2015

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School of Exercise & Health Science

The Regulation of Human Iron Metabolism in Hypoxia

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BSc. (Hons), Grad. Dip. Ed

This thesis is submitted for the award of Doctor of Philosophy (Sports Science) from the School of Exercise & Health Science, Faculty of Engineering & Health Science

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Date of Submission:

28/08/2015

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is not included in this version of the thesis

USE OF THESIS

The Use of Thesis statement is not included in this version of the thesis.

ACKNOWLEDGEMENTS

On the Process...

"Philosophy starts with wonder...

Indeed it does. Wonder, not merely in the academic sense, but also in life's bigger questions. What do you want? How far can you go? What are you willing to do to get there? In this respect, the PhD process has taught me many things, patience, persistence and determination. Quite often, it takes you to your limit, or beyond, but that is the very thing that keeps us progressing. In doing so, we learn much about our character. In essence, the most important teaching of the PhD is thus not academic at all. The most important lesson is perhaps not what we get, but who we become from our endeavours, that make them worthwhile.

The acknowledgements section was the most enjoyable part of the PhD to write. Where else can you be (almost) excused from writing in non-academic English? Consequently, I'll take this rare opportunity to be slightly rebellious and use the passive voice, split the infinitive, abbreviate words using apostrophes, start sentences with the word "but" and use the Oxford comma. More importantly, where else can you truly thank those who've helped you develop both academically and interpersonally? Through this process, we realise how far we've progressed in our life, the friendship we've made or strengthened and what is truly important to us. Therefore, as this journey nears its end, it fills me with nothing but gratitude for those who've walked this road with me.

...and, at the end, when philosophic thought has done its best, the wonder remains"

Alfred North Whitehead, Mathematician (1861-1847)

Yes, I think it does. On the other hand, perhaps a better state of confusion?

On those who have Shared the Journey...

Supervisory Panel

A/Prof Chris Abbiss: Thank you for your supervision, patience, understanding and guidance throughout the process. A fellow coffee lover, although I would have been hospitalised with heart palpitations if I consumed half as much as you did! I hope we can continue to do good things together.

Dr. Peter Peeling: You have been there from the very beginning. Thank you for believing in me and mentoring me every step of the way, both in sport and in life. Whether it was me falling off a treadmill at 18 $km \cdot h^{-1}$ during your own PhD, or me trying to beat your half marathon time (that took a while), or debating why 03:00 in the morning is definitely not the best time to go to the beach – it has been good fun. I am truly grateful for your commitment and efforts on my behalf.

Dr. Laura Garvican-Lewis: Laura, Laura, Laura. Thank you for keeping me on track throughout the process. You have gone from strength to strength in the four years I have known you. When we first met, you were almost at exactly this stage, ready to submit your PhD. Since then, you have got a PhD, run under 3:00 for a marathon, got married, had a daughter and continued to publish extensively. I'm not sure how you fit it all in. I think we have learnt a lot from each other during this thesis and I hope we continue to do so.

Prof. Chris Gore: A wholehearted thank you for giving me an opportunity at the Australian Institute of Sport as a physiology Occupational Trainee in 2011. Your guidance has been invaluable. You set a great example everyday with your integrity,

work ethic and attention to detail. I only hope I can emulate these qualities throughout my career.

Edith Cowan University

Dr. Greg Haff: Doc Haff. Your guidance, friendship and mentorship have been helped me immensely throughout the process. I know I could always count on you for a good chat about physiology and the scientific process. You even managed to get me to lift (albeit briefly), which is no mean feat, given my physique! I look forward to our friendship in the years to come, we've work to do!

Dr. Ihsan: A trusted friend and colleague. I am very grateful for guidance and advice. Even if you were constantly pestering me to play "wall-y" or telling why Arsenal will win the Premier League this year. However, you were always on hand with some sage advice or just to talking about running! I look forward to working together again in the future.

Dr. Chris Joyce: Joyle – my fellow Englishman, even if you are from Middlesbrough. Thanks for everything early on. Another one who has quietly achieved – PhD, job, got married and had children...Curry and beers, mate?

Dr. Carl Woods: Carlitos, I always enjoyed your insights and friendship. You worked very hard to be to be where you are now, so you very much deserve it. You have some great ideas and I know you will go from strength to strength in the coming years. I hope we can continue to share this journey.

Alan Metcalfe: Thank you for being a good friend. We've had a blast. So many memories, although I think half of them are "not suitable for print". No doubt, you'll retain your love of biscuits and baked beans, sometimes together in the same meal.

