins</li> <li>• Peristalsis</li> <li>• Production of biogenic amines</li> <li>• Production of cytokines: IL-4, IL-5, IL-9, IL-13 and TNF</li> </ul>
Mast cells	ND	<ul style="list-style-type: none"> <li>• Contractions of smooth muscle</li> <li>• Production of mucins</li> <li>• Peristalsis</li> <li>• Production of biogenic amines</li> <li>• Production of cytokines: IL-4, IL-5, IL-9, IL-13 and TNF</li> </ul>

This list of modules, sensors and responses is not comprehensive and has been simplified for clarity. It should be noted that the function of NALPs and NAIPs is not completely understood. In addition, the primary sensors that control expression of NK-cell-receptor ligands, as well as the sensors that activate antihelminthic responses by mast cells, eosinophils and basophils, have not been identified. Certain modules can be co-induced during infection; these modules are functionally linked (for example, phagocytes and the complement system) and co-regulated by the same cytokines. ND, not determined.

### **a) Mucosal Epithelial**

The mucosal epithelia, together with the skin, are defined as the primary interface between the host and the pathogenic and symbiotic microorganisms. Its main function relies not only in protecting the human body from pathogenic microorganisms, but also in allowing a symbiotic environment within the body. Mucosal epithelia cells are responsible for producing antimicrobial peptides, aiming to limit the sustainability and colonisation of pathogens (Medzhitov, 2007).

### **b) Phagocytes**

Phagocytic activity (the ingestion of particles) is crucial for host defence, by eliminating debris and pathogens (Frankenberg *et al.*, 2008). It represents the mechanism of engulfment and uptake of large ( $\geq 0.5 \mu\text{m}$ ) undesired particles (Aderem and Underhill, 1999) and is accomplished by a group of cells named phagocytic cells – monocytes/macrophages and neutrophils - which in turn have multiple antimicrobial mechanisms that are triggered by the presence of pathogens (Bellanti and Dayton, 1975). Phagocytosis is often accelerated by host products of the acute-phase response called opsonins, through the ability to bind to not only the cell walls of microorganisms, but also to opsonin receptors found on phagocytic cells (Medzhitov, 2007).

## **2.6.2 Adaptive Immune System**

The main mechanisms of the adaptive (or acquired) immune system are the so-called immune exclusion and immune elimination. These processes refer to the ability of the adaptive immune system to prevent the colonisation of pathogens by keeping them out of the human body, and to also find and destroy foreign, invading microorganisms, respectively (Gleeson, 2006b). Additionally, certain regulatory mechanisms are involved in



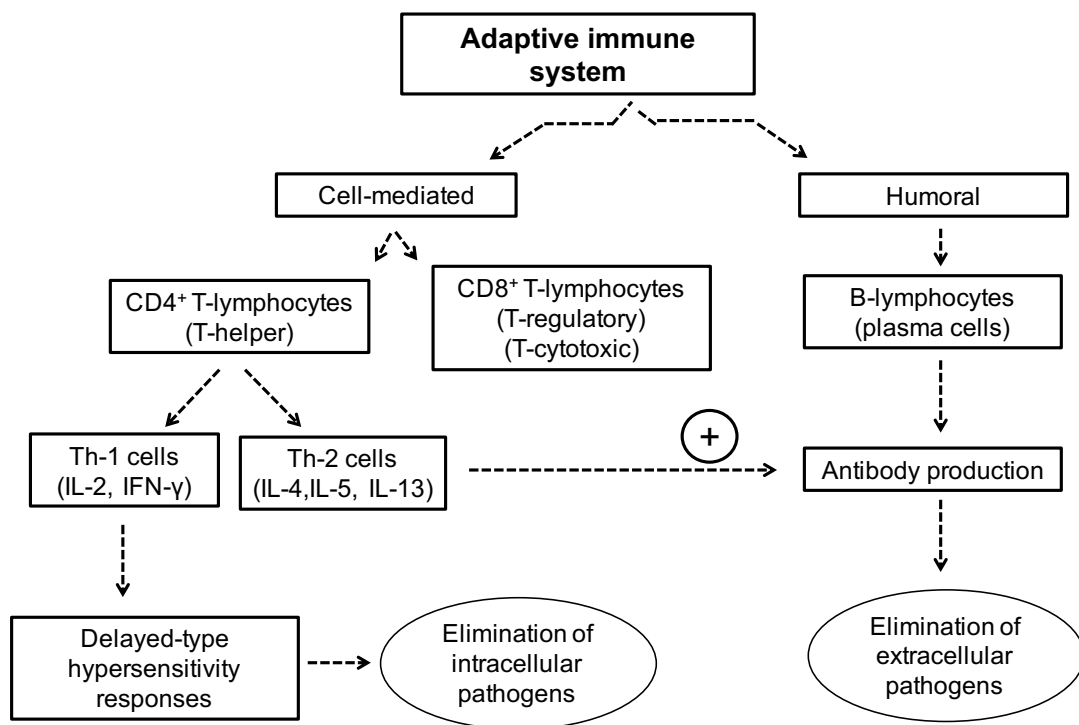
pacifying allergic reactions and distinguish symbiotic from pathogenic bacteria. Breaking down this control mechanism will eventually lead to the onset of autoimmune diseases (Rifkin *et al.*, 2005).

The adaptive immune response is opposed to the innate immune responses by three main characteristics: specificity, heterogeneity, and memory. Specificity refers to the fact that the immune products solely react to pathogens that share a similar configuration. It is the property of the immune response that distinguishes different antigens, regardless if species-, individual- or organ-specific (Ward, 1978). In contrast, heterogeneity is the ability of a large selection of cell types and products to interact with a multiplicity of responses proportionate with the array of cell types, whereas memory refers to the competence that results in amplification of the adaptive immune response through proliferation (increase in cell number) and differentiation (i.e. cells become more specialized) of cells following exposure to an antigen (Herscowitz, 1978). Adaptive immune recognition is mediated mainly by two types of antigen receptors, referred to as T-cell and B-cell receptors. The genes encoding antigen receptors are assembled from fragments through recombination-activating gene (RAG)-protein-mediated somatic recombination (Schatz *et al.*, 1992). This process yields an extensive array of receptors.

When an antigen (such as a population of bacteria or viruses) enters the human body, antibodies to that antigen may appear in the serum (Cunningham, 1978). These antibodies, y-shaped proteins produced by plasma cells (B cells of the adaptive immune system) that can act to neutralize pathogens, recognize a unique molecule of the antigen via the fragment antigen-binding (Fab) fragment (Litman *et al.*, 1993). Paratopes – antigen-binding bodies - of an antibody bind to specific three-dimensional antigen structures called epitopes, either tagging a microbe or an infected cell for attack by other components of the immune system, or neutralising its target directly, preventing its invasion and survival (Janeway *et al.*, 1997). The specificity of the antigen to which the

antibody is bound to will influence the type of immune mechanism being triggered, by defusing the biological process causing the disease or stimulating phagocytosis to obliterate the foreign substance (Janeway *et al.*, 1997).

There are two main types of lymphocyte that express antigen receptors. These are the conventional lymphocytes (conventional T cells - mostly  $\alpha\beta$  T cells - and B cells), and the innate-like lymphocytes (B-1 cells, NK T cells and subsets of  $\gamma\delta$  T cells). In turn, there are also two types of conventional  $\alpha\beta$  T cells: the T-helper ( $T_H$ ) cells, which are marked by the co-receptor CD4 on the cell surface; and cytotoxic T cells, which express CD8 (Bendelac *et al.*, 2001). These cells recognize antigenic peptides that are found bound to specific molecular complexes, called major histocompatibility complex (MHC). Conventional B cells are able to recognize almost any antigen by binding to a specific epitope (Medzhitov, 2007). The major components of the acquired immune system are presented in the figure below.



**Figure 2.11** The main components of the adaptive immune system. Adapted from Gleeson and Bosch (2013, p. 38).

## 2.7 Acute Effects of Exercise in Immunity

The responses of whole blood leucocyte subsets to bouts of acute exercise have been well described (Brahmi *et al.*, 1985, Gleeson and Bishop, 2005, Nieman *et al.*, 1994, Quindry *et al.*, 2003). However, the sensitivity of these cell subsets varies depending on the exercise stimulus. Overall, it is well established that exercise in general induces an elevation in total whole blood leucocytes when in a healthy state (McCarthy and Dale, 1988, Sand *et al.*, 2013). Different work rates will induce different rates of leucocytosis with significantly greater rates of leucocytosis being reported after a 45-min run at 80%  $\dot{V}O_{2max}$  when compared with a similar running test at 50%  $\dot{V}O_{2max}$  (Nieman *et al.*, 1994). Marked increases in neutrophils were also reported for example in response to a 2-hour cycle bout at 56%  $\dot{V}O_{2max}$  (Fielding *et al.*, 2000). An intermittent (alternating 1-min stages) running test to exhaustion at the speed and gradient at which  $\dot{V}O_{2max}$  was achieved, elicited a biphasic leucocytosis, with lymphocyte numbers (CD3<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup>, CD16<sup>+</sup>/56<sup>+</sup>), and granulocytes and monocytes increasing immediately post- and 6 h post-exercise in trained male athletes, respectively (Gray *et al.*, 1993). Furthermore, total whole blood leucocyte numbers have been reported to significantly increase by ~65% in healthy male and female individuals following a self-paced running test to exhaustion (60-90 min), with a 97% significant elevation in polymorphonuclear leucocytes (PMNs) but no alteration in lymphocyte numbers (Risøy *et al.*, 2003). Nonetheless, leucocytosis (increased CD4<sup>+</sup>, CD8<sup>+</sup>, neutrophils, and CD56<sup>+</sup> concentrations) has been reported to occur at the end of the second of two 75 min cycling bouts at ~75%  $\dot{V}O_{2max}$  separated by a 3 h recovery period (Ronsen *et al.*, 2001). Although all lymphocyte populations are known to increase in the circulation in response to strenuous exercise, NK cells are known to be the population that shows a more marked increase during/after high-intensity, long-duration exercise (Anane *et al.*, 2009, Gabriel *et al.*, 1991, Pedersen, 1991, Shek *et al.*, 1995). More recently, Anane *et al.* (2009) proposed for the first time that the  $\gamma\delta$  T-lymphocytes are stress-responsive cells, due to their mobilisation examined after completion of two 16-

min cycle bouts at 35% (low intensity) and 85% (high-intensity)  $\dot{W}_{max}$ . However, the low intensity bout has shown significantly less pronounced exercise-induced increases in the overall leucocyte populations. Similarly, a high-intensity (80%  $\dot{V}O_{2max}$ ), short-duration (45 min) treadmill-running exercise bout was intense enough to induce a marked leucocytosis, especially in the lymphocyte populations, which did not occur when compared with a similar duration, lower intensity (50%  $\dot{V}O_{2max}$ ) exercise bout (Nieman *et al.*, 1994).

Research has shown that this exercise-induced increase in total circulatory leucocytes may be attenuated with regular exercise (Blannin *et al.*, 1996), presumably by reducing the physical stress and fatigue levels provoked by the exercise bout itself (Vrabas *et al.*, 1999). Leucocyte counts usually return to baseline levels shortly after short-duration (30 min) exercise (McCarthy *et al.*, 1992b), nevertheless total leucocyte numbers may continue to rise during recovery following very high-intensity bouts of exercise, such as cycling for 4 min at 110-120%  $\dot{V}O_{2max}$  (Allsop *et al.*, 1992) and cycling at 80%  $\dot{W}_{max}$  to exhaustion (Field *et al.*, 1991).

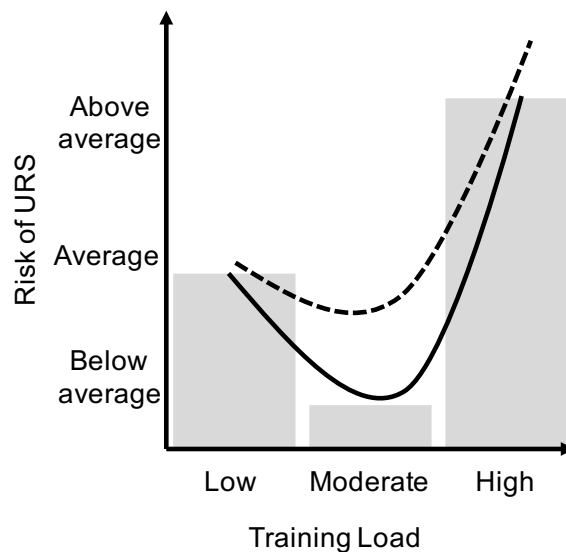
### 2.8 Overtraining and Susceptibility to Infection

As shown earlier in this chapter, different exercise regimens influence the immune system, either at a systemic, local or mucosal, and/or cellular level (Bermon *et al.*, 2017, Walsh *et al.*, 2011b). As explained in the Introduction of this Thesis, if sufficient recovery is not completed when intensifying exercise training, this may lead to an accumulation of fatigue levels with possible outcomes of overreaching/OTS. Developing NFOR/OTS has been associated with an increased vulnerability to numerous pathologies, with research suggesting that endurance runners are likely to experience an increased susceptibility for infectious episodes during heavy training or after a marathon race (Nieman *et al.*, 1990), bacterial or viral infections (Mackinnon, 1997), and URS (Nieman, 1994b), eventually

leading to weakness/severe fatigue (Fry *et al.*, 1994b, Lehmann *et al.*, 1996, Mackinnon *et al.*, 1997b), normally attributed to an impairment of the human body's defence mechanisms (MacKinnon, 2000). In fact, besides the incidence of injuries, URS has been suggested as the most usual medical presentation in elite athletes (Engebretsen *et al.*, 2013; Palmer-Green and Elliott, 2015). However, despite the prevalence of infectious pathogens has been commonly reported as the primary cause for URS in athletes, a study performed on a population of 32 elite triathletes, 31 recreational triathletes and 20 sedentary individuals which monitored URS over a period of 5 months, reported that only 30% of 37 episodes of illness had been caused by an infectious agent (Spence *et al.*, 2007). In addition, a bacterial or viral pathogen was not identified in 43% of URS episodes that occurred in 70 elite athletes from several sports over a prospective 14-month period (Cox *et al.*, 2008). Studies have proposed URS to be caused not only by pathogen or viral exposure, but also as an effect of exercise-induced bronchoconstriction (Bonsignore *et al.*, 2003) or an amplified exposure to aeroallergens causing a hypersensitivity response (Bonsignore *et al.*, 2001). Albeit the moderately low cohort of participants, these studies suggest that perhaps the incidence of URS has various aetiologies, all presenting with analogous signs and symptoms, and consequently effects on physical performance. Given the ambiguous aetiologies of the experienced respiratory symptoms in athletes, recommendations are that symptoms are referred to as URS instead of upper respiratory tract infections (URTI).

The relationship between exercise intensity and incidence of URS has long been described to have a J-shaped curve, suggesting that whilst the susceptibility for URS may decrease with moderate intensity exercise in sedentary individuals, the risk may rise above average following prolonged periods of intensified training (Nieman, 1994a, Nieman, 1994b). In addition, as physically fitter and more active athletes have shown to suffer less from URS events (Nieman *et al.*, 2011) a flatter curve has been proposed (Gleeson and Robson-Ansley, 2006), as there is still little evidence to indicate any

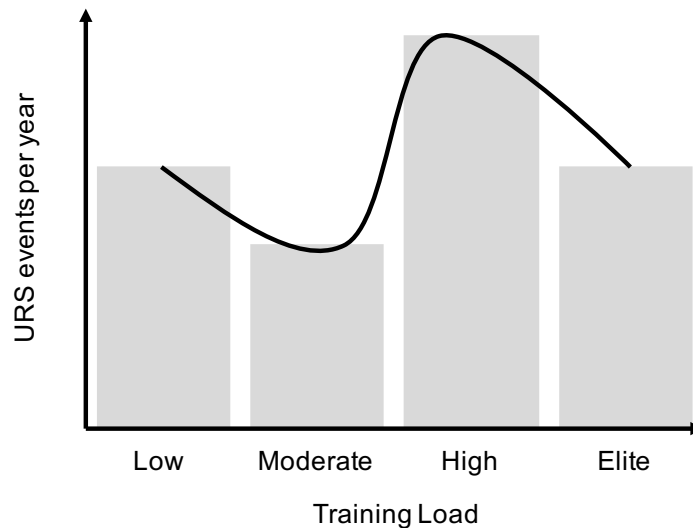
significant differences in immune function of sedentary individuals compared to moderately active people.



**Figure 2.12** Relationship between training volume and incidence of URS: the J-shaped curve model. (Adapted from Gleeson and Robson-Ansley in Gleeson (editor) 2006, p. 116).

It has been suggested that URS in sedentary individuals do not get aggravated with moderate exercise (Weidner and Schurr, 2003). Moreover, there is still insufficient knowledge to describe the immunological mechanism involved in the increased susceptibility to URS following prolonged, intensified physical training (Nieman, 2000). In fact, there have been inconsistent findings regarding overtrained athletes, with studies showing either a lower risk for URS in these athletes, when compared with well-trained athletes (Mackinnon and Hooper, 1996), or an increased incidence of URS following overreaching (Tiollier *et al.*, 2005). Nonetheless, elite athletes whose training load is considerably above average when compared to the rest of the population have been shown to respond differently to URS, with studies showing a negative correlation between training load (km/year or hour/year) and number of sick days (Malm, 2006). Elite endurance athletes are normally distinguished from non-elite according to their marathon race times or weekly training load, normally above 120 km/week (Billat *et al.*, 2001, Malm,

2006). Moreover, it is recognized that the nonexistence of infections is crucial in achieving the status of elite athlete and that these athletes whose training load is considerably above average when compared to the rest of the population have been shown to respond differently to URS, with studies showing a negative correlation between training load (km/year or hour/year) and number of sick days (Malm, 2006). Therefore, when including elite athletes in the URS /training volume model, this author proposes an extension of the traditional J-shaped curve model towards an S-shaped model (Figure 2.13). The URS may be plotted against training load by determining the odds ratio.



**Figure 2.13** The S-shaped curve model to describe the relationship between training volume and respiratory illness (Adapted from Malm (2006)).

Resistance to URS is accomplished mainly by an antibody found in the mucosal immune system, known as SIgA, which acts as the first line of defence against infectious agents on mucosal surfaces by neutralising and/or eliminating viral pathogens, such as cold-causing viruses (Bishop and Gleeson, 2009, Gleeson and Pyne, 2000, Tripodi *et al.*, 2011, Woof and Kerr, 2006). It has been proposed that low levels of SIgA, which may also be related to a decreased saliva flow rate may be a risk factor in increasing the incidence of URS (Gleeson *et al.*, 2012, Gleeson and Bishop, 2013). Endurance-trained athletes who have shown predisposition to illness following long periods of intensified training,

have also shown to have lower concentrations of SIgA and a higher release of anti-inflammatory cytokines, specifically IL-10 (Gleeson and Bishop, 2013). Furthermore, elite swimmers who experienced symptoms of overreaching/OTS after a 6-month training programme had significantly lower SIgA concentrations when compared to well-adapted athletes (Mackinnon and Hooper, 1994). In summary, low SIgA concentrations or secretion rate associated with elevated anti-inflammatory cytokine responses may be prone to an increased susceptibility to URS in athletes (Fahlman and Engels, 2005, Gleeson, 2000, Gleeson *et al.*, 2012). Furthermore, minimising pathogen transmission and maintaining immune capacity are key factors in helping athletes avoid illness, certainly those related with the respiratory tract (Bishop, 2012, Walsh *et al.*, 2011a).

### **2.8.1 Immune and Inflammatory Responses to Overreaching/OTS**

An elevation in the levels of certain hormones is known to occur during short-duration, high-intensity exercise (Bove, 1989, Hayes *et al.*, 2015a, Hough *et al.*, 2013, Hough *et al.*, 2011, Meeusen *et al.*, 2004), and these responses, particularly plasma and salivary cortisol and testosterone have been shown to blunt following chronic exercise-induced stress (Hough *et al.*, 2013, Hough *et al.*, 2015, Meeusen *et al.*, 2010, Meeusen *et al.*, 2004), as detailed earlier in this Thesis. Furthermore, it has been reported that abnormal hormonal responses following long periods of intensified training with limited recovery may cause chronic immunosuppression (Lancaster *et al.*, 2003) by decreasing the number of T-lymphocytes (Díaz *et al.*, 2003, Pinto *et al.*, 2006, Stites *et al.*, 1983), with flu-like symptoms of illness, such as sore throats and colds being more common in endurance athletes when compared to the general population (Peters, 1997, Pyne *et al.*, 2001, Walsh and Oliver, 2016).

Glucocorticoids such as cortisol have a key role in the regulation of circulating



eosinophils by decreasing its numbers (Lamas *et al.*, 1991), and inhibiting cytokine-mediated eosinophil survival (Wallen *et al.*, 1991), therefore potentially reducing allergic reactions. Furthermore, glucocorticoids bind to specific cytoplasmic receptors, which subsequently translocate to the nucleus, where will in turn bind to glucocorticoid response elements (GRE) on glucocorticoid-responsive genes. This mechanisms results in an increased transcription of genes coding for anti-inflammatory cytokines, such as IL-1 receptor antagonist (IL-1ra) and IL-10 (Barnes, 1998). Moreover, basophil apoptosis is a powerful anti-inflammatory mechanism induced by glucocorticoids (Yoshimura *et al.*, 2001), and particularly cortisol is known to have positive effects on neutrophil numbers (Davis *et al.*, 1991), which is further pronounced following short-duration, high-intensity exercise (normally above 60-70%  $\dot{V}O_{2max}$ ) when in a healthy state (Gabriel *et al.*, 1992, McCarthy *et al.*, 1992a). However, it is known that heavy exertion leading to overreaching may induce negative alterations in immunity and defence against pathogens (MacKinnon, 2000, Pedersen *et al.*, 1996, Svendsen *et al.*, 2016, Tiollier *et al.*, 2005), abnormal neuroendocrine responses (Hough *et al.*, 2013, Lehmann *et al.*, 1992b, Mackinnon *et al.*, 1997a, Meeusen *et al.*, 2010, Meeusen *et al.*, 2004) and pro- and anti-inflammatory cytokines (Smith, 2000, Svendsen *et al.*, 2016), and decreased granulocyte phagocytic activity (Baj *et al.*, 1994, Blannin *et al.*, 1996, Robson-Ansley *et al.*, 2007, Yaegaki *et al.*, 2007). Well-trained triathletes have experienced a 20% decrease in lipopolysaccharide (LPS)-stimulated neutrophil degranulation following a 2-week period of intensified training (Robson *et al.*, 1999a). Increased production of reactive oxygen species with consequent decreases in neutrophil phagocytic activity have been reported in female judoists following a 20-day pre-competition training period where the training sessions involved running, weight training and judo practice for 6 days a week (Yaegaki *et al.*, 2007). However, no differences in either reactive oxygen species production or phagocytic function at baseline has been reported in male judoists after a 7-day training camp also consisting of long-distance running, weight training and judo practice, with no alterations in total leucocytes or neutrophil counts (Yaegaki *et al.*, 2008). These studies suggest that long periods of

sustained heavy training may reduce neutrophil function, and therefore examining neutrophil functions may be a good predictive marker of overreaching.

When examining circulating leucocyte populations concentrations no differences have been reported when comparing elite swimmers diagnosed as overtrained with well-trained, healthy swimmers (Hooper *et al.*, 1995) or overtrained with healthy endurance athletes (Gabriel *et al.*, 1998). In contrast, Svendsen *et al.* (2016) have reported elevated numbers of resting circulating total leucocytes and neutrophil numbers after 8 days of intensified training in male cyclists. It is yet to conclude the effect overreaching/OTS has on the total leucocyte and individual leucocyte subsets circulating concentrations. However, leucocyte functions such as T-lymphocyte CD4<sup>+</sup>/CD8<sup>+</sup> ratio have been shown to be lower in long-distance runners following a 3-week period of intensified training (38% increase in training volume) (Verde *et al.*, 1992), and therefore, measuring these cells ratio may be a good indicator of the overreaching phenomena.

### 2.9 Aims and Structure of the Thesis

The main aims of this Thesis are:

1. To examine the effects of water consumption on the exercise-induced salivary cortisol and testosterone responses.
2. To establish the reproducibility of the salivary cortisol and testosterone responses to the short-duration, high-intensity cycling bout (55/80);
3. To develop a short-duration (30 min), high-intensity treadmill-run test to elicit a reproducible elevation in plasma and salivary steroid hormone responses when in a normal trained state (i.e. not suffering from FOR/NFOR or OTS).

## Chapter 2: Review of the Literature and Research Aims

4. To examine the plasma and salivary cortisol and testosterone responses to the developed treadmill running test before and after a 12-day period of intensified training;
5. To examine the responses of the immune system over the course of a 12-day intensified-training period. More specifically, to examine:
  - a. Mucosal immunity (SIgA);
  - b. Differences in exercise-induced leucocyte subset numbers and total leucocytes;
  - c. PMNs phagocytic function.

This current chapter focused on the pertinent background evidence that led to the design and development of the experimental chapters presented in this Thesis. Chapter 3 describes the methods followed in each of the experimental chapters. The following 4 chapters describe the main experimental studies of this Thesis. Chapter 4 focuses on the influence that water consumption has in diluting saliva, and consequently modifying the concentration of exercise-induced salivary hormone responses. This study uses a 30-min, high-intensity cycle bout (referred to as the 55/80) as the exercise tool. In Chapter 5, the reproducibility of the salivary hormone responses to the 55/80 is examined. Chapter 6 focuses on the development of a 30-min, high-intensity, treadmill-running test to elicit a robust elevation in plasma cortisol and testosterone when in a healthy state. In Chapter 7, an examination of the acute steroid hormone and immune responses to the  $RPE_{\text{treadmill}}$  was completed before and after a period of intensified training. This Chapter also examines whether the  $RPE_{\text{treadmill}}$  could be used as a suitable tool to highlight any exercise-induced alterations when in an overreached state. Ultimately, Chapter 8 merges the main findings of this Thesis in a general discussion.

## **CHAPTER 3.**

# **General Methods**

### 3.1 Ethical Approval

All studies presented in this Thesis were granted ethical approval by the University of Bedfordshire Research Ethics Committee and were completed in accordance with the World Medical Association (WMA) Declaration of Helsinki – Ethical Principles for Medical Research Involving Human Subjects (64<sup>th</sup> WMA General Assembly, Fortaleza, Brazil, October 2013). Prior to participation in the research studies, all participants completed a Physical Activity Readiness Questionnaire (PAR-Q) (Appendix 1) to confirm suitability to engage in physical activities. After comprehensive verbal and written descriptions of what would be required throughout the study, participants provided their written informed consent. In Chapters 6 and 7, participants also signed a blood screening form (Appendix 2). A health-screening questionnaire (Appendix 3) was completed by all participants in Chapter 7 only. All participants that took part in the experimental studies in Chapters 4 to 7 had a healthy lifestyle and exercised at least 3 hours per week in accordance with the UK Government Department of Health – Obesity and Healthy Eating guidelines (Department of Health, 2011). Participants who frequently work night shifts were also not allowed to participate, as these require around 4 days to adjust their circadian rhythm of cortisol secretion (Niu *et al.*, 2015). The baseline data for each participant's anthropometric measures were collected using a stadiometer (Holtain Ltd, Wales, UK) and a digital scale (Tanita BWB 0800, Allied Weighing, Japan).

### 3.2 Maximal Oxygen Uptake ( $\dot{V}O_{2max}$ ) Assessment

Participants  $\dot{V}O_{2max}$  was established in accordance with The British Association of Sports and Exercise Sciences' (BASES) Position Statement on the Physiological Assessment of the Elite Competitor (1992) (Bird and Davidson, 1997) criteria. Participants were required to achieve 3 of the following 5 recommended criteria for establishing  $\dot{V}O_{2max}$  presented below:

1. A plateau in the oxygen uptake ( $\dot{V}O_2$ ) with increasing exercise intensity, defined as a less than 2 mL.kg<sup>-1</sup>.min<sup>-1</sup> or 3% increase in  $\dot{V}O_2$  following an increase in exercise intensity.
2. A final respiratory exchange ratio (RER) of 1.15 or above.
3. A final heart rate of within 10 beats.min<sup>-1</sup> of the predicted age-related maximal heart rate (HR<sub>max</sub>). This can be estimated by subtracting the individual's age in years to 220.
4. Exercising until volitional exhaustion is achieved.
5. A rating of perceived exertion (RPE) of 19 or 20 on the 6-20 Borg scale.

### **3.2.1 Maximal Oxygen Uptake and Maximal Workload Test on a Cycle Ergometer**

In Chapters 4 and 5, participants completed a continuous, incremental  $\dot{V}O_{2max}$  test on a cycle ergometer. Before the start of the test, participants completed a 3-min warm-up at 120 W. The test started at an initial workload of 180 W and power output was then increased by 30 W every 3 min until volitional exhaustion, as suggested by the BASES guidelines for establishing exercise intensity for the determination of  $\dot{V}O_{2max}$  during leg cycling in adults (Cooke, 2009). The protocol was designed to be completed in the suggested time of 9-15 minutes (Bird and Davidson, 1997). Manually braked cycle ergometers (Monark 824E, Vansbro, Sweden) (Chapter 4) and electronically braked cycle ergometers (Lode Excalibur Sport, Groningen, The Netherlands) (Chapter 5) were used for these tests. Participants were asked to cycle at a pace of 70 revolutions per minute (rpm) during the test in the experimental study presented in Chapter 4. In both studies, participants used a facemask during the entire period of testing and expired gas was analysed throughout by the use of a breath-by-breath ergospirometry system (MetaLyzer 3B, Cortex, Leipzig, Germany) for the determination of the rates  $\dot{V}O_2$  and carbon dioxide

production ( $\dot{V}CO_2$ ). Participants' HR and RPE were monitored throughout the test and recorded at the last 15 s of each stage, using short-range radio telemetry (Polar FS1, Kempele, Finland) and the 6-20 Borg Rating of Perceived Exertion scale, respectively. Water was provided before and after completion of the required maximal exercise test.

Maximum workload ( $\dot{W}_{max}$ ) was determined using the equation:

$$\dot{W}_{max} = \dot{W}_{final} + (t/T) \times \dot{W}_{inc}, \text{ (Kuipers } et al., 1985)$$

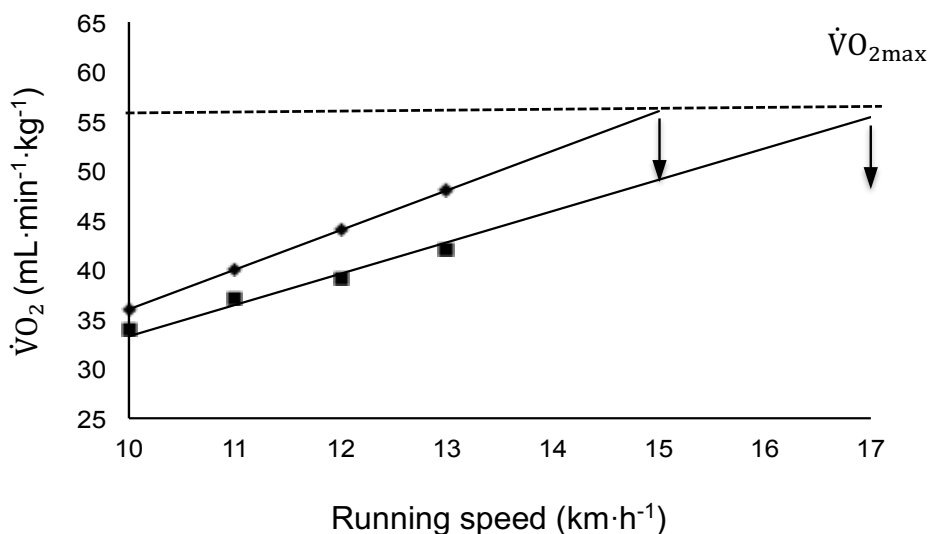
where  $\dot{W}_{final}$  is the workload at the last completed stage,  $t$  is the time in seconds reached in the final uncompleted stage,  $T$  is the duration of each stage (180 seconds), and  $\dot{W}_{inc}$  is the workload increment (30W). Workloads obtained from the determination of 55% and 80% of  $\dot{W}_{max}$  of each individual were used on the main trials undertaken in Chapters 4 and 5.

### 3.2.2 Maximal Oxygen Uptake on a Motorised Treadmill

For the experimental studies presented in Chapters 6 and 7, participants reported to the laboratories to undertake a  $\dot{V}O_{2max}$  test on a motorised treadmill (PPS55 Med-i, Woodway, Birmingham, UK). After familiarisation with the testing equipment and careful instructions on how to complete the protocol, a 3-min run at 7 km·h<sup>-1</sup> and 1% slope to reflect outdoor running (Jones and Doust, 1996) was used as a warm-up to the actual test. A 16-min submaximal, incremental treadmill-running test was then undertaken (4 stages of 4 min each) in order to determine the relationship between running speed and  $\dot{V}O_2$ . The initial speed was set between 6.5 – 12.0 km·h<sup>-1</sup> by the participants themselves, who were encouraged to set their own starting pace. The speed was then increased by 1 km·h<sup>-1</sup> every stage. After a recovery period of about 20 min, where participants were

asked to remain seated,  $\dot{V}O_{2\max}$  was assessed using an incremental treadmill-running test. The test started on a level treadmill (1% incline) and the gradient was increased by 1% every minute until volitional exhaustion. The initial speed was set at the speed corresponding to a HR of approximately 150 beats.min<sup>-1</sup> on the submaximal exercise test and remained constant throughout. Expired gas was analysed continuously during the test using a breath-by-breath ergospirometry exercise testing system (METALYZER 3B, Cortex, Leipzig, Germany) for the determination of the rates of  $\dot{V}O_2$  and  $\dot{V}CO_2$ . Participants' HR and RPE were recorded at the last 15 s of each stage, using short-range radio telemetry and the 6-20 Borg scale, respectively. Water was provided before and after completion of these exercise tests.

Velocity at  $\dot{V}O_{2\max}$  ( $v\dot{V}O_{2\max}$ ) was obtained from data collected in the  $\dot{V}O_{2\max}$  test. A linear equation was obtained and determination of  $v\dot{V}O_{2\max}$  was determined by regressing  $\dot{V}O_2$  on exercise intensity (i.e. increases in speed) for submaximal exercise and extrapolating this relationship to  $\dot{V}O_{2\max}$  (Jones *et al.*, 2009), as shown in Figure 3.1. Individuals with the same relative  $\dot{V}O_{2\max}$  (mL.min<sup>-1</sup>.kg<sup>-1</sup>) may have different  $v\dot{V}O_{2\max}$ , depending on their running economy levels.



**Figure 3.1** Graph depicting the relationship between  $\dot{V}O_2$  and submaximal running speed to calculate  $v\dot{V}O_{2\max}$  (adapted from Jones *et al.*, (2009)).



Individualized percentages of  $v\dot{V}O_{2\max}$ , specifically 50% and 75%  $v\dot{V}O_{2\max}$  were obtained to be used in the exercise trials in Chapter 7.

### **3.3 10-km Time Trial Performance Test**

A 10-km time trial test was used in Chapter 7 as a measure of athletic performance. Participants completed this test on a motorised treadmill (PPS55 Med-i, Woodway, Birmingham, UK). The treadmill screen was covered throughout so the participants did not know their running speed during the test. The treadmill slope was kept consistent at 1%. The scope of the test was explained to the participants before the start.

### **3.4 Urine Collection and Analysis of Osmolality**

Participants were asked to provide a urine sample immediately before starting each main experimental trial (exercise or resting trials). Urine osmolality is used to measure the number of dissolved particles per unit of water in the urine. This method has been suggested as an instrument to provide reliable information as to an athlete's hydration status (Shirreffs and Maughan, 1998). Urine samples were analysed for osmolality by pipetting a small volume of sample into a portable urine refractometer (Pocket PAL-OSMO™, Atago Vitech Scientific Ltd, Sussex, UK). Euhydration was confirmed when the sample osmolality was below 700 mOsmol·kg<sup>-1</sup> H<sub>2</sub>O (Sawka *et al.*, 2007).

### 3.5 General Procedures for Hormone Analysis

Commercially available enzyme-linked immunosorbent assay (ELISA) kits were used for quantitative determination of plasma and salivary hormone concentrations and the manufacturer's recommended procedures for analysis were followed. All assays were performed by the same researcher to avoid any inter-experimenter variability and inaccuracy of the results, and the ambient temperature of the laboratory was maintained between 20-25°C, as variations in temperature may alter the samples optical density values.

A microtiter plate shaker (Stuart SSM5, Stuart Scientific, UK) was used to thoroughly mix the contents of each well of the assay plates and a microtiter plate washing system (Asys Atlantis 4, Biochrom, UK) was used to wash ELISA plates where required.

Standards and controls were used to verify the accuracy of the results. A microtiter plate reader (Infinite® 200 PRO, Tecan GmbH, Switzerland) was used to quantitatively measure the absorbance values of each sample, standards and controls in each assay completed and extrapolate those values to obtain its respective concentrations. Contamination of reagents, pipettes, tubes and wells was avoided by using new disposable plastic pipette tips for each component and specimen. Samples were determined in duplicate to identify any possible pipetting errors and coefficients of variation (CVs) of all duplicates were calculated. Average values were used for analysis. Standard curves were determined automatically by use of the software Magellan™ version 7.1 (Tecan GmbH, Switzerland).

## 3.6 Saliva Collection, Handling and Analysis

### 3.6.1 Saliva Collection and Handling

Saliva samples were collected into 7 mL polystyrene sterile bijoux containers (Sterilin, Thermo Scientific, Loughborough, UK) by unstimulated passive drool, following the 'draining method' (Navazesh, 1993). To avoid the possibility of saliva dilution, participants were required not to drink any water within the 10 min preceding sampling, apart from Chapter 4 where the effect of the timing of water consumption before saliva sampling was measured. Samples were given with eyes closed, head tilted slightly forward and avoiding any orofacial movement. Minimum collection time was 3 min for each participant to allow for collection of sufficient sample volume (~2 mL). Participants were instructed to swallow all saliva, and then collect all consequently secreted saliva in the oral cavity before dripping it off the lower lip into the collection tube. After sample collection all saliva samples were kept on crushed ice during the entire period of testing, which did not exceed 1 h 20 min. All samples were weighed to the nearest milligram to estimate saliva volume and saliva density was assumed to be  $1.00 \text{ g}\cdot\text{mL}^{-1}$  (Cole and Eastoe, 1988). Saliva flow rate was determined by dividing the saliva volume by the collection time. Saliva samples were then centrifuged at  $14600 \text{ g}$  for 3 min (Espresso Microcentrifuge, Thermo Scientific, Loughborough, UK) and the supernatant was transferred into 1.5 mL aliquots (Eppendorf, Hamburg, Germany) to be stored at  $-80^{\circ}\text{C}$  until further analysis.

### 3.6.2 Enzyme-linked Immunosorbent Assays (ELISA)

#### a) *Salivary Cortisol*

Salivary cortisol concentrations were determined by the use of commercially available ELISA kits (Salimetrics, PA 16803, USA) and the recommended procedures for analysis were followed. On the day of the assay all samples were thawed, vortexed (Harris Vortex Mixer, Philip Harris Limited, UK) and centrifuged once again at 14600 *g* for 3 min (Espresso Microcentrifuge, Thermo Scientific, Loughborough, UK). The supernatant was then used on the assay.

Monoclonal antibody-coated 96-well plates were used. Antigens present in the plasma samples compete with the enzyme horseradish peroxidase for the free antibody binding sites. Following incubation, the plates were washed in order to stop the binding reaction and remove any unbound products. A tetramethylbenzidine (TMB) substrate solution was consequently added to each well, which in reaction with the peroxidase enzyme forms a blue liquid solution. Further incubation is completed, and the reaction is then stopped with a 3-molar solution of sulphuric acid, which turns the solution into a pale green colour.

Optical density values were read on a plate reader (Infinite® 200 PRO, Tecan GmbH, Switzerland). The amount of cortisol present is inversely proportional to the cortisol peroxidase detected (Chard, 1990). The intra-assay and inter-assay coefficients of variation are 4.6% and 6.0%, respectively.

**b) Salivary Testosterone**

Salivary testosterone concentrations were assessed by the use of commercially available ELISA kits (Salimetrics, PA 16803, USA) in an analogous way to salivary cortisol. Contrarily to the latter, the plate wells were coated with rabbit antibodies to testosterone and a 2-molar sulphuric acid solution was used to stop the TMB substrate reaction.

The intra-assay and inter-assay coefficients of variation are 4.6% and 9.8%, respectively.

**c) Salivary Immunoglobulin A**

Levels of SIgA were analysed using a sandwich-type ELISA method, adapted from previous research (Leicht *et al.*, 2011). Analysis of SIgA was done in collaboration with Dr Glen Davison and Miss Ellie Heynes, from the University of Kent, and it was completed across three days in 3 main stages.

On day one, a coating buffer (0.05M carbonate/bicarbonate, pH 9.6) was freshly prepared by dissolving 0.208g of sodium bicarbonate ( $\text{NaHCO}_3$ ) and 0.030g of sodium carbonate ( $\text{Na}_2\text{CO}_3$ ) in 55mL of deionized water ( $\text{dH}_2\text{O}$ ). This volume of solution is sufficient for up to four 96-well immunoplates. A capture antibody (mouse monoclonal anti-secretory component (IgA) antibody,  $8.7\text{mg}\cdot\text{mL}^{-1}$ ) (I6635, Sigma-Aldrich, Gillingham, Dorset, UK), 8.7 was diluted 1:1740 in the coating buffer obtaining a final working concentration of  $5\ \mu\text{g}\cdot\text{mL}^{-1}$  ( $5000\ \mu\text{g}\cdot\text{L}^{-1}$ ). Therefore, for four plates, 30  $\mu\text{L}$  of capture antibody were diluted in 52.2 mL of coating buffer. Consequently, 100  $\mu\text{L}$  of coating buffer/antibody solution were pipetted into each well of a 96-well microplate (Nunc™

MicroWell™, Thermo Fischer Scientific, Waltham, Massachusetts, USA) using a multichannel pipette. Plates were then sealed incubated overnight at 4°C in a flat position to ensure the coating antibody was evenly covering the wells.

On day two, all samples were thoroughly thawed and centrifuged at 13,400 rpm for 2 minutes. The resulting supernatant was then serially diluted, firstly 1:25 with phosphate-buffered saline (PBS) (20 µL of sample to 480 µL PBS). In a fresh eppendorf tube, the 25x-diluted samples were diluted further in a 1:30 dilution (i.e. 20 µL of 25x-diluted sample to 580 µL PBS). These final samples were then used in the assay. Plates were subsequently washed 4 x 200 µL with a solution of PBS, 0.3M sodium chloride (NaCl) and 1% Tween 20 (P9416 Sigma-Aldrich, Gillingham, Dorset, UK). A 'blocking solution' of 0.3 g of 2% bovine serum albumin (BSA) (A3059, Sigma-Aldrich, Gillingham, Dorset, UK) in 15 mL PBS was used in each plate, by dispensing 100 µL of solution into each well, to prevent samples binding to potentially non-antibody-coated areas in the well. Plates were then covered and incubated at room temperature for 60 minutes and washed again 4 x 200 µL as explained above. A diluted (1:1700) solution of IgA from human colostrum (I2636, Sigma-Aldrich, Gillingham, Dorset, UK) with PBS to give a final dilution of 1 µg·mL<sup>-1</sup> was used in the assay as the more concentrated standard. Serially dilutions were then performed on this standard to a total of six extra standards by adding 500 µL of standard to 500 µL of PBS in each tube. An IgA free standard (500 µL of PBS only) was used as a blank for the ELISA. All standards, blanks and samples were assayed in duplicate (50 µL/well) and incubated at 4°C overnight. This assay measures IgA in the concentration range 0.03-0.3 µg·mL<sup>-1</sup>.

On day three, the plates were washed again 4 x 200 µL as described above. Afterwards, 12 µL of horseradish peroxidase (HRP) conjugated polyclonal rabbit anti-human IgA (P021602, DAKO, Agilent, Santa Clara, USA) were mixed with 24 mL of PBS (enough volume of solution for four plates) (1:2000 dilution) and 50 µL of this final solution were dispensed into each well of the 96-well plate. A further 90-min incubation at room

temperature was completed. A final 4 x 200  $\mu\text{L}$  wash was accomplished and subsequently, 50  $\mu\text{L}$  of a solution of 10  $\mu\text{L}$  of 30% hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) in 24 mL of o-phenylenediamine dihydrochloride (OPD) (solution of 8 tablets previously dissolved in 24 mL of  $\text{dH}_2\text{O}$  for four plates) were added to each well. A final 10-min incubation period was completed in the dark as the colour develops. The reaction was then stopped by pipetting 75  $\mu\text{L}$ /well of 1M sulfuric acid ( $\text{H}_2\text{SO}_4$ ) in the same sequence as the OPD solution. The plate was read on an automated plate reader (ELx808™ Absorbance Reader, BioTek Instruments, Winooski, Vermont, USA) with a 490 nm filter. The concentrations obtained from the standard curve were then multiplied by the dilution factor (i.e. 750) to determine the actual amount of IgA in the original sample.

### **3.7 Blood Collection, Handling and Analysis**

#### **3.7.1 Blood Collection and Handling**

Whole blood samples were collected by venepuncture from an antecubital vein using a 21G needle with quickshield (Vacuette®, Greiner Bio-One, Stonehouse, Great Britain) into 5 mL tri-potassium ethylenediaminetetraacetic acid ( $\text{K}_3\text{EDTA}$ ) vacutainers (Vacuette®, Greiner Bio-One, Stonehouse, Great Britain). In Chapter 6, only 1 tube was obtained and after sample collection all samples were stored on crushed ice during the entire period of testing which did not exceed 80 min. Blood samples were then centrifuged at 1500 g for 10 min at 4°C (Heraeus Multifuge X3R, Thermo Scientific, Loughborough, UK) and the resulting plasma was then transferred into sterile 1.5 mL cryogenic tubes (Nalgene™, Thermo Scientific™, Thermo Fisher Scientific, Waltham, Massachusetts, USA) to be stored at -80°C (for no longer than 2 months) until further hormone analysis. In Chapter 7, whole blood was collected into 3 tubes (approximately 15 mL) and plasma was extracted

from one of these by following the protocol above. The remaining blood samples were used as described in the following subchapters 3.7.2 and 3.7.3.

