The importance of haemoglobin mass for cycling performance

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Bachelor of Science (Hons) in Sport and Exercise Science

University of Birmingham

A thesis submitted in total fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Exercise Physiology

Faculty of Education, Humanities, Law and Theology

Flinders University

Physiology

Australian Institute of Sport

Cycling Australia

February 23rd, 2011

Dedication

This thesis is dedicated to Professor Robert (Bob) Withers and Dr Aldo Sassi – two wonderful scientists who leave behind them a long lasting legacy for excellence in sport and exercise science.

THESIS CONTENTS

PUBLICATIONS

The work in this thesis has been presented at scientific meetings and / or published in peer reviewed journals as listed below:

International Journals

- Seasonal variation of haemoglobin mass in internationally competitive female road cyclists. **Garvican LA**, Martin DT, McDonald W, Gore CJ. Eur J Appl Physiol. 2010 May; 109(2):221-31.
- Stability of hemoglobin mass during a 6-day UCI ProTour cycling race. **Garvican LA**, Eastwood A, Martin DT, Ross ML, Gripper A, Gore CJ. Clin J Sport Med. 2010 May; 20(3):200-4
- Time course of the hemoglobin mass response to natural altitude training in elite endurance cyclists. **Garvican L**, Martin D, Quod M, Stephens B, Sassi A, Gore C. Scand J Med Sci Sports. 2010 Jun 18. [Epub ahead of print]
- Carbon monoxide uptake kinetics of arterial, venous and capillary blood during CO rebreathing. **Garvican LA**, Burge CM, Cox AJ, Clark SA, Martin DT, Gore CJ. Exp Physiol. 2010 Dec; 95(12):1156-66.
- The contribution of haemoglobin mass to increases in cycling performance induced by simulated LHTL. **Garvican LA**, Pottgiesser T, Martin DT, Schumacher YO, Barras M, Gore CJ. Eur J Appl Physiol. 2010 Nov 27. [Epub ahead of print]
- Haemoglobin mass in an anaemic endurance athlete before and after iron supplementati**on. Garvican LA**, Lobigs L, Telford R, Fallon K, Gore CJ.

Accepted for publicatio*n in International Journal of Sports Physiology and Performance, June 2011*

National / International Conferences

- **Garvican LA**, Martin DT, Clark MA, Quod MJ, Stephens B, Prommer N, Schmidt W, Impellizzeri FM, Rampinini E, Sassi A, Gore CJ The Time Course of the Erythropoietic Response to Natural Altitude Training in Elite Endurance Cyclists. *55th Annual meeting of the American College of Sports Medicine 2008*

- Martin DT, Quod MJ, **Garvican LA**, Etxebarria N, Stephens B, Impellizzeri FM, Rampinini E, Sassi A, Gore CJ Cycling Economy Following a 3-wk Natural Altitude Training Camp (~2700m) in Nationally Competitive Cyclists. *55th Annual meeting of the American College of Sports Medicine 2008*

- **Garvican LA**, Martin DT, Clark MA, Quod MJ, Stephens B, Prommer N, Schmidt W, Impellizzeri FM, Rampinini E, Sassi A, Gore CJ. Australian Cyclists living at Passo Stelvio: Physiological adaptations to a 3 week altitude training camp (~2700m). *Science of Cycling: Pre World Championships Congress, Varese 2008*

- **Garvican LA**, Martin DT, Eastwood A, Ross MLR, Abbiss CR, Gripper A, Zorzoli M, Schmidt W, Gore CJ. Haemoglobin Mass, Hct and [Hb] throughout a 6d UCI ProTour cycling race. *14th Annual Congress of the European College of Sports Science 2009*

- **Garvican LA**, Pottgiesser T, Martin DT, Schumacher YO, Fallon K, Barras M, Gore CJ. "The importance of hemoglobin mass for altitude-induced

increases in cycling performance" *15th Annual Congress of the European College of Sports Science 2010*

Awards

2010 recipient of the *Robert T Withers AIS Award* for excellence in sports physiology - Awarded for exercise physiology research that has had a substantial impact, or the potential to have a strong impact, on Australian sport.

FIGURES

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ABSTRACT

Delivery of oxygen during exercise is critically important for endurance athletes. Elite endurance athletes possess superior amounts of haemoglobin versus untrained counterparts, which enables a high rate of oxygen delivery during exercise. Haemoglobin mass (Hb_{mass}) can be measured via carbon monoxide (CO) rebreathing. The research in this thesis utilizes the recently 'optimised' method for the measurement of Hb_{mass} in elite endurance athletes, predominantly cyclists, with the aim of determining the importance of Hb_{mass} for endurance performance by: 1. examining factors which influence changes in Hb_{mass} in athletes, 2. the time course and magnitude of such changes and 3. the importance of changes in Hb_{mass} for cycling performance as reflected by maximal mean power (MMP).

The ability to accurately measure small changes in Hb_{mass} is essential; thus the first part of this thesis is concerned with the methodology underpinning Hb_{mass} measurement. Specifically, the uptake kinetics of CO during CO-rebreathing were investigated to identify potential sources of error associated with measurement in athletes. Compared with the conventional CO-rebreathing method, inhalation of a CO bolus during the optimised method resulted in faster carboxy-haemoglobin uptake, but did not shorten the time required for CO to mix completely throughout the circulation. Individual differences in circulatory mixing time and alterations of CO loss to extra-vascular compartments can confound the estimated Hb_{mass}.

The influence of stage-racing, iron supplementation, training load and altitude on Hb_{mass} was examined. Hb_{mass} remained stable throughout a 6-day pro-cycling stage race, despite plasma volume induced reductions in haemoglobin concentration and haematocrit. Iron supplementation of an anaemic female athlete had rapid and

marked effects on Hb_{mass} - increasing 49% within 2-weeks of supplementation and continuing to increase for 15-weeks. Hb_{mass} varied by ~3% throughout a competitive season in female cyclists, and was related to chronic training load. Additionally, changes in Hb_{mass} were associated with changes in MMP during training and racing.

The time course of the Hb_{mass} response during 3-weeks of natural altitude exposure (2760m) was determined, with a substantial increase (3%) in Hb_{mass} observed within 10-days. The time course was consistent with the hourly rate of increase previously documented for simulated altitude. Hb_{mass} and erythropoietin decreased on descent to sea level whilst ferritin increased (possibly indicative of neocytolysis), raising doubts as to the role of an enhanced Hb_{mass} for subsequent performance benefits at sea level. Therefore, in the final part of the thesis, the importance of hypoxia-induced increases in Hb_{mass} on cycling performance was examined. Using a unique study design, increases in Hb_{mass} induced by altitude exposure were removed, effectively 'clamping' the Hb_{mass} response. MMP_{4min} increased by ~4%, despite blocking a ~5% increase in Hb_{mass} suggesting that accelerated erythropoiesis is not the sole mechanism by which hypoxia improves performance. However, increases in Hb_{mass} appeared to influence the aerobic contribution to high-intensity exercise which may be important for subsequent high-intensity efforts. Overall this thesis confirms some existing observations regarding the influence of various external factors on Hb_{mass}, but challenges other notions regarding the importance of Hb_{mass} for traditional measures of endurance performance.

DECLARATION

I certify that this thesis does not incorporate, without acknowledgement, any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

.....

Laura Anne Garvican

We believe that this thesis is properly presented, conforms to the specifications for the thesis and is of sufficient standard to be worthy of examination.

....

Professor Christopher J Gore **Doctor David T Martin**

ACKNOWLEDGEMENTS

For me, this thesis encompasses far more than a series of research papers. This thesis has been an incredible journey, spanning many corners of the globe - from sea level to 2800 m, involving 21 airports, 4 continents and 8 trips around the world. What has made it even more memorable is that I was not alone, and I would like to extend my utmost appreciation to the many people and organisations who accompanied me along the way. Thanks for the ride!

To my supervisors, Chris Gore and David Martin, thank you for showing me the world of cycling, altitude training and Hb_{mass}! Thank you for the many hours of your time you have committed to me during this thesis, the thought processes, the comments, the heated debates – it was not possible without your faith, guidance and support. You have helped shape me into the sport scientist I am today. And to the late Bob Withers, thank you for having faith in me from the beginning and for ensuring that I stay true to my love of tea and the English language. I hope I have done you proud.

To all at Cycling Australia, particularly Shayne Bannan, Warren McDonald, Martin Barras, Brian Stephens and Dave McPartland, thank you for welcoming me into the cycling family and allowing me to share your passion for this amazing sport.

To Elsa Lepore, I don't know how you do what you do, but you make it easier for us all. Thank you.

To the other support staff: Beth Duryea, Christine O'Connor, Wayne Nichols and James Nitis, thank you for the good times (and the tough times) on tour. Now I know what hard work really is.

To Walter Schmidt and Nicole Prommer, thank you for ‘optimising’ the CO-rebreathing technique. Who would have thought carbon monoxide could take me so many places?

To AIS Performance Research, thank you for investing in the Sports Based PhD program. I hope you consider it as worthwhile as I.

To those who had gone before me, Tammie Ebert and Marc Quod – you could have warned me! Thank you for your wisdom, SRM lessons and the dos and don’ts. You ensured I would not be labelled an NTTA!

To all those in Physiology, past and present - what a fantastic place to work, thank you. You are the reason we are able to produce world leading research like nowhere else in the world. And to Marilyn Dickson (Maz) - everyday you continue to make me smile. Thank you for looking out for me!

To all those at Mapei, my Italian home, thank you for your hard work and hospitality. One day I will repay you. To Aldo, you will not be forgotten.

To all the Australian Cyclists, thank you for putting up with the capillary bloods, the broken cables and the nights with ‘no air.’

To Amanda Spratt, you are an inspiration and the most valuable and rewarding part of my PhD experience. I will see you at the movies.

To Megan Ross, we did it! Thank you for being a fantastic friend, but please come home soon – I’ll put the kettle on.

To Mum and Dad, thank you for the travel bug and inquisitive genes. Not the most conventional of PhD’s but it has certainly taken me places, given you something to write about in the Christmas letter and ensured another Garvican is listed on Pubmed.

And last, but of course no means least, to Shaun, thank you for your love, support and patience. We got there in the end.

Thank you.

LIST OF ABBREVIATIONS

AIS	Australian Institute of Sport
BLa	blood lactate
bpm	beats per minute
BV	blood volume
CL	confidence limit
CO	carbon monoxide
Δ	change
$\sum 7$	sum of seven (skinfold thicknesses)
EPO	erythropoietin
g	gram
Hb	haemoglobin
[Hb]	haemoglobin concentration
Hb_{mass}	haemoglobin mass
HbCO	carboxy-haemoglobin
Hct	haematocrit
HIF-1α	hypoxic inducible factor 1-alpha
HR	heart rate
kg	kilogram
L	litre
$L.min^{-1}$	litres per minute
LHTL	Live high: Train Low
Mb	myoglobin
$ml.kg.min^{-1}$	millilitres per kilogram of body mass per minute

$mmol.L^{-1}$	millimole per litre
MMP	maximum mean power output
min	minute
PV	plasma volume
Retics	reticulocytes
RBC	red blood cell
RCV	red cell volume
RPE	rating of perceived exertion
rpm	revolutions per minute
s	second
SD	standard deviation
SL	sea level
SRM	Schoeberer Resistance Measurement
SWC	smallest worthwhile change
TE	typical error
T_{lim}	ride time to exhaustion
T_{mix}	circulatory mixing time
VO_2peak	peak oxygen consumption
VO_2max	maximal oxygen consumption
vs.	versus
W	watt
$W.kg^{-1}$	watts per kilogram of body mass (power to mass ratio)
U23	Under 23 years of age
UCI	Union Cycliste Internationale

Introduction

Road cycling is an endurance sport, with success often accompanied by superior endurance physiology (Coyle, Coggan et al. 1988). Years of endurance training, combined with a favourable genetic disposition, result in a series of physiological adaptations, all designed to maximise endurance performance by increasing the amount of oxygen (O_2) which can be delivered and utilised by working muscles (Hawley and Stepto 2001). These adaptations can be broadly divided into two – those that occur *peripherally* such as increased vascularisation, increased mitochondrial density and increased enzyme activity, which enable O_2 to be extracted and metabolised at a high rate; and those that occur *centrally*, involving the cardiovascular system, and affecting the rate at which O_2 can be delivered around the body. Endurance training enhances this system via adaptations to heart contractility and function, blood volume and O_2 carrying capacity.

When both the peripheral and central systems are highly adapted, as in the case of an elite endurance athlete, high rates of work can be achieved for extended durations. The maximal amount of O_2 which can be consumed during exercise, defined as the maximal aerobic power (VO_2max), is dependent on both the delivery and utilisation of O_2, and as such will be limited by whichever system is least adapted In elite endurance athletes it is generally believed that VO_2max is centrally limited, that is, by the rate of O_2 delivery (Wagner 1996). Therefore, alterations to the O_2 transport system have the potential to enhance VO_2max further (Schmidt and Prommer 2010).

Haemoglobin (Hb), the O_2 carrying protein contained in red blood cells, is therefore critically important for endurance exercise. Total haemoglobin mass (Hb_{mass}) refers

to the total amount of Hb within the body, irrespective of compartmental fluid volumes. Elite endurance athletes are characterised by a high Hb_{mass}, and indeed, there is a strong relationship between Hb_{mass} and VO_2max. A 1 g increase in Hb_{mass} results in an increase in VO_2max of approximately 4 $ml.min^{-1}$ (Schmidt and Prommer 2010). It follows, that Hb_{mass} may also be closely related to endurance *performance;* and it is for this reason, that the goal of the majority of blood doping practices in endurance sport is to increase Hb_{mass}, either by inducing erythropoiesis (e.g. recombinant human erythropoietin administration) or via direct infusion of whole blood. Such practices are banned by the World Anti-Doping Agency (WADA 2011) due to their overwhelming affect on O_2 transport.

Interestingly, whilst the magnitude of changes to Hb_{mass} incurred though blood-doping has a significant impact on endurance performance (Gledhill 1982), the Hb_{mass}-performance relationship under physiological conditions is not as clearly defined as that for VO_2max. What is the affect of a 10 g increase in Hb_{mass} on individual pursuit time or on the ability to sustain a high power output whilst climbing? Much of the difficulty in attempting to answer this question depends upon the ability to define and measure endurance performance; since performance itself can be influenced by a multitude of factors. In addition, the plasticity and scope for alterations to Hb_{mass} via natural means remains to be determined. Therefore the primary aim of the research contained in this thesis is to extend previous investigations of Hb_{mass} in elite endurance athletes, with a specific focus on road cycling performance. Specifically, this thesis aims to quantify the stability and plasticity of Hb_{mass} in elite cyclists as well as to determine the importance of any changes for subsequent cycling performance.

The ability to accurately measure small changes in Hb_{mass} is critically important for longitudinal monitoring; thus, the first part of this thesis is concerned with the methodology underpinning the measurement of Hb_{mass}. In recent years, measurement of Hb_{mass} via carbon monoxide (CO) rebreathing has become a popular research tool, largely due to the recent "optimisation" of Burge and Skinner's method (1995) by Schmidt and Prommer in 2005. The optimised methodology greatly reduces the time required to complete a single test; which, combined with its relative portability has enabled measurement of Hb_{mass} in elite athletes on a more regular basis. In addition to its use in a research setting, the optimised rebreathing method has been proposed as a potential tool for doping detection (Prommer, Sottas et al. 2008). However, in order to guarantee successful application in either instance, the method must be shown to be both accurate and reliable – being able to detect small changes in Hb_{mass} with a low degree of error. It is with this respect, that the methods of Burge & Skinner (1995) and Schmidt & Prommer (2005) were critically compared. Specifically, the uptake kinetics of CO into vascular and non-vascular compartments during the above two protocols of CO-rebreathing were investigated in order to identify the potential sources of error associated with measurement in athletic populations, and therein to make any modifications to the method if required. The data presented serve to demonstrate the validity of the Schmidt & Prommer (2005) method and its suitability for the measurement of small but worthwhile changes in Hb_{mass} of elite endurance athletes.

Owing to the methodology previously available, there is a lack of longitudinal data obtained from elite endurance athletes, and as a result, the stability and plasticity of Hb_{mass} in this population has not been clearly defined. This thesis presents data showing the effect of periods of intense exercise (stage-racing), natural altitude

exposure, and iron supplementation on Hb_{mass} of elite endurance athletes. These data are important for an overall understanding of natural variations in Hb_{mass} which in turn, is an essential prerequisite for its potential application in the fight against blood doping in endurance sport. In addition, understanding the natural rhythms of the Hb_{mass} protein may assist in designing training programs for optimal aerobic adaptation. In this context, longitudinal data are presented which detail the variability of Hb_{mass} throughout a 6-month training and competition season in internationally competitive female cyclists. Changes in Hb_{mass} are analysed in relation to changes in training load and indices of performance, in an attempt to determine the relationship between Hb_{mass} and performance during training and racing.

Based on the current literature, the greatest potential for increasing Hb_{mass} by natural means in a healthy athlete arises from hypoxic exposure. Hypoxia, be it simulated or terrestrial altitude, is a popular training strategy amongst endurance athletes aiming to improve their performance at sea level. The mechanisms responsible for performance enhancements are still debated; however the most popular theory is that increases in VO_2max arising from an enhanced Hb_{mass} are responsible. Therefore, in the final part of the thesis, the importance of hypoxia-induced increases in Hb_{mass} on cycling performance will be examined. Specifically, using a unique study design, the role of Hb_{mass} following simulated-altitude training will be isolated in an attempt to establish the relative contribution of Hb_{mass} vs. non-haematological adaptations for cycling performance.

Overall, this thesis provides insight into the importance of Hb_{mass} for cycling performance – information which may be useful in the optimisation of athletic performance, as well as in the fight against blood – doping in endurance sport.

AIMS

The primary aim of this thesis was to determine the importance of Hb_{mass} for endurance performance by addressing the following key questions:

1. What factors influence changes in Hb_{mass} in elite endurance athletes (primarily cyclists), and what is the time course and magnitude of such changes?
2. How important are changes in Hb_{mass} for cycling performance as reflected by maximal mean power (MMP) for a fixed duration?

Specifically, the aims of each of the studies contained in this thesis are outlined below:

Study 1: CO uptake kinetics of arterial, venous and capillary blood during CO-rebreathing

1. Compare the uptake and distribution of CO throughout the circulatory system during two different methods of CO-rebreathing.
2. Determine the impact of differences in circulatory mixing time (t_{mix}), CO diffusion to myglobin (Mb), and CO wash-out following rebreathing on Hb_{mass}.

Study 2: Stability of haemoglobin mass during a 6 day UCI ProTour cycling race

1. Quantify the stability of Hb_{mass} throughout a UCI ProTour event in professional cyclists.
2. Assess the reliability of the method in a race setting and its viability as a potential anti-doping detection tool.

Study 3: Case study - Haemoglobin mass in an anaemic female middle –distance runner before and after iron supplementation

1. Document changes in Hb_{mass} following iron supplementation in a female endurance athlete diagnosed with iron-deficient anaemia.

Study 4: Time course of the haemoglobin mass response to natural altitude training in elite endurance cyclists

1. Determine the time course of Hb_{mass} changes to natural moderate altitude during and for 10 days after a 3 week altitude training camp in elite endurance athletes.

Study 5: Seasonal variation of haemoglobin mass in internationally competitive female road cyclists

1. Quantify the seasonal variation of Hb_{mass} in a group of internationally-competitive female endurance cyclists.
2. Examine the relationship between changes in training load and changes in Hb_{mass}.
3. Model the relationship between changes in Hb_{mass} with changes in road cycling performance, estimated from mean maximal power measured during training or racing.

Study 6: The importance of haemoglobin mass for increases in cycling performance induced by simulated LHTL

1. Investigate the importance of increases in Hb_{mass} for cycling performance following simulated normobaric LHTL, by removing any Hb_{mass} gained

throughout the period of hypoxic exposure and, thereby, effectively "clamping" the Hb_{mass} response.

CHAPTER 1:
Review of Literature
The Importance of haemoglobin mass for endurance performance

1.1 INTRODUCTION

The areas of Hb_{mass}, VO_2max and altitude training, and their interlinking relationships have been extensively reviewed (Gledhill, Warburton et al. 1999; Friedmann-Bette 2008; Schmidt and Prommer 2008; Schmidt and Prommer 2010). Most recently, Schmidt and Prommer (2010) published an excellent review on the impact of alterations to Hb_{mass} on VO_2max. However, despite the extent to which these topics have been reviewed, little attention has been placed on the relationship between Hb_{mass} and athletic performance. Therefore, the novel aspect of this review will focus on the importance of Hb_{mass} for endurance performance.

Maximal aerobic power (VO_2max) in man is defined by the Fick equation as a function of cardiac output (Q) and the arterial-venous oxygen difference (a-vDO_2):

$$VO_2max = Q \times a\text{-}vDO_2$$

Put simply, VO_2max is determined by the amount of oxygen (O_2) delivered to working muscles during exercise as well as the ability of these muscles to extract and utilise the O_2 available. In elite athletes, it is generally believed that the limiting factor for VO_2max is O_2 delivery rather than utilisation (Wagner 1996). O_2 delivery, in turn, is dependent on Q and arterial O_2 content (CaO_2) (Gledhill 1985). Haemoglobin (Hb) is the O_2 carrying protein contained in red blood cells, the total

content of which, largely determines CaO_2. Changes to the total Hb content of blood, can therefore have marked consequences for VO_2max in man (Gledhill 1985; Prommer, Sottas et al. 2008).

Hb content may be defined in a number of ways. *Haemoglobin concentration* ([Hb]) refers to the concentration of Hb within the entire blood volume and is commonly measured from a venous blood sample. By definition, concentrations rely on a fluid component, and therein lies a weakness of [Hb] measurement, since compartmental fluid shifts may confound results (Convertino 1991; Schumacher, Pottgiesser et al. 2008). Similarly, *haematocrit* (Hct) – the fraction of blood volume made up of red blood cells, may be influenced by plasma volume perturbations, and furthermore is a measure of erythrocyte fraction rather than Hb content directly (Schmidt, Biermann et al. 2000). *Total haemoglobin mass* (Hb_{mass}) refers to the total amount of Hb contained in the body (in grams) independent of fluid components, and therefore provides a strong indication of the O_2 carrying capacity of the blood (Gledhill 1985).

1.2 Measurement of Hb_{mass}

For over a century, scientists have been interested in the measurement of blood volume. Traditionally, the methodology for measurement involved the use of a radioactive label, for example Chromium 51 (^{51}Cr), which whilst considered the gold standard (Haematology 1980) in terms of accuracy, could not be repeated on a frequent basis due to the cost and time involved as well as the radioactive risk to the subject (Gore, Hopkins et al. 2005). The use of Evan's Blue dye (Gibson and Evans 1937) for the indirect measurement of red cell volume (RCV) subsequently gained popularity; however its accuracy and reliability have been questioned (Gore, Hopkins

et al. 2005). Carbon monoxide (CO) was first proposed as a tracer in 1882 (Grehant and Quinquard 1882). The high binding affinity of CO for Hb ensures that the tracer is quickly taken up and distributed throughout the circulation. However, early progress in the development of the CO rebreathing method was stalled due to the inability to accurately measure carboxy-haemoglobin (HbCO) fractions in sampled blood. Following the development of the CO-oximeter (Fogh-Andersen, Siggaard-Andersen et al. 1987) in the 1980's, Burge and Skinner revisited the CO rebreathing method, publishing a refined method in 1995, which displayed both high validity compared with the ^{51}Cr method and superior reliability to Evan's Blue (Burge and Skinner 1995; Gore, Hopkins et al. 2005).

The CO rebreathing method is based on dilution principles, with the equation below, fundamental to all subsequent calculations (Fairbanks 2000).

$$C_1V_1 = C_2V_2$$

Where:

C_1 = concentration of the solution at baseline, before dilution

C_2 = final concentration of the solution, after dilution.

V_1 = volume to be diluted

V_2 = final volume after dilution

First, the initial concentration of CO bound to Hb (HbCO) is determined, before a known volume of CO is administered. The CO dose is rebreathed though a closed circuit, enabling CO to be evenly distributed and mixed throughout the vascular system. Once equilibrium is reached throughout the entire circulation, the fraction of HbCO (%) is again determined. Hb_{mass} can then be calculated based on the volume of

CO in the blood (VCO) and the change in HbCO in the blood before and after rebreathing (Δ%HbCO) using the following equation:

$$Hb_{mass} = VCO \text{ in blood} / \Delta\%HbCO$$

Example: *According to Hufner's constant (Gorelov 2004), the binding capacity of CO to Hb is 1.39 ml.g^{-1}, that is, one gram of Hb binds 1.39 ml of CO. Therefore, 100 ml of CO will bind to 72 g of Hb. If Δ%HbCO is 7% (i.e, 7% of the total Hb in the blood is bound to CO), then 7% of Hb_{mass} = 72 g, and total body Hb_{mass} = 1028 g.*

The CO rebreathing method of Burge and Skinner has proven to be an accurate and reliable method of Hb_{mass} and blood volume determination (Burge and Skinner 1995; Gore, Hopkins et al. 2005), with the added benefit that repeated measurements can be performed longitudinally without adverse side effects. However, the original Burge and Skinner method involved at least 10 minutes of rebreathing, during which the subject was supine with a venous cannula for blood sampling inserted into an antecubital vein, and as a result the method was not easily incorporated during 'in-the-field' or 'routine' assessment of Hb_{mass} in athletic populations.

Schmidt and Prommer worked to further refine the CO rebreathing method, publishing the "optimised" rebreathing method in 2005 (Schmidt and Prommer 2005). The most notable changes to the technique involved redesigning the rebreathing spirometer to be more portable in nature and, importantly for the athlete, reducing the critical rebreathing step from 10-min to 2-min. In Schmidt and Prommer's method, CO is administered as a bolus in order to increase the rate of uptake into arterial circulation (Bruce and Bruce 2003). Following 2-min of rebreathing the subject is disconnected from the rebreathing apparatus and allowed to breathe room air. HbCO is measured from capillary blood samples obtained prior to

rebreathing and 7 minutes after inhalation of the CO bolus. Hb_{mass} is calculated using the equation presented earlier, however loss of CO via exhalation (minutes 2-7) and diffusion to myoglobin (Sawka, Convertino et al. 2000) must be accounted for as well as any CO remaining in the spirometer at the end of the rebreathing manoeuvre (Schmidt and Prommer 2005).

The 2-min optimised rebreathing method has been proposed as an efficient and practical tool for the regular assessment of Hb_{mass} in athletes. The sensitivity of Schmidt and Prommer's method to detect changes in Hb_{mass} has been assessed using phlebotomy. Schmidt and Prommer themselves, reported a 95 ± 19 g lower Hb_{mass} measured before and after a standard blood donation; with a mean error versus the expected calculated value of 9 ± 12 g (Schmidt and Prommer 2005). Pottgiesser et al (2007) confirmed and extended these results by demonstrating that changes in Hb_{mass} induced by withdrawal and reinfusion of packed red cells were detectable using the 2-min method. Changes ranging from -120 to -80 g following withdrawal and from +50 g to +100 g following reinfusion were detected. The mean error between measured and expected values (n=10) was 1 g ± 14 after donation and 2 g ± 19 g after reinfusion, although in individual cases both an over and underestimation were observed (Pottgiesser, Umhau et al. 2007). Taken together, these results confirm the sensitivity of the method to confidently detect changes in Hb_{mass} of at least 60 - 100 g, equating to ~7 - 10% in an athlete possessing a Hb_{mass} of 900 g. However, in sports science research, where small changes can be potentially meaningful, the ability to detect oscillations of Hb_{mass} of ~30 g in magnitude (~3% in the aforementioned example) is important. For this, a low typical error (TE) of measurement is essential. Indeed, as outlined in **Table 1-1**, many researchers have reported excellent reliability of ~2% with Schmidt and Prommer's method (Gore,

Bourdon et al. 2006; Pottgiesser, Umhau et al. 2007; Eastwood, Hopkins et al. 2008; Clark, Quod et al. 2009; Eastwood, Bourdon et al. 2009; Robertson, Saunders et al. 2010) and comparable results (~2%) to that of the Burge and Skinner method (Schmidt and Prommer 2005; Gore, Bourdon et al. 2006; Prommer and Schmidt 2007) provided aspects of the calculations used are similar (Steiner and Wehrlin 2010).

Table 1-1: Typical Error (TE) associated with the "optimised" CO rebreathing method for measurement of Hb_{mass}

Authors	Subjects	Method	TE %
Schmidt et al. 2005	11 healthy subjects (8 m, 3 f)	Schmidt	1.7 (3.3)
Gore et al. 2006	15 recreationally active (10 m, 5 f)	Schmidt	1.1 (0.9-1.8)
Gore et al. 2005	Meta analysis	Schmidt	2.2 (1.4-3.5)
Eastwood et al. 2008	23 adolescents (12 m, 11f)	Schmidt	2.3 (1.7-3.6)
Eastwood et al. 2008	6 active men	Schmidt	2.1 (1.7-2.6)
Prommer et al. 2008	24 trained athletes (20 m, 4 f)	Schmidt	1.4 (1.1-1.7)
Schumacher et al. 2007	7 male U23 cyclists	Schmidt	3.3
Pottgiesser et al. 2008	29 male volunteers	Schmidt	1.5
Clark et al. 2009	12 well trained male cyclists	Schmidt	1.9 (1.5-3.0)
Robertson et al. 2010	16 highly trained runners (11m, 5 f)	Schmidt	2.0(1.6-2.6)

Method: Schmidt – Schmidt and Prommer (2005), TE (90% CL) provided where available

The largest threat to the reliability of the method is associated with the accurate determination and delivery of the CO bolus, combined with its subsequent complete mixing throughout all vascular compartments. To date, the time required for CO to completely mix is debated, with only two studies documenting mixing times using capillary and venous blood (Gore, Bourdon et al. 2006; Prommer and Schmidt 2007). Before implementing the method, it is therefore important to conduct an independent comparison of the method as well as exploring the kinetics of CO uptake in arterial, venous and capillary blood.

Whilst the CO rebreathing method has become a popular tool in sports science research, the use of CO for the measurement of Hb_{mass} has been criticised (Sawka, Convertino et al. 2000), with the primary concern being that any loss of CO from the vascular bed (e.g. to myoglobin) may lead to an overestimation of Hb_{mass}. In 2007, Prommer and Schmidt (Prommer and Schmidt 2007) published data to the contrary, demonstrating minimal impact on Hb_{mass} calculations if an appropriate correction is made (Steiner and Wehrlin 2010). However, the impact of within subject variation in CO loss to myoglobin has not been investigated to date.

In summary, measurement of Hb_{mass} via CO rebreathing is a safe, accurate and reliable technique in experienced hands, which is well tolerated by the vast majority of athletes. Measurement error can be minimized by carefully controlling aspects of the methodology, e.g. the measurement and delivery of the CO dose, the number of replicates analysed for HbCO (Alexander, Garvican et al. 2010) and the timing of blood sampling (Gore, Bourdon et al. 2006; Prommer and Schmidt 2007). However, the influence of biological variation on the total error of measurement warrants further investigation.

1.3 Hb_{mass} of Athletic populations

Cross sectional studies of athletic populations reveal that endurance trained athletes display a higher Hb_{mass} than untrained subjects. The Hb_{mass} of elite middle and long distance runners (n=40) was reported to be on average 20% higher than that of age-matched non-athletes (n=12) (Brotherhood, Brozovic et al. 1975)

Total Hb_{mass} also appears in part to be related to competitive level, with elite populations having greater Hb_{mass} values than sub-elite (Heinicke, Wolfarth et al.

2001), and to training status (Kjellberg, Rudhe et al. 1949). In a comprehensive study, Heinicke et al (2001), investigated the Hb_{mass} characteristics (using the CO rebreathing method of Burge and Skinner, 1995) of 94 elite male athletes from 6 disciplines, and compared the results to 37 controls of varying activity status (Heinicke, Wolfarth et al. 2001). With the exception of downhill skiers, all disciplines displayed a significantly greater (+ 40%) relative Hb_{mass} than untrained controls. However, no significant differences were observed when the endurance disciplines of triathlon, running and cycling were compared. The lower Hb_{mass} observed in downhill skiers is believed to reflect the anaerobic nature of the training and competition demands of this discipline.

1.4 Stability of Hb_{mass}

Only a few longitudinal studies exist which document the stability of Hb_{mass} over time. Eastwood et al. (2008) documented the stability of Hb_{mass} over 100 days in 6 recreationally active men (Eastwood, Hopkins et al. 2008). Hb_{mass} was measured using the optimised rebreathing method on 42 ± 3 occasions over 100 - 114 days, with the mean error of measurement across all 6 subjects calculated to be 2.1%. The results indicate that biological variation exceeding 2% is not present over 100 days. Furthermore, pairwise comparisons did not reveal an increase in error over time as had previously been suggested (Gore, Hopkins et al. 2005). In addition, Schmidt and Prommer (2008) report longitudinal data spanning a 9 year period, over which Hb_{mass} was unchanged (1028 ± 184 g pre vs. 1023 ± 196 g post), despite changes in training volume and lifestyle. Again, indicating that Hb_{mass} is relatively stable in healthy adults, and possibly largely determined by genetics.

The effect of "growth" vs. "growth and training" on Hb_{mass} was investigated in a group of adolescents (~13 years old) (Eastwood, Bourdon et al. 2009). Following duplicate baseline measures, Hb_{mass} was measured every 3 months over a 12 month period, in 12 cyclists and 11 age matched controls. Absolute values of Hb_{mass} increased with time in both groups, however relative Hb_{mass} did not increase substantially in either group. The authors concluded that training does not increase Hb_{mass} of adolescents over and above that explained by growth, and attribute higher Hb_{mass} values in some subjects to an hereditary trait. However, indicators of maturation and puberty were not obtained and thus further investigations into the effects of maturation on Hb_{mass} are warranted.

1.5 Effects of endurance training on Hb_{mass}

The high Hb_{mass} reported in elite endurance athletes poses an intriguing question. Do years of endurance training culminate in an elevated Hb_{mass}, or is it a largely genetic trait which predisposes these individuals to excel at endurance sport via natural selection? To answer this question, researchers have attempted to document the affect of endurance training on blood volume using longitudinal designs.

To date, there have been few longitudinal studies conducted using truly elite athletes and in addition, much of the published research relates to measurement of RCV using indirect techniques (e.g. Evan's blue) and not Hb_{mass}. Nevertheless, following a comprehensive review, Sawka (2000) reports a positive effect of endurance training on total blood volume. Initial increases in blood volume during the first few days of training (up to ~2 weeks) are postulated to be almost entirely accounted for by plasma volume expansion, with increases in erythrocyte volume not evident for at

least 2-3 weeks. Erythrocyte volume increases at an "undetermined rate" before stabilizing at a new "steady state", which can be 8-10 % higher than in the untrained state (Sawka, Convertino et al. 2000). It should be noted however, that the time course of vascular volume alterations described by Sawka refers to the average response from 18 studies in which blood volume, plasma volume and erythrocyte volume were determined from pre and post measures only.

The scientific literature documenting the effects of training on Hb_{mass} and erythrocyte volume has produced some inconsistent results. Green et al (1991) monitored 7 untrained males during 8 weeks of progressive cycle training and observed no changes in RCV measured using ^{51}Cr (Green, Sutton et al. 1991). The researchers postulated that the 8 week training period was too short to elicit an erythropoietic response, but in a follow up study involving an 11 week training program, RCV was again unchanged (Shoemaker, Green et al. 1996). The authors were surprised by this finding and concluded that the higher RCV reported in trained athletes must either be genetically determined or the result of a markedly different type of training program undertaken by elite athletes compared with their subjects.

Two studies involving trained athletes have also failed to document an increase in RCV or Hb_{mass} following training. Gore et al. 1997, reported no change in Hb_{mass} (measured via CO rebreathing) following 12 weeks of intense training in 12 elite female rowers (819 ± 22 g vs. 817 ± 23 g), or in response to 4 weeks of heat training (32◦C) in 24 male and female rowers (Gore, Hahn et al. 1997). Similarly, four weeks of sea level training did not increase RCV in trained runners (Levine and Stray-Gundersen 1997). Some researchers have speculated that the absence of an erythropoietic response in highly trained athletes is due to either an insufficient

training stimulus (Sawka, Convertino et al. 2000) (evidenced by the lack of a training effect e.g. no change in VO_2max (Levine and Stray-Gundersen 1997)) or the result of a limited capacity for further adaptation imposed by pre-existing erythrocythemic hypervolemia (Gore, Hahn et al. 1997).

A few studies have however, reported an increase in RCV in response to training. Early work of Kjellberg et al (1949), using the CO rebreathing method of Sjostrand (Sjostrand 1948), reports a 10-19% increase of Hb_{mass} in 4 recreationally active subjects following 9 days of mountainous ski training (Kjellberg, Rudhe et al. 1949), yet the authors note that "other factors than the physical training may have played some part," and thus these results must be viewed with caution. Similarly, the ~5% increase in RCV (using Evan's Blue) after 3 weeks of aerobic cycle training observed by Schmidt in 6 healthy untrained males has been criticised due to potential errors arising in the calculation of RCV following indirect measurement (Schmidt, Maassen et al. 1988). The most convincing long term training study is that of Remes (1979), in which a ~4% increase in RCV measured with ^{51}Cr was reported, following 6 months of military training (Remes 1979). However, the untrained nature of the subjects again makes it difficult to extrapolate the results to elite athletic populations.

In perhaps the only comprehensive longitudinal training study available involving the "optimised" CO rebreathing method, Schmidt and Prommer (2008) report a 6.4% increase in Hb_{mass} of 16 leisure sportsmen following 9 months of marathon training (Schmidt and Prommer 2008). Specifically, in this study, Hb_{mass} increased from 932 ± 112 g to 992 ± 103 g ($p < 0.001$) between December and September and was accompanied by ~5.6% increase in VO_2max. Whilst this study demonstrates that Hb_{mass} can be modulated by long periods of training, two important aspects should be

noted. First, the subjects were relatively untrained at the start of the study, and thus undertook a large increase in training load over the course of the study. Secondly, even though a significant increase in Hb_{mass} was observed across the group, relative values did not reach the high values observed in elite distance runners (14 - 15 $g.kg^{-1}$ (Prommer, Thoma et al. 2009)), suggesting that other factors aside from training may be responsible for the high values reported in elite athletes.

If the high Hb_{mass} values observed in elite athletes are, indeed, predominantly a genetic trait, it would be expected that Hb_{mass} values would be relatively stable across a training year. Endurance athletes regularly undertake changes in training load during macro and micro training cycles, and the impact of such oscillations in training on Hb_{mass} has not been established. The reason for the lack of data in this respect, lies in the methodology previously available – radioactive tracers were expensive and could not be repeated on a regular basis; indirect methods lacked the sensitivity and reliability to measure small but potentially worthwhile changes in Hb_{mass}; and earlier versions of the CO rebreathing method were time consuming and not easily transportable. However, the optimised rebreathing technique is relatively portable, reliable and fast to administer, making frequent measurements on athletic populations much more accessible.

Prommer et al (2008) utilised the method to perform repeated measurements throughout a training year in trained athletes. Five measures were performed over 12 months, and timed to reflect different phases of the training year (e.g. off season, competition season) (Prommer, Sottas et al. 2008). Only minor oscillations in Hb_{mass} were observed, with the mean individual oscillation (highest-lowest value during the year) being 4.6%, leading the authors to conclude that the erythropoietic system of

elite athletes is largely insensitive to training (Schmidt and Prommer 2010). However, the methodology employed to quantify training load in this study was rather crude (hours per week) and the time between measures (~3 months) only allows a coarse assessment of Hb_{mass} stability over a competitive season. Further work is therefore warranted, in order to closely examine the relationship between changes in training load and Hb_{mass}, using elite level athletes and a more sophisticated approach to training load quantification.

In summary, studies examining the influence of training on Hb_{mass} are somewhat limited. Despite the observation that training can be associated with small increases in Hb_{mass} of a few percent, some researchers have observed no changes despite prolonged training. Preliminary evidence suggests that the very high levels of Hb_{mass} observed in elite athletes is either due to prolonged cumulative effects of training or is genetically predetermined and hence 'naturally selected.'

1.6 Factors influencing the stability of Hb_{mass}

Whilst currently available data indicates that Hb_{mass} remains relatively stable in elite athletes engaged in regular training, a number of external influences or circumstances have the potential to dramatically alter Hb_{mass} values. These may include: (i) periods of inactivity – either through illness and/or injury or voluntary detraining periods, (ii) periods of intensely strenuous exercise (e.g. Ironman triathlon, multi-stage racing), (iii) iron deficiency or supplementation, (iv) altitude exposure and (v) blood manipulation. Determining the effect of potential confounders on Hb_{mass} has two facets of importance – one from a monitoring perspective and the other being related to performance. If Hb_{mass} is to be used as long

term monitoring parameter (perhaps in an anti-doping setting), then it is important to understand the magnitude of change that may be expected following such influences. Alternatively, if coaches and athletes seek to optimise their Hb_{mass} values for endurance performance, then an understanding of the natural scope, variability and plasticity of the protein is important.

1.6.1 Inactivity due to illness / injury

Acquiring data pertaining to Hb_{mass} and illness / injury is difficult and often requires 'reactive' action. Therefore, with the exception of a few case studies, little data are presently available. Kjellberg et al. (1949) was one of the first to document a sudden change in Hb_{mass} due to inactivity, reporting a 15% decrease following 1.5 months of bed rest arising from a leg fracture. The response occurred rapidly, with a decrease observed after the first measurement at 10 days post injury (Kjellberg, Rudhe et al. 1949). More recently, Schumacher et al. published a case report, documenting the changes in Hb_{mass} of an elite cyclist immobilised for 4 weeks due to injury sustained from a collision with a car during training (Schumacher, Ahlgrim et al. 2008). Hb_{mass} decreased by 19%, and even when blood loss arising from surgery and the trauma itself were accounted for, a 14% decrease could still be attributed to inactivity. Furthermore, when physical activity was once again resumed, Hb_{mass} returned to pre injury values within 2 months.

A large decrease in erythrocyte volume (up to 40%) has also been documented in wounded soldiers, which could not be explained by the trauma itself, or by weight loss, limb atrophy or inactivity (Valeri and Altschule 1981). The military physicians who documented this "Missing Blood Syndrome" attributed the loss of erythrocytes in these soldiers to suppression of erythropoiesis by multifunctional cytokines,

thereby resulting in a form of the phenomenon known as "the anaemia of chronic disease" (Sawka, Convertino et al. 2000). These data indicate that the Hb_{mass} 'set point' of an individual is sensitive to some external factors and may be regulated accordingly.

To date, it is unclear whether illness and injury solely influence Hb_{mass} due to enforced periods of inactivity or whether underlying mechanisms associated with the presented condition regulate Hb_{mass}. Investigations which compare Hb_{mass} changes during periods of voluntary inactivity in healthy individuals with forced inactivity due to illness or injury are therefore warranted.

1.6.2 Intense exercise

Whilst currently available data suggest that Hb_{mass} is relatively stable across a training year, the impact of periods of very intense or strenuous exercise on Hb_{mass} has not been completely determined. Examples of intense exercise which may have the potential to cause perturbations in haematological parameters include multi-stage cycle racing, "Ironman" triathlon, marathon running or expedition style adventure races.

A few studies have focused on the impact of racing on various haematological parameters – predominately using cyclists. In these investigations, conventional haematological analysis of venous blood has been employed in order to assess the effect of both chronic and acute periods of competition.

Schumacher et al. (2002) evaluated the levels of [Hb] and Hct in highly trained cyclists over the course of a competitive season. 1628 samples from 224 German national team cyclists (1326 male samples, 302 female samples) were analysed and

revealed significant seasonal changes for [Hb] and Hct in both male and female cyclists. Most notably, Hct and [Hb] decreased during the summer months – coinciding with periods of intensified training or competition, leading the authors to suggest that heavy training may influence the erythropoietic system at least on a seasonal basis (Schumacher, Jankovits et al. 2002). In another study, Hct and [Hb] of the CSC Professional cycling team was analysed over the 2006/2007 competitive season. Again, both Hct and [Hb] decreased in-competition compared to the off season (Morkeberg, Belhage et al. 2009).