Keep plugging away and moving forward. My advice to you: 1) Don't buy any magic beans or book any holidays without asking me first, 2) avoid German girls, 3) why are you reading this? Don't you have work to do? ;-).

PhD Office: So many people are part of the bigger picture here. A sincere thank you those in the ECU PhD suite who have shared the journey with me or had to enduring my venting (everyone) or blindly walked into my humour (Tina – every time!). You've made it fun.

Australian Institute of Sport, Canberra

Lachlan Mitchell: We have already learnt so much from each other. Whenever I get an idea, as rare as it may be these days, I always think "if only Lach was here!" Similarly, if my R code wasn't working (pretty much daily), I always had you to bounce some ideas off, or to point out I'd missed a comma somewhere on line 3,567, hence why the code won't run. Indeed, we've had endless debates about everything, where of course, we are both ALWAYS right. We still do. When all is said and done, I am happy you could be part of this with me. Furthermore, who else would retrieve my bag from the police station after I've left it in a park during a half marathon, or organise my birthday party when I've managed to lock myself out of my house one hour before my birthday party is about to start. As you know, this is normal for me, so you just sigh and say "you idiot" but then help me out. We've come a long way together and I look forward to our friendship in the years to come.

Dr. Brad Clark: My partner in crime. I don't need to say anything to you, we've already solved the world's problems. At the end of the day, we're not saving lives here mate. Coffee?

Mel Shurrey (aka Mrs. Clark): Mel, it has been an absolute pleasure getting to know you throughout my PhD. They say behind every great man, is a great woman. That is certainly true here. Thanks your hospitality, cooking, patience (especially when Brad and I had taken over the lounge to play FIFA) and, most of all, your friendship.

Avish Sharma: An original A-Team member. You've a bright future ahead of you Avish. I really enjoyed watching you grow both professionally and personally during my time at the AIS. Thanks for all your help throughout data collection. We've had some great times haven't we? There are many more to come mate! #gotitdone.

Val Chan: Confusing at the best of times, but always willing to help out. You've overcome a lot yourself and I'm thankful for your listening ears and occasional piece of salacious gossip.

Dr. Philo Saunders: Philo, thanks for all your help with data collection and your mentorship over the years. I won't thank you for consisting taking me to pieces on every long run and track interval session, but keep doing what you're doing. You seem to be getting younger with age. I'm not sure how, but it works.

Prof. David Pyne: Pyney, I have always been thankful for your guidance and insights on all things sport. You always had time for me and steered me in the right direction. I do recall endless hours during my time at the AIS repairing many spreadsheets and doing all things Rugby League – a sport I am happy to admit I know absolutely nothing about and is the antithesis of my physique. I will never forget your pearling one-liners, mostly at AIS staff meetings but they could crop up just about anywhere. Only you can get away with those!

Dr. Chris Barnes: Much like the first Turning machine, somewhat of an enigma. When we can find you. However, a man with a big heart who was always willing to provide some statistical support and a quirky anecdote. Upon reflection, perhaps refrain from telling your story about when you spent time in the markets in Addis Ababa...Thank you very much for your patience and understanding.

Dr. Graeme Allbon: Absolute legend! Thank you very much for your assistance in running bioassays for this project and general life advice. Another who know the truly valuable things in life.

Institut für Sports-und Präventivmedizin, Saarbrücken

Dr. Sabrina Skorski: Frau Skorski. Be it in Perth, Adelaide, Canberra, Malmö, Paris, or Saarbrücken, I've always enjoyed your company and our heated debates. We've had some great times and I hope there are many more to come. Danke schön!

Caroline Schneider: From when we first met, to the present moment, I've enjoyed every minute. Your friendship, support and happy disposition has meant a lot to me. Merci beaucoup et j'attends avec impatience te voir encore

Murdoch University

Nathan Lawler: Half of this is yours too Nathan. Thanks for your help during to data collection. I couldn't have done it without you. I have an idea...;-).

Family

Mum & Dad: To you, I owe everything. You selflessly sacrifice so much on a daily basis to make sure I've always had what I needed. So many times, I've been so grateful to come home to Mum's cooking, or watch the football with Dad (Chelsea of course), or

just someone to chat to. You keep me balanced. You invested in my education to make sure I had a better life, so this thesis is for you. I very much hope I can make the most of the opportunities you've given me.

Daniel, Matthew, Luke: Boys, thank you for keeping me grounded and motivating me to be better. That said, we all know I'm still house FIFA champion.

Arthur: If you think you can, you might. In the end I did. Rest in peace. With a decent pint of real ale of course and with Fulham, Chelsea and Kingstonian playing good football. Well, maybe 1/3 ain't bad?