### 3.7.2 Haematology

In Chapter 7 all haematological analysis were performed in K<sub>3</sub>EDTA-treated whole blood. Total haemoglobin in whole blood was determined in duplicate using a HemoCue<sup>®</sup> Hb 201<sup>+</sup> analyser (HemoCue<sup>®</sup>, Ängelholm, Sweden). Whole blood was spun down in a microhaematocrit centrifuge (Haematospin 1300, Hawksley, Sussex, UK) and haematocrit was determined in duplicate by measuring packed cell volumes (PCV) using a tube reader (Hawksley, Sussex, UK). Plasma volume from resting blood samples was calculated from the haematocrit values, whereas exercise-induced changes in plasma volume were determined from the measured haemoglobin and haematocrit values, using the equation below:

$$PV_{Post} = BV_{Post} - CV_{Post} \text{ , (Dill and Costill, 1974)}$$

where  $PV_{Post}$ ,  $BV_{Post}$  and  $CV_{Post}$  are the plasma volume, blood volume and erythrocyte volume after exercise, respectively.  $BV_{Post}$  and  $CV_{Post}$  were calculated from the following equations:

$$BV_{Post} = BV_{Pre} (Hb_{Pre} / Hb_{Post})$$

$$CV_{Post} = BV_{Post} (Hct_{Post})$$

where  $Hb_{Pre}$  is the baseline haemoglobin concentration,  $Hb_{Post}$  is the haemoglobin concentration after exercise,  $Hct_{Post}$  is the haematocrit concentration after exercise, and  $BV_{Pre}$  is a standardized blood volume concentration set at 100.

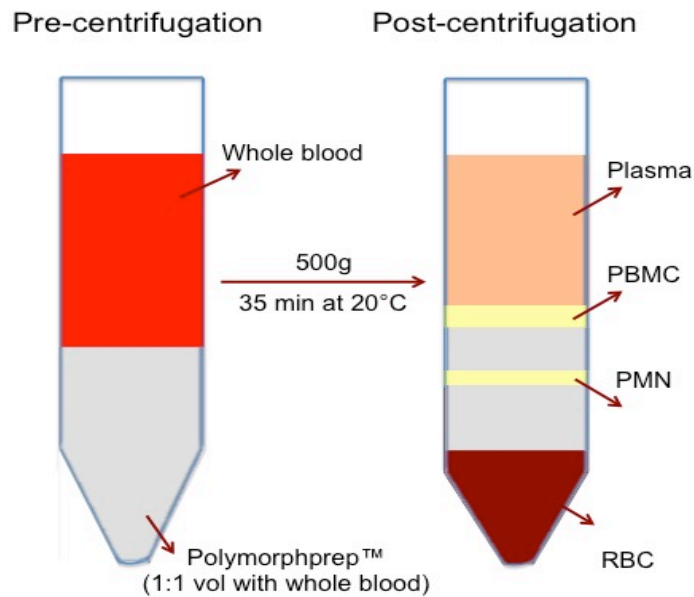


### 3.7.3 Leucocyte Isolation

#### a) *Peripheral Blood Mononuclear Cells (PBMCs) and Polymorphonuclear (PMN) granulocytes*

Polymorphoprep™ (PMP) is a ready-made, sterile and endotoxin-tested solution used for the isolation of pure PMNs from whole blood, containing sodium diatrizoate and polysaccharide. The cells separation is due to the rapid sedimentation of the leucocytes through the medium caused by its aggregation to the polysaccharide. As the leucocytes start losing water through the membrane to the medium (therefore diluting it significantly), the osmolality inside these cells increases. The leucocytes continue depositing at gradually less pronounced water loss rates as they travel through the medium from the top to the bottom. Thereby, the density of the medium is decreased most in the top zone of the tube, compared to the bottom zone, and a gradient is generated. As the leucocyte sedimentation and medium dilution alterations occur progressively and smoothly, this allows the formation of a continuous gradient. It is within this gradient that the PBMCs and PMNs are set, as shown in Figure 3.2.

For the isolation protocol K<sub>3</sub>EDTA-treated whole blood samples were used. 4 mL of PMP were dispensed into the base of a 15 mL polypropylene Falcon™ conical tube with screw caps (BD Biosciences, Oxford, UK) and carefully topped with 4 mL of whole blood avoiding mixing the contents. The tube was then centrifuged at 500 g for 30 min at 20°C, allowing the rotor to decelerate without the brake (i.e. acceleration and deceleration set at 2 and 1, respectively). The obtained plasma was removed and the PMNs (lower band) harvested and transferred to a clean Falcon tube (Figure 15). The PBMCs (upper band) were discarded.



**Figure 3.2** Purification of polymorphonuclear leucocytes (PMN) using Polymorphprep™.  
RBC – Red blood cells (i.e. erythrocytes)

The PMNs were subsequently cultured in a 1:2 solution of Dulbecco's Modified Eagle's Medium containing  $4500 \text{ mg}\cdot\text{L}^{-1}$  of glucose,  $110 \text{ mg}\cdot\text{L}^{-1}$  sodium pyruvate and L-glutamine (Sigma-Aldrich, Gillingham, Dorset, UK) and sterile-filtered water (BioReagent, Sigma-Aldrich, Gillingham, Dorset, UK) in a separate 15 mL Falcon tube. Both tubes were then centrifuged once more at  $400 \text{ g}$  for 10 min with the acceleration and deceleration back to full speed (i.e. 9). The supernatant was discharged, and the pellets re-suspended in 1 mL of AIM-V® serum free medium (1X) containing L-Glutamine,  $50 \mu\text{g}\cdot\text{mL}^{-1}$  of streptomycin sulphate and  $10 \mu\text{g}\cdot\text{mL}^{-1}$  of gentamicin sulphate, supplemented with bovine serum albumin (BSA, AlbuMAX®) (Gibco™, Thermo Fisher Scientific, Waltham, Massachusetts, USA). The tubes were then set aside for further cell counts, phagocytic activity assay, and staining for flow cytometry on the day.

**b) Total Leucocytes from Whole Blood**

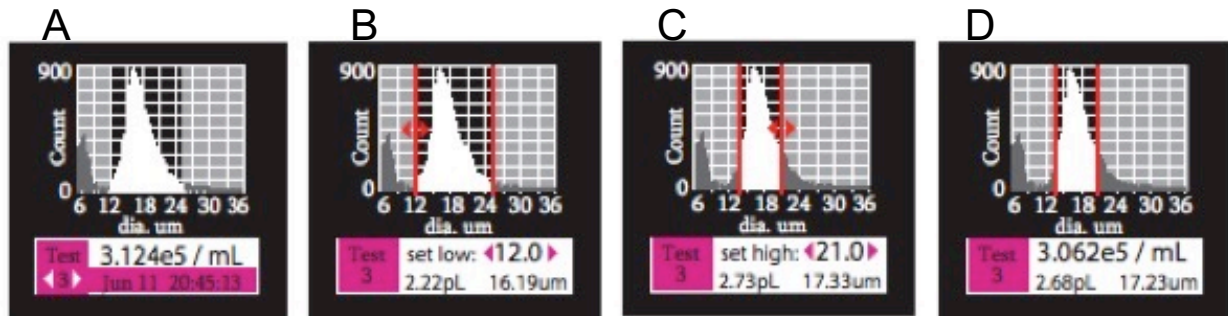
A 1X Red Blood Cell (RBC) Lysis Buffer containing ammonium chloride (eBioscience™, Thermo Fisher Scientific, Waltham, Massachusetts, USA) was mixed with whole blood in a 15 mL polypropylene Falcon™ conical tube with screw caps (BD Biosciences, Oxford, UK) in a 10:1 proportion (i.e. 5 mL of RBC lysis buffer to 500 µL of whole blood) by gentle inversion. A 10 min incubation period was completed, and the reaction was stopped by adding twice the volume of 10X, pH 7.4 Phosphate-Buffered Saline (PBS) (Gibco®, Thermo Fisher Scientific, Waltham, Massachusetts, USA) (i.e. 10 mL of PBS to 5 mL of 1X RBC). The sample was then centrifuged at 400 g for 5 min in a refrigerated centrifuge at 4°C. The resulting supernatant was subsequently discharged and the pelleted cells were re-suspended in 1 mL PBS before further analysis.

**3.7.4 Cell Counting using a Handheld Cell Counter**

Cell counting of whole blood leucocytes and PMNs in experimental Chapter 7 was performed using an automated handheld cell counter (Scepter™, Merck Millipore, Germany). For the cell count using the handheld cell counter, dilutions were prepared in 1.5 mL microcentrifuge aliquots in a ratio of 1:5 with 10X, pH 7.4 PBS. The minimal sample volume used and required for an accurate count was 100 µL. Disposable 60 µm (operating range between 10,000-500,000 cells·ml<sup>-1</sup>) microfluidic sensor tips (PHCC60050, Scepter, Merck Millipore, Billerica, Massachusetts, USA) were used.

To start the suspended cell count, a disposable tip was attached to the Scepter™ and after depressing the plunger it was submerged in the sample for analysis. The sample including the cells in suspension was drawn into the sensor by releasing the plunger. A count histogram appears on the display screen within 30 sec. Information about cell size

(diameter in  $\mu\text{m}$  and volume in  $\text{pL}$ ) and total cell count  $\cdot\text{mL}^{-1}$  is displayed, and the data can be managed by adjusting the upper and lower gates on the data screens (Figure 3.3). Total cell numbers were determined by multiplying the given cell number on the data screen by the dilution factor.



**Figure 3.3** Example of data screens from Scepter™; A) initial data screen following sample analysis; B) setting lower gate in cell diameter screen; C) setting upper gate in cell diameter screen; D) corrected cell count following gate adjustment.

### 3.7.5 Polymorphonuclear Leucocytes Phagocytic Activity Assay

Preparation of working stock dihydroethidium (wsDHE) and opsonized zymosan was done in advance and stored in a  $-20^{\circ}\text{C}$  and  $-80^{\circ}\text{C}$  freezers, respectively.

A 1.6 mM wsDHE was prepared by diluting dihydroethidium (DHE) in a 1:40 ratio in dimethyl sulfoxide (DMSO) (e.g. 5  $\mu\text{L}$  DHE to 195  $\mu\text{L}$  of DMSO).

Opsonized zymosan was obtained by suspending  $\sim 200$  mg of zymosan in 1 mL of a 1:2 solution of PBS with sterile-filtered human male serum (Sigma-Aldrich, Gillingham, Dorset, UK) and incubating for 1 hour at  $37^{\circ}\text{C}$ . The solution was then centrifuged at 7500 rpm for 5 minutes and the supernatant removed. The pellet was washed with 1 mL PBS and spun down again. The PBS wash was discarded and the opsonized zymosan was re-suspended at  $200 \text{ mg}\cdot\text{mL}^{-1}$  in fresh PBS.

The PMNs isolated from K<sub>3</sub>EDTA-treated whole blood were resuspended to roughly 2 million/mL in AIM-V<sup>®</sup> serum free cell medium as detailed in 3.7.3 a). 100 µL of PMN sample was placed into 3 wells of a 96-well polystyrene, sterile, flat bottom plate, to serve as non-stain control, baseline and treatment. To the non-stain control 100 µL of AIM-V<sup>®</sup> cell medium were added. A 2X DHE stain solution was prepared by mixing 5 µL of wsDHE with 250 µL of AIM-V<sup>®</sup> serum free cell medium. 100 µL of 2X DHE stain (16µM) were added to the other two wells. Finally, 2.5 µL of opsonized zymosan were added to the appropriate (treatment) well. The samples were incubated at 37°C in a humid 5% CO<sub>2</sub> environment for 30 minutes to allow for sufficient time for the DHE to label the PMNs (Saiki *et al.*, 1986). Contents were then transferred into 1.5 mL microcentrifuge tubes and 1 ml PBS was added into each. All samples were centrifuged in a microcentrifuge at 5000 rpm for 5 minutes, supernatant removed with a vacuum pump and pellet re-suspended in 200 µL of a FACS buffer (1 X PBS pH 7.2, containing calcium chloride, magnesium chloride, 2% foetal bovine serum and 0.01% azide), before acquisition via flow cytometry.

### 3.7.6 Leucocyte Subsets Activation and Acquisition

Total leucocytes from K<sub>3</sub>EDTA-treated whole blood and isolated PMNs were stained with fluorochrome-conjugated anti-human monoclonal antibodies for flow cytometric analysis (BD Accuri<sup>™</sup> C6 Plus, BD Biosciences, Oxford, UK). Controls for the flow cytometer were stained with 1µL mouse monoclonal antibodies (eBioscience<sup>™</sup>, Thermo Fisher Scientific, Waltham, Massachusetts, USA) against human cell surface markers in 50µL of isolated total leucocytes and 25µL of FACS buffer as follows: 1) Unstained cells; 2) FITC conjugated anti-human CD3 (cat no 11-00370-41); 3) PE conjugated anti-human CD3 (12-0037-41); 4) PerCP-Cyanine5.5 conjugated anti-human CD4 (45-0048-41); 5) APC conjugated anti-human CD4 (17-0048-41). For the experimental stains, 300 µL of sample (either whole blood leucocytes or PMNs, typically approximately 3.5 million and 2

million cells/mL, respectively) were transferred into separate aliquots and labelled appropriately. All aliquots were centrifuged at 500 *g* for 5 min and the supernatant was carefully discarded. 30µL of antibody cocktails (all antibodies were from eBioscience™, Thermo Fisher Scientific, Waltham, Massachusetts, USA, all cocktails contained antibody in FACS buffer at 1:50 dilution) were added directly onto the cell pellets in each tube as follows: A) T-lymphocyte surface markers: FITC conjugated anti-CD3 (11-0037-41), PE conjugated γδ T-cell receptor (TCR) (12-9959-41), PerCP-Cyanine 5.5 conjugated anti-CD4 (45-0048-41), APC conjugated anti-CD8a (17-0086-41); B) Monocyte/Macrophage, NK cells, B-cell and Granulocytes surface markers: FITC conjugated anti-CD11b (11-0113-41), PE conjugated anti-CD56 (12-0567-41), PerCP-Cyanine 5.5 conjugated anti-CD19 (45-0198-41), APC conjugated anti-CD11c (17-0128-41); C) Monocyte/Macrophage, Neutrophils, Eosinophils surface markers: FITC conjugated anti-CD16 (11-0168-41), PE conjugated anti-CD15 (12-0159-41), PerCP-Cyanine 5.5 conjugated anti-CD14 (45-0149-41), APC conjugated anti-CD23 (17-0238-41). All aliquots were incubated at 4°C for 30 min and cells were subsequently washed in 300 µL of FACS buffer and re-suspended in 200 µL of the same buffer for immediate flow cytometer acquisition. All samples were gated by morphology using side scatter (SSC) *versus* forward scatter (FSC) plots and 50,000 events were acquired per sample. Data files were generated through the use of BD C6 Analysis Software for PC (BD Biosciences, Oxford, UK) and were stored for later analysis as described in 3.7.7. The Flow Cytometer was calibrated every day before use and acquisition settings were defined before the start of the study and remained unchanged for all participant samples.

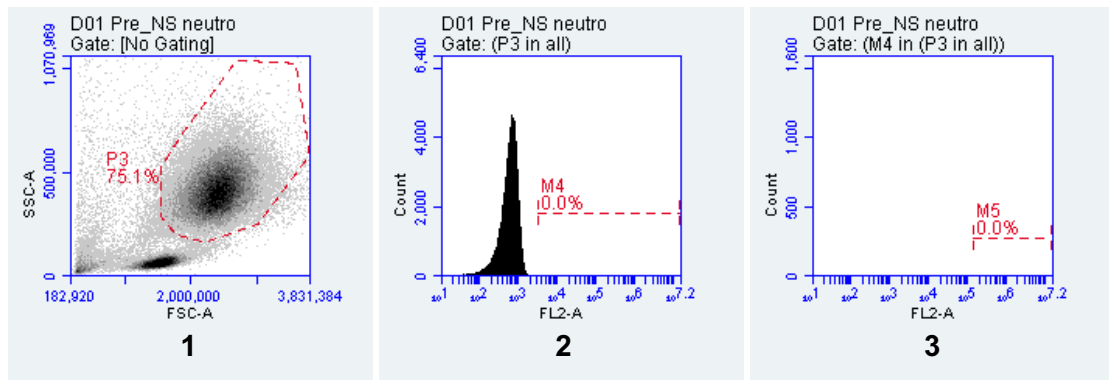
### 3.7.7 Flow Cytometry (FACS) Analysis

Flow cytometric analysis was performed using the software BD Accuri™ C6 Plus for Windows (BD Biosciences, San Jose, CA, USA).

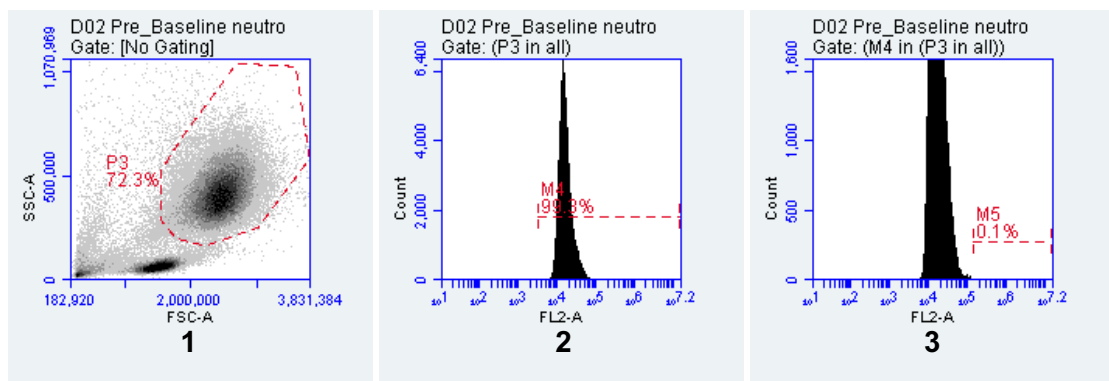
#### a) *Phagocytosis assay*

A side scatter (SSC) *versus* a forward scatter (FSC) plots of all acquired cells were used to gate on the isolated PMNs (Figure 3.4 A1, B1, C1). Histograms for counts (cell number) plotted against FL2 were created with gates for examination of the unstimulated PMNs (Figure 3.4 A), and fluorescence of dihydroethidium (DHE)-stained PMNs (Figure 3.4 B), and DHE-stained and opsonized zymosan-stimulated PMNs (Figure 3.4 C). DHE is a superoxide indicator that exhibits blue-fluorescence in the cytosol (Budd *et al.*, 1997) until it oxidizes and intercalates in the DNA present in the cells nucleus, staining it a bright red (Amir *et al.*, 2008). Neutrophils that take up DHE will fluoresce (FL2 positive) (Figure 3.4 B2). When stimulated with the opsonized zymosan which induces a sterile inflammation to which the PMNs will respond by activating its phagocytic capability, the DHE will react and turn into ethidium bromide which will fluoresce pronouncedly brighter, provoking a further shift to the right in FL2 (FL2 positive, high) (Figure 3.4 C3).

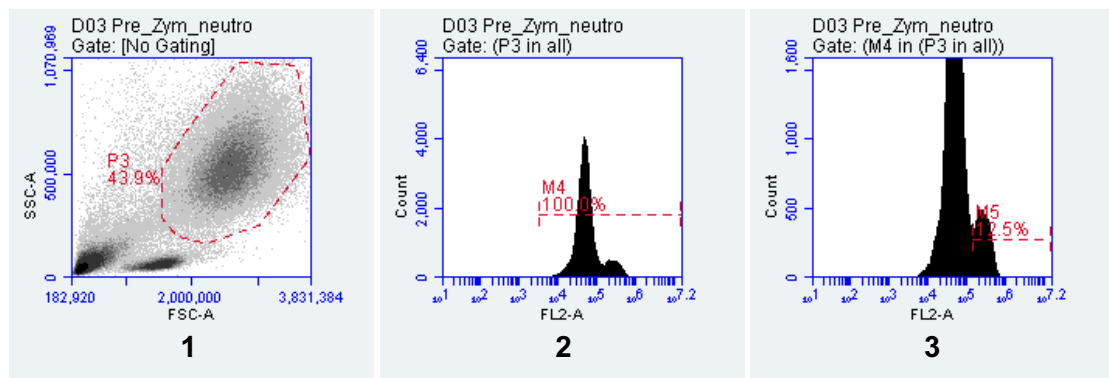
A



B



C

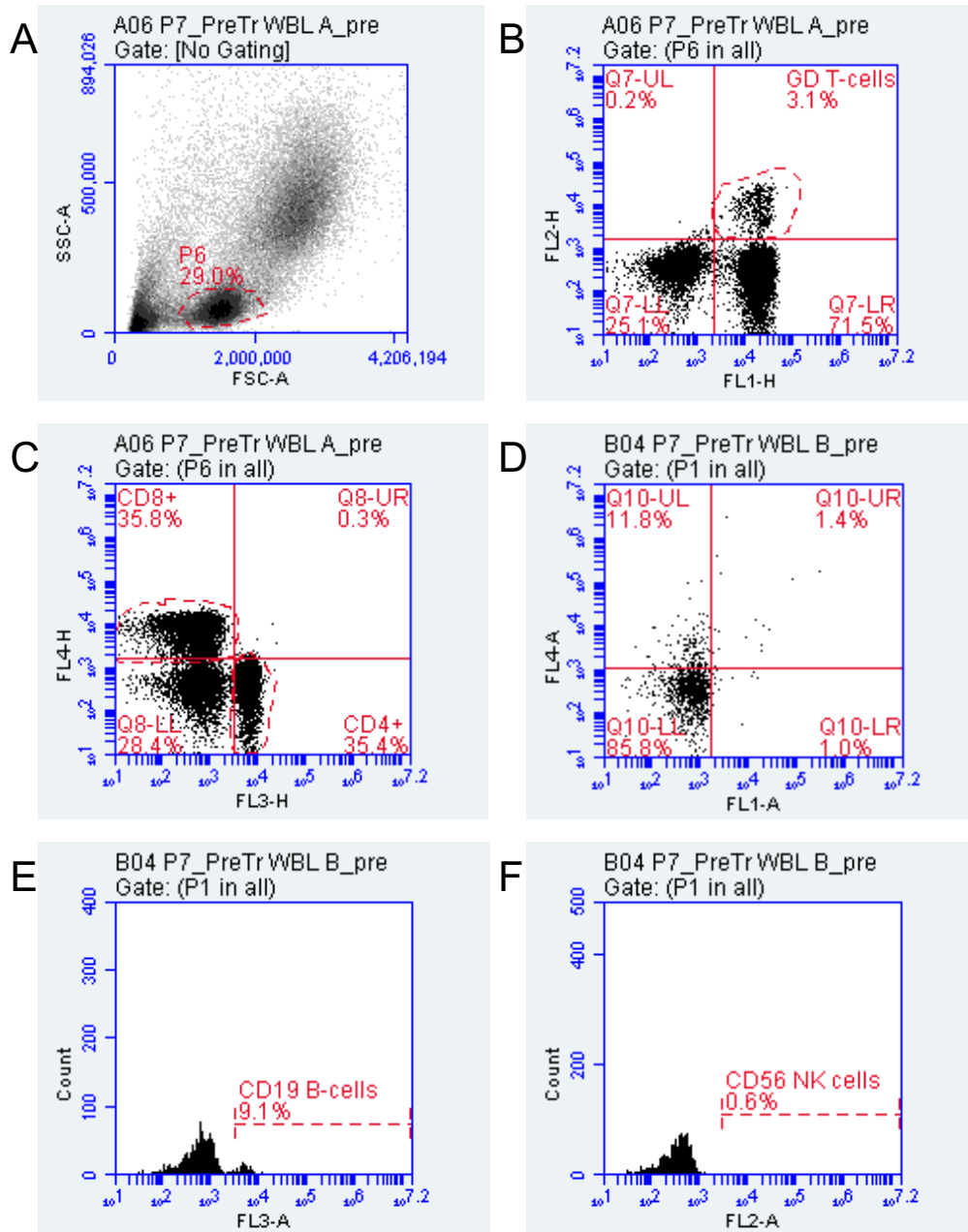


**Figure 3.4** Polymorphonuclear leucocytes (PMNs) phagocytic activity flow cytometric analysis. A) Unstimulated PMNs; B) Dehydroethidium-stained PMNs; C) Dehydroethidium-stained and stimulated with opsonized zymosan. P3 – Granulocyte population; FSC – Forward scatter (cell size); SSC – Side scatter (internal complexity/granularity); FL2-A – Fluorescence in FL2.



**b) T-lymphocyte analysis**

A side scatter (SSC) *versus* a forward scatter (FSC) plots of all acquired cells were used to gate on the lymphocyte population (Figure 3.5 A). Subsequently, FL2 *versus* FL1 (Figure 3.5 B) plots were used to gate on the  $\gamma\delta$  T-lymphocytes (stained with cocktail A as detailed in 3.7.6). Furthermore, it is known that the  $\alpha\beta$  T-cell population should express CD3 (Fowlkes *et al.*, 1987) and these will either be CD8 positive (CD8<sup>+</sup>, cytotoxic T-cells) or CD4 positive (CD4<sup>+</sup>, helper T-cells). For these, FL4 *versus* FL3 plots (Figure 3.5 C) of the lymphocyte population were used. The percentages shown in the plots were used for analysis in Chapter 7, after fluorescence compensation was performed when needed (Herzenberg *et al.*, 2006). An FL1 *versus* FL4 scatter plot was used to examine CD11b<sup>+</sup> and CD11c<sup>+</sup> populations, in which the percentage of CD11c<sup>+</sup> cells are likely to be dendritic cells (Figure 3.5 D). Finally, FL3 and FL2 histogram plots were used for the analysis of CD19<sup>+</sup> B-lymphocytes (Figure 3.5 E) and CD56<sup>+</sup> NK cells (Figure 3.5 F), respectively.

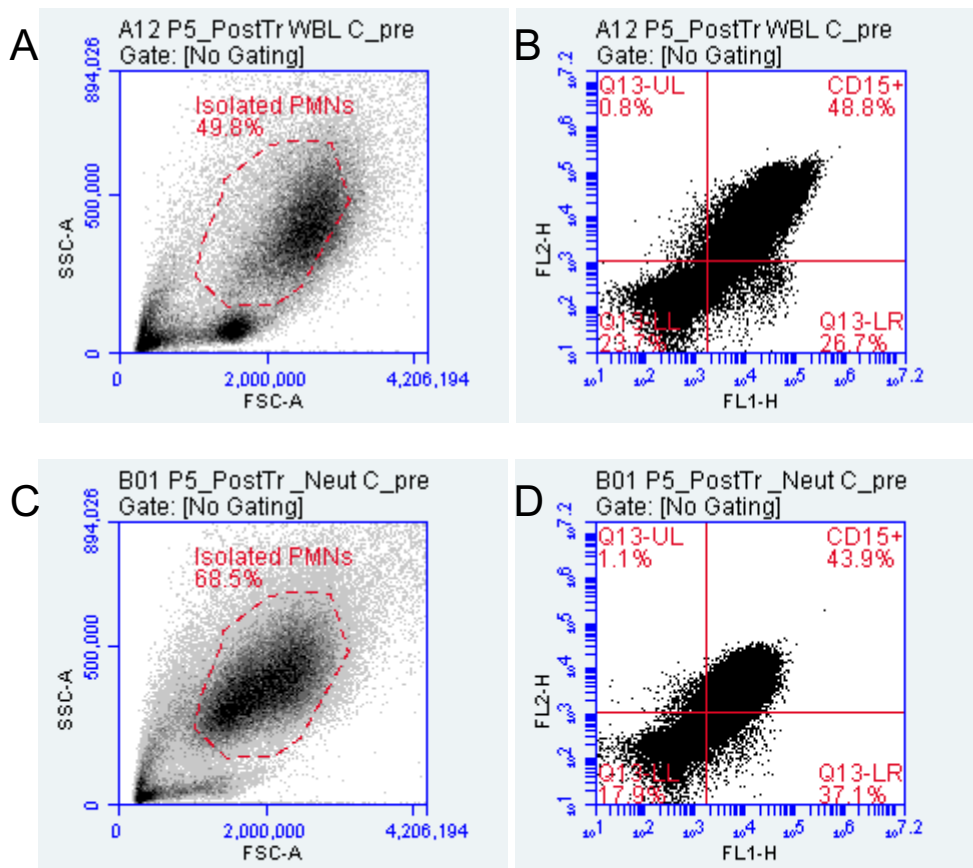


**Figure 3.5** Lymphocyte flow cytometric gating and analysis.

P6 – Lymphocyte population; FSC – Forward scatter (cell size); SSC – Side scatter (internal complexity/granularity); FL1-H – CD3 expression; FL2-H –  $\gamma\delta$  TCR expression; FL4-H – CD8 expression; FL3-H – CD4 expression; FL1-A – CD11c expression; FL4-A – CD11b expression; FL3-A – CD19 expression; FL2-A – CD56 expression.

### c) Granulocyte Analysis

A side scatter (SSC) *versus* a forward scatter (FSC) plots of all acquired cells were used to gate on the granulocyte population (Figures 3.6 A and 3.6 C). Subsequently, FL2 *versus* FL1 plots were used to gate on the granulocytes population in the whole blood leucocyte sample (Figure 3.6 B) that were stained for CD16<sup>+</sup> and CD15<sup>+</sup> neutrophils. The same scatter plot was created and used on the isolated PMNs sample (as detailed in 3.7.3) to examine the quality of the isolation by comparing the CD15<sup>+</sup> neutrophil population percentage in both plots (Figure 3.6 D).



**Figure 3.6** Granulocytes flow cytometric gating and analysis. FSC – Forward scatter (cell size); SSC – Side scatter (internal complexity/granularity); FL1-H – CD16 expression; FL2-H – CD15 expression.

### 3.7.8 Enzyme-linked Immunosorbent Assays

#### *a) Plasma Cortisol*

Plasma cortisol concentrations were assessed by the use of commercially available ELISA kits (IBL International, Hamburg, Germany) in an analogous way to the salivary cortisol ELISA method (subchapter 3.6.2 a)). Contrarily to the latter, the wells were coated with rabbit, polyclonal anti-cortisol antibodies and a 0.5 M sulphuric acid solution was used to stop the TMB substrate reaction. The intra-assay and inter-assay coefficients of variation are 2.9% and 3.5%, respectively.

#### *b) Plasma Testosterone*

Plasma testosterone concentrations were assessed by the use of commercially available ELISA kits (IBL International, Hamburg, Germany) in an analogous way to the salivary testosterone ELISA method (subchapter 3.6.2 b)). The main difference was that all wells were pre-coated with mouse anti-testosterone monoclonal antibodies and a 1 M sulphuric acid solution was used to stop the TMB substrate reaction. The intra-assay and inter-assay coefficients of variation are 4.3% and 6.5%, respectively.

## 3.8 Questionnaires and Diaries

### 3.8.1 Recovery-Stress Questionnaire for Athletes (RESTQ-76 Sport)

The RESTQ-76 Sport for athletes (Appendix 4) is a 76-statement recovery-stress questionnaire that discriminates 48 nonspecific and 28 sport-specific statements of stress

and recovery, consisting of 4 main scales in total (general stress, general recovery, sport stress and sport recovery) (Kellmann and Kallus, 2001). Each one of these main scales is subsequently divided into 7, 5, 3 and 4 subscales, respectively. Each of these subscales (19 in total) includes specific statements. The sum of scores (answer to each statement) in each of the 19 subscales is used to examine the overall responses to the questionnaire. Each answer ranges from never (0) to always (6) and covers the participants' past 3 days and nights. This questionnaire aids the practitioner/researcher confirming the athletes' physical and mental state by analysing their recovery state either from any exercise completed or from any stressful event that may have occurred within the 3-day period preceding the completion of the questionnaire. Greater scores of general recovery and sport recovery denote an increased sense of well-being, whereas lower rates of general and sport-specific stress emphasize a better predisposition to undertake physical activities (Kellmann and Kallus, 2001). The RESTQ-76 Sport was used in the studies presented in Chapters 4, 5, 6 and 7.

### **3.8.2 Upper Respiratory Symptoms (URS) Questionnaire**

In Chapter 7, all participants were required to complete a logbook in each main trial in which any subjective signs and symptoms of common cold experienced within the previous 3 days were documented and the scores of all symptoms were summed. The questionnaire applied in Chapter 7 in this Thesis was taken from Bishop (2013) and has been utilised in previous research (Hanstock *et al.*, 2016). The following symptoms were included in the log sheet: sneezing, headache, malaise, nasal discharge, nasal obstruction, sore throat, cough, ear ache, hoarseness, fever, chilliness, and joint aches and pains (Appendix 5). Participants were instructed to code their degree of discomfort in a 4-point scale from 0 to 3, using the Jackson score (either no symptoms at all, mild, moderate or severe) (Jackson *et al.*, 1958). The usefulness of a Jackson common cold

questionnaire has been documented in the recent International Society of Exercise and Immunology consensus statement (Bermon *et al.*, 2017).

### **3.8.3 Food Diary**

A 24-hour food diary was used as a method to standardize the caloric and macronutrient intake of participants in Chapters 4, 5, 6 and 7 (Appendix 6). All participants were instructed on weighing and recording their food intake and were asked to replicate their meals in the 24 hours before each main experimental trial. A digital scale (Salter 1036BKSSDR, HoMedics Group Ltd., Kent, UK) was given to each participant so they could complete the food diaries. A nutritional analysis software (Dietplan version 6.70.74, Forestfield Software, West Sussex, UK) was used to assess the average caloric and macronutrient (carbohydrate, protein, fat) intake, and the average amount of food consumed in the 24 hours preceding each main trial.

### **3.8.4 Training Diary**

In Chapter 7, a training diary was completed by the participants after each training session undertaken outside the laboratories (Appendix 7). Participants were instructed on measuring and recording their heart rate using a heart rate monitor. Resting, average and maximum heart rate, duration of training session and average RPE were recorded after each training session.

### 3.9 Measurement of Training Load

This training load measurement was used in the study presented in Chapter 7. A 12-day training diary was completed on two occasions and training load was measured by a training impulse (TRIMP) method. TRIMP will be calculated by the formula:

$$t(\text{min}) \cdot \Delta\text{HR} \cdot y, (\text{Banister, 1991})$$

where,  $t$  is the duration of each training session,  $y = 0.64e^{1.92x}$  (weighting factor),  $e$  is the base of the Napierian logarithms, and  $x = \Delta\text{HR}$  (Heart Rate Reserve). The  $\Delta\text{HR}$  is the difference between maximal and resting heart rate (HR).

Maximal HR ( $\text{HR}_{\text{max}}$ ) was expressed as the highest HR achieved during maximal intensity exercise to exhaustion. Resting heart rate ( $\text{HR}_{\text{rest}}$ ) was measured in the morning immediately after waking up and was assessed as being the lowest HR maintained during at least 5 seconds within a 10-minute period.

### 3.10 Statistical Analysis

Statistical analysis was accomplished using IBM® SPSS® Statistics version 23 (IBM® Corporation, Armonk, NY, USA). All procedures followed are described in the respective chapters.

## **CHAPTER 4.**

# **The Effects of Water Consumption on the Salivary Cortisol and Testosterone Responses to a Continuous 30-Min, High- Intensity Cycle Bout**



### 4.1 Introduction

Analysis of salivary biomarkers is becoming a standard and common measurement in field-based and laboratory based research, as it is a non-invasive measure that does not require any specific medical training and it can be easily performed (Chicharro *et al.*, 1998, Papacosta and Nassis, 2011). Two endocrine biomarkers commonly measured in saliva are cortisol and testosterone (Hayes *et al.*, 2016, Kirschbaum and Hellhammer, 1989, Sannikka *et al.*, 1983). Cortisol and testosterone concentrations are not influenced by saliva flow rate (Chicharro *et al.*, 1998, Lewis, 2006, Vining *et al.*, 1983) and cortisol has been shown moderate to strong positive correlations with its plasma concentrations (Khan-Dawood *et al.*, 1984, Perogamvros *et al.*, 2010, Port, 1991), as detailed in section 2.3.8 in this Thesis. Data obtained from a population of 122 individuals showed a significant positive correlation in salivary cortisol ( $r = 0.75$ ,  $p < 0.001$ ) when examined in a test-retest study design, by regressing the concentration values obtained in two measurement trials on a scatterplot (Liening *et al.*, 2010). The reliability of measuring testosterone in saliva has also been reported to be strong, with  $r = 0.87$  (males) and  $r = 0.78$  (females) when determined by the intraclass correlation coefficient (ICC) in samples from 100 healthy male and 100 healthy female individuals (Dabbs *et al.*, 1995). The findings from both these studies support the appropriateness of using salivary measurements of cortisol and testosterone as a non-invasive replacement for circulating levels.

A continuous 30-min, high-intensity cycle bout has been shown to elicit an acute salivary cortisol (~120%) and testosterone (~35%) increase in healthy, physically active male individuals (Hough *et al.*, 2011). Furthermore, Hough *et al.* (2013) and Hough *et al.* (2015) have reported blunted salivary cortisol and testosterone to the this exercise bout, after exposing healthy, trained athletes/triathletes through a period of intensified training.

#### Chapter 4: Effects of Saliva Dilution in Salivary Hormone Values

Therefore, it has been suggested that the exercise-induced responses of these salivary hormones may be used as biological markers of overreaching.

Most experimental studies measuring salivary hormone levels control for water consumption before sampling. Current procedures recommend interrupting water consumption at least 10 minutes before collecting saliva samples as the dilution of saliva samples may lead to an inaccurate concentration value of the hormones being examined. No experimental research has examined the impact of diluting saliva by consuming water on the salivary cortisol and testosterone has been completed to date. Further examination of the effects of water consumption in diluting saliva and consequent salivary hormone concentration is of interest. Therefore, the main aim of this experimental chapter is to examine whether consuming a fluid (specifically water) 10 min, 5 min or 1 min before providing a saliva sample would dilute samples and consequently provide an inaccurate concentration value, more specifically a lower concentration than is really present.

## 4.2 Methods

### 4.2.1 Procedures

In this experimental chapter, 10 participants volunteered to complete the study. As detailed in 3.1, all participants gave their written informed consent and completed a PAR-Q form before all experimental trials. The participants' anthropometric and physiological characteristics at baseline are outlined in Table 4.1.

**Table 4.1:** Participants' anthropometric and physiological characteristics at baseline.

	Mean $\pm$ SD
Age (years)	22 $\pm$ 2
Height (cm)	181 $\pm$ 4
Body mass (kg)	85.7 $\pm$ 9.4
HR <sub>max</sub> (beats·min <sup>-1</sup> )	186 $\pm$ 11
$\dot{V}O_{2max}$ (mL·kg <sup>-1</sup> ·min <sup>-1</sup> )	41 $\pm$ 8
$\dot{W}_{max}$ (Watts)	269 $\pm$ 45
n = 10	

All trials were undertaken at the University of Bedfordshire Sports Sciences Human Performance Laboratories and were completed at the same time of day (~12:00) to avoid diurnal variation. A standard breakfast chosen by each participant was consumed 4 hours before the exercise testing began and replicated before each main experimental trial, and each participant was requested to drink at least 500 mL of water during that period to ensure euhydration was achieved (Sawka *et al.*, 2007). Additionally, all participants reported to the laboratories approximately 30 min before the exercise test start time so all

necessary preliminary measurements could be collected. Participants remained fasted until the end of each main experimental trial.

Body mass measurements were carried out at three different time points (after sampling) to determine the rate of sweat lost during and after exercise. HR and RPE were monitored continuously throughout each exercise test and values were recorded at the last 15 s of each stage. Participants were asked to maintain their normal weekly training load in between each main experimental trial.

All participants were requested to consume a diet as similar as possible during the 24 h preceding each main experimental trial and were provided with a digital food scale to weight and record their food and fluid intake. By use of the Dietplan version 6.70.74 software (Forestfield Software, West Sussex, UK) mean energy intake 24 h prior to each trial was  $9439 \pm 3954$  kJ ( $2256 \pm 945$  kcal), and carbohydrate, ( $58\% \pm 12\%$ ), fat ( $27\% \pm 13\%$ ), and protein ( $14\% \pm 2\%$ ) intake were determined.

### 4.2.2 Preliminary Measurements and Main Experimental Trials

All participants completed 4 trials in total - three main experimental trials and one  $\dot{V}O_{2\max}$  test. In addition, 5 participants also completed a resting control trial (CTL). The CTL trial was added to the study design to allow for comparison of salivary cortisol and testosterone concentrations at rest and in response to exercise.

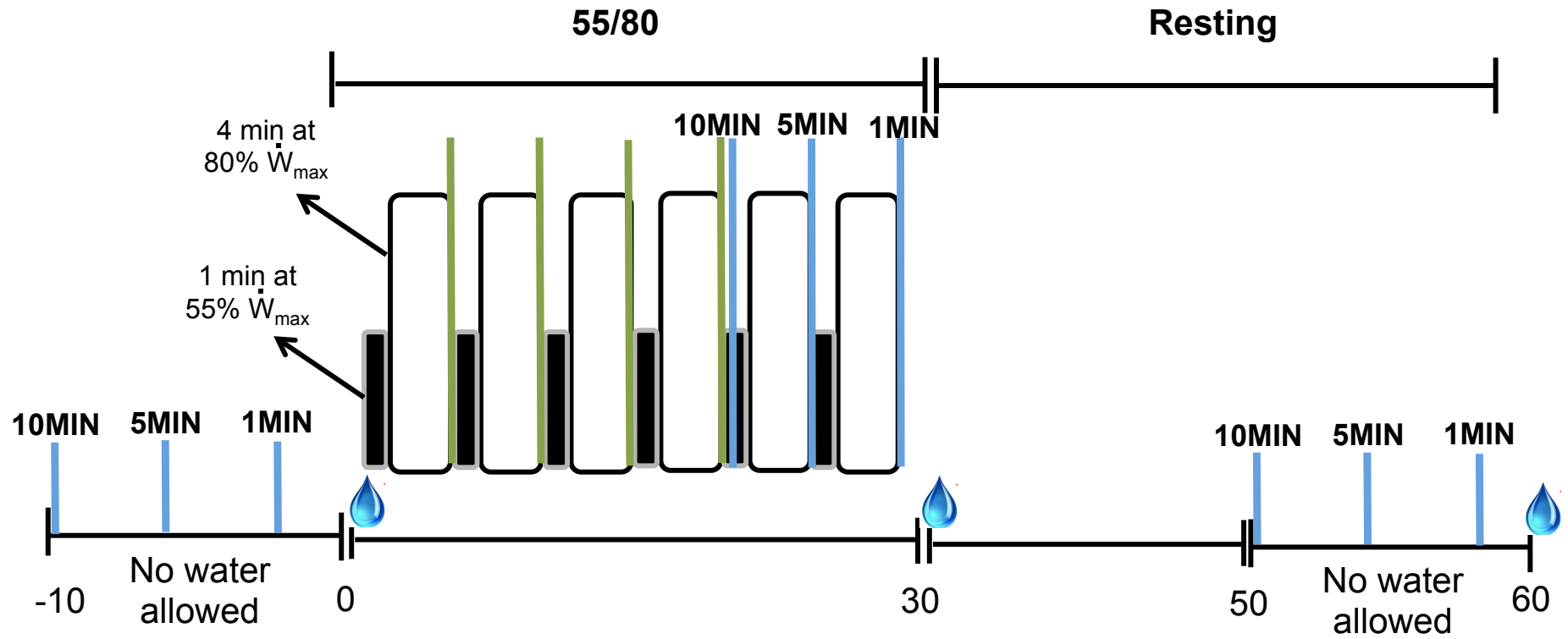
On the first visit to the lab, participants completed an incremental  $\dot{V}O_{2\max}$  test on a manually braked cycle ergometer (Monark 824E, Vansbro, Sweden). The protocol used to determine  $\dot{V}O_{2\max}$  is outlined in detail in 3.2.1. Concisely, the test started at 180 W and workload was increased by 30 W every 3 min until volitional exhaustion and gas samples

## Chapter 4: Effects of Saliva Dilution in Salivary Hormone Values

were collected throughout using a breath-by-breath ergospirometry exercise system (MetaLyzer 3B, Cortex, Leipzig, Germany). Participants' HR and RPE were recorded at the last 15 s of each stage. The  $\dot{V}O_{2\max}$  test was used to determine  $\dot{W}_{\max}$  (as detailed in subchapter 3.3), and consequently to calculate the exercise intensities to be used in the main exercise trials (i.e. 55% and 80%  $\dot{W}_{\max}$ ).

This was a crossover study, and all experimental trials were randomly completed. Saliva samples were collected at three different time points: pre-exercise, post-exercise, and 30 min post-exercise. The exercise test used in this experimental chapter was a continuous, 30-min, high-intensity cycle bout, designed of intercalated blocks of 1 min at 55%  $\dot{W}_{\max}$  and 4 min at 80%  $\dot{W}_{\max}$ . This cycle test is referred to as the 55/80.

All participants abstained from exercise, caffeine and alcohol intake in the 24 h before each trial (i.e. main experimental trials and CTL). The three main exercise trials were identical apart from the timing of water consumption before providing a saliva sample (Figure 4.1). Depending on the trial being completed, participants were required to consume 250 mL of water at either 10 min before every saliva sample (10MIN), 5 min before sampling (5MIN), or 1 min before sampling (1MIN). During completion of the 55/80 bout in the main exercise trials, a set volume of water was provided (100 mL) every 5 min up to and including 20 min. Participants were required to consume each 100 mL of water within 30 s. A 76-statement recovery-stress questionnaire for athletes (RESTQ-76 Sport) (Kellmann and Kallus, 2001) was completed at the start of each main trial to examine the participants' well-being and predisposition to undertake exercise (detailed in 3.8.1).



**Figure 4.1** Schematics of main experimental trial.  
 100 mL of water (green lines)  
 250 mL of water (blue lines)

### 4.2.3 Saliva Collection and Handling

All analytical procedures were described in detail in subchapter 3.6.

### 4.2.4 Statistical Analysis

Descriptive analysis was carried out to observe the central tendency and dispersion of the data. Then, data were checked for normality (Q – Q plots and Shapiro-Wilk tests) and for homogeneity of variance to avoid inaccuracy of the confidence intervals (CI) (Wilcox, 2010). If a data set was not normally distributed, a logarithmic transformation to base 10 was performed on the data and rechecked for normality. Logarithmic data sets were used for the analysis of the responses of salivary cortisol across all trials. When logarithmic data sets were still not normally distributed (salivary testosterone) a non-parametric Friedman's analysis of variance (ANOVA) was used. On finding a main effect, a two-related samples test (Wilcoxon signed-rank test) was used. A factorial (two-way) repeated measures ANOVA (Trial X Time) with Bonferroni adjustments was used to examine the salivary cortisol responses to exercise and *post hoc* tests for multiple comparisons were used. A one-way (Trial) repeated measures ANOVA with Bonferroni adjustments was used to compare the RESTQ-76 Sport questionnaire scores, urine osmolality and body mass, average HR responses and RPE scores during exercise, and hormone responses during CTL. Where Peak Post-Exercise values are presented, the data assume the highest post-exercise values observed. Data are reported as mean  $\pm$  SD. The level of significance was set at  $p < 0.05$ .