Haematological changes exhibited during competition show a similar trend. [Hb] and Hct during a 5 day stage race, decreased in 23 cyclists but not in 16 inactive controls (Schumacher, Temme et al. 2003). Similarly, repeated bouts of cycle-exercise over 10 days of racing, reduced venous Hct from 46.4 ± 1.5% to 41.3 ± 1.6% (Schmidt, Biermann et al. 2000). Finally, whilst not significant, Hct and [Hb] measured before and on the 4^{th} and 8^{th} morning of the Tour of Guadeloupe (a 9 day cycle race in a hot and humid environment) showed a trend towards lower values as the tour progressed, with [Hb] decreasing from 14.2 ± 0.6 to 13.5 ± 0.6 $g.dL^{-1}$ (Hue, Voltaire et al. 2006).

From these studies, it is apparent that measurements of Hct and [Hb] are sensitive to successive bouts of intense exercise. The consensus from researchers however, was that the observed decreases were related to an exercise-induced plasma volume expansion, rather than a breakdown of the Hb protein itself, yet without a direct measurement of Hb, the true impact of racing on Hb could only be inferred. To date, only one study has documented Hb_{mass} throughout a multi-stage cycling race (Schumacher, Pottgiesser et al. 2008). Hb_{mass} was measured in seven U23 German cyclists before, and daily following the first four stages of a professional cycling race

(UCI category 2.1). During the tour, venous blood samples were also obtained for measurement of [Hb] and Hct. Hb_{mass} was not significantly altered during 4 days of racing, whereas in contrast, a decrease in Hct and [Hb] was observed – consistent with a concomitant plasma volume expansion.

1.6.3 Iron deficiency / Supplementation

Endurance athletes may be at risk of iron deficiency, due to the high volume of training performed coupled with a calorie restricted diet (Burke and Read 1993). Insufficient iron intake or excessive iron loss, results in a negative iron balance which, if prolonged, can impair erythropoiesis; manifesting in iron-deficiency anaemia (Nielsen and Nachtigall 1998). The condition is typically diagnosed via low [Hb] and ferritin values obtained from venous blood (Thomas and Thomas 2002). If iron deficiency is deemed to be the cause of the anaemia, then iron supplementation is usually advised and is generally an effective treatment provided that there are no absorption issues. Follow-up analysis of [Hb] and ferritin values are often used to monitor recovery and ascertain the efficacy of supplementation.

Given that [Hb] increases with successful treatment of iron-deficiency anaemia, it could be assumed that Hb_{mass} will also increase. However, little data are available on the Hb_{mass} response to iron supplementation, with the majority of intervention studies only reporting [Hb]. In a recent case study, Treff et al. (2009) documented Hb_{mass} of an elite rower before and following diagnosis of iron deficiency anaemia. Hb_{mass} decreased dramatically with anaemia (-27.6%), but increased 50% in response to iron supplementation. In fact, post treatment values were 8% higher than pre-anaemia values. Interestingly, in this case ferritin levels did not change substantially at any time point and provided no indication of the athlete's condition (Treff, Schmidt et al.

2009). These results suggest that Hb_{mass} is very sensitive to iron stores, and that at least in severely depleted cases, iron supplementation can induce marked changes. The impact of iron supplementation in non-anaemic cases remains to be determined. Twelve weeks of twice daily oral iron supplementation did not increase Hb_{mass} in iron depleted (Ferritin <20 $\mu g.L^{-1}$) non anaemic athletes ([Hb] >11.7 $g.dL^{-1}$ for females, and > 13.5 $g.dL^{-1}$ for males) (Friedmann, Weller et al. 2001). However, in this instance, an increase in [Hb] was also not observed. In contrast, Wachsmuth reported an 8.6% increase in Hb_{mass} in 11 subjects who were deemed iron depleted (Ferritin <25 $\mu g.L^{-1}$) but non-anaemic ([Hb] >12.5 $g.dL^{-1}$ for females, and > 13.5 $g.dL^{-1}$ for males) following 10 weeks of iron supplementation and which was accompanied by an 8.5% increase in VO_2max (Wachsmuth, Aigner et al. 2010). The conflicting results may be largely due to the methodology employed, particularly with regard to the classification system adopted and the type of iron supplementation (Nielsen and Nachtigall 1998).

1.6.4 Altitude / Hypoxic exposure

The influence of altitude and hypoxic exposure on Hb_{mass} and RCV has previously been reviewed in depth (Berglund 1992; Rusko, Tikkanen et al. 2004; Levine and Stray-Gundersen 2006; Friedmann-Bette 2008), yet many unanswered questions remain that will be highlighted below.

Epidemiological research on lifelong altitude inhabitants provides an indication of the potential changes to Hb_{mass} which could occur when sea level residents are exposed to hypoxia (Beall 2007). In the Andes, the end result of five thousand years of adaptation to altitude living is an increased Hb_{mass} and [Hb] compared to typical values of sea level dwellers (Beall, Brittenham et al. 1998). These adaptations appear

to be relative to the altitude of residence, and occur over and above training adaptations. For example, compared with sea level residents, an 11% higher Hb_{mass} is observed at 2600 m, whereas at 3500 m Hb_{mass} can be 14% higher (Heinicke, Prommer et al. 2003; Beall 2007). Furthermore, professional cyclists native to 2600 m, displayed higher Hb_{mass} values than professional cyclists residing at sea level (Schmidt, Heinicke et al. 2002). These observations suggest that the erythropoietic system will readily adapt to hypoxic stress if the need arises.

Indeed, the stimulus arising from acute exposure to hypoxia when sea level residents ascend to altitude evokes a multifaceted adaptive response, of which the erythropoietic system is one part. The low partial pressure of oxygen at higher altitudes initiates a response cascade involving two key signalling proteins – HIF1α and erythropoietin, both of which are involved in erythropoiesis. Serum EPO levels increase dramatically in the first 1-2 days following ascent to altitude (Ge, Witkowski et al. 2002), and reach a peak after approximately 48 hours. If exposure is continued, EPO levels begin to decline, and stabilise just above baseline after 1-2 weeks of continuous exposure. Whilst a sustained elevation in serum [EPO] appears necessary for erythropoiesis to occur, the EPO response to altitude is highly variable both between (Chapman, Stray-Gundersen et al. 1998; Ge, Witkowski et al. 2002) and within (Garvican, Martin et al. 2007) individuals. Furthermore, only weak correlations have been reported between the magnitude of the EPO response and a change in Hb_{mass} (Chapman, Stray-Gundersen et al. 1998; Clark, Quod et al. 2009); highlighting the complexity of the entire pathway. Interestingly, the EPO response remains active to acute hypoxic stress, even after many years of regular exposure. Heinicke et al. (2003) documented a classic EPO response in Chilean soldiers on

ascent to 3550 m, even after 22 years of alternating between sea level and 3550 m every 3-11 days as part of their service (Heinicke, Prommer et al. 2003).

The effects of altitude exposure on Hb_{mass} levels of elite athletes is however, somewhat controversial (Schmidt and Prommer 2008), ranging from no effects (e.g. (Gore, Hahn et al. 1998; Ashenden, Gore et al. 1999; Friedmann, Jost et al. 1999) to ~10% increases (Levine and Stray-Gundersen 1997; Brugniaux, Schmitt et al. 2006). Gore et al. (1998) suggested that the lack of any observed increase in a group of world championship cyclists following 1 month of altitude training at ~2800 m, was the result of an already maximally adapted erythropoietic system, due to years of endurance training (Brotherhood, Brozovic et al. 1975). Certainly, the training status and physiological characteristics of the athletes in Gore's study are by far some of the most superior published to date. However, other possible explanations for the absent response may be due to illness, iron status or a potentially 'over trained' state during the exposure (Gore, Hahn et al. 1998; Saunders, Pyne et al. 2009).

Other studies failing to report an increase in Hb_{mass} (Ashenden, Gore et al. 1999; Friedmann, Jost et al. 1999; Saunders, Telford et al. 2004; Pottgiesser, Ahlgrim et al. 2009) have been later critiqued for being "too short" or "too low" and thereby failing to provide an adequate hypoxic stimulus (Rusko, Tikkanen et al. 2004; Levine and Stray-Gundersen 2006; Wilber, Stray-Gundersen et al. 2007). In contrast, increases in Hb_{mass} have been reported in trained subjects provided an apparent "threshold stimulus" is met. In fact, following numerous studies involving shorter hypoxic exposures which did not elicit a Hb_{mass} response (Ashenden, Gore et al. 1999; Saunders, Telford et al. 2004), Saunders and colleagues found that 46 nights of simulated LHTL at a mean altitude of 2860 m evoked a 4.9% increase in Hb_{mass} in

elite runners (Saunders, Telford et al. 2009). Similarly, a 5.3% increase in Hb_{mass} was reported in 10 national team orienteers following 24 days of natural altitude training at 2500 m (Wehrlin, Zuest et al. 2006). It should be noted however, that the magnitude of change observed in these studies is far below the ~10% changes reported by others (Levine and Stray-Gundersen 1997; Brugniaux, Schmitt et al. 2006; Robach, Schmitt et al. 2006). In the study of Levine et al (1997), red cell volume was measured using the Evan's Blue technique, and thus direct comparison to results obtained using CO rebreathing may not be possible due to differences in reliability of each technique.

Current recommendations suggest that a minimum of 3 - 4 weeks above 2000 - 2500 m are necessary for accelerated erythropoiesis to occur (Levine and Stray-Gundersen 2006; Wilber, Stray-Gundersen et al. 2007). In terms of simulated altitude exposure, it appears that at least 14 $h.d^{-1}$ at simulated altitudes above 2500 m are required (Rusko, Tikkanen et al. 2004; Saunders, Telford et al. 2009), and may explain the apparent absence of a response in earlier simulated LHTL studies in which an average daily dose of only $12h.d^{-1}$ was provided (Ashenden, Gore et al. 1999; Saunders, Telford et al. 2004; Neya, Enoki et al. 2007). Based on the data available, the Australian Institute of Sport has developed a model for simulated LHTL (> $14h.d^{-1}$, 3000 m, $\geq$ 3 weeks) which has been shown repeatedly to induce mean increases in Hb_{mass} in elite athletes in the region of 3 - 4% (Clark, Quod et al. 2009; Robertson, Saunders et al. 2010; Robertson, Saunders et al. 2010).

Whilst evidence in support of a "hypoxic threshold" for erythropoietic adaptation appears strong, it is not yet clear whether a dose-response relationship holds true for increasingly longer exposures. At some point, one must expect a ceiling to be

reached. Indeed, the stabilisation of Hb_{mass} in Andean natives at a higher set point demonstrates that at some point the adaptation is deemed "maximal" and no further increases are observed. However, data pertaining to the time course of adaptation are lacking, and thus it is not clear when the majority of the Hb_{mass} response is achieved during an altitude sojourn. Recently, the time course of the erythropoietic response to simulated LHTL has been published (Clark, Quod et al. 2009; Robertson, Saunders et al. 2010; Robertson, Saunders et al. 2010) indicating an increase in the region of 1% per week for an adequate dose of hypoxia. However, it remains to be determined whether the time course observed with LHTL simulated altitude holds true for natural altitude exposures, where the duration of daily exposure is greater. Nonetheless, based on the predictions above, a period of exposure close to 3 months would be required before Hb_{mass} adaptations to natural altitude reached a similar magnitude to those of long term altitude residents. Indeed, Brothers et al. (2010), report that Hb_{mass} of new recruits to the US Air Force Academy (USAFA) located at 2210 m above sea level, peaked 15 weeks after initial in-processing, reaching values 10.4% (+74 ± 39 g) above baseline (Brothers, Nelson et al. 2010). A slower time course was observed when iron supplementation was not given, with Hb_{mass} peaking at 28 weeks, but the magnitude of change was similar (+ 9.4%, 69 ± 9 g). However, retrospective haematological and performance data from USAFA, suggests that complete acclimatization to 2210 m exceeds 46 weeks, with differences between sea level and native residents still present after almost a year of exposure (Brothers, Doan et al. 2010).

Similarly, little information is available regarding the duration that these new found increases in Hb_{mass} persist once an individual returns to sea level. A phenomenon termed 'neocytolysis' has been described in astronauts during space flight and refers

to the selective destruction of young red blood cells (Alfrey, Udden et al. 1996) which are possibly deemed unnecessary for the current environment. It has been suggested that this phenomenon may also occur on decent from high altitude (Rice, Ruiz et al. 2001), but little data are available following brief soujorns at moderate altitudes. Recently, the Hb_{mass} of elite Kenyan runners was documented during a 6 week training camp in Germany; ~1800 m lower than their native training environment in Kenya. Hb_{mass} remained stable for the first 14 days at sea level, but was ~20 g lower when measured at 21 days, and dropped a total of 45 g (6%) after 33 days (Prommer, Thoma et al. 2010). Whilst these data suggest some form of adaptation to a more hyperoxic environment, the reported change in Hb_{mass} is not rapid enough to be indicative of neocytolysis. Indeed, since the cohort in this study were native altitude residents, it is unlikely that a significant proportion of neocytes were present, and thus neocytolysis in this instance is unlikely. However, recent data from the USAFA shows a 30 – 60% drop in the "newly acquired" Hb_{mass} of first year recruits, following the 3 week winter break at sea level (Brothers, Nelson et al. 2010); which in these newly acclimatised subjects may possibly be due to neocytolysis. However, without a more detailed time course of measurement (> weekly), it is unknown how rapidly de-acclimatisation occurs. If the pursuit of a natural means by which to increase Hb_{mass} provides that rationale for sea level athletes to engage in altitude training, then it must surely be important to understand the time course of re-adaptation to sea level. If altitude-induced Hb_{mass} gains are indeed quickly negated by neocytolysis on return to sea level, then the cost-benefit ratio of altitude training solely for this purpose may need to be reconsidered.

1.6.5 Blood Manipulation

The most obvious threat to the stability of Hb_{mass} comes in the form of blood manipulation. Clearly, if whole blood is removed or infused, Hb_{mass} will be altered (Pottgiesser, Umhau et al. 2007; Morkeberg, Sharpe et al. 2010). Pottgiesser investigated the recovery of Hb_{mass} after blood donation, reporting an immediate decrease by 75 ± 15 g (~8% if Hb_{mass} = 900 g) following donation of 1 unit (550 ml) of blood; and which was not recovered until 36 ± 11 days later (Pottgiesser, Specker et al. 2008). Serendipitously, Eastwood et al. (2007) also documented the time course of recovery following blood donation in a single subject during their investigation into the stability of Hb_{mass}, with a 12.5% increase in Hb_{mass} observed over the 100 d period.

The magnitude of change induced from infusion of blood of course depends on the volume of blood infused. One standard blood transfusion of packed red cells (PRC) if prepared from 1 unit of whole blood contains ~ 60 g of Hb_{mass} (Pottgiesser, Umhau et al. 2007), resulting in an increase of 6 – 7% in an individual possessing 900 g of Hb_{mass}. Worryingly, it has been predicted that up to 4 bags of blood may have been infused in the mid 1990's by professional cyclists – increasing Hb_{mass} by upwards of 240 g (Schmidt and Prommer 2010). Hence, the magnitude of change which is possible via blood manipulation far exceeds any of the changes induced by natural means described above.

Hb_{mass} increases can also be induced via recombinant Erythropoietin (rHuEPO) administration. Following 4 weeks of weekly EPO injections, Lundby and colleagues reported a mean increase in Hb_{mass} of 92 g (9.6%) (Lundby and Robach 2010). Again

it should be noted that in terms of a mean result, the magnitude of change is almost double that which can be expected via the altitude-induced EPO pathway.

1.6.6 Summary of factors influencing Hb_{mass} stability:

From the literature, it is clear that Hb_{mass} can be influenced by a variety of external factors. Of course, our understanding of the magnitude of change associated with each of these influences is dependent on the body of evidence available – data documenting the effect of injury, illness, iron status and competition are lacking. However, based on the available data, the magnitude of change in Hb_{mass} which could be expected due to each factor described above is summarised in **Figure 1-1.** It may also be pertinent to consider the rate of change associated with each of these effects. Some effects induce changes very rapidly, such as blood loss or infusion, whereas natural processes, which require the initiation of the erythropoietic cascade may require days, weeks or months to present. As previously discussed, the time course of Hb_{mass} changes in association to such perturbations also warrants further investigation.

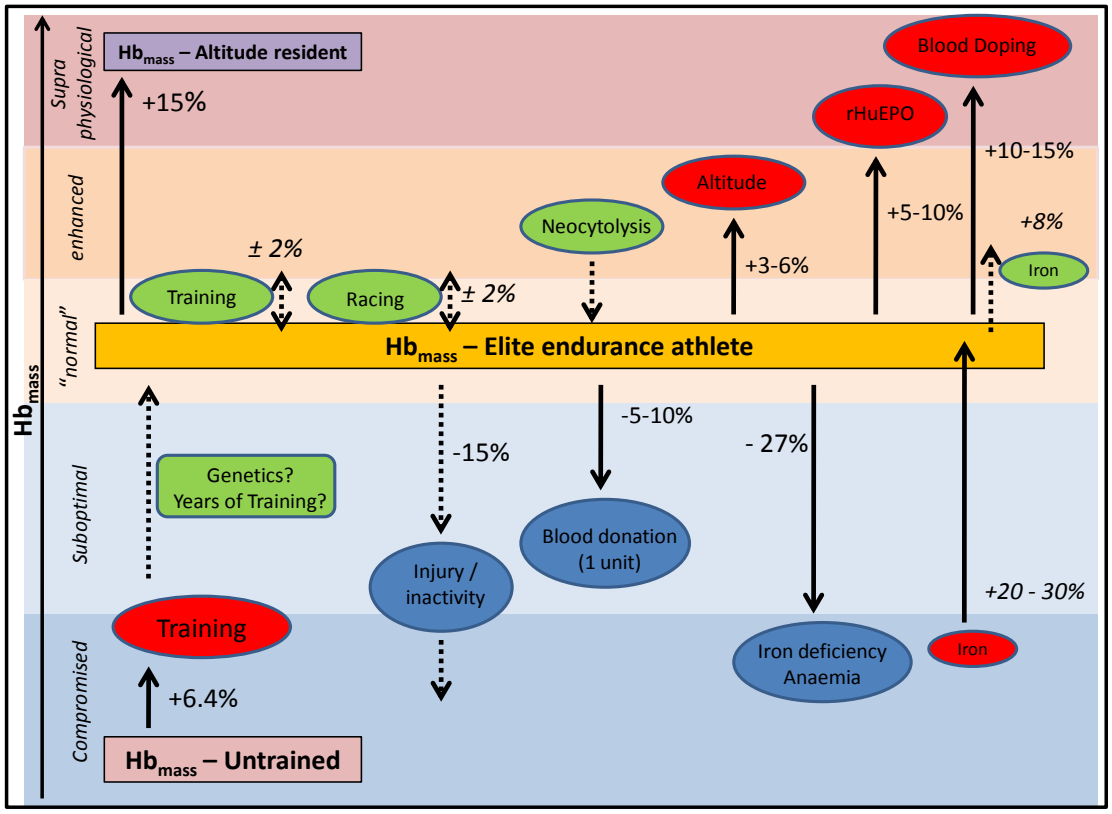

Figure 1-1: Summary of confounding factors to the stability of Hb_{mass} in endurance athletes

Red symbols indicate a positive change, blue symbols indicate a negative change. Green symbol indicates areas where more data are needed. Magnitudes of change indicated alongside directional arrows. Dotted arrows indicate areas for further investigation. Magnitudes of change obtained from (Schmidt, Heinicke et al. 2002; Prommer, Heckle et al. 2007; Schmidt and Prommer 2008; Schumacher, Ahlgrim et al. 2008; Schumacher, Pottgiesser et al. 2008; Clark, Quod et al. 2009; Treff, Schmidt et al. 2009; Lundby and Robach 2010; Robertson, Saunders et al. 2010; Schmidt and Prommer 2010; Wachsmuth, Aigner et al. 2010)

1.7 Hb_{mass} and VO_2max

The impact of alterations to Hb_{mass} on VO_2max has recently been extensively reviewed by Schmidt and Prommer (2010), but for the purpose of this review, the key areas related to elite athletes will be summarised. Under normoxic conditions, VO_2max of elite athletes is largely determined by [Hb] and *Q* (Schmidt and Prommer 2010). Alterations to Hb_{mass}, may therefore exert effects on VO_2max via two routes –

(i) via changes to total blood volume and hence *Q* and (ii) via changes to [Hb] and the associated changes to O_2 transport capacity (Schmidt and Prommer 2010).

Cross-sectional studies indicate a strong relationship between VO_2max and Hb_{mass}, which is independent of sex or age (Schmidt and Prommer 2010). Heinicke et al (2001) report a close association between VO_2max and absolute Hb_{mass} (r = 0.72) in 131 males of varying training status (Heinicke, Wolfarth et al. 2001). Similarly, both Gore et al. (1997) and Schmidt and Prommer (2008) report strong correlations between Hb_{mass} and VO_2max across a range of sports and degrees of training status, with the slope of the regression line ~4 in each instance. Unpublished data collected at the Australian Institute of Sport on 35 female cyclists also reveals a similar relationship between VO_2max and Hb_{mass}, with the slope of the regression line ~3 in this instance (**Figure 1-2**).

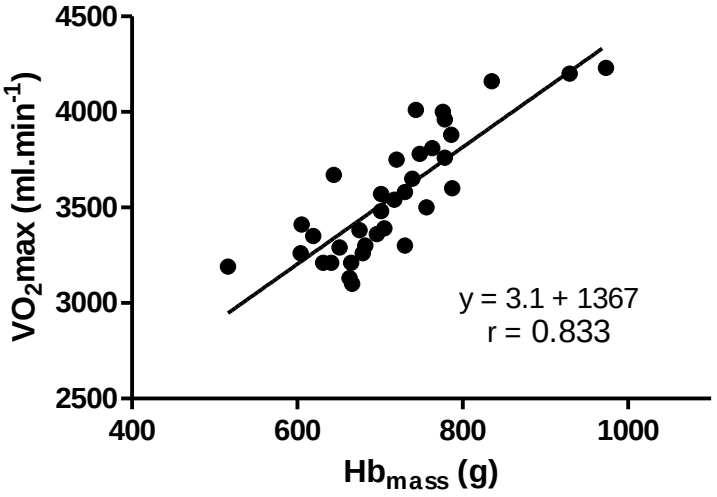

Figure 1-2: Relationship between Hb_{mass} and VO_2max in Australian female road cyclists

Data collected between 2005 and 2008 on 35 national level female road cyclists (unpublished observation)

In practical terms, this relationship translates to a change in VO_2max of ~4 mL.min^{-1} for every 1 g change in Hb_{mass} (Schmidt and Prommer 2008; Schmidt and Prommer 2010). Furthermore, the relationship is consistent with the theoretical calculation of O_2 transport during maximal aerobic exercise. Specifically, if an athlete possesses a total blood volume of 6 L, and *Q* at maximal exercise is 36 L.min^{-1}, then the entire blood volume is circulated around the body four times per min (Ekblom and Hermansen 1968; Rowell 1986; Schmidt and Prommer 2010). Thus, if 1 g of Hb binds 1.39 ml of O_2 (Hufner's constant, (Gorelov 2004)), and a-vDO_2 is assumed to be 75%, then an increase in Hb_{mass} of 1 g would be associated with an increase in VO_2max of ~4 mL.min^{-1} (0.75 x 1.39 x 4 = 4.17). Interestingly, and importantly, the association between [Hb] and VO_2max is much weaker (r = 0.25, slope = 1.7 (Schmidt and Prommer 2010)) than for Hb_{mass} and VO_2max under normal physiological conditions (Kanstrup and Ekblom 1984; Schmidt and Prommer 2010), highlighting the importance of the *total* amount of Hb available for oxygen transport as opposed to the concentration or viscosity of the blood. Furthermore, when the dual action of Hb_{mass} is considered (*Q* and O_2 transport), a higher correlation to VO_2max than [Hb] is expected (Schmidt and Prommer 2010).

The strong relationship between Hb_{mass} and VO_2max has two important implications; firstly that a high Hb_{mass} is an important prerequisite for a high VO_2max (Martino, Gledhill et al. 2002; Schmidt and Prommer 2010) and secondly that alterations to Hb_{mass} have the potential to alter VO_2max and possibly performance. Indeed, alterations to VO_2max have been demonstrated on a number of occasions using non-physiological models which serve to increase or decrease Hb_{mass} (e.g. phlebotomy, rhEPO administration). Prommer et al. (2007) removed ~550ml of blood (~75 g of Hb_{mass}) and observed a mean decrease in VO_2max of 255 ml.min^{-1} – equating to ~3

$ml.min^{-1}$ for each g of Hb removed (Prommer, Heckle et al. 2007). VO_2max was also measured at days 10, 20 and 40 after blood donation, and increased in proportion to the amount of Hb_{mass} recovered by each time point. Ekblom and colleagues used CO to "incapacitate" varying degrees of circulating Hb_{mass}, and thus reduced the O_2 transport capacity of the blood without removing Hb *per se*. Mean VO_2max decreased by 9.3 and 21.8% at 7 and 20% HbCO, respectively, with a linear relationship observed between HbCO and changes in VO_2max (Ekblom and Huot 1972). Similarly, a 3% decrease in VO_2max was reported 30 min after completion of the 2 min of CO rebreathing protocol at which time HbCO levels were ~5% (Schmidt and Prommer 2005).

Conversely, the effect of increasing Hb_{mass} by rhEPO on VO_2max is both positive and substantial. Following 24 days of rHuEPO administration, which increased Hb_{mass} by ~60 g and 105 g in two groups respectively, VO_2max increased by ~280 $ml.min^{-1}$ and 307 $ml.min^{-1}$ (Parisotto, Gore et al. 2000). However, 4 weeks after the cessation of treatment, both Hb_{mass} and VO_2max values had returned to baseline. Similarly, 13 weeks of micro-dose EPO administration resulted in an ~80 g increase in Hb_{mass}, which was accompanied by a 300 $ml.min^{-1}$ increase in VO_2max; again consistent with the hypothesis that 1 g of Hb is associated with a change in VO_2max of ~4 $ml.min^{-1}$ (Lundby, Robach et al. 2008; Lundby and Robach 2010).

The Hb_{mass}-VO_2max relationship has not been directly reported in an autologous blood-doping context; however, some data are available which describe the impact of infusing blood on VO_2max and [Hb]. From these data it could reasonably be inferred that Hb_{mass} was substantially increased due to the transfusion (Pottgiesser, Umhau et al. 2007). A 5% increase in VO_2max was observed 24 h and 7 days after an

autologous blood transfusion of 900 ml of whole blood (Buick, Gledhill et al. 1980). [Hb] was increased from 15.1 to 16.3 $g.dL^{-1}$; therefore assuming a [Hb] of 15.1 $g.dl^{-1}$, 900 ml would equate to 136 g of Hb_{mass} (900*(15.1/100)). Similarly, a "dramatic overnight increase" in VO_2max of 9% was observed following reinfusion of 800 ml of blood (Ekblom, Goldbarg et al. 1972). Thus, whether it be via alterations to *Q* or O_2 transport, there is strong evidence that changes to Hb_{mass} under non-physiological settings result in concomitant changes to VO_2max (Schmidt and Prommer 2010).

Of interest to the sporting community however, is whether the Hb_{mass}-VO_2max relationship holds true for Hb_{mass} changes induced by natural physiological processes, such as training, iron supplementation or altitude exposure. A 9 month marathon training program resulted in a mean increase in Hb_{mass} of 6.4% (60 g) in previously untrained runners, and was accompanied by a 250 $ml.min^{-1}$ increase in VO_2max (Schmidt and Prommer 2008). These data would suggest that the 1 g: 4 $ml.min^{-1}$ relationship exists for natural increases in Hb_{mass}; however, more data are needed from elite athletes to determine whether possible minor fluctuations associated with training (in the region of 2-3%) have an equal effect on VO_2max. Similarly, in the instance of iron supplementation of iron deficient anaemic (IDA) or iron depleted (IDN) athletes, the resulting increase in Hb_{mass} (IDA = 96 g, IDN = 53g) was also associated with a increase in VO_2max (IDA = 15.4 %, IDN = 8.5 %), with the slope of the regression line again indicating a ~4 $ml.min^{-1}$ increase in VO_2max per 1 g increase of Hb_{mass} (Wachsmuth, Aigner et al. 2010).

Interestingly, however, the Hb_{mass}-VO_2max relationship appears at times to uncouple following altitude training, with disproportionate changes in Hb_{mass} and VO_2max reported. Robertson et al. (2010) reported little association (r = 0.32) between

changes in Hb_{mass} and VO_2max following 3 weeks of LHTL simulated altitude training combined with hypoxic training (HT). In fact when the individual data are examined, some athletes displayed substantial increases in Hb_{mass} (> 5%) with no change in VO_2max, whereas others who experienced minor reductions in Hb_{mass}, increased VO_2max by ~5% (Robertson, Saunders et al. 2010). Similarly, despite a ~4 % increase in Hb_{mass} observed in elite runners following ~400 h of simulated LHTL (~2900 m), only a trivial change in VO_2max was observed (Saunders, Telford et al. 2009). Not surprisingly the relationship between changes in Hb_{mass} and VO_2max in this study was also trivial (r = 0.04). The authors suggest that the disproportionate changes may be attributed to a parallel reduction in *Q* or vascular regulation arising from the altitude exposure (Favret, Richalet et al. 2001). Wehrlin et al. (2006) observed a 5.3% increase in Hb_{mass} (~44 g) in a group of orienteers which was accompanied by a 4.1% increase in VO_2max (~145 $ml.min^{-1}$), which in terms of the mean data appears in line with the expected increase in VO_2max per g of Hb_{mass} (Wehrlin, Zuest et al. 2006). Indeed, the relationship between the change scores was ~0.7 when the group was divided into men (r = 0.75) and women (r = 0.68); however, when the group data are combined, the relationship becomes much weaker (r = 0.35, p = 0.29). Clark et al. (2009) report a trivial correlation between Hb_{mass} and VO_2max in well-trained cyclists following 21 days of simulated LHTL (r = 0.09, p = 0.32), however the slope of the regression line appears to indicate that a 1% increase in Hb_{mass} is associated with a 0.8% increase in VO_2max. The only study to report a significant (albeit weak) correlation (r = 0.37, p = 0.02) between changes in red cell volume and VO_2max is that of Levine and Stray-Gundersen (1997) who observed a 5% and 9% increase in red cell volume (measured using Evan's Blue) and VO_2max,

respectively, in collegiate runners following 4 weeks of LHTL (Levine and Stray-Gundersen 1997).

Overall, the balance of evidence available indicates that the nature of the increase in Hb_{mass} is important for the ensuing relationship between Hb_{mass} and VO_2max. In the majority of cases of blood gain or loss (e.g. blood-doping, phlebotomy), increasing or decreasing Hb_{mass} has a direct effect on VO_2max (Lundby, Robach et al. 2008). Interestingly, in the case of altitude exposure, other factors associated with adaptation to hypoxia may be equally if not more important than any Hb_{mass} changes, thereby overriding the importance of Hb_{mass} for VO_2max in contrast to other instances of erythropoiesis. Alternatively, where the changes in Hb_{mass} and VO_2max are small, the weak association between the two may allude to inaccuracy of the measures of Hb_{mass} (or RCV) or of VO_2max. In the latter case, reported values may in fact refer to VO_2peak as opposed to VO_2max. It remains to be determined whether the lack of a relationship is an illustration of the small signal to noise ratio or the lack of a relationship from a cause and effect perspective.

1.8 Hb_{mass} and Performance

Despite the clear relationship between changes in Hb_{mass} and VO_2max, the importance of Hb_{mass} for endurance performance is not an open and shut case, and surprisingly a topic which has been studied rarely. Certainly, in the case of blood-doping and EPO administration, large changes in Hb_{mass} of the order of 10% do coincide with marked effects on athletic performance (Brien and Simon 1987; Ekblom 1996). Cross country skiers, who were infused with 1350 ml of autologous blood (equating to ~200 g of Hb_{mass}), improved their 15 km race time by ~5%

(Berglund and Hemmingson 1987). Brien and Simon (1987) infused 400 ml of packed red blood cells (~130 g of Hb_{mass}) into male distance runners and observed significantly faster 10 km run times in all athletes (Brien and Simon 1987). In these examples, it is generally accepted that the enhanced O_2 transport capacity arising from the increased Hb_{mass} is the main contributor to enhanced performance in these primarily aerobic tasks. For these and a variety of other reasons, blood doping practices are now banned (WADA 2011).

The effects of small changes in Hb_{mass} (<5%) arising from natural means (e.g. training or altitude) on an athlete's performance however, are much harder to discern, largely because of the difficulty associated with measuring "performance" in elite athletes – where a change of just 0.5% in performance time may be deemed worthwhile in some sports (Hopkins, Hawley et al. 1999). As a result, many studies have essentially avoided the problem by using a VO_2max test as a surrogate measure of performance. Whilst a VO_2max test might provide a reliable measure of an athlete's maximal capabilities, its relevance to success in specific sporting events is questionable, and further, *"it should be emphasised that VO_2max is not equivalent to sports performance"* (Levine 2008). For example in the sport of road cycling, the percentage of VO_2max which can be sustained for extended durations is deemed more important for performance than VO_2max itself (Jeukendrup, Craig et al. 2000). Similarly, Roecker and colleagues have used a multitude of variables to predict running performances over different distances and found that the individual anaerobic threshold, running economy and speed at VO_2max have more relevance to sporting performance than VO_2max *per se* (Roecker, Schotte et al. 1998). In addition, in the studies where altitude training has resulted in an increase in performance but did not increase VO_2max (Gore, Hahn et al. 1998; Saunders,

Telford et al. 2009), using only a VO_2max test as a measure of performance would have resulted in performance gains essentially being "missed." The use of a VO_2max test as a measure of performance, also implicitly assumes that the primary mechanisms responsible for enhanced performance, which are associated with changes in Hb_{mass}, are indeed aerobic. Certainly in the case of altitude training, the role of Hb_{mass} for performance enhancement has been debated (Gore and Hopkins 2005; Levine and Stray-Gundersen 2005). Furthermore, other physiological adaptations which are related to the increase in Hb_{mass} e.g. augmented blood buffering (Gledhill 1985) or muscle buffering (Gore, Hahn et al. 2001), may also be important.

Obtaining a measure of athletic performance for research purposes, however, is not an easy task. Many athletes find it difficult to produce a maximal performance in an "artificial" laboratory setting, be it due to motivation or environmental factors (Noakes 2008), and despite the strict criteria that exist for quantifying VO_2max – there are no published criteria associated with measuring maximum performance. Therefore, real life competition performances may be the only way to truly gauge an athlete's capabilities, but due to the inability to carefully control all variables, as well as the number of trials, little data of this nature are available.

One study which has attempted to link altitude-induced Hb_{mass} changes with competition performances is that of Robertson and colleagues who studied nine swimmers throughout a coach-prescribed altitude training program in preparation for the national championships (Robertson, Aughey et al. 2010). Performance was assessed using both a 2000 m time trial in training as well as race performance at the national championships. Trivial changes in Hb_{mass} (0.9%) were moderately correlated

to the 1.2% mean improvement in time trial performance (r = 0.47); but the swimmers did not swim faster at the national championships when compared to swimmers who did not undertake any altitude training, raising doubt as to the efficacy of trivial changes in Hb_{mass} induced by altitude training for competition performance.

Wehrlin and Marti (2006) report a "double case" study of two elite German runners (1 x 5000 m, 1 x marathon) who produced "enhanced performances" following 26 days of altitude training. Hb_{mass} increased by ~4 and 7%, in the marathon and 5000 m runner, respectively. Performance was assessed using race times recorded before and after the altitude training period, culminating in the world championships 25 - 29 days following descent to sea level. Both athletes recorded faster times following LHTL, which the authors suggest may be associated with the increased Hb_{mass} (Wehrlin and Marti 2006). However, since Hb_{mass} was not measured in conjunction with race performance (rather only pre and post altitude training) it is not known for how long the altitude-induced gains in Hb_{mass} persisted. Indeed, it could be argued that Hb_{mass} may have returned to baseline values by the time of the world championship races (Rice, Ruiz et al. 2001; Schmidt and Prommer 2008; Robertson, Saunders et al. 2010), and thus its relative importance is questionable. In addition, as acknowledged by the authors themselves, the absence of a control group and the tactical nature of championship races weaken the performance aspect of the study. Nonetheless, the paper is one of the first to describe real world use of altitude training with a positive performance outcome in world class athletes.

In a bid to determine the effects of altitude training on performance, a few studies have employed a performance trial of some nature. When interpreting the results of

performance trials, it is important to note the following: the number of trials that were performed pre and post, the sport-specificity of the task, whether the athlete was familiar to the task, environmental conditions, pacing strategy, and any other confounding variables. The task should be as specific to the sport as possible and if not familiar to the athletes, then familiarisation trials should be performed. Some researchers have even attempted to encourage athletes to produce best performances by offering financial incentives. However, even the most rigorous of scientific investigations have struggled to detect performance changes – such is the multifaceted nature of athletic performance.

Many studies have utilised time trials as a performance task. Whilst these are perhaps the most sport specific, it is important to note that the length of the effort will partly determine the energy pathways which can be studied; that is, time trials of greater than 10 min in duration are predominantly aerobic in nature, whereas shorter efforts will involve a greater anaerobic component (Gastin 2001). If Hb_{mass} is presumed to predominantly influence the aerobic system (Levine and Stray-Gundersen 2005), then it would follow to employ a predominantly aerobic task as a measure of performance. However, if Hb_{mass} gains are utilised by alternate means, e.g. possibly in the case of altitude training, then it may be of use to assess anaerobic capabilities as well. Furthermore, performance tasks which are time-based, will also inherently be affected by the technical ability to transfer force application to movement speed, and as such make it harder to discern the effect of a physiological adaptation. In contrast, assessment of fitness traits which underpin a performance task, e.g. maximal mean power (MMP) for a given time duration, reduce the number of external variables which may influence the outcome. For example, in the sport of cycling, assessment of MMP for 4 min (MMP_{4min}) may enable more meaningful

interpretation in terms of the influence of Hb_{mass}, as opposed to 4 km individual pursuit time.

Levine and Stray-Gundersen employed a 5000 m time trial to assess running performance following 4 weeks of natural LHTL altitude training (Levine and Stray-Gundersen 1997). The group exhibited a mean increase in performance of 1.4%; but the response was varied with some individuals improving by 5% whilst others showed no improvement. When the group was divided into performance "responders" and "non-responders", it became apparent that the performance responders were also characterised by an increase in red cell volume and VO_2max, whereas the non-responders did not show an increase in either characteristic. Thus, the authors attributed the increase in performance to an enhanced aerobic pathway (Levine and Stray-Gundersen 2005). Improvements in 5000 m time trial performance (1.6%, ~18 s) were also observed in national team orienteers following 24 days of LHTL (Wehrlin, Zuest et al. 2006). Again, the authors suggest that the accompanying 5.3% increase in Hb_{mass} and 4.1% increase in VO_2max may explain the improvements in performance in the predominantly aerobic task.

Trivial to small improvements (~1%) in 3 and 4.5 km time trial performance were also observed in runners following simulated LHTL and LHTL + TH protocols, which also elicited increases in Hb_{mass} and VO_2max of ~3% and 2%, respectively (4.8% in VO_2max for LHTL + TH) (Robertson, Saunders et al. 2010; Robertson, Saunders et al. 2010). However, in these studies there was a lack of association between Hb_{mass}, VO_2max and performance changes, indicating that physiological adaptations to altitude exposure are not always directly transferred to performance improvements.

Controversially, performance improvements following altitude training have been reported without concomitant increases in Hb_{mass}. Gore et al. (1998) used a 5-min simulated Individual Pursuit to assess performance in 8 world class track endurance cyclists following one month of natural altitude training. Despite the absence of an increase in Hb_{mass} or VO_2max, cycling performance increased on at least one of three post testing trials (between 3 and 21 days post) by 4.3% (Gore, Hahn et al. 1998). Whilst those critical of the study have attributed the lack of a Hb_{mass} response to overtraining and illness, the significant performance gains observed cannot be ignored and raise questions as to the importance of Hb_{mass} for performance improvements following altitude training (Gore and Hopkins 2005).

Recently, Bonetti and Hopkins attempted to quantify the effect of altitude-induced gains in Hb_{mass} and red cell volume on performance by way of a meta-analysis, and although a clear, positive relationship between VO_2max and performance was found, no such relationship was observed between Hb_{mass} and performance (Bonetti and Hopkins 2009). Overall, the trivial effect of Hb_{mass} for performance was "unclear" (1.3%; 90% CL ± 2.4); and whilst the authors acknowledge that any effects could be attenuated by measurement error associated with measuring Hb_{mass} and red cell volume (Gore, Hopkins et al. 2005), this result is consistent with recently published findings (Robertson, Saunders et al. 2010; Robertson, Saunders et al. 2010). However, the effect on performance of increasing [Hb] was calculated as 4.8% (90% CL ± 2.7), with a > 50% chance of an increase which could hint towards a role for increased Hb_{mass} in performance enhancement (Bonetti and Hopkins 2009). Alternatively, the rise in [Hb] may be explained by plasma volume shifts associated with adaptation to hypoxia, and thereby independent of erythropoiesis (Schmidt and Prommer 2010).

Table 1-2 provides a summary of the studies which have reported changes in Hb_{mass}, VO_2max and, if applicable performance in response to a variety of interventions. As with VO_2max, it would appear that the nature of how Hb_{mass} is altered may be important for the impact of the adaptation on performance. Again, large increases due to blood manipulation have a clear effect on performance in predominantly aerobic tasks. However, when erythropoiesis is initiated via natural means (e.g. altitude or training); there may not be a direct transfer to performance. Perhaps, concomitant adaptations to the external stimuli have more of an impact on performance than changes in Hb_{mass} in these instances? Indeed, the HIF1-α / erythropoietin cascade initiated by hypoxia is complex and associated with variable outcomes including both aerobic and anaerobic adaptations (**Figure 1-3**). Hb_{mass} changes may therefore, be merely an indication that the upstream cascade has been activated, with non-haematological adaptations arising from hypoxic exposure contributing to performance enhancement (Gore, Clark et al. 2007).