To the Reader...

I recommend reading this thesis with a glass of red wine (for medicinal purposes of course!) or another suitably strong alcoholic beverage. Whilst this thesis may not be a literary masterpiece, I hope its outcomes inform you as much as they have informed me.

Are you sitting comfortably? Then I'll begin...

ABSTRACT

Athletes commonly use altitude exposure in an attempt to improve their aerobic performance at sea level. Altitude exposure enhances erythropoiesis and iron-dependent oxidative and glycolytic enzyme production, for this reason, athletes must maintain a healthy iron balance at altitude. A negative iron balance at altitude may limit such physiological adaptations, potentially reducing the performance benefits of altitude exposure.

This thesis examined the regulation of iron metabolism during acute (~31 min, Study One) and prolonged altitude exposure (14 days, Study Two). Finally, Study Three examined how daily oral iron supplementation influenced haemoglobin mass (Hb_{mass}) and iron parameter responses to prolonged, moderate altitude exposure in a large cohort of elite athletes. Specifically, Study One found acute (~31 min) interval exercise [5 × 4 min at 90% of the maximal aerobic running velocity (vVO_{2max})] increased post-exercise interleukin-6 (IL-6) production and elevated hepcidin production 3 h thereafter in both normoxia (fraction of inspired oxygen (F_IO_2) = 0.2093) and normobaric hypoxia (i.e. 3,000 m simulated altitude; F_IO_2 = 0.1450). These results suggest exercise performed in acute hypoxia does not alter the post-exercise hepcidin response, relative to exercise in normoxia, possibly owing to the short duration of the hypoxic stimulus.

Prolonged altitude exposure suppresses resting hepcidin levels in sojourning mountaineers, but its influence on the post-exercise hepcidin response exercise has not yet been investigated. Therefore, Study Two investigated how 14 days of live high: train low (LHTL) (exposure to 3,000 m simulated altitude for 14 $h{\cdot}d^{-1}$) influenced resting levels of hepcidin, erythropoietin (EPO) and blood iron parameters. Study Two also examined the post-exercise hepcidin and iron parameter responses to interval exercise

(5 × 1,000 m at 90% of the maximal aerobic running velocity) performed in normoxia (600 m natural altitude) and normobaric hypoxia (i.e. ~3,000 m simulated altitude), following 11 and 14 days of LHTL. The post-exercise hepcidin response was compared with interval exercise performed at a matched exercise intensity in normoxia or hypoxia before LHTL. Here, LHTL suppressed resting hepcidin levels after two days of exposure, but the post-exercise hepcidin response to interval exercise was similar in normoxia and hypoxia, both before and after LHTL. Additionally, Hb_{mass} increased by 2.2% and plasma ferritin levels decreased following LHTL. In conclusion, prolonged, moderate altitude exposure suppresses resting hepcidin levels, which likely ensures more iron can be transported to the erythron to support accelerated erythropoiesis.

Prolonged altitude exposure places a large burden on body iron stores because additional iron is required to support accelerated erythropoiesis. Accordingly, athletes often ingest oral iron supplements during altitude exposure to ensure they maintain a healthy iron balance. By analysing ten years of haematological data collected from well-trained athletes who undertook two-to-four weeks of LHTL at simulated (3,000 m) or natural (1,350-2,700 m) altitudes, Study Three established how oral iron supplement dose moderates the Hb_{mass}, serum ferritin and transferrin saturation response to prolonged moderate altitude exposure. In general, athletes supplemented with 105 $mg \cdot d^{-1}$ or 210 $mg \cdot d^{-1}$ of oral iron supplement increased their Hb_{mass} from pre-altitude levels by 3.3% and 4.0% respectively. Serum ferritin levels decreased by 33.2% in non-iron supplemented athletes and by 13.8% in athletes supplemented with 105 $mg \cdot d^{-1}$ of oral iron, however, those athletes who ingested 210 $mg \cdot d^{-1}$ markedly increased their iron storage compartment by 36.8% after moderate altitude exposure. Thus, daily oral iron supplementation at altitude assists athletes to maintain a healthy iron balance, providing them with sufficient iron to sustain accelerated erythropoiesis.

In conclusion, this thesis suggests exercise in acute hypoxia does not seem to alter the post-exercise hepcidin response relative to exercise in normoxia, but prolonged altitude exposure suppresses resting hepcidin levels and may attenuate the magnitude of post-exercise hepcidin response after 14 days of LHTL. Finally, daily oral iron supplementation may support iron balance and Hb_{mass} production in athletes undertaking prolonged moderate altitude exposure.