## 4.3 Results

Post-hoc tests for observed power analysis were performed and observed power for salivary cortisol and testosterone was 0.288 and 0.898, respectively.

### 4.3.1 Hydration Status

Euhydration was confirmed at the beginning of each experimental trial. The mean urine osmolality for all trials was  $354 \pm 211$  (10MIN),  $274 \pm 201$  (5MIN),  $261 \pm 219$  (1MIN) and  $556 \pm 158$  (CTL) mOsmol·kg<sup>-1</sup> of H<sub>2</sub>O, and did not alter across all trials ( $p > 0.05$ ).

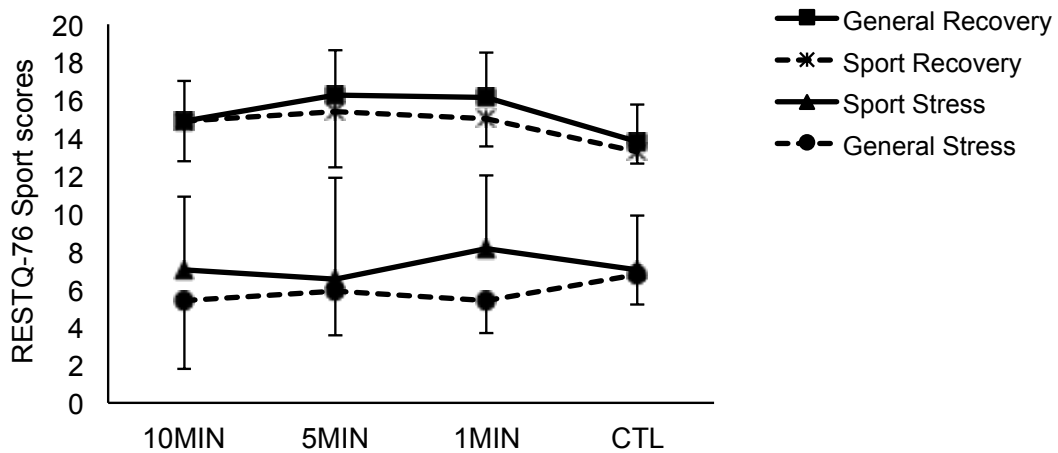
### 4.3.2 Total Weight Loss

Total loss in body mass when corrected for fluid intake was not different across trials ( $p > 0.05$ ) and was  $0.6 \pm 0.2$  kg (10MIN),  $0.7 \pm 0.2$  kg (5MIN) and  $0.5 \pm 0.1$  kg (1MIN).

### 4.3.3 Recovery-Stress Questionnaire

There was no trial effect in any of the 4 main scales, with the responses within each scale being similar across all trials ( $p > 0.05$  in all).





**Figure 4.2** RESTQ-76 Sport scores measured in all trials. Values are means  $\pm$  SD.

#### 4.3.4 Physiological Responses to Exercise

Average HR did not alter across trials ( $p > 0.05$ ) and was  $161 \pm 11$  beats $\cdot$ min $^{-1}$ ,  $163 \pm 11$  beats $\cdot$ min $^{-1}$  and  $161 \pm 14$  beats $\cdot$ min $^{-1}$  in the 10MIN, 5MIN and 1MIN trial, respectively. Moreover, RPE also remained unchanged ( $p > 0.05$ ), with average values of  $15 \pm 1$  in all 3 trials.

#### 4.3.5 Hormonal Responses During CTL

##### a) Salivary Cortisol

A time effect was shown ( $F_{2,3} = 9.558$ ,  $p = 0.05$ ), with follow up comparisons showing that salivary cortisol was lower at 30 min Post-CTL when compared to immediately Post-CTL ( $p = 0.36$ ) (Figure 4.3 A).

**b) Salivary Testosterone**

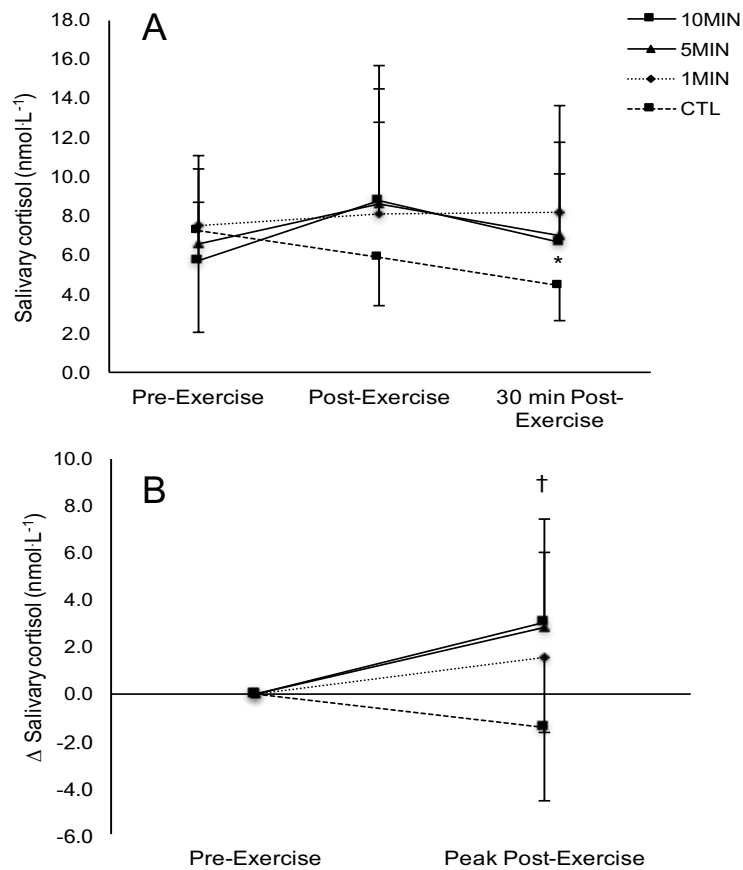
No time effect was shown in salivary testosterone during CTL ( $F_{2,3} = 0.717$ ,  $p = 0.56$ ) (Figure 4.4 A).

**4.3.6 Hormonal Responses to Exercise**

**a) Salivary Cortisol**

No trial effect was found ( $F_{2,8} = 0.953$ ,  $p = 0.43$ ). However, a time effect was shown from Pre- to Peak Post-Exercise ( $F_{2,8} = 14.279$ ,  $p = 0.004$ ), with salivary cortisol elevating from Pre- to Peak Post-Exercise in 10MIN and 5MIN only ( $p < 0.05$  and  $p < 0.01$ , respectively). Average absolute increases from Pre- to Peak Post-Exercise of  $3.01 \text{ nmol}\cdot\text{L}^{-1}$  (~52%),  $2.84 \text{ nmol}\cdot\text{L}^{-1}$  (~43%), and  $1.58 \text{ nmol}\cdot\text{L}^{-1}$  (~21%) were found in 10MIN, 5MIN, and 1MIN, respectively (Figure 4.3 B). No differences were shown when comparing each time point between trials (all  $p > 0.05$ ). The effect size for the salivary cortisol responses to exercise across trials was  $\eta^2 = 0.325$ .

## Chapter 4: Effects of Saliva Dilution in Salivary Hormone Values



**Figure 4.3** A) Salivary cortisol responses during 10MIN, 5MIN, 1MIN and CTL. B) Absolute changes in salivary cortisol from Pre to Peak Post-Exercise.

Values are means  $\pm$  SD.

\*Different than Post-Exercise values in CTL ( $p < 0.05$ ).

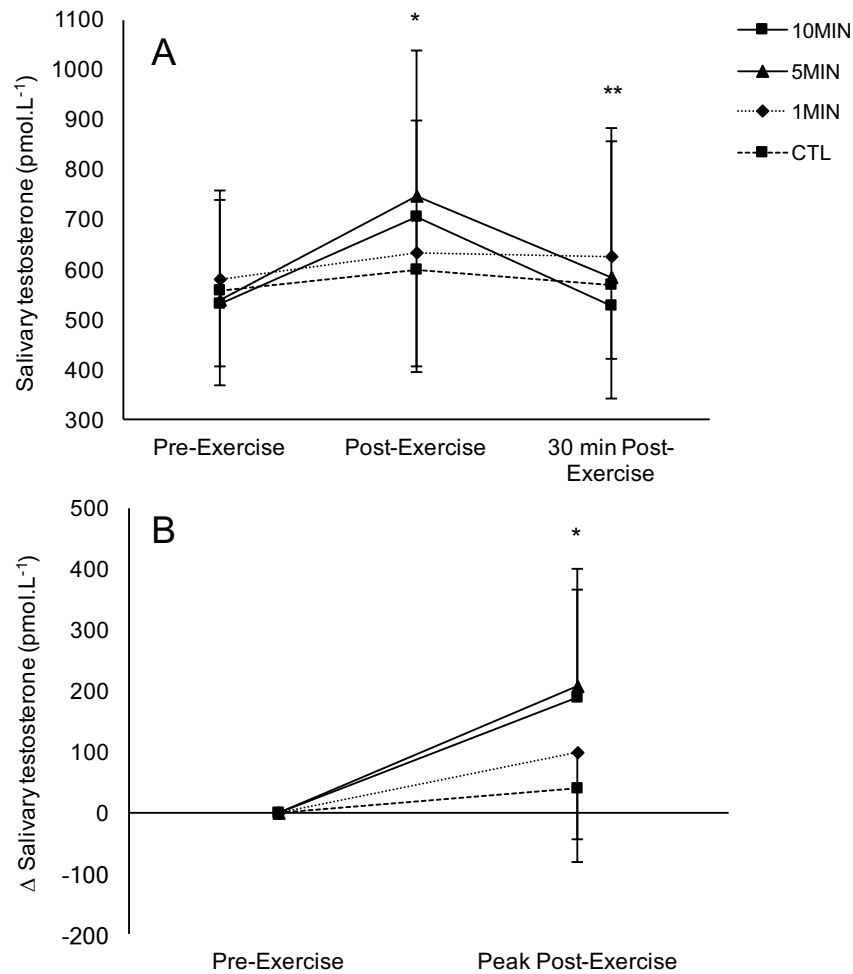
†Different than Pre-Exercise values in 10MIN ( $p < 0.05$ ) and 5MIN ( $p < 0.01$ ).

### b) Salivary Testosterone

A non-parametric Friedman's ANOVA with *post hoc* analysis with Wilcoxon signed-ranked test showed there were no differences when comparing the sample concentrations between trials ( $p > 0.05$  in all). However, a significant elevation was shown from Pre- to Post-Exercise in 10MIN and 5MIN trials ( $p < 0.05$  and  $p < 0.01$ , respectively), with exercise-induced responses returning to baseline levels at 30 min Post-Exercise. Average absolute increases from Pre- to Peak Post-Exercise of  $190 \text{ pmol}\cdot\text{L}^{-1}$  (~36%),  $208 \text{ pmol}\cdot\text{L}^{-1}$  (~39%), and  $99 \text{ pmol}\cdot\text{L}^{-1}$  (~17%) were shown in 10MIN, 5MIN, and 1MIN, respectively (Figure 4.4 B). No differences were shown when

## Chapter 4: Effects of Saliva Dilution in Salivary Hormone Values

comparing each time point between trials (all  $p > 0.05$ ). The effect size for the salivary testosterone responses to exercise across trials was  $\eta^2 = 0.703$ .



**Figure 4.4** A) Salivary testosterone responses during 10MIN, 5MIN, 1MIN and CTL. B) Absolute changes in salivary testosterone from Pre to Peak Post-Exercise. Values are means  $\pm$  SD.

\*Different than Pre-Exercise values in 10MIN and 5MIN ( $p < 0.05$ ).

\*\*Different than Post-Exercise values in 10MIN and 5MIN ( $p < 0.01$ ).

## 4.4 Discussion

This experimental chapter examined if consuming water 5 min and 1 min before saliva sampling would dilute samples leading to erroneously lower salivary cortisol and testosterone concentration values when compared with the recommended 10 min. No trial effect was shown when comparing the saliva cortisol and testosterone concentrations across all three trials. In addition, there are no differences in the concentration levels at each time point between trials. However, salivary cortisol and testosterone significantly elevated in response to the 55/80 in 10MIN and 5MIN only, with acute average absolute-changes of  $3.01 \text{ nmol}\cdot\text{L}^{-1}$  (~52%),  $2.84 \text{ nmol}\cdot\text{L}^{-1}$  (~43%), and  $1.58 \text{ nmol}\cdot\text{L}^{-1}$  (~21%) in salivary cortisol, and  $190 \text{ pmol}\cdot\text{L}^{-1}$  (~36%),  $208 \text{ pmol}\cdot\text{L}^{-1}$  (~39%), and  $99 \text{ pmol}\cdot\text{L}^{-1}$  (~17%) in salivary testosterone, in the 10MIN, 5MIN and 1MIN trials, respectively. In addition, this present study showed no differences in the recovery-stress questionnaire main scales, hydration status, HR or RPE when comparing all trials.

Measurement of salivary cortisol and testosterone have been proposed as a method that could largely replace plasma/serum concentrations of these hormones (Ahn *et al.*, 2007, Putignano *et al.*, 2001). Serum/plasma and salivary cortisol have been shown to be moderately correlated in both a population of 358 healthy individuals (Pearson's  $r = 0.59$ ,  $p < 0.0001$ ) (Ahn *et al.*, 2007) and in 290 women (Pearson's  $r = 0.64$ ,  $p < 0.0001$ ) (Putignano *et al.*, 2001). More recently, a study performed on 12 healthy male individuals reported a strong positive correlation ( $r = 0.81$ ,  $p < 0.05$ ) in the exercise-induced serum and saliva cortisol responses to a maximal treadmill-graded exercise to exhaustion (Powell *et al.*, 2015). The exercise-induced salivary and serum total testosterone to 3 different endurance and resistance exercise sessions have also shown to moderately correlate in endurance trained athletes ( $r = 0.60$ ,  $p < 0.001$ ) (Tanner *et al.*, 2014), but strong positive correlations have been reported in the resting samples in 20 male individuals ( $R^2 = 0.79$ ,  $p < 0.001$ ) (Shirtcliff *et al.*, 2002). However, this positive correlation has not always been observed, with research reporting poor levels of agreement between serum and salivary total and free testosterone concentrations in response to exercise in an aging male

#### Chapter 4: Effects of Saliva Dilution in Salivary Hormone Values

population (Hayes *et al.*, 2015b). However, despite the variability that may be observed across studies between methodological protocols, laboratory procedures and interpretation of results that may in turn, directly influence the salivary hormone data, the non-invasive character of saliva sampling makes it a method that can be practically used by researchers and practitioners who wish to measure these hormones. It is, however, important to take into account the standardisation of sample acquisition, analytical procedures and study designs, and the presence of technical and analytical error as there is still limited consistency between studies that have examined salivary cortisol and testosterone responses as surrogates for its circulating concentrations, as well documented in the Hayes *et al.* (2016) narrative review.

To reduce sample variability, a series of analytical controls should be in place. As salivary cortisol and testosterone vary diurnally, a standardisation of time of sample collection should be confirmed (Sinha-Hikim *et al.*, 2003, Weitzman *et al.*, 1971). In this study, all exercise tests began at 12:00 and sample collection was consistent across all trials at Pre-, immediately Post- (~12:30), and 30 min Post-Exercise (~13:00).

Cortisol is also known to be elevated during and after emotional and psychological stress (Burke *et al.*, 2005, Kirschbaum *et al.*, 1995, Skosnik *et al.*, 2000). As this study is examining and comparing salivary cortisol concentrations across several occasions, it is important to also control for the levels of psychological stress in the participants. The RESTQ-76 Sport questionnaire was completed by all participants before each main trial. Analysis of the questionnaire scores showed no disparities in any of the general or sport-specific stress and recovery scales, which emphasises the participants were in a similar state of wellbeing and predisposition to undertake physical activity at the start of each trial. Furthermore, these data suggest that the hormone responses have not been influenced by a change in psychological wellbeing in the participants.

Cortisol and testosterone concentrations may also change with food consumption (Gibson *et al.*, 1999), alcohol intake (Badrick *et al.*, 2008), smoking (Badrick *et al.*, 2007), caffeine (Lovallo

#### Chapter 4: Effects of Saliva Dilution in Salivary Hormone Values

*et al.*, 2006), and hydration state (Ali *et al.*, 2013, Karila *et al.*, 2008, Maresh *et al.*, 2006), and therefore examination of salivary hormones must follow appropriate controls. Consuming water (~5-7 ml·kg<sup>-1</sup> body mass within the hours prior to exercise will help to achieve euhydration, confirmed by a urine osmolality <700 mOsmol·kg<sup>-1</sup> of H<sub>2</sub>O (Sawka *et al.*, 2007). All participants were in a euhydrated state and abstained from exercise, alcohol and caffeine in the 24 h before, and food intake in the 4 h before each exercise trial. Fluid restriction during exercise has been reported to increase the circulating levels of cortisol (Brandenberger *et al.*, 1986, Francesconi *et al.*, 1985, Maresh *et al.*, 2006). In addition, a study completed on nine recreationally active male individuals has shown that plasma cortisol significantly elevated to a 2-h cycle bout at 65%  $\dot{V}O_{2max}$  (Bishop *et al.*, 2004). These authors reported that these responses were significantly higher when this exercise bout was completed with no fluid consumption, compared to when completed with a fluid (2 mL·kg<sup>-1</sup> body mass of artificially sweetened lemon-flavored water) provided every 15 min during exercise. These data support the importance of adequate hydration status in research examining hormone concentration levels at rest and in response to exercise, as well as an adequate fluid consumption during exercise. Similarly, Lovallo *et al.* (2006) examined the salivary cortisol responses to a 30-min cycle bout at an average 75 W. The authors reported that the exercise bout on its own did not have an influence in salivary cortisol responses. However, when completed after consumption of a 250 mg dose of caffeine, an acute elevation of salivary cortisol levels was observed, and these responses were significantly higher than following a placebo. These results suggest that caffeine intake may have an influence in salivary cortisol, and therefore it is important that studies examining this hormone control for caffeine intake.

Current guidelines propose that fluid consumption should cease at least 10 min before providing a saliva sample. However, this study intended to examine if consuming water during this period, specifically 5 min and/or 1 min before providing a saliva sample would dilute saliva and therefore provide lower, erroneous cortisol and testosterone concentrations. Participants were provided with 650 mL of water to be consumed during each exercise test (30 min in total). This volume was chosen, as guidelines propose that individuals whose body mass is around 90

#### Chapter 4: Effects of Saliva Dilution in Salivary Hormone Values

kg, should drink between 600-800 mL·h<sup>-1</sup> when running at a relatively low intensity (8.5-10 km·h<sup>-1</sup>), in order to avoid losing >2% body mass (Sawka *et al.*, 2007). A weight loss above 2% body mass has been reported to decrease aerobic capacity and mental performance (Cheuvront *et al.*, 2003). In this study, the average body mass of participants was 85.7 ± 9.4 kg. In order for the participants to have lost >2% body mass, average weight loss would have to be approximately 1.7 kg. The average weight loss in the main exercise trials when corrected for fluid intake did not change across trials and was 0.6 ± 0.2 kg (10MIN), 0.7 ± 0.2 kg (5MIN), 0.5 ± 0.1 kg (1MIN). These results suggest that the water consumption plan followed in this study may have been appropriate, as it did not induce a weight loss of above 2% body mass.

In this present study, no trial effect was observed when comparing the exercise-induced salivary cortisol and testosterone concentrations across all three main experimental trials. However, a significant elevation in salivary cortisol was shown in the 10MIN (+ 3.01 nmol·L<sup>-1</sup>) and 5MIN (+ 2.84 nmol·L<sup>-1</sup>) trials only. This acute elevation in salivary cortisol from Pre-Exercise in response to the 55/80 is in line with previous research (Hough *et al.*, 2013, Hough *et al.*, 2011). Detailed examination of the percentage-increase from baseline in all three trials shows that 10MIN (~52%) and 5MIN (~43%) elicited greater hormone responses than 1MIN (~21%, + 1.58 nmol·L<sup>-1</sup>). This may be due to a potential diluted Post-Exercise saliva sample in the 1MIN trial, which may erroneously reduce the real concentration level of cortisol in the sample. However, this percentage-elevation observed in the present study is below the 148% critical difference proposed for salivary cortisol alterations when determined by enzyme-linked immunosorbent assays (ELISA) to be considered meaningful (Hayes *et al.*, 2014). Concisely, critical difference is defined as the smallest change needed for an effect to be considered clinically meaningful, when considering the analytical error and the biological variation (Fraser and Fogarty, 1989). Additionally, Hayes *et al.* (2014) have determined the salivary cortisol and testosterone critical difference when examining the hormone values in laboratory-controlled conditions (as in this present study), which certainly reduces the level of uncontrolled variability when compared with research performed in more naturalistic environments.



#### Chapter 4: Effects of Saliva Dilution in Salivary Hormone Values

In this present study, salivary testosterone acutely increased from Pre-Exercise in the 10MIN and 5MIN trials only, with percentage-increases being higher in both (~36% and ~39%, respectively) when compared with the 1MIN trial (~17%). This acute elevation has also been reported by previous research using the 55/80 as the exercise tool to induce salivary testosterone changes either at 10 min Post-Exercise (Hough *et al.*, 2011) or immediately Post-Exercise (Hough *et al.*, 2013, Hough *et al.*, 2015). The critical difference for salivary testosterone as proposed by Hayes *et al.* (2014) is 90%, which again is greater than the percentage-increase observed in all three main trials. Additionally, Hayes *et al.* (2014) suggested that hormone measures in saliva may present a greater critical difference when compared with circulating levels, and that, therefore, may also be more inheritably variable, reducing the applicability in clinical diagnostics. It has been reported that salivary cortisol and testosterone may show great variability (Valero-Politi and Fuentes-Arderu, 1993; Stone *et al.*, 2001; Hayes *et al.*, 2012). Besides the variation that may be attributed to analytical and biological error, the statistically unaltered response of salivary testosterone to the 55/80 in 1MIN presented in this study may also be due to diluted saliva samples in the Post-Exercise sample. Previous research has reported an ~49% reduction in salivary testosterone in samples collected by passive drool 10 min after rinsing the mouth with water with no alteration in the cortisol levels, compared to samples collected upon rising before eating, drinking or performing any oral hygiene (Whembolua *et al.*, 2006). In the 1MIN trial, participants consumed 250 mL of water 1 min before providing a saliva sample. Although there is no trial effect when comparing all three exercise trials in both salivary cortisol and testosterone responses and no differences were observed in the Pre-Exercise and 30 min Post-Exercise samples a lower exercise-induced absolute change in both hormones concentrations in the 1MIN trial may be indicative of the inappropriateness of consuming water within such a short period before sampling. Consuming water 5 min before saliva sampling has not been shown to elicit significantly different salivary cortisol and testosterone concentrations than when ceasing water consumption within the recommended 10 min (i.e. 10MIN trial). This suggests that perhaps consuming water within 10 min or less (up to 5 min before providing a saliva sample) may not dilute saliva, and that the

#### Chapter 4: Effects of Saliva Dilution in Salivary Hormone Values

obtained hormone concentration will not be invalidly lower. Therefore, it may be suggested that the current 10 min barrier for ceasing water consumption could perhaps be reduced to 5 min.

No studies have previously examined the influence of water consumption within the recommended 10 min before sample collection on the salivary hormone responses. Analysis of the results presented in this chapter suggest that allowing water consumption for up to 5 min before saliva sampling does not appear to influence the salivary cortisol and testosterone concentrations at rest and in response to exercise. However, further examination of the influence of consuming water 5 min before providing a saliva sample is required and therefore, the subsequent chapters where saliva sampling methods are used will follow the recommended guidelines for ceasing water consumption 10 min before providing a saliva sample.

## **CHAPTER 5.**

# **The Reproducibility of Salivary Cortisol and Testosterone Responses to the 55/80 Bout**

## 5.1 Introduction

Symptoms of maladaptation associated with overreaching (FOR and NFOR) and OTS have been shown to occur in an athlete's career, with prevalence reported to range between 35% and 67% in elite distance runners (Morgan *et al.*, 1987), adolescent swimmers (Raglin *et al.*, 2000), and athletes from 26 different individual and team-sports (Birrer *et al.*, 2013, Kenttä *et al.*, 2001).

The 55/80, developed by Hough *et al.* (2011) has been proposed as a tool that can elicit an acute elevation in cortisol and testosterone when in a healthy state. Further to this Hough *et al.* (2013) reported blunted responses of salivary cortisol (by 166%) and testosterone (by 21%) to the 55/80 when comparing before to after an 11-day period of intensified aerobic training in active male individuals. A similar study with an elite level male triathlete population has shown a 44% blunted exercise-induced (to the 55/80 bout) response of salivary testosterone concentrations after a 10-day training camp (Hough *et al.*, 2015). These results suggest that there may be some promise in using the 55/80 bout as a tool to highlight blunted exercise-induced responses of cortisol and testosterone when an individual is in a state of overreaching (FOR/NFOR) or OTS. However, if this tool is to be used to highlight hormonal alterations when in an overreached/OTS state, then the reproducibility of the hormonal responses (specifically salivary cortisol and testosterone) to this cycle bout when in a healthy state is required.

Examining the reliability of a variable involves repeating the measurements on a number of occasions and examining the magnitude of the variation in those variables within an individual (Bagger *et al.*, 2003, Ross and Fraser, 1993). Reliability may be relative or absolute, depending on the measurement test being used. Intraclass correlation coefficient (ICC) is often proposed as a measurement of relative reliability, as it is a measurement of the individual variability relative to the inter-individual variability (Atkinson and Nevill, 1998, Šerbetar, 2015). As ICC includes this inter-individual variability it is dependent on sample heterogeneity (high inter-

## Chapter 5: Reproducibility of Salivary Hormones to the 55/80 Cycle

individual variability will generate a high ICC) (Shrout and Fleiss, 1979, Vincent and Weir, 2012). Therefore, ICC levels may be important to reflect the ability of a test to differentiate between subjects (McGraw and Wong, 1996). Guidelines propose that if an ICC value is found between 0.40 and 0.59, the level of correlation is considered to be fair, and if between 0.60 and 0.74, the correlation is good (Cicchetti, 1994, Cicchetti and Sparrow, 1981). Cicchetti (1994) and Cicchetti and Sparrow (1981) also propose that ICCs of or above 0.75 suggest an excellent correlation. However, ICC is not particularly informative for practitioners who wish to make inferences about single individuals from a test result (Vincent and Weir, 2012) and moreover, a high ICC may be erroneously interpreted when examining the reproducibility (within-subject variability) of the responses to a test. Therefore, it is important that an absolute measure of reliability is used. Absolute reliability refers to the variability observed in a measure when examined on repeated occasions, within an individual. Therefore, calculation of the within-subject variation may be used as a measure of absolute reliability, as it examines how comparable the responses of a variable to a test are when examined on a number of occasions within an individual (i.e. reproducibility of the measure) (Hopkins, 2000, Šerbetar, 2015). As it refers to the variation in a certain variable when one individual is tested on several occasions, the within-subject variation has been proposed as the ideal measurement of reproducibility for experimenters using exercise tools to monitor the performance/health status of athletes/patients (Hopkins, 2000). Determination of coefficients of variation (CV) is often used as a measure of the reproducibility of measurements in clinical trials and biochemistry assays (such as hormonal analysis) (Feltz and Miller, 1996, Strike, 1991). Atkinson and Nevill (1998) propose that an extension of this measure on a sample of individuals is to determine the mean CV from individual CVs. Therefore, determination of intra-individual coefficient of variation ( $CV_i$ ) may be used to measure the within-subject variation, as it establishes the differences in the amount by which the variables being measured vary within each individual (Sokal and Braumann, 1980). However, Atkinson and Nevill (1998), Hopkins (2000) and Šerbetar (2015) recommended that any reliability study should include more than one measurement of reliability, with focus on the use of a method of absolute reliability. In this present Chapter the use of  $CV_i$  was chosen. In addition, Atkinson and Nevill (1998) and Hopkins (2000) have also recommended the inclusion

## Chapter 5: Reproducibility of Salivary Hormones to the 55/80 Cycle

of ICC as a measure of relative reliability, as it provides information on the between-subject variability. For this reason, this current study will also utilise measurements of ICC.

Following the findings from Hough *et al.* (2013) and Hough *et al.* (2015) confirming the reproducibility of salivary cortisol and testosterone to the 55/80 may corroborate the utility of this exercise test as a suitable tool to be used when highlighting any salivary cortisol and testosterone alterations that may occur during an overreached/OTS period. Therefore, the aim of this study is to examine the reproducibility of the physiological responses and the salivary cortisol and testosterone responses to the 55/80. It is hypothesized that HR and RPE to the 55/80 will not differ across trials, and that both hormones will acutely elevate to the 55/80. It is also hypothesized that the HR, RPE and hormonal responses will be reproducible.

## 5.2 Methods

### 5.2.1 Procedures

This study was a randomized, crossover designed experiment and 11 recreationally active male individuals volunteered to take part. The participants' anthropometric and physiological characteristics at baseline are outlined in Table 5.1. Each participant came to the University of Bedfordshire Sports Sciences Human Performance Laboratories on 5 different occasions separated by at least 7 days, to undertake a  $\dot{V}O_{2\max}$  test and subsequent measurement of individual physiological characteristics, 3 main experimental trials and one resting control trial were completed.

**Table 5.1** Participants' anthropometric and physiological characteristics at baseline.

	<b>Mean <math>\pm</math> SD</b>
Age (years)	21 $\pm$ 2
Height (cm)	178 $\pm$ 4
Body mass (kg)	80.8 $\pm$ 12.2
HR <sub>max</sub> (beats·min <sup>-1</sup> )	188 $\pm$ 6
$\dot{V}O_{2\max}$ (mL·kg <sup>-1</sup> ·min <sup>-1</sup> )	45 $\pm$ 3
$\dot{W}_{\max}$ (Watts)	266 $\pm$ 24
n = 11	

A  $\dot{V}O_{2\max}$  test was completed on the first visit to the laboratories. This was used as the preliminary measurement trial, and it was completed within 3-5 days before the start of the main experimental trials. The  $\dot{V}O_{2\max}$  test was a continuous, incremental test on an electronically braked cycle ergometer (Lode, Groningen, The Netherlands) as detailed in 3.2.1. In brief, the test started at 180W with power output increased by 30W every 3 min until volitional exhaustion.

## Chapter 5: Reproducibility of Salivary Hormones to the 55/80 Cycle

Expired gas was analysed continuously via a breath-by-breath ergospirometry exercise testing system (MetaLyzer 3B, Cortex, Leipzig, Germany). Exercise intensities for the 55/80 used in the main experimental trials were obtained from the determination of 55% and 80% of  $\dot{W}_{max}$  of each individual.

Each main experimental trial followed the same protocol, however 1 of the experimental trials (CTL) did not contain any exercise session. Participants reported to the laboratory ~30 min before the exercise bout start time (i.e. 11:30). Hydration status (by measuring urine osmolality), body mass measurement, and completion of the RESTQ-76 recovery-stress questionnaire were completed. The 55/80 cycle bout was used in the main exercise trials and was started at the same time of day (12:00) to avoid diurnal variation influencing the hormonal concentrations being examined. Saliva samples were collected pre-exercise, immediately post-exercise, and 30 min post-exercise. A standard breakfast chosen by each participant was consumed 4 hours before the exercise testing began and was replicated before each main trial. Each participant was requested to drink at least ~500 mL of water during that period to help to ensure a euhydrated state on arrival at the laboratory.

Participants remained fasted until the end of each main experimental trial, but were allowed to drink water *ad libitum* throughout, except within the 10 min period before saliva sampling. HR and RPE were recorded at the last 15 s of each stage. Participants were asked to undertake their normal weekly training in between each main experimental trial to avoid training load alterations influencing the responses of the hormones examined in the current study. The participants abstained from exercise, caffeine and alcohol intake and were also requested to consume a diet as similar as possible in the 24 h leading up to each main experimental trial. Instructions on weighing and recording their food intake were given individually. Mean energy intake 24 hours prior to each trial was  $9661 \pm 3050$  kJ ( $2309 \pm 729$  kcal), and carbohydrate ( $55\% \pm 12\%$ ), fat ( $27\% \pm 12\%$ ), and protein ( $18\% \pm 6\%$ ) consumed (Dietplan version 6.70.74, Forestfield Software, West Sussex, UK) were determined.



## 5.2.2 Analytical Procedures

Saliva collection and handling, and analysis procedures are described in Chapter 3, section 3.6.1 and 3.6.2, respectively.

## 5.2.3 Statistical Analysis

Data were checked for normality (Shapiro-Wilk test) prior to further statistical analysis. If a data set was not normally distributed, a logarithmic transformation (log base 10) was performed on the data and rechecked for normality. Logarithmic data sets were used for salivary cortisol analysis only. When data sets were still not normally distributed (salivary testosterone was the only data set not normally distributed) a non-parametric Friedman's ANOVA was used. On finding an effect, *post hoc* Wilcoxon signed-rank tests were used on the original data set. A factorial (two-way - Trial x Time) repeated measures ANOVA was used to analyse salivary cortisol values. For all comparisons where the assumption of sphericity was violated, Greenhouse-Geisser corrections were used. The RESTQ-76 Sport scores were analysed by running a one-way repeated measures ANOVA with Bonferroni adjustments. A one-way (Trial) repeated measures ANOVA was used to compare average HR responses and RPE scores during exercise and hormone responses during CTL. The  $CV_i$  were presented as a percentage and were used to examine the reproducibility of the physiological and hormone responses to the 55/80 bout.  $CV_i$  were calculated by hand using the equation  $CV_i = (SD_t/\bar{X}_t)*100$ , where  $CV_i$  is the intra-individual coefficient of variation,  $SD_t$  is the standard deviation of the hormone responses to all 3 main experimental trials averaged, and  $\bar{X}_t$  is the average of the hormone concentrations at Pre-, Post- and 30 min Post-Exercise in all 3 main experimental trials averaged (Sale, 1991). The ICC used was a two way model, based on the examination of single measures, and will therefore be referred to as ICC (2,1). This ICC model includes both systematic and random error in its equation. The alpha significance was set as  $p < 0.05$ . Data are reported as mean  $\pm$  standard deviation (SD).

## 5.3 Results

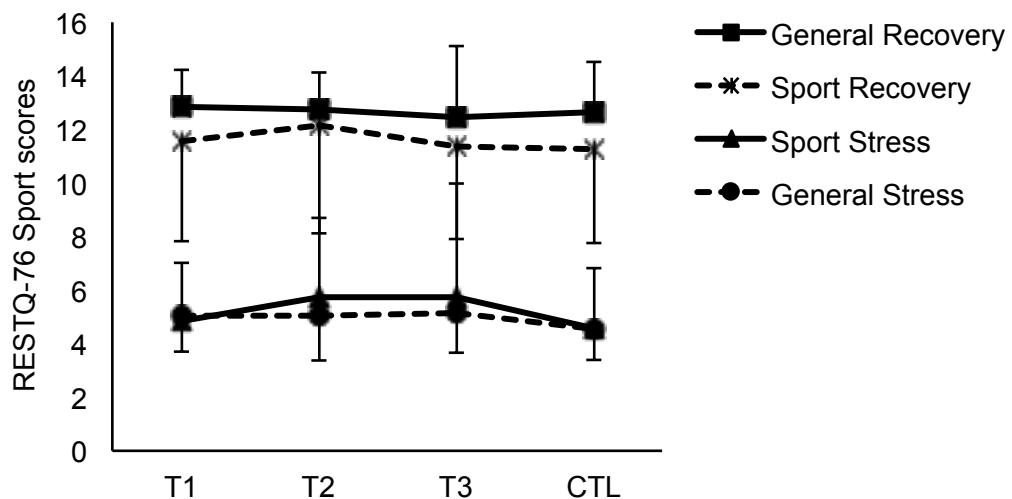
Post-hoc power analysis demonstrated that the observed power for salivary cortisol and testosterone were 0.212 and 1.000, respectively.

### 5.3.1 Hydration Status

The hydration status of the participants did not alter across all trials ( $p > 0.05$ ) and mean urine osmolality was  $357 \pm 121$  mOsmol·kg<sup>-1</sup> of H<sub>2</sub>O.

### 5.3.2 Recovery-Stress Questionnaire

Analysis of the RESTQ-76 Sport showed no significant disparities in any of the four main stress or recovery scales.



**Figure 5.1** RESTQ-76 Sport scores measured in all trials. Values are means  $\pm$  SD.

### 5.3.3 Physiological Responses to Exercise

No trial effect was shown for HR ( $F_{2,9} = 3.537$ ,  $p = 0.07$ ) or RPE ( $F_{2,9} = 1.522$ ,  $p = 0.27$ ). Average HR in T1, T2 and T3 was  $169 \pm 3$  beats·min<sup>-1</sup>,  $167 \pm 2$  beats·min<sup>-1</sup> and  $169 \pm 3$  beats·min<sup>-1</sup>, respectively. The RPE scores were  $15 \pm 1$  in T1,  $15 \pm 1$ , in T2, and  $14 \pm 2$  in T3. The CV<sub>i</sub> for HR and RPE was  $2 \pm 1\%$  and  $5 \pm 5\%$ , respectively.

### 5.3.4 Hormonal Responses During CTL

#### a) Salivary Cortisol

A time effect was shown for salivary cortisol during CTL ( $F_{2,9} = 30.386$ ,  $p < 0.001$ ). *Post hoc* analysis indicates that salivary cortisol decreased over time with Post- and 30 min Post-CTL being lower than Pre-CTL (both  $p < 0.01$ ) (Figure 5.2).

#### b) Salivary Testosterone

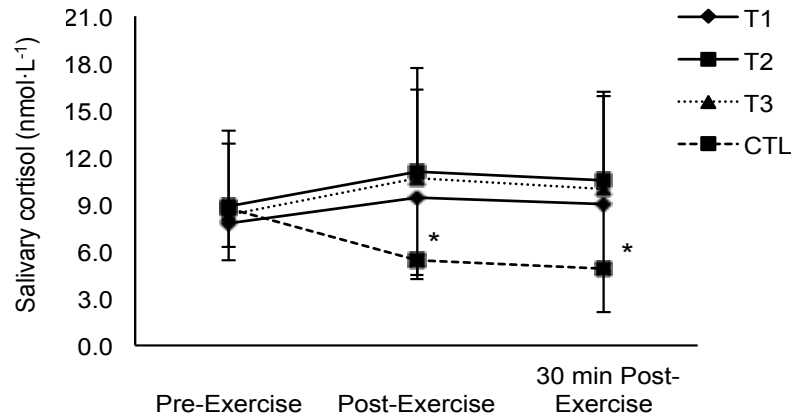
Salivary testosterone did not alter during the CTL trial ( $F_{2,9} = 0.504$ ,  $p = 0.62$ ) (Figure 5.4).

### 5.3.5 Hormonal Responses to Exercise

#### a) Salivary Cortisol

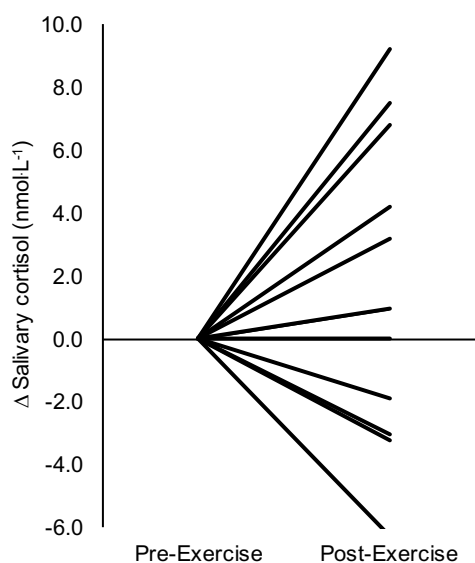
No trial effect ( $F_{2,9} = 1.428$ ,  $p = 0.29$ ) or time effect ( $F_{2,9} = 0.134$ ,  $p = 0.88$ ) was found when comparing T1, T2 and T3. However, although not significant, average percentage-increases

from Pre- to Post-Exercise of 29%, 44% and 38% were found in T1, T2 and T3, respectively (Figure 5.2). The effect size for the salivary cortisol responses to exercise across trials was  $\eta^2 = 0.222$ .



**Figure 5.2** Salivary cortisol responses to the 55/80 at Pre-, Post- and 30 min Post-Exercise. Values are means  $\pm$  SD. \*Different from Pre-Exercise values in CTL ( $p < 0.01$ ).

There was an individual nature to the salivary cortisol responses to the 55/80 bout with exercise-induced percentage-changes ranging from -58% (-4.9 nmol·L<sup>-1</sup>) to +228% (+12.1 nmol·L<sup>-1</sup>) (Figure 5.3). The CV<sub>i</sub> for salivary cortisol across all exercise trials was 20  $\pm$  11%. ICC values are presented in Table 5.2.

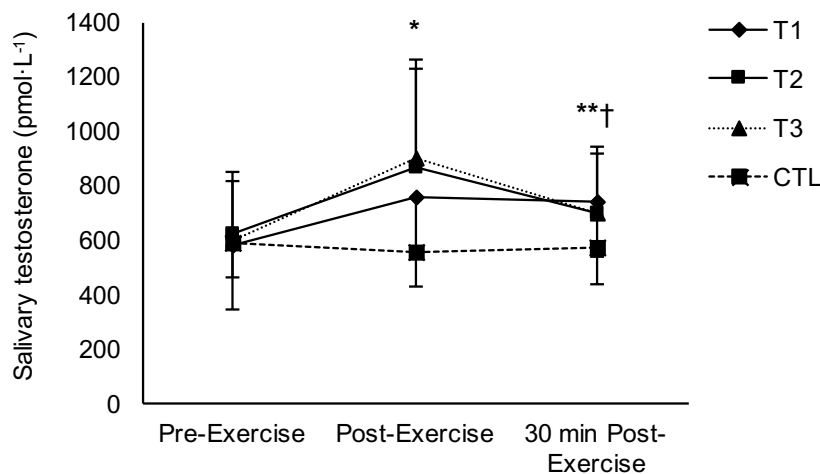


**Figure 5.3** Individual average absolute change in salivary cortisol (nmol·L<sup>-1</sup>) from Pre- to Post-Exercise across all trials.

### b) Salivary Testosterone

A non-parametric Friedman's ANOVA with *post hoc* analysis via a Wilcoxon signed-ranked test showed no effect of trial, as there were no differences when comparing each time point between trials ( $p > 0.05$  in all). However, a robust exercise-induced elevation from Pre- to Post-Exercise was shown in all exercise trials ( $Z = -2.803$ ,  $p = 0.005$  in all) (Figure 5.4 B).

Average absolute-change from Pre- to Post-Exercise in T1, T2 and T3 was  $+171.5 \pm 154.9$   $\text{pmol}\cdot\text{L}^{-1}$  (+34%),  $+239.4 \pm 233.5$   $\text{pmol}\cdot\text{L}^{-1}$  (+35%), and  $+301.8 \pm 127.3$   $\text{pmol}\cdot\text{L}^{-1}$  (+53%), respectively. Individual absolute changes range from  $56.3$   $\text{pmol}\cdot\text{L}^{-1}$  to  $508$   $\text{pmol}\cdot\text{L}^{-1}$  (Figure 5.5). The  $\text{CV}_i$  for salivary testosterone across all exercise trials was  $10 \pm 5\%$ . ICC values are presented in Table 5.2. The effect size for the salivary testosterone responses to exercise across trials was  $\eta^2 = 0.892$ .



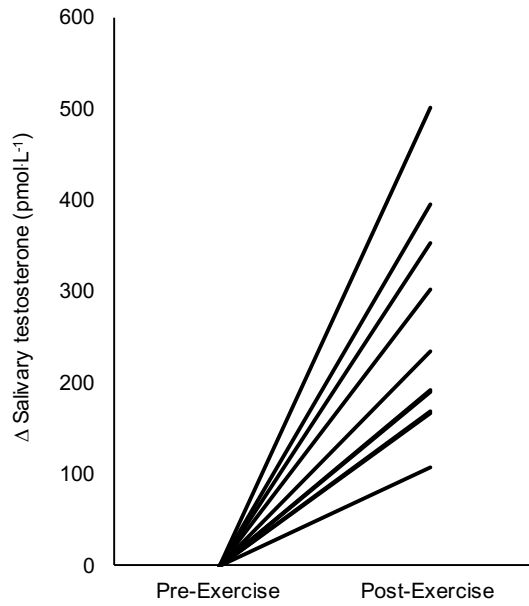
**Figure 5.4** Salivary testosterone responses to the 55/80 at Pre-, Post- and 30 min Post-Exercise.

Values are means  $\pm$  *SD*.

\*Different from Pre-Exercise values in all trials ( $p < 0.01$ ).

\*\*Different from Pre-Exercise values in T2 and T3 ( $p < 0.05$ ).

† Different from Post-Exercise values in T2 and T3 ( $p < 0.05$ ).



**Figure 5.5** Individual average absolute change in salivary testosterone (pmol·L<sup>-1</sup>) from Pre- to Post-Exercise across all trials.

**Table 5.2** CV<sub>i</sub> and ICC values for the physiological and hormonal variables.

	HR	RPE	Salivary cortisol	Salivary testosterone
CV <sub>i</sub>	2 ± 1 %	5 ± 5%	20 ± 11%	10 ± 5%
ICC	0.873	0.565	0.479	0.627

CV<sub>i</sub> = Intra-individual coefficient of variation; ICC = Intraclass correlation coefficient, model 2,1. Values are means ± SD.

## 5.4 Discussion

This study aimed to examine the reproducibility of the salivary cortisol and testosterone responses to the 55/80 cycle bout. The  $CV_i$  were used as a measure of absolute reliability and these were shown to be  $20 \pm 11\%$  and  $10 \pm 5\%$  for salivary cortisol and testosterone, respectively. ICC values for these hormones were 0.479 (salivary cortisol) and 0.627 (salivary testosterone). Salivary cortisol did not elevate in response to the 55/80, and these exercise-induced responses were shown to have an individualised nature. In contrast, exercise-induced salivary testosterone acutely elevated to the 55/80 immediately Post-Exercise in all three trials. Salivary testosterone decreased from Post-Exercise to 30-min Post-Exercise, but remained elevated when compared to baseline in T2 and T3, but not in T1. The variables of HR and RPE did not alter when compared across all trials and have been shown to be reproducible, with a  $CV_i$  of  $2 \pm 1\%$  and  $5 \pm 5\%$ , respectively. ICC (2,1) for these variables was 0.873 and 0.565, respectively. Euhydration, measured via urine osmolality, was confirmed and it was not different across all experimental trials. The responses to the RESTQ-76 Sport questionnaire were also unaltered during the study.