Table 1-2: Changes in Hb_{mass}, VO_2max and Performance

Authors	Treatment	ΔHb_{mass}	ΔVO_2max	Performance task	ΔPerformance
Prommer et al. 2007	Blood donation (550 ml)	* *(-75 g)*	* - 255 ml.min^{-1}	-	-
Schmidt & Prommer 2008	9 months endurance training	6.4 % (60 g)	5.9% (250 ml.min^{-1})	-	-
Wachsmuth et al. 2010	Iron supplementation	17.9 % (96 g)	15.4 % *	-	-
Parisotto et al. 2000	24 d rHuEPO administration	6.9 & 12 % *(60 & 105 g)*	6.3 & 6.9 % *(280 & 307 ml.min^{-1})*	-	-
Lundby et al. 2007, 2009	13 wks rHuEPO administration	8.3% (79 g)	7.6 % (~300 ml.min^{-1})	-	-
Berglund & Hemmingson 1987	1350 ml autologous blood transfusion	* ~200 g	-	15 km XC ski race	5% (-3 min)
Wehrlin et al. 2006	26 days natural LHTL	3.9 %, 7.6 % (36 g, 67 g)	-	World championship race (run)	11th and 14th place
Robertson et al. 2010	4 x 2 wks simulated and natural LHTL	0.9 % *	-	2000m TT and National Championships (swim)	1.2% TT, ↔ Race performance
Levine and Stray Gundersen 1997	28 d natural LHTL	5 % (RCV)	9 % *	5000 m TT (run)	1.3 % (-13 s)
Gore et al. 1998	31 d natural LHTH	↔ *	↔ *	5 min Individual Pursuit (cycle)	4.3 % *
Wehrlin et al. 2006	24 d natural LHTL	5.3 % (44 g)	4.1 % *(~145 ml.min^{-1})*	5000 m TT (run)	1.6 % (-18 s)
Clark et al. 2009	21 d simulated LHTL	3.3% *(~45 g)*	↔ 0.4% *(18 ml.min^{1})*	-	-
Saunders et al. 2009	~400 h simulated LHTL	3.8% 36 g	↔, -0.7% (-30 ml.min^{-1})	-	-
Robertson et al. 2010	21 d simulated LHTL	2.8 % (25 g)	2.1 % *(~95 ml.min^{-1})*	4.5 km TT (run)	1.4 % (-6 s)
Robertson et al. 2010	21 d simulated LHTL (+TH)	3.6 % (28 g)	4.8 % *(~200 ml.min^{-1})*	3000 m TT (run)	1.1 % (-12 s)

↔ Indicates lack of observed change, * Raw data not provided, data in *Italics* estimated from mean raw data

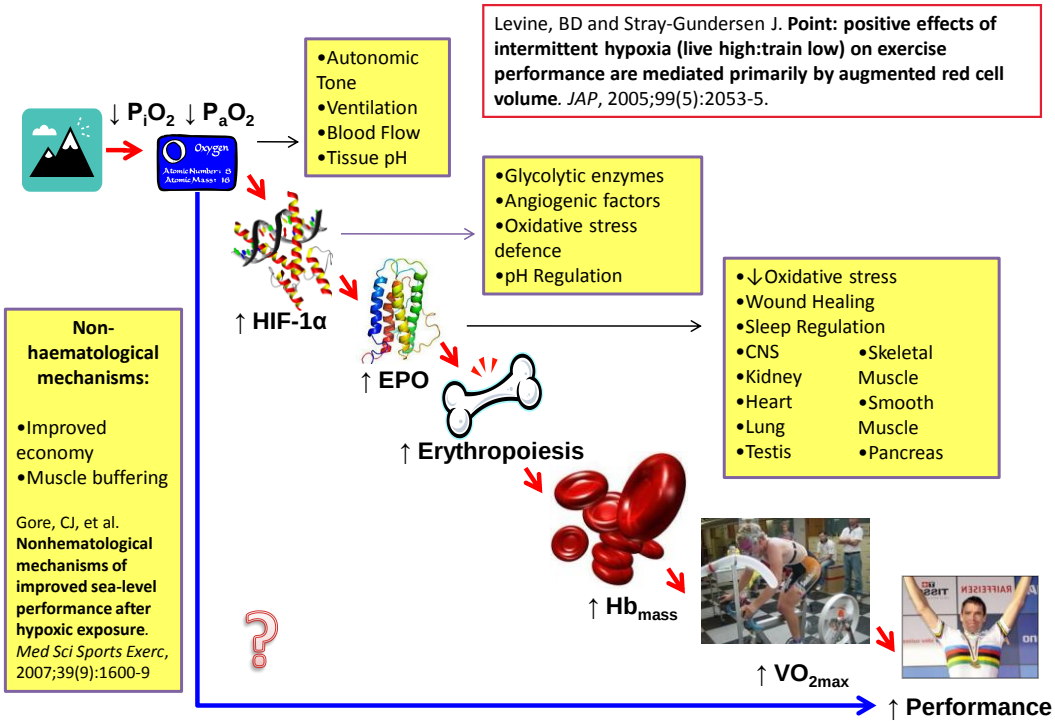

Figure 1-3: Simplistic overview of the HIF 1- α / Erythropoietin response cascade to hypoxia

Altitude-induced erythropoiesis has been proposed as the primary mechanism by which altitude training increases endurance performance (due to increases in VO_2max) (Levine and Stray-Gundersen 2005). However non haematological mechanisms may be equally if not more important (Gore and Hopkins 2005; Gore, Clark et al. 2007). In addition, several aspects of the response cascade may have significant physiological effects which are unrelated to erythropoiesis, but nonetheless important for endurance performance.

Thus, with the Hb_{mass} – performance relationship so complex, there remain many unanswered questions. Is it possible that Hb_{mass} increases may contribute to performance enhancements by other means aside from aerobic pathways? If this is the case, performance assessment will need to require tasks which involve both an aerobic and anaerobic contribution. Are Hb_{mass} changes at altitude a marker of other adaptations occurring within the athlete that may contribute to performance enhancements? It may not be the Hb_{mass} *per se* that contributes to performance

enhancement in elite endurance athletes, but measurable changes in this protein may indicate that an athlete is in an "adaptive" state, with many other signalling pathways activated by the stimulus. Are the reasons which prevent Hb_{mass} changes at altitude in some athletes the same reasons which result in performance stagnation at sea level? If the erythropoietic cascade is partially or completely blocked for some reason (e.g. inadequate energy availability, illness, or inflammation), it is possible that other adaptive pathways are also negatively affected. Therefore, future research, encompassing these themes is warranted if we are to better understand the importance of modest changes in Hb_{mass} for endurance performance of elite athletes.

1.9 CONCLUSION

The CO rebreathing method is a valid and reliable method for measuring Hb_{mass}, with routine measurement in athletic populations now possible, due in part to the refinements made to the technique by Schmidt and Prommer. Endurance athletes possess higher Hb_{mass} values than untrained individuals, but it is unclear how much of their elevated Hb_{mass} is due to training or genetic predisposition. Hb_{mass} is relatively stable in recreational adults, but more data are required to discern the effects of external factors such as intense exercise, illness, injury, iron supplementation and altitude exposure on Hb_{mass} in elite athletes. A strong relationship has been observed between Hb_{mass} and VO_2max, with a 1 g change in Hb_{mass} associated with ~4 $ml.min^{-1}$ change in VO_2max. However, despite the many excellent review papers written about Hb_{mass} and VO_2max, the Hb_{mass}-performance relationship has not been clearly defined, raising questions as to the importance of Hb_{mass} for endurance performance of elite athletes. Specifically, the popular belief that altitude-induced increases in Hb_{mass} are responsible for performance enhancements,

via an increase in VO_2max, is intuitively appealing, but not necessarily proven. In fact, this concept is challenged by the results of studies which have documented an improvement in performance following altitude training in the absence of an increase in Hb_{mass}. Furthermore, in many studies, altitude-induced increases in Hb_{mass} do not appear to be directly transferred to changes in VO_2max or performance, again indicating that non-haematological adaptations to hypoxia are potentially important for performance enhancement. Thus, the role of Hb_{mass} in supporting or enabling improvements in endurance performance warrants further investigation.

CHAPTER 2: CO uptake kinetics of arterial, venous and capillary blood during CO-rebreathing

2.1 ABSTRACT

The uptake and distribution of CO throughout the circulatory system during two different methods of CO-rebreathing (2-min 'Schmidt' and 40-min 'Burge') was determined in nine healthy volunteers. Specifically, the impact of i) differences in circulatory mixing time (t_{mix}), ii) CO diffusion to myoglobin (Mb) and iii) CO wash-out was assessed on calculated haemoglobin mass (Hb_{mass}). Arterial (*a*), muscle venous (*vm*) and capillary (*c*) samples were obtained simultaneously at 0, 1, 2, 3.5, 5, 7.5, 10, 12.5, 15, 20, 30 and 40 min for determination of carboxyhaemoglobin (HbCO). CO wash-out was measured from expired air following rebreathing. The rate of CO diffusion to Mb was calculated using the change in HbCO after t_{mix}, and the rate of CO wash-out. In both methods, HbCO*a* and HbCOc followed a near identical time course, peaking within the first two minutes and decreasing thereafter. HbCO*vm* increased slowly and was significantly lower at min 1, 2 and 3.5 in both methods ($p<0.01$). HbCO*a* peaked significantly higher in the Schmidt method ($p=0.03$). t_{mix} occurred by min 10 in most but not all subjects. The rate of CO wash-out was $0.25 \pm 0.13 ml.min^{-1}$ in Schmidt and $0.25 \pm 0.16 ml.min^{-1}$ in Burge. The rate of CO diffusion to Mb was 0.22 ± 0.11 $ml.min^{-1}$ and $0.16 \pm 0.13 ml.min^{-1}$ ($p=0.63$) in Schmidt and Burge, respectively. Inhalation of a CO bolus during the Schmidt

method results in faster HbCO*a* uptake but does not greatly shorten t_{mix} or influence rates of CO wash-out and flux to Mb. The calculated Hb_{mass} depends substantially on the plateau level of HbCO; therefore it is paramount to ensure HbCO is mixed completely prior to blood sampling, as well accounting for potential within-subject alterations of CO exhalation and CO flux to Mb.

2.2 INTRODUCTION

The high affinity of carbon monoxide (CO) for haemoglobin (Hb) (Gorelov 2004) makes it a suitable tracer for the measurement of total Hb mass (Hb_{mass}) in humans (Burge and Skinner 1995; Schmidt and Prommer 2005). Since the inception of CO as a potential erythrocyte label in 1882 (Grehant and Quinquard 1882), the CO-rebreathing method has undergone a number of refinements (Thomsen, Fogh-Andersen et al. 1991; Burge and Skinner 1995; Schmidt and Prommer 2005; Prommer and Schmidt 2007) in order to improve its accuracy, reliability and practical application. Today, measurement of Hb_{mass} via CO-rebreathing is considered one of the more superior methods available for blood volume measurement (Gore, Hopkins et al. 2005), with the technique now commonly used in clinical and athletic populations (Prommer, Sottas et al. 2008; Schumacher, Ahlgrim et al. 2008; Clark, Quod et al. 2009; Garvican, Eastwood et al. 2010; Robertson, Saunders et al. 2010).

CO enters the circulatory system via diffusion at the alveoli, and binds to Hb in competition with oxygen (O_2), forming carboxy-haemoglobin (HbCO). In addition, CO may diffuse to extra-vascular compartments, binding with other molecules e.g.

myoglobin (Mb), although all to a lesser extent than Hb (Stewart 1975). Over time, the distribution of HbCO throughout all circulatory compartments equilibrates and sampling of HbCO from this point on can be used to estimate Hb_{mass}. Once CO-rebreathing is terminated, CO is slowly removed from circulation via exhalation.

The duration of rebreathing varies between the two most common methods; up to 40 min in the method of Burge & Skinner (1995), whilst the "optimised" method of Schmidt & Prommer (2005) involves just 2 min of rebreathing. Regardless of the duration used, the accuracy of the method is based on assumptions that first, HbCO has equilibrated throughout the circulatory system and secondly, that at the time of blood sampling, all CO administered into the system can be accounted for. Potential sources of error therefore lie in the timing of blood sampling relative to HbCO equilibration, as well as the quantification of CO loss to extra-vascular compartments or via exhalation.

The multi-compartment model of Bruce and Bruce (2003) predicts that HbCO concentration sampled during CO-rebreathing is sensitive to a number of external parameters including – application of the CO dose, the type of blood sampled, the rate of diffusion to other compartments and the duration of rebreathing (Bruce and Bruce 2003; Schmidt and Prommer 2005). Previously, the circulatory uptake kinetics of CO during the Schmidt and Burge methods have been compared using venous and arterialised-capillary blood samples (Gore, Bourdon et al. 2006; Prommer and Schmidt 2007) in an attempt to determine the optimal time and site for blood sampling. Whilst the mean response has yielded a 'recommended' sampling time, these prior results indicate substantial individual variation which could have

important consequences for the calculation of Hb_{mass}. A more complete profile of the time course of HbCO distribution throughout the circulatory system involving multiple vascular compartments may allow a greater understanding of any physiological differences between the CO kinetics of the two methods and assist in further refinements. In addition to potentially influencing circulatory uptake and mixing times, the method of CO-rebreathing employed may also affect subsequent rates of CO diffusion to Mb or CO washout via exhalation, which as yet, has not been determined. If different, Hb_{mass} calculations should ultimately not be affected, as long as these losses are accounted for accurately.

The aim of the present study was to compare the uptake and distribution of CO throughout the circulatory system during two different methods of CO-rebreathing. Specifically, the potential impact of differences in circulatory mixing time (t_{mix}), CO diffusion to Mb, and CO wash-out following rebreathing on Hb_{mass} will be assessed in relation to the underlying physiology of these differences, so that recommendations to improve the accuracy of the method can be made.

2.3 METHODS

2.3.1 Ethical Approval

The study was approved by the Australian Institute of Sport Human Ethics Committee. All subjects were informed of the risks and procedures involved and provided written consent before participating.

2.3.2 Study Design

The time course of HbCO during two methods of CO-rebreathing was determined from multiple, simultaneous assessments of arterial (*a*), capillary (*c*) (pre-warmed finger tip) and muscle venous (*vm*) blood in nine recreationally-active, healthy subjects (**Table 2-1**). All subjects were non-smokers and completed two CO-rebreathing tests at the same time of day, one to three days apart, in a randomised counterbalanced order. One test involved the 2-min method of Schmidt & Prommer (2005), whilst the other involved the Burge & Skinner (1995) method, with the subject semi-recumbent throughout both tests. For each subject, the same volume of CO was administered during each test, equivalent to 1.5 $ml.kg^{-1}$ body mass.

Table 2-1: Subject Characteristics

	Age (yr)	Height (cm)	Body mass (kg)	Sum of 7 skinfolds† (mm)
Men (n = 7)	38.4 (9.4)	183.3 (6.7)	80.4 (7.1)	67.6(21.8)
Women (n = 2)	25.1 (0.5)	166.7 (4.2)	53.4 (5.1)	59.6 (0.3)

Values are means (SD)

† biceps, triceps, subscapular, mid-abdominal, supraspinale, mid-thigh and calf skinfolds

2.3.3 Schmidt method

The 2-min CO-rebreathing procedure was first described by Schmidt & Prommer (2005) and a modified version has been described in detail by Prommer & Schmidt (2007). Briefly, following stabilisation in room air, a bolus of 99.5% chemically pure CO (BOC gasses, Sydney, Australia) was inhaled and rebreathed for 2-min through a closed circuit consisting of a glass spirometer and 3 L anaesthetic bag containing

100% O_2. A portable CO gas meter (Fluke CO-220, Germany) was used to test for any leaks from the system during the test.

2.3.4 Burge and Skinner method

Each subject also performed a 40-min CO-rebreathing procedure according to the protocol of Burge & Skinner (1995), using a 2 L anaesthetic bag. The rebreathing period was preceded by 5-min of breathing 100% O_2 in order to flush nitrogen from the airways (Burge and Skinner 1995). Before and upon completion of the test, the entire breathing apparatus was immersed under water to verify that there were no leaks in the system.

2.3.5 Blood sampling

a, *vm* and *c* blood was sampled simultaneously during both CO-rebreathing tests. *a* samples (2 mL) were collected from an arterial line inserted into the radial artery into 3 mL syringes (Rapidlyte TM Arterial Blood Sampler, Bayer Corporation, East Walpole MA, USA). *vm* samples (2 mL) were collected from an indwelling cannula in an antecubital vein into 5 mL ground glass syringes prepared with heparin (Enterna-Matic model, Sanitex, Switzerland). *c* samples (200 μL) were collected into glass pre-heparinised capillary tubes (Clinitubes, Radiometer, Copenhagen, Denmark). All samples were stored on an ice slurry and analysed within 3 h of collection after thorough mixing. HbCO(*a*, *vm* and *c*) (%), [Hb] and O_2 saturation (%) of each blood sample were measured using an OSM-3 Hemoximeter (Radiometer, Copenhagen, Denmark). Five replicates from each sample were analysed where possible, with the mean value used for subsequent calculations.

HbCO*vm* was corrected according to Hutler (2000) to account for changes in O_2 saturation (Hutler, Beneke et al. 2000).

For both methods, *a* and *vm* was sampled at 0 (baseline), 1, 2, 3.5, 5, 7.5, 10, 12.5, 15, 20, 30 and 40 min after administration of the CO dose. Arterialised-*c* samples for both methods were obtained from a pre-warmed finger tip (achieved by warming the hand in a bucket containing 45^{o}C water) at 0, 2, 5, 7.5, 12.5, 20, 30, 40 min.

2.3.6 CO exhalation

Expired gas was collected following disconnection from the rebreathing apparatus in both methods in order to quantify the amount of CO exhaled. Subjects were connected to a Douglas bag (aluminised Mylar bags) via an R2700 respiratory valve (Hans Rudolph, Shawnee, KS, USA) exactly 5.5 min after cessation of rebreathing, and all exhaled air was collected for 12.5 min. The collection periods were: 7.5 – 20 min following the Schmidt method and 45.5 – 58 min following the Burge method. The delay in collection was imposed so as not to interfere with the normal time line of the Schmidt method, particularly the measurement of end-tidal [CO] before the 7.5 min blood sample. The [CO] in the Douglas bag at the end of the collection period was measured in parts per million (ppm) using a portable CO gas meter (Pac 7000, Drager, Pittsburg, PA, USA). The volume of each bag was determined using a 350 L Tissot spirometer (Warren E. Collins, Braintree, MA, USA), corrected for temperature and ambient barometric pressure. The volume of CO (ml) exhaled was calculated as the product of end-tidal [CO] and the volume of expirate, and corrected for dead space.

The volume of CO exhaled prior to gas collection in the Schmidt method (min 2-7.5) was estimated according to the method of Schmidt & Prommer (2005). End-tidal [CO] was measured 7 min after inhalation of the CO dose using a portable CO gas meter (Pac 7000, Drager, Pittsburg, PA, USA), and multiplied by alveolar ventilation (V_A - estimated according to height (cm) as [0.075 x height] – 7.75) and time (min).

2.3.7 CO diffusion to myoglobin

The multi-compartment model of Bruce and Bruce (2003) predicts that CO inhaled will also diffuse to Mb during and following rebreathing. The volume of CO lost from the vascular bed to Mb has been calculated by Prommer & Schmidt (2007), using the decrease in HbCO over time together with the volume of CO exhaled. Fundamental to their calculation is the proviso that CO is equilibrated throughout the vascular system. In the present study, CO loss to Mb during both methods was calculated using the formulae described in detail by Prommer & Schmidt (2007). Since t_{mix} varied for each subject, the starting point and time span for the calculated CO flux was determined for each individual, e.g. if complete mixing was achieved by min 10, the CO flux to Mb between 10 and 20 min was calculated.

2.3.8 Calculation of Hb_{mass}

Hb_{mass} was calculated for *a, vm and c* samples at min 7.5, 10 and 12.5 as follows:

$$Hb_{mass} = K \times MCO \times 100/(\Delta HbCO \times 1.39)$$

- K = (ambient barometric pressure mmHg x 273°K) / (760 mm Hg x ambient temperature °K)

- MCO = Volume of CO administered minus the CO volume remaining in the spirometer and lung at the end of rebreathing, as well as the amount of CO exhaled between the end of rebreathing and the time of blood sampling, and the amount of CO lost to Mb (Prommer and Schmidt 2007). For the Burge method, the subject remains connected to the rebreathing apparatus and thus CO exhalation is zero. For the Schmidt method, the amount of CO exhaled (after disconnection) = end-tidal [CO] x V_A x time, whereas the amount of CO remaining in the spirometer and the lung = [CO] in the spirometer x (spirometer volume + lung residual volume) (Schmidt and Prommer 2005). The amount of CO remaining in the spirometer in the Burge method was not measured directly as it is assumed to be 2.2% of the administered volume (Burge and Skinner 1995), with a range of 1.8-2.7% (Thomsen, Fogh-Andersen et al. 1991).

- ΔHbCO = the difference in HbCO between the pre and post rebreathing sample. For *c* only, the min 10 sample was calculated from the mean of the min 7.5 and 12.5 sample.

- 1.39 = the CO binding capacity for Hb as determined by Hufner (Gorelov 2004).

The calculated Hb_{mass} values were adjusted to account for the amount of Hb removed in the preceding test.

2.3.9 Statistical Analysis

A repeated measures ANOVA with main effects of type of blood (*a, vm, c)*, time and method (Schmidt vs. Burge) was used to compare the time course of HbCO. A second repeated measures ANOVA for type of blood and time (7.5, 10, 12.5 min) was used to compare the resultant Hb_{mass} calculated at min 7.5, 10, and 12.5 with each type of blood, as well as to compare the Hb_{mass} values calculated at the recommended time points for each method (Schmidt 7.5 min vs. Burge 10 min) for each blood type. Tukey HSD *post hoc* tests were used to identify differences between cell means. Mean and individual coefficients of variation (CV) were calculated for the Hb_{mass} value obtained for the two methods using the three blood types at time points 7.5, 10 and 12.5 min. Bland and Altman (Bland and Altman 1986) plots were used to compare the change in %HbCO (ΔHbCO) in *a, vm, c* blood at different time points, as well as the Hb_{mass} calculated using the three blood types at different time points and using the two rebreathing methods. Differences between CO exhalation and CO loss to Mb during the two methods were assessed using a paired t-test. The impact of errors arising in ΔHbCO, the volume of CO exhaled or the volume of CO lost to Mb on the subsequent calculation of Hb_{mass} was assessed by altering one variable at a time whilst keeping all other variables constant. Linear regression analysis was performed using the values obtained for a range of degrees of error, to determine the relationship between a percent change in ΔHbCO, the CO exhaled or CO lost to Mb and the resultant percent change in Hb_{mass}. All analyses were completed using Statistica (version 6.0, Statsoft, Tulsa, OK, USA). The level of significance was set to $p \leq 0.05$. Values in the text and figures are reported as means ± standard deviation (SD) unless otherwise stated.

2.4 RESULTS

2.4.1 CO Kinetics and mixing time

Overall, the time course of ΔHbCO was different between methods, the type of blood and the time of sampling ($F_{(10,70)}$ = 3.04, p = 0.003), (**Figure 2-1**). ΔHbCO*a* was significantly higher than ΔHbCO*vm* at min 1, 2 and 3.5 for both the Burge and Schmidt methods (p < 0.01). ΔHbCO*c* closely tracked ΔHbCO*a* throughout both procedures.

In the Burge method, ΔHbCO*a* and ΔHbCO*c* peaked in the first two minutes before decreasing and reaching a plateau after ~10 min (**Figure 2-1**). In comparison, during the Schmidt method ΔHbCO*a* peaked at min 1, and decreased thereafter. During both methods, mean ΔHbCO*vm* increased slowly, rising steadily from the onset of rebreathing, until converging with the plateau of *a* and *c* between min 7.5 and 10. For the Burge method, the mean difference between ΔHbCO of *a* and *vm* remained between -0.09 and 0.02%, from min 10 onwards, consistent with complete mixing. Similarly, in the Schmidt method the mean difference between *a* and *vm* was 0.06 ± 0.21% at min 10 indicating complete mixing in the majority of subjects.

For the same CO dose, mean ΔHbCO*a* peaked higher during the Schmidt method (7.79 ± 1.11% vs. 7.06 ± 1.03%, p = 0.03) but decreased thereafter, falling below the values for the Burge method from min 5 onwards and significantly so at min 20, 30 and 40 (**Figure 2-1**). In contrast, during the Burge method, mean ΔHbCO*a* reached a plateau of ~6.7 ± 1.1% at min 10; only decreasing 0.22 ± 0.08 over the following 30 min. Mean ΔHbCO*vm* increased more slowly during the Schmidt method (**Figure 2-**

1) and was significantly lower at min 1. At min 30 and 40, ΔHbCO was significantly lower in the Schmidt method for all blood types (**Figure 2-1**).

2.4.2 Individual Cases

Individual variation was apparent in t_{mix} during each method (**Figure 2-2**). At min 5 of the Burge method, ΔHbCO*vm* differed from *a* and *c* by > 0.1% (the smallest increment provided by the OSM3 hemoximeter) in 7 subjects, with the difference in 3 subjects exceeding 1.0% (**Figure 2-3**). By min 10, ΔHbCO differed by > 0.1% in 5 subjects, and in only 4 by min 12.5. One subject failed to reach equilibrium (**Figure 2-2c**). In the Schmidt method, ΔHbCO*vm* differed from *a* and *c* by > 0.1% in 8/9 subjects at min 5, but decreased to 5 subjects at min 7.5, three at min 10 and one subject at min 12.5 (**Figure 2-3**).

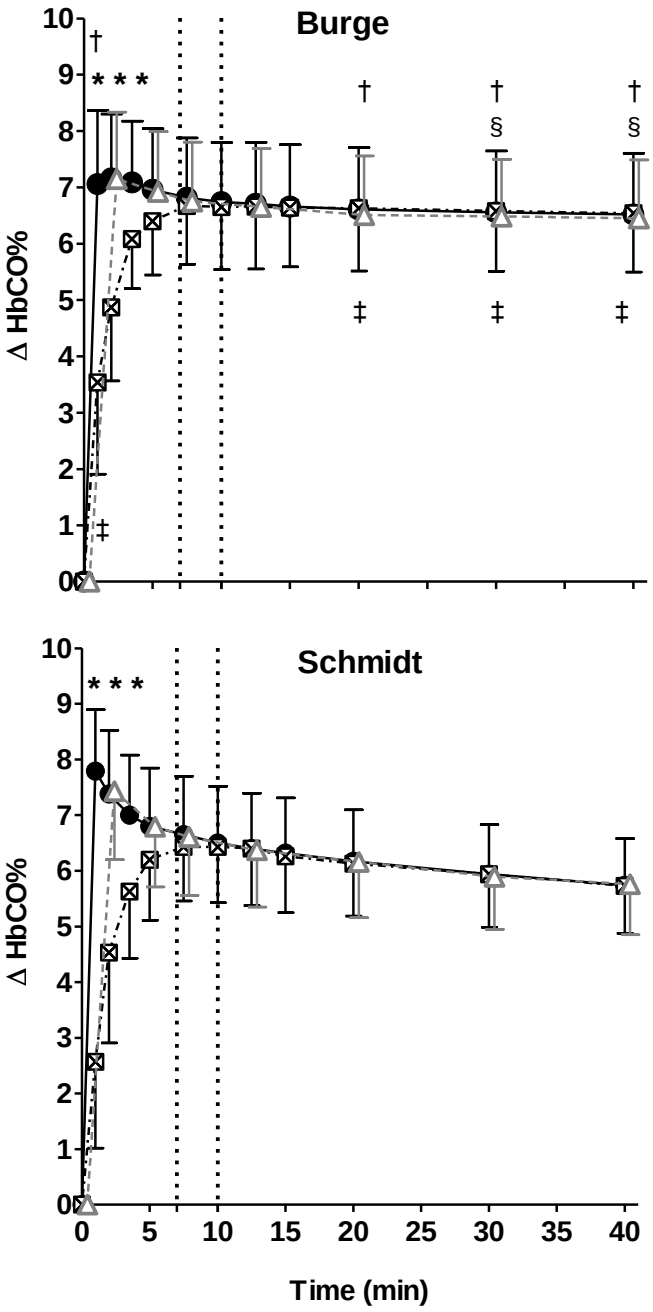

Figure 2-1: Changes in HbCO (%) in arterial (closed circles), venous (squares) and capillary (open triangles) blood during 40 min of rebreathing (Burge method) and 2 min of rebreathing a CO bolus (Schmidt method)

Values are means ± SD. * denotes significant difference between arterial and venous values within a method, $p \leq 0.05$. †, § and ‡ denotes significant difference between methods for arterial, capillary and venous blood, respectively. The vertical dotted lines indicate the time of blood sampling recommended for Hb_{mass} calculations in each method (Burge = min 10, Schmidt = min 7.5).

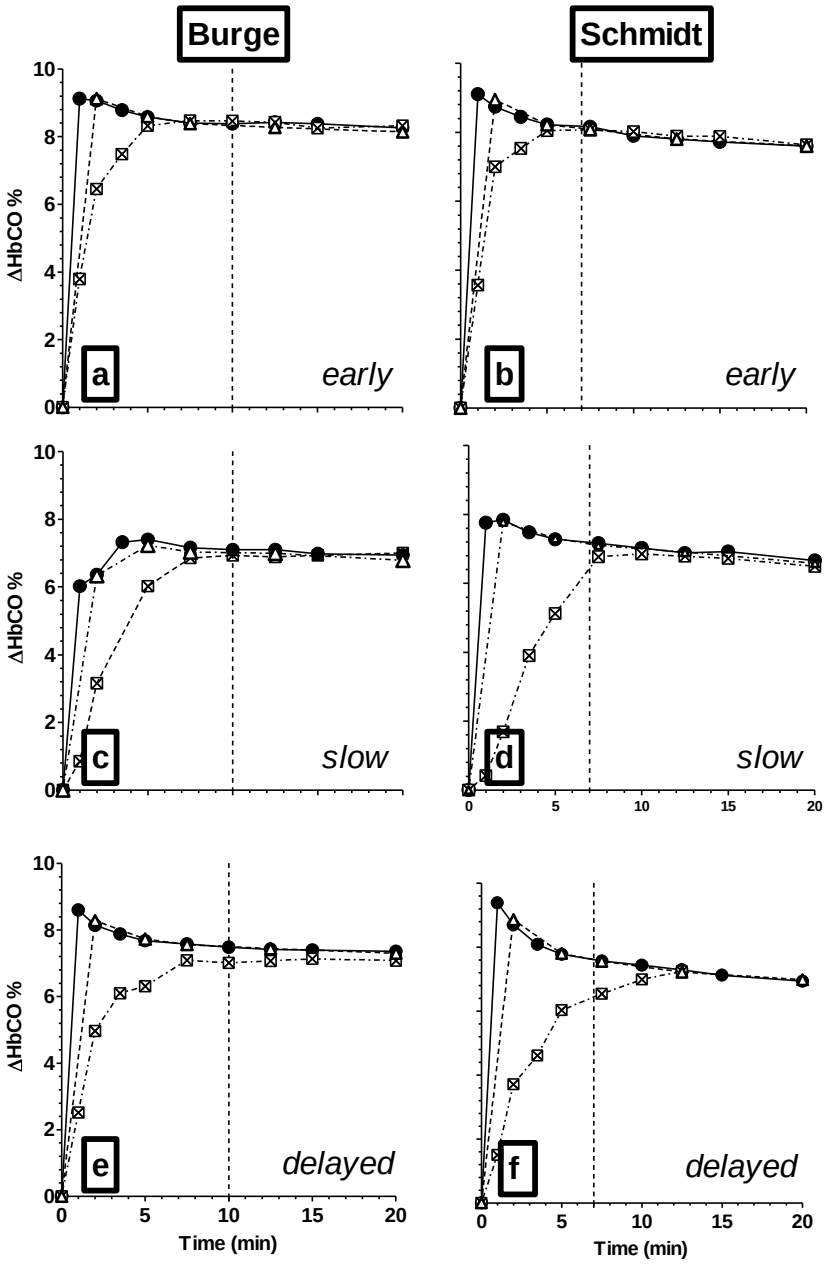

Figure 2-2: Individual HbCO curves for 3 subjects who show markedly different mixing times

Each row represents the same subject: top panels - subject X showing early mixing time in both Burge (a) and Schmidt (b) method; middle panels - subject Y showing slow mixing time in both Burge (c) and Schmidt (d) method, but complete by 10 mins; bottom panels – subject Z showing very slow mixing time in both Burge (e) and Schmidt (f) method. The vertical dotted lines indicate the time of blood sampling recommended for Hb_{mass} calculations in each method (Burge = min 10, Schmidt = min 7.5).

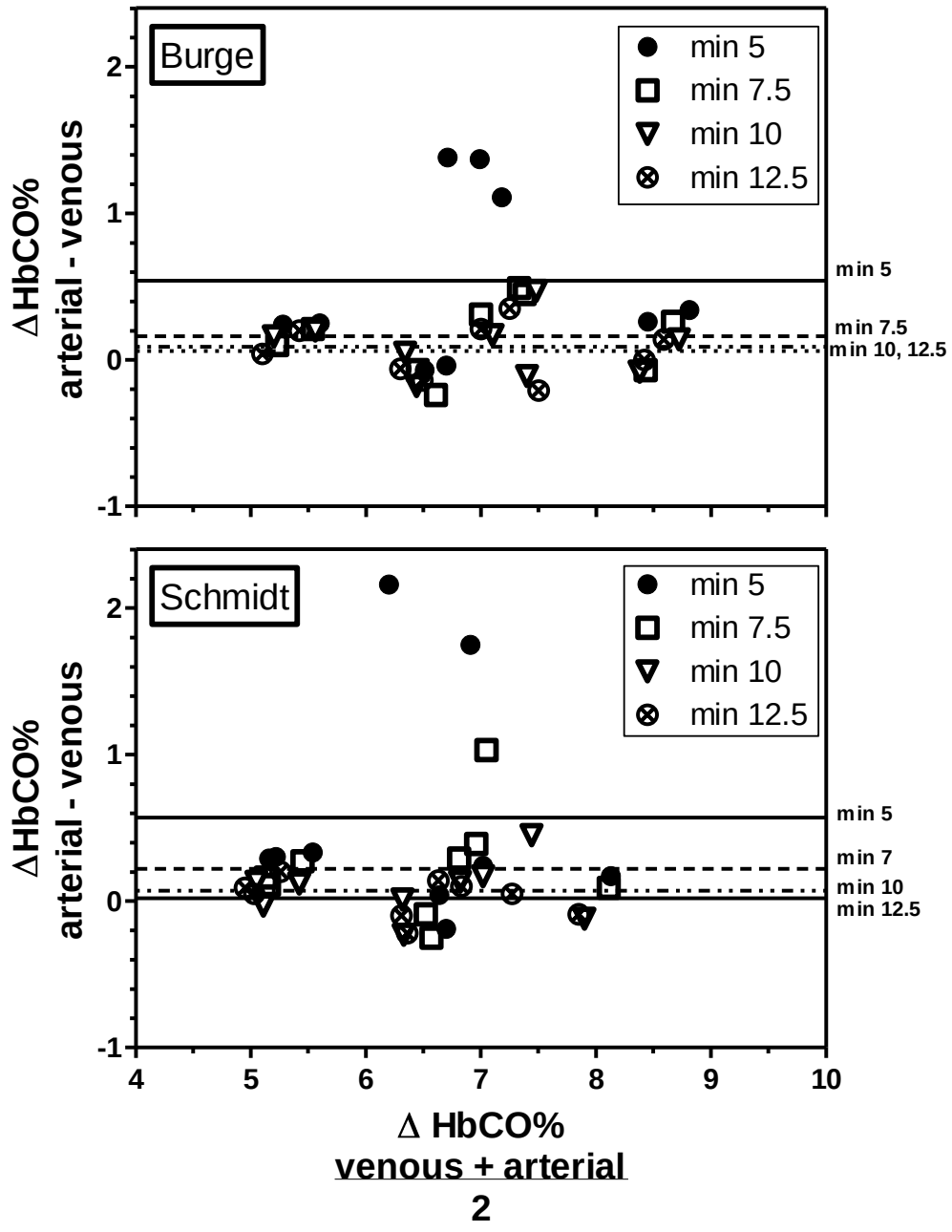

Figure 2-3: Differences in ΔHbCO (%) (for each individual) between arterial and venous blood during the Burge and Schmidt methods of CO rebreathing.

Horizontal lines indicate the mean difference at 5, 7.5, 10 and 12.5 min.

2.4.3 CO exhalation

The mean cumulative volume of CO exhaled in 12.5 min was not significantly different between methods; Schmidt: 3.15 ± 1.69 ml or 0.25 ± 0.13 ml.min^{-1} and Burge: 2.98 ± 1.80 ml or 0.25 ± 0.16 ml.min^{-1}. The calculated CO exhalation rate (between min 2 - 7.5), following the Schmidt method was 0.34 ± 0.08 ml.min^{-1} (p=0.26).

2.4.4 CO diffusion to myoglobin

The mean diffusion rate of CO to Mb was 0.22 ± 0.11 ml.min^{-1} following the Schmidt method and 0.16 ± 0.13 ml.min^{-1} during the Burge method (p = 0.63). Based on the calculated diffusion rates, the estimated volume of CO bound to Mb at the time of blood sampling (Schmidt = min 7, Burge = min 10), was 1.54 ± 0.74 ml and 1.63 ± 1.26 ml, respectively, equating to 1.68% and 1.78% of the administered CO dose.

2.4.5 Calculated Hb_{mass}

Hb_{mass} calculated from *a, vm* and *c* was different between the method used ($F_{(1,8)}$ = 5.38, p = 0.049) and time of sampling ($F_{(2,16)}$ = 4.42, p = 0.03). The mean calculated Hb_{mass} for all blood types was consistently lower in the Schmidt than the Burge method at min 7.5, 10 and 12.5. Specifically, the difference between the mean Hb_{mass} calculated at min 7.5 for the Schmidt method and at min 10 for the Burge method was -3.7 ± 3.2% for *a*, -1.7 ± 4.9% for *vm* and -4.6 ± 3.8% for *c* (**Figure 2-4**).

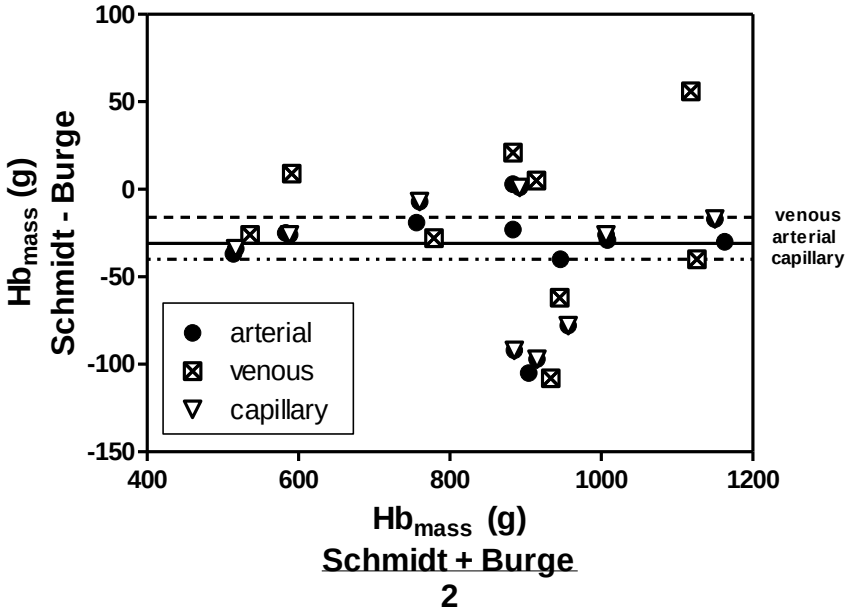

Figure 2-4: Difference between Hb_{mass} values calculated from the Schmidt and Burge method (for each individual)

Horizontal values are the mean difference using arterial, venous and capillary samples obtained at the recommended time points for each method - minute 7.5 for the Schmidt method and minute 10 for the Burge method.

For the Schmidt method, the mean CV for Hb_{mass} calculated from the pooled data of all three blood types was 2.7 ± 2.5%, 1.7 ± 1.2% and 2.1 ± 1.7% at min 7.5, 10 and 12.5, respectively. If subject Z who did not completely equilibrate until min 12.5 (**Figure 2-2f**) is removed from the analysis, the mean CV at min 7.5 is reduced to 2.0 ± 1.3% and the corresponding values at min 10 and 12.5 are 1.5 ± 1.1% and 2.1 ± 1.8%. For the Burge method, the mean CV for Hb_{mass} calculated from all three blood types was 2.1 ± 1.1% at min 7.5 and, 1.8 ± 0.8% at both min 10 and 12.5.

Increasing ΔHbCO by +1% resulted in a -1% change in calculated Hb_{mass}. The slope of the regression line was -0.997 ($r=0.9997$, $p<0.0001$). For CO loss to Mb, a +1%

change in the calculated volume resulted in a -0.02% change in Hb_{mass}, with the slope of the regression line -0.022 ($r=0.9992$, $p<0.0001$). Changing the volume of CO exhaled by +1% resulted in a -0.03% change in Hb_{mass} (slope = -0.033, $r=0.910$, $p<0.0001$).

2.5 DISCUSSION

The primary aim of the study was to compare the uptake and distribution of CO throughout the circulatory system during two different methods of CO-rebreathing. Inhalation of a CO bolus resulted in faster uptake of CO during the Schmidt method but did not greatly shorten t_{mix}. CO exhalation and loss to Mb were not different between the methods, but together with ΔHbCO have substantial implications for the calculation of Hb_{mass}.

2.5.1 CO kinetics

The uptake kinetics of CO throughout the vascular system, including loss to extra-vascular compartments, has been modelled by Bruce & Bruce (2003). The model consists of five compartments, four of which are vascular: 1. the lungs, 2. arterial blood (*a*), 3. mixed venous blood (*MV*), 4. muscle tissue (*M*), and 5. non-muscle tissue (*NM*). CO enters the system via V_A, where it diffuses into pulmonary *c* blood at a rate driven by the lung diffusion capacity for CO (DL_{CO}). CO in end-capillary blood quickly mixes with *a*, resulting in a rapid rise and overshoot of HbCO*a* (Fig 1). Next, CO enters either *M* or *NM* where further mixing occurs. Both compartments contain extra-vascular as well as vascular sub-compartments, and thus at this stage, some CO may escape from the vascular bed either in dissolved form, or in binding to

Mb. Lastly, blood leaving *M* and *NM* combines and enters *MV*; mixing further before returning to the lungs (Bruce and Bruce 2003).

The time for blood to pass through each compartment is governed by the ratio of the compartmental volume to the blood flow through each compartment. At rest, Bruce & Bruce attribute 40% of total blood volume to *M* and 25% to *NM*, resulting in a longer transit time through *M*. In addition, since CO must pass through these compartments before reaching venous circulation, the rate of appearance of HbCO*v* is slower than HbCO*a* (Bruce and Bruce 2003). It is important to distinguish between venous blood sampled from *M* (*vm*) as opposed to *MV* (*mv*). HbCO*mv* may equilibrate with HbCO*a* after ~3 min (Bruce & Bruce 2003), however due to the slower transit time through *M* vs. *NM*, HbCO*vm* does not converge with HbCO*a* until ~6 min. Venous blood sampled from an antecubital vein more closely refers to *M*, and as such provides an estimate of t_{mix}, (Bruce & Bruce 2003). In the present study, the mean time for HbCO*vm* levels to converge with HbCO*a* was closer to 10 min.

2.5.2 CO Mixing time (t_{mix})

Despite several attempts, t_{mix} has not been clearly defined, yet the time taken for CO to equilibrate has critical consequences for the calculation of Hb_{mass}. If incomplete, premature sampling of either *c* or *vm* could yield spurious results (Wennesland, Brown et al. 1962; Burge and Skinner 1995), with the falsely inflated HbCO*c* resulting in an underestimation of Hb_{mass}, whereas *vm* sampling would yield too low HbCO and consequently overestimate Hb_{mass}. Previous studies have reported t_{mix} during longer durations of rebreathing, as in the Burge method, to be 8-10 min

(Benignus, Hazucha et al. 1994; Burge and Skinner 1995; Hutler, Beneke et al. 2000; Schmidt and Prommer 2005; Gore, Bourdon et al. 2006), whereas for short rebreathing durations (e.g. the Schmidt method), t_{mix} has ranged from 2-10 min (Tikuisis, Buick et al. 1987; Schmidt and Prommer 2005; Gore, Bourdon et al. 2006; Prommer and Schmidt 2007), with the current recommendations for blood sampling 7 and 10 min in the Schmidt and Burge methods, respectively.

The rationale for the shorter rebreathing time adopted by the Schmidt method is that the application of a bolus CO dose will result in faster uptake and distribution of CO, thereby significantly shortening t_{mix} (Schmidt & Prommer 2005). Indeed, the time course of HbCO during exposure to either 1500 or 7500 ppm CO, where the duration of exposures was manipulated so that the total CO dose was equal, suggests that t_{mix} may be shorter when CO *concentration* is higher (Tikuisis, Buick et al. 1987). In the present study, the initial peak in ΔHbCO*a* was almost 1.0% (unit value) higher during the Schmidt method, even though the volume of CO administered was the same. The rapid inhalation of the CO bolus and subsequent breath hold may serve to increase alveolar pCO, resulting in a greater CO flux from the lungs to the blood (Bruce & Bruce 2003). However, whilst the initial rise of HbCO*a* was increased as expected (Bruce and Bruce 2003; Schmidt and Prommer 2005), t_{mix} was not greatly shortened vs. the Burge method. Furthermore, contrary to expectations HbCO*vm* increased more slowly during the Schmidt method. Another importance difference between the two methods which may affect the kinetics of CO uptake at the start of rebreathing is the level of O_2 that is inhaled immediately prior to rebreathing (room air in the Schmidt method vs. 100% O_2 in the Burge method). Thus CO uptake in the

Schmidt method may be affected by changes in Pa_{O2} arising from breathing 100% O_2 during the rebreathing procedure (Bruce and Bruce 2003).