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suppressing resting hepcidin levels. A reduction in whole body iron stores during prolonged altitude exposure also suppresses resting hepcidin production. A post-exercise increase in inflammation may override hypoxic hepcidin suppression during prolonged hypoxic exposure, regardless of the hypoxic stimulus.

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LIST OF PUBLICATIONS

The following chapters have been published in, or submitted to, academic journals for peer-review:

Chapter Three

Govus, A. D., Abbiss, C. R., Garvican-Lewis, L. A., Swinkels, D. W., Laarakkers, C. M., Gore, C. J., & Peeling, P. (2014). Acute hypoxic exercise does not alter post-exercise iron metabolism in moderately trained endurance athletes. *European Journal of Applied Physiology,* 114(10): 2183-2191. Impact factor: [2.3].

Chapter Four

Govus, A. D., Peeling, P, Abbiss, C. R., Lawler, N.G., Thompson, K., Peiffer, J.J., Swinkels, D. W., Laarakkers, C. M., Gore, C. J., Garvican-Lewis, L. A., (2015). Live high: train low - influence on resting and post-exercise hepcidin levels (*In review: Scandinavian Journal of Medicine and Science in Sport,* Impact Factor [3.1]).

Chapter Five

Govus, A. D., Garvican-Lewis, L. A., Abbiss, C. R., Peeling, P., Gore, C.J. (2015) Pre-altitude serum ferritin levels and daily oral iron supplement dose mediate iron parameter and hemoglobin mass responses to altitude exposure. *PLoS One.* 10(8):e0135120. Impact factor: [3.5]

Additional Publications Related to this Thesis:

Peeling, P., Sim, M., Badenhorst, C.E., Dawson, B.T., **Govus, A.D.**, Abbiss, C.R., Swinkels, D.W., Trinder, D. (2014) Iron status and the acute post-exercise hepcidin response in athletes *PLoS One* 9(3): e93002. Impact factor [3.5]

Conference Presentations:

Govus, A.D., Peeling, P., Abbiss, C.R., Lawler, N., Swinkels, D.W., Laarrakkers, C.M., Thompson. K.G., Peiffer, J., Gore, C.J., Garvican-Lewis, L.A. (2015) Fourteen days of live high: train low altitude exposure does not alter the post-exercise hepcidin response. *20th Annual Congress of the European College of Sports Science. Malmö, Sweden*

Competitive Research Grants ($25,556):

Govus, A.D., Abbiss, C.R., Peeling, P., Garvican, L.A., Gore, C.J. (2013) Iron regulation at altitude: and investigation into the influence of acute exposure, chronic responses and best practice iron supplementation. *ECU Higher Degrees by Research – Mobility Grant* ($5,000).

Garvican-Lewis, L.A., **Govus, A.D.**, Abbiss, C.R., Peeling, P., Gore, C.J. (2013) The effect of acute hypoxic training on post-exercise iron status, inflammatory cytokine and hepcidin levels in well-trained male endurance runners. *Australian Institute of Sport – High Performance Sports Research Fund* ($20,556).

1 CHAPTER ONE

INTRODUCTION

1.1 Overview

This doctoral thesis contains three studies investigating iron metabolism during either acute or prolonged hypoxia. Specifically, the first two studies (Study One and Two) of this thesis examined the regulation of iron metabolism following exercise performed in normoxia and hypoxia during acute (~31 min, Study One) and prolonged (two weeks, Study Two) hypoxic exposure. Finally, the third study analysed the effect of oral iron supplement dose on the haemoglobin mass (Hb_{mass}) and iron parameter responses to prolonged, moderate altitude exposure in a large cohort of well-trained athletes.

1.2 Background

Endurance athletes use two main methods of hypoxic exposure to enhance their aerobic performance: 1) acute hypoxic exposure, which involves intermittent exposure to, or exercise in, hypoxia several times per week, and 2) prolonged hypoxic exposure, consisting of several weeks exposure to natural or simulated, moderate, or high altitudes (3). Acute hypoxic exposure (<48 h) is considered too short to stimulate erythropoiesis, but may instead improve aerobic performance by enhancing oxidative and glycolytic enzyme concentrations (4). In contrast, given a sufficient hypoxic dose (i.e. altitude duration × elevation) (5), moderate altitude (i.e. >2,200 m) enhances Hb_{mass} by ~1% per 100 h of exposure (6). In addition, two-to-four weeks of moderate altitude exposure improves exercise economy, skeletal muscle buffer capacity, oxidative and glycolytic enzyme concentrations, in turn enhancing aerobic and anaerobic metabolism (7).