In studies measuring the reproducibility of any variable there is a requirement to have some control measures in place. Plasma cortisol has been reported to elevate when an individual is in a hypohydrated state. Francesconi *et al.* (1985) and Maresh *et al.* (2006) have reported elevations in plasma cortisol when comparing individuals in a hypohydrated state to a euhydrated state. More specifically, Francesconi *et al.* (1985) found an increase of ~155% in plasma cortisol, elevating from  $14.2 \mu\text{g}\cdot\text{mL}^{-1}$  (euhydrated) to  $36.2 \mu\text{g}\cdot\text{mL}^{-1}$  (hypohydrated by 7% body mass) in a group of six adult healthy males. Maresh *et al.* (2006) supported this finding with an elevation of ~25% in plasma cortisol (increased from  $\sim 400 \text{ nmol}\cdot\text{L}^{-1}$  to  $\sim 500 \text{ nmol}\cdot\text{L}^{-1}$ ) when comparing individuals in a euhydrated state with a hypohydrated state (hypohydrated by 5% body mass). It must be noted that Maresh *et al.* (2006) also examined the influence of hydration status on plasma testosterone concentrations and did not observed any effect of

## Chapter 5: Reproducibility of Salivary Hormones to the 55/80 Cycle

hydration status on plasma testosterone. This finding was supported by Hoffman and colleagues who reported that pre-exercise moderate levels of hypohydration (-3.8% body mass) did not influence the exercise-induced or resting concentrations of plasma testosterone when compared to a euhydrated state (Hoffman *et al.*, 1994). These findings suggest that testosterone may not be sensitive to changes in hydration status. Taken this information as a whole emphasizes the importance of a euhydrated state when examining cortisol concentrations. In this current study the participants consumed at least 500 mL of water within the hours preceding the main experimental trials in order to ensure a state of euhydration (confirmed by a urine osmolality measurement of below 700 mOsmol·kg<sup>-1</sup> H<sub>2</sub>O) (Sawka *et al.*, 2007). In this present study, mean urine osmolality was 357 ± 121 mOsmol·kg<sup>-1</sup> H<sub>2</sub>O, confirming that hydration status has not impacted the results reported in this current study. In addition, as detailed in Chapter 4, caffeine intake has also been reported to influence cortisol concentrations (Lovallo *et al.*, 2006). In this present study, caffeine consumption was not allowed in the 24 h leading up to each main trial. Additionally, cortisol is known to be elevated during and after emotional and psychological stress (Burke *et al.*, 2005, Kirschbaum *et al.*, 1995, Skosnik *et al.*, 2000). Therefore, on the morning of each main trial, participants completed a RESTQ-76 recovery-stress questionnaire. High scores of general and sport recovery in the RESTQ-76 Sport denote an increased sense of well-being, whereas lower scores of general and sport-specific stress emphasize a better predisposition to undertake physical activity (Kellmann and Kallus, 2001). Analysis of the questionnaire scores in this current study highlight no significant alterations in any of the recovery or stress scales when compared across all main experimental trials. These findings suggest that any alterations seen in the hormonal concentration values have not been influenced by a change in psychological and emotional wellbeing, and it also indicates that participants were in a similar state of wellbeing at the start of each main experimental trial.

Cortisol is known to have a high intra-individual variability, with research reporting that 78% of total variation in the cortisol awakening response is due to intra-individual variability (Almeida *et al.*, 2009). As stated in the introduction in this chapter, the within-subject variation is considered



## Chapter 5: Reproducibility of Salivary Hormones to the 55/80 Cycle

an absolute measurement of reproducibility, as it gives an indication of the magnitude of change in a variable examined on several occasions within an individual (Hopkins, 2000, Šerbetar, 2015). This current study reports a ~20% and ~10%  $CV_i$  in the salivary cortisol and testosterone concentration levels, respectively. At first examination, these results may seem high, however, this finding is in line with previous research, which reported  $CV_i$  values of 22% and 13% in resting circulating cortisol and testosterone in healthy male individuals, respectively (Maes *et al.*, 1997). These data suggest that the variability observed in this present study in salivary cortisol and testosterone fall within the intra-individual variation associated with these hormones. Therefore, the  $CV_i$  obtained in this current study may indicate that the salivary cortisol and salivary testosterone responses to the 55/80 when completed on several occasions in a similar state of wellbeing will be reproducible and within the normal variability associated with these hormones. In addition, the ~20% (salivary cortisol) and ~10% (salivary testosterone)  $CV_i$  reported in this study are lower than the 166% and 21% blunted response of these hormones observed in active men following a period of intensified training, as reported by Hough *et al.* (2013). These blunted responses reported to occur after a training period in Hough *et al.* (2013), were considerably higher than the intra-individual variability associated with the salivary cortisol and testosterone responses when in a healthy state. Therefore, this suggests that a period of intensified training is likely to alter the exercise-induced salivary cortisol and testosterone responses to a magnitude above the intra-individual variability observed when in a healthy state, and therefore these blunted responses may be observed as an effect of exercise training stress.

The ICCs obtained in this study for salivary cortisol and testosterone were 0.479 and 0.627, respectively. It is important to remember that ICCs are a measure of relative reliability as it is a ratio of the between-subject variability to the within-subject variability, and therefore the magnitude of ICC is dependent on the between-subject variability (Hopkins, 2000, Shrout and Fleiss, 1979). Thus, the ICCs presented in this current study may provide an indication of the variability in the hormone responses amongst the individuals who completed this study. As ICCs are dependent on sample heterogeneity, high ICCs may indicate a greater variability between

## Chapter 5: Reproducibility of Salivary Hormones to the 55/80 Cycle

the individuals examined (Šerbetar, 2015, Vincent and Weir, 2012). The average between-subject variability calculated by CV in response to the 55/80 was seen to be high in this current study at  $55.4 \pm 2.2\%$  (salivary cortisol) and  $46.8 \pm 18.1\%$  (salivary testosterone).

Hough *et al.* (2011) and Hough *et al.* (2013) have reported a significant increase in salivary cortisol in response to the 55/80 of approximately  $6 \text{ nmol}\cdot\text{L}^{-1}$  (120%) and  $11 \text{ nmol}\cdot\text{L}^{-1}$  (210%), respectively. This present study was not able to replicate the findings from Hough *et al.* (2011) and Hough *et al.* (2013), as salivary cortisol did not acutely elevate in response to the 55/80 in any of the trials. However, there was an average percentage-change in salivary cortisol from Pre- to Post-Exercise of 29%, 44% and 38% in T1, T2 and T3, respectively. In an attempt to explain this unexpected finding, the hormonal responses in the CTL need to be examined. During the CTL trial a significant decrease of 35% in salivary cortisol was seen from Pre- to Post-CTL, and it decreased further within the following 30-min. Peak concentrations of cortisol occur shortly after awakening and start decreasing from this point forward until it reaches its nadir concentrations during the first hours of night sleep (Born *et al.*, 1999, Van Cauter, 1990, Weibel *et al.*, 1995). The response of the saliva cortisol values suggest that the circadian rhythm has led to the reduction in saliva cortisol reported during the CTL trial. Yet, individual exercise-induced responses of salivary cortisol were varied. These individual responses range from  $-4.9 \text{ nmol}\cdot\text{L}^{-1}$  to  $+12.1 \text{ nmol}\cdot\text{L}^{-1}$ . Davies and Few (1973) have reported that exercise of short-duration (20 to 30 min) at an intensity of or above  $60\% \dot{V}O_{2\text{max}}$  will induce an acute elevation in circulating cortisol levels. In addition, Hough *et al.* (2011), Hough *et al.* (2013) and Hough *et al.* (2015) have all reported an acute elevation in salivary cortisol in response to the 55/80. With this in mind, it was hypothesized from the findings in this present study that the contrasting individual responses to the 55/80 could be due to the different fitness levels of the participants. Participants in Hough *et al.* (2011) and Hough *et al.* (2013) presented an average  $\dot{V}O_{2\text{peak}}$  of  $\sim 52 \text{ mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ . However, the participants in this present study show considerably lower  $\dot{V}O_{2\text{max}}$  levels ( $45 \pm 3 \text{ mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ ). The data from this present study may suggest that perhaps athletes with lower fitness levels may have a dampened exercise-induced cortisol

## Chapter 5: Reproducibility of Salivary Hormones to the 55/80 Cycle

activity, however further examination of this anecdotal relationship needs to be completed. Yet, a study done on 82 male individuals of which 32 were endurance athletes and 50 were untrained individuals has reported contrasting results (Virus *et al.*, 1992). All participants (both athletes and untrained) in Virus *et al.* (1992) cycled for 2 hours at 60%  $\dot{V}O_{2max}$ . The authors reported a high between-subject variability in the exercise-induced plasma cortisol responses with only half of the participants, regardless of training status, showing an increase in plasma cortisol concentrations to exercise, whereas the other half did not respond to it. A study by Hough *et al.* (2015) completed in elite triathletes was not able to replicate the blunted cortisol responses following a period of intensified training found by Hough *et al.* (2013). Hough *et al.* (2015) has reported no alterations in the exercise-induced salivary cortisol responses after a 10-day training camp. The authors proposed that these unaltered responses found in cortisol concentrations when compared before and after an intensified training period were perhaps due to a low number of participants ( $n = 7$ ) or the ability of the elite triathletes to cope with the intensification of the training sessions. The findings from this present study suggest that perhaps the unpredictable/individualised nature of cortisol (a between-subject variability of 55.4%) may be a reasonable explanation for Hough *et al.* (2015) being unable to highlight a blunted response in the elite triathlete population. This may be supported by the findings from Virus *et al.* (1992) who have reported a high between-subject variability in cortisol, with only half of the participants showing an elevation in cortisol to a 2-h cycle bout at 60%  $\dot{V}O_{2max}$ .

Conversely, in this study salivary testosterone acutely elevated in response to the 55/80 in all main exercise trials by 34%, 35% and 53% in T1, T2 and T3, respectively. This is in line with other studies that have reported significant exercise-induced elevations in salivary testosterone to the 55/80 in the magnitude of 53% and 35% in normal trained male individuals (Hough *et al.*, 2013, Hough *et al.*, 2011) and of 114% in an elite male triathlete population (Hough *et al.*, 2015). Hough *et al.* (2013) have previously suggested that the 55/80 may be a useful tool to highlight blunted salivary cortisol and testosterone responses when an individual is overreached. The blunted salivary cortisol and testosterone responses reported by Hough *et al.* (2013) were in the magnitude of 166% and 21% when compared to pre-training responses,

## Chapter 5: Reproducibility of Salivary Hormones to the 55/80 Cycle

respectively. However, Hough *et al.* (2015) have suggested for the first time that exercise-induced salivary testosterone responses are more sensitive to an intensification of training stress than the exercise-induced salivary cortisol. In addition, the authors observed that a blunted salivary testosterone response to a 55/80 after a period of intensified training occurred independently of a perceived increase in training stress. Hough *et al.* (2015) have also suggested that blunted salivary testosterone to a 55/80 bout may be a predictor of the incidence of overreaching. The acute increase in salivary testosterone observed in all three exercise trials suggests that this hormone may be indeed more sensitive to the 55/80 than salivary cortisol. Therefore, salivary testosterone may be a more reliable endocrine biomarker to be used to examine any alterations expected to occur following a period of intensified training.

In conclusion, the CV<sub>i</sub> in the exercise-induced responses of salivary cortisol and testosterone found in this present study are within the intra-individual variation that has been reported for the resting concentrations of these hormones previously. In addition, these CV<sub>i</sub> are lower than the blunted response reported to happen following periods of intensified training. These data suggest that the 55/80 elicits consistent, reproducible salivary cortisol and testosterone responses within the normal variability associated with these hormones. The previously identified blunted responses of these hormones when comparing before and after an intensified period of training (Hough *et al.*, 2013, Hough *et al.*, 2015), implies that this exercise protocol may be a worthwhile test when diagnosing a potential dysfunction of the HPA and HPG axis demonstrated in athletes suspected to be overreached. Detailed examination of the exercise-induced salivary cortisol and testosterone may suggest that perhaps testosterone, due to its sensitivity to exercise stress, lower individual variability, and consistent, reproducible, acute elevation in response to the 55/80, may be a more reliable endocrine biomarker compared to cortisol to be utilized when highlighting any hormonal alterations in athletes on the edge of overreaching.

## **CHAPTER 6.**

# **Reproducibility of Exercise-Induced Plasma Cortisol and Testosterone Responses: The Development of a New Tool to Highlight Hormonal Alterations During Overreaching**

### 6.1 Introduction

As detailed previously in this Thesis, the 55/80 cycle bout has been proposed to be a suitable tool to highlight blunted cortisol and testosterone exercise-induced responses which may be associated with overreaching. In addition, Chapter 5 of this Thesis suggests that the intra-individual variability of the salivary cortisol and testosterone responses to the 55/80 lies at or below  $20 \pm 11\%$  and  $10 \pm 5\%$ , respectively. The intra-individual variability reported in cortisol and testosterone examined in Chapter 5 is within the intra-individual variability of these hormones reported in previous studies. Maes *et al.* (1997) has suggested that the resting intra-individual variability in plasma cortisol is 21.7%, and in plasma testosterone is 12.6%. These findings suggest that the salivary cortisol and testosterone responses found in response to the 55/80 are within the variability associated with these hormones and are therefore reproducible to the extent to which this variability allows.

Hough *et al.* (2011) and Hough *et al.* (2013) reported robust elevations of salivary cortisol and testosterone concentrations in response to the 55/80 bout. However, as reported in Chapter 5 we were unable to reproduce these elevations of cortisol concentrations in response to the 55/80 bout. This may be due to the influence of a circadian rhythm on the cortisol responses. However, the  $CV_i$  for both cortisol and testosterone presented in Chapter 5 have supported the reproducibility of these hormonal responses to the 55/80.

Despite the promise of using the 55/80 bout as a tool to highlight altered biomarkers during a period of overreaching (Functional or Non-Functional), this exercise tool simply may not be an ideal tool for non-cyclists. Therefore, to expand the population that can be tested for overreaching utilising an exercise stress test to highlight blunted exercise-induced hormonal responses, this study seeks to design a running test capable of inducing an acute and reproducible elevation in plasma and salivary cortisol and testosterone in physically active, healthy males. A 30-min running bout at  $80\% \dot{V}O_{2max}$  has been reported to elevate plasma

## Chapter 6: Reproducibility of Steroid Hormone Responses to a Running Bout

cortisol by ~20% (Verde *et al.*, 1992). In addition, an acute elevation in plasma cortisol of ~97% and total testosterone of ~31% in response to a running test to exhaustion at 100% ventilatory threshold has been reported by Daly *et al.* (2005). These results suggest that a short duration (30 min) running test may be appropriate to induce robust elevations in both cortisol and testosterone when in a normal trained state (i.e. not a FOR, NFOR or OTS state). It is also reasonable to suggest that, like the 55/80 bout, a running bout could highlight alterations in cortisol and testosterone following a period of intensified training. To use the 55/80 cycle bout it is required to complete preliminary exercise testing for determination of the exercise intensities. This may sometimes not be practical, and therefore, this present study proposes to develop a self-paced running bout based on the individuals' perceived exertion using the 6-20 Borg scale. This self-paced running bout could therefore be more practically applied in an athletic population. As it has been proposed that exercise intensity needs to be above a certain threshold ( $> 60\% \dot{V}O_{2max}$ ) for cortisol to significantly elevate (Davies and Few, 1973), the running bout developed in this present study was designed in an attempt to reproduce these exercise intensities. The 6-20 Borg scale was designed so that perceived exertion (ranging from 6 to 20) matched HR, and consequently exercise intensity (Borg, 1970). Therefore, Borg (1970) proposed that the 6-20 scores on the RPE scale would designate a HR ranging from 60 to 200  $beats \cdot min^{-1}$ . The relationship between the 6-20 Borg scale, HR and exercise intensity has been examined (Eston and Williams, 1988, Smutok *et al.*, 1980). Smutok *et al.* (1980) completed a study on 10 healthy men who undertook 3 different trials. On the first visit to the labs, all participants run on a treadmill at 4.7, 6.5, 11.3 and 12.9  $km \cdot h^{-1}$  and reported their perceived exertion on the 6-20 Borg scale. On the two following visits, the participants were asked to regulate their pace on the treadmill to run at a speed corresponding to the RPE scores they had reported at each speed on trial 1. Based on the results obtained by regression analyses, Smutok *et al.* (1980) concluded that treadmill-run exercise mediated by RPE elicits a consistent HR response if above an average 150  $beats \cdot min^{-1}$  (~80%  $HR_{max}$ ) ( $r = 0.83$ ). This suggests that the exercise bout used in the current study should be able to elicit consistent HR responses if the intensity leads the athlete to a HR above 150  $beats \cdot min^{-1}$ . In this present study, it was hypothesized that RPE scores of 11 (light) and 15 (hard) could replicate the design of the 55/80

## Chapter 6: Reproducibility of Steroid Hormone Responses to a Running Bout

(alternating blocks of 1 min at low intensity, and 4 min at high intensity).

However, if the running tool designed in this present study is to be used by practitioners to highlight hormonal alterations after periods of intensified training, the reproducibility of the hormonal responses to the bout must be examined. As described in Chapter 5, absolute and relative reliability are two different types of reliability testing. Relative reliability refers to the ratio of total between-subject variability to the within-subject variability, and therefore gives an indication of the magnitude of association of the between-subjects responses. In contrast, absolute reliability describes the intra-individual variability of a measure, independently of sample heterogeneity and should therefore be used as a measure of the reproducibility of a variable (Atkinson and Nevill, 1998, Šerbetar, 2015). Atkinson and Nevill (1998) recommended that a measure of relative reliability could be used to support absolute reliability, as it may give an indication of the inter-individual variability of the variable being examined. The  $CV_i$  and ICC (2,1) were used to examine the reproducibility of the physiological and hormonal responses to the running bout.

The main aim of this study was to design a self-paced, short-duration (30 min), high-intensity running bout to elicit reproducible and robust elevations in plasma and salivary cortisol and testosterone when completed in a healthy state.



## 6.2 Methods

### 6.2.1 Procedures

This study was a randomized, crossover experiment and was conducted on 13 recreationally active male volunteers. The baseline physiological and anthropometric data are presented in Table 6.1.

**Table 6.1** Participants' anthropometric and physiological characteristics at baseline.

	<b>Mean ± SD</b>
Age (years)	22 ± 6
Height (cm)	177 ± 5
Body mass (kg)	77.7 ± 13.4
HR <sub>max</sub> (beats·min <sup>-1</sup> )	190 ± 8
$\dot{V}O_{2max}$ (mL·kg <sup>-1</sup> ·min <sup>-1</sup> )	53 ± 5
n = 13	

On the first visit to the laboratories, participants completed a preliminary  $\dot{V}O_{2max}$  test on a motorised treadmill, as detailed in section 3.2.2 of this Thesis. On the following visits, participants completed 4 more trials in total – three main exercise trials and one resting trial (CTL). The main experimental trials and CTL were completed at 12:00 to avoid the influence of diurnal variation of the hormones being examined. A standard breakfast was consumed before 09:00 and was replicated before each main trial. Participants were requested to consume ~500 mL of water in the morning of the trial and euhydration was confirmed by a urine osmolality <700 mOsmol·kg<sup>-1</sup> H<sub>2</sub>O (Sawka *et al.*, 2007). All participants reported to the laboratory at ~11:30 and completed a RESTQ-76 Sport questionnaire. Participants remained fasted until the end of each main experimental trial, but were allowed to drink water *ad libitum* throughout the exercise bout, except within the 10 min period before providing a saliva sample. Body mass was

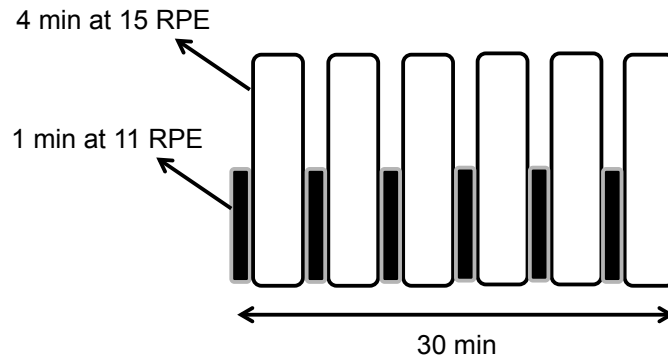
## Chapter 6: Reproducibility of Steroid Hormone Responses to a Running Bout

measured pre- and post-exercise and HR and RPE were measured in the last 15 s of each stage. The  $\dot{V}O_{2\max}$  test was used to determine the levels of physical fitness of the participants.

Participants were requested to consume a similar diet during the 24 hours preceding each trial, which was measured via a weighed food diary as detailed in 3.8.3 in this Thesis. Mean energy intake 24 hours prior to each trial was  $8941 \pm 2565$  kJ ( $2137 \pm 613$  kcal), and carbohydrate ( $57\% \pm 15\%$ ), fat ( $23\% \pm 9\%$ ), and protein ( $20\% \pm 6\%$ ) consumed were determined.

### 6.2.2 Main Experimental Trials

In 3 of the main experimental trials a running bout was completed. The first main exercise trial consisted of a running bout to familiarise the participant with the stress of the running bout to be used in the two other main exercise trials (T1 and T2). The running bout was referred to as the  $RPE_{\text{treadmill}}$  and consisted of continuously alternating blocks of 1 min at a perceived exertion equivalent to 11 (fairly light) and 4 min at 15 (hard) on the 6-20 Borg's RPE scale on a motorised treadmill for 30 min (Figure 6.1). The treadmill speed could be adjusted but not seen by the participant to maintain the RPE in the target range. A 30-min resting trial (CTL) where no exercise was undertaken was also completed. In all exercise trials, the treadmill slope was set at 1% gradient to more accurately reflect the energetic cost of outdoor running (Jones and Doust, 1996). Participants were required to abstain from exercise, caffeine and alcohol intake in the 24 hours leading up to the trial, and from food ingestion four hours before each main trial. To avoid baseline peak circulating cortisol levels due to circadian rhythm, all participants were asked to wake up no later than 08:00 on the morning of the trial. Each of the main experimental trials was separated by 4-7 days. Saliva and blood samples were collected Pre-Exercise, Post-Exercise, and 30 min Post-Exercise in T1 and T2, and Pre-, Post- and 30 min Post-CTL.



**Figure 6.1** Schematic presentation of the RPE<sub>treadmill</sub> running bout.

### 6.2.3 Analytical Procedures

Collection, handling and analysis of blood and saliva samples were accomplished as described in Chapter 3.

### 6.2.4 Statistical Analysis

All data were checked for normality and for homogeneity of variance. Non-normally distributed data sets (plasma cortisol, and salivary cortisol and testosterone) were log transformed (to base 10) and rechecked for normality. Normally distributed data sets were analysed using a two-way repeated measures ANOVA. On finding an effect, subsequent paired sample *t*-tests were used with Bonferroni adjustments. A one-way repeated measures ANOVA with subsequent paired-sample *t* test with Bonferroni corrections was used to examine HR and speed in CTL and exercise trials, and hormonal responses during CTL. Reproducibility analysis was accomplished by determining the  $CV_i$  of all physiological and hormonal measurements as detailed in the previous chapter (section 5.2.3). The two-way ICC model, based on single measures was determined. Effect sizes (ES) were determined by hand as detailed in Vincent and Weir (2012, p. 165) to examine the magnitude of hormonal change between trials (Hopkins *et al.*, 2009). The alpha level of significance was set as  $p < 0.05$ . Data is reported as mean  $\pm$  SD.

## 6.3 Results

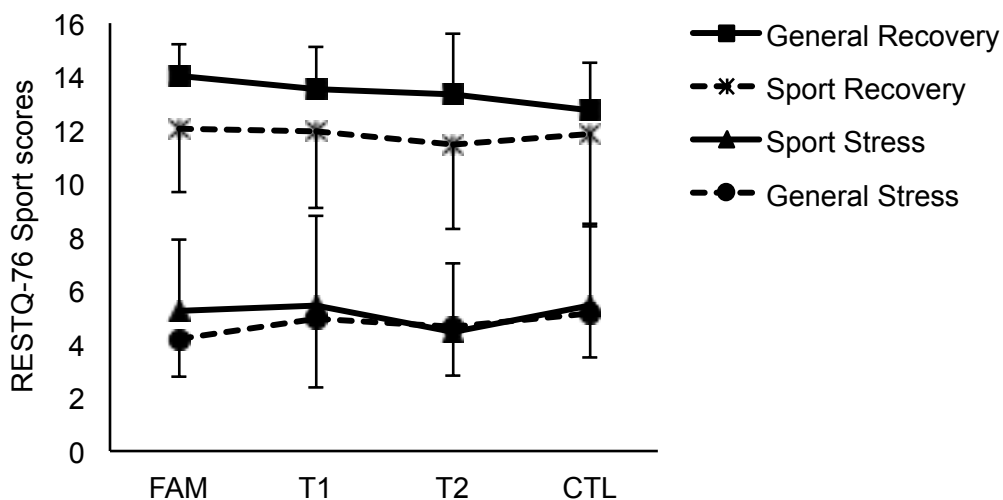
Post-hoc power analysis demonstrated that the observed power for plasma cortisol and testosterone was 0.326 and 1.000, respectively. The observed power for salivary cortisol and testosterone was 0.376 and 0.713, respectively.

### 6.3.1 Hydration Status

Urine osmolality did not differ across all trials and was  $345 \pm 198$  mOsmol $\cdot$ kg $^{-1}$  H $_2$ O in T1,  $310 \pm 168$  mOsmol $\cdot$ kg $^{-1}$  in T2, and  $301 \pm 166$  mOsmol $\cdot$ kg $^{-1}$  H $_2$ O in CTL ( $p > 0.05$ ).

### 6.3.2 Recovery-Stress Questionnaire

No changes in the RESTQ-76 Sport scores were found in any of the stress or recovery scales across all trials ( $p > 0.05$ ).



**Figure 6.2** RESTQ-76 Sport scores measured in all trials. Values are means  $\pm$  SD.

### 6.3.3 Physiological Responses to Exercise

No differences in HR or speed were found when comparing FAM, T1 and T2 ( $p < 0.05$ ). Average HR was  $169 \pm 6$  beats·min<sup>-1</sup> in FAM,  $171 \pm 8$  beats·min<sup>-1</sup> in T1, and  $169 \pm 13$  beats·min<sup>-1</sup> in T2. Average speed was  $12.1 \pm 2.0$  km·h<sup>-1</sup> in FAM,  $12.1 \pm 1.9$  km·h<sup>-1</sup> in T1, and  $12.0 \pm 1.8$  km·h<sup>-1</sup> in T2. Reliability data for speed and heart rate are presented in Table 6.2.

**Table 6.2** CV<sub>i</sub> and ICC values for speed and heart rate to both running bouts.

	HR	Speed
<b>CV<sub>i</sub></b>	$1.8 \pm 1.3\%$	$2.2 \pm 1.8\%$
<b>ICC</b>	0.912	0.955

CV<sub>i</sub> = Intra-individual coefficient of variation (%); ICC = Intraclass Correlation Coefficient, model 2,1.

### 6.3.4 Hormonal Responses During CTL

#### a) Plasma Cortisol and Testosterone

Plasma cortisol significantly decreased from Pre- to Post-CTL ( $p < 0.01$ ) by  $\sim 18\% \pm 16\%$  (Figure 6.3). Plasma testosterone did not alter over time ( $p > 0.05$ ) (Figure 6.5).

#### b) Salivary Cortisol and Testosterone

Salivary cortisol significantly decreased from Pre- to Post-CTL ( $p < 0.01$ ) by  $\sim 32\% \pm 16\%$  (Figure 6.3). Salivary testosterone did not alter over time ( $p > 0.05$ ) (Figure 6.5).

### 6.3.5 Hormonal Responses to Exercise

#### a) *Plasma Cortisol*

No trial effect ( $F_{1,12} = 0.131$ ,  $p = 0.72$ ) or time effect ( $F_{1,12} = 3.943$ ,  $p = 0.07$ ) were found when comparing T1 and T2 (Figure 6.3). However, although not significantly plasma cortisol increased from Pre- to Peak Post-Exercise by 34% in T1, and by 47% in T2.

The plasma cortisol responses were showed to be variable between individuals, with individual exercise-induced changes ranging from -40% to +304% (Figure 6.4). The  $CV_i$  was  $12 \pm 7\%$ . The effect size for the plasma cortisol responses to exercise across trials was  $\eta^2 = 0.266$ .

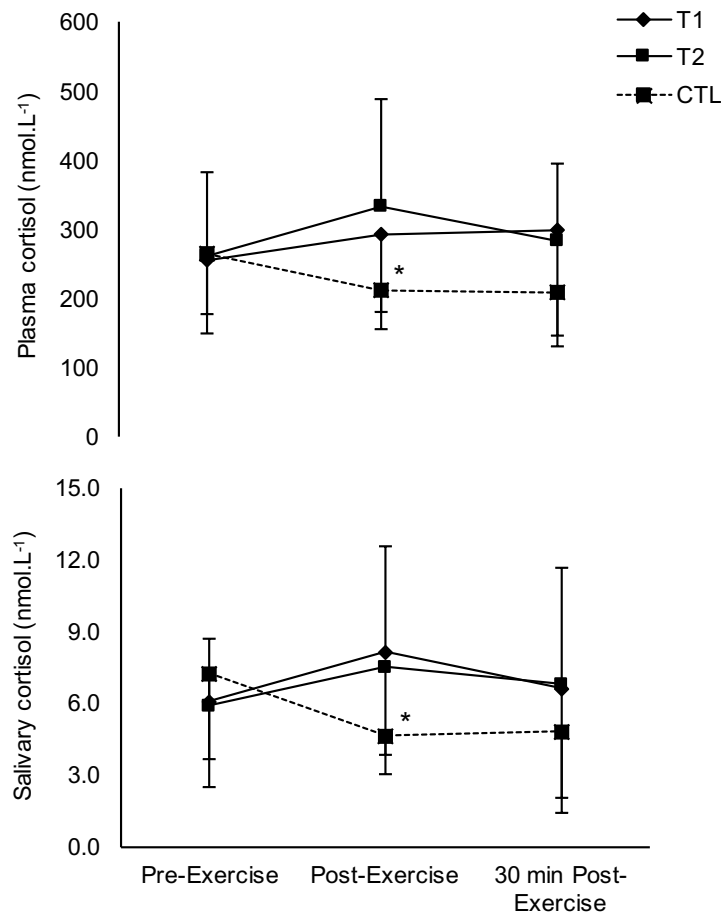
#### b) *Salivary Cortisol*

No trial effect ( $F_{1,12} = 0.134$ ,  $p = 0.72$ ) or time effect ( $F_{1,12} = 2.84$ ,  $p = 0.10$ ) were found when comparing T1 and T2 (Figure 6.3). However, although not a significant alteration, salivary cortisol increased from Pre- to Peak Post-Exercise by 58% in T1, and by 70% in T2.

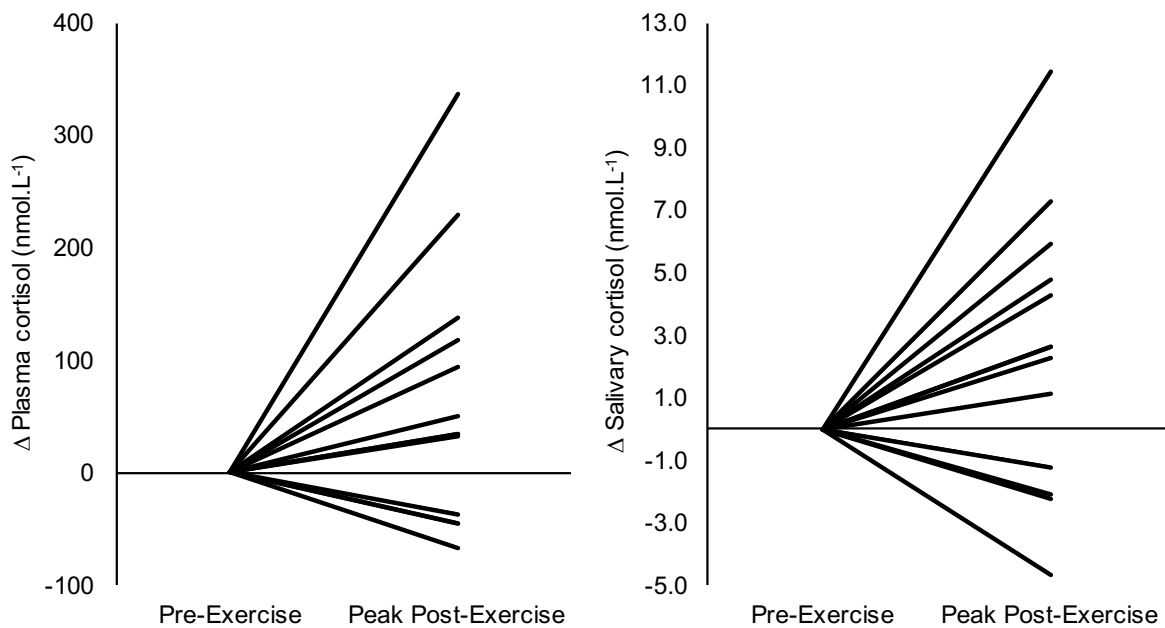
The salivary cortisol responses were showed to be variable between individuals, with individual exercise-induced changes ranging from -55% to +370% (Figure 6.4). The  $CV_i$  was  $17 \pm 17\%$ . The effect size for the salivary cortisol responses to exercise across trials was  $\eta^2 = 0.164$ .

Pearson's correlation coefficient revealed that the exercise-induced salivary cortisol moderately correlated with its plasma measures ( $r = 0.578$ ,  $p = 0.217$ ).

Chapter 6: Reproducibility of Steroid Hormone Responses to a Running Bout



**Figure 6.3** Plasma and salivary cortisol responses to exercise and CTL. Values are means  $\pm$  SD. \*Different from Pre-Exercise values ( $p < 0.05$ ).



**Figure 6.4** Individual average absolute changes in plasma and salivary cortisol from Pre- to Peak Post-Exercise.

**c) Plasma Testosterone**

No trial effect ( $F_{1,12} = .504$ ,  $p = .49$ ) was found when comparing T1 and T2 (Figure 6.5). However, a time effect was present ( $F_{2,11} = 44.403$ ,  $p < .001$ ). Pairwise comparisons showed that plasma testosterone acutely elevated in both exercise trials ( $p < 0.01$ ) and returned to baseline levels at 30 min Post-Exercise ( $p < 0.05$ ). On average plasma testosterone elevated from Pre- to Peak Post-Exercise by 46% in T1, and by 38% in T2.

Individual responses of plasma testosterone range between +6% and +82% (Figure 6.6). The  $CV_i$  was  $12 \pm 9\%$ . The effect size for the plasma testosterone responses to exercise across trials was  $\eta^2 = 0.890$ .

**d) Salivary Testosterone**

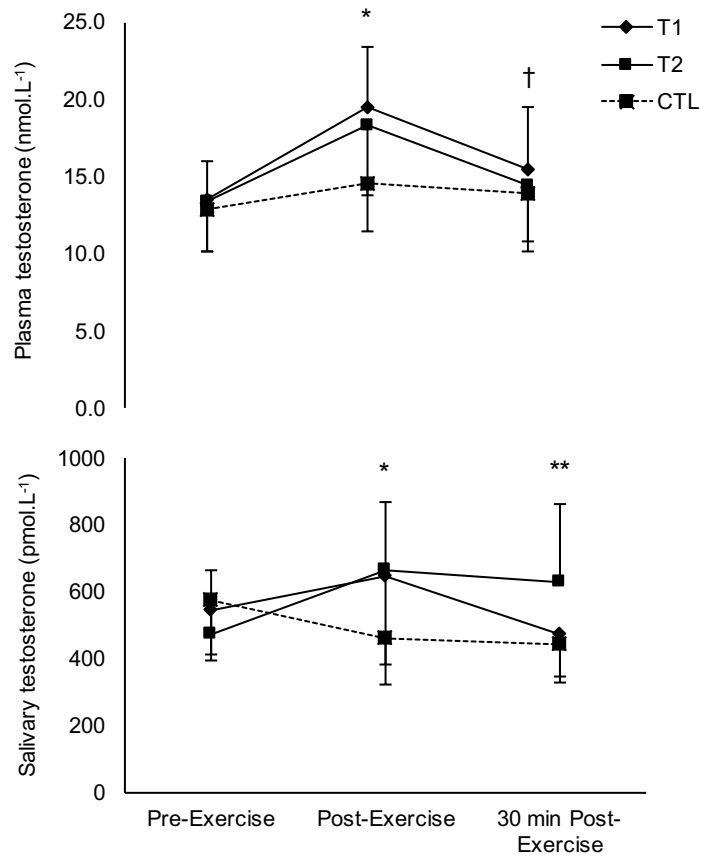
No trial effect ( $F_{1,12} = .328$ ,  $p = .58$ ) was found when comparing T1 and T2 (Figure 6.5). A time effect was found ( $F_{2,11} = 4.962$ ,  $p < .05$ ) with pairwise comparisons showing salivary testosterone elevating in both exercise trials ( $p < 0.01$ ) and remained elevated above Pre-Exercise values at 30 min Post-Exercise in T2 only ( $p < 0.05$ ). Salivary testosterone increased from Pre- to Peak Post-Exercise by 26% in T1, and by 57% in T2.

Individual responses of salivary testosterone range from +1% to +114% (Figure 6.6). The  $CV_i$  was  $14 \pm 10\%$ . The effect size for the salivary testosterone responses to exercise across trials was  $\eta^2 = 0.304$ .

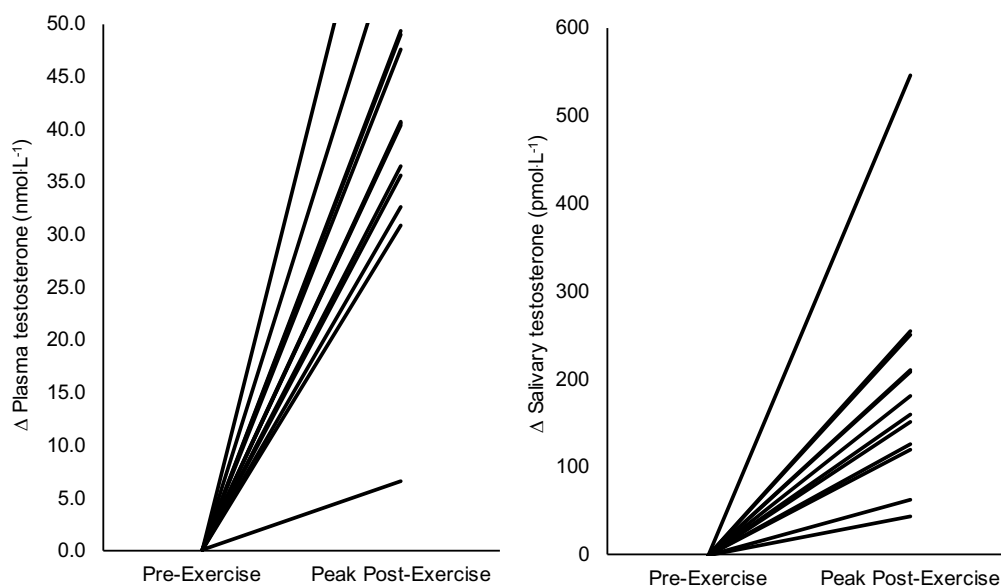
Pearson's correlation coefficient revealed that the exercise-induced salivary testosterone moderately correlated with its plasma measures ( $r = 0.538$ ,  $p = 0.051$ ).



## Chapter 6: Reproducibility of Steroid Hormone Responses to a Running Bout



**Figure 6.5** Plasma and salivary testosterone responses to exercise and CTL. Values are means  $\pm$  SD.  
 \*Different from Pre-Exercise values in the exercise trials ( $p < 0.01$  and  $p < 0.05$ . for plasma and salivary values).  
 †Different from Post-Exercise values in the exercise trials ( $p < 0.05$ ).  
 \*\*Different from Post-Exercise values in T2 ( $p < 0.05$ ).



**Figure 6.6** Individual absolute changes in plasma and salivary testosterone from Pre- to Peak Post-Exercise.

**Table 6.3** CV<sub>i</sub>, ICC and ES values for the hormone responses to the RPE<sub>treadmill</sub> bouts.

	<b>CV<sub>i</sub></b>	<b>ICC</b>	<b>ES</b>
<b>Plasma Cortisol (nmol·L<sup>-1</sup>)</b>	12 ± 7%	0.62	0.03
<b>Plasma Testosterone (nmol·L<sup>-1</sup>)</b>	12 ± 9%	0.58	0.04
<b>Salivary Cortisol (nmol·L<sup>-1</sup>)</b>	17 ± 17%	0.49	0.1
<b>Salivary Testosterone (pmol·L<sup>-1</sup>)</b>	14 ± 10%	0.44	0.01

CV<sub>i</sub> = Intra-individual coefficient of variation (%); ICC = Intraclass Correlation Coefficient, model 2,1; ES = Cohen's Effect Size.

## 6.4 Discussion

This study aimed to examine the reproducibility of plasma and salivary cortisol and testosterone responses to a continuous, self-paced, 30-min, high-intensity running bout, referred to as the RPE<sub>treadmill</sub>. It was hypothesized that the RPE<sub>treadmill</sub> would be stressful enough to induce an acute elevation in plasma and salivary cortisol and testosterone and that these responses would be reproducible. Despite an average percentage-increase in exercise-induced plasma cortisol concentrations from Pre- to Peak Post-Exercise of 34% in T1, and 47% in T2, and in salivary cortisol (58% in T1 and 70% in T2), these changes were not significant in any trial. Contrastingly, plasma testosterone (46% in T1 and 38% T2) and salivary testosterone (26% in T1 and 57% in T2) markedly elevated in response to both exercise trials from Pre- to Peak Post-Exercise. The intra-individual variability in plasma and salivary cortisol ( $12 \pm 7\%$ , and  $17 \pm 17\%$ , respectively) and testosterone ( $12 \pm 9\%$ , and  $14 \pm 10\%$ , respectively) observed in this study are within or close to the normal variability associated with these hormones, and therefore support the reproducibility of the hormonal and physiological responses to the RPE<sub>treadmill</sub>.

As explained in Chapter 5, in measuring the reproducibility of the hormonal responses to exercise some important control measures must be in place. Hydration status has been reported to influence cortisol and testosterone concentrations. As outlined in Chapter 5 cortisol concentrations have been shown to elevate when in a hypohydrated state compared to a euhydrated state (Francesconi *et al.*, 1985, Maresh *et al.*, 2006). In this current study hydration status was measured at the beginning of each main experimental trial. All participants were confirmed to be in a euhydrated status with a urine osmolality measure of below 700 mOsmol.kg<sup>-1</sup> H<sub>2</sub>O (Sawka *et al.*, 2007). This confirms that hydration status has had no influence on the salivary and plasma cortisol and testosterone responses reported in this current study. In addition to the control of hydration status, caffeine and alcohol intake was also restricted in the 24 h period before each main experimental trial. As detailed in the previous Chapter, Lovallo *et*

## Chapter 6: Reproducibility of Steroid Hormone Responses to a Running Bout

*al.* (2006) have reported an acute elevation in plasma cortisol concentrations following a 30-min cycle bout at an average 75 W combined with consumption of a 250 mg dose of caffeine, when compared to exercise on its own. In addition, alcohol consumption has also been shown to have an influence on the cortisol concentrations (Ireland *et al.*, 1984). In their study, Ireland *et al.* (1984) have shown that consumption of a moderate dose of alcohol (740 mL of cold non-alcoholic beer to which were added 0.5 mL of ethanol per kg body mass) has induced elevated plasma cortisol concentrations when compared to the cortisol concentrations examined after consumption of a similar volume of water. Similarly, plasma testosterone has also been proposed to be altered following acute alcohol intake (Mendelson *et al.*, 1977). These authors reported a marked fall in testosterone levels occurring in parallel with a gradual elevation of alcohol concentrations in blood, during a period of acute alcohol intoxication. In this present study, alcohol consumption was not allowed in the 24 h preceding each main experimental trial. This suggests that the responses of cortisol and testosterone have not been influenced by alcohol consumption. In addition, cortisol is known to be a stress-related hormone that is elevated during and after emotional and psychological stress (Burke *et al.*, 2005, Kirschbaum *et al.*, 1995, Skosnik *et al.*, 2000). The RESTQ-76 Sport was used to examine the participants' general- and sport-specific recovery and stress scores before each main trial. In this study, analysis of the RESTQ-76 Sport scores showed no significant disparities in any of the stress and recovery scales, which details that the participants were in a similar state of wellbeing and predisposition to undertake physical activity on every trial and therefore the hormonal responses reported have not been influenced by a change in wellbeing in the participants.

The reproducibility of the physiological responses to the  $RPE_{\text{treadmill}}$  was examined in the current study. Monitoring HR during exercise testing is a common method used to categorize exercise intensities (Kenny, 1990). Being a self-paced exercise bout, the  $RPE_{\text{treadmill}}$  could provoke different HR responses if the speeds chosen by the runners were different when completing the running bout on different occasions. In this study, HR responses and speed in the  $RPE_{\text{treadmill}}$  did not alter across all main exercise trials. These results are important, as an alteration in the speeds would be indicative of a subsequent alteration in exercise intensity, and

## Chapter 6: Reproducibility of Steroid Hormone Responses to a Running Bout

therefore influence the response of both cortisol and testosterone. Additionally, the HR and speed responses were shown to be reproducible, with  $CV_i$  of  $1.8 \pm 1.3\%$  for HR, and  $CV_i$  of  $2.2 \pm 1.8\%$  for speed. The ICCs obtained for these measures were 0.912 and 0.955, respectively. As detailed in Chapter 5, ICC is a measure of the between-subject variability relative to the within-subject variability. Therefore, a high between-subject variability will generate a high ICC. The ICCs calculated for the HR and speed responses in this present study may give an indication of the varied HR and speed responses when comparing the participants between each other. However, the low  $CV_i$  show that HR and speed to both trials did not show to be varied. These data suggest that the  $RPE_{\text{treadmill}}$  bout induces a similar physiological strain and therefore elicits similar HR and running speed. This finding strengthens the use of this exercise stress test may induce a similar physiological stress on individuals when completed on different occasions.