In the present study, circulatory mixing was complete in most subjects by min 10 during both methods. However, marked differences in t_{mix} were observed in some subjects (**Figure 2-2**), which may be related to cardiac output (*Q*) and muscle blood flow during rebreathing. Bruce & Bruce (2003) demonstrate that alterations to the fraction of *Q* directed through *M* have substantial implications for the modelled rise of HbCO*vm*. If muscle blood flow is increased, so too is the turnover of blood through *M*; resulting in a faster rise in HbCO and a shorter t_{mix}. This highlights the need to standardise exercise and body temperature prior to and during rebreathing. In the present study, subjects were supine and rested, however we cannot discount that *Q* or muscle blood flow were different between the tests since neither were directly measured.

The contrasting results relating to t_{mix} may also be associated with differences in the peripheral circulation of the subjects used in each study. In healthy individuals, differences in regional circulation patterns, perhaps due to various training modalities, have been suggested to affect CO kinetics during rebreathing (Smith, Hazucha et al. 1994). For example, a greater muscle capillary density may increase the transit time through *M* and hence increase t_{mix}, as might medical or environmental conditions which delay compartmental blood flow (Brown, Hopper et al. 1951). In the present study, one subject reported a history of poor peripheral circulation on follow-up, whilst another reported feeling cold, despite the experiment being

performed at an ambient temperature of 23°C. Both subjects displayed prolonged t_{mix}, presumably as the fraction of *Q* to *M* was altered.

2.5.3 Impact of t_{mix} on Hb_{mass}

Small errors relating to ΔHbCO have significant implications for the calculation of Hb_{mass}. An error in ΔHbCO of just -0.1 unit value (equating to -1.7% at a ΔHbCO of 6%), results in a +1.7% error in Hb_{mass} (17 g if Hb_{mass} = 1000 g). Since the OSM-3 hemoximeter only provides HbCO to one decimal place, the potential for large errors relating to ΔHbCO is magnified. The impact of longer t_{mix} on the *a – vm* HbCO difference can be seen in **Figure 2-2f**, where sampling at min ~7 would result in a 0.3% higher ΔHbCO, and a resultant 20 g (~3%) lower Hb_{mass} than sampling at min 12.5.

Furthermore, Bruce & Bruce (2003) indicate that the HbCO plateau level is not only influenced by t_{mix}, but also strongly affected by the ratio of *M* to *NM* blood volume, with larger *M* blood volumes resulting in a lower plateau. Studies using Evan's Blue indicate that in some cases of shock or hypothermia, the circulating dye does not reach all portions of the vascular tree, and thus even though a plateau is reached, it represents the circulating, but not the *entire* red cell volume (Wennesland, Brown et al. 1962). Clearly, an understanding of the influence of blood volume distribution on ΔHbCO at the time of sampling is of critically importance for the accuracy of both methods.

Errors arising from variation in t_{mix} can be overcome via simultaneous sampling of *c* and *vm* in order to individually determine t_{mix} (Hutler, Beneke et al. 2000). Such a scenario is invasive, negating the 'optimisations' made by the Schmidt method, but

could be employed in a research setting. Nonetheless, researchers should make adjustments to the timing of blood sampling under circumstances when t_{mix} is predicted to be prolonged – e.g. in cases of poor peripheral circulation (Raynaud's phenomenon), or possibly in colder environments (Charkoudian 2010). Whether such adjustments should involve strategies to increase muscle blood flow, e.g. through exercise or body warming, should be investigated.

2.5.4 CO exhalation

Once rebreathing is terminated, CO is removed from circulation and HbCO slowly returns to baseline values. CO wash-out is evident if the two HbCO curves of the Schmidt and Burge methods (**Figure 2-1**) are compared – a plateau is not reached in the Schmidt method, and furthermore HbCO*vm* does not reach the same level even though an equal CO dose was administered. Bruce & Bruce (2006) have demonstrated that CO wash-out in room air is biphasic. Immediately post exposure, and in addition to the continued diffusion to Mb, CO is removed from the vascular compartment via exhalation, resulting in a rapid rate of CO removal. The second phase is slower since it requires CO bound to Mb to first diffuse back into the vasculature before it can be exhaled (Bruce and Bruce 2006). Measurement of CO exhalation began 5.5 min after rebreathing (when CO equilibration was largely complete) and was not different between the methods. Our finding confirms Bruce & Bruce's model which predicts that changing DL_{CO} will alter the rise of the HbCO curve but not its later decay. However, for the accuracy of the Schmidt method, the amount of CO lost immediately following disconnection must be quantified; although this is complicated by the fact that CO in the vascular system is still

equilibrating. CO exhalation between 2 - 7.5 min (estimated via measurement of end-tidal [CO] and estimation of V_A), was slightly higher than the measured rate between 7.5 - 20 min. Indeed, Prommer & Schmidt (2007) note that the exhaled volume of CO in the first 2 min after disconnection also includes CO remaining in the lungs from the procedure itself, which may account for the higher calculated rate. For simplicity, Prommer & Schmidt (2007) recommend calculating CO exhalation as opposed to measuring it directly, but it should be noted that variation in V_A or in the measurement of end-tidal [CO] will introduce an additional source of error (Schmidt and Prommer 2005). The resultant difference in the volume of CO exhaled at min 7.5 using the theoretical rate of 0.34 $ml.min^{-1}$ vs. the measured rate of 0.25 $ml.min^{-1}$ was ~0.7 ml (+36%), resulting in ~1.2% lower Hb_{mass}.

2.5.5 CO diffusion to Myoglobin

The CO-rebreathing method has been criticised in the past for not properly accounting for CO loss to Mb, and thereby overestimating Hb_{mass} (Sawka, Convertino et al. 2000). Bruce & Bruce's model (2003), provides compelling evidence that a "significant fraction of inspired CO can be bound to muscle Mb," and predicts that 1.3 – 1.9% of CO was bound to Mb after 10 min of rebreathing. We calculated a similar *total* loss to Mb during both methods (~1.8% of the CO dose), which was comparable to the findings of Prommer & Schmidt (2007) who report a loss of 2.3% of the CO dose.

CO flux out of *M* is determined by the difference between the pCO of arterial blood (Pa_{CO}) entering *M* and pCO of *M* itself, in addition to the CO diffusion capacity of *M* (DM_{CO}) (Bruce and Bruce 2003). Therefore, it should be noted that calculation of

loss to Mb using the average flux may be incorrect since Pa_{CO} will vary until t_{mix}. The somewhat higher rate of CO loss calculated for the Schmidt method might be expected due to higher Pa_{CO} arising from the bolus application. However, since Prommer & Schmidt's calculations cannot be applied before t_{mix}, early higher rates of CO loss are not accounted for. The difference between the methods is therefore likely due to a systematic error in the calculation, perhaps associated with the quantification of CO exhalation which is not required in the Burge method. If CO exhalation is underestimated, then CO diffusion to Mb will be falsely inflated. Thus, whilst calculation of CO loss to Mb enables an estimate within physiological expectations, precise measurement *in vivo* is warranted.

DM_{CO} represents all the mechanisms by which CO may 'leak out' of the vascular system and is determined by a number of factors including: diffusion rate across the capillary wall, diffusion rate within the tissue, rate of MbCO formation and the total surface area for diffusion (Bruce & Bruce 2003). Therefore, differences in muscle mass (especially between men and women), capillary density (endurance trained vs. resistance trained), and total Mb content between subjects may account for the variation in reported rates of CO loss to Mb. DM_{CO} also affects the plateau level and rate of decline of HbCO – an increased DM_{CO} results in a greater CO flux to Mb, therefore decreasing HbCO (Bruce and Bruce 2003), which has implications for calculation of Hb_{mass}, as described above. In this respect, physiological factors which may alter DM_{CO} (e.g. changes to muscle morphology) need to be considered when monitoring Hb_{mass} longitudinally.

Based on the CO flux to Mb currently reported, failure to account for any loss to Mb will result in a ~2.2% overestimation of Hb_{mass}. The Schmidt method (2005) currently adopts a 'one size fits' all approach for calculated CO loss to Mb, which appears quite robust within an individual unless muscle mass (and subsequently Mb content) varies by ± 10kg (Prommer and Schmidt 2007). However, to date we are unaware of any investigations regarding the rate of CO binding to free Mb, which may have implications for measurements of Hb_{mass} performed after muscle damage.

2.5.6 Hb_{mass} Calculations

In accordance to previous findings, the use of *a*, *vm* or *c* blood did not impact on the subsequent calculation of Hb_{mass}, provided sampling was performed after t_{mix} (Hutler, Beneke et al. 2000; Gore, Bourdon et al. 2006; Prommer and Schmidt 2007). However, due to inconsistencies in t_{mix}, (e.g. in our subjects) the timing of blood sampling in the Schmidt method has been questioned (Gore, Bourdon et al. 2006; Prommer and Schmidt 2007). In the present study, t_{mix} was complete in most subjects by min 10, and consistent with the lower CV for Hb_{mass} calculated at this time point. Interestingly, increasing the time to blood sampling beyond 10 min did not reduce the CV of Hb_{mass}, since the impact of errors associated with the estimation of CO lost to Mb and through exhalation also apparently increases over time (Prommer and Schmidt 2007). It should be noted, that the high CV calculated at min 7.5 in the Schmidt method, is reduced when the very slow mixing individual is removed. Therefore, sampling at min 7 may be sufficient as recommended by Prommer & Schmidt (2007), provided there is no reason to suspect a delay in t_{mix}, although sampling at min 10 would ensure complete mixing in the majority of subjects.

Hb_{mass} calculated using the Schmidt method was consistently lower than the value calculated using the Burge method, and comparable to the results of Gore et al (2006) who report a 2.9% lower Hb_{mass} using the Schmidt method. Our finding is largely attributable to the fact that CO loss to Mb is not accounted for in the Burge method (Burge and Skinner 1995), and based on our calculations would result in a ~2.2% overestimation. The remainder of the error may be attributable to an overestimation of CO loss via exhalation or inadequate mixing (resulting in a higher $\Delta HbCO_c$) in the Schmidt method.

Nevertheless, despite the additional potential for error in the Schmidt method, it appears that these errors have been adequately accounted for in the calculation of Hb_{mass}. On numerous occasions, researchers have demonstrated that the Schmidt method can be executed with excellent reliability (Schmidt and Prommer 2005; Gore, Bourdon et al. 2006; Prommer and Schmidt 2007; Eastwood, Hopkins et al. 2008; Garvican, Eastwood et al. 2010; Robertson, Saunders et al. 2010), which when combined with its relative portability and minimal invasiveness, makes it a highly attractive tool for regular monitoring of Hb_{mass} including in the field (Garvican, Eastwood et al. 2010).

2.5.7 Recommendations

Based on the findings of the present study and in conjunction with the model of Bruce & Bruce, a number of recommendations can be made to improve the accuracy and reliability of Hb_{mass} measurement using CO-rebreathing. To ensure complete mixing of CO, simultaneous samples of *vm* and *c* should be taken. Where this is impractical, sampling at min 10 in both methods should ensure complete mixing in

the majority of subjects, provided subjects are comfortably warm and report no history of circulatory disorders. Prior exercise (type and duration) should be documented and standardised where possible. An attempt to quantify individual variation in the size of the muscle compartment and its Mb content should be made by estimating muscle mass, with the rate of CO flux to Mb adjusted accordingly. The impact of prior exercise and muscle damage, in relation to blood flow and free Mb warrants further investigation.

2.6 CONCLUSION

The CO uptake kinetics of *a*, *c* and *vm* sampled simultaneously during CO-rebreathing are consistent with previous predictions based on a multi-compartment model. t_{mix} at rest varies between subjects, but is largely complete 10 min after the commencement of rebreathing in the Schmidt and Burge methods. Rates of CO diffusion to extra-vascular compartments and wash out via exhalation are important in the calculation of Hb_{mass}, and must be considered in conjunction with t_{mix} when determining the optimal time for blood sampling. The plateau level of HbCO has a significant impact on Hb_{mass} calculation; therefore it is paramount to ensure HbCO is equilibrated prior to blood sampling, as well as accounting for potential within-subject alterations of muscle blood flow, CO exhalation and CO flux to Mb.

CHAPTER 3: Stability of haemoglobin mass during a 6 day UCI ProTour cycling race

3.1 ABSTRACT

Objective: Blood doping in endurance sport is a growing problem. The purpose of this study was to determine the reliability of Hb_{mass} measurement in the field, and to establish the variability of Hb_{mass} during a cycling race, in order to assess its viability as an additional anti-doping detection parameter. **Design:** Control-matched longitudinal. **Setting:** UCI ProTour stage race. **Participants:** Six professional cyclists and five recreationally-active controls. **Interventions:** 72 Hb_{mass} tests using the optimised CO-rebreathing method were performed over 7 consecutive days, before and throughout the tour. Fasted venous blood was obtained for measurement of Haematocrit (Hct) and Haemoglobin concentration [Hb] in the morning prior to D_1, D_3 and D_6. **Main Outcome Measures:** Reliability of Hb_{mass} measurement was established using Typical Error (TE) calculated from two baselines measures. Individual change scores and coefficients of variation were used to assess stability during racing. **Results:** TE for Hb_{mass} was 1.3% (95% Confidence limits (CL); 0.9-2.5%). Calculated 95% and 99.99%CL for percent change in Hb_{mass} were ±3.6% and ±7.2%, respectively. Mean Hb_{mass} remained within ±1.9% of baseline in cyclists and ±0.5% in controls. In all cases, individual change scores for both cyclists and controls fell within the 95%CL. There was a decrease in Hct (8.1±2.8%) and [Hb] (9.7±3.2%) throughout the tour in cyclists but not controls. **Conclusion:** We

demonstrate that Hb_{mass} can be measured reliably via CO-rebreathing during a cycling tour. Unlike [Hb] and Hct, Hb_{mass} remains stable over six days of racing in professional cyclists and may have potential in an anti-doping context.

3.2 INTRODUCTION

Blood doping in endurance sport is a growing problem. Multiple generations of recombinant human erythropoietin (rhEPO) and other erythropoietic stimulants, coupled with systematic blood removal and reinfusion (Borrione, Mastrone et al. 2008), are making it increasingly difficult for anti-doping authorities to achieve a clean sport. Currently, autologous blood transfusions cannot be detected and a simple test to detect all synthetic stimulants at once is near impossible.

In an attempt to combat blood doping from a broader perspective, the Biological Passport (Ashenden 2002) was introduced by the International Cycling Union (UCI) in 2008. The premise of the passport is to monitor an individual athlete's blood profile over time, with any substantial deviations in selected parameters from those expected based on prior measurements, deemed suspicious and followed up with further testing. The passport relies on measurement of haematological parameters sensitive to blood doping, e.g. haemoglobin concentration [Hb], haematocrit (Hct) and reticulocytes. Monitoring of these parameters is not without limitations however, especially in lieu of the influence of plasma volume (PV), which in itself can be affected by many factors (Sawka, Convertino et al. 2000). [Hb] and Hct generally decrease in response to multiple days of cycle racing due to PV expansion (Schumacher, Pottgiesser et al. 2008; Morkeberg, Belhage et al. 2009). A recent longitudinal study of professional cyclists in Team CSC also reports seasonal

changes in [Hb] and Hct, with a decrease in these parameters observed during the competitive season and an increase in the off-season (Morkeberg, Belhage et al. 2009). These natural variations in response to training, racing and rest can make interpretation of the data in an anti-doping context difficult and confusing.

The aim of most blood manipulations is to increase total haemoglobin mass (Hb_{mass}). Therefore, the direct measurement of this oxygen carrying protein in an anti-doping context is appealing due to its direct correlation with maximum aerobic power (Schmidt and Prommer 2008). Recent optimization of the carbon monoxide (CO)-rebreathing method for determination of Hb_{mass} (Prommer and Schmidt 2007) has made measurement in athletic populations more feasible, as the entire test can be completed in <15mins. In fact, due to the reliability and sensitivity of this method (Gore, Bourdon et al. 2006), the use of Hb_{mass} as an additional anti-doping parameter has recently been proposed (Prommer, Sottas et al. 2008).

If Hb_{mass} is to be incorporated into the Biological Passport, then the method for measurement must be relatively easy to administer and portable, as well as accurate and repeatable. The parameter of interest (e.g. Hb_{mass}) must also be noticeably influenced by doping practices but be relatively unchanged by periods of training and racing. Pottgiesser demonstrated that removal and subsequent reinfusion of one unit of blood results in changes of Hb_{mass} of ~6%, which can be detected using the CO-rebreathing method (Pottgiesser, Umhau et al. 2007). In contrast, Hb_{mass} appears to be relatively stable (±3%) over an entire training year in endurance athletes, despite fluctuations in training load (Prommer, Sottas et al. 2008).

The acute effects of periods of intense exercise on Hb_{mass}, such as multi-stage cycling races, are still to be elucidated. Schumacher reported Hb_{mass} to be stable in German

U23 cyclists over 4 days of racing (Schumacher, Pottgiesser et al. 2008). In this study, the Typical Error (TE) of measurement was 3.3% - higher than typically reported for the CO-rebreathing method (Gore, Hopkins et al. 2005; Prommer, Sottas et al. 2008). The authors suggest that the higher TE may be due to the field setting in which the measures were performed, but do not discount increased variability due to racing. If 95% confidence limits (CL) for the error associated with a change (%) from baseline values are calculated using a TE of 3.3% (TE x 1.96 x √2), then the 95%CL = ±9.4%. With this degree of uncertainty, potential manipulations using microdoses of rhEPO or small blood transfusions which could induce beneficial changes in Hb_{mass} (40 - 80g) within these limits would unfortunately go undetected. In contrast, if the TE is closer to 2%, as typically reported in a laboratory setting (Gore, Hopkins et al. 2005; Prommer, Sottas et al. 2008), then the 95%CL would be reduced to ±5.5%. Therefore, it is necessary to establish whether Hb_{mass} can be measured with the same degree of reliability in the field as in a laboratory. In addition, a lower TE would allow more meaningful inferences to be made on the stability of Hb_{mass} during stage racing.

The aim of the present study was to determine the stability of Hb_{mass} throughout a UCI ProTour event in professional cyclists. A secondary aim was to assess the reliability of the method in a race setting and its viability as an anti-doping detection tool.

3.3 METHODS

Hb_{mass} was measured in six male professional cyclists (CYC: mean (SD); age 24 (5) y, height 179 (5) cm, mass 71 (4) kg) and five recreationally-active male controls

(CON: 31 (6) y, 179 (6) cm, 74 (6) kg) on seven consecutive days before and throughout a multi-stage cycling race. All cyclists were members of the same team. Individual stage finishes and final General Classification placing ranged from 10^{th} – 120^{th}. Control subjects were healthy, active non-smokers and were instructed to continue normal activities and training (<2 $h.d^{-1}$) during the course of the investigation. The study was approved by the Human Ethics Committee at the Australian Institute of Sport. The procedures and risks involved were fully explained to all participants and written informed consent was obtained prior to commencement of the study.

3.3.1 Race details and logistics

All measurements were performed at the Tour Down Under, a UCI ProTour race held in Adelaide, Australia during the southern hemisphere summer. The 2009 race covered 802 km over 6 days, totalling 19.5 h of racing. The power output demands of this race have been described previously (Ebert, Martin et al. 2006). Details of each stage can be found in **Table 3-1**. Daily temperatures in central Adelaide according to the Australian Bureau of Meteorology ranged from 24 - 39 C. Hb_{mass} measures were performed by the same researcher at the event hotel within 4 h of the stage completion. All measurements involving athletes were completed by 19:00 h; 16 h before the start of the subsequent stage. As the half life of CO in the blood is 2.5 h (Schmidt and Prommer 2005), HbCO would have returned to normal levels prior to the start of the next stage and thus would not affect the athlete's performance. Random doping controls were performed by anti-doping authorities on two occasions, and no positive cases were reported. Diet and fluid intake were not controlled.

Table 3-1: Topographic, climatic and race characteristics of the 2009 Tour Down Under

	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5	Stage 6
Distance (km)	140	145	136	143	148	90
Winner's Time (h:m)	3:45	3:46	3:15	3:29	3:28	1:42
Profile	Flat	Flat	Hilly	Hilly	Hilly	Criterium
Temperature (^{O}C)	39	29	28	28	24	30

3.3.2 Haemoglobin mass

The optimised CO-rebreathing method (Schmidt and Prommer 2005; Prommer and Schmidt 2007), was used to determine Hb_{mass}. Briefly, a CO bolus of 1.2 $ml.kg^{-1}$ was injected into a glass spirometer and rebreathed for two minutes. HbCO (%) in the blood was measured via capillary finger-tip samples (200 µL) before and seven minutes after administration of the CO dose. Samples were analysed immediately in quintuplet using an OSM-3 hexiometer (Radiometer, Copenhagen, Denmark). Duplicate baseline measures were performed on consecutive days before the start of the race and averaged to determine baseline (D_0). During racing, measurements were performed after stages 1-5 (D_{1-5}). No measures were performed on the last race day because of logistical complications associated with the cyclists' departure.

3.3.3 Haematology

Fasted, venous blood samples were obtained upon waking two days prior to racing (D_0), after two stages (D_3) and before the last stage (D_6) in the cyclists. The same time points were used for controls with the exception of the first sample which was obtained on D_1. Whole blood (~4 ml) was collected from a forearm vein under stasis into a K_3EDTA vacutainer in accordance with UCI protocol. [Hb] and Hct were

measured via Spectrophotometry and cumulative pulse height detection, respectively using an XE-2100 analyser (Roche Diagnostics, Switzerland) at an accredited UCI laboratory within 12 h of collection.

3.3.4 Statistics

Data were analysed using a contemporary statistical approach (Hopkins, Marshall et al. 2009) and are presented as mean (SD) unless otherwise stated. To assess the reliability of Hb_{mass} measurement, TE was calculated using the two pre-race measures for each participant (n=11). TE is calculated as the standard deviation of the change scores between measurements divided by √2 and expressed in percent. TE was used to calculate CL for the error associated with the change score from D_0 as follows: TE x z-score x √2, using a z-score of 1.96 and 3.89 for the 95% and 99.99% CL, respectively.

To assess mean changes from D_0 of Hb_{mass}, Hct and [Hb] over the course of the race, as well as differences between cyclists and controls, all raw data were firstly log transformed to reduce bias arising from non-uniformity of error. Mean effects, and the precision of estimate (95%CL), from D_0 and the effect of racing versus recreational activity were estimated using the unequal-variances t statistic. The smallest worthwhile change was derived from Cohen's scale for Effect Sizes (1988) in which a small effect size is ≥ 0.2.

The percentage likelihood of the observed differences is expressed using the following descriptors: <1%, almost certainly not; 1-5%, very unlikely; 5-25%, unlikely; 25-75%, possibly; 75-95%, likely; 95-99%, very likely; >99%, almost certainly. The effect was deemed "unclear" if its confidence interval overlapped the

thresholds for both positive and negative change. Differences in the change scores from D_0 between cyclists and controls were deemed substantial if the probability of the true change was greater than 75% and if $p<0.05$ (Hopkins, Marshall et al. 2009).

Individual daily change scores were calculated for all cyclists and controls. In addition, individual and mean coefficients of variation were calculated to assess within-subject daily variability.

3.4 RESULTS

3.4.1 Haemoglobin mass

The CO-rebreathing procedure was successfully implemented on all testing days, with complete compliance from both cyclists and controls. One cyclist experienced discomfort during his first test and terminated the test before completion of the 2-min rebreathing procedure. However, this test was repeated successfully several hours later.

The TE for Hb_{mass} measurement was 1.3% (95%CL; 0.9-2.5%). Nine of the participants had a difference score <2% between the two baseline measures with the largest difference score 3.5%. The calculated 95% and 99.99% CL for the change scores from D_0 were ±3.6% and ±7.2%, respectively.

Mean Hb_{mass} of the cyclists remained within ±1.9% of baseline (D_0 = 997 (67) g) throughout the tour, ranging from -1.9% on D_5 to +1.3% on D_2 (**Figure 3-1**). In the controls, mean Hb_{mass} remained within 0.5% of baseline (D_0 = 901 (113) g) during the same period. Daily change scores tended to be slightly more variable in cyclists vs. controls, although no substantial differences were detected. The mean CV for

Hb_{mass} during the tour was 1.6% (95%CL; 1.3-2.1%) in cyclists vs. 1.3% (1.0-1.7%) in controls. Individual CVs ranged from 0.3-2.3% in cyclists and from 0.7-1.7% in controls.

Individual Change scores from D_0 for Hb_{mass} are shown in **Figure 3-2**. All measurements for cyclists and controls fell within the 95% CL calculated from the TE at D_0.

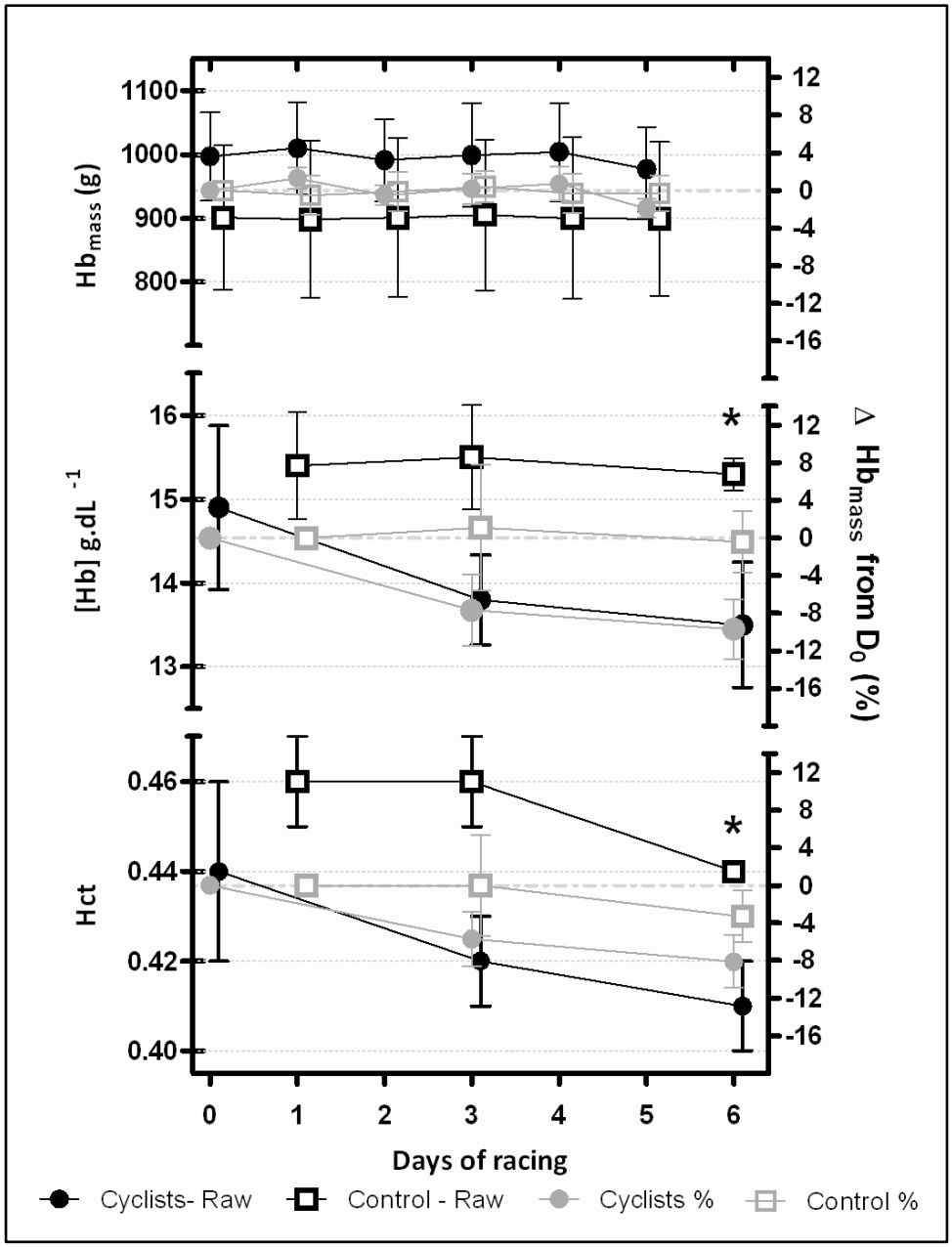

Figure 3-1: Hb_{mass}, [Hb] and Hct (mean ± SD) before and throughout a 6 day cycling race in six professional cyclists (circles) and five recreationally-active controls (squares)

Raw units are shown on the Left Y axis (black symbols), and change (%) from baseline (D_0) shown on the Right Y axis (grey symbols). (* denotes a substantial difference between the groups in the change scores from D_0, $p<0.05$)

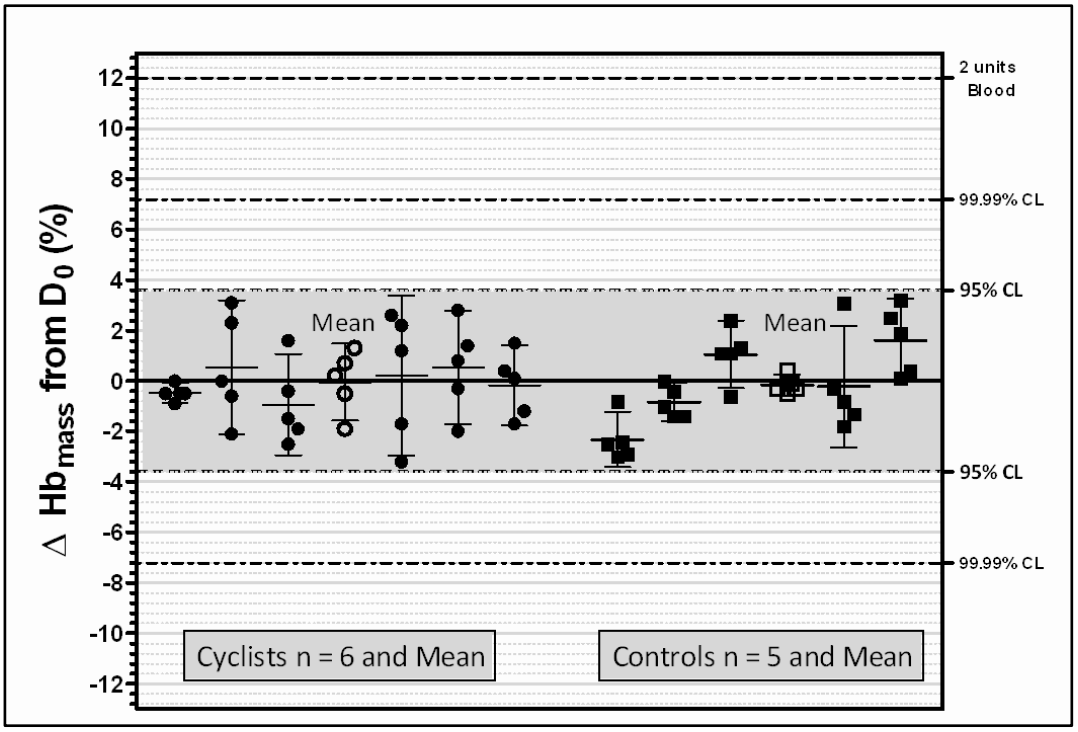

Figure 3-2: Individual (closed symbols) and mean (open symbols) changes (± SD) in Hb_{mass} (%) from D_0 during a 6 day cycling race in six professional cyclists (circles) and five controls (squares)

95% and 99.99% Confidence limits (CL) associated with measurement error for a change score are indicated by dotted lines. For reference, expected changes due to blood doping are indicated in upper right hand corner.

3.4.2 Haemoglobin Concentration and Haematocrit

Both [Hb] and Hct decreased substantially (**Figure 3-1**) during the period of investigation in cyclists but not controls. This decrease was progressive over the three time points: Cyclists, [Hb] (D_0 = 154 (6.4) $g \cdot L^{-1}$), D_3 = -7.7 (3.8) %, p=0.005; D_6 = -9.7 (3.2) %, p=0.000; Hct (D_0 = 0.44 (0.02)), D_3 = -5.7 (2.9)%, p=0.005; D_6 = -8.1 (2.8)%, p=0.000), and observed in all six cyclists. At D_6, [Hb] and Hct were "almost certainly" lower in cyclists than controls; Hct = -4.7% (95%CL: -8.8 to -0.5%), p=0.036; [Hb] = -8.9% (-13.3 to -4.2%), p=0.004.

3.5 DISCUSSION

3.5.1 Changes in Hb_{mass}, Hct and [Hb] during a cycling stage race

Hb_{mass} remained stable in both professional cyclists racing in hot conditions and recreationally-active controls throughout the week of racing. The individual CV for Hb_{mass} was less than 2.5% in the 11 cases measured, with the largest change from baseline on any one day ±3.2% (34 g). In contrast, and similar to previous observations (Morkeberg, Belhage et al. 2008; Schumacher, Pottgiesser et al. 2008; Morkeberg, Belhage et al. 2009), stage racing resulted in a decrease in Hct (~8%) and [Hb] (~10%) compared to recreationally-active controls.

The observed decrease in Hct and [Hb] without concomitant changes in Hb_{mass} is consistent with an acute PV expansion induced by periods of increased training load such as cycling stage racing (Neumayr, Pfister et al. 2002; Schumacher, Pottgiesser et al. 2008). We were unable to calculate PV directly in the present study, as venous blood samples were collected in the morning and Hb_{mass} measured in the evening on completion of each stage. However, a 9.5% expansion in PV has been observed following four days of cycling racing (Schumacher, Pottgiesser et al. 2008) and similarly, an 11.9% increase in PV in amateur cyclists one day after an ultra-marathon (Neumayr, Pfister et al. 2002).

The direct measurement of Hb_{mass} via CO-rebreathing has the advantage that it is not affected by fluid shifts, thus observed changes in Hb_{mass} which exceed measurement error at a specified level of uncertainty can be considered ‘real’. No substantial increases or decreases in Hb_{mass} were observed in any individual during the week of racing. This finding is not surprising if the potential ‘legal’ means by which Hb_{mass}

can be altered are considered. Natural increases in Hb_{mass} arising from training induced erythropoiesis (Sawka, Muza et al. 2009) are unlikely to present in a detectable magnitude during the short time frame of investigation (<1 week). Short term release of erythrocytes from the spleen following periods of exercise 'stress' may lead to an increased amount of circulating haemoglobin (Stewart and McKenzie 2002). However, Prommer has shown that even at rest, complete mixing of CO in the blood includes erythrocytes contained in the spleen and thus subsequent release would not lead to an increased Hb_{mass} measurement (Prommer, Ehrmann et al. 2007). Potential causes for a decrease in Hb_{mass} during racing include trauma, exercise-induced haemolysis, and in female athletes, menstrual blood loss. Some athletes were involved in minor crashes during the race, however all lacerations were superficial and any blood loss would have been negligible. Although not measured, exercise-induced haemolysis is unlikely during cycling racing since it is the 'foot strike' during running, as opposed to strenuous exercise *per se,* that is deemed to be the major contributing factor (Schumacher, Schmid et al. 2002; Telford, Sly et al. 2003). Finally, menstrual loss in female athletes is again unlikely to result in detectable changes in Hb_{mass}, since average menstrual blood loss is only 35ml (Fraser, Warner et al. 2001), equating to <5g of Hb (assuming a [Hb] of 15g/dl) over one week.

3.5.2 Measurement of Hb_{mass} in a race setting

The TE for Hb_{mass} measurement in the present study is comparable to previous studies from our laboratory and others (Gore, Hopkins et al. 2005; Prommer, Sottas et al. 2008; Eastwood, Bourdon et al. 2009; Robertson, Saunders et al. 2010) and demonstrates that it is possible to measure Hb_{mass} in the field with the same degree of reliability as in a controlled laboratory environment. During a similar race setting,

Schumacher reported a higher TE for Hb_{mass} during stage racing and attributed this larger error in part due to the field location (Schumacher, Pottgiesser et al. 2008). Schumacher highlighted a number of possible methodological explanations for their inflated TE, most of which we were able to improve in the current research as described below.

The greatest sources of error in the CO-rebreathing method lie in the volume determination and delivery of the CO dose, and in the analysis of HbCO (Burge and Skinner 1995; Gore, Hopkins et al. 2005). In the present study, a larger dose of CO was administered; 1.2 $ml.kg.min^{-1}$ versus 1.0 $ml.kg.min^{-1}$ in the study of Schumacher et al. Calculation of Hb_{mass} is dependent on the change in HbCO in the blood before and after rebreathing. A higher change score in %HbCO (delta) is associated with a higher degree of accuracy (Burge and Skinner 1995) and can be achieved using a greater dose of CO. A delta of 6% is deemed desirable in terms of accuracy and still remains well within safe limits for HbCO concentrations. Due to the expected large blood volumes of the athletes measured in the present study, such a delta can only be achieved using a higher dose of CO. Despite a mean dosage of 86 ml, the mean delta of HbCO in our study was 5.6%. Schumacher reported a mean delta of 4.5% using a dose of 70 ml of CO and indeed, suggest that a higher dosage may have improved the TE in their study.

By using the mean of duplicate baseline measures to establish D_0, we were able to gain more confidence in our initial value, and therefore improve our sensitivity to detect real changes. Multiple measures of a sample reduce the error of measurement as a function of the square root of *n* replicates (Hopkins 2000). However, duplicate baseline measures employing the current Hb_{mass} methodology may not always be

possible within (stage) racing and therefore the integrity of the 'pre' value to which the change score is derived must be carefully considered.

3.5.3 Application to anti doping

The improved TE of the present study enabled 95% CL and 99.99% CL of 3.6 and 7.2% respectively, to be derived for a change in Hb_{mass} from baseline. For an athlete with a Hb_{mass} of 1200 g, this equates to 1:20 cases when Hb_{mass} is expected to change by >43 g and 1:10,000 cases when Hb_{mass} could be expected to change by >86 g. One unit of whole blood contains ~60 g of Hb (Pottgiesser, Umhau et al. 2007). Thus the infusion of one unit of blood could result in a change in Hb_{mass} of 5%, falling outside the 95% CL of the present study, and therefore could potentially be flagged as 'suspicious'. Importantly, all measurements of Hb_{mass} in the present study fell within the 95% CL during the week of racing confirming the stability of Hb_{mass} in the context of racing.

Whilst this study again demonstrates that Hb_{mass} is stable to within ~3% during periods of intense cycling exercise up to one week in duration (Schumacher, Pottgiesser et al. 2008), further research is needed to assess stability during races of 1-3 weeks, especially in view of a potential application during the Grand Tours. Furthermore, whilst this study has addressed the use of CO-rebreathing in a cycling context, further research is needed before the results can be directly applied to other endurance populations such as rowing, running and cross-country skiing. The logistics of incorporating Hb_{mass} into the Biological Passport should also be systematically investigated including the effect of multiple testers and analysers around the world. Lastly, the variability and sensitivity associated with measurement

of Hb_{mass} points towards its use as an additional parameter for the Biological Passport rather than a standalone detection test (Morkeberg, Sharpe et al. 2010).

3.6 CONCLUSION

We have demonstrated that measurement of Hb_{mass} via CO-rebreathing can be performed reliably 'on location' during a professional cycling tour. Unlike [Hb] and Hct, Hb_{mass} remained stable over six days of racing in hot conditions in professional cyclists; demonstrating its potential as an additional parameter for the Biological Passport.

CHAPTER 4:
Case study - Haemoglobin mass in an anaemic female middle–distance runner before and after iron supplementation

4.1 ABSTRACT

Haemoglobin mass (Hb_{mass}) of a female endurance athlete was measured via carbon monoxide (CO)-rebreathing upon diagnosis of iron-deficiency anaemia (Haemoglobin concentration = 8.8 $g.dL^{-1}$, Ferritin = 9.9 $ng.ml^{-1}$) and regularly during treatment thereafter. Hb_{mass} increased by 49%, two weeks following an intramuscular iron injection and continued to increase with oral iron supplementation for 15 weeks. The presented case illustrates that Hb_{mass} is readily responsive to iron supplementation in a severely iron-deficient anaemic athlete and that changes can be tracked efficiently using the CO-rebreathing method.

4.2 INTRODUCTION

Female distance runners may be at greater risk of iron depletion due to iron loss through foot-strike haemolysis, menstrual irregularities and insufficient iron intake. In severe cases, iron depletion may develop into iron-deficiency anaemia, with endurance performance severely impaired due to a decreased oxygen transport capacity (Eichner 1992). In clinical settings, haemoglobin concentration [Hb] and serum ferritin are typically used for diagnosis, yet have limitations in elite athletes since both can be affected by training (Fallon 2008; Schumacher, Pottgiesser et al.

2008). Direct measurement of haemoglobin mass (Hb_{mass}) via carbon monoxide (CO)-rebreathing has the advantage that is not affected by plasma volume shifts (Prommer, Sottas et al. 2008), thereby providing an additional and robust diagnostic tool for the investigation of anaemia in athletes. The aim of the present study was to document changes in Hb_{mass} following iron supplementation in a female endurance athlete diagnosed with iron-deficiency anaemia.

4.3 CASE REPORT

A female middle-distance runner (age 19 y, height 171 cm, mass 63 kg) presented with high levels of fatigue which were accompanied by a steady decrease in running performance and training ability over ~3 months. Following medical examination, blood tests revealed severe iron-deficiency anaemia characterised by low serum ferritin and accompanied by low [Hb], decreased serum iron concentration, and decreased percent transferrin saturation (**Table 4-1**) (Rodenberg and Gustafson 2007).

Table 4-1: Haematological and Iron profile of a 19 year old female endurance athlete before and following iron supplementation

	Baseline	*2 weeks post injection*	*7 weeks post injection*	*15 weeks post Injection*
Hb_{mass} (g)	389^	580^	690	710^
[Hb] ($g.dL^{-1}$)	8.8	12.0	13.1	13.0
Haematocrit	0.270	0.347	0.368	0.362
Mean Cell volume (fL)	70.0	81.0	86.1	91.7
Mean Corpusuclar Haemoglobin (pg)	22.8	28.1	30.7	33.1
Mean Cell [Hb] ($g.dL^{-1}$)	32.6	34.7	35.7	36.1
%Microcytosis (%)	17.3	18.6	13.3	1.0
%Macrocytosis (%)	0	0.7	0.6	1.1
%Hypochromasia (%)	13.5	2.1	0.9	0.1
%Hyperchromasia (%)	0.7	1.3	2.3	2.2
%microcytic/%Hypochromic	1.3	8.9	14.8	10.0
Iron ($\mu g.dL^{-1}$)	24.6	71.0	148.1	86.6
Ferritin ($ng.ml^{-1}$)	9.9	23.4	35.7	27.0
Transferrin ($g.L^{-1}$)	3.39	2.64	2.59	2.38
Saturation (%)	6	21	45	29

(^ Mean of two measures)

Hb_{mass} was measured in duplicate before and repeatedly following iron supplementation using the optimised CO-rebreathing technique (Prommer, Sottas et al. 2008; Schumacher, Pottgiesser et al. 2008), until Hb_{mass} was deemed to have stabilised. The Typical Error of the method is 1.1 - 2.0 % (Prommer, Sottas et al. 2008; Schumacher, Pottgiesser et al. 2008). Briefly, a CO bolus of 0.8 - 1.2 $ml.kg^{-1}$ was rebreathed for two minutes. HbCO (%) in the blood was measured via capillary finger-tip samples (200 µL) before and seven minutes after administration of the CO dose and analysed immediately in quintuplet using an OSM-3 hexiometer (Radiometer, Copenhagen, Denmark). Due to the very low [Hb] of the initial blood screen, the volume of CO administered at the start of the monitoring period was 0.8

ml.kg^{-1}, but was increased to the optimal dose of 1.2 ml.kg^{-1} in accordance with changes in Hb_{mass}. [Hb] and iron status were determined from venous blood collected from a forearm vein. In our laboratory, the TE for [Hb] and Ferritin is 2.1% and 6.9%, respectively. The timeline of Hb_{mass}, [Hb] and iron supplementation are shown in **Figure 4-1**.