Haematological adaptations to prolonged altitude exposure are highly variable and are influenced by factors such as training volume, nutrition, illness, injury and pre-

altitude iron stores (8). Sufficient iron stores are required to support a three-to-five-fold increase in erythropoiesis as well as increased iron-dependent oxidative enzyme production at altitude (9). Athletes who are unable to maintain a healthy iron balance at altitude may develop a functional iron deficiency and form microcytic and/or hypochromic red cells, thus blunting the haematological benefits of altitude exposure (10). Additionally, low iron availability may compromise the synthesis of several iron-dependent non-haem proteins (11). Maintaining a healthy iron balance is therefore extremely important in supporting the hypoxic-mediated haematological and non-haematological adaptations to prolonged altitude exposure.

In addition to low pre-altitude iron stores, erythropoiesis may be compromised if iron is not rapidly mobilised from the labile iron pool to meet erythroid iron demands during initial altitude adaptation (12). The body's master iron regulatory hormone, hepcidin, regulates iron mobilisation from the labile iron pool (consisting mostly of iron contained in reticuloendothelial macrophages) (13). Hepcidin is a 25 amino-acid peptide hormone synthesised by liver hepatocytes that regulates systemic iron availability by internalising and degrading ferroportin iron export channels located on the cellular surface of iron storage cells such as reticuloendothelial macrophages and hepatocytes (14). Elevated hepcidin levels reduce iron efflux from storage cells into the blood plasma (15) consequently limiting iron delivery to the erythron. An increase in the inflammatory myokine, interleukin-6 (IL-6), immediately post-exercise has recently been shown to up-regulate hepcidin 3-6 h thereafter (16). Exercise intensity (17), duration (18) and pre-exercise serum ferritin levels (19) also moderate the post-exercise hepcidin response. Increased hepcidin levels post-exercise may impair intestinal iron absorption and iron recycling from senescent red blood cells, thereby transiently reducing plasma iron availability. In the long-term, such post-exercise

elevations in hepcidin may require athletes to mobilise iron from storage cells to maintain daily iron balance, which could reduce an athlete's iron stores if daily iron losses are not replenished.

Exercise during acute and prolonged hypoxic exposure presents two opposing mechanisms of hepcidin regulation. Whilst hypoxic exposure and exercise both increase IL-6 production (20), increased erythropoietin (EPO) production at high altitude suppresses hepcidin levels within 24-48 h (21-23). Hepcidin suppression is a favourable response to early altitude exposure since it enhances intestinal iron absorption and promotes the mobilisation of iron from storage sites to the blood plasma to support accelerated erythropoiesis. Conversely, since free (unbound) iron catalyses reactive oxygen and nitrogen specie formation (24), increased post-exercise hepcidin production is thought to protect the body from oxidative damage by sequestering iron in reticuloendothelial macrophages, thereby transiently reducing iron available to catalyse the formation of free oxygen and nitrogen radicals via the Fenton-Haber-Weiss reaction (25). To date, hepcidin's contribution to post-exercise iron metabolism during acute or prolonged hypoxic exposure has not been investigated. The dominant hepcidin regulatory pathway during either acute or prolonged hypoxic exposure protocols is therefore unknown. Further investigation into the hormonal control of post-exercise iron metabolism in acute and prolonged hypoxia may thus help athletes to maintain a healthy iron balance when using hypoxia as a complementary training method.

Oral iron supplementation may help athletes to maintain a healthy iron balance when undertaking prolonged, moderate altitude exposure. To date, no clear oral iron supplementation guidelines exist for athletes planning to undertake either acute or prolonged altitude exposure. Generally, oral iron supplements are prescribed to ensure

athletes have sufficient iron stores to sustain the three- to five-fold increase in erythropoiesis associated with prolonged altitude exposure (9) and are especially important in individuals with low pre-altitude serum ferritin levels (i.e. <35 $\mu g \cdot L^{-1}$). Oral iron supplementation, however, may not be required in those athletes with otherwise healthy pre-altitude serum ferritin levels (i.e. >35 $\mu g \cdot L^{-1}$), or if the hypoxic dose is low [for example, short sojourns (2 weeks or less) to low or moderate altitude (12)]. Furthermore, the optimal oral iron supplement dose necessary to support erythropoiesis during moderate altitude exposure is highly individual and iron uptake is influenced by an individual's current iron status and/or their ferrokinetics (26). Yet, despite the widespread use of hypoxic exposure methods, it is unknown how oral iron supplement dose influences Hb_{mass} production in athletes undertaking prolonged altitude exposure.