Similar studies to this one have reported a significant elevation of salivary cortisol and testosterone in response to a continuous 30-min, cycle bout when in a healthy state, as stated in previous chapters in this Thesis (Hough *et al.*, 2013, Hough *et al.*, 2011, Hough *et al.*, 2015). It has been established that duration and intensity of exercise sessions are two important factors known to cause an exercise-induced increase in plasma and salivary cortisol concentrations (Bloom *et al.*, 1976, Galbo *et al.*, 1977, Kuoppasalmi *et al.*, 1980, Nieman *et al.*, 1994). It has been proposed that the exercise intensity needs to exceed a threshold of 60%  $\dot{V}O_{2\text{max}}$  for at least 20-30 min for cortisol to significantly elevate (Davies and Few, 1973). More recently, Hill *et al.* (2008) supported these findings by reporting no change in plasma cortisol levels to a 30-min cycle bout at 40%  $\dot{V}O_{2\text{max}}$ . Plasma and salivary cortisol did not significantly increase in response to the  $RPE_{\text{treadmill}}$  in this current study. There was, however, a percentage-elevation from Pre- to Peak Post-Exercise in both trials of 34% in T1, and 47% in T2 (plasma cortisol), and 58% in T1 and 70% in T2% (salivary cortisol). Detailed examination of the individual cortisol responses shows contrasting responses, ranging from moderate decreases to marked acute increases. As the  $RPE_{\text{treadmill}}$  is a self-paced running bout based on the 6-20 Borg scale, each participant will exercise at an intensity dependant of an individual perceived

## Chapter 6: Reproducibility of Steroid Hormone Responses to a Running Bout

exertion. Although the RPE<sub>treadmill</sub> bout was designed to elicit an RPE of 15 (hard) for the majority of the test (24 min), it was not confirmed whether this would provoke an exercise intensity stressful enough to acutely elevate cortisol levels. A robust and consistent exercise-induced elevation in plasma and salivary testosterone was seen in both trials. Furthermore, testosterone levels did not change with time during CTL, whereas cortisol significantly decreased from Pre- to Post-CTL (within 30 minutes). Similarly to the study presented in Chapter 5, the circadian rhythm of cortisol is likely to have led to RPE<sub>treadmill</sub> being unable to induce the hypothesised elevations in plasma and salivary concentrations. In this present study, all participants were asked to rise at least 4 h before the start of the experimental trial to circumvent elevated cortisol concentrations associated with diurnal variation. However, we did not confirm with the participants whether they have indeed awakened at this time as required. Not following this procedure may influence the cortisol concentrations at the time of testing, as cortisol is known to be at its peak concentrations shortly after awakening (Debono *et al.*, 2009). In addition, cortisol has also been shown to have a high intra-individual variability (Bertagna *et al.*, 1994), with research reporting that 78% of total variation in the cortisol awakening response is due to intra-individual variability (Almeida *et al.*, 2009), as detailed in the previous chapter. When examining the intra-individual variation across trials, as presented in Table 6.3, this study shows an intra-individual variation of ~12% and ~17% in the plasma and salivary cortisol responses, respectively. At first examination, these data may seem a little high, however, the within subject variability in cortisol has already been reported to be 21.7% (Maes *et al.*, 1997). However, the CV<sub>i</sub> for salivary testosterone reported in this chapter is above the previously reported 12.6% (Maes *et al.*, 1997) and the 11.8% (Sartorius *et al.*, 2012) average intra-individual variability in serum testosterone. This may be due to the fact that these studies have examined the variability of these hormones while at rest, while the present chapter has looked at the exercise-induced responses. Overall, the data from this experimental chapter suggest that the variability found falls or tends to fall within normal biological variability values reported previously, certainly for plasma and salivary cortisol and plasma testosterone. The results from the repeated measures ANOVA showed no differences between trials. The ES were used to examine the magnitude of change between trials within the running test (Cohen, 1988). Cohen

## Chapter 6: Reproducibility of Steroid Hormone Responses to a Running Bout

(1988, p. 21) has proposed that an effect size value of 0.8 represents large differences and that the lower this value, the less pronounced the differences, with small differences reported if  $ES = 0.2$ . The ES for plasma cortisol, plasma testosterone, salivary cortisol and salivary testosterone were 0.03, 0.04, 0.1 and 0.01, respectively. The ES values presented in this experimental chapter indicate that the differences between T1 and T2 were trivially small. These data supports the trivial changes in the responses of the hormones examined in this study when compared across trials.

It was hypothesized that plasma and salivary cortisol and testosterone would acutely elevate in response to the  $RPE_{\text{treadmill}}$  and that this running bout would provoke reproducible hormonal and physiological responses. The examination of the hormonal responses measured in this study, suggests that testosterone may be a better indicator of a hypothalamic-pituitary activation following short-duration, high-intensity exercise when compared to cortisol responses. This study has shown a robust elevation of plasma and salivary testosterone in response to the  $RPE_{\text{treadmill}}$  with a reasonable intra-individual variability of  $12 \pm 9\%$  and  $14 \pm 10\%$ , respectively. This study suggests that the  $RPE_{\text{treadmill}}$  bout may elicit reproducible plasma and salivary cortisol responses, but does not significantly elevate the cortisol concentrations from pre- to post-exercise. This means it may be difficult to use the  $RPE_{\text{treadmill}}$  in an attempt to measure cortisol responses as an endocrine biomarker to highlight exercise-induced stress levels. However, both plasma and salivary testosterone significantly elevated to this running tool and these responses were reproducible. The advantage of using the  $RPE_{\text{treadmill}}$  is that it will not require a  $\dot{V}O_{2\text{max}}$  to determine exercise intensities and may be practically used in the field, as a suitable tool to be used to induce a significant elevation certainly in testosterone levels. It is expected that the  $RPE_{\text{treadmill}}$  will also induce blunted testosterone responses following a period of intensified training, as reported by Hough *et al.* (2013) and Hough *et al.* (2015), highlighting its usefulness to prevent the incidence of NFOR/OTS. This study also proposes that cortisol is individualised and that the exercise-induced responses may be influenced by a circadian rhythm. However, the intra-individual variability of plasma and salivary cortisol and testosterone responses is at a

## Chapter 6: Reproducibility of Steroid Hormone Responses to a Running Bout

level that suggests that the  $RPE_{\text{treadmill}}$  could highlight blunted exercise-induced cortisol and testosterone responses following a period of intensified training.



## **CHAPTER 7.**

# **Effects of a 12-Day Intensified-Training Period on the Endocrine and Immune Systems: Using a Novel Short-Duration Running Test to Prevent the Incidence of the NFOR/OTS**

## 7.1 Introduction

Chapter 6 proposes that the RPE<sub>treadmill</sub> bout induces reproducible HR, running speed and plasma and salivary cortisol and testosterone responses. In addition, the intra-individual variability of plasma and salivary cortisol and testosterone responses is at a level that suggests that the RPE<sub>treadmill</sub> could highlight blunted exercise-induced cortisol and testosterone responses following a period of intensified training.

As a reminder, the incidence overreaching (FOR/NFOR) and the OTS commonly occur during an athlete's competitive career. Morgan *et al.* (1987) have reported that 60% of female and 64% of male elite distance runners have reported being in an overreached/OTS state at least once in their career. Birrer *et al.* (2013) reported similar prevalence rates (60%) of overtraining symptoms in mid to long distance runners. The prevalence of these states is not isolated to individual endurance sports as highlighted by Matos *et al.* (2011) who reported that a total of 110 out of 376 athletes (29%) from individual and team-sports self-reported symptoms associated with overtraining at least once in their career. In conclusion, the prevalence rates of overreaching/OTS are high and can have an immense impact on an athlete's health and wellbeing.

Signs of overreaching have been reported to occur within a period as short as 7 days (Halsen *et al.*, 2002). The early detection of signs of overreaching is important in preventing the incidence of an FOR/NFOR/OTS. The early recognition of overreaching is difficult but athletes in this state normally experience acute decrements in athletic performance, which is sometimes accompanied with increased levels of fatigue together with disturbed mood states (Meeusen *et al.*, 2004, Urhausen *et al.*, 1995). For this reason, it has been proposed that the use of a performance test should be considered when tracking individuals in danger of overreaching (Budgett, 1998, Meeusen *et al.*, 2013, Urhausen *et al.*, 1995). Time-trial tests may be a good tool to indicate any alterations in athletic performance when in an overreached state, as

## Chapter 7: Endocrine and Immune Impairments After Intensified Training

significant increases in time to completion have been reported in several studies (Halson *et al.*, 2002, Halson *et al.*, 2003). Halson *et al.* (2002) have reported a significant increase in the time to complete a simulated time trial performance test ( $65.3 \pm 2.6$  min) after a 2-week period of intensified training, when compared with a 2-week of normal training ( $59.4 \pm 1.9$  min). This time trial test involved completing an individualised predefined amount of work, determined by multiplying their workload at  $75\% \dot{V}O_{2max}$  by 3600 as described by Jeukendrup *et al.* (1996).

As reported in the previous chapters in this Thesis, overreaching/OTS may be accompanied by endocrine dysfunction, the most commonly examined occurring within the HPA and HPG axes. Meeusen *et al.* (2004) were the first to propose that examination of the exercise-induced responses of cortisol and its precursor ACTH, could provide an indication of an overreached state. These researchers reported blunted exercise-induced responses of these hormones to the second of a double cycle to fatigue test separated by a 4 h recovery period when trained cyclists were overreached, in comparison to when in a normal-trained, healthy state. Despite the interesting findings, the duration of the protocol used by Meeusen *et al.* (2004) may not be suitable to practically apply in an athletic population, and therefore a shorter exercise test could be more appropriate. Following on from these studies, Hough *et al.* (2013) reported exercise-induced blunted responses of salivary cortisol (~166%) and testosterone (~21%) to a 30-min cycle bout following an 11-day period of intensified training suspected to have induced overreaching. However, Hough *et al.* (2015) were not able to reproduce these blunted exercise-induced salivary cortisol responses following a period of intensified training, when tested in an elite male triathlete population. It was proposed that this could be due to the capability of the elite athletes to cope with an intensification in training load.

Almost every cell in the human body contains receptors for cortisol, and therefore cortisol has a wide-range of functions depending on the cells it is acting upon. One of these actions of cortisol is an immunosuppressive effect in the human body (Le Tulzo *et al.*, 2004, Voorhis *et al.*, 1989). Malm *et al.* (2004) have reported that a marked decrease in T-lymphocytes (specifically  $CD3^+ CD4^+ CD8^+$  and  $CD3^+ CD4^- CD8^-$ ) and NK cell numbers to two consecutive 45 min football

## Chapter 7: Endocrine and Immune Impairments After Intensified Training

games were associated with an elevation in serum cortisol concentration levels. In addition, strenuous exercise of long-duration (~90 min) has also been associated with immunosuppression, by reducing circulating lymphocytes, SIgA concentration levels, and suppressing NK cell activity (Mackinnon and Hooper, 1994, Pedersen *et al.*, 1996). As overtraining is characterized by increased-volume training sessions with limited recovery episodes, it has been suggested that immunosuppression may occur in overreached athletes and that, therefore preventive measures should be considered (Hackney, 2012). Again, this exercise-induced immunosuppression is often associated with elevated cortisol concentrations, with consequent inhibition of anti-inflammatory cytokine production (Munck and Guyre, 1991). In addition, leucocyte functions such as T-lymphocyte CD4<sup>+</sup>/CD8<sup>+</sup> ratio have been reported to drop, although not significantly, in long-distance runners following 3 weeks of intensified training (38% increase in training volume) (Verde *et al.*, 1992). These heavy training periods leading to a decreased immunity state are associated with an elevated risk of URS. Increased symptoms of URS have been reported within the first weeks following marathon and ultra-marathon competitive events (Nieman, 2009). It has been recognized that symptoms of URS may arise with the occurrence of overreaching. Increased symptoms of URS have been associated with decreased concentrations of the marker of mucosal immunity SIgA (Gleeson *et al.*, 1999b, Nieman and Nehlsen-Cannarella, 1991). SIgA has been extensively examined in response to exercise of different duration and intensity as a marker of immune status (Bishop and Gleeson, 2009). Acute decreases of SIgA (52%) have been observed in competitive swimmers following a 2-hour training session (D'Ercole *et al.*, 2016), as well as following heavy exertion (e.g. 140-km ultramarathon), with an increase in the susceptibility to URS (Nieman *et al.*, 2006). Previous research had already proposed that low levels of SIgA are commonly associated with an elevated occurrence of URS (Hanson *et al.*, 1983). However, it is still unclear what the effects of overreaching on SIgA are, as increases (Gleeson *et al.*, 2000), decreases (Gleeson *et al.*, 1999b, Tharp and Barnes, 1990) and no changes (Mackinnon and Hooper, 1994, Tiollier *et al.*, 2005) have been reported following periods of intensified training. This may be due to the different training protocols, exercise duration and intensity, the fitness level of participants, and even the method used to express IgA data (Bishop and Gleeson, 2009). However, the

## Chapter 7: Endocrine and Immune Impairments After Intensified Training

fluctuations in SIgA in response to exercise and its association with URS in athletes may be a good indicator of mucosal immunity in athletes suspected to be overreached.

Another important indicator of immunity status is phagocytic activity. A decreased phagocytic function has been associated with intensified training leading to overreaching, with neutrophil activity decreasing independently of unchanged cortisol levels (Robson *et al.*, 1999a). An increase in the production of reactive oxygen species following prolonged, high-intensity exercise and activation of the immune system have been shown to potentially induce an elevation in oxidative stress as reviewed by Bermon *et al.* (2017) in their latest Consensus Statement. In addition, an 80% increase in oxidative stress per cell has been associated with a consequent 20% decrease in phagocytic activity in resting samples in female judoists following a 20-day pre-competition training period (Yaegaki *et al.*, 2007).

It was hypothesized that a 12-day period of intensified training would be stressful and intense enough to induce a state of FOR. The aim of this experimental chapter is to examine the exercise induced plasma and salivary cortisol and testosterone responses to the RPE<sub>treadmill</sub> running bout before and after a 12-day period of intensified training. It is hypothesized that this exercise bout may be a suitable tool to highlight blunted exercise-induced hormonal (certainly testosterone) responses associated with overreaching. Furthermore, this chapter aims to examine the effect of a 12-day training period on immunity. It was hypothesized that after the training period, resting and exercise-induced SIgA concentrations and secretion rate will be reduced. Resting total whole blood leucocyte and PMN counts, and PMN phagocytic activity are also expected to be reduced following the intensified training period compared to before the intensified training period.

## 7.2 Methods

### 7.2.1 Procedures

For this experimental study, 8 healthy young males who exercised at least 3 hours a week visited the laboratories on 17 occasions in total. The participant's anthropometric and physiological characteristics at baseline are outlined in Table 7.1 presented below.

**Table 7.1** Participants' physiological and anthropometric characteristics at baseline.

	<b>Mean <math>\pm</math> SD</b>
Age (years)	21 $\pm$ 5
Height (cm)	178 $\pm$ 5
Body mass (kg)	72.8 $\pm$ 6.6
HR <sub>max</sub> (beats·min <sup>-1</sup> )	187 $\pm$ 8
$\dot{V}O_{2max}$ (mL·kg <sup>-1</sup> ·min <sup>-1</sup> )	59 $\pm$ 6
$v\dot{V}O_{2max}$ (km·h <sup>-1</sup> )	16.6 $\pm$ 2.2
<hr/>	
n = 8	

A familiarisation trial was completed at least three days prior to the start of the study to acquaint the participants with the exercise bout completed in the main experimental trials. Subsequently, each participant completed a main experimental trial on two separate occasions (followed by a  $\dot{V}O_{2max}$  test the day after), i.e. before and after a 12-day period of intensified training. The  $\dot{V}O_{2max}$  tests were completed to examine if the training period had an influence in the fitness level of the participants. During the 12 days of training, all participants completed three different exercise sessions in an intercalated manner. This strategy was used to reduce the monotony during the training period and the exercise sessions were designed as follows:

## Chapter 7: Endocrine and Immune Impairments After Intensified Training

A) A 90-min continuous treadmill-run, subdivided in one block of 70 min at 55% of the individuals'  $\dot{V}O_{2\max}$  and one 20-min block at 75%  $\dot{V}O_{2\max}$ . The  $\dot{V}O_{2\max}$  was calculated as detailed in 3.2.2 in the methods chapter;

B) A 5-km time-trial;

C) A 70-min, self-paced, continuous treadmill-run at a speed corresponding to an RPE of 12 (light) on the 6-20 Borg scale for the first 30 min, at 13 (somewhat hard) during the following 30 min, and at 15 (hard) for the final 10 min.

Each session was completed 4 times over the 12-day period. This training programme was designed to increase the participants' normal training load. All participants completed a training diary during a 12-day period of normal training. During the 12-day intensified-training period, the TRIMP scores increased by 124% (713 to 1598) and the duration of training by 143% (7 h to 17 h), when compared to the normal training period. Participants' body mass, rating of sweat loss, HR and RPE were monitored on every training day and water was provided *ad libitum*. Training diaries were completed to monitor the training sessions completed outside the lab environment during this period. A 24-h food diary was completed the day before the first main experimental trial and replicated in the 24 hours leading up to main trial completed after the training period. Participants were required to abstain from exercise, and alcohol and caffeine intake in the 24 hours preceding each main experimental trial. A schematic presentation of the study design is presented in Figure 7.1.

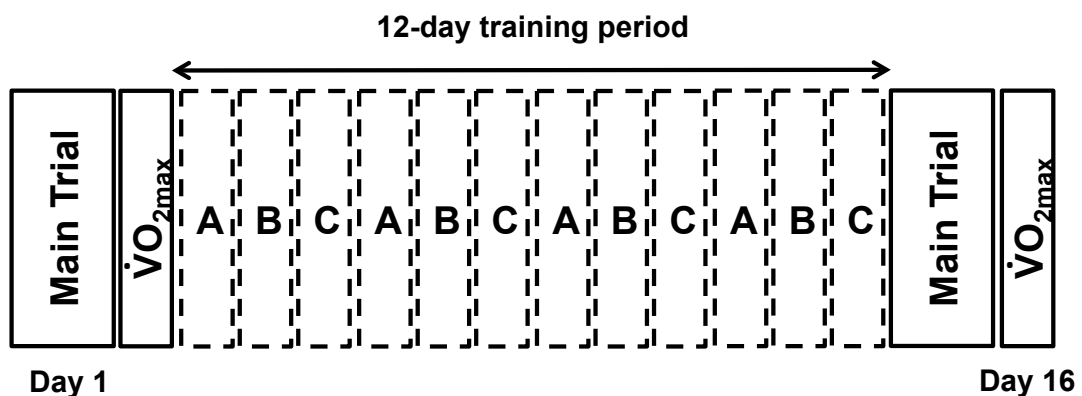
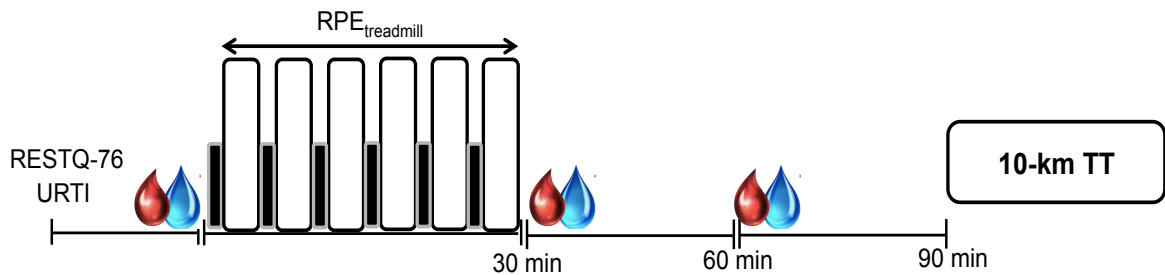


Figure 7.1 Schematic presentation of the study design.

## Chapter 7: Endocrine and Immune Impairments After Intensified Training

All main experimental trials started at 11:30. Participants gave a urine sample and sat down for the remainder 20 minutes. During this period, a RESTQ-76 Sport questionnaire and a URS log using the Jackson score were completed as detailed in the methods section in this Thesis. The 30-min running bout, i.e. the  $RPE_{\text{treadmill}}$ , started at ~12:00. Saliva and blood samples (collection methods detailed in 3.6.1 and 3.7.1, respectively) were collected before, up to 1 minute after, and 30 minutes after the running bout. Saliva samples were collected immediately after blood was drawn. Each main trial ended with the completion of a 10-km time-trial test (10TT), which was started 60 minutes after the end of the running bout. This test was used as a measure of athletic performance. A schematic presentation of this sequence is outlined below. A  $\dot{V}O_{2\text{max}}$  test was undertaken the day after each main trial.



**Figure 7.2** Schematic presentation of each main experimental trial.

### 7.2.2 Maximal Oxygen Uptake Assessment

The protocol used for determination of  $\dot{V}O_{2\text{max}}$  is explained in detail in the section 3.2.2 in this Thesis.

### 7.2.3 Analytical Procedures

All analytical procedures were detailed in Chapter 3. As a reminder, 3-min unstimulated saliva samples and 15 mL of whole blood were drawn Pre-, Post-, and 30 min Post- $RPE_{\text{treadmill}}$  in the main experimental trials. In summary,  $K_3\text{EDTA}$ -treated whole blood samples were used for



## Chapter 7: Endocrine and Immune Impairments After Intensified Training

plasma extraction, and isolation of whole blood leucocytes and PMNs. Total leucocytes were used for subsequent leucocyte phenotyping, and the harvested PMNs were used for examination of phagocytic activity. As a reminder, 50,000 events were acquired for leucocyte phenotyping via flow cytometry, and percentage of monoclonal antibody-stained leucocyte subsets was examined.

Saliva samples were weighed to the nearest milligram and saliva volume was estimated assuming saliva density to be  $1.0 \text{ g}\cdot\text{mL}^{-1}$  (Cole and Eastoe, 1988). From this, saliva flow rate was determined by dividing the sample volume by the collection time, and SIgA secretion rate was subsequently calculated by multiplying the obtained concentration by saliva flow rate.

### 7.2.4 Statistical Analysis

Data were checked for normality (Shapiro-Wilk test) and homogeneity of variance. If normality was not achieved, logarithmic transformations of base 10 were performed on the data prior to re-assessment of normality. When logarithmic data sets violated normality (saliva flow rate, haemoglobin, whole blood leucocyte counts, CD19 B-cells, CD11c<sup>+</sup> dendritic cells and phagocytosis data), multiple Wilcoxon signed rank tests were completed on the original data to compare Pre-, Post-, and 30 min Post-Exercise at Pre- and Post-Training. Logarithmic data sets were used for the examination of salivary cortisol, SIgA,  $\gamma\delta$  T-lymphocytes, CD4<sup>+</sup>/CD8<sup>+</sup> ratio, and NK cells. A one-way (trial) repeated measure ANOVA with Bonferroni adjustments was applied to urine osmolality, RESTQ-76 Sport,  $\dot{V}O_{2\text{max}}$  and 10-km time-trial data. The URS questionnaires were analysed with Student's paired sample *t test* (Pre-Training vs. Post-Training for each scale). A two-way (trial x time) repeated measures ANOVA was used with Bonferroni corrections on all other variables. For all comparisons where the assumption of sphericity was violated, Greenhouse-Geisser corrections were used. When required, Student's paired sample *t tests* were used. The HR and speed data were examined over time at the end

## Chapter 7: Endocrine and Immune Impairments After Intensified Training

of each 1 min and 4 min stages, and average HR and speed for trial (Pre- and Post-Training) were determined taking into account the duration of each stage (i.e. ((average of data in the 1 min stages x 6) + (average of data in the 4 min stages x 24))/30). The presented effect sizes were calculated by hand, by dividing the difference between the means of the groups by the pooled SD (Cohen, 1988). Statistical significance was accepted at  $p < 0.05$ . All data were presented as mean  $\pm$  SD unless stated otherwise.

## 7.3 Results

Post-hoc power analysis demonstrated that the observed power for plasma cortisol and testosterone was 0.457 and 0.980, respectively. The observed power for salivary cortisol and testosterone was 0.550 and 0.694, respectively. The SIgA analyses showed an observed power of 0.637, whereas SIgA secretion rate showed an observed power of 0.414. Phagocytic function analysis showed an observed power of 0.930.

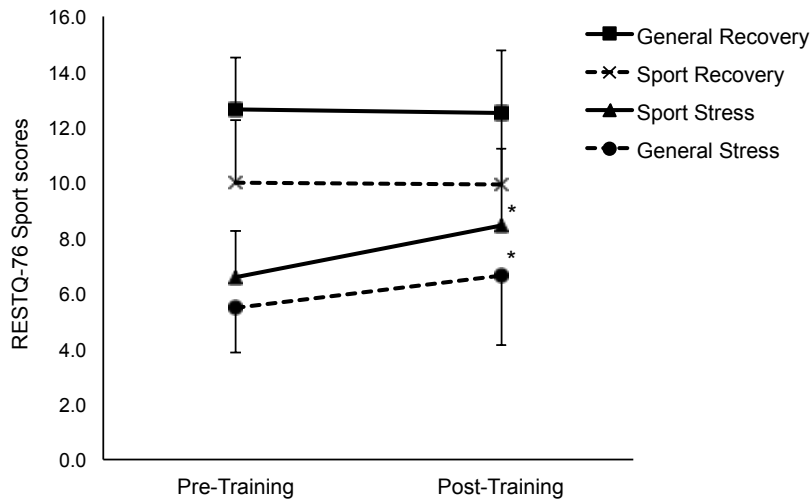
### 7.3.1 Hydration Status

Urine osmolality did not differ across all trials and was  $345 \pm 198$  mOsmol $\cdot$ kg<sup>-1</sup> H<sub>2</sub>O Pre-Training and  $301 \pm 166$  mOsmol $\cdot$ kg<sup>-1</sup> H<sub>2</sub>O Post-Training ( $p > 0.05$ ).

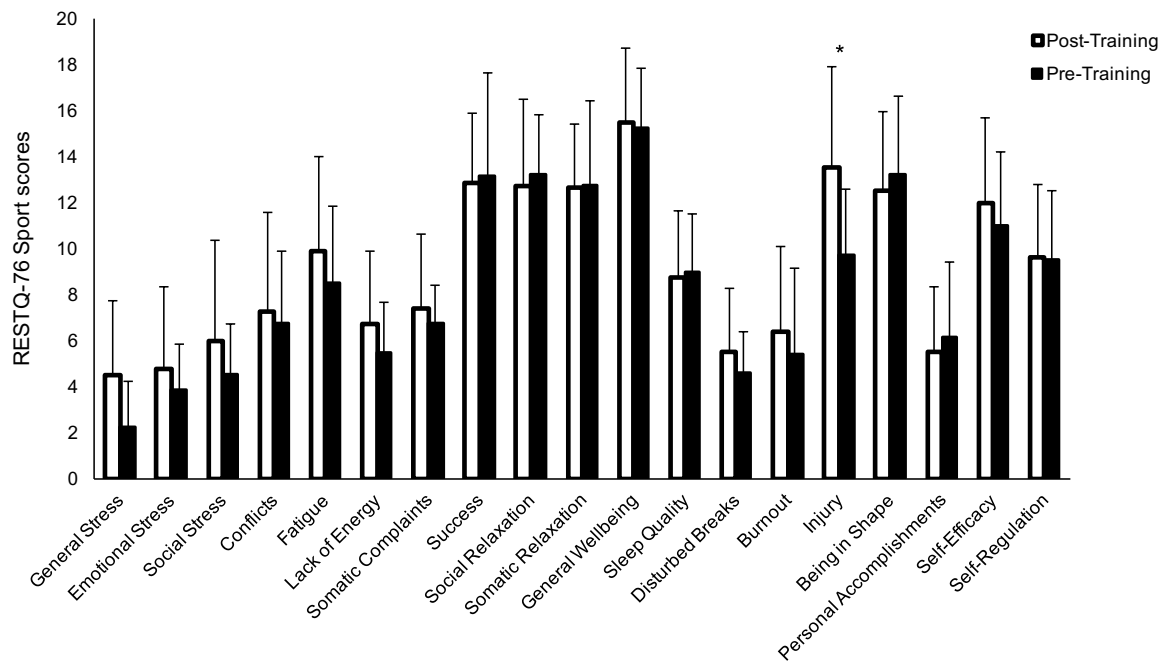
### 7.3.2 Recovery-Stress Questionnaire

There is a trial effect when comparing Pre- to Post-Training in the General Stress ( $F_{1,7} = 7.083$ ,  $p < 0.05$ ) and in the Sport Stress scales ( $F_{1,7} = 7.250$ ,  $p < 0.05$ ), with an increase of these scales at Post-Training, compared to Pre-Training (Figure 7.3). Further examination shows 38% greater scores on the subscale *Injury* at Post-Training ( $p < 0.01$ ) (Figure 7.4).

## Chapter 7: Endocrine and Immune Impairments After Intensified Training



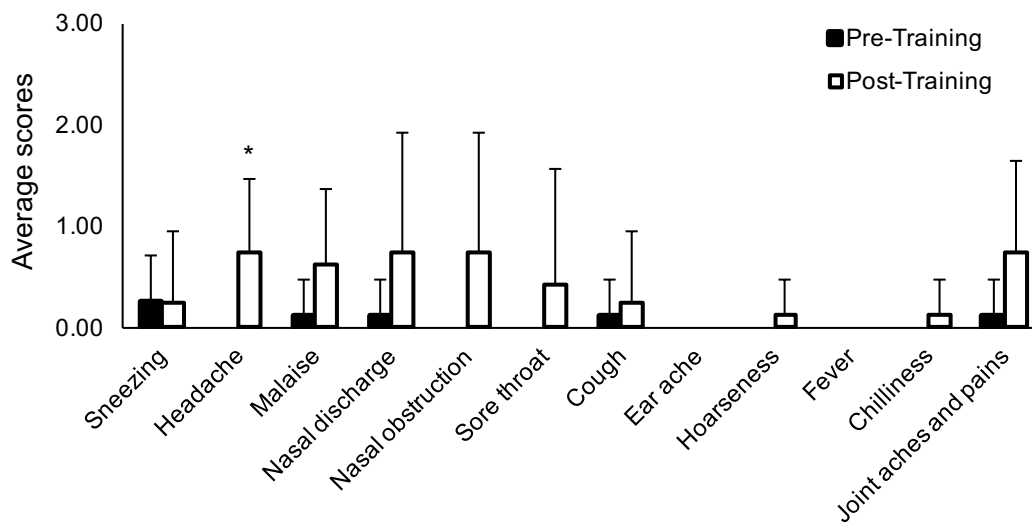
**Figure 7.3** RESTQ-76 Sport scores before and after a 12-day period of intensified training. Values are means  $\pm$  SD. \*Different from Pre-Training ( $p < 0.05$ ).



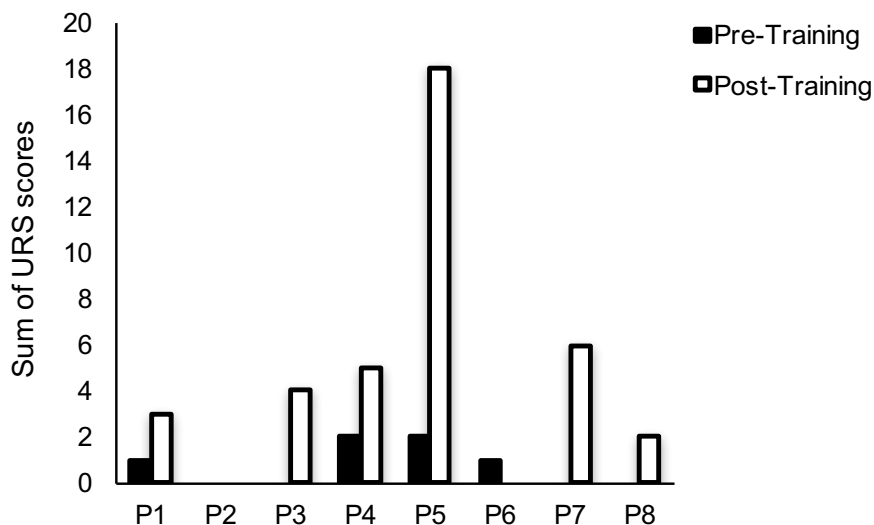
**Figure 7.4** RESTQ-76 Sport scores in all subscales. Values are means  $\pm$  SD. \*Different from Pre-Training ( $p < 0.01$ ).

### 7.3.3 Upper Respiratory Symptoms (URS)

There are no differences in average URS scores when comparing Pre- with Post-Training in any of the URS scales ( $p > 0.05$ ), apart from the *Headaches* scale, in which increased scores were observed Post-Training, compared to Pre-Training ( $p < 0.01$ ) (Figure 7.5). However, 5 individuals have reported increased URS, from which 3 have reported more severe symptoms after the 12 days of intensified training (Figure 7.6).



**Figure 7.5** Average upper respiratory symptoms (URS) scores at Pre- and Post-Training. Values are means  $\pm$  SD. \*Different from Pre-Training ( $p < 0.01$ ).

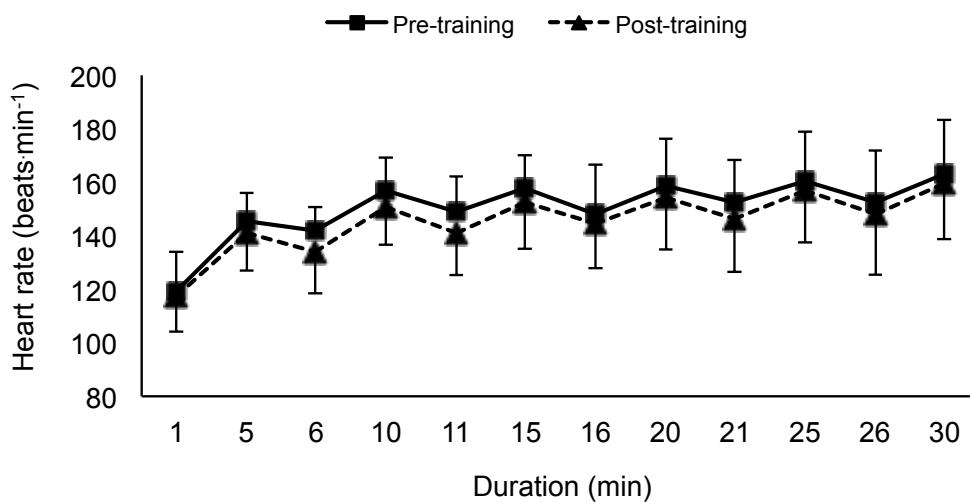


**Figure 7.6** Sum of individual upper respiratory symptoms (URS) scores at Pre- and Post-Training. P# refers to participant number.

### 7.3.4 Physiological Responses to Exercise

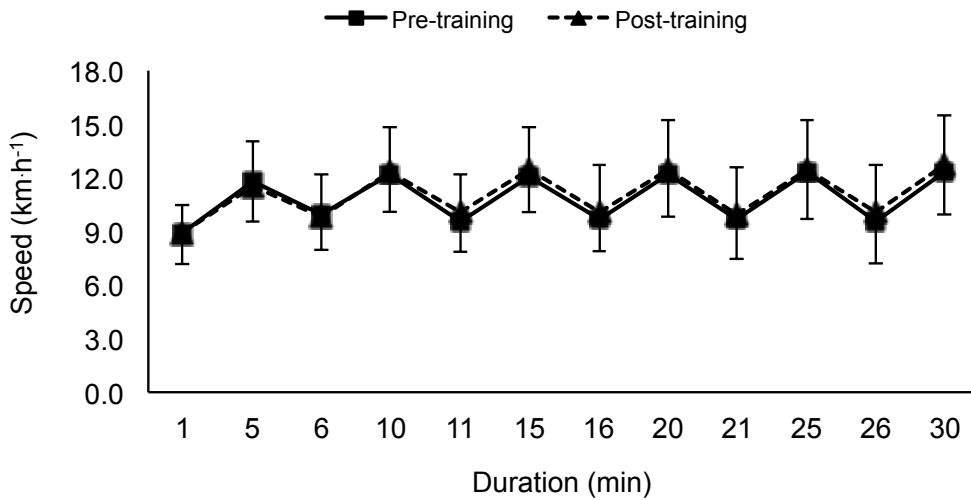
There was no trial effect when comparing HR responses ( $F_{1,7} = 1.326$ ,  $p = 0.29$ ) or speed ( $F_{1,7} = 0.279$ ,  $p = 0.61$ ) across time at Pre- and Post-Training.  $\dot{V}O_{2\max}$  was also unaffected by the training period ( $F_{1,7} = 0.013$ ,  $p = 0.91$ ), being  $59 \pm 6 \text{ mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$  and  $59 \pm 7 \text{ mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$  before and after the 12 days of training, respectively.

Average HR for trial was  $154 \pm 15 \text{ beats}\cdot\text{min}^{-1}$  at Pre-Training and  $150 \pm 18 \text{ beats}\cdot\text{min}^{-1}$  at Post-Training. Heart rate responses to each stage during the  $\text{RPE}_{\text{treadmill}}$  is presented in Figure 7.7.



**Figure 7.7** Heart rate responses during the  $\text{RPE}_{\text{treadmill}}$  at Pre- and Post-Training. Values are means  $\pm$  SD.

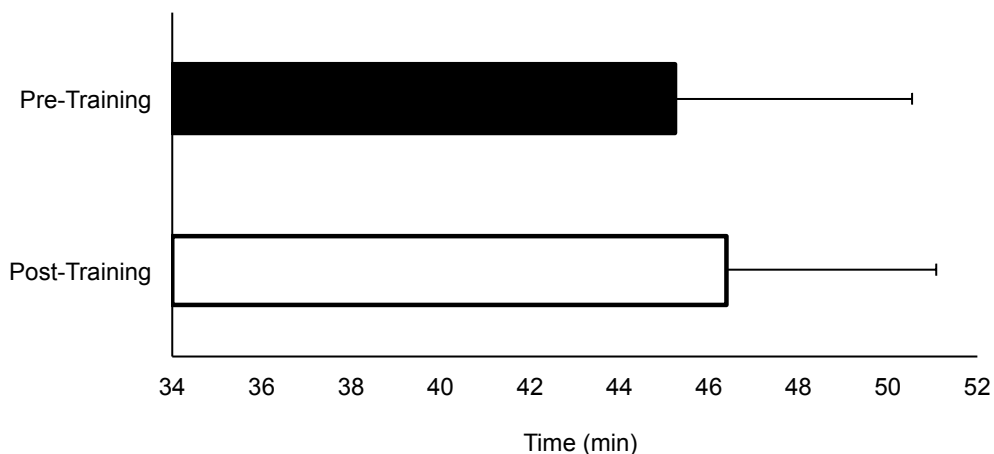
Average speed for trial was  $12.0 \pm 2.9 \text{ km}\cdot\text{h}^{-1}$  at Pre-Training and  $12.2 \pm 2.5 \text{ km}\cdot\text{h}^{-1}$  at Post-Training. Average speed at each stage during the  $\text{RPE}_{\text{treadmill}}$  is presented in Figure 7.8.



**Figure 7.8** Average speed at each stage during the RPE<sub>treadmill</sub> at Pre- and Post-Training. Values are means  $\pm$  SD.

### 7.3.5 10-km Time Trial Performance Test

There was no trial effect when comparing the time to completion in 10-km time-trial at Pre- and Post-Training ( $F_{1,5} = 0.356$ ,  $p = 0.577$ ) (Figure 7.9). Although the difference in time to completion was not significant, time to completion was  $1:21:04 \pm 00:39:39$  (min:s:ms) higher at Post-Training ( $p > 0.05$ ), with ES = 0.3.



**Figure 7.9** Time to completion in 10-km time-trial test. Values are means  $\pm$  SD.

### 7.3.6 Hormonal Responses to Exercise

#### **a) Plasma Cortisol**

No trial effect ( $F_{1,7} = 0.564$ ,  $p = 0.48$ ), or time effect ( $F_{2,6} = 0.553$ ,  $p = 0.09$ ) was found (Figure 7.10). Absolute change from Pre- to Post-Exercise was  $-41.7 \text{ nmol}\cdot\text{L}^{-1}$  (-14%) Pre-Training, and  $31.7 \text{ nmol}\cdot\text{L}^{-1}$  (+15%) Post-Training (Figure 7.11). Plasma cortisol showed to be individualised with individual absolute changes ranging between  $-88.5 \text{ nmol}\cdot\text{L}^{-1}$  and  $+169.5 \text{ nmol}\cdot\text{L}^{-1}$ . The effect size for the plasma cortisol responses was  $\eta^2 = 0.553$ .

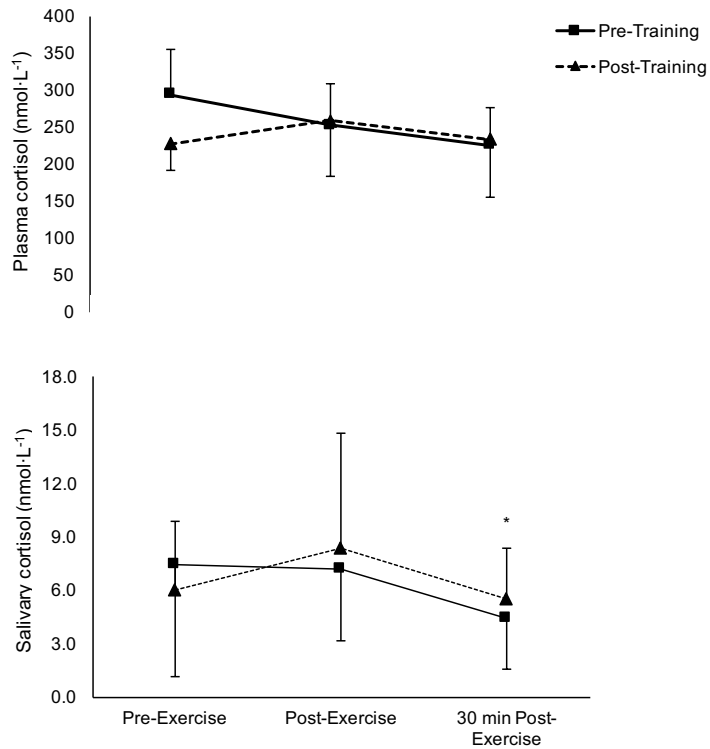
#### **b) Salivary Cortisol**

No trial effect was found ( $F_{1,7} = 0.042$ ,  $p = 0.84$ ). However, a time effect was found ( $F_{2,6} = 6.854$ ,  $p < 0.05$ ), with salivary cortisol decreasing from Post- to 30 min Post-Exercise at Pre-Training (Figure 7.10). Absolute change from Pre- to Post-Exercise was  $-0.5 \text{ nmol}\cdot\text{L}^{-1}$  (0%) Pre-Training, and  $2.3 \text{ nmol}\cdot\text{L}^{-1}$  (+46%) Post-Training (Figure 7.11). Salivary cortisol also showed to be very individualised with absolute changes ranging between  $-8.0 \text{ nmol}\cdot\text{L}^{-1}$  and  $+19.2 \text{ nmol}\cdot\text{L}^{-1}$ . The effect size for the salivary cortisol responses was  $\eta^2 = 0.609$ .

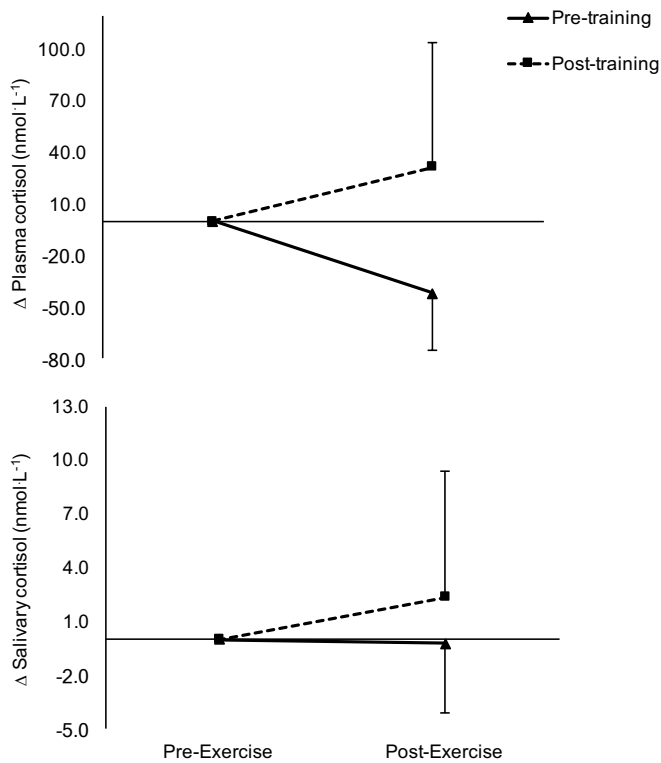
Pearson's correlation coefficient revealed that the exercise-induced salivary cortisol correlated strongly with its plasma measures ( $r = 0.836$ ,  $p = 0.005$ ).



## Chapter 7: Endocrine and Immune Impairments After Intensified Training



**Figure 7.10** Plasma and salivary cortisol at Pre- and Post-Training. Values are means  $\pm$  *SD*.  
\*Different from Pre-Exercise values Pre-Training ( $p < 0.01$ ).



**Figure 7.11** Absolute change from Pre- to Post-Exercise in plasma and salivary cortisol at Pre- and Post-Training. Values are means  $\pm$  *SD*.