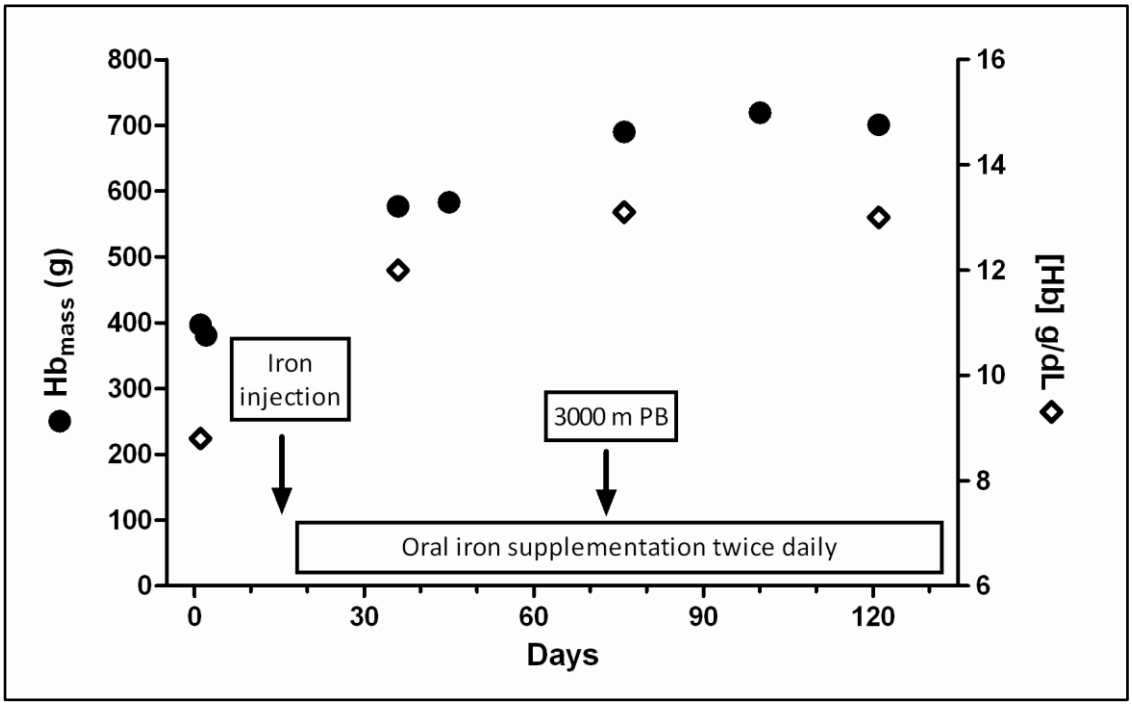

Figure 4-1: Haemoglobin mass and Haemoglobin concentration before and following iron supplementation in a 19 year old female endurance athlete.

4.4 DISCUSSION

Hb_{mass} increased markedly and rapidly in response to intra-muscular iron supplementation, and thereafter continued to increase with oral iron supplementation. Upon initial presentation, the athlete's Hb_{mass} was 389 g, equivalent to 6.2 g.kg^{-1} and consistent with the diagnosis of iron-deficiency anaemia – the normal range for a female endurance athlete being 10 - 13 g.kg^{-1} (Heinicke, Wolfarth et al. 2001). Two weeks after the iron injection, Hb_{mass} increased by 49% to 580 g or 9.3 g.kg^{-1}. Seven

weeks post-injection, Hb_{mass} increased an additional 110g to 690 g, or 77% above the initial value. At 10.9 $g.kg^{-1}$ the athlete's Hb_{mass} was within the normal range for a female endurance athlete. The athlete was gradually able to resume her usual training volume (~80 $km.wk^{-1}$) and intensity over the intervention period and recorded a personal best time over 3000 m ~70 days post-injection. Both Hb_{mass} and [Hb] appeared to reach a plateau 15 weeks after treatment began at ~700 g and 13.0 $g.dL^{-1}$, respectively.

The influence of changes in training load during the monitoring period should be considered in relation to the observed increase in Hb_{mass}. Changes in Hb_{mass} have been related to changes in 6 week training load in elite cyclists (Garvican, Martin et al. 2010). During the early stages of treatment, only minimal low-intensity training sessions were completed; however, at 6 weeks post-injection training load was progressively increased with 'normal' training resumed by 15 weeks. Therefore, as the athlete responded to treatment and was able to increase her training load, it is possible that an additional erythropoietic stimulus was gained from training *per se*. However, changes in Hb_{mass} arising from even dramatic (100%) changes in training load over 6 weeks are unlikely to exceed ~10%, (Garvican, Martin et al. 2010) implying that the majority of changes observed in this runner, and certainly those in the initial intervention period, can be attributed to iron supplementation. In addition, whilst the performance improvement is very likely due to the change in Hb_{mass}, the minimal low-intensity training performed at the start of treatment could be considered a 'taper' that may partially explain some of the performance recovery (Mujika, Padilla et al. 2004).

The present case illustrates that Hb_{mass} is readily responsive to iron supplementation in a severely iron-deficient anaemic athlete and that changes are tracked efficiently using the CO-rebreathing method. Measurement of Hb_{mass}, in addition to [Hb] and iron parameters, represents another valuable diagnostic and monitoring tool for anaemic conditions. Hb_{mass} measurement has the advantage that it is independent of changes in plasma volume and therefore can be performed at any time of day, irrespective of training or nutritional status. Furthermore, the technique may be more sensitive to subtle changes in total haemoglobin content, and thus oxygen carrying capacity, which may have significant effects for endurance performance. This is illustrated by the changes in Hb_{mass} and [Hb] observed in the athlete in the later stages of treatment, when Hb_{mass} continued to increase whereas [Hb] remained constant.

The presented case confirms the effectiveness of iron supplementation in the treatment of iron-deficiency anaemia and provides some additional insight into the time course of the erythropoietic response to treatment. From an anti-doping perspective, iron status and supplementation, particularly in female athletes, should be considered in reference to longitudinal monitoring of Hb_{mass} and blood parameters over time (Prommer, Sottas et al. 2008).

CHAPTER 5: Time course of the haemoglobin mass response to natural altitude training in elite endurance cyclists

5.1 ABSTRACT

To determine the time course of haemoglobin mass (Hb_{mass}) to natural altitude training, Hb_{mass}, erythropoietin [EPO], reticulocytes, ferritin and soluble transferrin receptor (sTfR) were measured in 13 elite cyclists during, and 10 days after, three weeks of sea level (SL, n=5) or altitude (ALT, n=8, 2760m) training. Mean Hb_{mass}, with a typical error of ~2%, increased during the first 11 days at altitude (Mean±SD 2.9±2.0%) and was 3.5 ± 2.5% higher than baseline after 19 days. [EPO] increased 64.2 ± 18.8% after 2 nights at altitude but was not different from baseline after 12 nights. Hb_{mass} and [EPO] did not increase in SL. Reticulocytes (%) were slightly elevated in ALT at days 5 and 12 (18.9 ± 17.7% and 20.4 ± 25.3%), sTfR was elevated at day 12 (18.9 ± 15.0%), but both returned to baseline by day 20. Hb_{mass} and [EPO] decreased on descent to sea level whilst ferritin increased. The mean increase in Hb_{mass} observed after 11 days (~300 h) of altitude training was beyond the measurement error and consistent with the mean increase after 300 h of simulated Live high:train low altitude. Our results suggest that in elite cyclists, Hb_{mass} increases progressively with 3 weeks of natural altitude exposure, with greater increases expected as exposure persists.

5.2 INTRODUCTION

Erythropoietin (EPO) induced increases in total haemoglobin mass (Hb_{mass}) may partially explain improvements in endurance performance following 4 wks of training at moderate altitude (~2000 - 3000 m) (Levine and Stray-Gundersen 2005). Recent scientific debate has focused on whether improvements in endurance performance following altitude training may also be partially explained by non-haematological adaptations (Gore and Hopkins 2005). Researchers supporting the paradigm that altitude training improves endurance performance by increasing Hb_{mass} have predominately used experimental designs involving measures of Hb_{mass} and performance before and after altitude exposure. Following a comprehensive review of the altitude training literature, Rusko (2004) concluded that 2 weeks of moderate altitude exposure is insufficient to elicit an erythropoietic response, but spending more than 12 hours per day for 3 - 4 weeks at > 2000 m altitude elicits a 3 - 5% increase in Hb_{mass} (Rusko, Tikkanen et al. 2004). It is now common for sport scientists to recommend that athletes spend at least 3 weeks and ideally 4 weeks at altitude, because this dose is believed necessary for accelerated erythropoiesis to occur (Wilber, Stray-Gundersen et al. 2007). However, in those studies that have documented an increase in Hb_{mass} following 3 or 4 weeks of natural altitude exposure, the time course of the Hb_{mass} response was not determined, with measurements performed only pre and post altitude exposure, not during. Thus, whilst four weeks of altitude exposure has been shown to elicit gains in Hb_{mass} (~7 - 8%) of nearly twice that of 3 week sojourns (Wilber, Stray-Gundersen et al. 2007), the minimal duration required to evoke a measureable increase in Hb_{mass} has not been clarified. For elite cyclists, heavy competition schedules can make it difficult to include a 4 week altitude training camp *during* the season. Research documenting the

time course of Hb_{mass} during moderate altitude exposure is therefore important for coaches and athletes who believe haematological changes signify a successful altitude training program, since the time course may reveal the minimum duration of altitude exposure required to obtain measurable haematological benefits.

Recently, Clark et al. (2009) published the time course of Hb_{mass} during 21 days of live high:train low (LHTL) simulated altitude, where an increase of ~1% per week was observed. The athletes in this study were required to spend 14 hours per day at a simulated altitude of 3000 m and accumulated a total of 294 h over a 3 week period. A similar and reproducible time course has been observed in elite runners using a similar LHTL protocol (Robertson, Saunders et al. 2009). Data from natural altitude sojourns suggests a similar time course for adaptation, that is, at least 10 weeks of continuous exposure is needed to achieve optimal haematological adaptation to moderate altitude, assuming an increase in total haemoglobin of ~1.6 g per day or 1% per week (Berglund 1992). To our knowledge, we are unaware of any research that has directly measured Hb_{mass} during a period of *natural* altitude exposure where the total exposure to altitude in hours is often longer than for LHTL. If the time course of the erythropoietic response to altitude is directly related to the hours of exposure (Levine and Stray-Gundersen 2006) it is possible that a faster rate of accelerated erythropoiesis may be observed during natural altitude training compared with simulated LHTL. As previously discussed, conclusions from previous research are based on the comparison of pre- and post-altitude data only, and therefore implicitly assume that the Hb_{mass} response is linear with respect to time. Of course, such a calculation results in an over-simplistic description of the time course, since one expects the initial response to hypoxia to be delayed by several days to allow for the appearance and maturation of extra reticulocytes from the bone marrow (Banfi

2008). However, it remains to be investigated as to how long such a delay persists before measurable increases in Hb_{mass} are detected and whether from that point onward greater haematological adaptations are accumulated with longer periods of exposure.

Data pertaining to the duration of which altitude induced gains in Hb_{mass} persist on descent to sea level are also lacking. A marked and rapid decrease in Hb_{mass} has been observed in Peruvian natives in the first 3 - 7 days following descent from high altitude (Rice, Ruiz et al. 2001), yet altitude induced gains in Hb_{mass} following simulated LHTL have been found to persist for at least one week following cessation of exposure (Clark, Quod et al. 2009; Robertson, Saunders et al. 2009). The time course of Hb_{mass} *following* natural altitude training is of particular relevance for athletes who seek to gain haematological benefits through altitude training, particularly prior to major competition.

The purpose of this study was therefore, to determine, for the first time, the time course of Hb_{mass} changes to natural moderate altitude during, and for 10 days after, a 3 week altitude training camp in elite endurance athletes.

5.3 METHODS

16 internationally competitive cyclists from the same Union Cycliste Internationale (UCI) continental team were recruited to participate in the study, which was approved by the Australian Institute of Sport Ethics Committee. The procedures and risks involved were fully explained to the cyclists before the study began, and written informed consent was obtained. The cyclists were assigned to a sea level or altitude training group, depending on their upcoming race schedule. Nine of the sixteen

cyclists participated in a 3 week natural altitude training camp (Stelvio, Italy, ~2800m); whilst the remaining 7 cyclists were engaged in a training camp with similar training loads near sea level (Kangaroo Valley, NSW ~600 m). All cyclists presented injury and illness free at the start of the study, however three cyclists (1 x altitude, 2 x sea level) were unable to complete the full training camp due to injury or personal commitments and withdrew from the study. The physical characteristics of the remaining cyclists are outlined in **Table 5-1**. Starting one week before, and throughout the duration of both training camps, each cyclist was supplied with oral iron (305 mg ferrous sulphate + 1000 mg Vitamin C) which they were requested to ingest on a daily basis.

In the six months prior to the study, both groups of cyclists were engaged in international road or track-endurance racing. Two weeks prior to the commencement of the study, both groups received a mid-year break (~10-day) and thus were commencing a rebuild phase in preparation for the U23 World Road Cycling Championships at the end of the season. All cyclists were UCI registered, had completed relevant 'whereabouts' documentation and were thus subject to potential random anti-doping controls prior to and throughout the study period.

Table 5-1: Physical characteristics of the cyclists before the start of the training camp

Physical Characteristics	Altitude (n=8)	Sea level (n=5)
Age (years)	20.7 ± 0.4	20.5 ± 1.8
Mass (kg)	65.8 ± 3.7	70.9 ± 8.5
VO_{2peak} ($L.min^{-1}$)	4.9 ± 0.3	5.2 ± 0.3
VO_{2peak} ($ml.kg^{-1}.min^{-1}$)	74.8 ± 3.1	73.8 ± 7.1

Values are mean ± standard deviation

5.3.1 Experimental Design

All 13 cyclists were monitored for a period of 5 weeks which included the three week training camp (**Figure 5-1**). Pre and post intervention measures were performed in the first and fifth weeks. Logistical constraints made it difficult to perform all measures at exactly the same time point in both groups, thus some time points vary by one day.

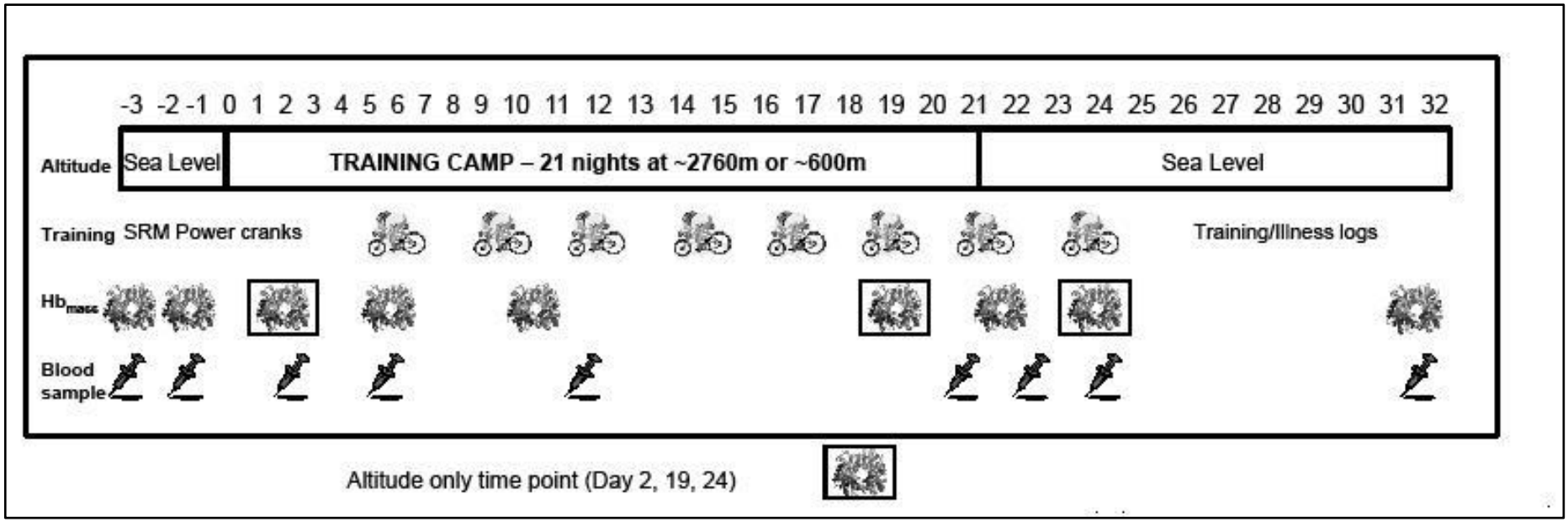

Figure 5-1: Schematic outline of testing and training before, during and after the 3 week altitude or sea level training camp

Note: sea level time points varied by one day due to logistical reasons.

The altitude group participated in a 3 week natural altitude training camp at Passo dello Stelvio, Italy; living at 2760 m and training at 1000 – 3000 m in the surrounding mountains for 2 - 6 $h.d^{-1}$. Whilst training rides included descent to valleys situated at ~1000 m, daily routes encompassed numerous mountain passes, resulting in the majority of ride time > 1800 m. Altitude during each ride was monitored using a Polar Heart Rate Monitor with an on-board altimeter (725S, Polar Electro, Kempele, Finland). Training for the sea level group was conducted at or near sea level in Australia (Kangaroo Valley, NSW and Canberra, ACT). Training schedules for the two groups were designed in a manner to attempt to match total climbing duration within the constraints of the environment. Typically this consisted of 500 - 600 km per week of total training, with frequent low intensity climbing.

Cycling training during the three week training camp was monitored using a mobile power measuring device fitted to each athlete's bicycle (SRM Training System, Professional Version, Schoberer Rad Messtechnik, Julich-Weldorf, Germany). In addition, cyclists were asked to complete a training, illness and injury log on a daily basis, which included perceptual information about the day's training. Commercially available software (TrainingPeaks WKO+ Version 2.2, PeaksWare, Lafayette, Colorado, USA) was used to collate and analyse training data.

5.3.2 Haemoglobin mass

Hb_{mass} was measured using the optimised carbon monoxide (CO) rebreathing method (Schmidt and Prommer 2005). Briefly, a CO dose of 1 ml per kg body weight was administered and rebreathed for 2 minutes through a glass spirometer. Capillary finger tip blood samples (200 μL), for determination of % HbCO, were taken before administration of the CO dose and at 6 and 8 minutes after the initial inhalation of

CO. Blood samples were analysed using a CO-oximeter (OSM3, Radiometer, Denmark) and run in 5 replicates where possible. Hb_{mass} was calculated from the mean change in %HbCO as described previously (Gore, Bourdon et al. 2006) .

Duplicate baseline measures were performed for all cyclists before the start of the training camp. The typical error (TE), calculated from these baseline measures was 1.7% (90% CL: 1.2 – 3.6%) for the altitude group and 2.3% (1.5 – 5.2%) for the sea level group. During the training camp, subsequent Hb_{mass} measures were performed on Day 5/6, 10/11, 19/20. Duplicate measures were performed in the altitude group on return to sea level (Day 22 and 24). The TE calculated from the duplicate measures on return to seal level was 1.7% (1.1-3.2%). A final measure was performed 10/11 days after the end of the training camp, equating to Day 31 in the altitude group and Day 32 in the sea level group (**Figure 5-1**). All Hb_{mass} measurements in the altitude group were performed by the same researcher; however measurements on the sea level group were performed by three experienced researchers at different time points (researcher 1 at D_0 and D_5; researcher 2 at D_{10}, researcher 3 at D_{21} and D_{32}).

An additional measurement was performed on Day 2 of the training camp in the altitude group, to verify that atmospheric pressure differences and/or the transportation of equipment did not adversely affect the method. The short duration of altitude exposure between baseline measures and this time point is insufficient to induce erythropoiesis; therefore any differences observed at this point should be attributed to measurement error. The volume of CO administered during the rebreathing procedure at altitude was corrected for the ~30% decrease in partial

pressure due to the higher altitude in order to maintain a consistent 'dose', resulting in ~30% larger volume for each subject at altitude than at sea level.

5.3.3 Haematology

Resting fasted venous blood samples were collected by trained phlebotomists from a forearm superficial vein under stasis for determination of haematocrit (Hct), haemoglobin concentration [Hb], percent reticulocytes (%retics) and serum erythropoietin concentration [EPO] as well serum ferritin and soluble transferrin receptor (sTfR). All samples were collected in the morning (0630 - 0730) with the cyclists in a seated upright position in accordance with standard UCI protocol. Duplicate measures were collected before the training camp, following the 2^{nd}, $5^{th}/6^{th}$, $10^{th}/12^{th}$ and $20^{th}/21^{st}$ nights of the training camp and twice on return to sea level (**Figure 5-1**). A final sample was obtained 10/11 days after the end of the training camp, equating to Day 31 in the altitude group and Day 32 in the sea level group. A total volume of 67.5 ml of blood was obtained over the 9 samples, equating to ~ 10 g of Hb.

Whole blood was analysed within 8 hours of collection using an ADVIA flow cytometer (Bayer, Tarrytown, NY), with separate analysers in Australia (Canberra for the sea level group) and Italy (Sondrio for the Altitude group). Serum was separated by centrifugation at a speed of 1531 *g* for 5 min and aliquoted into separate tubes for analysis. Serum ferritin and sTfR were determined within 8 hours using a Hitachi 911 biochemistry automatic analyser (Boehringer Mannheim GmbH Limited, Germany) in either the Australian or Italian UCI accredited laboratory. The remaining serum was stored at –80 $^{\circ}$C and analysed as a single batch in Australia. [EPO] analysis was performed using an Immulite automated solid-phase, sequential

chemiluminescent Immulite assay (Diagnostic Product Coorperation, Los Angeles, CA, USA).

5.3.4 Statistical analysis

Data were analysed using a contemporary statistical approach (Hopkins, Marshall et al. 2009). Measured variables were log transformed for analysis to reduce bias arising from non-uniformity of error, and back transformed to obtain changes in means and variation as percents. Mean effects relative to baseline, were estimated using an Excel spreadsheet for standard crossover trials (http://www.sportsci.org/resource/stats/xcrossover.xls). Mean effects of altitude vs. sea level training were estimated using the spreadsheet for standard controlled trials (http://www.sportsci.org/resource/stats/xcontrial.xls). The smallest worthwhile change (SWC) for haematological parameters was derived from Cohen's scale for Effect Sizes (1988) in which a small effect size is ≥ 0.2. The SWC adopted for Hb_{mass} was 1%, consistent with previous of Hb_{mass} changes during simulated LHTL (Clark, Quod et al. 2009).

The magnitude of differences between change scores of Hb_{mass} and haematological parameters is expressed as an effect of time at altitude compared to baseline and the effect of altitude vs. sea level training. The percentage likelihood of the observed differences is expressed using the following descriptors: <1%, almost certainly not; 1-5%, very unlikely; 5-25%, unlikely; 25-75%, possibly; 75-95%, likely; 95-99%, very likely; >99%, almost certainly. The effect was deemed "unclear" if its confidence interval overlapped the thresholds for both positive and negative change.

Linear regression and subsequent correlation analysis (GraphPad PRISM Version 5) were used to determine the relationships between the following parameters in the altitude group: a) % change in [EPO] on ascent (D_2: Δ[EPO]↑) and % change in Hb_{mass} (ΔHb_{mass}↑), b) % change in [EPO] on descent (D_{23}: Δ[EPO]↓) and % change in Hb_{mass} (D_{23}: ΔHb_{mass}↓), and c) % change in serum ferritin 10 days after descent (D_{21} vs. D_{31}: ΔFe) and % change in Hb_{mass} 10 days after descent (ΔHb_{mass}↓↓). The following classification system refined by Hopkins et al 2009 was used to interpret the magnitude of the relationship: trivial 0.0-0.1; small 0.1-0.3; moderate 0.3-0.5; large 0.5-0.7; very large 0.7-0.9; almost perfect 0.9-1; perfect 1.

Data are expressed as the mean and standard deviation (SD) unless otherwise stated. Data in graphs are presented as percent changes from mean of duplicate baseline measures.

5.4 RESULTS

5.4.1 Training

Characteristics of cycling training during the 3 week sea level or altitude camp are described in **Table 5-2**. Based on current recommendations, training duration and intensity during the first three days at altitude were reduced in an attempt to avoid illness and overtraining (Saunders, Pyne et al. 2009), thus training load in the altitude group was lower than the sea level group in week 1. Training was predominantly characterised by low intensity, low cadence climbing, with total time spent in power bands associated with climbing similar in both groups.

Table 5-2: Characteristics of cycling training during 3 weeks of sea level or altitude training

Training Characteristic		Altitude	Sea Level
Distance (km)	*Week 1*	534	586
	Week 2	605	607
	Week 3	548	530
Training Stress Score™ (Arbitrary units)	*Week 1*	800	1334
	Week 2	1197	1080
	Week 3	1085	1176
Intensity Factor™ (Arbitrary units)	*Week 1*	0.59	0.72
	Week 2	0.67	0.60
	Week 3	0.67	0.76
Time in Power Bands (%)	*0-2 w.kg^{-1}*	35	32
	2-4 w.kg^{-1}	46	38
	4-6 w.kg^{-1}	19	24
	>6 w.kg^{-1}	0.5	6

Training Stress Score™ and Intensity Factor™ derived from SRM Power files and analysed using Training Peaks WKO+ Software Version 2.2

5.4.2 Haemoglobin mass

The mean Hb_{mass} and haematological parameters of both groups at baseline (D_0) are displayed in **Table 5-3**. The additional Hb_{mass} measure performed at D_2 (941 ± 68 g) in the altitude group was not substantially different from D_0.

Table 5-3: Hb_{mass} and haematological parameters at baseline

Baseline Value	Altitude (n=8)	Sea Level (n=5)
Hb_{mass} (g)	936 ± 66	993 ± 97
Hb_{mass} (g.kg-^{1})	14.2 ± 1.5	14.0 ± 0.4
Reticulocytes (%)	0.9 ± 0.2	1.6 ± 0.3
[EPO] (mU.ml^{-1})	15.4 ± 4.0	15.9 ± 3.9
Serum Ferritin (ng/mL)	65.9 ± 19.8	110.7 ± 33.3
sTfR (mg.l^{-1})	2.9 ± 0.5	2.4 ± 0.6

Values are the mean of duplicate measures ± standard deviation

The time course of changes in Hb_{mass} observed in response to sea level and altitude training is shown in **Figure 5-2**. At D_{11} the mean increase in Hb_{mass} was 2.9% (90% Confidence Limits: 1.5 - 4.2%) and by D_{19} was 3.5% (1.6 - 5.3%). Compared with the mean of the two baseline measures, Hb_{mass} at both of these time points was "very likely" higher.

On return to sea level, mean Hb_{mass} (duplicate measure) decreased in 6 out of 8 cyclists. Mean Hb_{mass} at D_{23} was 2.0% (0.2 - 3.8%) but was still "likely" higher than at baseline. Following an additional 10 days at sea level, mean Hb_{mass} remained stable (D_{31} = 2.3, 0.6 - 4.1%) and was still "likely higher" than at baseline. In contrast, three weeks of sea level training did not substantially change Hb_{mass} (D_{21}: -0.8, -4.0 - 2.6%) in the sea level group. At D_{32}, mean Hb_{mass} was "possibly" higher than at baseline (1.5%, -8.0 - 3.9%), but remained within the 'noise' of the method.

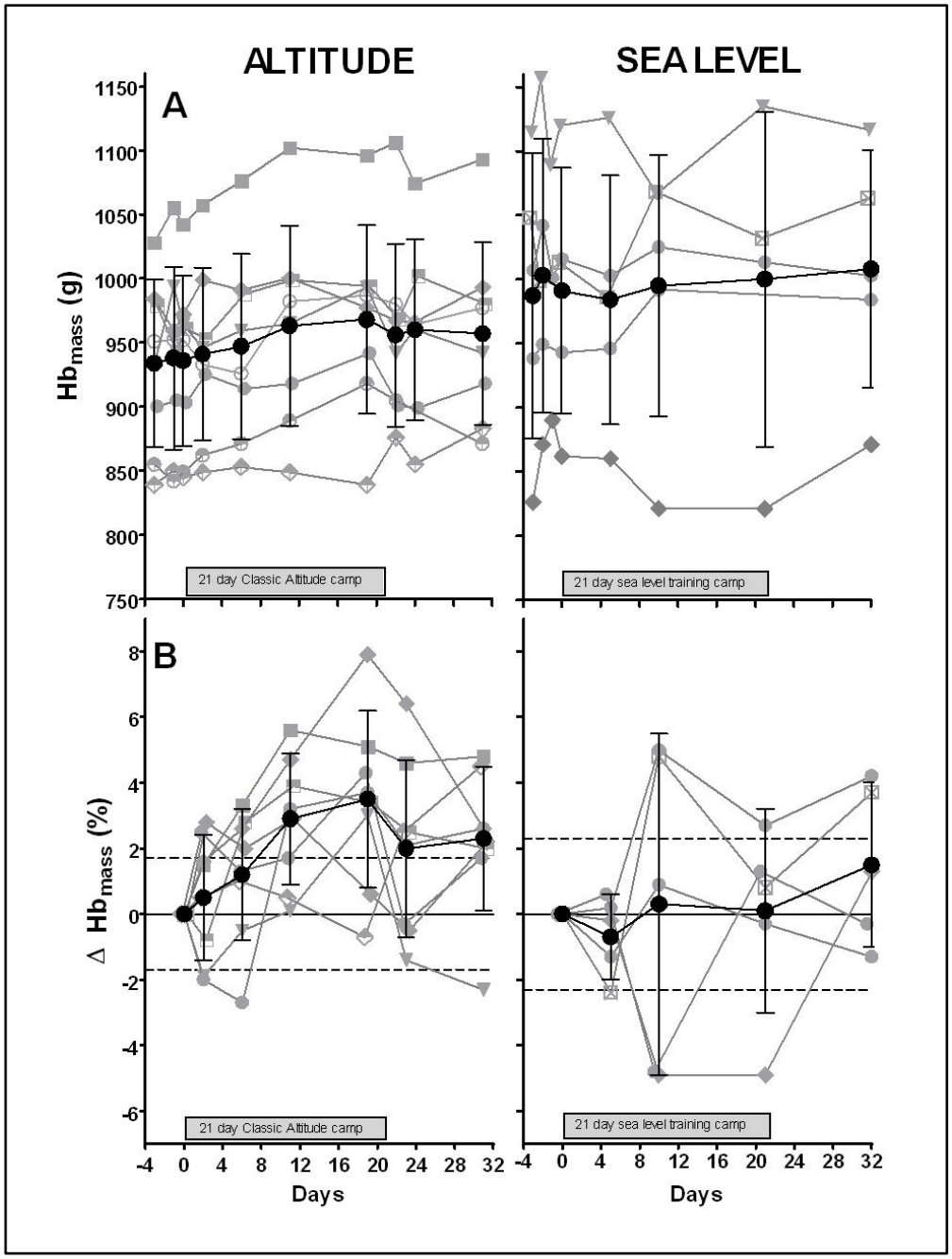

Figure 5-2: Mean and Individual data for **(a)** absolute changes in Hb_{mass}, **(b)** percentage change (Δ) in Hb_{mass} from the mean of two baseline measures following altitude training and sea level training

Dark shaded circle mean ± SD, *light symbols* individual values. Duplicate pre and post measures are displayed for absolute values. Dotted line indicates TE from duplicate baseline measures for each group.

Compared with the sea level group, the mean increase in Hb_{mass} of the altitude group was "possibly" greater at D_{11} (+2.6%, 90%CL = -2.4 – 7.7%) and "likely" to be greater by the end of the altitude period (D_{19}: +3.3%, 0.2 - 6.5%). Ten days following the altitude training period, differences in Hb_{mass} between the two groups were "unclear" (D_{31}: +0.8%, -1.7 - 3.3%).

5.4.3 Haematology

Mean [EPO] increased 64.2 ± 18.8% from D_0 in response to two nights of altitude exposure and was "almost certainly" higher compared to the sea level group (**Figure 5-3**). [EPO] decreased thereafter and was not substantially different from D_0 values by D_{12} at altitude. On descent to sea level, [EPO] decreased in all 8 cyclists, with the mean of duplicate measures 41.1 ± 31.8% below baseline values and "almost certainly" lower. Ten days later, [EPO] increased towards baseline but remained "likely" lower (D_{31}: -22.9 ± 59.6%). [EPO] in the sea level group was variable but did not substantially increase or decrease over the three week training period. The correlation between Δ[EPO] and ΔHb_{mass} was "small", both on ascent to altitude (Δ[EPO]↑ vs. ΔHb_{mass}↑; r=0.16, p=0.70) and on descent to sea level (ΔEPO↓ vs. ΔHb_{mass}↓; r=0.20, p=0.64).

Reticulocytes (%) increased slightly from D_0 during altitude training and were "very likely" higher at D_5 (18.9 ± 17.7%) and D_{12} (20.4 ± 25.3% – relative changes) compared to the mean baseline value and "almost certainly" higher than sea level training at D_5. By D_{21}, % retics were not different from D_0 but were "likely" lower at D_{31}. Due to technical reasons, it was not possible to measure % retics after 2 nights of sea level training. Following 10 nights of sea level training, % retics were "likely"

higher than D_0 (23.8 ± 31.3%) and the effect of altitude versus sea level training was "unclear".

sTfR increased with altitude training and was 18.9 ± 15.0% higher than D_0 at D_{12}. Thereafter, sTfR decreased and was not substantially different from D_0 at D_{21} (11.8 ± 18.6%). Ten days following altitude, sTfR was 6.6 ± 10.2 % lower than baseline and "likely" not beneficial. During sea level training, sTfR was elevated from D_0 only at D_{20} (10.1 ± 5.4%).

After an initial increase (D_2), serum ferritin decreased during the first 12 days of altitude training but by D_{21} the difference from baseline was "trivial" (**Figure 5-3**). On return to sea level, serum ferritin increased in all athletes and was "almost certainly" higher than baseline by D_{31}. There was a "moderate" correlation between ΔFe and ($\Delta Hb_{mass}\downarrow\downarrow$) (r = 0.47, p=0.29) with increases in serum ferritin associated with a decrease in Hb_{mass}. By contrast, serum ferritin decreased with sea level training.

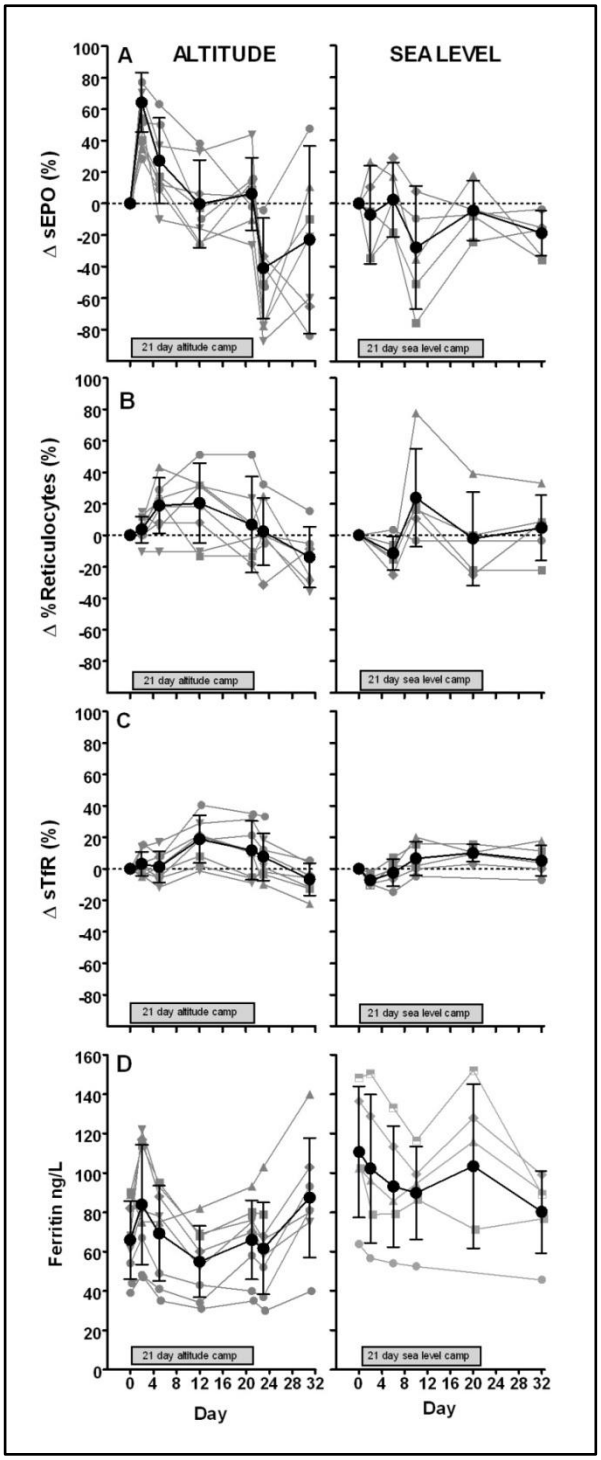

Figure 5-3: Mean and percentage change (Δ) in (a) [EPO], (b) % Reticulocytes (relative changes) and (c) sTfR following altitude and sea level training, (d) Serum ferritin values before, during and after the altitude or sea level training camp

Dark shaded circle mean ± SD, *light symbols* individual values.

5.5 DISCUSSION

5.5.1 Hb_{mass} during natural altitude training

We observed a ~4% increase in Hb_{mass} in elite endurance cyclists following 3 weeks of altitude training at 2760 m; a gain of similar magnitude to that observed by others (Rusko, Tikkanen et al. 2004; Wilber, Stray-Gundersen et al. 2007). This finding supports the recent recommendation that a 'minimum of 3 - 4 weeks of altitude exposure is required for accelerated erythropoiesis to occur' (Wilber, Stray-Gundersen et al. 2007). However, the novel aspect of this study is that we were able to document the time course of the Hb_{mass} response to a *natural* altitude training sojourn, showing that measureable increases in the group mean Hb_{mass} (~3%) could be detected after 11 days of altitude training. The time course of this response was faster than expected based on recently published research. Clark et al. (2009) documented the time course of the Hb_{mass} response during simulated LHTL at a similar moderate altitude (3000 m) and using a somewhat simplistic linear model reported a group mean increase of 1% per week. A similar and repeatable increase in Hb_{mass} in elite runners has been observed during two separate periods of simulated LHTL (Robertson, Saunders et al. 2009) using a similar protocol to that of Clark et al. (2009). The disparity between the results of the present study and the observations during simulated LHTL may lie in the total number of exposure hours accumulated in one week. The simulated LHTL time course is based on an exposure of 14 hours per day, thus it is likely more pertinent to describe time based changes in terms of accumulated hours rather than weeks. One week of simulated LHTL exposure equates to ~100 h, assuming the minimum requirement of at least 12 hours per day is met (Rusko, Tikkanen et al. 2004), and results in a total duration of ~300 h over 3

weeks. In the present study, the nature of natural altitude training resulted in 18 - 22 hours of exposure per day, enabling hours to be accumulated much faster; ~150 h after 7 days, ~300h after 14 days, ~450 h after 21 days. Thus, a similar number of hours at altitude were accumulated after 14 days of altitude training in the present study compared to 21 days of LHTL. In fact, the group mean change in Hb_{mass} observed after 300 h (14 days) of exposure in the present study is similar to that reported by others after 3 weeks (~300 h) of simulated LHTL (Clark, Quod et al. 2009; Robertson, Saunders et al. 2009). In combination, these data suggest that an increase in the group mean Hb_{mass} of 1.0 – 1.2 % per 100 hours of exposure at 2700 – 3000 m may be expected.

The progressive increase in Hb_{mass} observed throughout the altitude training camp is consistent with the notion of a dose-response relationship (Rusko, Tikkanen et al. 2004; Wilber, Stray-Gundersen et al. 2007). Indeed, altitude native cyclists have been shown to possess an 11% greater Hb_{mass} than lowland cyclists (Schmidt, Heinicke et al. 2002) indicating that long term residence at moderate altitude results in greater adaptations even in highly trained athletes. Future research involving regular measurement of Hb_{mass} during several months of exposure may be needed to identify the time course for optimal haematological adaptation in elite athletes. Regardless, the potential benefit of longer exposures relative to non-haematological adaptations to altitude training may also be important and beneficial for performance (Gore and Hopkins 2005; Gore, Clark et al. 2007).

The training status and condition of the athletes prior to altitude exposure may have altered the time course of erythropoietic response. In the present study, the athletes were returning to training following a 10 day mid-season break during which little or

no training was performed. Whilst recent research indicates that Hb_{mass} remains stable over a training year (Prommer, Sottas et al. 2008), a subtle interaction between individual training loads and oscillations in Hb_{mass} has not been ruled out (Garvican, Martin et al. 2010). Drastic losses in Hb_{mass} have been reported following acute injury (Schumacher, Ahlgrim et al. 2008) and data from our laboratory also shows a loss in Hb_{mass} with a sudden drop in training load (Garvican, Martin et al. 2010). Furthermore, a decrease in red cell volume was observed in elite runners following a 7 day 'rest only' taper (Shepley, MacDougall et al. 1992) indicating that sudden changes in training volume may regulate blood volume. If small losses of Hb_{mass} had occurred during the mid-season break, the rapid rise observed during altitude exposure may be indicative of a return to 'erythropoietic homeostasis.' Recommencement of 'normal' training at sea level did not increase Hb_{mass} however, indicating that the observed response was indeed altitude and not training based. With this in mind, altitude exposure at the beginning or middle of the season could potentially be used to expedite adaptations of the oxygen transport system that are acquired from 'base' endurance training (Sawka, Muza et al. 2009).

5.5.2 Hb_{mass} on return to sea level

We observed a 1.5% decrease in Hb_{mass} within 3 days of descent from altitude and which persisted when measured 10 days after descent. Whilst measurement error cannot be discounted, duplicate measures were performed on the first and third day post altitude, with the mean of the two showing a 1.5% drop from values measured on day 19 of the training camp. Further, the same equipment and analyser were used for all measures, despite the change in location, and care was taken to ensure calibration procedures were conducted properly.

The rapid decrease in Hb_{mass} is contrary to that of studies using simulated LHTL, which have described that the resulting increase in Hb_{mass} persists for at least one week following the completion of the hypoxic intervention (Clark, Quod et al. 2009; Robertson, Saunders et al. 2009). The effects of LHTL on Hb_{mass} are however still relatively transient, with a number of studies reporting a return to pre altitude values after 4-6 weeks (Brugniaux, Schmitt et al. 2006; Robertson, Saunders et al. 2009). Similarly, when natural altitude dwellers reside at sea level for sustained durations, a steady reduction in Hb_{mass} has been observed (Prommer, Thoma et al. 2009) suggesting that removal of the altitude stimulus results in an adaptation to the normoxic environment. By contrast, a rapid decrease in Hb_{mass} has been reported on descent from high altitude (Rice, Ruiz et al. 2001), a phenomenon termed neocytolysis, and first described during space flight (Alfrey, Udden et al. 1996). Preferential destruction of young erythrocytes in these instances is believed to be stimulated by a sudden drop in EPO concentration upon descent to sea level, and can be prevented via erythropoietin administration (Rice, Ruiz et al. 2001).

A marked drop in [EPO] was observed in all 8 cyclists, and whilst not as great as the ~80% drop observed by Rice et al (2001), was twice that observed following the cessation of simulated LHTL in the study of Clark et al. (2009). If neocytolysis is driven by changes in [EPO], then the difference in the time course of Hb_{mass} losses following simulated LHTL and the natural altitude training performed in the present study may be explained by the magnitude of the EPO response following exposure. Since the oscillating nature of simulated LHTL involves 'descent' to sea level daily, the stimulus of sustained normoxic exposure may result in a less dramatic fall in [EPO]. Further support for the role of EPO as a mediator of neocytolysis can be seen at the final time point; Hb_{mass} remains stable and is accompanied by a rise in EPO

towards baseline values. It should be noted however, that the magnitude of EPO decrease on return to sea level was weakly related to the magnitude of loss in Hb_{mass}, highlighting the highly variable nature of the EPO response (Garvican, Martin et al. 2007). The time course of serum ferritin in the present study is also indicative of neocytolysis and reduced red cell production. Similar to the observations of Rice et al. (2001), changes in serum ferritin 'mirrored' changes in Hb_{mass}, with serum ferritin levels rising markedly following 10 days at sea level.

The presence of neocytolysis following natural altitude training has potential consequences for the use of natural altitude training, especially in relation to timing of exposure prior to important competition. Future research should more closely examine the time course of changes in Hb_{mass} following both simulated LHTL and natural altitude and whether the onset of neocytolysis is reduced after longer durations of exposure or a more gradual descent to sea level.