1.3 Statement of the Problem

Low pre-altitude iron stores and/or an inability to rapidly mobilise iron from storage sites to service erythropoiesis may blunt the haematological adaptations associated with prolonged altitude exposure (12). Hypothetically, an exercise-induced increase in hepcidin expression during the post-exercise recovery period could also compromise haem synthesis at altitude by limiting the transport of iron from storage cells to the bone marrow. Furthermore, elevated post-exercise hepcidin levels at altitude may transiently impair intestinal iron absorption during exercise recovery, thereby reducing plasma iron availability and iron incorporation into storage cells. Consequently, athletes may be unable to replenish their iron stores via dietary means, and thus become reliant on iron mobilised from the labile iron storage pool and hepatocytes to maintain iron status, further reducing their iron stores.

Exercise in hypoxia may augment post-exercise IL-6 production since exercise and hypoxia both promote IL-6 production (27). Theoretically, increased post-exercise IL-6 production following exercise in acute hypoxia could augment the magnitude of the post-exercise hepcidin response compared with similar exercise in normoxia. Practically, augmented post-exercise hepcidin production after exercise in hypoxia could make it difficult for athletes to maintain a healthy iron balance when using acute hypoxic exposure protocols since iron availability may be reduced. Alternatively, acute hypoxia may alternatively attenuate the post-exercise hepcidin response. Study One of this thesis therefore investigated how interval exercise performed in acute hypoxia influenced IL-6, hepcidin and iron parameter responses both immediately post-exercise and 3 h post-exercise in well-trained endurance athletes.

In contrast to acute hypoxic exposure, prolonged altitude exposure (>1 week) stimulates a three-to-five-fold increase in erythropoiesis (9). Insufficient iron delivery to the bone marrow during prolonged altitude exposure may therefore limit the maturation of erythroid precursor cells into erythrocytes. High altitude exposure suppresses hepcidin production within 24-48 h of (21-23) and likely helps to improve iron availability for erythropoiesis by promoting iron mobilisation from the labile iron pool. In comparison, increased post-exercise hepcidin production at altitude may reduce intestinal iron uptake and iron recycling from reticuloendothelial macrophages, potentially limiting iron available for haem synthesis. The post-exercise hepcidin and iron responses during prolonged altitude exposure, however, are currently unknown. To address this question, Study Two investigated how live high: train low (LHTL) influenced resting hepcidin, erythropoietin (EPO) and iron parameters levels after two and 14 days exposure to normobaric hypoxia (14 $h \cdot d^{-1}$ at 3,000 m simulated altitude).

Additionally, Study Two compared the post-exercise hepcidin and iron parameter response to a standardised interval running session performed in normoxia and simulated hypoxia both before and after two weeks of LHTL.

Athletes require sufficient iron stores to support accelerated erythropoiesis during prolonged altitude exposure, but may be unable to provide sufficient iron to synthesise haemoglobin if pre-altitude iron stores are too low (10), iron intake is inadequate, or iron transport to the erythron is insufficient (12). Athletes are accordingly prescribed oral iron supplements several weeks before, and during, prolonged altitude exposure to raise their iron stores to cope with accelerated erythropoiesis. The influence of different oral iron supplement doses on Hb_{mass} production and iron parameter responses, however, has not yet been investigated in a large cohort of well-trained athletes. Furthermore, a better understanding of how different oral iron supplement doses influence Hb_{mass} and iron parameter response to prolonged altitude exposure may allow sport scientists to develop altitude-specific iron supplementation guidelines for athletes planning to undertake moderate altitude exposure, in turn ensuring athletes can better maintain a healthy iron balance at altitude.

1.4 Significance of the Research

This thesis aimed to further the current understanding of how athletes regulate iron metabolism following interval exercise performed during acute and prolonged hypoxic exposure. Specifically, the findings of this thesis could enable medical practitioners, sports physiologists and dieticians to refine the current guidelines for dietary iron intake and oral iron supplementation relevant to acute and prolonged altitude exposure, which may in turn enable athletes to better regulate their iron balance when undertaking altitude exposure. Additionally, improving iron availability

by providing oral iron supplements during prolonged altitude exposure could reduce an athlete's risk of developing a functional iron deficiency by ensuring their pre-altitude iron stores can support accelerated erythropoiesis without depleting the body's iron storage pools. Finally, maintaining a healthy iron balance at altitude may ensure sufficient iron is available to support iron-dependent, non-haematological adaptations to altitude, potentially enhancing aerobic performance in normoxia or hypoxia.

Occupations requiring workers to perform physical and cognitive tasks at high altitude, such as military personnel, may also be interested in the outcomes of this thesis. Maintaining a healthy iron balance at altitude may assist these individuals to adapt to high altitudes more effectively since low serum ferritin levels are associated with reduced work capacity and cognitive ability (28). Furthermore, iron supplementation at high altitude may protect against acute altitude sickness (29).