**c) Plasma Testosterone**

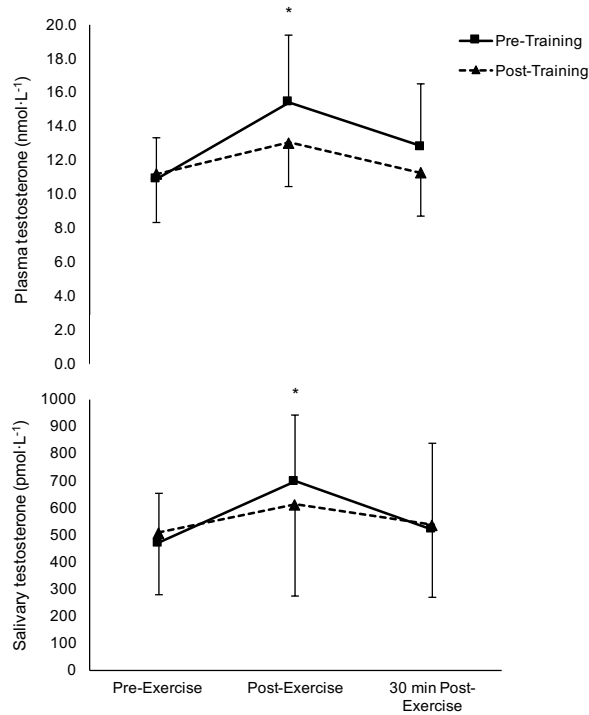
No trial effect was found ( $F_{1,7} = 1.692$ ,  $p = 0.24$ ). However, a time effect was observed ( $F_{2,6} = 16.882$ ,  $p = 0.003$ ), with plasma testosterone acutely elevating from Pre- to Post-Exercise at Pre-Training only (Figure 7.12). Absolute change from Pre- to Post-Exercise decreased from  $4.6 \text{ nmol}\cdot\text{L}^{-1}$  (43%) at Pre-Training to  $1.9 \text{ nmol}\cdot\text{L}^{-1}$  (19%) at Post-Training (Figure 7.13). The effect size for the plasma testosterone responses was  $\eta^2 = 0.849$ .

**d) Salivary Testosterone**

No trial effect was found ( $F_{1,7} = 0.108$ ,  $p = 0.75$ ). However, a time effect was found ( $F_{2,6} = 6.854$ ,  $p < 0.05$ ), with salivary testosterone elevating from Pre- to Post-Exercise at Pre-Training only (Figure 7.12). Absolute change in salivary testosterone decreased from  $227.8 \text{ pmol}\cdot\text{L}^{-1}$  (55%) at Pre-Training to  $105.3 \text{ pmol}\cdot\text{L}^{-1}$  (24%) at Post-Training (Figure 7.13). The effect size for the salivary testosterone responses was  $\eta^2 = 0.683$ .

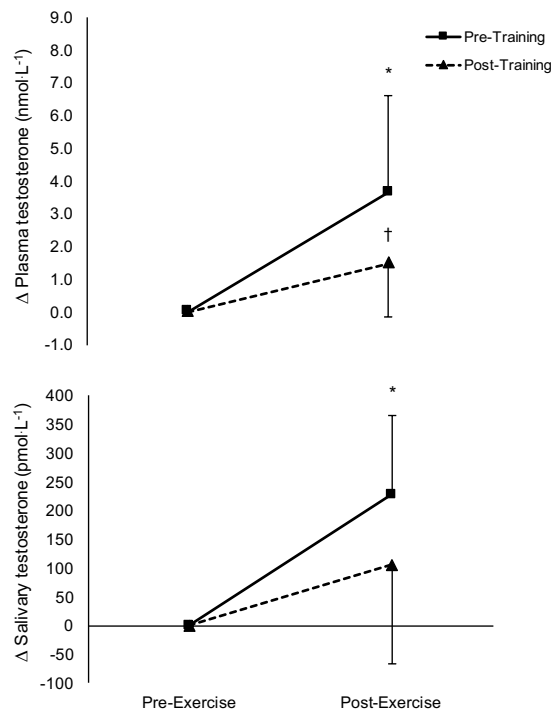
Pearson's correlation coefficient revealed that the exercise-induced salivary testosterone moderately correlated with its plasma measures ( $r = -0.514$ ,  $p = 0.124$ ).

## Chapter 7: Endocrine and Immune Impairments After Intensified Training



**Figure 7.12** Plasma and salivary testosterone at Pre- and Post-Training. Values are means  $\pm$  SD.

\*Different from Pre-Exercise values at Pre-Training ( $p < 0.01$ ).



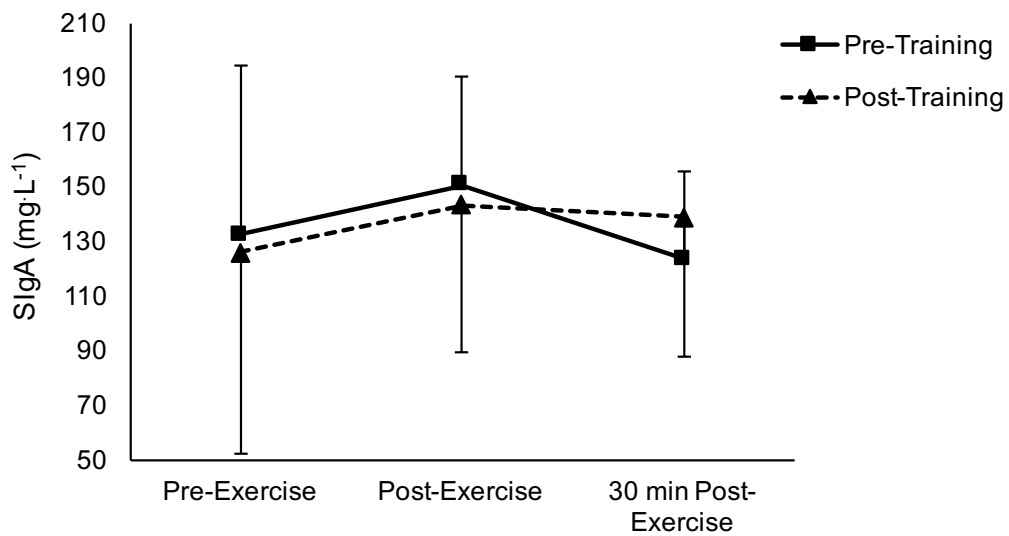
**Figure 7.13** Absolute change from Pre- to Post-Exercise in plasma and salivary testosterone at Pre- and Post-Training. Values are means  $\pm$  SD.

\*Different from Pre-Exercise values at Pre-Training ( $p < 0.01$ ).

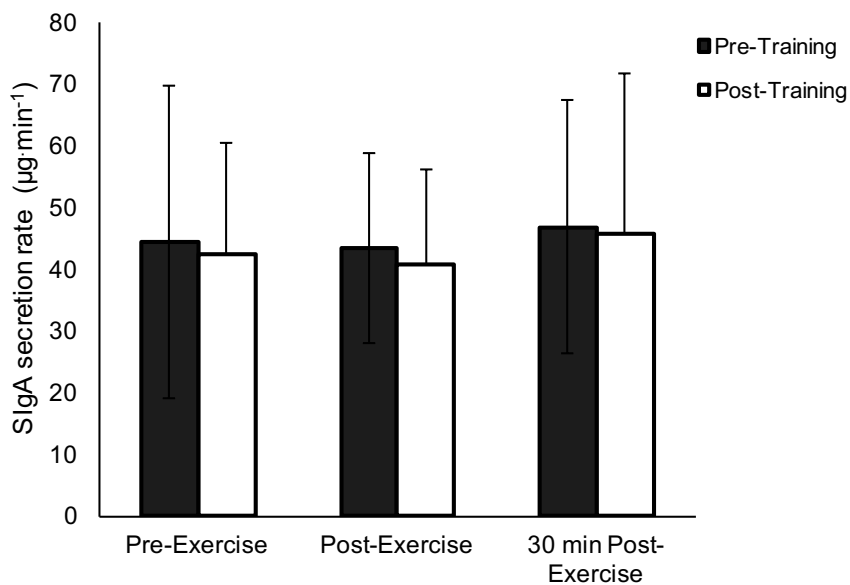
†Different from Pre-Training values ( $p < 0.05$ ).

### 7.3.7 Salivary Immunoglobulin A (SIgA)

No trial ( $F_{1,7} = 0.181$ ,  $p = 0.68$ ) or time effect were observed ( $F_{1,7} = 1.989$ ,  $p = 0.17$ ), with SIgA remaining unchanged at Pre-Training and Post-Training (Figure 7.14). Saliva flow rate and SIgA secretion rate were both unaffected by exercise or training period ( $p > 0.05$  in all). The SIgA secretion rate is presented in Figure 7.15. The effect sizes for the SIgA concentration and secretion rate were  $\eta^2 = 0.655$  and  $\eta^2 = 0.524$ , respectively.



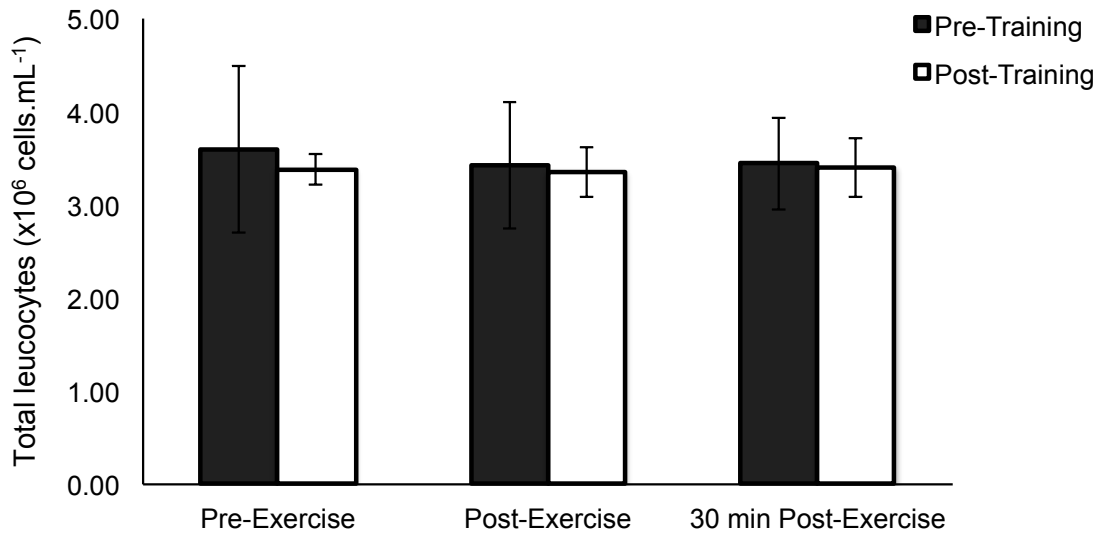
**Figure 7.14** SIgA responses at Pre- and Post-Training. Values are means  $\pm$  SD.



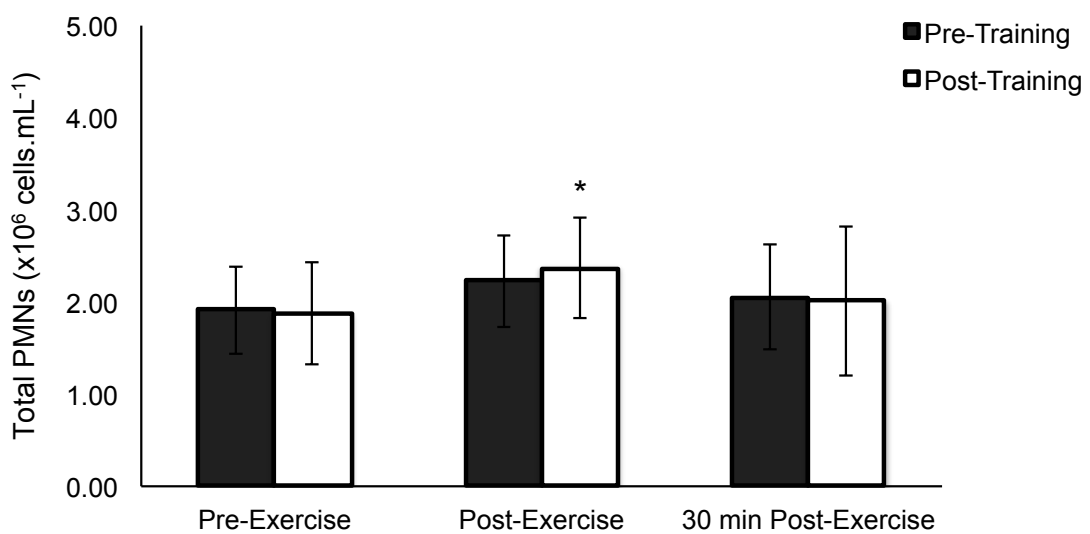
**Figure 7.15** SIgA secretion rate over time at Pre- and Post-Training. Values are means  $\pm$  SD.

### 7.3.8 Total Leucocytes and Polymorphonuclear Leucocytes

Total whole blood leucocytes were unaffected by exercise or the training period ( $p > 0.05$  in all) (Figure 7.16). However, an exercise-induced mobilisation of PMN numbers was seen at Post-Training ( $p < 0.05$ ) (Figure 7.17). Average cell numbers are presented in Table 7.2.



**Figure 7.16** Total whole blood leucocyte counts. Values are means  $\pm$  SD.



**Figure 7.17** Total polymorphonuclear leucocyte (PMN) counts. Values are means  $\pm$  SD.

\*Different from Pre-Exercise values at Post-Training ( $p < 0.05$ ).

### 7.3.9 Leucocyte Phenotyping

The 12-day training period had no effect on any of the leucocyte populations at rest ( $p > 0.05$ ). Yet, exercise had an effect on  $\gamma\delta$  T-lymphocytes by acutely increasing its circulating levels by 3.1% to 9.2%, and on CD19 B-lymphocytes by decreasing its levels from 6.4% to 4.6% at Pre-Training ( $p < 0.05$ ). Post-Training, percentage of circulating CD56 NK cells has acutely elevated ( $p < 0.05$ ).

Haematological parameters (i.e. haemoglobin, haematocrit and plasma volume) did not alter with exercise or training ( $p > 0.05$ ), apart from an exercise-induced increase in haemoglobin observed at Post-Training ( $p < 0.05$ ). Table 7.2 presented below outlines all haematological data, total leucocytes and PMN counts, and leucocyte subset percentages in a 50,000-cell population.

**Table 7.2** Haematological, total and differential leucocyte counts and leucocyte phenotyping at Pre- and Post-Training.

	Pre-Training			Post-Training		
	Pre-Exercise	Post-Exercise	30 min Post	Pre-Exercise	Post-Exercise	30 min Post
<b>General haematology</b>						
Haematocrit (%)	44 ± 1	44 ± 2	43 ± 1	44 ± 1	44 ± 2	42 ± 2
Haemoglobin (g·L <sup>-1</sup> )	152 ± 3	155 ± 4	151 ± 4	148 ± 6	153 ± 6**	151 ± 11
Plasma volume (%)	56 ± 1	56 ± 2	57 ± 1	57 ± 1	56 ± 2	58 ± 2
<b>Leucocyte counts</b>						
Total (x 10 <sup>6</sup> ·mL <sup>-1</sup> )	3.6 ± 0.9	3.4 ± 0.7	3.4 ± 0.5	3.4 ± 0.2	3.4 ± 0.3	3.4 ± 0.3
PMNs (x 10 <sup>6</sup> ·mL <sup>-1</sup> )	1.9 ± 0.5	2.2 ± 0.5	2.1 ± 0.6	1.9 ± 0.5	2.4 ± 0.5**	2.0 ± 0.8
<b>Leucocyte phenotyping (% in 50,000 events)</b>						
γδ T-lymphocytes	3.1 ± 3.8	9.2 ± 14.3*	N/A	8.5 ± 15.1	8.8 ± 15.5	N/A
CD4 <sup>+</sup>	28.9 ± 6.6	23.1 ± 10.9	N/A	27.9 ± 9.1	24.3 ± 8.0	N/A
CD8 <sup>+</sup>	23.8 ± 8.6	21.9 ± 12.8	N/A	19.4 ± 7.1	21.5 ± 8.9	N/A
CD4 <sup>+</sup> /CD8 <sup>+</sup>	1.3 ± 0.3	1.2 ± 0.3	N/A	1.6 ± 0.7	1.3 ± 0.8	N/A
CD19 <sup>+</sup> B-cells	6.4 ± 3.1	4.6 ± 3.0*	N/A	5.0 ± 2.0	4.4 ± 2.2	N/A
CD56 <sup>+</sup> NK cells	1.8 ± 2.3	1.9 ± 2.2	N/A	1.6 ± 1.2	2.4 ± 1.9**	N/A
CD11c <sup>+</sup>	8.3 ± 4.5	9.3 ± 5.1	N/A	5.0 ± 3.5	9.4 ± 7.9	N/A
CD15 <sup>+</sup>	55.7 ± 27.6	46.6 ± 32.6	N/A	55.8 ± 30.5	71.0 ± 22.6	N/A

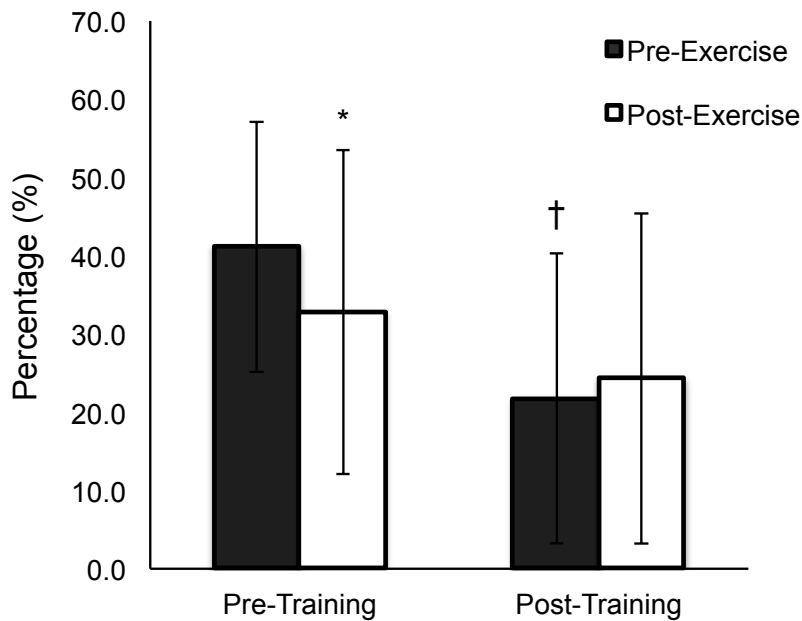
PMN – Polymorphonuclear leucocytes; CD4<sup>+</sup> - Helper T-cells; CD8<sup>+</sup> - Cytotoxic T-cells; CD11c<sup>+</sup> - Dendritic cells; CD15<sup>+</sup> - Neutrophils. Values are means ± SD.

\*Different from Pre-Exercise values at Pre-Training ( $p < 0.05$ ).

\*\*Different from Pre-Exercise values at Post-Training ( $p < 0.05$ ).

### 7.3.10 Phagocytic Function

An exercise-induced 20% decrease in phagocytic function was observed at Pre-Training ( $p < 0.05$ ). In addition, baseline phagocytic function was reduced by 47% Post-Training, when compared to Pre-Training ( $p < 0.05$ ) (Figure 7.18). The effect size for the PMN phagocytic function was  $\eta^2 = 0.742$ .



**Figure 7.18** Phagocytic activity of zymosan-stimulated PMNs at Pre- and Post-Training. Values are means  $\pm$  *SD*.

\*Different from Pre-Exercise values at Pre-Training ( $p < 0.05$ ).

†Different from Pre-Training values ( $p < 0.05$ ).



## 7.4 Discussion

The main findings of this study were that plasma and salivary cortisol were unaffected in response to the RPE<sub>treadmill</sub> when completed before and after the 12-day training period. There was no significant elevation of testosterone (saliva or plasma) to the RPE<sub>treadmill</sub> Post-Training, while the RPE<sub>treadmill</sub> caused an acute elevation in the hormone Pre-Training. Mucosal immunity, measured by SIgA concentration levels and secretion rate analysis was unaffected by exercise or training. However, a tendency for increased URS symptoms was found (Post-Training when compared with Pre-Training). A 197% acute elevation of  $\gamma\delta$  T-lymphocytes was shown Pre-Training. However, unchanged responses were observed Post-Training. CD56 NK cells have acutely elevated by 50% Post-Training, but no change was observed Pre-Training. Baseline phagocytic function significantly decreased by 47% Post-Training, when compared to Pre-Training.

Increased scores of General-, and Sport-related stress were observed after the training period when compared to Pre-Training, with further examination showing higher scores on the 'Fitness/Injury' subscale. These scores were calculated by adding together the responses to the four statements: 'Parts of my body were aching'; 'My muscles felt stiff or tense during performance'; 'I had muscle pain after performance'; and 'I felt vulnerable to injuries'. Hough *et al.* (2013) used a shorter version of the recovery-stress questionnaire used in this study (RESTQ-56) and have reported elevated scores of Burnout and Fatigue in overreached individuals following an 11-day cycling training period. In this study, despite the greater scores in the stress-related scales and perceived susceptibility to injuries, the athletic performance levels were unaffected. A 10-km time-trial test was completed before and after training, and no statistically significant differences were observed in time to completion when comparing Pre- to Post-Training. These results suggest that the increased psychological and social stress levels did not influence athletic performance. However, detailed examination of the data shows that time to completion was 1:21:04  $\pm$  00:39:39 (min:s:ms) higher at Post-Training compared to Pre-

## Chapter 7: Endocrine and Immune Impairments After Intensified Training

Training. Cohen's ES was calculated and was 0.3. Although this ES represents small differences, as proposed by Cohen (1988), it is recognized that a difference of this magnitude may be considered an impactful difference in athletic performance.

No differences in any of the physiological characteristics examined have been observed, with HR and speed in the  $RPE_{\text{treadmill}}$  being similar at Pre- and Post-Training. In addition,  $\dot{V}O_{2\text{max}}$  was also unaffected, which suggests that any differences that may be seen in endocrine or immune markers have not been influenced by physiological stress or fitness level. Furthermore, these findings also suggest that the  $RPE_{\text{treadmill}}$  was of similar intensity before and after training, and consequently the imposed exercise-induced stress was also analogous.

Hough *et al.* (2013) reported a 166% and 21% blunted exercise-induced salivary cortisol and testosterone responses, respectively after completion of 11 days of intensified aerobic training (cycling) in physically active, young and healthy males. It has been proposed that exercise of  $> 60\% \dot{V}O_{2\text{max}}$  for at least 20-30 min is required for cortisol levels to acutely increase (Davis *et al.*, 1991). This present study shows an absolute change of  $-41.7 \text{ nmol}\cdot\text{L}^{-1}$  (-14%) Pre-Training, with individual absolute changes ranging from  $-88.5 \text{ nmol}\cdot\text{L}^{-1}$  to  $+169.5 \text{ nmol}\cdot\text{L}^{-1}$ . A similar response of salivary cortisol was shown, with concentrations being significantly lower at 30-min Post-Exercise, compared to Post-Exercise. Individual acute responses of salivary cortisol range between  $-8.0$  to  $19.2 \text{ nmol}\cdot\text{L}^{-1}$ . However, plasma and salivary cortisol increase from Pre- to Post-Exercise at Post-Training (although not significantly) by 15% and 40%, respectively. As the previous chapter confirmed the reproducible character of cortisol to the  $RPE_{\text{treadmill}}$ , this opposite response of cortisol Post-Training may be due to increased stress levels associated with a period of intensified training. It has been suggested that one of the HPA patterns during exercise and psychological chronic stress may be a hyperresponsiveness of the pituitary gland inducing a higher secretion of ACTH levels with concomitant elevated levels of circulating glucocorticoids (Aguilera, 1994). However, this study shows significantly lower resting levels of plasma cortisol, which have also been previously reported as an effect of a 6-week, 6 days a week training programme in trained cyclists (Lehmann *et al.*, 1993). These lower levels of

## Chapter 7: Endocrine and Immune Impairments After Intensified Training

resting circulating cortisol levels were suggested to be due to an exhaustion of the adrenal gland, observed in professional cyclists over the course of a 3-week tour race (Lucia *et al.*, 2001). However, it has been recognized that examination of resting levels of cortisol may not be ideal when highlighting overreaching/OTS as incongruent results have been reported to date, and therefore should not be taken as a marker of overreaching (see Table 2.2, section 2.3.5 b). As a reminder, the RPE<sub>treadmill</sub> is composed of 6 blocks of 1 min at an RPE of 11 (light), and 6 blocks of 4 min at 15 (hard) continuously intercalated. Although it was designed to include 24 min of hard running exercise, the RPE<sub>treadmill</sub> is a self-paced, running bout based on self-perception of exertion using the 6-20 Borg scale. All participants in this study had a high fitness level ( $59 \pm 6 \text{ mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ ). Detailed examination of the average running speeds in the Pre-Training trial ( $12.0 \pm 2.9 \text{ km}\cdot\text{h}^{-1}$ ) may suggest that perhaps the RPE<sub>treadmill</sub> may not have been stressful enough to induce an acute elevation in plasma and salivary cortisol, as hypothesized based on the findings from Hough *et al.* (2011) and Hough *et al.* (2013). However, plasma and salivary testosterone both showed to significantly elevate in response to the RPE<sub>treadmill</sub> before the training period. This exercise-induced elevation of testosterone has been associated with a concomitant elevation in serum LH, both reported to acutely respond to 20 min of progressive maximal intensity exercise (Cumming *et al.*, 1986). In addition, the absolute change in plasma testosterone presented in this study showed to be blunted with absolute changes of  $4.6 \text{ nmol}\cdot\text{L}^{-1}$  (43%) at Pre-Training, and  $1.9 \text{ nmol}\cdot\text{L}^{-1}$  (19%) at Post-Training. Absolute change in salivary testosterone, although not significantly different, was  $227.8 \text{ pmol}\cdot\text{L}^{-1}$  (55%) at Pre-Training, and  $105.3 \text{ pmol}\cdot\text{L}^{-1}$  (24%) at Post-Training. This is in line with the findings from Hough and colleagues (2013 & 2015), who have reported a 21% and 44% blunted salivary testosterone response following an 11-day period of training and a 10-day training camp in healthy male individuals and male elite triathletes, respectively. Cumming *et al.* (1983) reported that dysfunctional gonad production of testosterone could be associated with high levels of cortisol in the circulation. This was proposed following drug administration- (insulin or hydrocortisone) induced hypercortisolism, resulting in an acute increase of cortisol occurring alongside with a depressed response of testosterone, perhaps due to an inhibitory effect of cortisol upon the LH receptors present in the Leydig cells in the testes. This mechanism may explain the results

found in this present study, where elevated exercise-induced plasma cortisol at Post-Training, was associated with a blunted plasma testosterone response.

It has been proposed that temporary mucosal immune deficiencies are associated with an increased susceptibility to experience URS (Tomasi *et al.*, 1982). This has been supported by several studies (Fahlman and Engels, 2005, Gleeson *et al.*, 1999a, Gleeson *et al.*, 1999b, Novas *et al.*, 2003). In this study, SIgA concentrations and secretion rate were both unaffected by exercise or training. This may be due to the fitness level of the participants who have completed this present study. In a cohort of 54 competitive swimmers a 52% decrease in SIgA was observed after a 2-hour training session (D'Ercole *et al.*, 2016). However, D'Ercole *et al.* (2016) reported no changes in SIgA in 69 non-competitive swimmers following a similar training session. Low levels of SIgA have been suggested to be related to a decreased saliva flow rate and, consequently considered as a potential risk factor in increasing the incidence of URS (Gleeson *et al.*, 2012, Gleeson and Bishop, 2013). Although low levels of SIgA may be expected following periods of intensified training (Fahlman and Engels, 2005, Gleeson *et al.*, 2012, Gleeson *et al.*, 1999b, Neville *et al.*, 2008), research has shown that this is not always the case. Significant increases in resting (peak increase was ~100%) and exercise-induced (peak increase was ~50%) SIgA have been reported over a 12-week training programme in elite swimmers, with the researchers proposing that perhaps the 12-week training period may not have been of sufficient duration to examine the hypothesized low levels of mucosal immunity, specifically SIgA (Gleeson *et al.*, 2000). In contrast, Tiollier *et al.* (2005) have reported unchanged SIgA levels after a 3-week period of high-intensity, military combat-like training. The different findings reported so far, including the results from this present study, may be due to the different training protocols, exercise duration and intensity and the fitness level of participants (Bishop and Gleeson, 2009). These authors also suggest that the reported inconsistent findings may be partially due to the different methods used to report IgA data. SIgA data may be erroneously interpreted if saliva flow rate is not determined, as a decrease in saliva volume has been associated with exercise (Bishop *et al.*, 2000, Walsh *et al.*, 1999). In addition, Bishop and Gleeson (2009) suggest that perhaps determination and examination of SIgA secretion rate

## Chapter 7: Endocrine and Immune Impairments After Intensified Training

may be more appropriate, as it takes into account saliva flow rate, calculated from sample volume and collection time. Concluding, the fluctuations in SIgA in response to exercise and its association with URS in athletes may be a good indicator of mucosal immunity in athletes suspected to be overreached. With this in mind, although no changes in SIgA have been observed in this study, there was a propensity for a slight exacerbation of the symptoms consistent with airway inflammation Post-Training compared with Pre-Training. Individual examination of URS indicates that 5 participants have experienced increased URS, from which 3 have reported considerably higher symptoms at Post-Training when compared to before the training period. However, and despite the more frequent and statistically significant increased headaches experienced by the participants Post-Training, this alone is not a strong indicator of the existence of respiratory infection or illness. Moreover, these data reinforce that extrapolating data from small cohorts may lead to wrongful conclusions, and therefore potentially providing unreliable URS data.

It has been well established that leucocytosis may occur in response to exercise and that the magnitude of leucocytosis (total and differential) will depend on the exercise intensity and duration (McCarthy and Dale, 1988, Sand *et al.*, 2013). Whole blood leucocyte numbers have been reported to markedly elevate by ~65% in healthy male and female individuals in response to a self-paced running test to exhaustion (time to completion was 60-90 min), explained by a 97% significant elevation in PMNs, but no change in lymphocyte numbers (Risøy *et al.*, 2003). Unexpectedly, total leucocyte counts did not alter acutely in response to exercise or when comparing Pre- with Post-Training. To the author of this Thesis knowledge, only one study has reported no changes in total circulating leucocytes in response to exercise. This referred study, was completed on 37 individuals who completed a graded exercise test to exhaustion in a motorized treadmill where the speed and gradient were increased every 3 min (Wardyn *et al.*, 2008). Wardyn *et al.* (2008) report that it is unclear why this inexistent leucocytosis is present and speculate that this irresponsiveness not seen in other studies may be due to the different exercise stimulus used in the different studies. As for the present experimental chapter, total leucocytes were isolated from whole blood samples using a 1x RBC lysis buffer (the incubation

## Chapter 7: Endocrine and Immune Impairments After Intensified Training

times were respected and replicated every time) and the harvested leucocytes were counted using a handheld cell counter. Interestingly, Wardyn *et al.* (2008) reported a significant increase in haemoglobin in the individuals. The present study also shows a significant increase in haemoglobin from Pre-Exercise at Post-Training. Haemoglobin levels have been reported to increase to 30 minutes of cycling at 65%  $\dot{V}O_{2max}$  (Bruunsgaard *et al.*, 1997). This may be due mainly due to haemoconcentration (a decrease in plasma volume in relation to red blood cells) due to intracellular fluid shifts and fluid lost with sweating (Wasserman *et al.*, 1987). When considering the differential blood counts, it was hypothesized that neutrophils would significantly elevate in response to the  $RPE_{treadmill}$  when in a healthy state, and that exercise-induced neutrophilia would be greater following a period of intensified training. Again, no significant changes were found in exercise-induced PMNs from whole blood at Pre-Training, but a modest 16% percentage increase was seen. However, a significant 26% acute elevation was shown after the 12 days of training. PMNs are a class of leucocytes also referred to as granulocytes, and therefore include basophils, eosinophils and neutrophils, the latter accounting for the majority (~90%) of PMNs (Gleeson, 2006a). However, no significant differences were found when examining the percentage of granulocytes expressing CD15, which are likely to be neutrophils. This increase in PMNs at Post-Training may be due to a more marked elevation in eosinophils and basophils numbers than neutrophils. Basophil and eosinophil circulating numbers have been shown to elevate by ~61% and ~36%, respectively in healthy adolescents, following a 6-min cycling bout at 80-90%  $HR_{max}$  (Schwindt *et al.*, 2007). However, neutrophil numbers have been extensively reported to elevate in response to exercise of different duration and intensity (Anane *et al.*, 2009, Nieman *et al.*, 1994, Pedersen, 1991, Ronsen *et al.*, 2001). In this study, although not significantly, the percentage of CD15<sup>+</sup> cells examined in a population of 50,000 cells increased by 27%. Therefore, the increase in PMNs reported after the 12-day period of intensified training in this study may be due to an increase in the circulating numbers of neutrophils as well.

Previous research has shown that an exercise-induced increase in total circulatory leucocytes may be attenuated with regular exercise (Blannin *et al.*, 1996), presumably by reducing the physical stress and fatigue levels provoked by the exercise bout itself (Vrabas *et al.*, 1999).

## Chapter 7: Endocrine and Immune Impairments After Intensified Training

Blannin *et al.* (1996) has demonstrated that endurance trained individuals ( $\dot{V}O_{2max}$  was  $61 \pm 9$  mL·kg<sup>-1</sup>·min<sup>-1</sup>) had lower neutrophil counts when compared to sedentary individuals ( $\dot{V}O_{2max}$  was  $37 \pm 7$  mL·kg<sup>-1</sup>·min<sup>-1</sup>). In addition, albeit neutrophil counts elevated in response to a 15 min submaximal cycling test in both groups, this change was significantly less accentuated in the trained individuals. All participants in this study presented a good fitness level (average  $\dot{V}O_{2max}$  was  $59 \pm 6$  mL·kg<sup>-1</sup>·min<sup>-1</sup>) and exercised regularly. It may be reasonable to suggest that perhaps the modest increase in PMNs at Pre-Training may be due to the fitness level of the participants, and that a greater neutrophilia occurred as an effect of increased stress levels after the 12 days of training. In addition, the unchanged levels of total leucocytes and PMNs at 30 min Post-Exercise may be due to the previously reported return of leucocyte counts to baseline levels within 30 minutes of exercise cessation (McCarthy *et al.*, 1992b).

The resting CD4<sup>+</sup>/CD8<sup>+</sup> ratio has been proposed to fall (although not significantly) in endurance-trained runners who underwent a 3-week period of intensified training (Verde *et al.*, 1992). The present study did not show any changes in either resting, or exercise-induced CD4<sup>+</sup>/CD8<sup>+</sup> ratio. Anane *et al.* (2009) have been the first to identify the cytotoxic  $\gamma\delta$  T-lymphocytes as stress responsive cells (either psychological or physical stress). The authors reported a significant elevation in this cell population to an acute speech stress task (~50% elevation) and to a 16-min cycling test at 85%  $\dot{W}_{max}$  (~200% elevation), in combination with increased numbers of NK (CD3<sup>-</sup>CD56<sup>+</sup>) cells (~200% and ~900%, respectively) and CD8<sup>+</sup> T cells (~100% change to exercise only). No change was reported in B cells or CD4<sup>+</sup> T cells. This present study has shown a significant elevation in cytotoxic  $\gamma\delta$  T-lymphocytes of 197% from Pre- to Post-Exercise at Pre-Training, which is in line with the stress responsiveness firstly reported by Anane *et al.* (2009). Also before the training period, there was an exercise-induced reduction in CD19<sup>+</sup> B-cells of 28%. B-lymphocytes isolated through the CD19 antigen (Funderud *et al.*, 1990) have been shown to be unaffected after a 120 min treadmill-run bout at 65%  $\dot{V}O_{2max}$  (Shek *et al.*, 1995) or after eccentric one-legged exercise (Palmø *et al.*, 1995). In contrast, Bruunsgaard *et al.* (1997) have reported an increased proliferation of CD19<sup>+</sup> cells to 30 minutes

## Chapter 7: Endocrine and Immune Impairments After Intensified Training

of cycling at 65%  $\dot{V}O_{2max}$ . These contrasting findings are inconclusive as to understanding the responsiveness of CD19<sup>+</sup> B-cells to exercise. However, lymphocyte concentrations, including CD19<sup>+</sup>, have been reported to rapidly decrease in the recovery period following high-intensity (>75%  $\dot{V}O_{2max}$ ), long-duration (> 1 h) exercise (McCarthy and Dale, 1988).

Although most lymphocyte populations are known to increase in the circulation in response to strenuous exercise, NK cells have been reported to be the population that shows a more marked increase during/after high-intensity, long-duration exercise (Anane *et al.*, 2009, Gabriel *et al.*, 1991, Pedersen, 1991, Shek *et al.*, 1995). Pre-Training, exercise did not have an effect on NK cell numbers. A study performed on trained and sedentary individuals who underwent an incremental cycling test to exhaustion has reported similar results, with no changes in the absolute number of NK cells (Brahmi *et al.*, 1985). However, the 12 days of training had an effect on the CD56<sup>+</sup> NK cells, with this population increasing by 50% from Pre- to Post-Exercise when compared to Pre-Training. This finding is in contrast with common findings from previous research, showing decreased total CD56 NK cells in elite swimmers being observed over the course of a winter training season (Rama *et al.*, 2013). Considering an elevation in overall stress in the participants occurring during the 12 days, this increased percentage of NK cells in the circulation may be considered as an adaptive effect caused by the training period.

A marked effect that overreaching has in immunity is a decreased neutrophil function. Robson *et al.* (1999a) has observed that athletes who were overreached following a period of intensified training have shown substantial decrements in neutrophil function, independent of cortisol responses. Supporting these findings, Yaegaki *et al.* (2007) have reported an 80% increase in oxidative stress per cell at rest occurring after 20 days of intensified training in preparation for a competition in association with a subsequent 20% decrease in phagocytic activity. The present study shows a 20% decrease in PMNs phagocytic function in response to the RPE<sub>treadmill</sub> occurring at Pre-Training. Similar findings have been shown in 18 healthy male individuals who completed an exercise session at 55%  $\dot{V}O_{2max}$  for up to 3 hours, with indices of



## Chapter 7: Endocrine and Immune Impairments After Intensified Training

decreased neutrophil function regardless of an elevation in circulating neutrophils (Robson *et al.*, 1999b). In parallel with the findings from Robson *et al.* (1999a) and Yaegaki *et al.* (2007) the current study has shown that the 12-day intensified-training period has induced a significant 47% decrease in phagocytic function when at rest. This is an impactful finding as it reinforces the negative effects that intensified periods of training with limited recovery may have in immunity.

In conclusion, it has been hypothesized that the 12 days of training would be stressful enough to induce a state of overreaching in the participants and that this would be supported by a blunted response of plasma and salivary cortisol and testosterone responses to the RPE<sub>treadmill</sub>. Plasma and salivary cortisol did not show to acutely elevate to the RPE<sub>treadmill</sub> at Pre-Training, and these responses were certainly not blunted as hypothesized following on the findings from Hough *et al.* (2013) and Hough *et al.* (2015). As Chapter 6 has confirmed that cortisol and testosterone are reproducible in response to the RPE<sub>treadmill</sub>, the results from this present study may suggest that either the RPE<sub>treadmill</sub> is not intense enough to induce an acute elevation of cortisol concentration levels, which will therefore not show the expected blunted response when overreached, or the cortisol may not be an ideal endocrine marker to be used to highlight overreaching due to its anecdotal nature and intra-individual variability. In contrast, plasma and salivary testosterone have shown to consistently acutely elevate to the RPE<sub>treadmill</sub>, with blunted plasma and considerably lower saliva (although not significant) values occurring at Post-Training. With this in mind, the responses observed in plasma and salivary testosterone and its more reliable character suggest that perhaps this may be a more reliable indicator of a HPG dysfunction associated with overreaching. In addition, albeit SIgA was unaffected by exercise or period of training, increased symptoms consistent with URS have been observed in some participants, with headache symptoms being significantly higher at Post-Training, compared with Pre-Training. Increased scores of general stress and sport stress in the RESTQ-76 Sport after the 12 days also suggest that perhaps the training period may have induced overreaching in the participants. As reported by Anane *et al.* (2009) who for the first time have proposed that  $\gamma\delta$  T-lymphocytes are stress responsive cells, this present study has also shown a significant

## Chapter 7: Endocrine and Immune Impairments After Intensified Training

197% exercise-induced increase in this leucocyte population at Pre-Training. A significant 47% decrease in the capability of PMNs (mainly neutrophils) to undergo phagocytosis suggests that the training period designed in this experimental chapter induced detrimental effects in immunity associated with overreaching. In conclusion, the training period in this study may have induced a state of overreaching. Additionally, testosterone may be a suitable endocrine biomarker to highlight the early incidence of overreaching, and certainly the  $RPE_{\text{treadmill}}$  may be a suitable tool to be used to examine these exercise-induced responses of testosterone when tracking individuals at risk of suffering from overreaching in an attempt to reduce the incidence of OTS. Future studies may require the completion of a recovery period to help confirm overreaching (certainly FOR) was achieved.

## **CHAPTER 8.**

# **General Discussion**

This present chapter discusses the novelty of the data reported and its applications in the field of sport and exercise science. The sequence of studies presented in the experimental Chapters 4 to 7 were designed to examine the effects of short-duration (30 min), high-intensity exercise on the acute plasma and salivary cortisol and testosterone responses (Chapters 4-7), the effects of water consumption in the 10 min period before saliva sampling on the salivary cortisol and testosterone concentration levels (Chapter 4), the confirmation of the reproducibility of salivary hormone responses to a cycling test designed by Hough *et al.* (2011) to highlight overreaching, and the design and development of a self-paced, short-duration running test to be practically used in the field as a tool to highlight the onset of overreaching in an athletic population (Chapters 6 and 7). The final experimental chapter (Chapter 7) also examines the effects of a period of intensified-training (12 days) on several parameters of immunity status. The main aim of this Thesis was to develop a running test capable of highlighting abnormal endocrine responses associated with early stages of overreaching, in an attempt to reduce the incidence of NFOR/OTS. This test was designed to be short in duration (30 min) and self-paced, in order to allow practitioners and exercise scientists to use this tool throughout an athlete's career to track individuals at risk of developing such symptoms associated with overreaching, with no requirement for preliminary testing for determination of exercise intensities. In addition, it was also intended to identify any immune impairments associated with overreaching.

Meeusen *et al.* (2004) were the first to propose that the examination of the exercise-induced responses of particular hormones may be a more reliable method to observe any abnormal endocrine responses associated with overreaching compared to the sole examination of resting concentrations alone. They examined the responses of plasma ACTH, cortisol, GH and prolactin to a double incremental cycle to fatigue test separated by a 4h resting period, before and after a training camp. Meeusen *et al.* (2004) aimed to establish a difference in hormonal responses between healthy trained athletes and those diagnosed as overreached (overreached athletes showed a greater decrease in physical performance compared to a healthy state). Meeusen *et al.* (2004) reported an exercise-induced increase of ACTH of 310% in trained,

healthy athletes to the second of a 2-cycle to exhaustion test separated by 4 hours, which was reduced to 83% when in an overreached state. In contrast, plasma cortisol showed an exercise-induced increase of 45% in trained, healthy athletes to the second cycle to exhaustion test, with a 6% decrease observed when in an overreached state. Despite the interesting findings, the duration of the protocol used in Meeusen *et al.* (2004) may not be practical when used in overreached athletes, or indeed to fit in a busy schedule of healthy athletes and coaching sessions. Following on from these findings, Hough *et al.* (2011) designed a 30-min, high-intensity cycling bout (the 55/80) capable of acutely elevating plasma and salivary cortisol and testosterone responses in male individuals when in a healthy, normal-trained state, with blunting responses occurring after a period of intensified training. In addition, Hough *et al.* (2015) reported a similar blunting in salivary testosterone, but not in salivary cortisol in elite male triathletes following a 10-day training camp. The data presented in Chapter 5 in this Thesis suggest that the variability examined in both hormones to the 55/80 are within their normal hormonal variability. To support this inference, the blunted responses reported by Hough *et al.* (2013) exceed the intra-individual variability found for cortisol and testosterone. Therefore, it is reasonable to suggest that the 55/80 cycle bout could highlight blunted cortisol and testosterone that will not be influenced by the normal random variability in this hormone measurement.

Despite the usefulness of the 55/80 developed by Hough *et al.* (2011, 2013 & 2015) and the reproducible character of salivary cortisol and testosterone to this exercise bout, as examined in Chapter 5 in this Thesis, the 55/80 may not be an ideal tool to be used on an athlete who is not accustomed or familiar with cycling exercise. Therefore, Chapter 6 focused on designing a similar intensity and duration treadmill-running bout that could be readily applied to a running population. As with the 55/80 cycling protocol it was necessary to examine the reproducibility of plasma and salivary cortisol and testosterone responses to the treadmill bout. The running bout designed was self-paced and exercise intensities were based on the 6-20 Borg scale. This running test, the  $RPE_{\text{treadmill}}$ , was designed to replicate the effects of the 55/80 cycle bout developed by Hough *et al.* (2011) on the hormones examined. As referred in Chapter 6, an acute plasma and salivary cortisol elevation in response to the  $RPE_{\text{treadmill}}$  was not observed.

Importantly during a resting trial completed in this study both plasma and salivary cortisol decreased significantly over time. This finding indicates that the exercise-induced responses of cortisol to the  $RPE_{\text{treadmill}}$  have been influenced by the diurnal variation of this hormone perhaps leading to the lower than expected hormone responses found. However, it cannot be discounted that the  $RPE_{\text{treadmill}}$  simply was not stressful enough to induce an acute elevation in cortisol concentration levels. Although this running bout (30 min in duration) was designed so the athletes completing it run for 24 min at an RPE of 15 (hard), this study did not examine if these intensities would be equivalent to an exercise intensity sufficient to induce an acute elevation in this hormone, as described by Davies and Few (1973). With this in mind, another important challenge of measuring cortisol concentrations is its individualised and variable character, and therefore, the data from this Thesis proposes that this may not be an ideal endocrine biomarker to be used to highlight acute effects of exercise. As reported in Chapter 6, the absolute changes to the  $RPE_{\text{treadmill}}$  ranged from -40% to +304% (plasma cortisol) and -55% to +370% (salivary cortisol). In contrast, the low intra-individual variability observed in plasma and salivary testosterone may give an indication of the more appropriate usefulness of this hormone as a biomarker to highlight acute changes to physical stressors. The intra-individual variation in plasma and salivary testosterone was  $12 \pm 9\%$  and  $15 \pm 10\%$ , respectively. Despite the  $CV_i$  of plasma and salivary testosterone responses to the  $RPE_{\text{treadmill}}$  are slightly above the 12.6% and 11.8% described by Maes *et al.* (1997) and Sartorius *et al.* (2012), respectively, these researchers have examined the intra-individual variability of these hormones at rest. The  $CV_i$  presented in this present study include the exercise-induced responses and for this reason, the  $CV_i$  may need to be adjusted. At first examination of the responses of cortisol and testosterone, it was suspected that any differences in the exercise-induced response of testosterone or cortisol could be due to an individual's ventilatory or lactate thresholds. A 40-week cycling training programme has elicited a 12.6% improve in individual's anaerobic threshold (Fellmann *et al.*, 1985), with these researchers reporting that this increase in anaerobic threshold post-training, occurred in parallel with an elevation in the exercise-induced responses of cortisol and testosterone, when compared to before training, with no alteration in the baseline concentrations. These findings support this present study's speculative findings that the

differences in the individual exercise-induced hormonal responses may be due to the individual anaerobic thresholds. Despite the inconclusive findings regarding the exercise-induced responses of cortisol to the RPE<sub>treadmill</sub>, this Thesis proposes that the RPE<sub>treadmill</sub> elicits reproducible plasma and salivary cortisol and testosterone responses when completed on different occasions. It is, however, unlikely that this exercise tool will highlight blunted cortisol responses following a period of intensified training. However, both plasma and salivary testosterone elevate in response to the RPE<sub>treadmill</sub> when in a normal trained state. Therefore, the data from this Thesis support the suitability of the RPE<sub>treadmill</sub> as an appropriate tool to induce a reproducible acute elevation of plasma and salivary testosterone when in a healthy (non-overreached) state. However, the use of a control, resting trial is highly advised not only for determination of the individualised exercise-induced responses, but also due to the influence diurnal variation is known to have in both hormones.