5.5.3 Individual variation of Hb_{mass}

In experienced hands, the optimised CO rebreathing method is a robust tool for assessing Hb_{mass} in athletic populations (Prommer, Sottas et al. 2008). The 1.7% TE reported here for the altitude group (attained both before and after altitude exposure), is substantially less than the 2.2% reported in a recent meta-analysis (Gore, Hopkins et al. 2005) and again demonstrates that error associated with the technique does not increase over time (Eastwood, Hopkins et al. 2008). Therefore, based on the 1.7% TE of the method in our hands, the 95% confidence limits of the random error associated with an *individual* percent change in Hb_{mass} = $\sqrt{2}$ x 1.96 x %TE, which equates to ± 3.9% and should be considered when interpreting individual responses. In practical terms, if an athlete in the current study was measured as having a 7%

increase in Hb_{mass} after 19 days of hypoxic exposure, then there is a 95% likelihood that that their 'true' change lies between 3.1 and 10.9 %. Likewise, whilst a 3% measured increase may in fact be as high as 6.9 %, it could also be as low as -0.9 % and should be interpreted with caution. Greater confidence in individual measures can be obtained if duplicate measures are performed in very close succession; with measurement error attenuated by a factor of √2. Thus duplicate measures at critical time points are highly recommended for future research assessing individual Hb_{mass} responses to altitude training. Despite the uncertainty of the individual changes associated with a TE of 1.7%, the mean Hb_{mass} response after 19 days was "very likely" higher than at baseline, with 90% confidence limits of +1.6 to +5.3%, which provides assurance of a real increase in Hb_{mass} following natural altitude training.

5.5.4 Perspectives

The use of the optimised CO-rebreathing method in the present study has enabled a more detailed description of the Hb_{mass} response to natural altitude to be documented. The time course of the mean Hb_{mass} response is typically related to the total hours of exposure, with greater increases expected with longer durations of exposure. In some endurance athletes, natural altitude training camps of ~14 days may be sufficient to induce measureable erythropoietic adaptations, although the longer hypoxic dose of 4 weeks is desirable (Wilber, Stray-Gundersen et al. 2007) and should be strived for where possible in order to achieve greater haematological gains. Whilst short duration altitude sojourns may be more easily incorporated into a competition season and may be useful in early season and the rebuilding phase mid-season to expedite oxygen transport adaptations to endurance training, shorter durations should be used with caution and preferably in conjunction with careful monitoring of Hb_{mass} to

ensure a desirable erythropoietic benefit is achieved. Regardless, the increase in Hb_{mass} following natural altitude training appears quite transient and may follow a different time course to that of simulated LHTL. If altitude induced increases in Hb_{mass} are specifically sought by athletes and coaches, then the timing of altitude training in relation of important competition should be considered carefully.

CHAPTER 6: Seasonal variation of haemoglobin mass in internationally competitive female road cyclists

6.1 ABSTRACT

Purpose: To quantify the seasonal variability of haemoglobin mass (Hb_{mass}) in cyclists during a competitive season, and investigate whether variability is associated with changes in training load or performance. **Methods:** Hb_{mass} was measured in 10 internationally-competitive female road cyclists approximately once per month for 2 - 10 months via CO-rebreathing. Power meters were used to quantify daily load (Training Stress Scores) during training and racing, from which cumulative training load units for 7, 14, 28 and 42 days were calculated. Maximal Mean Power (MMP) for 1, 4, 10 and 25 min, performed during training or racing was used as a surrogate for performance. The relationship between changes in training load (%ΔTraining) and changes in Hb_{mass} (%ΔHb_{mass}), and between %ΔHb_{mass} and changes in MMP (%ΔMMP) was established via regression analysis. **Results:** Individual coefficients of variation (CV) in Hb_{mass} ranged from 2.0% to 4.4%. The weighted mean CV in Hb_{mass} was 3.3% (90% Confidence Limits: 2.9 - 3.8%) or 23 g over the average 6.6 ± 2.3 month period monitoring period. The effect of %ΔTraining on %ΔHb_{mass} was small for 7 d and 14 d (r=0.22 and 0.29), moderate for 42 d (r=0.35) and large for 28 d (r=0.56). The regression slope was greatest for 42 d, with a 10% change in training associated with a ~1% change in Hb_{mass}. The relationship between %ΔHb_{mass} and

%ΔMMP was ~0.5:1 for MMP_{1min}, $_{10min}$ and $_{25min}$ and ~1:1 for MMP_{4min}. **Conclusions:** Hb_{mass} varies by ~3% in female cyclists during a competitive season. Some of the variation may be related to oscillations in chronic training load.

6.2 INTRODUCTION

Research indicates that haemoglobin mass (Hb_{mass}) is a key determinant of VO_{2max} (Schmidt and Prommer 2008), and is relatively stable (~2.1% within subject variation) in recreationally active men over 100 days (Eastwood, Hopkins et al. 2008). The extent to which Hb_{mass} varies throughout a competitive season in elite endurance athletes who train for many hours each day is however, not well established. Although it has been reported that Hb_{mass} remains stable during a 5-day cycling stage race (Schumacher, Pottgiesser et al. 2008), the cumulative effects of months of training and racing may contribute to subtle fluctuations in Hb_{mass} via training induced erythropoiesis (Sawka, Muza et al. 2009). Furthermore, whether any changes in Hb_{mass} are associated with changes in training load or performance remains to be established.

The lack of longitudinal data in athletes has been primarily due to the technique available for measuring Hb_{mass}. Until recently, measurement of Hb_{mass} required nearly an hour of an athlete's time, (Burge and Skinner 1995; Ashenden, Gore et al. 1999; Gore, Rodriguez et al. 2006) a commitment that interfered with routine measurements in elite athletes. The recently validated optimised carbon monoxide (CO) rebreathing technique of Schmidt and Prommer (Schmidt and Prommer 2005; Prommer and Schmidt 2007) provides an improved technique for monitoring Hb_{mass} in athletes because the entire procedure can be completed within 15 minutes.

The performance of elite cyclists in a competitive phase can vary by as little as ~2% (Paton and Hopkins 2005), and an understanding of factors that influence these fluctuations is of critical interest to coaches and athletes striving for world-class performances. It is possible that minor fluctuations (of a few percent) in Hb_{mass} are related to training load which in turn influences the fitness and, possibly, the performance of road cyclists. Consequently, Hb_{mass} may provide a physiological basis for part of the performance changes of elite athletes that are imperfectly modelled as a function of their fitness and fatigue levels (Busso 2003).

The primary aim of the study was to quantify the seasonal variation of Hb_{mass} in a group of internationally-competitive female endurance cyclists. Specifically, we sought to examine the relationship between changes in training load and changes in Hb_{mass}. A secondary aim was to attempt to model the relationship between changes in Hb_{mass} with changes in road cycling performance, with the latter estimated from mean maximal power measured during training or racing.

6.3 METHODS

6.3.1 Subjects

Ten Australian female cyclists were monitored during the 2008 competitive road cycling season. All were members of either the Australian National Team or a UCI registered professional women's team and characterised at the beginning of the season as follows (mean ± SD): age 23.9 ± 4.5 y, height 170.9 ± 5.0 cm, body mass 57.5 ± 5.3 kg, sum of 7 skinfolds 60.2 ± 12.7 mm, VO_{2max} 63.3 ± 5.0 $ml.kg.min^{-1}$. The methodology used to assess the initial physiological and anthropometrical characteristics of the female cyclists has been described previously (Lee, Martin et

al. 2002). For the current research, the incremental exercise test was performed on an electromagnetically braked cycle ergometer (Lode Excalibur, Groningen, The Netherlands), beginning at 125 W and increasing 25 W every 3 min until volitional exhaustion. All procedures used in this study were approved by the Australian Institute of Sport Ethics Committee and subjects provided written informed consent.

Pre-season training (Nov – Jan) and the initial racing period (Jan – Mar) were conducted in Australia. From April to August, training and racing were predominantly based in Europe. The racing calendar consisted of both one-day 'classics' and stage races ranging from 4 - 10 days in duration. Monthly training volume and individual race distances are displayed in **Figure 6-1**. Mean (±SD) monthly training time and distance was 67.8 ± 5.9 h and 1940 ± 264 km, respectively. All athletes were given a mid-season break in late May / early June for 2 - 3 weeks. One athlete presented with a chronic sciatic nerve injury in the second half of the season which prevented her from training and she returned to Australia where her Hb_{mass} continued to be measured.

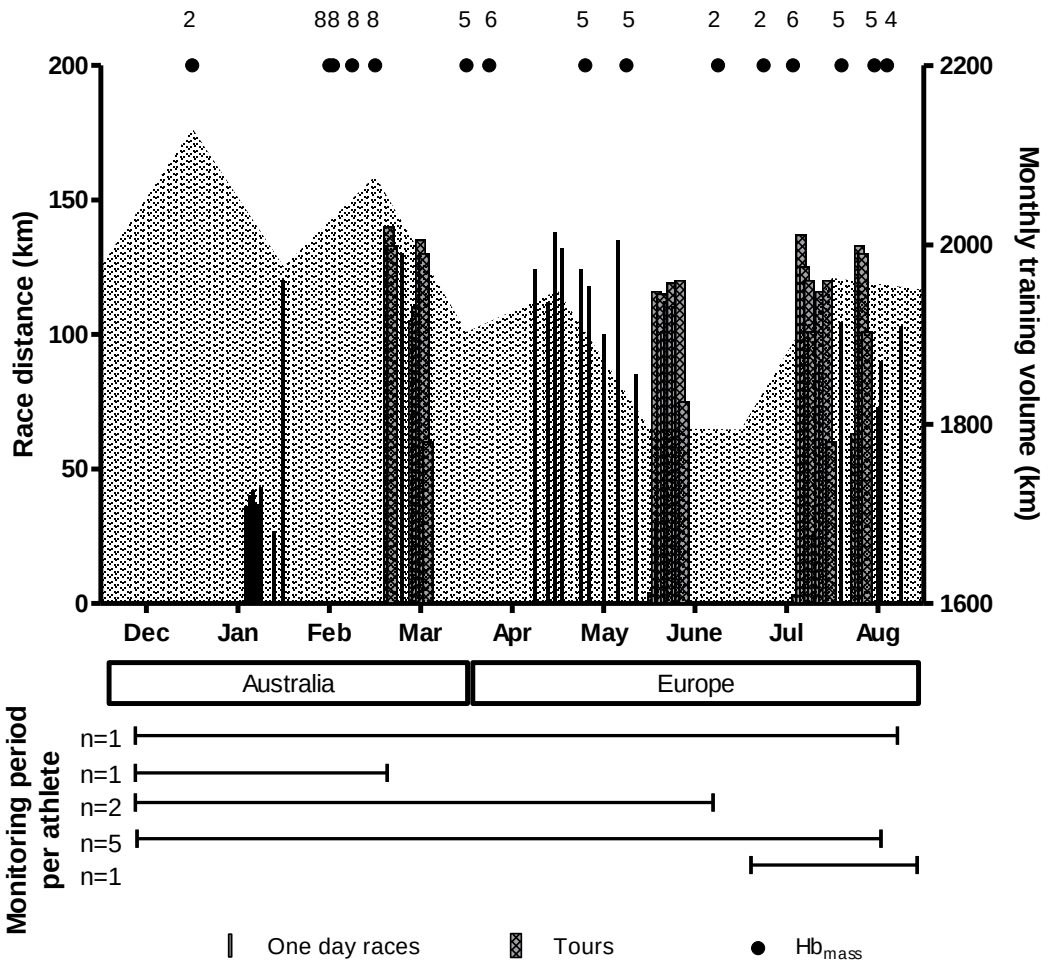

Figure 6-1: Racing and testing schedule for late 2007 to mid-2008

A schematic representation of the study period indicating monthly training volume in km on right Y-axis (shaded area), distance (km) and type of cycling races undertaken (one day races or multi-stage tours) on left Y-axis, the monitoring period of each athlete and the timing of Hb_{mass} measurements (*). Note that not all cyclists were measured on each occasion; the number of athletes measured on each occasion is indicated below each time point.

6.3.2 Haemoglobin Mass

Hb_{mass} was measured using the optimised CO rebreathing technique (Schmidt and Prommer 2005). Briefly, a CO dose of 1.0 $ml.kg^{-1}$ body weight was administered and rebreathed for 2 minutes. Capillary fingertip blood samples, for determination of %HbCO, were taken before the start of the test and at 6 and 8 mins post

administration of the CO dose. Blood samples were analysed a minimum of 5 times using an OSM-3 hemoximeter (Radiometer, Copenhagen). Hb_{mass} was calculated from the mean change in HbCO before and after re-breathing CO. Due to logistical constraints, two separate OSM-3 analysers were used – one in Australia and one in Europe. The inter-analyser standard deviation for physiological values of HbCO is 0.5%, as listed in Table K on page A.2.9 in version 2C of the OSM-3 Hemoximeter Operator's Manual (Radiometer, Copenhagen, Denmark). The same technician performed all measurements of Hb_{mass}, with the exception of one instance in Europe when 4 athletes were tested by another highly experienced technician. The Typical Error (TE) for Hb_{mass} quantification using the 2 min CO rebreathing protocol, based on duplicate measures performed on 8 of the 10 cyclists in February, was 1.8% (90% confidence limits = 1.3 - 2.1%).

Attempts to quantify Hb_{mass} occurred every month, but testing frequency was modified in accordance with the athletes' travel and racing schedules. The monitoring period of each athlete and the timing of Hb_{mass} measures are shown in **Figure 6-1**. The duration of monitoring for each athlete was influenced by their availability and proximity to the National Team. Consequently, monitoring of one athlete ceased following the pre season training and initial racing period due to a change in team, whilst monitoring of her replacement did not commence until later in the European racing block. The minimum duration for monitoring was 2 months with the maximum 10 months. The minimum number of measures performed on any one cyclist was 4 with the maximum number 14. The mean number of measures performed per athlete was 7.9 ± 2.9 over a 6.6 ± 2.3 month period. On two occasions, the timing of Hb_{mass} measurement coincided with the start and finish of a 9-day and a

5-day multi-stage race. Typically, measurements were performed during a 'recovery' day following a loading block.

One athlete spent a 3 week block in simulated altitude (14 $h.d^{-1}$ at 3000 m) prior to one measurement. Her datum point, two days post-altitude was removed from the analysis to avoid altitude acting as a confounding variable. Her next data point was 4.5 weeks post altitude, by which time any residual effect on Hb_{mass} would be negligible (Heinicke, Heinicke et al. 2005; Brugniaux, Schmitt et al. 2006).

4.3.3 Training Load and Performance (Maximal Mean Power)

Training and racing data were recorded on a daily basis using mobile power measuring devices (SRM Training System, Professional Version, Schoberer Rad Messtechnik, Julich-Weldorf, Germany) fitted to each athlete's bicycle. Each SRM crank was calibrated at the beginning of the season using a custom built calibration rig previously described by Gardner and colleagues (Gardner, Stephens et al. 2004). Training monitoring began in late November 2007 and preceded the first Hb_{mass} measurement (**Figure 6-1**).

Power data files were collated and processed in TrainingPeaks WKO+ software (Version 2.2, PeaksWare, Lafayette, Colorado, USA). Daily training load was quantified from cycling power meter data and expressed as a Training Stress Score (TSS) as described by Allen and Coggan (Allen and Coggan 2006). The TSS load unit is analogous to a Banister's training impulse (TRIMP) (Banister and Calvert 1980) as it combines exercise duration and intensity but uses power output instead of HR. More specifically, the TSS score is based on an arbitrary load of 100 which is assigned to a well-paced, maximal 1-hour time trial effort. Highly variable power

output profiles are "normalised" (via an exponential weighting technique used to estimate the average power output for a given duration assuming perfect pacing) and then weighted according to the maximal 1-hour power TSS of 100. As an example, if 300 W can be sustained for 1 h (TSS=100) then a normalised power of 300 W for 30 min would accumulate a TSS of 50.

Cumulative training load units were then calculated from an exponentially weighted rolling average of daily training TSS units using four different time constants – 42 days (6 weeks), 28 d (4 wk), 14 d (2 wk) and 7 d (1 wk) (Allen and Coggan 2006) (*http://www.cyclingpeakssoftware.com/power411/performancemanagerscience.asp*). The different time constants were chosen to reflect time periods long enough to induce a change in fitness (42 d, 28 d) and short enough to examine periods of training responsible for heavy fatigue (e.g. a stage race), or freshness (e.g., following a taper). The software computed a training load value for each day of the monitoring period, via rolling averages, using the TSS scores of the previous 42, 28, 14 and 7 days from any given date. The exponential weighting of these rolling averages places a greater importance on the TSS scores achieved closest to the target date, with the impact of a single training session on the training load unit for a given day greatest for the shorter time constants. For example, a single hard or easy training day will have a relatively large impact on the 7 day training load, but relatively little effect over 42 days. Accumulated training load units were calculated for every relevant day of the monitoring period, thereby allowing an estimate of load to be aligned with Hb_{mass} measured on a particular date (**Figure 6-2**).

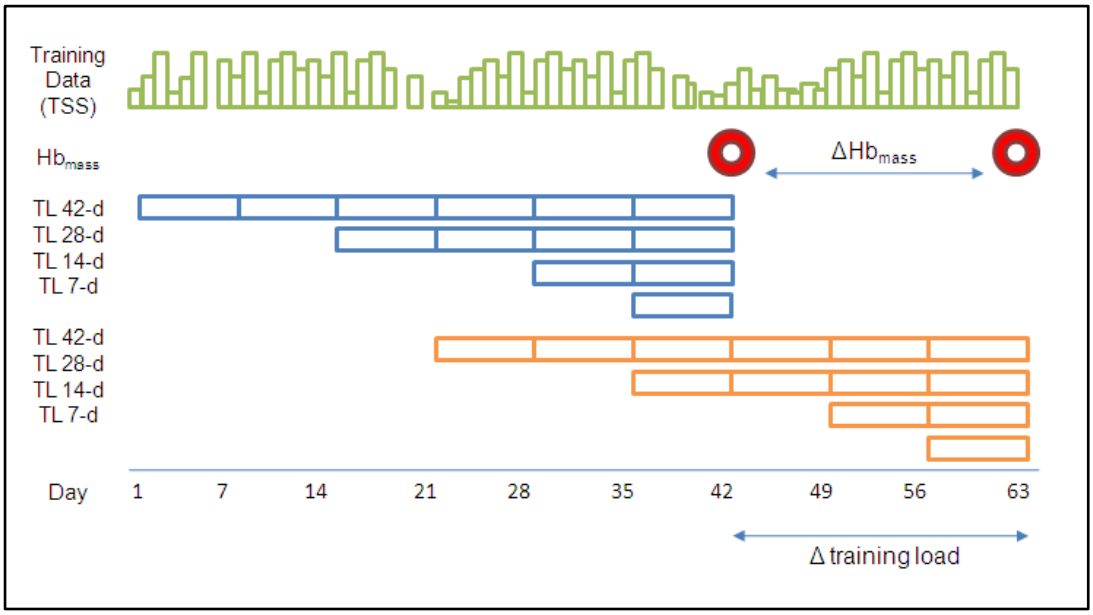

Figure 6-2: A schematic representation of training load quantification in relation to the timing of Hb_{mass} measurement

For simplicity, just two measures of Hb_{mass} are illustrated as well as the corresponding training load (TL) for 7, 14, 28 or 42 d. Changes in training load refer to the difference in training load expressed as a percentage on the dates of two consecutive measures of Hb_{mass}, irrespective of the period between these two measures. Similarly, changes in Hb_{mass} between the two dates can be expressed as a percentage.

In addition to power meter files, training diaries were completed daily which included a reference to training type, duration and distance performed each day. These data served as important reference material that allowed TSS units to be estimated via the software's manual entry function in the instances when the power meter did not record properly. Specifically, there were 127 out of a total of 1178 cases where training logs were used to estimate a TSS for the day. SRM power data from at least 80% of each athlete's monitoring period was required for inclusion in the regression aspect of the study described in the Statistics sub-section. Eight of the 10 cyclists successfully completed daily training logs for the duration of their monitoring period. SRM power data were successfully obtained for 89 ± 5% (range

84 – 100 %) of the monitoring period in 8/10 cyclists, therefore, subsequent regression analysis was performed using only these eight data sets.

Maximal Mean Power (MMP), defined as the highest average power (W) produced for a given duration, was used as a surrogate of cycling performance. MMP for 1-min (MMP_{1min}), 4-min (MMP_{4min}), 10-min (MMP_{10min}) and 25-min (MMP_{25min}) produced during training or racing in the 2 weeks preceding each Hb_{mass} measurement were identified using Training Peak WKO+ software.

6.4 STATISTICAL ANALYSIS

6.4.1 Seasonal Variation

An overall weighted co-efficient of variation based on the sample size for each athlete (Eastwood, Hopkins et al. 2008) was calculated using all Hb_{mass} data points to quantify the within-subject variability of Hb_{mass} in the cyclists throughout the season. Weighted CVs were also calculated for each training load and MMP using the same method.

6.4.2 Correlations: Training Load, Hb_{mass} and Maximal Mean Power

Percent changes in training load, Hb_{mass} and MMP were calculated in series, such that the percent change reflected an increase or decrease from the previous Hb_{mass} measurement and corresponding training load / MMP. For example; if Hb_{mass} measurements were performed two months apart, the percent change in Hb_{mass} from the first of the two measures to the second was calculated, as were the corresponding percent changes in training load and MMP between the date of the first and second measure of Hb_{mass} (**Figure 6-2**). Linear regression analysis was used to evaluate the

relationship between 1) change in training load (%) and change in Hb_{mass} (%) and 2) change in Hb_{mass} (%) and percent change in MMP (1-min, 4-min, 10-min, and 25-min).

Regression analysis was performed for each athlete. Due to differences in sampling number between athletes, a weighted mean slope and correlation coefficient (with their 90% confidence limits) was calculated from the individual slopes and correlations of each athlete. Individual weighting for each subject was attributed as follows:

$$Weight = (a-2) * n / A$$

Where:

a = number of data points for subject,

n = number of subjects and,

A = total number of data points

Effect sizes of correlation coefficients were defined as follows: trivial, 0.0; small, 0.1-0.3; moderate, 0.3-0.5; large, 0.5-0.7; very large, 0.7-0.9; nearly perfect 0.9 and perfect, 1.0 (Hopkins, Marshall et al. 2009). Confidence limits (90%CL) were used to determine ranges in which the true value of an effect size would lie.

6.4.3 Interpretation of the Slope

The slope of the regression line was used to evaluate the training load- Hb_{mass} and Hb_{mass}-MMP relationships. Because the units of both axes are in percent, the slope equals the magnitude of change associated with a one percent change in training load or Hb_{mass}; for instance, if the slope of Hb_{mass} versus MMP = 0.9, then a 1% increase in Hb_{mass} would be associated with a 0.9% increase in MMP.

The slope of the regression line is attenuated by the error of measurement associated with the predictor variable (Hopkins, Marshall et al. 2009). This attenuation is equivalent to a factor equal to the reliability of the intra-class correlation, thus the slope can be adjusted by the reciprocal of the intra-class correlation for which the formula is:

$$Intra\text{-}class\ correlation = (SD^2\text{-}sd^2)/SD^2$$

Where:

SD = between-subject standard deviation for the predictor variable

sd = error of measurement ('noise') in the predictor variable ($\sqrt{2}$*TE)

TE = typical error of measurement (%), which is defined as the within-subject standard deviation of two baseline measures.

6.4.4 Stage racing

Paired t-tests were performed to assess differences in Hb_{mass} before and after multi-day stage races.

6.5 RESULTS

6.5.1 Seasonal Variation

Overall, mean Hb_{mass} for all measures of the 10 Australian internationally-competitive female cyclists studied was 711 ± 86 g (12.3 ± 0.9 $g.kg^{-1}$). Individual coefficients of variation (CV) ranged from 2.0% to 4.4% during the 2 - 10 month periods of training and competition monitored for each athlete. The weighted CV for all Hb_{mass} measures performed (n=79 samples from 10 cyclists) was 3.3% (90%

Confidence Limits: 2.9 - 3.8%) or 23 g over the average 6.6 ± 2.3 month period training and racing. The mean maximal oscillation between the highest and lowest values was 8.5 ± 2.7% (60 ± 14 g). The largest individual oscillation was 89 g and occurred in the athlete whose training load was markedly decreased due to injury.

6.5.2 Training

Cyclists completed an average of 68 ± 6 h of cycling training per month, and an average distance of 1940 ± 264 km. The greatest training volume in hours and distance was recorded in December (75 h, 2130 km) and the lowest in May for hours (60 h, 1796 km) and June for distance (65 h, 1794 km). Mean training loads were as follows: 7 d 107 ± 38, 14 d 114 ± 32, 28 d 108 ± 29, 42 d 112 ± 23 (arbitrary units (Allen and Coggan 2006)). The weighted CV for training load was greatest for 7 d (30.4%, 90%CL: 25.9-37.1%) and smallest for 42 d (13.9%, 90%CL: 11.9 - 16.8%). The weighted CV for training load for 14 d and 28 d was similar, 23.2% (90%CL: 19.9 - 28.1%) and 21.4% (90%CL: 18.3 - 25.9%), respectively. Training loads were highest in February, March and July (7d ≥ 135, 14 d ≥130, 28 d ≥ 120, 42 d ≥ 115) during periods of intensified training or racing and lowest in May or June (7d ≤ 90, 14 d ≤ 90, 28 d ≤ 100, 42 d ≤ 100).

The mean MMPs and weighted CVs were as follows: $MMP_{1min:}$ 382 ± 58 W (11.5%; 90%CL: 9.8 - 14.1%), $MMP_{4min:}$ 307 ± 33 W (6.7%; 90%CL: 5.7 - 8.2%), $MMP_{10min:}$ 273 ± 30 W (7.4%; 90%CL: 6.2 - 9.0%) and $MMP_{25min:}$ 239 ± 28 W (8.8%; 90%CL: 7.5 - 10.9%).

6.5.3 Training load and Hb_{mass}

The relationship between changes in training load and changes in Hb_{mass} is shown in **Figure 6-3**. A small effect of training load was found for 7 and 14 day time constants (r = 0.22 and 0.29, respectively), a moderate effect for 42 d (r =0.35) and a large effect for 28 d (r=0.56), while the true value of the effect might lie between 0.55 and 0.57 for 28 d.

Although the relationship between training load and Hb_{mass} was strongest for the 28 d time constant, the weighted mean slope was greatest for the 42 d training time constant (Slope, (Lower-Upper 90%CL)): 7 d: 0.009 (0.008-0.010), 14 d: 0.066 (0.065-0.068), 28 d: 0.076 (0.072 - 0.079), 42 d: 0.114 (0.105 - 0.124), which reflects a ~1% increase in Hb_{mass} for a 10% increase in the 42 d average training load or a ~0.8% increase in Hb_{mass} for a 10% increases in the 28 d average training load.

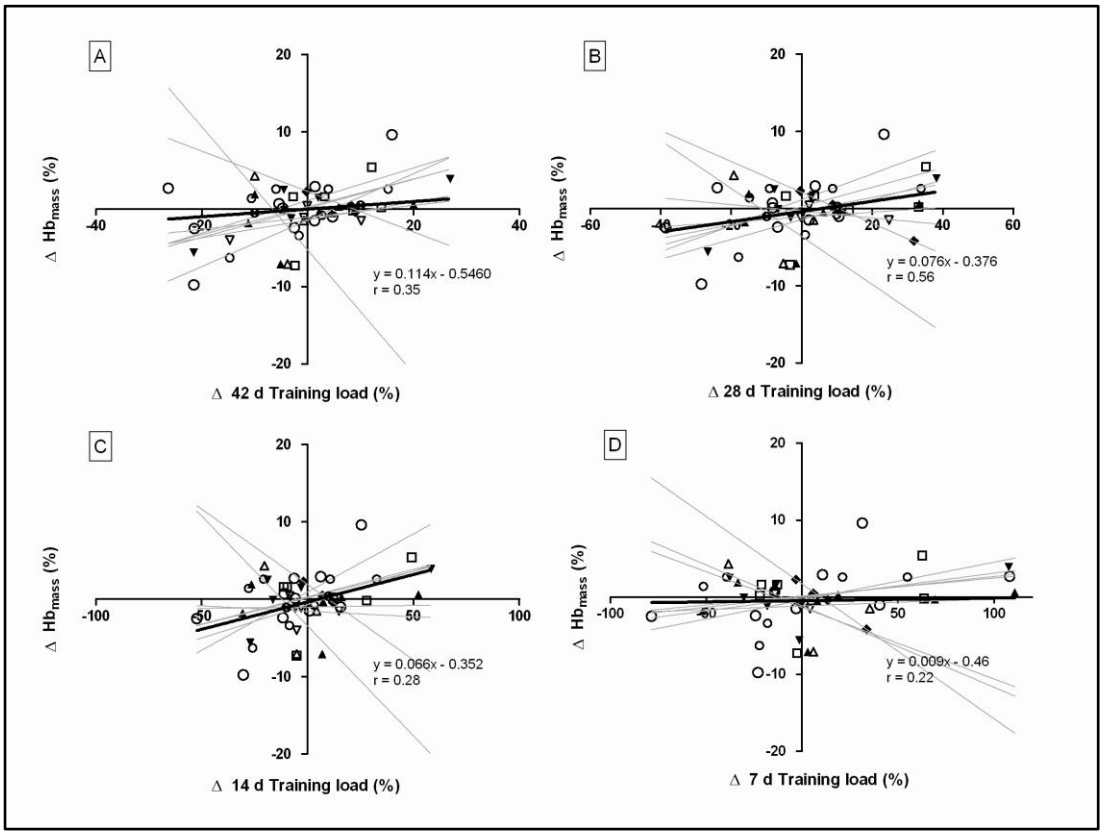

Figure 6-3: The relationship between changes in training load and changes in Hb_{mass}

Data are % changes from the preceding measurement. Panels show regression analysis for four different time constants of training; A: 42-day, B: 28-day, C: 14-day, D:7-day. Regression lines for each athlete are shown with the solid line indicating the weighted mean slope of all athletes (n=8). Different symbols are used for each individual athlete's data

6.5.4 Hb_{mass} and Mean Maximal Power

The relationship between changes in Hb_{mass} and changes in MMP is shown in **Figure 6-4**. There were small effects for MMP_{10min} and MMP_{25min} (r=0.29 and 0.28, respectively) with large effects for MMP_{1min} and MMP_{4min} (r=0.57 for both). The relationship was ~0.5:1 for MMP_{1min}, MMP_{10min} and MMP_{25min} (Slope, (Lower - Upper 90%CL)): MMP_{1min}: 0.436 (0.069 - 0.938), MMP_{10min}: 0.491 (0.442 - 0.541), MMP_{25min}: 0.607 (0.546 - 0.672) and ~1:1 for MMP_{4min}:1.029, (1.006 - 1.053). If attenuation of the slope due to noise in the predictor variable is considered, this

relationship may be ~1:1 for most cases (non-attenuated slope: MMP_{1min}: 0.793, MMP_{4min}: 1.871, MMP_{10min}: 0.935, MMP_{25min}: 1.173).

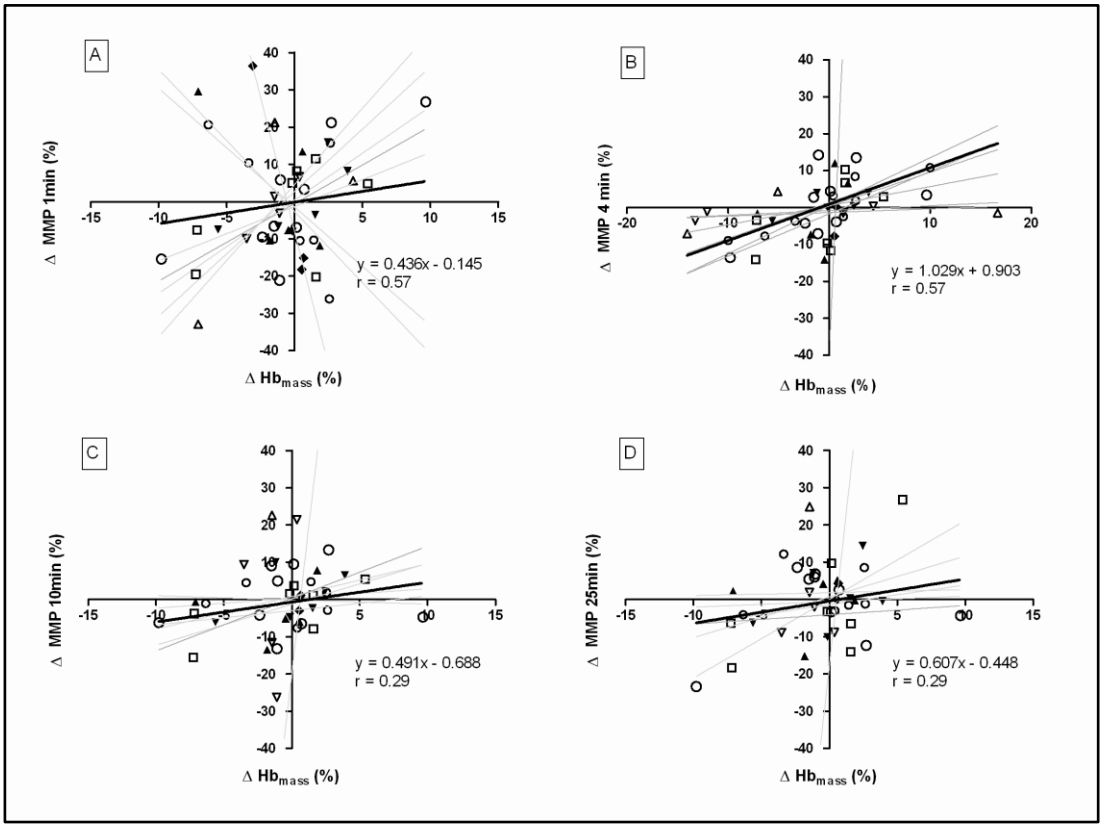

Figure 6-4: The relationship between changes in Hb_{mass} and changes in Maximal Mean Power of different durations

Data are % changes from the preceding measurement. Panels show regression analysis for four different power durations; A: MMP_{1min}, B: MMP_{4min}, C: MMP_{10min}, D: MMP_{25min}. Regression lines for each athlete are shown with the solid line indicating the weighted mean slope of all athletes (n=8). Different symbols are used for each individual athlete's data.

6.5.5 Stage racing and Hb_{mass}

The mean change in Hb_{mass} following stage racing was less than 2% (~15 g). More specifically, mean (±SD) Hb_{mass} was 691 ± 118 g before and 683 ± 115 g after a 9-day stage race (% change = -1.1 ± 0.7%, n=4, p=0.05) and 712 ± 68 g before and 725 ± 68 g after a 5-day stage race (% change = 1.8 ± 2.2%, n=3, p=0.31). During the 5 d

stage race, 3 cyclists who were part of the same team did not race and completed normal training. Hb_{mass} for these cyclists before and after the same 5 d period was 703 ± 108 g at the start and 701 ± 110 g at the end (% change = -0.4 ± 0.6%, n=3, p=0.48).

6.6 DISCUSSION

The main finding of the present study is that Hb_{mass} varies by ~3 % in a group of internationally-competitive female cyclists during a competitive season. This fluctuation may be partly related to changes in training load, as a 10% change in training load over 6 weeks was associated with a 1% change in Hb_{mass}.

6.6.1 Stability of Hb_{mass} during a season

In an attempt to prevent doping in cyclists, the concept of a 'biological passport' has been adopted by the International Cycling Union (UCI). Normal variation in a range of important blood parameters is profiled to allow for abnormal variations due to doping practices to be detected (Malcovati, Pascutto et al. 2003). Hb_{mass} has been suggested for inclusion into the passport (Prommer, Sottas et al. 2008) and for this reason, normative data describing the magnitude of natural fluctuations of Hb_{mass} in non-doping elite athletes are important.

In a small sample of internationally-competitive female cyclists, we report Hb_{mass} to be relatively stable to within 3.3% over a 6 month competitive season. Based on our data, an individual female cyclist with a Hb_{mass} of 700 g would have an expected 95% normal variation of ± 3.3 x 1.96 = ± 6.5% (or ± 45 g) throughout a season, with values ranging between 655 and 745 g. In our data set, there is only one instance out

of 79 measures when Hb_{mass} exceeds ± 6.5% of the athlete's mean value. The relative stability of Hb_{mass} is consistent with the findings of Prommer et al (2008) who also report Hb_{mass} in a group of endurance athletes to be stable over a one year training period, with individual oscillations less than 7%.

Variation in Hb_{mass} during a competition season could arise from a variety of sources, both methodological and biological. Prommer et al (2008) conclude that "methodological noise" is primarily responsible for the individual oscillations observed over a training year. The TE of the CO rebreathing method in the current study was 1.8%, in line with the ~2% previously reported (Gore, Hopkins et al. 2005), yet it should be appreciated that this results in 95% confidence limits for a change in Hb_{mass} between two measures of $\pm 1.96 \times \sqrt{2} \times 1.8\%$, or ± 5.0%. However, there still remains some uncertainty as to what component of a TE is biological variation and what is measurement error (Gore, Hopkins et al. 2005). In addition, it cannot be discounted that inter-analyser error arising from the use of two OSM-3 analysers may account for a substantial fraction of the overall weighted CV. Fortunately, when comparing the changes in Hb_{mass} over time (as in the regression analysis) the effect of two analysers is attenuated because there are only 9 instances out of 79 measures when 2 measures from 2 different analysers are compared *in series*. In future, careful inter-analyser validation and quality assurance protocols, e.g. the use of three known standards of %HbCO, should be adopted to limit this source of uncertainty. Unfortunately, such standards are not available from Radiometer, the manufacturer of the OSM-3. Regardless, even if all of our typical error (1.8%) is methodological noise, this is substantially less than the 3.3% seasonal variation that we observed during 6 months of training and racing.

The effect of the daily training environment on the stability of Hb_{mass} is unclear. Prommer et al. (2008) report no significant effects of training on Hb_{mass} throughout a season, but do not "completely exclude the influence of training." Interestingly, the greatest variation in mean Hb_{mass} from baseline in Prommer's study was observed in October at the start of the off-season – when variation in training load was also greatest. Quantification of training load in the study of Prommer et al. was based on the number of hours of training per week in the 3 months preceding each measurement, and whilst sufficient to categorise the phases of a training year (off-season, training and competition phases), lacks the sensitivity required to detect subtle oscillations in training cycles which may be of physiological importance for Hb_{mass} and performance. The use of power meters and more frequent measurement of Hb_{mass} in the present study, attempted to provide a more sophisticated approach to a complex problem. Indeed, the slightly higher individual oscillations observed in our study may be reflective of the frequency and timing of Hb_{mass} measurements in relation to training load.

6.6.2 Hb_{mass} and Training load

Changes in training load were associated with changes in Hb_{mass} when training load (TSS unit) was expressed as a 28 d and 42 d rolling average. The regression model indicates that increasing training load by ~22% over 6 weeks (2SD) could increase Hb_{mass} by 2.5%, equating to an average increase of 0.4% per week. Similarly, an increase in training load of 36% (2SD) over 4 weeks may result in an increase of 2.7% in Hb_{mass} (~0.7% per week).

Conversely, the model also predicts a decrease in Hb_{mass} following a decrease in training load. A 14% decrease in Hb_{mass} has recently been reported in a female cyclist

following inactivity due to surgery (Schumacher, Ahlgrim et al. 2008); but Hb_{mass} was recovered over 8 weeks at an average rate of 1.8% per week when rehabilitation and subsequent sport-specific training were resumed. In the present study, the regression model predicts that a 100% increase (or decrease) in 6 week training load would yield a 11.4% increase (or decrease) in Hb_{mass}, ~ 1.9% (~14 g) per a week; a magnitude not too dissimilar from the changes reported by Schumacher et al. (2008).

In elite endurance cyclists, increases of 10-20% in training load over a 6 week training block can occur during periods of intensified training and / or (stage racing). In the two instances when Hb_{mass} was measured before and after stage racing, when training load was increased, subtle changes of < 2% were observed (1.3% after 9-day stage race, 1.8% after 5-day stage race). These fluctuations are within measurement error (TE = 1.8%) and are consistent with reports that Hb_{mass} does not notably change following stage racing (Schumacher, Pottgiesser et al. 2008). However, the increased training load arising from multiple stage races completed in short succession (over 1 month), may provide a stimulus for erythropoiesis (Sawka, Muza et al. 2009) which does not manifest in a detectable increase in Hb_{mass} (> TE of the method) until 4 - 6 weeks later. Nevertheless, our data suggests that sustained increases in Hb_{mass} of > 5%, without the confounding influence of altitude training (Levine and Stray-Gundersen 1997) would exceed the realm of normal training induced fluctuations in already highly endurance trained athletes since this would require dramatic increases in training load over 6 weeks of > 50%.

In contrast, in our model, changes in training load over 1 – 2 weeks had little effect on Hb_{mass}, with even changes in the order of 83% (2 SD) over one week associated with a change in Hb_{mass} of just 0.8% (~6 g). The acute effects of large changes in

training load over a week on Hb_{mass} are relevant to situations where training is disrupted, for example during travel, mild sickness / injury or a taper, yet our modelling highlights the robustness of Hb_{mass} in these instances. Further research is warranted to establish the impact of severe illness or injury on Hb_{mass}.

6.6.3 Hb_{mass} and Maximal Mean Power

A relationship between changes in Hb_{mass} and changes in MMP of different durations was observed, with a 1% change in Hb_{mass} associated with a 1% change MMP_{4min}. Power output over four minutes has been identified as a key determinant of performance in Women's World Cup cycling races (Ebert, Martin et al. 2005), and thus changes of as little as 1% in this trait are deemed "worthwhile" (Paton and Hopkins 2005).

If maximal cycling power over ~4 min is limited by endurance capacity then even subtle changes in Hb_{mass} could be of physiological importance. Following altitude training, increases in performance have been directly attributed to increases in VO_{2max} via increases in Hb_{mass} (Levine and Stray-Gundersen 2005). Whilst this causal relationship has been debated in the context of altitude training (Gore and Hopkins 2005), a high correlation between Hb_{mass} and endurance capacity is well established (Kanstrup and Ekblom 1984; Schmidt and Prommer 2008) and has been confirmed by manipulation of Hb_{mass} in humans (Parisotto, Gore et al. 2000; Prommer, Heckle et al. 2007). Erythropoiesis stimulated by cycling training (Sawka, Muza et al. 2009) may therefore partially explain improvements in MMP through a variety of mechanisms including an enhanced oxygen supply (Wagner 1996).

In our model, the relationship between Hb_{mass} and MMP becomes of increasing physiological importance with greater changes in Hb_{mass}; e.g. a 3% increase in Hb_{mass} (~ 21g) could increase MMP_{4min} by 9 – 17 W. Such changes, both in Hb_{mass} and MMP, are attainable via natural training adaptations and present an appealing case for optimising Hb_{mass} through training. However, given that changes to cycling performance are multi factorial, our observation of a relationship between Hb_{mass} and MMP is likely to be somewhat coincidental rather than the direct and sole determinant of performance, as illustrated by the fact that each raw correlation coefficient is < 1. It is possible that changes in Hb_{mass} merely reflect the completion of an adaptive phase in the athlete, resulting in the accumulation of a number of proteins, including haemoglobin. Increases in Hb_{mass} would therefore not directly mediate changes in MMP, but rather indicate that other adaptations have occurred. Indeed improvements in 4000 m individual pursuit performance have been observed without concomitant changes in Hb_{mass} (Gore, Hahn et al. 1998), highlighting that other training adaptations, e.g. changes to skeletal muscle morphology, acid-base status and fuel supply, must also be contributing to improvements in cycling performance (Hawley and Stepto 2001). Hb_{mass} is therefore just one of many training adaptations and its relative contribution to cycling performance is yet to be fully elucidated.

6.6.4 Limitations

One of the primary limitations of this study is the lack of a ‘true’ performance test. Indeed, our method for monitoring cycling fitness using MMPs detected during normal racing and training departs from conventional measurements of cycling performance that are typically quantified in the laboratory during a maximal effort.