1.5 Research Aims

This thesis aimed to investigate how acute (single session) and prolonged (two weeks) altitude exposure (~3,000 m simulated altitude) affects the body's master iron regulatory hormone, hepcidin, and to determine its influence on iron metabolism in well-trained endurance athletes undertaking different forms of altitude exposure. This thesis also aimed to determine how oral iron supplement dose moderated the Hb_{mass} and iron parameter responses during two-to-four weeks of altitude exposure in well-trained athletes.

Specifically, the studies presented in this thesis aimed to investigate:

1.5.1 Study One

- The post-exercise inflammatory, hepcidin and iron parameter response to acute exercise in hypoxia (3,000 m simulated altitude).

1.5.2 Study Two

- The influence of 14 days of LHTL on resting and post-exercise hepcidin and iron parameter levels.

1.5.3 Study Three

- The influence of oral iron supplement dose on Hb_{mass} and iron parameter responses in well-trained athletes during prolonged (two-to-four weeks), moderate altitude exposure.

1.6 Research Questions & Hypotheses

The research question (denoted "Q") and corresponding hypotheses (denoted "H") for each study of this thesis are outlined below:

1.6.1 Study One

Acute hypoxic exercise does not alter post-exercise iron metabolism in well-trained endurance athletes

Q1: How does an interval running session (5 × 4 min at 90% $v\dot{V}O_{2max,}$ separated by 1.5 min passive recovery), performed in hypoxia [3,000 m simulated altitude (F_IO_2 = 0.1408)] influence iron parameter [serum iron, serum ferritin, soluble transferrin receptor (sTfR)], inflammatory cytokine (IL-6) and hepcidin levels during exercise

recovery (immediately post-exercise and 3 h post-exercise) compared with an equivalent session performed in normoxia (F_IO_2 = 0.2093)?

H1: Compared with interval exercise in normoxia, interval exercise in hypoxia will result in:

a) higher IL-6 levels immediately post-exercise;

b) suppressed hepcidin levels 3 h post-exercise, independent of a post-exercise rise in IL-6;

1.6.2 Study Two

Live high: train low - Influence on resting and post-exercise hepcidin levels

Q1: How does two nights of LHTL at 3,000 m simulated altitude affect resting hepcidin and erythropoietin levels in well-trained distance runners?

H1: Compared with baseline levels, two nights of LHTL will:

a) suppress resting hepcidin levels;

b) elevate resting erythropoietin levels.

Q2: How does 14 days of LHTL affect resting hepcidin, iron parameter (plasma iron, plasma ferritin, plasma transferrin, plasma transferrin saturation) and Hb_{mass} levels in well-trained distance runners?

H2: Compared with baseline levels, 14 days of LHTL will:

a) suppress resting hepcidin levels;

b) decrease resting ferritin levels;

c) increase Hb_{mass} from baseline levels.

Q3: How does the 3 h post-exercise hepcidin and iron parameter response differ after interval exercise (5 × 1,000 m at 90% $vVO_{2max,}$ 1.5 min passive recovery) performed in normoxia (600 m natural altitude) and normobaric hypoxia (3,000 simulated altitude) on the 11^{th} and 14^{th} day of LHTL compared with post-exercise responses to equivalent exercise performed before LHTL?

H3: Compared with interval exercise performed before LHTL, interval exercise performed in normoxia and hypoxia on the 11^{th} and 14^{th} day of LHTL will:

a) attenuate hepcidin levels 3 h post-exercise;

b) result in a comparable iron parameter response between the two exercise conditions, 3 h post-exercise.

1.6.3 Study Three

Pre-altitude serum ferritin levels and daily oral iron supplement dose mediate iron parameter and hemoglobin mass responses to altitude exposure

Q1: How does daily oral iron supplement dose (none, 105 $mg{\cdot}d^{-1}$, 210 $mg{\cdot}d^{-1}$) mediate the Hb_{mass} and iron parameter (serum ferritin and serum transferrin saturation) response to two-to-four weeks of LHTL in well-trained athletes?

H1: Athletes who ingest a higher daily oral iron supplement dose will have a greater increase in Hb_{mass} and a lower reduction in serum ferritin levels following LHTL, compared with those athletes who ingested a lower dose of oral iron supplement daily.

Q2: How do three different iron supplement doses (none, 105 $mg{\cdot}d^{-1}$ and 210 $mg{\cdot}d^{-1}$) influence erythroid and storage iron incorporation following LHTL?