Chapter 7 has subsequently examined the effects of a 12-day period of intensified training on both the plasma and salivary cortisol and testosterone responses to the RPE<sub>treadmill</sub> bout. The results from this experimental chapter suggest that the 12 days of training have increased the overall stress levels in the participants, which was supported by the increased scores of general and sport-specific stress found on examination of the recovery-stress questionnaire, which was observed alongside an exercise-induced blunted response of testosterone in both the circulation and in saliva. These results are in line with previous research that reported a decrease in HPG activity following a period of intensified training (Hough *et al.*, 2013, Hough *et al.*, 2015, Lucia *et al.*, 2001). This downregulation of the HPG activity may be explained by the findings of Cumming *et al.* (1983). These authors suggested that high levels of circulating cortisol were potentially associated with a dysfunctional testosterone production following drug-induced hypercortisolism resulting in an inhibition of the LH receptors in the Leydig cells. The impactful reduction in physical performance and increased fatigue levels, lead the researcher to consider that the training period was stressful enough to induce a state of overreaching. These data, together with the observed blunted exercise-induced plasma and salivary testosterone after the training period direct to a recommendation to implement the RPE<sub>treadmill</sub> bout as an appropriate

exercise test that may be used to highlight any dysfunction in the HPG axis during overreaching, in an attempt to avoid more prolonged states of maladaptation. However, the RPE<sub>treadmill</sub> bout may not be stressful enough to induce an acute elevation in plasma or salivary cortisol levels, and therefore, should future researchers wish to use this tool to examine acute changes in the latter hormone, it may be appropriate to design and develop a similar duration, but more intense running protocol. Following in depth examination of the data in this Thesis and the existing literature, it is hypothesised that perhaps increasing the intensity on the 4-min stage to 17 on the RPE Borg scale may be more appropriate to significantly witness an elevation in cortisol levels in plasma and saliva.

One of the main findings of Chapter 7 was that baseline granulocyte phagocytic function was impaired by 47% following the 12 days of intensified training. These results are in line with the results from Robson *et al.* (1999a) and Robson *et al.* (1999b) who reported decreased neutrophil function in overreached endurance athletes, and from Yaegaki *et al.* (2007) who have observed a decreased phagocytic function in female judoists after a pre-competition training programme. Yaegaki *et al.* (2007) proposed that this decreased phagocytic function may be due to increased levels of oxidative stress provoked by an elevation in the production of reactive oxygen species. Future directions should perhaps focus on understanding the molecular mechanism leading to the reduction in phagocytic function, potentially due to an increased oxidative stress, aiming to highlight early signs of the onset of a decrement in phagocytic function, in attempt to avoid it.

In addition, future directions also lead to the examination of  $\gamma\delta$  T-lymphocytes as stress responsive cells. An impactful increase in the mobilisation of this population has been observed firstly and solely by Anane *et al.* (2009), with Chapter 7 being, to this Thesis' author's knowledge, the second experimental study to focus on this cell subset in response to a physical stress. An impactful acute elevation in the  $\gamma\delta$  T-lymphocyte numbers when in a healthy state was presented in this Thesis, with markedly elevated baseline numbers being observed after 12 days of intensified exercise training. This may suggest that  $\gamma\delta$  T-lymphocytes will acutely



elevate in response to stress when in a healthy state only, and that following a period of excessive exercise or chronic stress this population mobilisation will be greater. This current finding may be presented as novel data, with potential for future applications not only in sports performance, but also and especially in the medical field.

This Thesis provides compelling evidence that a 12-day period of intensified training with limited recovery may develop symptoms associated with overreaching, which is in turn associated with increased overall stress levels (psychological, social and physiological), a dysfunction of endocrine, hypothalamic-pituitary axes, especially the HPG axis, and decreased immunity, supported by a decreased capacity of PMNs (mainly neutrophils) for phagocytosis. This Thesis also suggests that testosterone may be a reliable endocrine biomarker to be examined when tracking individuals on the edge of overreaching, and that the  $RPE_{\text{treadmill}}$  may be a suitable tool to be used to examine testosterone responses in an attempt to avoid the incidence of overreaching. The  $RPE_{\text{treadmill}}$  is a short-duration, high-intensity and self-paced treadmill-running test that elicits reproducible hormonal responses and can be practically used in the field by practitioners willing to highlight an early stage overreaching, with no need for preliminary testing for determination of individual exercise intensities. It may be important to also assess the usability of the  $RPE_{\text{treadmill}}$  in a population of female athletes, to determine if the findings in this Thesis would be applicable to this population as well, would the expectation that similar results would be found. However, a disadvantage of using the present exercise test is the cost of hormonal and immune markers analysis and the need for highly specialised staff, and therefore there are a number of variables that are recommended as useful when tracking overreaching in a non-elite population. These recommendations include the use of time-trial tests to track for athletic performance decrements (Halsen *et al.*, 2002, Meeusen *et al.*, 2013), recovery-stress questionnaires and URS logs to track for psychological distress, physical wellbeing and the onset of respiratory illness (Meeusen *et al.*, 2013), and eventually examination of the maximal and resting heart rate and heart rate variability (HRV) of the individuals (Makivić, Nikić and Willis, 2013) as preventive measures to diagnose early stage overreaching.

## References

- Aderem, A. & Underhill, D. M. (1999) 'Mechanisms of phagocytosis in macrophages', *Annual Review of Immunology*, 17, 593-623.
- Aguilera, G. (1994) 'Regulation of pituitary ACTH secretion during chronic stress', *Frontiers in Neuroendocrinology*, 15, 321-350.
- Ahn, R. S., *et al.* (2007) 'Salivary cortisol and DHEA levels in the Korean population: age-related differences, diurnal rhythm, and correlations with serum levels', *Yonsei Medical Journal*, 48, 379-388.
- Akana, S. F., *et al.* (1992) 'Feedback and facilitation in the adrenocortical system: unmasking facilitation by partial inhibition of the glucocorticoid response to prior stress', *Endocrinology*, 131, 57-68.
- Alberts, B., *et al.* (2002) *Molecular Biology of the Cell*, New York: Garland Science.
- Alghadir, A. H., Gabr, S. A. & Aly, F. A. (2015) 'The effects of four weeks aerobic training on saliva cortisol and testosterone in young healthy persons', *Journal of Physical Therapy Science*, 27, 2029-2033.
- Ali, M. A., *et al.* (2013) 'Effects of dehydration and blockade of angiotensin II AT1 receptor on stress hormones and anti-oxidants in the one-humped camel', *BioMed Central Veterinary Research*, 9, 232.
- Allsop, P., *et al.* (1992) 'Intrasplenic blood cell kinetics in man before and after brief maximal exercise', *Clinical Science*, 83, 47-54.
- Almeida, D. M., Piazza, J. R. & Stawski, R. S. (2009) 'Interindividual differences and intraindividual variability in the cortisol awakening response: an examination of age and gender', *Psychology and Aging*, 24, 819-27.
- Amir, Y., Edward, O. & Utpal, B. (2008) 'A protocol for in vivo detection of reactive oxygen species', *Protocol Exchange*.

- Anane, L. H., *et al.* (2009) 'Mobilization of gammadelta T lymphocytes in response to psychological stress, exercise, and beta-agonist infusion', *Brain, Behaviour and Immunity*, 23, 823-829.
- Aps, J. K. & Martens, L. C. (2005) 'Review: the physiology of saliva and transfer of drugs into saliva', *Forensic Science International*, 150, 119-131.
- Argus, C. K., *et al.* (2009) 'Changes in strength, power, and steroid hormones during a professional rugby union competition', *The Journal of Strength & Conditioning Research*, 23, 1583-1592.
- Argus, C. K., *et al.* (2012) 'Effects of two contrast training programs on jump performance in rugby union players during a competition phase', *International Journal of Sports Physiology and Performance*, 7, 68-75.
- Armstrong, L. E. & VanHeest, J. L. (2002) 'The unknown mechanism of the overtraining syndrome: clues from depression and psychoneuroimmunology. / Le mecanisme inconnu du syndrome de surentrainement: cle de la depression et de l' immunologie psycho-neuronale', *Sports Medicine*, 32, 185-209.
- Atkinson, G. & Nevill, A. M. (1998) 'Statistical Methods For Assessing Measurement Error (Reliability) in Variables Relevant to Sports Medicine', *Sports Medicine*, 26, 217-238.
- Badrick, E., *et al.* (2008) 'The relationship between alcohol consumption and cortisol secretion in an aging cohort', *The Journal of Clinical Endocrinology & Metabolism*, 93, 750-757.
- Badrick, E., Kirschbaum, C. & Kumari, M. (2007) 'The relationship between smoking status and cortisol secretion', *The Journal of Clinical Endocrinology & Metabolism*, 92, 819-824.
- Bagger, M., Petersen, P. H. & Pedersen, P. K. (2003) 'Biological variation in variables associated with exercise training', *Internal Journal of Sports Medicine*, 24, 433-440.
- Baj, Z., *et al.* (1994) 'Immunological status of competitive cyclists before and after the training season', *International Journal of Sports Medicine*, 15, 319-324.
- Baker, D. (2001) 'The effects of an in-season of concurrent training on the maintenance of maximal strength and power in professional and college-aged rugby league football players', *The Journal of Strength & Conditioning Research*, 15, 172-177.

- Baker, H., *et al.* (1976) 'Changes in the pituitary-testicular system with age', *Clinical Endocrinology*, 5, 349-372.
- Balsalobre-Fernández, C., Tejero-González, C. M. & del Campo-Vecino, J. (2014) 'Relationships between training load, salivary cortisol responses and performance during season training in middle and long distance runners', *PLoS One*, 9, e106066.
- Banister, E. (1991) Modeling elite athletic performance. *In*: MACDOUGALL, J. D., WENGER, H. A. & GREEN, H. J. (eds.) *Physiological Testing of Elite Athletes*. Champaign, Illinois: Human Kinetics.
- Barnes, P. J. (1998) 'Anti-inflammatory actions of glucocorticoids: molecular mechanisms', *Clinical Science*, 94, 557-572.
- Barrett, K. E., Barman, S. M. & Boitano, S. (2005) *Ganong's Review of Medical Physiology*. New Delhi: McGraw Hill, 2010.
- Barrett, K. E., *et al.* (2010) Immunity, Infection and Inflammation. *Ganong's Review of Medical Physiology*. 23rd ed.: The McGraw Hill Medical.
- Beato, M. (1989) 'Gene regulation by steroid hormones', *Cell*, 56, 335-344.
- Bellanti, J. A. (1978a) Anatomic organization of the immune system. *In*: BELLANTI, J. A. (ed.) *Immunology II*. Philadelphia: W. B. Saunders Company.
- Bellanti, J. A. (1978b) Introduction to Immunology. *In*: BELLANTI, J. A. (ed.) *Immunology II*. Philadelphia: W. B. Saunders Company.
- Bellanti, J. A. & Dayton, D. H. (1975) *The Phagocytic Cell in Host Resistance*, New York: Raven Press.
- Bendelac, A., Bonneville, M. & Kearney, J. F. (2001) 'Autoreactivity by design: innate B and T lymphocytes', *Nature Reviews Immunology*, 1, 177-186.
- Bermon, S., *et al.* (2017) 'Consensus Statement: Immunonutrition and Exercise', *Exercise Immunology Review*, 23, 8-50.
- Bertagna, X., *et al.* (1994) 'The combined corticotropin-releasing hormone/lysine vasopressin test discloses a corticotroph phenotype', *The Journal of Clinical Endocrinology & Metabolism*, 79, 390-394.

- Beutler, B. & Rietschel, E. T. (2003) 'Innate immune sensing and its roots: the story of endotoxin', *Nature Reviews Immunology*, 3, 169-176.
- Bhasin, S., *et al.* (1996) 'The effects of supraphysiologic doses of testosterone on muscle size and strength in normal men', *New England Journal of Medicine*, 1996, 1-7.
- Billat, V. L., *et al.* (2001) 'Physical and training characteristics of top-class marathon runners', *Medicine & Science in Sports & Exercise*, 33, 2089-2097.
- Bird, S. & Davidson, R. (1997) *Guidelines for the Physiological Testing of Athletes*, Leeds: British Association of Sport and Exercise Sciences.
- Birrer, D., *et al.* (2013) 'Prevalence of non-functional overreaching and the overtraining syndrome in Swiss elite athletes', *Schweiz Z Sportmedizin Sporttraumatol*, 61, 23-29.
- Bishop, N. & Gleeson, M. (2009) 'Acute and chronic effects of exercise on markers of mucosal immunity', *Frontiers in Bioscience*, 14, 4444-4456.
- Bishop, N. C. (2012) 'Overcoming microbial hurdles: keeping the Olympics infection-free', *Future Microbiology*, 7, 913-915.
- Bishop, N. C. (2013) The influence of exercise on infection risk. *In*: GLEESON, M., BISHOP, N. C. & WALSH, N. (eds.) *Exercise Immunology*. Oxon, UK: Routledge.
- Bishop, N. C., *et al.* (2000) 'Carbohydrate and fluid intake affect the saliva flow rate and IgA response to cycling', *Medicine and Science in Sports and Exercise*, 32, 2046-2051.
- Bishop, N. C., *et al.* (2004) 'No effect of fluid intake on neutrophil responses to prolonged cycling', *Journal of Sports Sciences*, 22, 1091-8.
- Blannin, A. K., *et al.* (1996) 'Effects of submaximal cycling and long-term endurance training on neutrophil phagocytic activity in middle aged men', *British Journal of Sports Medicine*, 30, 125-129.
- Bloom, S., *et al.* (1976) 'Differences in the metabolic and hormonal response to exercise between racing cyclists and untrained individuals', *The Journal of Physiology*, 258, 1.
- Bompa, T. O. (1996) 'Variations of Periodization of Strength', *Strength & Conditioning Journal*, 18, 58-61.
- Bompa, T. O. & Haff, G. G. (2009) *Periodization: Theory and Methodology of Training*: Human Kinetics Publishers.

- Borg, G. (1970) 'Perceived exertion as an indicator of somatic stress', *Scandinavian Journal of Rehabilitation Medicine*, 2, 92.
- Born, J., *et al.* (1999) 'Timing the end of nocturnal sleep', *Nature*, 397, 29-30.
- Bonsignore, M. R. *et al.* (2001) 'Airway inflammation in nonasthmatic amateur runners', *American Journal of Physiology - Lung Cellular and Molecular Physiology*, 281(3), pp. L668–L676.
- Bonsignore, M. R. *et al.* (2003) 'Increased airway inflammatory cells in endurance athletes: What do they mean?', *Clinical and Experimental Allergy*, pp. 14–21. doi: 10.1046/j.1365-2222.2003.01557.x.
- Bove, A. (1989) 'Hormonal responses to acute and chronic exercise', *Physiology*, 4, 143-146.
- Brahmi, Z., *et al.* (1985) 'The effect of acute exercise on natural killer-cell activity of trained and sedentary human subjects', *Journal of Clinical Immunology*, 5, 321-328.
- Branden, C. & Tooze, J. (1993) 'Introduction to Protein Structure, Garland, New York, 1991', *Search PubMed*, 161-177.
- Brandenberger, G., *et al.* (1986) 'Vascular fluid shifts and endocrine responses to exercise in the heat', *European Journal of Applied Physiology and Occupational Physiology*, 55, 123-129.
- Bruunsgaard, H., *et al.* (1997) 'Exercise-induced increase in serum interleukin-6 in humans is related to muscle damage', *The Journal of Physiology*, 499, 833.
- Budd, S. L., Castilho, R. F. & Nicholls, D. G. (1997) 'Mitochondrial membrane potential and hydroethidine-monitored superoxide generation in cultured cerebellar granule cells', *FEBS Letters*, 415, 21-24.
- Budgett, R. (1998) 'Fatigue and underperformance in athletes: the overtraining syndrome', *British Journal of Sports Medicine*, 32, 107-110.
- Burke, H. M., *et al.* (2005) 'Depression and cortisol responses to psychological stress: a meta-analysis', *Psychoneuroendocrinology*, 30, 846-856.

- Cadore, E. L., *et al.* (2012) 'Hormonal responses to concurrent strength and endurance training with different exercise orders', *The Journal of Strength & Conditioning Research*, 26, 3281-3288.
- Cassatella, M. A. (1995) 'The production of cytokines by polymorphonuclear neutrophils', *Immunology Today*, 16, 21-26.
- Chan, S. & Debono, M. (2010) 'Replication of cortisol circadian rhythm: new advances in hydrocortisone replacement therapy', *Therapeutic Advances in Endocrinology and Metabolism*, 1, 129-38.
- Chard, T. (1990) *Introduction to Radioimmunoassay and Related Techniques*, Amsterdam: Elsevier.
- Chen, Y.-C., Huang, Y.-L. & Huang, B.-M. (2005) 'Cordyceps sinensis mycelium activates PKA and PKC signal pathways to stimulate steroidogenesis in MA-10 mouse Leydig tumor cells', *The International Journal of Biochemistry & Cell Biology*, 37, 214-223.
- Chevront, S. N., Carter, R. & Sawka, M. N. (2003) 'Fluid balance and endurance exercise performance', *Current Sports Medicine Reports*, 2, 202-208.
- Chicharro, J. L., *et al.* (1998) 'Saliva composition and exercise', *Sports Medicine*, 26, 17-27.
- Cicchetti, D. V. (1994) 'Guidelines, criteria, and rules of thumb for evaluating normed and standardized assessment instruments in psychology', *Psychological Assessment*, 6, 284.
- Cicchetti, D. V. & Sparrow, S. A. (1981) 'Developing criteria for establishing interrater reliability of specific items: applications to assessment of adaptive behavior', *American Journal of Mental Deficiency*.
- Cohen, J. (1988) *Statistical Power Analysis for the Behavioral Sciences*, Hillsdale, NJ: Lawrence Earlbaum Associates.
- Cole, A. S. & Eastoe, J. E. (1988) *Biochemistry and Oral Biology*, London (UK): Wright.
- Cooke, C. B. (2009) Maximal oxygen uptake, economy and efficiency. *In*: ESTON, R. & REILLY, T. (eds.) *Kinanthropometry and Exercise Physiology Laboratory Manual: Tests, Procedures and Data*. 3rd ed. Oxon: Routledge.

- Cooke, R., McIntosh, J. & McIntosh, R. (1993) 'Circadian variation in serum free and non-SHBG-bound testosterone in normal men: measurements, and simulation using a mass action model', *Clinical Endocrinology*, 39, 163-171.
- Costill, D., *et al.* (1991) 'Adaptations to swimming training: influence of training volume', *Medicine and Science in Sports and Exercise*, 23, 371-377.
- Coutts, A. J., *et al.* (2007) 'Monitoring for overreaching in rugby league players', *European Journal of Applied Physiology*, 99, 313-24.
- Cox, A. J. *et al.* (2008) 'Clinical and laboratory evaluation of upper respiratory symptoms in elite athletes', *Clinical Journal of Sport Medicine*, 18(5), pp. 438-445. doi: 10.1097/JSM.0b013e318181e501.
- Crofford, L. J., *et al.* (1997) 'Circadian relationships between interleukin (IL)-6 and hypothalamic-pituitary-adrenal axis hormones: failure of IL-6 to cause sustained hypercortisolism in patients with early untreated rheumatoid arthritis', *The Journal of Clinical Endocrinology & Metabolism*, 82, 1279-1283.
- Cumming, D., Quigley, M. & Yen, S. (1983) 'Acute suppression of circulating testosterone levels by cortisol in men', *The Journal of Clinical Endocrinology & Metabolism*, 57, 671-673.
- Cumming, D. C., *et al.* (1986) 'Reproductive hormone increases in response to acute exercise in men', *Medicine and Science in Sports and Exercise*, 18, 369-373.
- Cunningham, A. J. (1978) *Understanding Immunology*, New York: Academic Press.
- Czeisler, C. A. & Klerman, E. B. (1998) 'Circadian and sleep-dependent regulation of hormone release in humans', *Recent Progress in Hormone Research*, 54, 97-130; discussion 130-2.
- D'Ercole, S., *et al.* (2016) 'The effect of swimming on oral health status: competitive versus non-competitive athletes', *Journal of Applied Oral Science*, 24, 107-13.
- Dabbs, J. M., *et al.* (1995) 'Reliability of salivary testosterone measurements: a multicenter evaluation', *Clinical Chemistry*, 41, 1581-1584.
- Daly, W., *et al.* (2005) 'Relationship between stress hormones and testosterone with prolonged endurance exercise', *European Journal of Applied Physiology*, 93, 375-380.



- Davies, C. & Few, J. (1973) 'Effects of exercise on adrenocortical function', *Journal of Applied Physiology*, 35, 887-891.
- Davis, J. M., *et al.* (1991) 'Increased neutrophil mobilization and decreased chemotaxis during cortisol and epinephrine infusions', *Journal of Trauma and Acute Care Surgery*, 31, 725-732.
- Debono, M., *et al.* (2009) 'Modified-release hydrocortisone to provide circadian cortisol profiles', *The Journal of Clinical Endocrinology & Metabolism*, 94, 1548-1554.
- Department of Health (2011) *UK physical activity guidelines* [Online]. Available: <https://www.gov.uk/government/publications/uk-physical-activity-guidelines>.
- Dhabhar, F. S., *et al.* (1994) 'Diurnal and acute stress-induced changes in distribution of peripheral blood leukocyte subpopulations', *Brain, Behavior, and Immunity*, 8, 66-79.
- Di Blasio, A., *et al.* (2016) 'Acute and delayed effects of high intensity interval resistance training organization on cortisol and testosterone production', *The Journal of Sports Medicine and Physical Fitness*, 56, 192-199.
- Díaz, P., *et al.* (2003) 'Increased plasma cortisol might explain the depressed Th-1 type lymphocyte function in severe respiratory syncytial virus infected infants', *Journal of Allergy and Clinical Immunology*, 111, S281.
- Dill, D. B. & Costill, D. L. (1974) 'Calculation of percentage changes in volumes of blood, plasma, and red cells in dehydration', *Journal of Applied Physiology*, 37, 247-8.
- Dillon, E. L., *et al.* (2010) 'Hormone treatment and muscle anabolism during aging: androgens', *Clinical Nutrition*, 29, 697-700.
- Diver, M. J., *et al.* (2003) 'Diurnal rhythms of serum total, free and bioavailable testosterone and of SHBG in middle-aged men compared with those in young men', *Clinical Endocrinology*, 58, 710-717.
- Duclos, M., *et al.* (1998) 'Corticotroph axis sensitivity after exercise in endurance-trained athletes', *Clinical Endocrinology (Oxford)*, 48, 493-501.

- Duclos, M., *et al.* (1997) 'Trained versus untrained men: different immediate post-exercise responses of pituitary adrenal axis', *European Journal of Applied Physiology and Occupational Physiology*, 75, 343-350.
- Dupuy, O., *et al.* (2010) 'Effect of Functional Overreaching on Executive Functions', *Internal Journal of Sports Medicine*, 31, 617-623.
- Edgar, R. S., *et al.* (2012) 'Peroxiredoxins are conserved markers of circadian rhythms', *Nature*, 485, 459-464.
- Edington, D. W. & Edgerton, V. R. (1976) *Instructor's Manual - The Biology of Physical Activity*, Boston: Houghton Mifflin Company.
- Eik-Nes, K. B. (1975) Biosynthesis and secretion of testicular steroids. *In*: GREEP, R. O., B., A. E., HAMILTON, D. W. & GEIGER, S. (eds.) *Handbook of Physiology*. Washington DC: American Physiological Society.
- Engelbrechtsen, L. *et al.* (2013) 'Sports injuries and illnesses during the London Summer Olympic Games 2012.', *British Journal of Sports Medicine*, 47(7), pp. 407–414. doi: 10.1136/bjsports-2013-092380.
- Eston, R. & Williams, J. (1988) 'Reliability of ratings of perceived effort regulation of exercise intensity', *British Journal of Sports Medicine*, 22, 153-155.
- Evans, N. A. (2004) 'Current concepts in anabolic-androgenic steroids', *The American Journal of Sports Medicine*, 32, 534-542.
- Evans, P., *et al.* (1994) 'Stress, arousal, cortisol and secretory immunoglobulin A in students undergoing assessment', *British Journal of Clinical Psychology*, 33, 575-576.
- Fahlman, M. M. & Engels, H.-j. (2005) 'Mucosal IgA and URTI in American college football players: a year longitudinal study', *Medicine and Science in Sports and Exercise*, 37, 374-380.
- Fellmann, N., *et al.* (1985) 'Effects of endurance training on the androgenic response to exercise in man', *International Journal of Sports Medicine*, 6, 215-219.
- Feltz, C. J. & Miller, G. E. (1996) 'An asymptotic test for the equality of coefficients of variation from k populations', *Statistics in Medicine*, 15, 647-658.

- Ferrini, R. L. & Barrett-Connor, E. (1998) 'Sex hormones and age: a cross-sectional study of testosterone and estradiol and their bioavailable fractions in community-dwelling men', *American Journal of Epidemiology*, 147, 750-754.
- Field, C. J., Gougeon, R. & Marliss, E. B. (1991) 'Circulating mononuclear cell numbers and function during intense exercise and recovery', *Journal of Applied Physiology*, 71, 1089-1097.
- Fielding, R. A., *et al.* (2000) 'Effects of prior exercise on eccentric exercise-induced neutrophilia and enzyme release', *Medicine and Science in Sports and Exercise*, 32, 359-364.
- Fitts, R. H. (1994) 'Cellular mechanisms of muscle fatigue', *Physiological Reviews*, 74, 49-94.
- Fowlkes, B., *et al.* (1987) 'A novel population of T-cell receptor  $\alpha\beta$ -bearing thymocytes which predominantly expresses a single V $\beta$  gene family', *Nature*, 329, 251-254.
- Francesconi, R. P., *et al.* (1985) 'Plasma hormonal responses at graded hypohydration levels during exercise-heat stress', *Journal of Applied Physiology*, 59, 1855-1860.
- Frankenberg, T., *et al.* (2008) 'Phagocytosis-induced apoptosis of macrophages is linked to uptake, killing and degradation of bacteria', *European Journal of Immunology*, 38, 204-215.
- Fraser, C. G. and Fogarty, Y. (1989) 'Interpreting laboratory results', *British Medical Journal (Clinical Research Ed.)*, 298, pp. 1659-1660.
- Friedmann, B., Goodman Jr., E. H. & Weinhouse, S. (1967) 'Effects of glucose feeding, cortisol, and insulin on liver glycogen synthesis in the rat', *Endocrinology*, 81, 486-496.
- Fry, A. C., *et al.* (1994a) 'Endocrine responses to overreaching before and after 1 year of weightlifting', *Canadian Journal of Applied Physiology*, 19, 400-410.
- Fry, R., *et al.* (1994b) 'Psychological and immunological correlates of acute overtraining', *British Journal of Sports Medicine*, 28, 241-246.
- Fry, R. W., Morton, A. R. & Keast, D. (1991) 'Overtraining in athletes', *Sports Medicine*, 12, 32-65.

- Funderud, S., *et al.* (1990) 'Functional properties of CD19 B lymphocytes positively selected from buffy coats by immunomagnetic separation', *European Journal of Immunology*, 20, 201-206.
- Gabriel, H., *et al.* (1992) 'Immunoregulatory hormones, circulating leucocyte and lymphocyte subpopulations before and after endurance exercise of different intensities', *International Journal of Sports Medicine*, 13, 359-366.
- Gabriel, H., Urhausen, A. & Kindermann, W. (1991) 'Circulating leucocyte and lymphocyte subpopulations before and after intensive endurance exercise to exhaustion', *European Journal of Applied Physiology and Occupational Physiology*, 63, 449-457.
- Gabriel, H., *et al.* (1998) 'Overtraining and immune system: a prospective longitudinal study in endurance athletes', *Medicine and Science in Sports and Exercise*, 30, 1151-1157.
- Galbo, H., *et al.* (1977) 'Thyroid and testicular hormone responses to graded and prolonged exercise in man', *European Journal of Applied Physiology and Occupational Physiology*, 36, 101-106.
- Ganong, W. F. (1991) *Review of Medical Physiology*: McGraw-Hill Companies.
- Gibson, E. L., *et al.* (1999) 'Increased salivary cortisol reliably induced by a protein-rich midday meal', *Psychosomatic Medicine*, 61, 214-224.
- Gleeson, M. (2000) 'Mucosal immune responses and risk of respiratory illness in elite athletes', *Exercise Immunology Review*, 6, 5-42.
- Gleeson, M. (2006a) *Immune Function in Sport and Exercise*: Churchill Livingstone Elsevier.
- Gleeson, M. (2006b) Introduction to the immune system. In: GLEESON, M. (ed.) *Immune Function in Sport and Exercise*. Philadelphia: Churchill Livingstone: Elsevier.
- Gleeson, M., *et al.* (2012) 'Respiratory infection risk in athletes: association with antigen-stimulated IL-10 production and salivary IgA secretion', *Scandinavian Journal of Medicine & Science in Sports*, 22, 410-417.
- Gleeson, M. & Bishop, N. C. (2005) 'The T cell and NK cell immune response to exercise', *Annals of Transplantation*, 10, 44.

- Gleeson, M. & Bishop, N. C. (2013) 'URI in athletes: are mucosal immunity and cytokine responses key risk factors?', *Exercise and Sport Sciences Reviews*, 41, 148-153.
- Gleeson, M. & Bosch, J. (2013) The human immune system. In: GLEESON, M., BISHOP, N. C. & WALSH, N. (eds.) *Exercise Immunology*. Oxon, UK: Routledge.
- Gleeson, M., *et al.* (1999a) 'Salivary IgA subclasses and infection risk in elite swimmers', *Immunology and Cell Biology*, 77, 351-355.
- Gleeson, M., *et al.* (2000) 'Immune status and respiratory illness for elite swimmers during a 12-week training cycle', *International Journal of Sports Medicine*, 21, 302-307.
- Gleeson, M., *et al.* (1999b) 'Salivary IgA levels and infection risk in elite swimmers', *Medicine and Science in Sports and Exercise*, 31, 67-73.
- Gleeson, M. & Pyne, D. B. (2000) 'Exercise effects on mucosal immunity', *Immunology and Cell Biology*, 78, 536-544.
- Gleeson, M. & Robson-Ansley, P. (2006) Immune responses to intensified training and overtraining. In: GLESSON, M. (ed.) *Immune Function in Sport and Exercise*. Churchill Livingstone Elsevier.
- Grandys, M., *et al.* (2009) 'Endurance training of moderate intensity increases testosterone concentration in young, healthy men', *International Journal of Sports Medicine*, 30, 489-495.
- Granger, D., *et al.* (2004) 'The "trouble" with salivary testosterone', *Psychoneuroendocrinology*, 29, 1229-40.
- Gray, A. B., *et al.* (1993) 'The response of leukocyte subsets and plasma hormones to interval exercise', *Medicine and Science in Sports and Exercise*, 25, 1252-1258.
- Greaves, M. & West, H. (1963) 'Cortisol and cortisone in saliva of pregnancy', *Journal of Endocrinology*, 26, 189-195.
- Guezennec, Y., *et al.* (1986) 'Hormone and metabolite response to weight-lifting training sessions', *International Journal of Sports Medicine*, 7, 100-105.
- Guglielmini, C., Paolini, A. & Conconi, F. (1984) 'Variations of serum testosterone concentrations after physical exercises of different duration', *International Journal of Sports Medicine*, 5, 246-249.

- Hackney, A., Sinning, W. & Bruot, B. (1990) 'Hypothalamic-pituitary-testicular axis function in endurance-trained males', *International Journal of Sports Medicine*, 11, 298-303.
- Hackney, A. C. (2012) 'Clinical management of immunosuppression in sportsmen-women: recommendations for sports medicine physicians and physiotherapists', *Acta Kinesiologiae Universitatis Tartuensis*, 18, 20-28.
- Hackney, A. C., Szczepanowska, E. & Viru, A. (2003) 'Basal testicular testosterone production in endurance-trained men is suppressed', *European Journal of Applied Physiology*, 89, 198-201.
- Häkkinen, K., *et al.* (1987) 'Relationships between training volume, physical performance capacity, and serum hormone concentrations during prolonged training in elite weight lifters', *International Journal of Sports Medicine*, 8, S61-S65.
- Häkkinen, K., *et al.* (1988) 'Neuromuscular and hormonal responses in elite athletes to two successive strength training sessions in one day', *European Journal of Applied Physiology and Occupational Physiology*, 57, 133-139.
- Halson, S. I., *et al.* (2002) 'Time course of performance changes and fatigue markers during intensified training in trained cyclists', *Journal of Applied Physiology*, 93, 947-956.
- Halson, S. I. & Jeukendrup, A. E. (2004) 'Does overtraining exist? An analysis of overreaching and overtraining research.', *Sports Medicine*, 34, 967-981.
- Halson, S. L., *et al.* (2003) 'Immunological responses to overreaching in cyclists', *Medicine and Science in Sports and Exercise*, 35, 854-61.
- Hanson, L. Ä., Björkander, J. & Oxelius, V. A. (1983) *Selective IgA Deficiency*, Edinbrugh: Churchill Livingstone.
- Hanstock, H. G., *et al.* (2016) 'Tear Fluid SIgA as a Noninvasive Biomarker of Mucosal Immunity and Common Cold Risk', *Medicine and Science in Sports and Exercise*, 48, 569-77.
- Harman, S. M., *et al.* (2001) 'Longitudinal effects of aging on serum total and free testosterone levels in healthy men', *The Journal of Clinical Endocrinology & Metabolism*, 86, 724-731.
- Hayes, L. D., *et al.* (2015a) 'Exercise-induced responses in salivary testosterone, cortisol, and their ratios in men: a meta-analysis', *Sports Medicine*, 45, 713-726.

- Hayes, L. D., *et al.* (2016) 'Salivary Testosterone and Cortisol Measurement in Sports Medicine: a Narrative Review and User's Guide for Researchers and Practitioners', *International Journal of Sports Medicine*.
- Hayes, L. D., *et al.* (2014) 'Critical difference applied to exercise-induced salivary testosterone and cortisol using enzyme-linked immunosorbent assay (ELISA): distinguishing biological from statistical change', *Journal of Physiology and Biochemistry*, 70(4), pp. 991–996. doi: 10.1007/s13105-014-0368-6.
- Hayes, L. D., *et al.* (2012) 'Diurnal variation of cortisol, testosterone, and their ratio in apparently healthy males', *Sport SPA*, 9(1), pp. 5–13.
- Hayes, L. D., *et al.* (2015b) 'Poor levels of agreement between serum and saliva testosterone measurement following exercise training in aging men', *Aging Male*, 18, 67-70.
- Heath, G., Macera, C. & Nieman, D. (1992) 'Exercise and upper respiratory tract infections. Is there a relationship?', *Sports Medicine New Zealand*, 14, 353-365.
- Herscowitz, H. B. (1978) Immunophysiology: cell function and cellular interactions. *In*: BELLANTI, J. A. (ed.) *Immunology II*. Philadelphia: W. B. Saunders Company.
- Herz, J. E. & Fried, J. 1958. *Synthesis of Steroids*. USA patent application. 8th July 1958.
- Herzenberg, L. A., *et al.* (2006) 'Interpreting flow cytometry data: a guide for the perplexed', *Nature Immunology*, 7, 681-685.
- Hill, E., *et al.* (2008) 'Exercise and circulating cortisol levels: the intensity threshold effect', *Journal of Endocrinological Investigation*, 31, 587-91.
- Hinson, J., Raven, P. & Chew, S. L. (2010) *The Endocrine System: Basic Science and Clinical Conditions*: Churchill Livingstone/Elsevier.
- Hoffman, J., *et al.* (1994) 'Effects of hydration state on plasma testosterone, cortisol and catecholamine concentrations before and during mild exercise at elevated temperature', *European Journal of Applied Physiology and Occupational Physiology*, 69, 294-300.
- Hollander, D., Meyers, M. C. & LeUnes, A. (1995) 'Psychological factors associated with overtraining: Implications for youth sport coaches', *Journal of Sport Behavior*, 18, 3.

- Hoogeveen, A. & Zonderland, M. (1996) 'Relationships between testosterone, cortisol and performance in professional cyclists', *International Journal of Sports Medicine*, 17, 423-428.
- Hooper, S., *et al.* (1993) 'Hormonal responses of elite swimmers to overtraining', *Medicine and Science in Sports and Exercise*, 25, 741-747.
- Hooper, S. L., *et al.* (1995) 'Markers for monitoring overtraining and recovery in elite swimmers', *Medicine & Science in Sports & Exercise*, 27, 106-112.
- Hopkins, W., *et al.* (2009) 'Progressive statistics for studies in sports medicine and exercise science', *Medicine and Science in Sports and Exercise*, 41, 3.
- Hopkins, W. G. (2000) 'Measures of reliability in sports medicine and science', *Sports Medicine*, 30, 1-15.
- Horrocks, P., *et al.* (1990) 'Patterns of ACTH and cortisol pulsatility over twenty-four hours in normal males and females', *Clinical Endocrinology*, 32, 127-134.
- Hough, J., *et al.* (2013) 'Salivary cortisol and testosterone responses to high-intensity cycling before and after an 11-day intensified training period', *Journal of Sports Sciences*, 31, 1614-1623.
- Hough, J., *et al.* (2011) 'Plasma and salivary steroid hormone responses of men to high-intensity cycling and resistance exercise', *Journal of Strength and Conditioning Research*, 1, 23-31.
- Hough, J., Robertson, C. & Gleeson, M. (2015) 'Blunting of Exercise-Induced Salivary Testosterone in Elite-Level Triathletes With a 10-Day Training Camp', *International Journal of Sports Physiology & Performance*, 10.
- Hurley, J. H. (1999) 'Structure, mechanism, and regulation of mammalian adenylyl cyclase', *Journal of Biological Chemistry*, 274, 7599-7602.
- Ireland, M. A., *et al.* (1984) 'Acute effects of moderate alcohol consumption on blood pressure and plasma catecholamines', *Clinical Science (London, England: 1979)*, 66, 643-648.
- Jackson, G. G., *et al.* (1958) 'Transmission of the common cold to volunteers under controlled conditions: I. The common cold as a clinical entity', *AMA Archives of Internal Medicine*, 101, 267-278.



- Janeway, C. A., Jr. (1989) 'Approaching the asymptote? Evolution and revolution in immunology', *Cold Spring Harbor Symposia on Quantitative Biology*, 54 Pt 1, 1-13.
- Janeway, C. A., *et al.* (1997) *Immunobiology: The Immune System in Health and Disease*: Current Biology Singapore.
- Jasper, M. S. & Engeland, W. C. (1997) 'Splanchnicotomy increases adrenal sensitivity to ACTH in nonstressed rats', *American Journal of Physiology-Endocrinology And Metabolism*, 273, E363-E368.
- Jemmott, J. B. & Magloire, K. (1988) 'Academic stress, social support, and secretory immunoglobulin A', *Journal of Personality and Social Psychology*, 55, 803.
- Jeukendrup, A., *et al.* (1996) 'A new validated endurance performance test', *Medicine and Science in Sport and Exercise*, 28, 266-270.
- Jones, A. M. & Doust, J. H. (1996) 'A 1% treadmill grade most accurately reflects the energetic cost of outdoor running', *Journal of sports sciences*, 14, 321-327.
- Jones, A. M., Vanhatalo, A. T. & Doust, J. H. (2009) From Severe to Extreme Exercise: VO<sub>2</sub>max and exercise economy. In: ESTON, R. & REILLY, T. (eds.) *Kinanthropometry and Exercise Physiology Laboratory Manual: Tests, Procedures and Data*. 3rd ed. Oxon: Routledge.
- Jovanovic, D. V., *et al.* (1998) 'IL-17 stimulates the production and expression of proinflammatory cytokines, IL- $\beta$  and TNF- $\alpha$ , by human macrophages', *The Journal of Immunology*, 160, 3513-3521.
- Kageta, T., *et al.* (2015) 'Influences of increased training volume on exercise performance, physiological and psychological parameters', *The Journal of Sports Medicine and Physical Fitness*, 56, 913-921.
- Karila, T., *et al.* (2008) 'Rapid weight loss decreases serum testosterone', *International Journal of Sports Medicine*, 29, 872-877.
- Kellmann, M. & Kallus, K. W. (2001) *Recovery-Stress Questionnaire for Athletes: User Manual*, Champaign, IL: Human Kinetics.
- Kelly, R. B. (1987) 'From organelle to organelle', *Nature*, 326, 14-15.

- Kenny, G. 1990. *A Comparative Analysis of Physiological Responses at Submaximal Work loads Between Simulated Performances of Cycling*. Master of Sciences, University of Ottawa.
- Kenttä, G., Hassmén, P. & Raglin, J. (2001) 'Training practices and overtraining syndrome in Swedish age-group athletes', *International Journal of Sports Medicine*, 22, 460-465.
- Khan-Dawood, F. S., Choe, J. K. & Dawood, M. Y. (1984) 'Salivary and plasma bound and "free" testosterone in men and women', *American Journal of Obstetrics and Gynecology*, 148, 442-445.
- Khani, S. & Tayek, J. A. (2001) 'Cortisol increases gluconeogenesis in humans: its role in the metabolic syndrome', *Clinical Science*, 101, 739-747.
- Kirschbaum, C. & Hellhammer, D. H. (1989) 'Salivary cortisol in psychobiological research: an overview', *Neuropsychobiology*, 22, 150-169.
- Kirschbaum, C., *et al.* (1995) 'Persistent high cortisol responses to repeated psychological stress in a subpopulation of healthy men', *Psychosomatic Medicine*, 57, 468-474.
- Kirwan, J. P., *et al.* (1988) 'Physiological responses to successive days of intense training in competitive swimmers', *Medicine and Science in Sports and Exercise*, 20, 255-259.
- Koutedakis, Y., Metsios, G. S. & Stavropoulos-Kalinoglou, A. (2006) Periodization of Exercise Training in Sport. In: WHYTE, G. (ed.) *The Physiology of Training*. Philadelphia: Churchill Livingstone, Elsevier.
- Kraemer, W. J., *et al.* (1999) 'Acute hormonal responses to a single bout of heavy resistance exercise in trained power lifters and untrained men', *Canadian Journal of Applied Physiology*, 24, 524-537.
- Kraemer, W. J. & Ratamess, N. A. (2005) 'Hormonal responses and adaptations to resistance exercise and training', *Sports Medicine*, 35, 339-361.
- Kudielka, B. M., *et al.* (2007) 'Circadian cortisol profiles and psychological self-reports in shift workers with and without recent change in the shift rotation system', *Biological Psychology*, 74, 92-103.
- Kuipers, H., *et al.* (1985) 'Variability of aerobic performance in the laboratory and its physiologic correlates', *International Journal of Sports Medicine*, 6, 197-201.

- Kuoppasalmi, K., *et al.* (1980) 'Plasma cortisol, androstenedione, testosterone and luteinizing hormone in running exercise of different intensities', *Scandinavian Journal of Clinical and Laboratory Investigation*, 40, 403-409.
- Lamas, A. M., Leon, O. G. & Schleimer, R. P. (1991) 'Glucocorticoids inhibit eosinophil responses to granulocyte-macrophage colony-stimulating factor', *The Journal of Immunology*, 147, 254-259.
- Lancaster, G. I., *et al.* (2003) 'Effect of acute exhaustive exercise and a 6-day period of intensified training on immune function in cyclists', *Proceedings of The Physiological Society*.
- Landman, A., *et al.* (1976) 'Testosterone in human saliva', *Experientia*, 32, 940-941.
- Laursen, P. B. & Jenkins, D. G. (2002) 'The scientific basis for high-intensity interval training', *Sports Medicine*, 32, 53-73.
- Le Meur, Y., *et al.* (2014) 'Maximal exercise limitation in functionally overreached triathletes: role of cardiac adrenergic stimulation', *Journal of Applied Physiology*, 117, 214-222.
- Le Tulzo, Y., *et al.* (2004) 'Monocyte human leukocyte antigen-DR transcriptional downregulation by cortisol during septic shock', *American Journal of Respiratory and Critical Care Medicine*, 169, 1144-1151.
- Lehmann, M., *et al.* (1992a) 'Training-overtraining: influence of a defined increase in training volume vs training intensity on performance, catecholamines and some metabolic parameters in experienced middle-and long-distance runners', *European Journal of Applied Physiology and Occupational Physiology*, 64, 169-177.
- Lehmann, M., *et al.* (1993) 'Influence of 6-week, 6 days per week, training on pituitary function in recreational athletes', *British Journal of Sports Medicine*, 27, 186-192.
- Lehmann, M., *et al.* (1996) 'Unaccustomed high-mileage vs intensity training-related changes in performance and serum amino acid levels', *International Journal of Sports Medicine*, 17, 187-192.
- Lehmann, M., *et al.* (1992b) 'Decreased nocturnal catecholamine excretion: parameter for an overtraining syndrome in athletes?', *International Journal of Sports Medicine*, 13, 236-242.