Instead, performance indices (MMP) were reliant on the efforts given during racing and training, with an important assumption being that the athlete actually produced a maximal effort over the durations of interest during the two weeks preceding a Hb_{mass} measurement. We have observed a close association between MMPs produced in the field and maximal effort MMPs produced in a controlled laboratory environment. Specifically, MMPs produced by elite male U23 cyclists during an international stage race were not different and within 1% of those produced in maximal laboratory efforts prior to racing (Quod, Martin et al. 2010). In the present study, the weakest relationships between Hb_{mass} and MMP were observed for 10 and 25 min indicating that these efforts may be more greatly influenced by factors other than Hb_{mass}, not least of which the psychological / motivational component of how they are performed. That is, we cannot discount that the weaker relationships could be due, in part, to the intent with which these efforts were performed – maximal 4 min efforts are more common in training and racing than 10 and 25min efforts.

Another key limitation lies in the inability to fully estimate 'training load.' In the present study, only training performed on the bicycle was included in the calculations using data collected from power meters. Many other factors including nutrition, resistance training, travel and sleep may contribute to an athlete's overall training load and thus may also influence Hb_{mass} and performance. Physiological parameters directly related to erythropoietic stimulation, such as erythropoietin concentration and arterial oxygen saturation, were not measured during training in the present study, and in future could shed light on the impact of different training modalities on Hb_{mass}.

The present study is unique in its sole involvement of female athletes. It could be postulated that oscillations in Hb_{mass} may be attributed to menstrual blood loss, which was not quantified in the present study. However, such losses are likely to be too small to influence Hb_{mass} based on the error associated with the CO-rebreathing method. Menstrual blood loss can average 35 ml with a typical range from 10 - 80 ml (Fraser, Warner et al. 2001), and even with moderately heavy blood loss (> 60 ml) only ~ 9 g of Hb would be lost over one week. An average of 5 g (< 1%) is more likely in women with normal menstrual flow (assuming a [Hb] of $15g.dL^{-1}$). Changes of this magnitude are well within the TE for determining Hb_{mass}, which is ~2.2% (Gore, Hopkins et al. 2005) or 1.8% in this study.

6.7 CONCLUSION

On average, Hb_{mass} varies by 3.3% in internationally-competitive female cyclists during a competitive season. It is possible that some of the variation is related to variation in chronic training load. Additionally, changes in Hb_{mass} were associated with changes in MMP 1, 4, 10 and 25 min. This observation suggests that Hb_{mass} changes should be considered in addition to other physiological adaptations arising from changes in training load when changes in performance are analysed.

CHAPTER 7: The importance of haemoglobin mass for increases in cycling performance induced by simulated LHTL

7.1 ABSTRACT

We sought to determine whether improved cycling performance following 'Live High-Train Low' (LHTL) occurs if increases in haemoglobin mass (Hb_{mass}) are prevented via periodic phlebotomy during hypoxic exposure. Eleven, highly-trained, female cyclists completed 26-nights of simulated LHTL (16 $h.d^{-1}$, 3000 m). Hb_{mass} was determined in quadruplicate before LHTL and in duplicate weekly thereafter. After 14-nights, cyclists were pair-matched, based on their Hb_{mass} response (ΔHb_{mass}) from baseline, to form a response group (Response, n=5) in which Hb_{mass} was free to adapt, and a Clamp group (Clamp, n=6) in which ΔHb_{mass} was negated via weekly phlebotomy. All cyclists were blinded to the blood volume removed. Cycling performance was assessed in duplicate before and after LHTL using a maximal four-minute effort (MMP_{4min}) followed by a ride time to exhaustion test at peak power output (T_{lim}). VO_2peak was established during the MMP_{4min}. Following LHTL, Hb_{mass} increased in Response (Mean ± SD, 5.5 ± 2.9%). Due to repeated phlebotomy, there was no ΔHb_{mass} in Clamp (-0.4 ± 0.6%). VO_2peak increased in Response (3.5 ± 2.3%) but not in Clamp (0.3 ± 2.6%). MMP_{4min} improved in both groups (Response 4.5 ± 1.1%, Clamp 3.6 ± 1.4%) and was not different between groups (p=0.58). T_{lim} increased only in Response, with Clamp substantially worse than Response (-37.6 %;

90%CL -58.9 to -5.0, p=0.07). Our novel findings, showing a ~4% increase in MMP_{4min} despite blocking a ~5% increase in Hb_{mass}, suggest that accelerated erythropoiesis is not the sole mechanism by which LHTL improves performance. However, increases in Hb_{mass} appear to influence the aerobic contribution to high-intensity exercise which may be important for subsequent high-intensity efforts.

7.2 INTRODUCTION

Maximal aerobic power (VO_2max) of elite athletes can be limited by maximal oxygen supply to working muscles (Wagner 1996). Therefore, changes to systemic oxygen transport via alterations to blood volume (BV) and haemoglobin concentration ([Hb]) can have significant implications for VO_2max and potentially endurance performance (Gledhill, Warburton et al. 1999). Erythrocythemia, induced either by red cell infusion or erythropoietin administration, has been shown repeatedly to increase both VO_2max and endurance performance (Berglund and Hemmingson 1987; Brien and Simon 1987; Ekblom 1996), whilst conversely, reducing oxygen transport capacity via blood loss or partial blocking of haemoglobin by carbon monoxide has detrimental effects for VO_2max and performance (Ekblom and Huot 1972; Kanstrup and Ekblom 1984).

With the benefits of increasing oxygen transport capacity so evident, it is of no surprise that many elite endurance athletes seek to increase their total haemoglobin mass (Hb_{mass}) via hypoxic exposure. An increase in Hb_{mass} in response to both normobaric and hypobaric hypoxia has been documented on many occasions (Levine and Stray-Gundersen 1997; Clark, Quod et al. 2009; Robertson, Saunders et al. 2010), although changes are somewhat lower in magnitude to those possible via

blood doping (Gledhill, Warburton et al. 1999; Schmidt and Prommer 2010). Today, Live high: Train Low (LHTL) (Levine and Stray-Gundersen 1997) has become a popular training strategy for elite endurance athletes due to the widespread paradigm that small gains in Hb_{mass} of approximately 5% arising from hypoxic exposure are responsible for increases in high-intensity endurance performance via increases in VO_2max (Levine and Stray-Gundersen 1997; Levine and Stray-Gundersen 2005).

The complex response cascade initiated by a lower partial pressure of oxygen in hypoxia may also result in non-haematological adaptations, such as improved muscle efficiency (Saunders, Telford et al. 2004) and greater muscle buffering capacity (Gore, Hahn et al. 2001), which may be equally as important for sea level performance as an increase in Hb_{mass} (Gore and Hopkins 2005; Gore, Clark et al. 2007). The relative importance of haematological and non-haematological adaptations to hypoxia for endurance performance has, in the past, been difficult to discern since both may occur concomitantly. However, in many cases, a hypoxia-induced Hb_{mass} response may not be detectable, because the hypoxic dose is insufficient (Levine and Stray-Gundersen 2006), or possibly because the athlete has limited potential for adaptation (Gore, Hahn et al. 1998). Nevertheless, "worthwhile" performance enhancements are still reported after hypoxic exposure of various durations (Bonetti and Hopkins 2009), leading some researchers to question the sole dependence of performance improvements following LHTL on increases in Hb_{mass} (Gore and Hopkins 2005).

The aim of the present study was to investigate the importance of increases in Hb_{mass} for cycling performance following simulated normobaric LHTL. We employed, a 'subject-blind' design, by removing any Hb_{mass} gained throughout the period of hypoxic

exposure and, thereby, effectively "clamping" the Hb_{mass} response. If performance gains are still observed despite a Hb_{mass} "clamp", this would support the concept of a non-haematological component to performance enhancement following LHTL (Gore and Hopkins 2005; Gore, Clark et al. 2007).

7.3 METHODS

Fourteen, highly-trained female cyclists were monitored during a 6 week training camp consisting of 26 nights of simulated normobaric LHTL. Cyclists were pair-matched after 14 nights based on their change in Hb_{mass} from baseline and randomly assigned to either a 'Response' group in which Hb_{mass} was free to adapt, or a 'Clamp' group in which any increase in Hb_{mass} was negated via repeated phlebotomy to ensure Hb_{mass} for each cyclist remained equal to baseline throughout LHTL. Both groups trained together near sea level (Canberra, ~600 m ambient altitude) and were tested under normoxic conditions at the Australian Institute of Sport (AIS) before and after the LHTL period. The study was approved by the AIS Human Ethics committee and all cyclists provided written informed consent before participating.

7.3.1 Study Design

The study design is outlined in Figure **7-1**. Prior to simulated hypoxic exposure, all cyclists completed baseline testing consisting of: four Hb_{mass} measurements, one incremental graded exercise test to exhaustion for determination of Peak Power Output (PPO) and two dual-stage cycling performance tests (see Performance Tests sub-section for details). Following baseline testing, the athletes began a 26 night LHTL protocol, sleeping at simulated normobaric altitude of 3000 m and training at ~600 m. Simulated normobaric hypoxia was created via nitrogen dilution using a

purpose–built, five-bedroom facility at the AIS. The cyclists were required to spend at least 14 $h.d^{-1}$ inside the facility and to record their hours of exposure in a log book. All cyclists were supplemented with oral iron daily (305mg ferrous sulphate), commencing two weeks prior to and throughout LHTL in an attempt to establish equal preconditions and to prevent bias arising from iron deficiency. In addition, an iron screen (ferritin) was performed prior to inclusion in the study.

Performance testing was performed again on completion of the LHTL (Day 27 - 30) and involved all athletes completing the full performance testing protocol on two occasions, separated by one day (**Figure 7-1**).

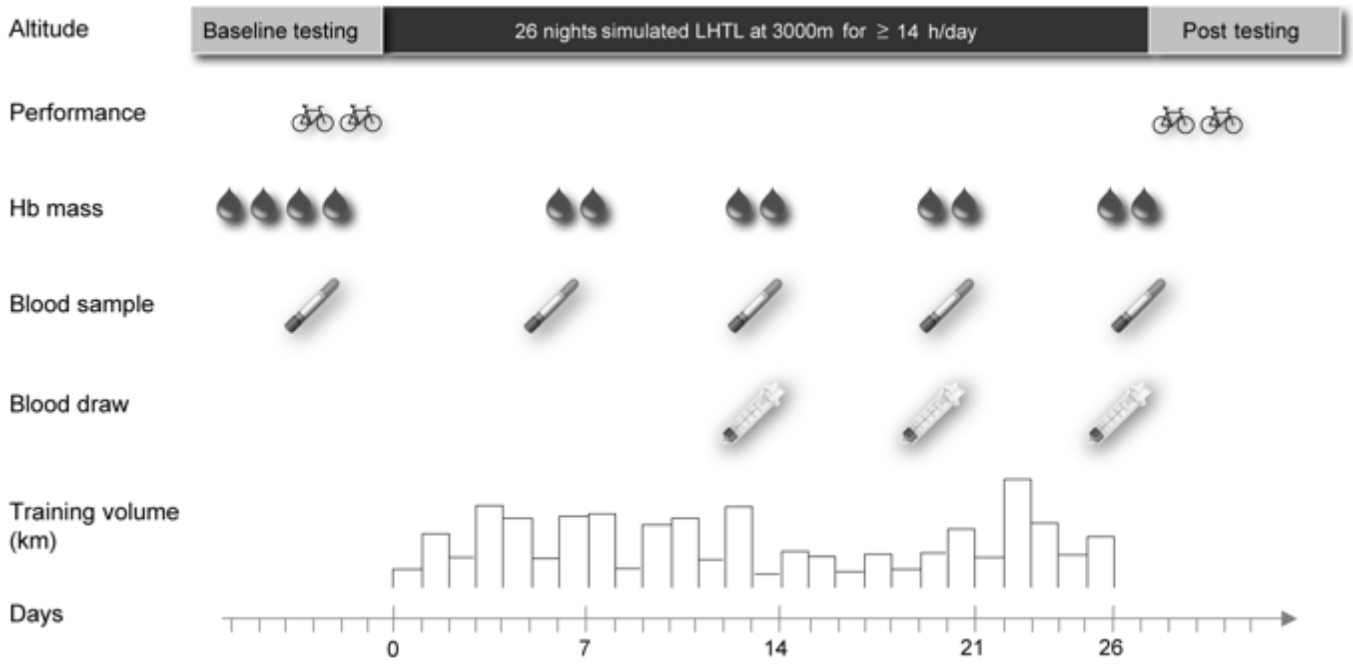

Figure 7-1: Schematic outline of the study design illustrating the timeline (days) of Live High/Train Low (LHTL) hypoxic exposure (Altitude), haemoglobin mass assessments via CO rebreathing (Hb_{mass}), cycling-ergometer performance tests (Performance), venous blood collection (Blood sample), venous blood withdrawal (Blood draw) and daily training volume in km (Training volume)

Cyclists were assigned to one of two groups based on their Hb_{mass} response. In one group ("Response"), Hb_{mass} was 'free' to respond to hypoxia. In the other group ("Clamp"), the Hb_{mass} response was 'clamped' by repeatedly removing any Hb_{mass} increases during the hypoxic exposure, such that Hb_{mass} at the end of the simulated LHTL period was equal to baseline (see below for details). The rationale for 'clamping' Hb_{mass} throughout the course of LHTL, as opposed to a single blood removal at the end of LHTL, was to reduce any potential benefits that athletes might have from training with a progressively enhanced Hb_{mass}.

7.3.2 Subjects and Group Allocation

Thirteen out of the 14 cyclists completed the full training camp. One cyclist withdrew after 10 days of LHTL due to reasons unrelated to experimental protocols or requirements. Prior to the start of the study, it was decided that the allocation of cyclists into groups (Response vs. Clamp group) would not occur until after 14 nights of simulated hypoxic exposure, as previous studies (Clark, Quod et al. 2009; Robertson, Saunders et al. 2010) have shown that the time course of the Hb_{mass} response to hypoxia is unlikely to present in a measurable magnitude after only 7 days of exposure. Once the percent difference of Hb_{mass} compared to baseline was calculated (from the mean of duplicate measures each week), cyclists were assigned in a pair-matched manner so that both groups would include cyclists with a comparable change in Hb_{mass} in response to LHTL after 14 nights. A prerequisite for blood removal in the clamp group was that only cyclists whose Hb_{mass} increase exceeded 2% compared with baseline values at the respective time point were selected for blood removal. This criterion was based on the typical error (TE) for measurement of Hb_{mass} (described below).

One subject lost 3.1 kg (~6%) of body mass and showed a consistent decrease in Hb_{mass} throughout the training camp. She was therefore neither allocated into a 'group' nor included in any part of the statistical analysis. Another cyclist disclosed that she experimented with caffeine supplementation prior to her final performance tests. It was not possible to repeat the tests within the study timeframe, thereby contaminating her results with an additional source of variation. Thus, only data from the remaining 11 cyclists were included in further analysis (Response = 5, Clamp = 6). The physical characteristics of the remaining 11 cyclists are outlined in **Table 7-1**.

Table 7-1: Characteristics of the cyclists at baseline

	Clamp (n=6)	Response (n=5)	All (n=11)
Age (y)	22.4 ± 4.8	20.1 ± 2.1	21.4 ± 3.9
Height (cm)	167.9 ± 5.2	171.1 ± 3.6	169.3 ± 4.6
Mass (kg)	56.9 ± 6.7	61.3 ± 4.6	58.9 ± 6.0
∑7 Skinfolds (mm) †	60.3 ± 16.7*	83.9 ± 12.0	71.0 ± 18.7
Peak Power Output (W)	295.2 ± 39.1	295.8 ± 20.2	295.5 ± 30.5
Peak Power Output ($W.kg^{-1}$)	5.2 ± 0.5	4.8 ± 0.3	5.0 ± 0.4
VO_2max ($L.min^{-1}$)	3.54 ± 0.38	3.71 ± 0.26	3.62 ± 0.33
VO_2max ($ml.kg.min^{-1}$)	62.4 ± 5.7	61.3 ± 4.6	61.6 ± 4.3
Hb mass (g)	635 ± 92	676 ± 60	654 ± 78
Hb mass ($g.kg^{-1}$)	11.1 ± 0.8	11.0 ± 0.3	11.1 ± 0.6
Ferritin $ng.L^{-1}$	68.5 ± 14.8	45.9 ± 24.7	58.2 ± 22.2

Values are means ± SD

† biceps, triceps, subscapular, mid-abdominal, supraspinale, mid-thigh and calf skinfolds

* denotes significant difference between Response and Clamp group $p<0.05$

7.3.3 Haematology

In all cyclists, venous haemoglobin concentration (v[Hb]) ($g.dL^{-1}$) and venous haematocrit (vHct) (%) were determined via flow cytometry using a fully automated

analyser (ADVIA 120 Hematology Analyzer, Bayer Diagnostics, Tarrytown, USA) while serum ferritin ($ng.L^{-1}$) was measured using ELISA technologies (Dade Behring BN ProSpec, Dade Behring, Inc., Deerfield, IL, USA).

7.3.4 Haemoglobin mass

Hb_{mass} was measured using the optimised carbon monoxide (CO) rebreathing method (Schmidt and Prommer 2005). Briefly, a CO dose of 1.2 $ml.kg^{-1}$ body mass was administered and rebreathed for 2 minutes through a glass spirometer. Capillary finger tip blood samples (200 μL), for determination of % carboxy-haemoglobin (HbCO) and capillary haemoglobin concentration (c[Hb]), were taken before and 7 minutes after the initial inhalation of the CO dose. Blood samples were analysed in quintuplet using a CO-oximeter (OSM-3, Radiometer, Copenhagen, Denmark). Hb_{mass} was calculated from the mean change in %HbCO as described previously (Schmidt and Prommer 2005). c[Hb] was determined from the mean of five replicates performed on the pre test capillary sample. In addition, two further capillary samples were obtained at the start of the test for measurement of capillary haematocrit (cHct) using microcentrifugation technique (Hawksley England, Micro-Hematocrit Centrifuge. Lancing, Sussex, England). The results of both measurements were averaged.

Hb_{mass} was measured on four consecutive days at the start of the study period, with the mean value of all measures calculated to obtain a 'true' baseline value. Thereafter, the weekly individual increase in Hb_{mass} was determined through duplicate measures on consecutive days each week, with the mean of the two measures used to establish changes from baseline for each cyclist (**Figure 7-1**). In an attempt to minimise measurement error associated with the CO rebreathing method,

the same experienced researcher conducted all tests and all blood analyses were performed on one OSM-3 analyser. The TE for the CO rebreathing method, calculated from the four baseline measures at the start of the study was 2.0% (90% confidence limit: 1.7 – 2.5%). Multiple measurements reduce the uncertainty of a measurement by a factor of √n, where n is the number of measurements (Hopkins 2000). Therefore, given the TE of 2% at baseline, by performing quadruplicate measures our TE for a change in Hb_{mass} is reduced to ± 1.4% ((2.0% / √4) x √2) at a 68% level of confidence, or to ±2.3% (1.4% x 1.645) with 90% confidence. TE calculated from weekly duplicate measures thereafter was: Week 1: 2.0% (1.5 – 3.1%); Week 2: 2.2% (1.7 – 3.4%); Week 3: 2.1 (1.6 – 3.1%); Week 4: 1.5% (1.1 – 2.3%). Similarly, the use of duplicate measures each week reduces the error associated with individual change scores by √2.

Blood volume (BV), red cell volume (RCV) and plasma volume (PV) were calculated using c[Hb], cHct and Hb_{mass} determined during the weekly CO rebreathing tests as described previously (Schumacher, Pottgiesser et al. 2008). A factor of 0.91 was used to correct for whole body Hct (Chaplin, Mollison et al. 1953).

7.3.5 Blood removal

In order to obtain a 'subject-blind'-design, the venepuncture for blood sampling and removal was standardised. More precisely, the duration of the puncture of an antecubital vein from insertion to extraction of the needle was timed to be equal and, importantly, all cyclists were prevented from observing the procedure by placing their arm through a visual screen (opaque curtain) for the entire duration of the procedure. Furthermore, cyclists were not informed of their Hb_{mass} response at any

time during the study, nor were they informed of the amount of blood removed at any time point.

Following 20 minutes of supine rest (to allow plasma volume to stabilize (Ahlgrim, Pottgiesser et al. 2010)) two tubes (BD Vacutainer EDTA (2 ml) and BD Vacutainer Serum (4 ml), BD Australia, North Ryde, Australia) of venous blood were drawn from all cyclists using a 19 G needle (Wing-Flo, Intermedica GmbH, Mainz, Germany) for determination of v[Hb] ($g.dL^{-1}$), vHct (%) and serum ferritin ($\mu g.L^{-1}$). Following a single flush with normal saline (3 ml) after the first sample, the needle remained inserted intravenously in all cyclists whilst the EDTA sample was simultaneously analysed on site for individual v[Hb] (ADVIA 120 Hematology Analyzer, Bayer Diagnostics, Tarrytown, USA) in those cyclists selected for the Hb_{mass} removal procedure. If necessary, the amount of whole-blood to be removed was then quantified as follows:

$$\textit{Volume of venous withdrawal (ml)} = \Delta\, Hb_{mass}\ (g) \,/\, (v[Hb]\ \textit{(individual subject and day)}\ (g.dL^{-1}) \,/\, 100\ (ml.dL^{-1}))$$

For example, if a cyclist showed a Hb_{mass} increase of 11 g after 14 nights of simulated hypoxia, corresponding to a 2.1% increase from baseline, and on site measured v[Hb] was 13.9 $g.dL^{-1}$, then the blood volume to be removed was calculated to be 79 ml. Blood was removed from the selected cyclists using a 3-way-stop-cock (Discofix C-3, B. Braun, Melsungen, Germany) and 50 ml syringes (Original-Perfusor-Spritze OPS 50mL Luer Lock, B. Braun, Melsungen, Germany) under medical supervision. The volume of blood already removed for analysis (6 ml) was accounted for in the removal process. Cyclists in the Response group received a 'sham' withdrawal in which the needle remained inserted inside the vein but no

further blood was removed, apart from the initial 6 ml. Plasma volume was not corrected via saline infusion to abide by anti-doping regulations. Instead, cyclists in both groups were asked to increase their oral fluid intake by 250 – 500 ml after the procedure.

7.3.6 Performance Tests

Cycling performance was assessed in duplicate both before and after simulated LHTL using an electromagnetically braked cycle ergometer (Lode Excalibur Sport, Groningen, The Netherlands). The ergometer was calibrated before, and verified after, the study period using a custom-built dynamic calibration rig described previously (Gardner, Stephens et al. 2004). On arrival, all cyclists first completed an incremental graded exercise test to exhaustion on the test ergometer for familiarisation and determination of Peak Power Output (PPO), beginning at 125 W and increasing 25 W every 3 min until volitional exhaustion. PPO (W) was calculated as follows (Kuipers, Verstappen et al. 1985):

$$PPO\ (W) = W_L + [(t/a)*b],$$

where W_L = power output of the last complete workload, t = time (min) for the final incomplete work load, a = workload time increments (min), b = workload power increments (W).

Each performance test consisted of two stages: stage 1. maximal 4-min effort, stage 2. ride time to exhaustion at PPO (T_{lim}). The protocol was designed to discern the effects of LHTL on both a single maximal performance as well as the cyclist's ability to recover for a subsequent maximal task shortly after (Meeusen, Nederhof et al. 2008). Such a scenario is often present in road cycling races and therefore has high

practical relevance. Following a standardized warm up, the cyclists began a 4-min maximal effort. Power output was fixed for the first 2 minutes at 105% of PPO. In the final 2 minutes, the linear factor (gearing) of the ergometer was set to elicit 105% of PPO at 98 rpm and cyclists were instructed to complete as much work (kJ) as possible by altering their cadence. If cadence deviated from 98 rpm during the final 2 minutes, power output was adjusted as follows: Power output (W) = rpm^2 x linear factor. Maximal mean power elicited over the four minutes (MMP_{4min}) was calculated from the total work performed. Based on the two baseline tests, the TE for MMP_{4min} was 1.4% (90% CL: 1.0 - 2.0%). The MMP_{4min} was chosen as a measure of performance instead of a traditional VO_2max test for two reasons: 1) 4-min efforts are highly important in the sport of cycling, with an individual pursuit in track cycling typically lasting ~4 min and 2) data collected during women's world cup racing has identified MMP_{4min} as a key determinant of top 20 performances vs. non top 20 (Ebert, Martin et al. 2005). Since the 4-min task requires both an aerobic and anaerobic contribution (Craig, Norton et al. 1993; Gastin 2001), any non-haematological adaptations to hypoxia may also be important for overall performance.

Oxygen consumption (VO_2) during MMP_{4min} was measured continuously using a custom-built automated Douglas bag system with associated in-house software (Australian Institute of Sport, Belconnen, Australia) as described previously (Saunders, Telford et al. 2004). VO_2 values were calculated using standard algorithms for consecutive 30-s periods, with VO_2peak determined as the sum of the highest two consecutive measurements. Previous work has shown that VO_2peak achieved during a 4 minute maximal effort is not significantly different from the VO_2max achieved during a graded exercise test (Gore, Hahn et al. 1998). The

maximal accumulated oxygen deficit (MAOD) (Medbo, Mohn et al. 1988) arising from the 4-minute maximal effort was calculated as the difference between estimated oxygen requirements of the work achieved (derived from the power – VO_2 regression for each athlete) and the total VO_2 measured during the effort (Roberts, Clark et al. 2003). The relative aerobic and anaerobic contributions of the effort were calculated as the percentage of measured VO_2 compared with the predicted VO_2. Blood lactate concentration (Lactate Pro, Akray, Japan) was measured via capillary sampling one minute following the completion of the effort.

Ten minutes after the completion of the 4-minute maximal effort, the cyclists were instructed to ride for as long as possible at PPO (T_{lim}). The rationale for including this novel aspect of the test was to mimic scenarios in road racing in which the duration of an effort is not pre-determined, as well as an attempt to assess the full impact of the LHTL training block on the cyclists' performance capabilities. Indeed, Meeusen et al. have suggested that certain aspects of fatigue appear to be better discerned when using a two bout exercise protocol (Meeusen, Nederhof et al. 2008). Cyclists were given a warning when their cadence dropped below 80 rpm and were stopped following the 3rd warning. The cyclists were not able to see elapsed time, with cadence the only feedback available. Based on the two baseline tests, the TE for T_{lim} was 17.0% (90% CL: 12.9 - 25.8%). During this final part of the performance testing, VO_2 was not measured, to ensure that there were no additional sources of distraction for the athletes. Blood lactate concentration was again assessed one minute after completion of the effort. The typical duration of this effort was 3 - 5 min and thus the metabolic demands of the task were similar to that of the four minute maximal test (Craig, Norton et al. 1993; Gastin 2001).

Performance testing was completed in duplicate (with one rest day between tests) on all occasions. The 'best test' was deemed to be the test in which the highest MMP_{4min} was obtained regardless of the T_{lim} work completed, and all corresponding data were used for analysis.

7.3.7 Training

Cycling training for the entire duration of the camp was prescribed and supervised by the Australian National Team coach. Training was reverse-periodised to include a focus on short duration power development at the start of the training block and followed by longer duration rides of low intensity (**Table 7-2**). One week following the completion of LHTL, the cyclists competed in a 2-day cycling race, the taper for which was incorporated into the final week of training (**Figure 7-1**). Training was conducted as a single group, with the exception of testing days that were randomly allocated. All training rides were monitored using a calibrated mobile power measuring device fitted to each athlete's bicycle (SRM Training System, Professional Version, Schoberer Rad Messtechnik, Julich-Weldorf, Germany or PowerTap, Saris Cycling Group, USA). In addition, cyclists were asked to complete a training, illness and injury log on a daily basis, which included perceptual information about the day's training. Cycling training was quantified using commercially available software (TrainingPeaks WKO+ Version 2.2, PeaksWare, Lafayette, Colorado, USA). The software calculates a Training Stress Score^{TM} (TSS) and the associated Intensity Factor^{TM} (IF) using SRM power data (Allen and Coggan 2006). The TSS is analogous to a Banister Training Impulse (TRIMP) (Banister and Calvert 1980) and is a combination of training duration and relative training intensity referenced to a cyclist's maximum power output for one hour. The IF is a relative

intensity scale with 1.00 reflecting the power output that can be maintained during a maximal 1 hour time trial. The software "normalises" variable power output in an attempt to estimate the intensity of the effort as if it was produced using a constant power output profile.

Table 7-2: Characteristics of training during 26 nights of LHTL simulated hypoxia

	Week	Clamp (n=6)	Response (n=5)	All (n=11)
Distance (km)	1	615 ± 117	640 ± 75	629 ± 96
	2	467 ± 89	471 ± 38	469 ± 67
	3	330 ± 28	344 ± 80	337 ± 54
	4	511 ± 40	463 ± 51	489 ± 50
Duration (h)	1	21.2 ± 3.8	21.9 ± 2.4	21.5 ± 3.1
	2	16.6 ± 2.8	16.7 ± 1.6	16.6 ± 2.2
	3	11.8 ± 1.5	12.3 ± 3.6	12.0 ± 2.6
	4	19.2 ± 3.7	16.4 ± 2.0	17.9 ± 3.2
Training Stress Score™	1	1563 ± 353	1425 ± 362	1500 ± 347
	2	1290 ± 309	1127 ± 234	1216 ± 277
	3	610 ± 169	577 ± 195	595 ± 172
	4	1002 ± 147	934 ± 273	971 ± 205
Intensity Factor™	1	0.83 ± 0.06	0.78 ± 0.09	0.80 ± 0.07
	2	0.78 ± 0.10	0.71 ± 0.09	0.75 ± 0.09
	3	0.65 ± 0.08	0.63 ± 0.07	0.64 ± 0.07
	4	0.64 ± 0.13	0.64 ± 0.13	0.67 ± 0.10
% of training time spent in power bands ($W.kg^{-1}$)	0-2	50 ± 3.3	52 ± 3.5	51 ± 3.4
	2-4	37 ± 2.4	38 ± 1.7	37 ± 2.0
	4-6	11 ± 1.7	9 ± 2.0	10 ± 2.0
	>6	2 ± 0.3*	1 ± 0.3	2 ± 0.4

Values are means ± SD, calculated using Training Peaks WKO software (*see Methods*)

Distance – cumulative distance ridden per week (km);

Duration – cumulative duration of training ride per week (h);

Training Stress Score – sum of daily training load per week arbitrary units);

Intensity Factor – average daily training intensity per week (arbitrary units);

% of training time spent in power bands – percentage of overall training time spent in each power band ($W.kg^{-1}$)

7.3.8 Statistical analysis

Data were analysed using a contemporary statistical approach (Hopkins, Marshall et al. 2009) since many conventional approaches can be insensitive to small but practically important changes – performance changes of only 0.5% in magnitude can be important to elite athletes (Hopkins, Hawley et al. 1999). Measured variables were log transformed prior to analysis in order to reduce bias arising from non-uniformity of error, and back transformed to obtain changes in means and standard deviations (SD) as percents (Hopkins, Marshall et al. 2009). Mean effects of LHTL in the Clamp versus Response group were estimated via the unequal variances t statistic, using a spreadsheet for standard controlled trials (http://www.sportsci.org/resource/stats/xcontrial.xls), which accounts for the observed difference, and the smallest worthwhile change. The smallest worthwhile change for MMP_{4min} and VO_2peak was 1% and 2% for Hb_{mass} whereas for other physiological parameters (HR_{pk}, Blood Lactate, etc) the smallest worthwhile change was derived from Cohen's scale for Effect Sizes in which a small effect size is ≥ 0.2 (Cohen 1988). The percentage likelihoods of the observed differences between the Clamp and Response groups are expressed using the following descriptors: <1%, almost certainly not; 1-5%, very unlikely; 5-25%, unlikely; 25-75%, possibly; 75-95%, likely; 95-99%, very likely; >99%, almost certainly. Effects were deemed substantial if the percentage likelihood that the true value was practically positive (or negative) was > 75%. The effect was deemed "unclear" if its confidence interval overlapped the thresholds for both positive and negative change.

Data are expressed as the mean (± SD) unless otherwise stated. Data in graphs are presented as percent changes from the mean of baseline measures (± SD), using the

back-transformed values for individuals and groups. Differences between the two groups for baseline and training characteristics were assessed using an unpaired t-test, and for these the level of significance was set to $p<0.05$.

7.4 RESULTS

7.4.1 Simulated Hypoxic Exposure

The cyclists accumulated a total of (mean ± SD) 423 ± 17 h of exposure and an average of 16.3 ± 0.7 $h.d^{-1}$. There was no substantial difference in duration of exposure between the groups (Response: 16.6 ± 0.4 $h.d^{-1}$; Clamp: 16.0 ± 0.8 $h.d^{-1}$).

7.4.2 Haemoglobin mass, blood volume and blood removal

Hb_{mass} substantially increased with simulated hypoxic exposure in both groups (**Figure 7-2**). After 26 nights of LHTL, mean Hb_{mass} in the Response group was 5.5 ± 2.9% higher than baseline (**Figure 7-3**). In the Clamp group, three cyclists increased Hb_{mass} greater than the 2% threshold after 14 nights, and subsequently had blood removed (**Table 7-3**), with further blood removal for two of these cyclists after 26 nights. Blood removal for the remaining three cyclists occurred after 21 nights. The Hb_{mass} of these three cyclists after 26 nights demonstrate our effectiveness in returning Hb_{mass} to baseline (**Table 7-3**). The maximum volume of blood removed on any one occasion was 314 ml; with the mean volume removed 180 ml. Individual serial values of Hb_{mass} are displayed in **Figure 7-2**.

Table 7-3: Individual blood removal of "Clamp" group throughout live high train low

Subject ID	Timepoint	Δ Hb_{mass} from baseline (g)	Δ Hb_{mass} from baseline (%)	[Hb] (g/dl)	Volume removed (ml)	Sum removed (ml)
1	altitude 14d	-1	-0.3	-	0	
	altitude 21d	14	2.7	14.1	101	
	altitude 26d	-1	-0.2	-	0	101
2	altitude 14d	11	2.1	13.9	82	
	altitude 21d	3	0.6	-	0	
	altitude 26d	16	2.9	14.1	112	194
5	altitude 14d	12	2.0	-	0	
	altitude 21d	34	5.5	14.7	235	
	altitude 26d	-9	-1.5	-	0	235
7	altitude 14d	9	1.4	-	0	
	altitude 21d	43	6.7	14.6	295	
	altitude 26d	-4	-0.6	-	0	295
9	altitude 14d	18	2.3	14.9	120	
	altitude 21d	9	1.1	-	0	
	altitude 26d	1	0.1	-	0	120
10	altitude 14d	50	7.3	16.0	314	
	altitude 21d	-1	-0.1	-	0	
	altitude 26d	24	3.5	14.9	163	477

If the total amount of Hb_{mass} removed is considered, the theoretical additive Hb_{mass} response of the Clamp group was 4.5 ± 2.1% (**Figure 7-3**), and was not substantially different from the response observed in the Response group (p=0.55). However, at the time of post-LHTL testing (following blood removal), the mean change in Hb_{mass} of the Clamp group compared to baseline was -0.4 ± 0.6%, and was "very likely" lower (-5.6%; 90%CL -8.1 to -3.0 , p=0.01) than the Response group and also well within our TE for the CO rebreathing method.

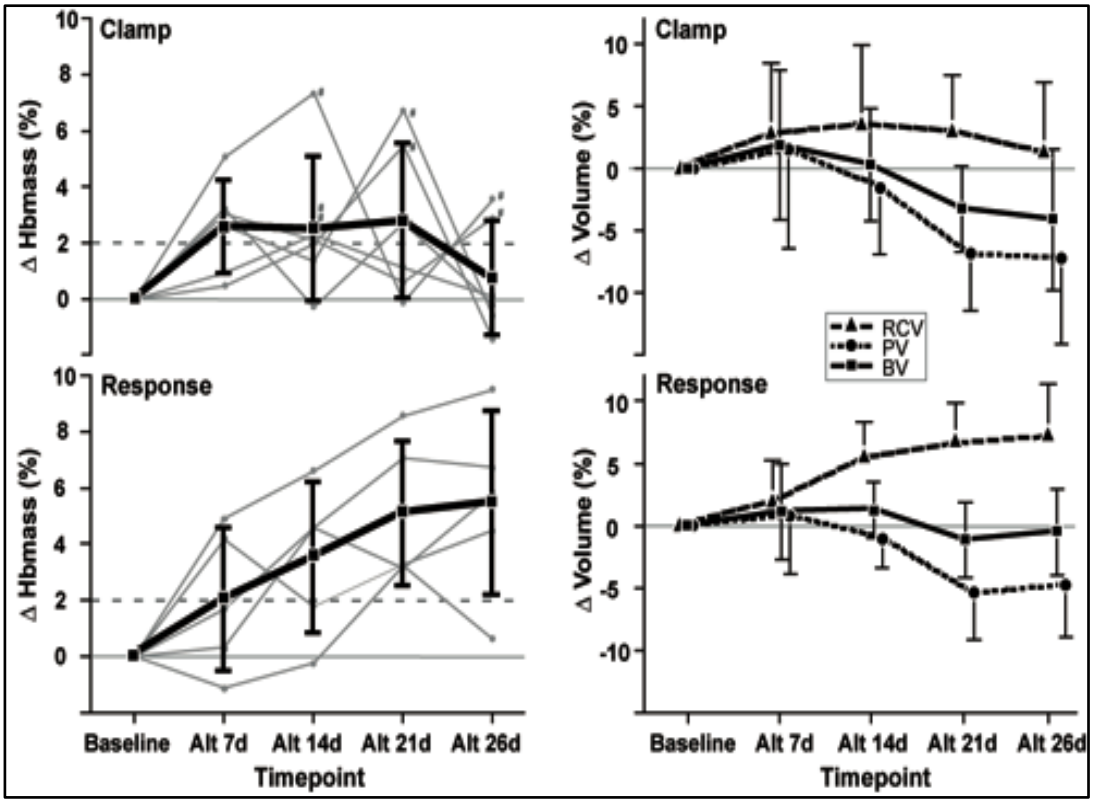

Figure 7-2: Weekly individual and mean (± SD) changes (%) from baseline in Hb_{mass} and blood volume compartments during 26 nights of live high train low simulated hypoxia

# denotes blood removed from this individual at this time point. Note: blood was removed from 2 subjects after 26 nights of LHTL in order to return Hb_{mass} to baseline values.

Mean changes in RCV were consistent with changes in Hb_{mass} (**Figure 7-2**), with the Response group displaying a 6.8 ± 4.1% increase, which was "likely" higher than the 1.1 ± 5.5% increase in the Clamp group (p=0.08). In the Response group, PV changes mirrored RCV, decreasing by 5.0 ± 4.3% in order to conserve BV, which was not substantially different from baseline following 26 nights of hypoxic exposure (-0.6 ± 3.4%). In the Clamp group, a similar decrease in PV was observed (-7.7 ± 7.3, p=0.46), however due to the loss of RCV through phlebotomy, total BV of the Clamp group was "likely" lower (-4.3 ± 6.0%, p=0.13) compared with baseline, with the mean change "possibly" lower than the Response group (p=0.22).

7.4.3 VO_2peak and Cycling Performance

Following LHTL, MMP_{4min} increased in all athletes (**Figure 7-3**). Mean increases in the groups were: Response = 4.5 ± 1.1%, Clamp = 3.6 ± 1.4% with "trivial" differences between the groups (-0.8%; 90%CL -2.2 to 0.6, p=0.58). Mean VO_2peak substantially increased in the Response group (3.5 ± 2.3%) but not in the Clamp group (0.3 ± 2.6%), with the magnitude of change from baseline "likely" lower within the Clamp group (-3.2%; 90%CL -5.7 to 0.5, p=0.056), (**Figure 7-3**). Cyclists in the Response group rode substantially longer (+28.5 ± 20.8%) at PPO compared with baseline (**Figure 7-3**). In comparison, mean T_{lim} substantially decreased in the Clamp group following LHTL (-19.4 ± 64.5 %) and their performance was "likely" worse than the Response group (-37.6 %; 90%CL -58.9 to -5.0, p=0.07).

MAOD increased substantially in Clamp (18.8 ± 17.6%, p=0.048) but not in Response (11.6 ± 10.9%, p=0.076). Similarly, the anaerobic contribution increased substantially in the Clamp group (Pre 16.5 ± 3.5%, Post 18.9 ± 4.1%) but not in the Response group (Pre 14.0 ± 3.6%, Post 14.8 ± 2.5%), with the increase observed in the Clamp group "possibly" higher than the Response group (6.5%; 90%CL -7.1 to 22.5, p=0.41). There were no substantial effect of LHTL on mean post-test lactate concentration following either the MMP_{4min} (Response: 13.0 ± 2.4 vs. 12.9 ± 1.9 $mmol.L^{-1}$; Clamp 14.4 ± 1.0 vs. 14.2 ± 1.2 $mmol.L^{-1}$) or the T_{lim} test (Response: 13.1 ± 2.5 vs. 13.6 ± 1.5 $mmol.L^{-1}$; Clamp 14.6 ± 1.6 vs. 14.6 ± 1.2 $mmol.L^{-1}$); with differences between the groups "unclear."

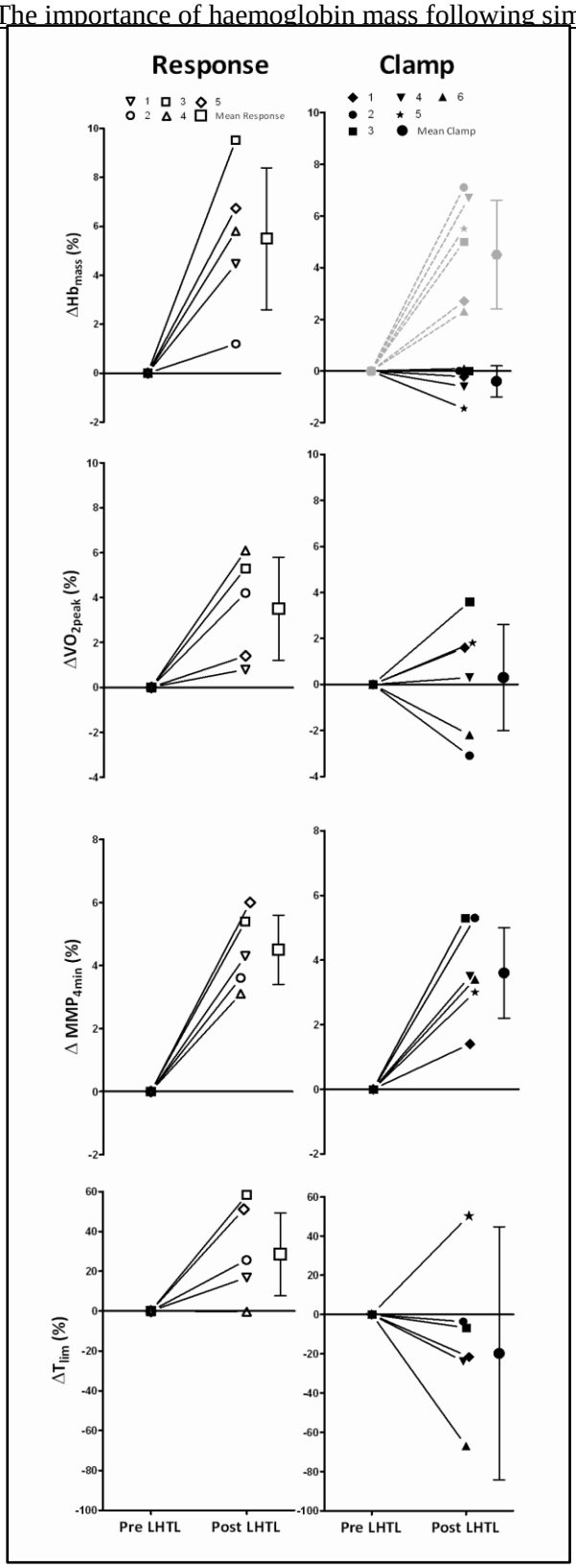

Figure 7-3: Individual and mean (± SD) changes (%) in Hb_{mass} (both actual in black & theoretical in grey), VO_2peak, MMP_{4min} and T_{lim} following 26 nights of live high train low

All values are back-transformed from log-data effects compared with baseline (± SD)

7.4.4 Training

Retrospective analysis of the allocated groups (Response vs. Clamp) revealed no substantial differences in training load during LHTL with the exception of the percentage of training time spent above 6 $W.kg^{-1}$ (**Table 7-2**).

7.5 DISCUSSION

The main finding of the present study is that removal of hypoxia-induced increases in Hb_{mass} during LHTL did not prevent increases in maximal four-minute cycling performance in highly-trained female cyclists following 26 nights of simulated hypoxic exposure. Furthermore, enhancements to four-minute cycling performance were not distinguishable between the cyclists whose Hb_{mass} was 'clamped' and those in which Hb_{mass} was allowed to increase (~5%). However, the performance of the Clamp group was ~40% worse in a subsequent ride to exhaustion, which reinforces the role of Hb_{mass} for repeated high-intensity efforts.

The relationship between Hb_{mass} and VO_2max, is well established with increases in Hb_{mass} directly related to increases in VO_2max via enhanced oxygen transport (Gledhill, Warburton et al. 1999; Schmidt and Prommer 2010). In fact, a 1 g increase in Hb_{mass} results in an ~4 $ml.min^{-1}$ increase in VO_2max (Schmidt and Prommer 2010). Therefore, the dominant paradigm which suggests that increases in VO_2max secondary to increases in Hb_{mass} following hypoxia are responsible for improvements in sea level performance (Levine and Stray-Gundersen 2005) is intuitively appealing. Indeed, athlete's who have shown no performance response to altitude training – termed "non-responders" (Levine and Stray-Gundersen 1997), equally did not exhibit changes in red cell volume. However, unlike the classic blood doping studies

in which only Hb_{mass} and/or blood volume are manipulated (Berglund and Hemmingson 1987; Brien and Simon 1987; Ekblom 1996), hypoxic exposure may initiate a multitude of responses of which augmented Hb_{mass} is just one (Gore, Clark et al. 2007). Therefore, whilst hypoxia-induced increases in Hb_{mass} may partially explain changes in sea level performance; other, non-haematological adaptations may be equally if not more important (Gore and Hopkins 2005). Furthermore, it is postulated that changes in Hb_{mass} may simply indicate an athlete's 'adaptive' state and that other adaptations to hypoxia are occurring independently of changes in Hb_{mass}.

In the present study we attempted to isolate the role of Hb_{mass} on cycling performance following simulated LHTL by repeatedly removing the hypoxia-induced increase in Hb_{mass}. The fundamental aspect of this unique study design was that the EPO and HIF-1 signalling cascades remained 'active', with only the end result of erythropoiesis (i.e. increased Hb_{mass}) being blocked, and not the upstream pathways. Using this design, we were able to first, identify which athletes were 'responding' haematologically to hypoxia and secondly, remove the Hb_{mass} response without preventing any other adaptive processes associated with the upstream pathway (Sasaki, Masuda et al. 2000). Previous studies which have documented a performance response without an accompanying increase in Hb_{mass} (Gore, Hahn et al. 1998) have been criticized for the lack thereof, which has been attributed to either an insufficient hypoxic 'dose' (Levine and Stray-Gundersen 2006) or an unsatisfactory state for erythropoietic adaptation in the athlete; e.g. due to illness, inflammation or iron deficiency. It was therefore important in the present study to demonstrate that the athletes were adapting to the hypoxic environment before any blood removal took place. By pair-matching the cyclists based on their Hb_{mass} response (after 14 nights of exposure), we were also able to ensure that both groups displayed a similar haematological response. Due to the error

of measurement associated with the CO rebreathing method, and even using duplicate measures, it was not possible to confidently detect changes in Hb_{mass} of < 2%. As a result it was not possible to maintain a true Hb_{mass} 'clamp' on a daily basis throughout the entire period of exposure, with our study design limited to, at best, weekly blood removals. Therefore the confounding effect of performing some training sessions with 'extra' Hb_{mass} on the subsequent performance tests cannot be dismissed. However, since blood removal was determined on an individual basis, the maximum time frame an athlete in the Clamp group could train with an enhanced Hb_{mass} was limited to one week, as opposed to the full exposure period in the Response group. Our data do indicate however, that we were able to effectively remove the hypoxia-induced increases in Hb_{mass} in the Clamp group at several time-points such that at post-LHTL performance testing, their Hb_{mass} was equivalent to baseline measures. In this way, we were able to assess the importance of 'extra' Hb_{mass}, induced by hypoxia, for a cycling-specific performance task.

7.5.1 Role of Hb_{mass} during maximal four minute cycling performance

Improved four-minute cycling performance and VO_2peak were observed after an adequate dose of LHTL that increased Hb_{mass} by ~5% in the Response group; consistent with the paradigm that increases in sea-level performance following LHTL are primarily mediated by increases in Hb_{mass} (Levine and Stray-Gundersen 2005). However, whilst a strong correlation between the total amount of Hb available and VO_2max is evident at sea-level (Kanstrup and Ekblom 1984; Schmidt and Prommer 2010), the relationship between changes in Hb_{mass} and VO_2max following LHTL is not clearly defined (Friedmann-Bette 2008; Schmidt and Prommer 2010). Indeed, in the present study, performance improved to a similar extent in the Clamp

group without an enhanced Hb_{mass} or VO_2peak; raising new questions with respect to the principal role of Hb_{mass} on sea-level performance following LHTL. As often demonstrated in other aspects of physiology, a system is rarely "limited" by solely one component (Schumacher and Roecker 2006), rather adaptive processes serve to compensate for weaker components so that functional capacity is maintained. For example, myoglobin knockout mice are able to somehow compensate for the absence of a key component of the oxygen delivery system; showing indistinguishable exercise capacities to their wild type counterparts during a highly metabolically demanding endurance task (Garry, Ordway et al. 1998).

Our data indicate that alternate mechanisms for enhanced performance exist following LHTL. Enhanced oxygen transport via increased Hb_{mass}, if available, appears to be the dominant mechanism for increased work during a maximal cycling performance task – as demonstrated by the Response group. However, in its absence, alternative adaptive pathways may be utilised - a scenario demonstrated by our Clamp group, who were able to produce a greater performance without a concomitant increase in VO_2peak. Unfortunately, our measurements do not allow us to definitively comment on the mechanisms responsible for the performance improvements in the Clamp group. However, the changes in both MAOD and the relative anaerobic contribution to the task indirectly point toward an increased reliance on anaerobic pathways in this group (Bangsbo, Michalsik et al. 1993). Roberts and colleagues reported increases in both MMP_{4min} and MAOD during an identical cycling performance task in a group of well trained cyclists following 5, 10 and 15 days of LHTL (8-10 $h.d^{-1}$) at 2,650 m (Roberts, Clark et al. 2003). Whilst haematological changes were not measured in this study, it is unlikely that even the highest 'dose' of LHTL used would elicit a sufficient hypoxic dose to induce substantial erythropoietic adaptations (Levine and Stray-Gundersen

2006). Again, the authors were unable to present evidence of the mechanism responsible for the increase in MAOD, but suggest an increased muscle buffering capacity (Mizuno, Juel et al. 1990) or changes to lactate transport (Zoll, Ponsot et al. 2006) may explain the hypoxia-induced improvement in performance observed. Further, whilst our postulate of increased anaerobic reliance in the current study is not supported by the blood lactate data (which did not differ between the groups), an improvement in muscle buffering capacity has been documented without a concomitant up-regulation of anaerobic metabolism during intense exercise (Gore, Hahn et al. 2001). Therefore, it is possible that early performance adaptations to LHTL (when the hypoxic dose is insufficient to initiate remarkable changes in Hb_{mass}) are anaerobic, with aerobic adaptations arising later if hypoxic exposure is continued. Such adaptations would allow for 2 - 4% increases in a single high-intensity cycling performance task, independent of Hb_{mass}.

7.5.2 Role of Hb_{mass} during ride time to exhaustion (Tlim)

The contrasting results of the two groups during the second part of the performance test, may offer further insight into the energy systems utilised by the athletes in the preceding task. The striking increase T_{lim} exhibited by the Response group supports a preferential role of aerobic metabolism during multiple maximal efforts. These data are consistent with previous work showing an increase in performance following induced erythrocythemia (Berglund and Hemmingson 1987; Brien and Simon 1987; Gledhill, Warburton et al. 1999). The increased Hb_{mass} of the Response group not only served to enhance oxygen transport during the maximal effort (as demonstrated by the increased VO_2peak), but may also have contributed to improved rates of recovery in terms of lactate and metabolite clearance following the effort (Kanstrup

and Ekblom 1984; Brosnan, Martin et al. 2000). In contrast, whilst the Clamp group was able to draw on alternate mechanisms to produce a similar single effort to the Response group in the MMP_{4min} test, when asked to repeat a maximal effort, they displayed a marked amount of fatigue and were unable to reach even their pre-LHTL standards. Kanstrup reported similar findings after 'blood loss' with both VO_2max and performance time decreased following an induced decrease in both [Hb] and blood volume (Kanstrup and Ekblom 1984). Gledhill has suggested that "improvements to aerobic performance may be attributed in part to physiological alterations related to increase in [Hb] e.g. augmented buffering" (Gledhill, Warburton et al. 1999), therefore the role of a modest (~5%) increase in Hb_{mass} upon sea level performances post-LHTL, may also be related to recovery processes associated with the effort, as opposed to defining the effort itself. The disparate efforts of the Clamp group in the two all-out performance tests suggest that, in the absence of an enhanced Hb_{mass}, the 10 min recovery period was inadequate to dissipate the residual fatigue arising from the elevated anaerobic contribution of the prior maximal effort. However, it is important to note the unique design of the performance tests, in which the two tasks were juxtaposed. It is highly likely that the preceding MMP_{4min} effort influenced the athlete's capabilities in the subsequent T_{lim} test, and therefore the results of a single ride time to exhaustion may be somewhat different. In summary, the increases in Hb_{mass} and VO_2max of the Response group allowed the athletes to adopt a more favourable metabolic strategy during the performance tests, which meant they were more capable of producing repeated maximal efforts. In a cycling road-race context, where multiple all out efforts are required, our results highlight the clear benefit of a modestly increased Hb_{mass} after LHTL.

7.5.3 Limitations

It must be acknowledged that the design employed for blood removal may have exerted confounding effects that we were unable to capture. A single blood removal at the end of simulated LHTL may have minimised such effects; however the impact of a larger blood removal on total blood volume would have been difficult to manage since reinfusion of plasma was not possible due to anti-doping regulations. Plasma losses associated with the blood volumes removed in the present study are typically recovered within 24 hours (http://www.givelife2.org/donor/faq.asp#3) and thus acute alterations to blood volume should not have affected performance testing in the following days. However, whilst blood removal was effective in clamping the erythropoietic response, when combined with the reduction in plasma volume induced by hypoxia (which serves to increase [Hb] in order to maintain oxygen supply (Schmidt and Prommer 2010)), total blood volume of the Clamp group was in fact reduced compared to baseline. The effect of mild hypovolemia on performance therefore, cannot be discounted (Gledhill, Warburton et al. 1999). It may also be speculated that dehydration induced from living in normobaric hypoxia may have further confounded plasma volume and influenced performance. Whilst hydration status on the morning of performance testing was not determined, individual body mass changes did not indicate that any athletes were dehydrated, with both groups showing a modest 0.4 kg decrease in body mass following LHTL.

The central and neural adaptations to the LHTL period and the subsequent effect on performance must also be considered (Millet, Roels et al. 2010), especially with regard to the Clamp group. Despite the fact that all training was conducted as one group, the Clamp group spent a significantly greater percentage of training time at

the highest training intensity (> 6 $W.kg^{-1}$) and thus the neural effects of training may have been greater in this group, therein contributing to the improved performance at sea level (Friedmann-Bette 2008).

The study is limited by the absence of a classic control group, however the positive effects of LHTL compared to sea level training have been demonstrated previously (Levine and Stray-Gundersen 1997), and thus in the present study, the Response group serve as a control group of sorts. The small sample size of our groups – due to the elite population studied, is also a limitation, but our design is strengthened by the multiple measurements performed. Furthermore, whilst follow up performance testing in the weeks following cessation of LHTL may have provided further insight into the benefits for each group, this was not possible due to the training and competition demands of the cyclists. Lastly, whilst our measurements do not allow us to discern the mechanisms responsible for the improved performance in the Clamp group, our data provides important information for athletes engaged in normobaric simulated LHTL about the contribution of Hb_{mass} for subsequent performance improvement, which may assist in the planning and implementation of hypoxic training.

7.6 CONCLUSION

Improved 4-min performance and VO_2peak were observed after an adequate dose of simulated normobaric LHTL that increased Hb_{mass} by ~5%. However, 4-min performance improved to a similar extent in a matched group without an enhanced Hb_{mass} or VO_2peak. On one hand, Hb_{mass} remains an important factor for overall performance following simulated LHTL, since only the group who increased Hb_{mass}

was able to improve performance in a ride time to exhaustion test following a maximal 4-min effort. Nevertheless, our novel findings contest the widespread paradigm that modest increases in Hb_{mass} are a prerequisite for enhanced performance following LHTL and suggest that accelerated erythropoiesis is not the sole mechanism by which LHTL improves performance. Future research should therefore focus further on the exploration of the non-haematological mechanisms that determine enhanced performances following LHTL, or incorporate performance tests of sufficient duration (e.g. 30 min) to have an over-whelming dependence on aerobic metabolism.

CHAPTER 8: Summary, future directions and conclusions

8.1 SUMMARY

This thesis examined the influence and importance of changing Hb_{mass} on cycling performance. There are a number of factors that may potentially influence Hb_{mass} in elite endurance athletes – some effects on Hb_{mass} may be relatively small, if even existent, whereas others may have large and potentially meaningful effects on Hb_{mass}. This thesis has documented a variety of factors that produce both subtle (e.g. stage racing) and profound (e.g. altitude exposure) influences on Hb_{mass} and has also attempted to untangle the complex relationship between changes in Hb_{mass} and cycling performance, all the while, fully aware that correlation does not confirm causality.

8.1.1 CO rebreathing methodology

Hb_{mass} can be accurately and reliably measured using the CO rebreathing technique, and recent modifications to the Burge & Skinner (1995) method by Schmidt & Prommer (2005) have increased the ease of measurement in athletic populations during periods of important training and competition. Compared to the Burge & Skinner method, the 2-min method has been shown to produce valid results, with excellent reliability (< 2%) (Gore, Hopkins et al. 2005; Schmidt and Prommer 2005). When discussing sources of error associated with the method, much attention has been placed on methodological issues, such as the measurement and delivery of the

CO bolus and the measurement of the HbCO fraction before and after rebreathing. In addition, researchers have previously discussed and debated the ideal timing of blood sampling following rebreathing as well as the magnitude of CO loss to Mb, with the aim of minimising measurement error and increasing the precision of the test (Gore, Bourdon et al. 2006; Prommer and Schmidt 2007). However, these methodological issues tend to be discussed based on population data with little attention to possible individual differences. Are some individuals prone to producing an over or under estimation of Hb_{mass} when tested with the CO-rebreathing technique? If so, is this related to underlying physiological differences rather than the application of method *per se*?

The kinetics and distribution of CO throughout the human body have been theoretically modelled by Bruce and Bruce (2003). Based on their model, and using data obtained during CO rebreathing, the CO kinetics of the Burge & Skinner and Schmidt & Prommer methods were compared in order to obtain a greater understanding of possible underlying physiological sources of error. The findings presented in Chapter 2 support previous suggestions that some physiological variables within an individual can influence the results under specific circumstances (Brown, Hopper et al. 1951; Gore, Bourdon et al. 2006; Prommer and Schmidt 2007). Previously, these potential confounders to 'normal' CO kinetics were deemed only relevant to individuals possessing pathological conditions which delay or prevent complete mixing of CO throughout the circulation (e.g. shock, Raynaud's phenomenon). However, as summarised in **Figure 8-1**, there are a number of factors which can influence the HbCO curve during rebreathing - demonstrating that CO kinetics can be altered even in healthy individuals.

The curve is characterised by three key sections: 1 – the rate of increase, which determines how quickly HbCO appears in the muscle venous compartment, 2 – the plateau level, which is fundamental for the subsequent calculation of Hb_{mass}, and 3 – the rate of decay, which is determined by CO loss out of the muscle compartment (either through exhalation or in binding to Mb). Changes to muscle morphology (e.g. capillary density, muscle fibre size and Mb concentration) may affect the muscle diffusion capacity for CO, and therefore could influence the plateau height (2) and rate of decay (3). Alterations to breathing rate, muscle blood flow and blood volume distribution – all of which may be influenced by environmental factors (e.g. ambient temperature, ambient O_2 concentration) or prior exercise, also have the potential to affect the rate of increase (1) in %HbCO (and hence the time required for complete mixing throughout circulation to occur (t_{mix})) as well as the plateau height (2). Importantly, these factors can be adequately controlled, provided researchers have an understanding and appreciation of CO kinetics, as well as the influence of aspects of human physiology on the accuracy of the test. Failure to do so may result in values which indicate a change in Hb_{mass} has falsely occurred. For example, if an athlete were to be tested following an ice bath, when a larger proportion of blood flow was directed to the core, the time taken for HbCO to appear and mix throughout the muscle venous compartment could be increased, with the plateau level likely lower. The resulting effect would be a spuriously higher Hb_{mass}. Similarly, if Mb concentration or the binding affinity for Mb in the extra-vascular compartment were to increase, (perhaps due to muscle damage) this would result in a greater rate of decay and again a lower plateau level at the time of sampling. These errors could be avoided by standardising the procedure prior to the test, delaying the time of blood

sampling to ensure HbCO is completely mixed throughout circulation, and accounting for an increased loss of CO to Mb during the calculation of Hb_{mass}.

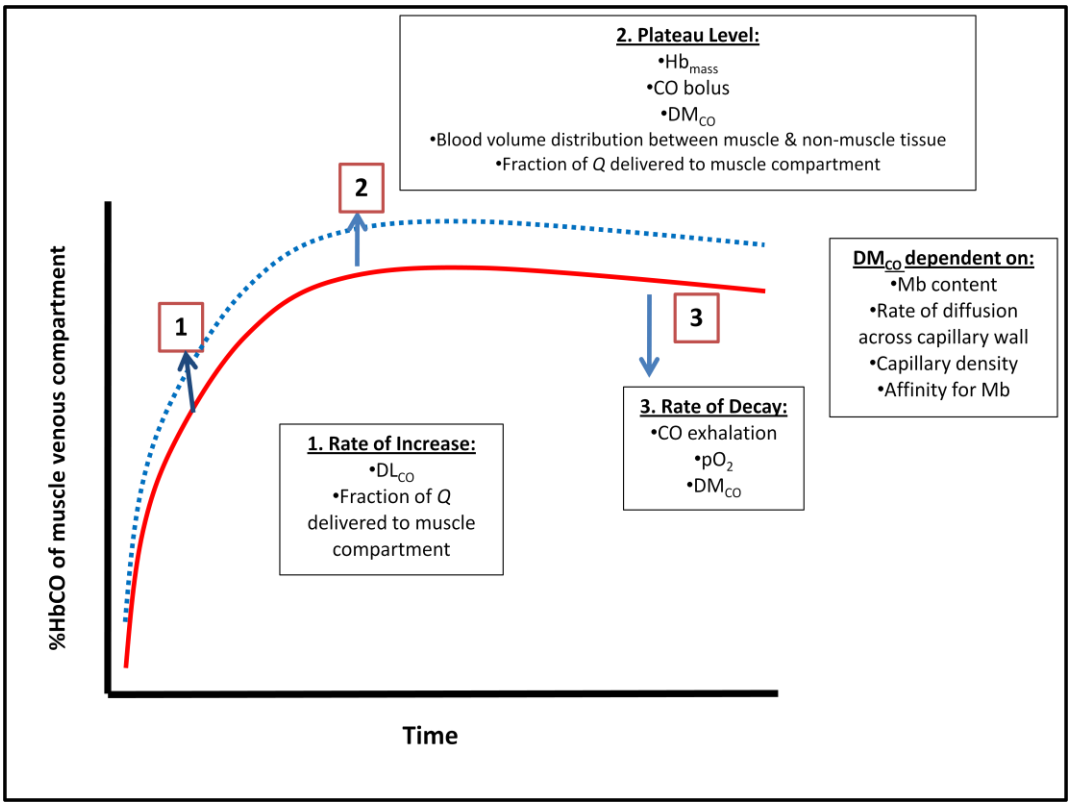

Figure 8-1: Factors influencing CO kinetics during CO rebreathing

Schematic representation of %HbCO of muscle venous compartment (Bruce & Bruce 2003, Chapter 2) with respect to time (unspecified scale) during CO rebreathing. 1. Rate of increase – the rate of appearance of HbCO in muscle venous blood, 2. Plateau level – the level at which no further increase in HbCO is achieved and equilibrium is reached between all compartments of circulation, 3. Rate of decay – the rate at which CO escapes from the muscle venous compartment, either via exhalation at the lungs, or through diffusion to extravascular compartments where it is bound to Mb. Q = Cardiac output; DL_{CO} = Lung diffusion capacity for CO; DM_{CO} = Muscle diffusion capacity for CO

8.1.2 Factors influencing Hb_{mass}

The Hb_{mass} of endurance athletes has previously been well documented and there is a clear distinction between elite endurance athletes (who possess an elevated Hb_{mass}) compared with untrained counterparts (Heinicke, Wolfarth et al. 2001; Schmidt and Prommer 2010). However, many unanswered questions remain which are associated

with the ability of Hb_{mass} to adapt to training and other external stimuli, as well as the importance of changes in Hb_{mass} in endurance athletes. The research in this thesis contributes to our understanding of the influence of a number of external factors on Hb_{mass}. More specifically, the data presented reveal the influence of stage racing, iron supplementation, training load, hypoxic exposure and removal of the hypoxic stimulus on Hb_{mass} in elite cyclists. **Figure 8-2** provides a summary of these findings within the context of elite endurance athletes.

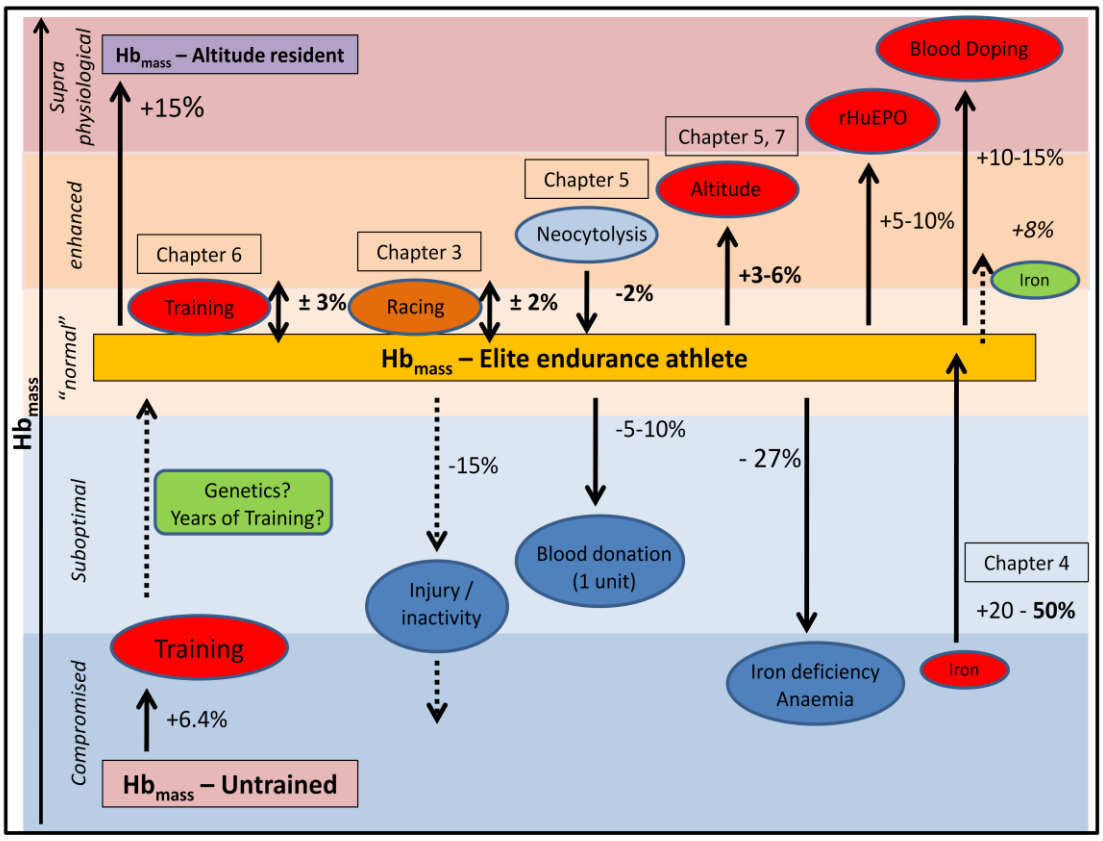

Figure 8-2: Summary of confounding factors to stability of Hb_{mass} in endurance athletes

Red symbols indicate a positive change, blue symbols indicate a negative change. Orange symbol indicates stable values. Magnitudes of change indicated alongside directional arrows. Data in **bold** contained in the thesis chapters indicated. Dotted arrows indicate areas for further investigation. Magnitudes of change obtained from data in Chapters 3-7 of this thesis and (Schmidt, Heinicke et al. 2002; Prommer, Heckle et al. 2007; Schmidt and Prommer 2008; Schumacher, Ahlgrim et al. 2008; Schumacher, Pottgiesser et al. 2008; Clark, Quod et al. 2009; Treff, Schmidt et al. 2009; Lundby and

Robach 2010; Robertson, Saunders et al. 2010; Schmidt and Prommer 2010; Wachsmuth, Aigner et al. 2010)

In this model, the reference point for 'normal' Hb_{mass} is deemed that of an elite endurance athlete; such that a highly active individual, with adequate iron stores and who is not sick or injured will be characterised by a Hb_{mass} within a 'normal' range. Perturbations to Hb_{mass} may result in substantial and meaningful changes in either a positive or negative direction. For example, should the athlete stop training due to injury or illness, Hb_{mass} may decrease to a 'suboptimal' or 'below normal' level, whilst severe blood loss or iron deficiency anaemia may decrease Hb_{mass} to such an extent that endurance capabilities are compromised. In contrast, endurance training or altitude exposure may serve as tools to optimise or enhance Hb_{mass} above the normal range, whereas blood manipulations have the potential to increase Hb_{mass} to supra physiological levels. It is important to note that in this model, untrained individuals possess a 'sub optimal' or compromised Hb_{mass}, for which the scope for improvement remains to be determined (Schmidt and Prommer 2008).

The influence of stage racing on Hb_{mass} was investigated in Chapter 3. Similar to previously published research (Schumacher, Pottgiesser et al. 2008), our data indicate that in elite cyclists, Hb_{mass} remains stable during periods of intense exercise (i.e., 1 week cycling stage race). Furthermore, the successful application of the CO rebreathing technique during a sanctioned UCI event provides support for its potential use as an anti-doping tool. However, further investigation following similarly demanding endurance events involving other sports (e.g. marathon running, Ironman triathlon) or less conditioned athletes is warranted. As described earlier, physical trauma, muscle damage and long lasting changes to muscle blood flow associated with other endurance events have the potential to influence the HbCO

curve (**Figure 8-1**) and must therefore be carefully investigated before applying the CO rebreathing method in these settings.

Blood manipulation remains the largest and most rapid influence on Hb_{mass}. Interestingly, the magnitude of change in Hb_{mass} induced by iron supplementation in anaemic or iron deficient athletes may exceed changes associated with autologous blood transfusions (up to 50 %, Chapter 4); however, importantly, these changes serve to restore Hb_{mass} to homeostatic "normal" rather than "supra-physiological" levels. The effect of iron supplementation in athletes with 'normal Hb_{mass}' values is yet to be determined and could potentially pose an ethical dilemma if IV iron administration is found to noticeably enhance Hb_{mass} via 'natural' means.

For endurance athletes seeking to enhance Hb_{mass} via legal and natural means, hypoxic exposure induces a greater erythropoietic effect than training *per se* and in some individuals can invoke a Hb_{mass} response close to the lower boundary of some blood manipulations (**Figure 8-2**), albeit somewhat transiently. In Chapter 5, data are presented which document the time course of the Hb_{mass} response to natural altitude training. Substantial increases in Hb_{mass} were observed in a group of elite cyclists following only 10 days of exposure – revealing a much faster time course than previously anticipated (Wilber, Stray-Gundersen et al. 2007). These results indicate that even in terms of Hb_{mass}, short duration altitude camps may have some efficacy, especially if they are more easily incorporated into the training and competition schedule than traditional longer exposures (3 - 4 weeks).

It is possible that the time course of Hb_{mass} observed in Chapter 5 may be influenced by the timing of the altitude exposure relative to the yearly training cycle. The athletes involved in the study commenced the altitude training block immediately

following a mid-season break, and as such may have presented with a suboptimal or slightly lower than 'normal' Hb_{mass} (**Figure 8-2**). Therefore in this case, altitude exposure may simply have served to accelerate Hb_{mass} back to normal values, raising a potentially alternative use for altitude training – as a means of optimising Hb_{mass} at various times of the season (e.g. pre season, or mid season) or when returning from injury, as opposed to directly before competition. Furthermore, whilst epidemiological data indicate that long-term adaptation to hypoxia can result in a sustained elevation in Hb_{mass} (Schmidt, Heinicke et al. 2002; Brothers, Doan et al. 2010), the effect of repeated short-term exposures over time is not well established. Repeated exposures could potentially drive a gradual upward adaptation in a similar manner to fitness and training adaptations observed with progressive overload and recovery. Research on this topic should be more readily available in the coming years, due to in part to the relative ease of Hb_{mass} measurement in elite athletes, as well as the increasing popularity of altitude training.

8.1.3 Hb_{mass} and Cycling Performance

The ability to answer the question of "*how important are changes in Hb_{mass} for performance?*" hinges on our ability to define and measure performance. Cycling performance is influenced by a multitude of variables, including environmental conditions (barometric pressure, temperature and wind speed), rolling resistance, mass, tyre pressure, drag, pacing and motivation (Olds, Norton et al. 1995; Olds, Norton et al. 1995). In contrast, cycling specific fitness, e.g. maximal mean power over 4 minutes (MMP_{4min}) is a far more tangible and measurable trait (Quod, Martin et al. 2010). Therefore, for the purposes of the research in this thesis, cycling

performance was defined in relation to cycling specific fitness as opposed to how fast the bike travels.

In Chapter 6, an attempt was made to determine the interaction between training load and Hb_{mass} across a competitive season. Subtle interactions were observed, which lay on the cusp of measurement error when changes in training load were small. However, over time a progressive overload associated with training and racing during a season resulted in a measurable change in Hb_{mass}. Similarly, oscillations in cycling performance (reflected by MMP assessed using cycling power meters during training and racing) were observed, which were positively associated with changes in Hb_{mass}. These findings suggest that Hb_{mass} can indeed be influenced by the typical periodisation of training load experienced by elite cyclists, and further, in a manner which mirrors changes in performance. It is therefore, intuitively appealing to suggest that athletes and their coaches actively pursue ways to maximise Hb_{mass}. Certainly, currently available research does not indicate any negative effects of an enhanced Hb_{mass} within a range that does not noticeably increase blood viscosity (Telford, Kovacic et al. 1994), and the reported strong relationship between Hb_{mass} and VO_2max certainly presents strong rationale that aerobic capacity benefits from increases in Hb_{mass} (Heinicke, Wolfarth et al. 2001; Schmidt and Prommer 2010). However, whilst a positive correlation between changes in Hb_{mass} and cycling performance was observed, the longitudinal design adopted does not allow us to assume nor prove that the observed changes in Hb_{mass} *per se* directly influence cycling fitness and subsequently performance. Rather, concomitant physiological adaptations arising from the changes in training load may occur and these adaptations may be more important for supporting improved performance than the observed changes in Hb_{mass}.

The value and importance of the transiently acquired Hb_{mass} in response to altitude exposure has been widely debated (Gore and Hopkins 2005; Levine and Stray-Gundersen 2005). From a practical perspective, many sport scientists have been lulled into the belief that altitude training is only effective if it is accompanied by an increase in Hb_{mass} and similarly, that performance gains following altitude training are solely due to increases in Hb_{mass} (Levine and Stray-Gundersen 2005). The data presented in Chapter 7 question both of these assumptions. Cyclists who had their Hb_{mass} response to LHTL 'clamped' via repeated phlebotomy, were surprisingly able to produce similar improvements in performance in a cycling specific task (MMP_{4min}) compared with those possessing altitude-induced increases in Hb_{mass}. In this study, it appears that the 'clamped' cyclists supported their improved performance using O_2-independent metabolism as VO_2max was unchanged, whereas the athletes who possessed an altitude-induced increase in Hb_{mass} were able to utilise their newfound improvements in aerobic capacity. These results support previous findings which hint at an uncoupling between the Hb_{mass} – VO_2max and/or performance relationship following altitude training (Saunders, Telford et al. 2009; Robertson, Saunders et al. 2010). However, the most intriguing finding of the study described in Chapter 7, was the effect of clamping Hb_{mass} on a repeat performance task. Following ten minutes of recovery, the cyclists were instructed to ride until exhaustion at peak power output, yet the clamp group were not able to achieve even baseline standards. This observation suggests that enhanced Hb_{mass} may play a role in recovery between efforts, and increases the robustness of any performance enhancement achieved via altitude training. Our data only allow us to speculate on the mechanisms involved, but it is possible that the enhanced Hb_{mass} acts via a feed forward mechanism which allows the most appropriate metabolic pathway or pacing

strategy to be utilised at the time, as well as a feedback mechanism via blood buffering and lactate clearance.

8.2 FUTURE DIRECTIONS

The majority of altitude research has examined performance responses only immediately after cessation of hypoxic exposure. A detailed investigation of the time course of Hb_{mass} and performance responses for a prolonged period of time following the altitude training period may provide more insight into the interaction between Hb_{mass} and endurance performance. Anecdotally, it has been suggested that the best performances following altitude exposure present themselves 4 - 6 weeks later (Millet, Roels et al. 2010), by which time Hb_{mass} will most likely have returned to baseline values (Robertson, Saunders et al. 2010). Such a finding would contradict the belief that increases in Hb_{mass} are primarily responsible for increases in performance following altitude training (Levine and Stray-Gundersen 2005). Alternatively, it is possible that Hb_{mass} increases associated with altitude training result in additional adaptations which support and underpin improvements in endurance performance. Thus, Hb_{mass} increases may be observed when good training environments transpire, but Hb_{mass} *per se* is not required for most traditional measurements of performance. Perhaps only with repeat tests, are we able to observe the type of fitness gains that may be supported by an enhanced Hb_{mass}. In addition, it follows that after a period of altitude training, the additional Hb_{mass} acquired may support a period of more intense or better quality training – allowing an athlete to train harder, for longer or more frequently. The fruits of such training may present as performance gains 4 - 6 weeks later, as anecdotes suggest, even though Hb_{mass} may no longer be elevated. Investigation of this concept by incorporating regular Hb_{mass}

measurement with careful and detailed training monitoring techniques would provide important and extremely useful information to coaches looking to optimise the timing of altitude exposure prior to competition.

Finally, a novel theme which has emerged based on observations made throughout this thesis and which warrants additional research, is that changes in Hb_{mass} may serve as a marker of an athlete's "adaptive state." In other words, an athlete who is adapting to a training or hypoxic stimulus may demonstrate an increase in protein synthesis relating to a number of different proteins, including Hb. Thus an increase in Hb_{mass} may reflect that many important adaptive pathways have been activated, with associated increases in related proteins. For example, positive changes in Hb_{mass} associated with endurance training, may indicate that favourable metabolic adaptations are occurring which subsequently impact on fitness and performance, but which are far more difficult or invasive to measure. If this is the case, Hb_{mass} could be a surrogate for protein synthesis and longitudinal monitoring of Hb_{mass} may be a potential means for not only assessing fitness traits but also training effectiveness, fatigue state and performance readiness in conjunction with other endocrine markers. More specifically, in the case of altitude exposure, increases in Hb_{mass} may reflect that the HIF-1α and erythropoietin primed cascade have been activated, which in its self may result in a multitude of adaptations throughout the body.

The individual data presented in this thesis highlights the variability of the Hb_{mass} response to the same stimulus, e.g. some athletes exhibited a very large increase in Hb_{mass} in response to LHTL whereas others did not respond at all. Furthermore, whilst the mean Hb_{mass} response to LHTL has shown to be reproducible in a group of runners (Robertson, Saunders et al. 2010), individual responses were not always of

the same magnitude. Therefore, investigation of the best physiological and nutritional state in which to present for favourable Hb_{mass} adaptation, as well as the most suitable type of training to employ during the proposed period of adaptation, would be both exciting and challenging, as well as providing valuable information for coaches and athletes alike.

8.3 CONCLUSION

Overall, this thesis aimed to investigate the factors which can influence Hb_{mass} in elite endurance athletes and to determine the importance of such changes for cycling performance. It is hoped that the data presented offers additional insight into the complexities of Hb_{mass} - performance relationship and is of use to sports scientists, coaches and athletes in their quest for athletic success. This thesis confirms some existing beliefs, particularly regarding the influence of a variety of external factors on Hb_{mass}, but challenges other notions regarding the importance of Hb_{mass} for traditional measures of endurance performance of elite athletes. Indeed, the traditional concept that increasing Hb_{mass} results in an increase in VO_2max and thereby endurance performance, may be somewhat simplistic. Perhaps instead of a direct cause and effect relationship between Hb_{mass} and performance, it may be more intuitive to consider Hb_{mass} changes as an indicator of training and adaptive states which lead to improved performances.

APPENDIX

Appendix I

Conference Poster:

Garvican LA, Martin DT, Eastwood A, Ross MLR, Abbiss CR, Gripper A, Zorzoli M, Schmidt W, Gore CJ.

Haemoglobin Mass, Hct and [Hb] throughout a 6d UCI ProTour cycling race.

14th Annual Congress of the European College of Sports Science 200

Haemoglobin Mass, Hct and [Hb] throughout a 6 day UCI ProTour cycling race

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Introduction:

Haemoglobin concentration [Hb] and haematocrit (Hct) can decrease in response to multiple days of cycle racing due to a plasma volume expansion.[3] However, total haemoglobin mass (tHb-mass) appears to remain stable during short stage races in amateur cyclists.[3]

Can tHb-mass be used as an additional anti-doping detection parameter? [2]

Criteria:

1. Test time <15min, Typical Error <2%[1]
2. Stable during 1-3wk stage races.

Questions:

How stable is tHb-mass during a hot 6 day cycling stage race?

Can tHb-mass be quantified daily during a UCI ProTour event?

Methods:

The Race: Tour Down Under, UCI ProTour, Australian summer **(Table 1)**

Study Design: tHb-mass measured using carbon monoxide rebreathing[1] within 4 h of stage finish on D1-5.

Duplicate measures averaged to establish baseline (D0).

[Hb] and Hct measured on D0, D3, D6 from morning, fasted, venous blood samples following UCI guidelines.

Subjects: Professional Cyclists (n=6♂) on the same team (age 24 ± 5 y, height 179 ± 5 cm, mass 71 ± 4 kg; mean ± SD). Stage and final General Classification ranged from 7th – 114th.

Control subjects (n=5♂) recreationally-active (31 ± 6 y, 179 ± 6 cm, 74 ± 6 kg)

Results:

Reliability of tHb-mass measurement:

Typical Error (TE) from two baseline measures 1.3% (95% CL; 0.9-2.5%)

95% Confidence Limit for the change scores in tHb-mass was 3.6% calculated from the baseline Typical Error (TE x √2 x Z-score) **(Figure 1)**

Table 1: Topographic, climatic and race characteristics of the 2009 Tour Down Under.

	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5	Stage 6
Distance (km)	140	145	136	143	148	90
Winner's Time (hour:min)	3:45	3:46	3:15	3:29	3:28	1:42
Profile	Flat	Flat	Hilly	Hilly	Hilly	Criterium
Temp (C)	40	34	30	34	31	36
Mean Power (W)	161	196	235	181	211	241

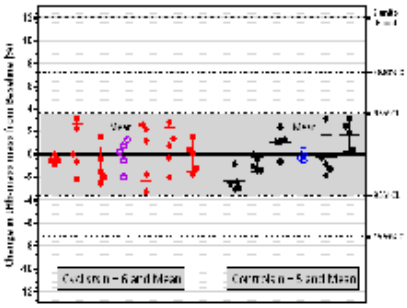

Figure 1: Individual and mean changes (± SD) in tHb-mass (%) from baseline (D0) during the race. Shaded area indicates 95% confidence limits (CL) associated with measurement error for a change score. For reference, expected changes due to blood doping are indicated.[3]

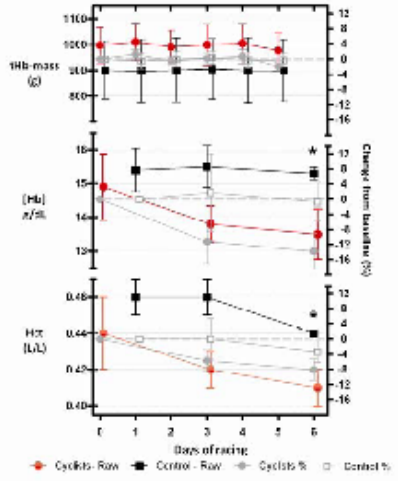

Figure 2: tHb-mass, [Hb] and Hct (mean ± SD) before and during the race. Change from baseline (%) shown on right Y axis. (*denotes substantial difference between the groups in the change scores from D0).

Within Subject variability:

Individual coefficient of Variation (CV):

Cyclists = 0.3-2.3%

Controls = 0.7-1.7%

Mean CV (95% CL):

Cyclists = 1.6% (1.3-2.1%)

Controls = 1.3% (1.0-1.7%)

Daily change scores (Figure 2):

Mean tHb-mass in Cyclists was within ± 1.9% (20 g) of D0 throughout the tour.

Mean change scores in Cyclists ranged from -1.9% at D5 to +1.3% at D2.

Controls (901 ± 113 g) = ± 0.5% (5 g).

No substantial difference in the daily change scores for tHb-mass between Cyclists and Controls.

At D6, mean change scores for [Hb] and Hct were substantially lower in the Cyclists vs. Controls:

[Hb] = -8.9%; (95% CL: -13.3 to -4.2%)

Hct = -4.7%; (95% CL: -8.8 to -0.5%)

Discussion:

Individual CV for tHb-mass was <2.5% in the 11 cases that were measured.

The largest individual change from baseline on any one day was ± 3.2% (34 g).

A change in tHb-mass from baseline values > ± 3.6% during a 1 week stage race exceeds the 95% CL; i.e. 1:20. Changes > ± 7.2% would exceed the 99.99% CL; i.e. 1:10,000.

Further research is required to establish the normal variability of tHb-mass during longer, 1-3 wk stage races in elite cyclists.

Conclusion:

- **tHb-mass was stable over 6 days of racing in hot environments**
- **tHb-mass can accurately be measured during a cycling tour**
- **tHb-mass has potential as an additional anti-doping detection tool**

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Photos courtesy of cyclingnews.com and M Ross

Appendix II

Photographic summary of this thesis:

(Clock wise from top left)

1. *Altitude group at Passo dello Stelvio, 2007*
2. *Vicki Whitelaw warming up for the World Championship Time Trial, Varese 2008*
3. *Easter egg hunt with AIS women's road team, 2008*
4. *Analysing capillary bloods on OSM – 3, 2008*
5. *Will Walker performing the CO rebreathing test at 2760 m, Stelvio, 2007*
6. *Valverde performing a VO2max test before Tour Down Under, 2010*
7. *Laura and Nick Brown discussing the menu at the Science of Cycling Congress, Varese, 2008*
8. *Dinner time at Passo dello Stelvio, 2007*
9. *Laura participating as a subject in the comparison of CO kinetics in the Burge and Schmidt methods (Chapter 2), 2008*
10. *Laura prepares a slushie and towels to 'pre-cool' Michael Rogers at pre-Olympic camp, Varese, 2008*
11. *Laura, with supervisors Chris Gore and David Martin at presentation of the Robert T Withers – AIS Award, Canberra, 2010*

Always walk through life as if you have something new to learn and you will.
~Vernon Howard

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