- Leicht, C. A., Bishop, N. C. & Goosey-Tolfrey, V. L. (2011) 'Mucosal immune responses to treadmill exercise in elite wheelchair athletes', *Medicine and Science in Sports and Exercise*, 43, 1414-1421.
- Leite, R., *et al.* (2011) 'Acute effect of resistance training volume on hormonal responses in trained men', *Journal of Sports Medicine and Physical Fitness*, 51, 322.
- Lewis, J. G. (2006) 'Steroid analysis in saliva: an overview', *Clinical Biochemist Reviews*, 27, 139.
- Liening, S. H., *et al.* (2010) 'Salivary testosterone, cortisol, and progesterone: two-week stability, interhormone correlations, and effects of time of day, menstrual cycle, and oral contraceptive use on steroid hormone levels', *Physiology & Behavior*, 99, 8-16.
- Lin, D., *et al.* (1995) 'Role of steroidogenic acute regulatory protein in adrenal and gonadal steroidogenesis', *Science*, 267, 1828.
- Litman, G. W., *et al.* (1993) 'Phylogenetic diversification of immunoglobulin genes and the antibody repertoire', *Molecular Biology and Evolution*, 10, 60-72.
- Lovallo, W. R., *et al.* (2006) 'Cortisol responses to mental stress, exercise, and meals following caffeine intake in men and women', *Pharmacology Biochemistry and Behavior*, 83, 441-447.
- Lucia, A., *et al.* (2001) 'Hormone levels of world class cyclists during the Tour of Spain stage race', *British Journal of Sports Medicine*, 35, 424-30.
- Lundblad, J. R. & Roberts, J. L. (1988) 'Regulation of proopiomelanocortin gene expression in pituitary', *Endocrine Reviews*, 9, 135-158.
- MacGlashan, D., *et al.* (1999) 'Upregulation of FcεRI on human basophils by IgE antibody is mediated by interaction of IgE with FcεRI', *Journal of Allergy and Clinical Immunology*, 104, 492-498.
- Mackinnon, L. (1997) 'Immunity in athletes', *International Journal of Sports Medicine*, 18, S62-8.
- MacKinnon, L. T. (2000) 'Overtraining effects on immunity and performance in athletes', *Immunology and Cell Biology*, 78, 502-509.

- Mackinnon, L. T. & Hooper, S. (1994) 'Mucosal (secretory) immune system responses to exercise of varying intensity and during overtraining', *International Journal of Sports Medicine*, 15, S179-S183.
- Mackinnon, L. T., *et al.* (1997a) 'Hormonal, immunological, and hematological responses to intensified training in elite swimmers', *Medicine and Science in Sports and Exercise*, 29, 1637-1645.
- Mackinnon, L. T. & Hooper, S. L. (1996) 'Plasma glutamine and upper respiratory tract infection during intensified training in swimmers', *Medicine and Science in Sports and Exercise*, 28, 285-90.
- Mackinnon, L. T., *et al.* (1997b) 'Hormonal, immunological, and hematological responses to intensified training in elite swimmers', *Med Sci Sports Exerc*, 29, 1637-45.
- Maes, M., *et al.* (1997) 'Components of biological variation, including seasonality, in blood concentrations of TSH, TT3, FT4, PRL, cortisol and testosterone in healthy volunteers', *Clinical Endocrinology*, 46, 587-598.
- Maestroni, A. (2010) DNA, RNA e sintesi proteica muscolare. *In: LUZI, L. (ed.) Biologia Cellulare Nell'Esercizio Fisico*. 1st Edition ed. Milan: Springer.
- Mäestu, J., Jürimäe, J. & Jürimäe, T. (2005) 'Hormonal response to maximal rowing before and after heavy increase in training volume in highly trained male rowers', *Journal of Sports Medicine and Physical Fitness*, 45, 121.
- Makivić, B., Nikić, M. and Willis, M. (2013) 'Heart Rate Variability (HRV) as a Tool for Diagnostic and Monitoring Performance in Sport and Physical Activities', *Journal of Exercise Physiology*, 16(3), pp. 103–131.
- Malm, C. (2006) 'Susceptibility to infections in elite athletes: the S-curve', *Scandinavian Journal of Medicine and Science in Sports*, 16, 4-6.
- Malm, C., Ekblom, O. & Ekblom, B. (2004) 'Immune system alteration in response to two consecutive soccer games', *Acta Physiologica Scandinavica*, 180, 143-55.
- Maresh, C., *et al.* (2006) 'Effect of hydration state on testosterone and cortisol responses to training-intensity exercise in collegiate runners', *International Journal of Sports Medicine*, 27, 765-770.

- Martin, C. R. (1976) *Textbook of Endocrine Physiology*, Baltimore, Maryland: Williams & Wilkins Co.
- Maso, F., *et al.* (2004) 'Salivary testosterone and cortisol in rugby players: correlation with psychological overtraining items', *British Journal of Sports Medicine*, 38, 260-263.
- Matos, N. F., Winsley, R. J. & Williams, C. A. (2011) 'Prevalence of nonfunctional overreaching/overtraining in young English athletes', *Medicine and Science in Sports and Exercise*, 43, 1287-94.
- McCarthy, D. & Dale, M. (1988) 'The leucocytosis of exercise. A review and model', *Sports Medicine*, 6, 333-363.
- McCarthy, D., *et al.* (1992a) 'Studies on the immediate and delayed leucocytosis elicited by brief (30-min) strenuous exercise', *European Journal of Applied Physiology and Occupational Physiology*, 64, 513-517.
- McCarthy, D., *et al.* (1992b) 'Changes in the leucocyte count during and after brief intense exercise', *European Journal of Applied Physiology and Occupational Physiology*, 64, 518-522.
- McCaulley, G. O., *et al.* (2009) 'Acute hormonal and neuromuscular responses to hypertrophy, strength and power type resistance exercise', *European Journal of Applied Physiology*, 105, 695-704.
- McClelland, D. C., Ross, G. & Patel, V. (1985) 'The effect of an academic examination on salivary norepinephrine and immunoglobulin levels', *Journal of Human Stress*, 11, 52-59.
- McConnel, T. R. & Sinning, W. E. (1984) 'Exercise and temperature effects on human sperm production and testosterone levels', *Medicine and Science in Sports and Exercise*, 16, 51-55.
- McGraw, K. O. & Wong, S. P. (1996) 'Forming inferences about some intraclass correlation coefficients', *Psychological Methods*, 1, 30.
- Medzhitov, R. (2001) 'Toll-like receptors and innate immunity', *Nature Reviews Immunology*, 1, 135-145.
- Medzhitov, R. (2007) 'Recognition of microorganisms and activation of the immune response', *Nature*, 449, 819-826.

- Meehan, H. L., *et al.* (2004) 'The overtraining syndrome: A multicontextual assessment', *The Sport Psychologist*, 18, 154-171.
- Meeusen, R., *et al.* (2013) 'Prevention, diagnosis and treatment of the overtraining syndrome: Joint consensus statement of the European College of Sport Science (ECSS) and the American College of Sports Medicine (ACSM)', *European Journal of Sport Science*, 13, 1-24.
- Meeusen, R., *et al.* (2010) 'Diagnosing overtraining in athletes using the two-bout exercise protocol', *British Journal of Sports Medicine*, 44, 642-648.
- Meeusen, R., *et al.* (2004) 'Hormonal responses in athletes: the use of a two bout exercise protocol to detect subtle differences in (over) training status', *European Journal of Applied Physiology*, 91, 140-146.
- Mendelson, J. H., Mello, N. K. & Ellingboe, J. (1977) 'Effects of acute alcohol intake on pituitary-gonadal hormones in normal human males', *Journal of Pharmacology and Experimental Therapeutics*, 202, 676-682.
- Miloski, B., *et al.* (2015) 'Does testosterone modulate mood states and physical performance in young basketball players?', *The Journal of Strength & Conditioning Research*, 29, 2474-2481.
- Miyawaki, T., *et al.* (1984) 'Circadian changes of T lymphocyte subsets in human peripheral blood', *Clinical and Experimental Immunology*, 55, 618.
- Mohr, M., *et al.* (2016) 'Muscle damage, inflammatory, immune and performance responses to three football games in 1 week in competitive male players', *European Journal of Applied Physiology*, 1-15.
- Mooren, F., *et al.* (2005) Inter- and Intracellular Signaling. In: MOOREN, F. & VÖLKER, K. (eds.) *Molecular and Cellular Exercise Physiology*. Champaign, Illinois: Human Kinetics.
- Morgan, W., *et al.* (1987) 'Psychological characterization of the elite female distance runner', *International Journal of Sports Medicine*, 8, 124-131.
- Morton, R. H. (1997) 'Modelling training and overtraining', *Journal of Sports Sciences*, 15, 335-340.

- Mujika, I., *et al.* (1995) 'Effects of training on performance in competitive swimming', *Canadian Journal of Applied Physiology*, 20, 395-406.
- Mujika, I., *et al.* (1996) 'Hormonal responses to training and its tapering off in competitive swimmers: relationships with performance.', *European Journal of Applied Physiology and Occupational Physiology*, 74, 361-366.
- Munck, A. & Guyre, P. M. (1991) Glucocorticoids and immune function. *In: ADER, R., FELTEN, D. L. & COHEN, N. (eds.) Psychoneuroimmunology*. 2nd ed. San Diego: Academic Press.
- Navazesh, M. (1993) 'Methods for collecting saliva', *Annals of the New York Academy of Sciences*, 694, 72-77.
- Neville, V., Gleeson, M. & Folland, J. P. (2008) 'Salivary IgA as a risk factor for upper respiratory infections in elite professional athletes', *Medicine and Science in Sport and Exercise*, 40, 1228-1236.
- Nichols, B. A., Bainton, D. F. & Farquhar, M. G. (1971) 'Differentiation of monocytes. Origin, nature, and fate of their azurophil granules', *Journal of Cell Biology*, 50, 498-515.
- Nieman, D. (1994a) Physical activity, fitness, and infection. *In: BOUCHARD, C. (ed.) Exercise, Fitness, and Health: A Consensus of Current Knowledge*. Champaign, IL: Human Kinetics Books.
- Nieman, D., *et al.* (2006) 'Relationship between salivary IgA secretion and upper respiratory tract infection following a 160-km race', *Journal of Sports Medicine and Physical Fitness*, 46, 158.
- Nieman, D., *et al.* (1994) 'Effect of high-versus moderate-intensity exercise on lymphocyte subpopulations and proliferative response', *International Journal of Sports Medicine*, 15, 199-206.
- Nieman, D. C. (1994b) 'Exercise, upper respiratory tract infection, and the immune system', *Medicine and Science in Sports and Exercise*, 26, 128-39.
- Nieman, D. C. (1997) 'Immune response to heavy exertion', *Journal of Applied Physiology*, 82, 1385-1394.



- Nieman, D. C. (1998) 'Influence of carbohydrate on the immune response to intensive, prolonged exercise', *Exercise Immunology Review*, 4, 64-76.
- Nieman, D. C. (2000) 'Is infection risk linked to exercise workload?', *Medicine and Science in Sports and Exercise*, 32, S406-11.
- Nieman, D. C. (2009) 'Immune function responses to ultramarathon race competition', *Medicina Sportiva*, 13, 189-196.
- Nieman, D. C., *et al.* (2011) 'Upper respiratory tract infection is reduced in physically fit and active adults', *British Journal of Sports Medicine*, 45, 987-992.
- Nieman, D. C., *et al.* (1990) 'Infectious episodes in runners before and after the Los Angeles Marathon', *Journal of Sports Medicine and Physical Fitness*, 30, 316-328.
- Nieman, D. C. & Nehlsen-Cannarella, S. L. (1991) 'The effects of acute and chronic exercise on immunoglobulins', *Sports Medicine*, 11, 183-201.
- Niu, S.-F., *et al.* (2015) 'Differences in cortisol profiles and circadian adjustment time between nurses working night shifts and regular day shifts: A prospective longitudinal study', *International Journal of Nursing Studies*.
- Norman, A. W. & Litwack, G. (1997) *Hormones*: San Diego: Academic Press.
- Novas, A., Rowbottom, D. & Jenkins, D. (2003) 'Tennis, incidence of URTI and salivary IgA', *International Journal of Sports Medicine*, 24, 223-229.
- Olefsky, J. M. & Kimmerling, G. (1975) 'Effects of glucocorticoids on carbohydrate metabolism', *The American Journal of the Medical Sciences*, 271, 202-210.
- Palmer-Green, D. and Elliott, N. (2015) 'Sports injury and illness epidemiology: Great Britain Olympic Team (TeamGB) surveillance during the Sochi 2014 Winter Olympic Games', *British Journal of Sports Medicine*, 49(1), pp. 25–29. doi: 10.1136/bjsports-2014-094206.
- Palmø, J., *et al.* (1995) 'Effect of eccentric exercise on natural killer cell activity', *Journal of Applied Physiology*, 78, 1442-1446.
- Papacosta, E. & Nassis, G. P. (2011) 'Saliva as a tool for monitoring steroid, peptide and immune markers in sport and exercise science', *Journal of Science and Medicine in Sport*, 14, 424-434.

- Pedersen, B. (1991) 'Influence of physical activity on the cellular immune system: mechanisms of action', *International Journal of Sports Medicine*, 12, 23-S29.
- Pedersen, B., Rohde, T. & Zacho, M. (1996) 'Immunity in athletes', *The Journal of Sports Medicine and Physical Fitness*, 36, 236-245.
- Pedersen, B., *et al.* (1997) 'Exercise-Induced Immunomodulation - Possible Roles of Neuroendocrine and Metabolic Factors', *International Journal of Sports Medicine*, 18(S 1), pp. S2–S7. doi: 10.1055/s-2007-972695.
- Pedersen, B. K. and Ullum, H. (1994) 'NK cell response to physical activity: possible mechanisms of action.', *Medicine and Science in Sports and Exercise*, 26(2), pp. 140–6.
- Pederson, B. K. (2005) Exercise and the cell: Leukocytes. *In*: MOOREN, F. & VÖLKER, K. (eds.) *Molecular and Cellular Exercise Physiology*. Champaign, Illinois: Human Kinetics.
- Perogamvros, I., *et al.* (2010) 'Salivary cortisone is a potential biomarker for serum free cortisol', *The Journal of Clinical Endocrinology & Metabolism*, 95, 4951-4958.
- Peters, E. (1997) 'Exercise, immunology and upper respiratory tract infections', *International Journal of Sports Medicine*, 18, S69-77.
- Pinto, R. A., *et al.* (2006) 'T helper 1/T helper 2 cytokine imbalance in respiratory syncytial virus infection is associated with increased endogenous plasma cortisol', *Pediatrics*, 117, e878-e886.
- Plas, C. & Nunez, J. (1976) 'Role of cortisol on the glycogenolytic effect of glucagon and on the glycogenic response to insulin in fetal hepatocyte culture', *Journal of Biological Chemistry*, 251, 1431-1437.
- Plymate, S. R., Tenover, J. S. & Bremner, W. J. (1989) 'Circadian variation in testosterone, sex hormone-binding globulin, and calculated non-sex hormone-binding globulin bound testosterone in healthy young and elderly men', *Journal of Andrology*, 10, 366-371.
- Port, K. (1991) 'Serum and saliva cortisol responses and blood lactate accumulation during incremental exercise testing', *International Journal of Sports Medicine*, 12, 490-494.
- Powell, J., *et al.* (2015) 'Salivary and serum cortisol levels during recovery from intense exercise and prolonged, moderate exercise', *Biology of Sport*, 32, 91.

- Putignano, P., *et al.* (2001) 'Salivary cortisol measurement in normal-weight, obese and anorexic women: comparison with plasma cortisol.', *European Journal of Endocrinology*, 145, 165-171.
- Pyne, D. B., *et al.* (2001) 'Mucosal immunity, respiratory illness, and competitive performance in elite swimmers', *Medicine and Science in Sports and Exercise*, 33, 348-53.
- Quindry, J. C., *et al.* (2003) 'The effects of acute exercise on neutrophils and plasma oxidative stress', *Medicine and Science in Sports and Exercise*, 35, 1139-1145.
- Raglin, J. (1993) Overtraining and staleness: psychometric monitoring of endurance athletes. In: SINGER, R. N., MURPHY, M. & TENNANT, L. K. (eds.) *Handbook of Research on Sport Psychology*. New York: Macmillan.
- Raglin, J., *et al.* (2000) 'Training practices and staleness in 13-18-year-old swimmers: A cross-cultural study', *Pediatric Exercise Science*, 12, 61-70.
- Rakoff-Nahoum, S., *et al.* (2004) 'Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis', *Cell*, 118, 229-241.
- Rama, L., *et al.* (2013) 'Changes in natural killer cell subpopulations over a winter training season in elite swimmers', *European Journal of Applied Physiology*, 113, 859-868.
- Rasmussen, H. & Barrett, P. Q. (1984) 'Calcium messenger system: an integrated view', *Physiological Reviews*, 64, 938-984.
- Rifkin, I. R., *et al.* (2005) 'Toll-like receptors, endogenous ligands, and systemic autoimmune disease', *Immunological Reviews*, 204, 27-42.
- Risøy, B. A., *et al.* (2003) 'Delayed leukocytosis after hard strength and endurance exercise: aspects of regulatory mechanisms', *BiomedCentral Physiology*, 3, 14.
- Robson, P., *et al.* (1999a) 'The effect of an acute period of intense interval training on human neutrophil function and plasma glutamine in endurance-trained male runners', *Journal of Physiology*, 515, 84-85.
- Robson, P. J., *et al.* (1999b) 'Effects of exercise intensity, duration and recovery on in vitro neutrophil function in male athletes', *International Journal of Sports Medicine*, 20, 128-130.

- Robson-Ansley, P., Blannin, A. & Gleeson, M. (2007) 'Elevated plasma interleukin-6 levels in trained male triathletes following an acute period of intense interval training', *European Journal of Applied Physiology*, 99, 353-360.
- Roitt, I. M. (1977) *Essential Immunology*, Oxford: Blackwell Scientific Publications.
- Ronsen, O., *et al.* (2001) 'Leukocyte counts and lymphocyte responsiveness associated with repeated bouts of strenuous endurance exercise', *Journal of Applied Physiology*, 91, 425-434.
- Rosenberg, H. F. & Domachowske, J. B. (2001) 'Eosinophils, eosinophil ribonucleases, and their role in host defense against respiratory virus pathogens', *Journal of Leukocyte Biology*, 70, 691-698.
- Ross, J. W. & Fraser, M. (1993) 'Analytical goals developed from the inherent error of medical tests', *Clinical Chemistry*, 39, 1481-1493.
- Rubens, R., DHONT, M. & Vermeulen, A. (1974) 'Further studies on Leydig cell function in old age', *The Journal of Clinical Endocrinology & Metabolism*, 39, 40-45.
- Sabag, N., Castrillon, M. A. & Tchernitchin, A. (1978) 'Cortisol-induced migration of eosinophil leukocytes to lymphoid organs', *Experientia*, 34, 666-667.
- Saez, J. M. (1994) 'Leydig cells: endocrine, paracrine, and autocrine regulation', *Endocrine Reviews*, 15, 574-626.
- Saez, J. M., *et al.* (1989) 'Sertoli-Leydig Cell Communicationsa', *Annals of the New York Academy of Sciences*, 564, 210-231.
- Saiki, I., *et al.* (1986) 'Quantitative Fluorescent Microassay for Identification of Antiproliferative Compounds 2', *Journal of the National Cancer Institute*, 77, 1235-1240.
- Sale, D. (1991) Testing strength and power. *In: MACDOUGALL, J. D., WENGER, H. A. & GREEN, H. J. (eds.) Physiological Testing of the High-Performance Athlete*. Champaign, IL: Human Kinetics.
- Sand, K. L., *et al.* (2013) 'Effects of exercise on leukocytosis and blood hemostasis in 800 healthy young females and males', *World Journal of Experimental Medicine*, 3, 11.

- Sannikka, E., *et al.* (1983) 'Testosterone concentrations in human seminal plasma and saliva and its correlation with non-protein-bound and total testosterone levels in serum', *International Journal of Andrology*, 6, 319-330.
- Santoro, N., *et al.* (1988) 'Alterations of the hypothalamic GnRH interpulse interval sequence over the normal menstrual cycle', *American Journal of Physiology-Endocrinology and Metabolism*, 255, E696-E701.
- Santoro, N., Filicori, M. & Crowley Jr, W. F. (1986) 'Hypogonadotropic disorders in men and women: diagnosis and therapy with pulsatile gonadotropin-releasing hormone', *Endocrine Reviews*, 7, 11-23.
- Sartorius, G., *et al.* (2012) 'Serum testosterone, dihydrotestosterone and estradiol concentrations in older men self-reporting very good health: The healthy man study', *Clinical Endocrinology*, 77, 755-763.
- Sawka, M. N., *et al.* (2007) 'American College of Sports Medicine position stand. Exercise and fluid replacement', *Medicine and Science in Sports and Exercise*, 39, 377-390.
- Schatz, D. G., Oettinger, M. A. & Schlissel, M. S. (1992) 'V (D) J recombination: molecular biology and regulation', *Annual Review of Immunology*, 10, 359-383.
- Schwindt, C. D., *et al.* (2007) 'Do circulating leucocytes and lymphocyte subtypes increase in response to brief exercise in children with and without asthma?', *British Journal of Sports Medicine*, 41, 34-40.
- Selby, C., Lobb, P. A. and Jeffcoate, W. J. (1988) 'Sex hormone binding globulin in saliva', *Clinical Endocrinology*, 28(1), pp. 19-24.
- Selye, H. (1936) 'A syndrome produced by diverse noxious agents', *Nature*, 138, 32.
- Selye, H. (1946) 'The general adaptation syndrome and the diseases of adaptation 1', *The Journal of Clinical Endocrinology & Metabolism*, 6, 117-230.
- Šerbetar, I. (2015) 'Establishing Some Measures of Absolute and Relative Reliability of a Motor Test', *Croatian Journal of Education*, 17, 37-48.

- Sgrò, P., *et al.* (2014) 'Testosterone responses to standardized short-term sub-maximal and maximal endurance exercises: issues on the dynamic adaptive role of the hypothalamic-pituitary-testicular axis', *Journal of Endocrinological Investigation*, 37, 13-24.
- Shek, P. N., *et al.* (1995) 'Strenuous exercise and immunological changes', *International Journal of Sports Medicine*, 16, 466-474.
- Sherman, B., Wysham, W. & Pfoh, B. (1985) 'Age-related changes in the circadian rhythm of plasma cortisol in man', *The Journal of Clinical Endocrinology & Metabolism*, 61, 439-443.
- Shirreffs, S. M. & Maughan, R. J. (1998) 'Urine osmolality and conductivity as indices of hydration status in athletes in the heat', *Medicine and Science in Sports and Exercise*, 30, 1598-602.
- Shirtcliff, E. A., Granger, D. A. & Likos, A. (2002) 'Gender Differences in the Validity of Testosterone Measured in Saliva by Immunoassay', *Hormones and Behaviour*, 42, 62-69.
- Shrout, P. E. & Fleiss, J. L. (1979) 'Intraclass correlations: uses in assessing rater reliability', *Psychological Bulletin*, 86, 420.
- Sinha-Hikim, I., *et al.* (2003) 'Testosterone-induced muscle hypertrophy is associated with an increase in satellite cell number in healthy, young men', *American Journal of Physiology-Endocrinology and Metabolism*, 285, E197-E205.
- Skosnik, P. D., *et al.* (2000) 'Modulation of attentional inhibition by norepinephrine and cortisol after psychological stress', *International Journal of Psychophysiology*, 36, 59-68.
- Smith, L. L. (2000) 'Cytokine hypothesis of overtraining: a physiological adaptation to excessive stress?', *Medicine and Science in Sports and Exercise*, 32, 317-31.
- Smutok, M. A., Skrinar, G. S. & Pandolf, K. B. (1980) 'Exercise intensity: subjective regulation by perceived exertion', *Archives of Physical Medicine and Rehabilitation*, 61, 569-574.
- Snyder, A., *et al.* (1995) 'Overtraining following intensified training with normal muscle glycogen', *Medicine and Science in Sports and Exercise*, 27, 1063-1070.
- Sokal, R. R. & Braumann, C. A. (1980) 'Significance tests for coefficients of variation and variability profiles', *Systematic Biology*, 29, 50-66.

- Spence, L., *et al.* (2007) 'Incidence, etiology, and symptomatology of upper respiratory illness in elite athletes', *Medicine and Science in Sports and Exercise*, 39(4), pp. 577–586. doi: 10.1249/mss.0b013e31802e851a.
- Sperlich, B., *et al.* (2010) 'High-intensity interval training improves VO<sub>2</sub>peak, maximal lactate accumulation, time trial and competition performance in 9–11-year-old swimmers', *European Journal of Applied Physiology*, 110, 1029-1036.
- Stites, D. P., Bugbee, S. & Siiteri, P. K. (1983) 'Differential actions of progesterone and cortisol on lymphocyte and monocyte interaction during lymphocyte activation—relevance to immunosuppression in pregnancy', *Journal of Reproductive Immunology*, 5, 215-228.
- Stocco, D. M. & Clark, B. J. (1996) 'Regulation of the acute production of steroids in steroidogenic cells', *Endocrine Reviews*, 17, 221-244.
- Stone, A. A. *et al.* (2001) 'Individual differences in the diurnal cycle of salivary free cortisol: A replication of flattened cycles for some individuals', *Psychoneuroendocrinology*, 26(3), pp. 295–306. doi: 10.1016/S0306-4530(00)00057-3.
- Strike, P. W. (1991) *Statistical Methods in Laboratory Medicine*, Oxford, UK: Butterworth-Heinemann.
- Sutton, J., *et al.* (1973) 'Androgen responses during physical exercise', *British Medical Journal*, 1, 520-522.
- Svendsen, I. S., *et al.* (2016) 'Impact of intensified training and carbohydrate supplementation on immunity and markers of overreaching in highly trained cyclists', *European Journal of Applied Physiology*.
- Tabata, I., *et al.* (1991) 'Effect of low blood glucose on plasma CRF, ACTH, and cortisol during prolonged physical exercise', *Journal of Applied Physiology*, 71, 1807-1812.
- Takeda, K., Kaisho, T. & Akira, S. (2003) 'Toll-like receptors', *Annual Review of Immunology*, 21, 335-376.
- Tanner, A. V., Nielsen, B. V. & Allgrove, J. (2014) 'Salivary and plasma cortisol and testosterone responses to interval and tempo runs and a bodyweight-only circuit session in endurance-trained men', *Journal of Sports Sciences*, 32, 680-689.

- Tharp, G. D. & Barnes, M. W. (1990) 'Reduction of saliva immunoglobulin levels by swim training', *European Journal of Applied Physiology and Occupational Physiology*, 60, 61-64.
- Thomson, S. P., McMahon, L. J. & Nugent, C. A. (1980) 'Endogenous cortisol: a regulator of the number of lymphocytes in peripheral blood.', *Clinical Immunology and Immunopathology*, 17, 506-514.
- Tiollier, E., *et al.* (2005) 'Intense training: mucosal immunity and incidence of respiratory infections', *European Journal of Applied Physiology*, 93, 421-428.
- Tomasi, T. B., *et al.* (1982) 'Immune parameters in athletes before and after strenuous exercise', *Journal of Clinical Immunology*, 2, 173-178.
- Traish, A. M., *et al.* (2011) 'Testosterone deficiency', *The American Journal of Medicine*, 124, 578-587.
- Tremblay, M. S., Copeland, J. L. & Van Helder, W. (2004) 'Effect of training status and exercise mode on endogenous steroid hormones in men', *Journal of Applied Physiology*, 96, 531-539.
- Tremblay, M. S., Copeland, J. L. & Van Helder, W. (2005) 'Influence of exercise duration on post-exercise steroid hormone responses in trained males', *European Journal of Applied Physiology*, 94, 505-513.
- Tripodi, D., *et al.* (2011) 'Inflammatory and immunitary modifications in saliva of subjects with labial and tongue piercing', *European Journal of Inflammation*, 9, 175-183.
- Trochimiak, T. & Hübner-Woźniak, E. (2012) 'Effect of exercise on the level of immunoglobulin a in saliva', *Biology of Sport*, 29, 255.
- Tsigos, C. & Chrousos, G. P. (1994) 'Physiology of the hypothalamic-pituitary-adrenal axis in health and dysregulation in psychiatric and autoimmune disorders', *Endocrinology and Metabolism Clinics of North America*, 23, 451-66.
- Tsigos, C. & Chrousos, G. P. (2002) 'Hypothalamic-pituitary-adrenal axis, neuroendocrine factors and stress', *Journal of Psychosomatic Research*, 53, 865-871.
- Tsujita, S. & Morimoto, K. (1999) 'Secretory IgA in saliva can be a useful stress marker', *Environmental Health and Preventive Medicine*, 4, 1-8.



- Urhausen, A., Gabriel, H. & Kindermann, W. (1995) 'Blood hormones as markers of training stress and overtraining', *Sports Medicine*, 20, 251-276.
- Urhausen, A., Gabriel, H. & Kindermann, W. (1998a) 'Impaired pituitary hormonal response to exhaustive exercise in overtrained endurance athletes', *Medicine and Science in Sports and Exercise*, 30, 407-414.
- Urhausen, A., *et al.* (1998b) 'Ergometric and psychological findings during overtraining: a long-term follow-up study in endurance athletes', *International Journal of Sports Medicine*, 19, 114-120.
- Urhausen, A. & Kindermann, W. (2002) 'Diagnosis of Overtraining: What Tools Do We Have?', *Sports Medicine*, 32, 95-102.
- Vale, W., *et al.* (1981) 'Characterization of a 41-residue ovine hypothalamic peptide that stimulates secretion of corticotropin and beta-endorphin', *Science*, 213, 1394-1397.
- Valero-Politi, J. and Fuentes-Arderu, X. (1993) 'Within- and between-subject biological variations of follitropin, lutropin, testosterone, and sex-hormone-binding globulin in men', *Clinical Chemistry*, 39(8), pp. 1723–1725.
- Van Cauter, E. (1990) 'Diurnal and ultradian rhythms in human endocrine function: a minireview', *Hormone Research in Paediatrics*, 34, 45-53.
- Van Cauter, E., Leproult, R. & Kupfer, D. J. (1996) 'Effects of gender and age on the levels and circadian rhythmicity of plasma cortisol', *The Journal of Clinical Endocrinology & Metabolism*, 81, 2468-2473.
- VanBruggen, M. D., *et al.* (2011) 'The relationship between serum and salivary cortisol levels in response to different intensities of exercise', *International Journal of Sports Physiology and Performance*, 6, 396-407.
- Verde, T., Thomas, S. & Shephard, R. (1992) 'Potential markers of heavy training in highly trained distance runners', *British Journal of Sports Medicine*, 26, 167-175.
- Vermeulen, A., Rubens, R. & Verdonck, L. (1972) 'Testosterone secretion and metabolism in male senescence', *The Journal of Clinical Endocrinology & Metabolism*, 34, 730-735.

- Vesterinen, V., *et al.* (2016) 'Predictors of individual adaptation to high-volume or high-intensity endurance training in recreational endurance runners', *Scandinavian Journal of Medicine & Science in Sports*, 26, 885-893.
- Vincent, W. & Weir, J. (2012) *Statistics in Kinesiology: Human Kinetics*.
- Vining, R. F., McGinley, R. A. & Symons, R. G. (1983) 'Hormones in saliva: mode of entry and consequent implications for clinical interpretation', *Clinical Chemistry*, 29, 1752-1756.
- Viru, A., Karelson, K. & Smirnova, T. (1992) 'Stability and variability in hormonal responses to prolonged exercise', *International Journal of Sports Medicine*, 13, 230-235.
- Vitaterna, M. H., Takahashi, J. S. & Turek, F. W. (2001) 'Overview of circadian rhythms', *Alcohol Research and Health*, 25, 85-93.
- Vitteck, J., *et al.* (1985) 'Direct radioimmunoassay (RIA) of salivary testosterone: correlation with free and total serum testosterone', *Life Sciences*, 37, 711-716.
- Voorhis, B. J. V., Anderson, D. J. & Hill, J. A. (1989) 'The effects of RU 486 on immune function and steroid-induced immunosuppression in vitro', *The Journal of Clinical Endocrinology & Metabolism*, 69, 1195-1199.
- Vrabas, I. S., *et al.* (1999) 'Endurance training reduces the rate of diaphragm fatigue in vitro', *Medicine and Science in Sports and Exercise*, 31, 1605-1612.
- Wallen, N., *et al.* (1991) 'Glucocorticoids inhibit cytokine-mediated eosinophil survival', *The Journal of Immunology*, 147, 3490-3495.
- Walsh, D. A. & Van Patten, S. M. (1994) 'Multiple pathway signal transduction by the cAMP-dependent protein kinase', *The FASEB Journal*, 8, 1227-1236.
- Walsh, N., *et al.* (1999) 'The effects of high-intensity intermittent exercise on saliva IgA, total protein and alpha-amylase', *Journal of Sports Sciences*, 17, 129-134.
- Walsh, N. P., *et al.* (2011a) 'Position statement part two: Maintaining immune health', *Exercise Immunology Review*, 17, 64-103.
- Walsh, N. P., *et al.* (2011b) 'Position statement part one: Immune function and exercise', *Exercise Immunology Review*, 17, 6-63.

- Walsh, N. P. & Oliver, S. J. (2016) 'Exercise, immune function and respiratory infection: An update on the influence of training and environmental stress', *Immunology and Cell Biology*, 94, 132-9.
- Walton, M. J., *et al.* (2007) 'A diurnal variation in testicular hormone production is maintained following gonadotrophin suppression in normal men', *Clinical Endocrinology*, 66, 123-129.
- Ward, P. A. (1978) Mechanisms of tissue injury: inflammation. *In*: BELLANTI, J. A. (ed.) *Immunology II*. Philadelphia: W. B. Saunders Company.
- Wardyn, G. G., *et al.* (2008) 'Effects of exercise on hematological parameters, circulating side population cells, and cytokines', *Experimental Hematology*, 36, 216-223.
- Wasserman, K., *et al.* (1987) 'Principles of exercise testing and interpretation', *Journal of Cardiopulmonary Rehabilitation and Prevention*, 7, 189.
- Weibel, L., *et al.* (1995) 'Comparative effect of night and daytime sleep on the 24-hour cortisol secretory profile', *Sleep*, 18, 549-556.
- Weidner, T. & Schurr, T. (2003) 'Effect of exercise on upper respiratory tract infection in sedentary subjects', *British Journal of Sports Medicine*, 37, 304-306.
- Weitzman, E. D., *et al.* (1971) 'Twenty-four hour pattern of the episodic secretion of cortisol in normal subjects', *The Journal of Clinical Endocrinology & Metabolism*, 33, 14-22.
- Wheeler, G. D., *et al.* (1991) 'Endurance training decreases serum testosterone levels in men without change in luteinizing hormone pulsatile release', *The Journal of Clinical Endocrinology & Metabolism*, 72, 422-425.
- Wheeler, G. D., *et al.* (1984) 'Reduced serum testosterone and prolactin levels in male distance runners', *Jama*, 252, 514-516.
- Whembolua, G.-L. S., *et al.* (2006) 'Bacteria in the oral mucosa and its effects on the measurement of cortisol, dehydroepiandrosterone, and testosterone in saliva', *Hormones and Behavior*, 49, 478-483.
- Wierman, M. E. (2007) 'Sex steroid effects at target tissues: mechanisms of action', *Advances in Physiology Education*, 31, 26-33.

- Wilcox, R. R. (2010) *Fundamentals of Modern Statistical Methods : Substantially Improving Power and Accuracy*, New York, NY: Springer Science+Business Media, LLC.
- Williams, R. H. (1962) *Textbook of Endocrinology*.
- Woof, J. M. & Kerr, M. A. (2006) 'The function of immunoglobulin A in immunity', *The Journal of Pathology*, 208, 270-282.
- Wyatt, F. B., Donaldson, A. & Brown, E. (2013) 'The Overtraining Syndrome: A Meta-Analytic Review', *Journal of Exercise Physiology*, 16, 12-23.
- Yaegaki, M., *et al.* (2007) 'Change in the capability of reactive oxygen species production by neutrophils following weight reduction in female judoists', *British Journal of Sports Medicine*, 41, 322-7.
- Yaegaki, M., *et al.* (2008) 'Measuring neutrophil functions might be a good predictive marker of overtraining in athletes', *Luminescence*, 23, 281-286.
- Yoshimura, C., *et al.* (2001) 'Glucocorticoids induce basophil apoptosis', *Journal of Allergy and Clinical Immunology*, 108, 215-220.
- Zeier, H., Brauchli, P. & Joller-Jemelka, H. I. (1996) 'Effects of work demands on immunoglobulin A and cortisol in air traffic controllers', *Biological Psychology*, 42, 413-423.

## Appendices

**Appendix 1: Physical activity readiness questionnaire (PAR-Q)**



Please circle the correct answer:

1. Have you ever been told by your doctor that you have a heart condition and advised only to participate in physical activity approved by your doctor?

Yes / No

2. Do you experience any chest pains when you participate in physical activity?

Yes / No

3. Have you recently experienced any chest pains whilst not participating in physical activity?

Yes / No

4. Do you ever lose consciousness?

Yes / No

5. Do you ever lose your balance as a result of dizziness?

Yes / No

6. Do you have any problems with your bones and joints that could cause further problems if you participate in physical activity?

Yes / No

7. Are you aware of any reasons as to why you should not participate in physical activity?

Yes / No

Name: .....

Signature: ..... Date: .... / .... / .....

**Appendix 2: Blood screening analysis form**



Please read the following:

- a. Are you suffering from any known active, serious infection?
- b. Have you had jaundice within the previous year?
- c. Have you ever had any form of hepatitis?
- d. Have you any reason to think you may be HIV positive?
- e. Have you ever been involved in intravenous drug use?
- f. Are you a haemophiliac?
- g. Is there any other reason you are aware of why taking blood might be hazardous to your health?
- h. Is there any other reason you are aware of why taking your blood might be hazardous to the health of the technician?

Can you answer **Yes** to any of questions a-g? Please tick your response in the box below:

Yes  No

Samples of your blood will be collected in the manner outlined to you by the qualified laboratory technician. All relevant safety procedures will be strictly adhered to during all testing procedures (as specified in the Risk Assessment document available for inspection in the laboratory).

I declare that this information is correct, and is for the sole purpose of giving the tester guidance as to my suitability for the test.

Name .....

Signed .....

Date .... / .... / .....

If there is any change in the circumstances outlined above, it is your responsibility to tell the person administering the test immediately.

**Appendix 3: Health-related daily routine questionnaire**



To be completed by the participant:

Name:.....

Date:...../...../.....

Please read the following carefully and answer as accurately as possible. Your answers will be treated as strictly confidential. If you have any doubts or difficulties with any of the questions please feel free to ask the researcher responsible for the study.

Are you asthmatic?

Yes  No

Do you have any allergies (e.g. Hay Fever)?

Yes  No

**In your regular daily routine:**

Do you smoke?

Daily  Occasionally  Never

Do you drink alcohol?

Daily  Occasionally  Never

You consider your diet is...

Balanced  Unhealthy  Specific

Hours of sleep?

Below 5  5-7 hours  Above 7

***Thank you very much!***

**The Researcher:** \_\_\_\_\_

\_\_\_\_\_/\_\_\_\_\_/\_\_\_\_\_

**The Participant:** \_\_\_\_\_



## Appendix 4: Recovery-stress questionnaire for athletes (RESTQ-76)



## RESTQ-76 Sport

Participant no. \_\_\_\_\_

Age: \_\_\_\_\_ Gender: M / F Date: \_\_/\_\_/\_\_\_\_ Time: \_\_:\_\_

This questionnaire consists of a series of statements. These statements possibly describe your psychic or physical well-being or your activities during the past few days and nights.

Please select the answer that most accurately reflects your thoughts and activities. Indicate how often each statement was right in your case in the past days.

The statements related to performance should refer to performance during competition as well as during practice.

For each statement there are seven possible answers. Please make your selection by marking the number corresponding to the appropriate answer.

**Example:*****In the past (3) days/nights***

... I read a newspaper.

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	<del>very often</del>	always

In this example, the number 5 is marked. This means that you read a newspaper very often in the past three days.

Please do not leave any statements blank.

If you are unsure which statement to choose, select the one that most closely applies to you.

Please turn the page and respond to the statements in order without interruption.

***In the past (3) days/nights*****1) ... I watched TV**

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**2)... I did not get enough sleep**

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**3)... I finished important tasks**

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**4)... I was unable to concentrate well**

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**5)... everything bothered me**

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**6)... I laughed**

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**7)... I felt physically bad**

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**8)... I was in a bad mood**

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**9)... I felt physically relaxed**

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**10)... I was in good spirits**

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**11)... I had difficulties in concentrating**

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**12)... I worried about unresolved problems**

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**13)... I felt at ease**

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**14)... I had a good time with friends**

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**15)... I had a headache**

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**16)... I was tired from work**

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**17)... I was successful in what I did**

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**18)... I couldn't switch my mind off**

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**19)... I fell asleep satisfied and relaxed**

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**20)... I felt uncomfortable**

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**21)... I was annoyed by others**

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**22)... I felt down**

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**23)... I visited some close friends**

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**24)... I felt depressed**

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**25)...** *I was dead tired after work*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**26)...** *other people got on my nerves*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**27)...** *I had a satisfying sleep*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**28)...** *I felt anxious or inhibited*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**29)...** *I felt physically fit*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**30)...** *I was fed up with everything*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**31)...** *I was lethargic*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**32)...** *I felt I had to perform well in front of others*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**33)...** *I had fun*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**34)...** *I was in a good mood*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**35)...** *I was overtired*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**36)...** *I slept restlessly*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**37)... I was annoyed**

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**38)... I felt as if I could get everything done**

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**39)... I was upset**

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**40)... I put off making decisions**

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**41)... I made important decisions**

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**42)... I felt physically exhausted**

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**43)... I felt happy**

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**44)... I felt under pressure**

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**45)... everything was too much for me**

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**46)... my sleep was interrupted easily**

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**47)... I felt content**

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**48)... I was angry with someone**

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**49)...** *I had some good ideas*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**50)...** *parts of my body were aching*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**51)...** *I could not get rest during the breaks.*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**52)...** *I was convinced I could achieve my set goals during performance*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**53)...** *I recovered well physically*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**54)...** *I felt burned out by my sport*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**55)...** *I accomplished many worthwhile things in my sport*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**56)...** *I prepared myself mentally for performance*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**57)...** *my muscles felt stiff or tense during performance*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**58)...** *I had the impression there were too few breaks*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**59)...** *I was convinced that I could achieve my performance at any time*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**60)...** *I dealt very effectively with my teammates' problems*

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**61)... I was in a good condition physically**

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**62)... I pushed myself during performance**

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**63)... I felt emotionally drained from performance**

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**64)... I had muscle pain after performance**

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**65)... I was convinced that I performed well**

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**66)... too much was demanded of me during the breaks**

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**67)... I psyched myself up before performance**

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**68)... I felt that I wanted to quit my sport**

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**69)... I felt very energetic**

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**70)... I easily understood how my teammates felt about things**

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**71)... I was convinced that I had trained well**

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**72)... the breaks were not at the right times**

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**73)... I felt vulnerable to injuries**

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**74)... I set definite goals for myself during performance**

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**75)... my body felt strong**

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**76)... I felt frustrated by my sport**

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

**77)... I dealt with emotional problems in my sport very calmly**

0	1	2	3	4	5	6
never	seldom	sometimes	often	more often	very often	always

Thank you very much!



## Appendix 5: Jackson score upper respiratory tract illness questionnaire

**JACKSON SCORE UPPER RESPIRATORY TRACT ILLNESS QUESTIONNAIRE**

Name ..... Subject Number.... Date.....

Trial.....


**Do you think that you are suffering from a common cold or flu today?****Fill in the circle if your answer is YES**    

If yes please complete all the questions below-

**Are any of the following symptoms of the common cold or flu present today? Please indicate your response by filling in one circle for each of the following symptoms:**

<b><u>SYMPTOM</u></b>	<b><u>DEGREE OF DISCOMFORT</u></b>			
	<b>None at all</b>	<b>Mild</b>	<b>Moderate</b>	<b>Severe</b>
<b>Sneezing</b>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
<b>Headache</b>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
<b>Malaise</b> (feeling of being generally unwell, run down or out of sorts)	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
<b>Nasal discharge</b> (runny nose)	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
<b>Nasal obstruction</b> (blocked nose)	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
<b>Sore throat</b>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
<b>Cough</b>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
<b>Ear ache</b>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
<b>Hoarseness</b>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
<b>Fever</b>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
<b>Chilliness</b>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
<b>Joint aches and pains</b>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>

Appendix 6: Food intake diary


<b>Effects of a 12-day intensified-training period on the endocrine and immune systems</b>				
<b>FOOD INTAKE DIARY (24 h before each main trial)</b>				
Name:			Age:	
Height:	cm	Body mass:	kg	
Date				
<b>Breakfast</b> (day before testing)	Time	Food		Weight
	:			
<b>Lunch</b> (day before testing)	Time	Food		Weight
	:			
<b>Dinner</b> (day before testing)	Time	Food		Weight
	:			
Daily totals:				
<b>Breakfast</b> (morning of the trial)	Time	Food		Weight
	:			

## Appendix 7: Training diary

**Rating of Perceived Exertion (RPE) scale**

Numerical rating	How hard you are exercising
6	No exertion at all
7	Extremely light
8	
9	Very light
10	
11	Light
12	
13	Somewhat hard
14	
15	Hard
16	
17	Very hard
18	
19	Unbearably hard
20	Maximal exertion

- ✓ You will need to use this scale to measure your perceived exertion every time you finish your daily training, so you can fully fill out your training diary;
- ✓ A heart rate monitor will also be worn at all time during your training session.

Effects of a 12-day intensified-training period on the endocrine and immune systems				
TRAINING DIARY				
Name:		Age:		
Height:		Weight:		
Type of exercise:				
Resting Heart Rate (HR <sub>rest</sub> ) / Maximal Heart Rate (HR <sub>max</sub> )				
<b>SESSION</b> _____	Date / /	Time :	HR <sub>rest</sub>	bpm
Exercise completed			HR <sub>max</sub>	bpm
			Average HR	bpm
			Duration	min
			Average RPE:	
Comments:				
<b>SESSION</b> _____	Date / /	Time :	HR <sub>rest</sub>	bpm
Exercise completed			HR <sub>max</sub>	bpm
			Average HR	bpm
			Duration	min
			Average RPE:	
Comments:				
<b>SESSION</b> _____	Date / /	Time :	HR <sub>rest</sub>	bpm
Exercise completed			HR <sub>max</sub>	bpm
			Average HR	bpm
			Duration	min
			Average RPE:	
Comments:				
<b>SESSION</b> _____	Date / /	Time :	HR <sub>rest</sub>	bpm
Exercise completed			HR <sub>max</sub>	bpm
			Average HR	bpm
			Duration	min
			Average RPE:	
Comments:				
<b>SESSION</b> _____	Date / /	Time :	HR <sub>rest</sub>	bpm
Exercise completed			HR <sub>max</sub>	bpm
			Average HR	bpm
			Duration	min
			Average RPE:	
Comments:				