H2: Athletes who ingest a higher dose of oral iron supplements daily will have greater erythroid and storage iron incorporation compared with athletes who ingested a lower oral iron supplement dose daily.

1.7 Definition of Terms

ANOVA: Analysis of variance

CL: Confidence limit

DMT-1: Divalent metal transporter-1

DCytB: Duodenal cytochrome-B

EPO: Erythropoietin

F_IO_2: Fraction of inspired oxygen

GXT: Graded exercise test

HIF: Hypoxia inducible factor

HR: Heart rate

HYP: Hypoxic exercise trial

IHE: Intermittent hypoxic exposure

IHT: Intermittent hypoxic training

IDA: Iron deficiency anaemia

IL-6: Interleukin-6

INT: Interval exercise

IRE: Iron responsive element

IRP: Iron responsive protein

LHTH: Live high: train high

LHTL: Live high: train low

mTOR: Mammalian target of rapamycin

NORM: Normoxic exercise trial

PDGF-BB: Platelet-derived growth factor-BB

RBC: Red blood cells

ROS: Reactive oxygen species

RPE: Rating of perceived exertion

SD: Standard deviation

sTfR: Soluble transferrin receptor

Tf: Transferrin

TfR-1: Transferrin receptor-1

TSAT: Transferrin saturation

VO_{2peak}: Peak oxygen consumption

vVO_{2peak}: Running velocity attained at peak oxygen consumption

VO_{2max}: Maximal oxygen consumption

vVO_{2max}: Running velocity attained at maximal oxygen consumption

V_E (ATPS): Volume of expired gas (atmospheric temperature and pressure saturated)

V_E (STPD): Volume of expired gas (standard temperature and pressure dry)

WCX-TOF MS: Weak cation-exchange, time-of-flight mass spectroscopy

2 CHAPTER TWO

REVIEW OF THE LITERATURE

2.1 Introduction

Hypoxia is a common state in the body characterised by a reduction in tissue oxygen availability. Whilst an acute reduction in oxygen availability compromises energy production via aerobic metabolism, prolonged exposure to hypoxia stimulates several physiological adaptations necessary to maintain oxygen homeostasis. As a result, acute (several hours) and/or prolonged (several weeks) exposure to hypoxia has become an important supplementary training tool used by athletes to enhance their aerobic and anaerobic performance (7). Two key adaptations to prolonged hypoxia are an increase in blood oxygen carrying capacity, namely an increase in haemoglobin mass (Hb_{mass}) and an increase in energy production via aerobic and anaerobic mechanisms as a result of enhanced oxidative and glycolytic enzyme concentrations (30). Such adaptations to oxygen delivery and utilisation mechanisms in healthy individuals following prolonged hypoxic exposure have been shown to enhance both aerobic (31) and possibly anaerobic (32) exercise performance at sea level.

It is important to ensure individuals are healthy before undertaking altitude exposure to maximise their adaptations to hypoxia. Accordingly, athletes should be free from injury and illness, whilst also ensuring they maintain both energy and iron balance (8). Low pre-altitude iron stores (10) and/or reduced iron availability during altitude exposure may however jeopardise the body's adaptive response to hypoxia (8) because iron is essential for the synthesis of proteins involved in oxygen transport (i.e. haemoglobin) and oxygen storage (i.e. myoglobin). Furthermore, iron plays a key role in energy production via aerobic and anaerobic metabolism (33).

A large amount of iron is required to support an increase in the synthesis of iron-dependent haem and non-haem proteins during hypoxic exposure. Athletes may find it

difficult to maintain a healthy iron balance during hypoxic exposure owing to several exercise-related mechanisms of iron loss such as haemolysis (i.e. the destruction of red blood cells), haematuria, gastrointestinal bleeding and sweating (34). Daily iron losses may be higher in female athletes depending on the volume of blood lost during menstruation. Yet, the amount of iron lost through these mechanisms is rather minimal (~2-3 $mg \cdot d^{-1}$) and an increase in intestinal iron absorption during hypoxic exposure can often compensate for this (34). Recently, an exercise-induced, inflammatory-mediated rise in the iron regulatory hormone, hepcidin, 3-6 h post-exercise has been suggested to reduce intestinal iron absorption and the recycling of iron from senescent erythrocytes by reticuloendothelial macrophages during the post-exercise recovery period (16). In addition to low iron stores, an exercise-related rise in hepcidin levels during acute and prolonged hypoxic exposure could therefore compromise the benefits associated with hypoxic exposure modalities, since less iron is available for the synthesis of iron-dependent haem and non-haem proteins.

The regulation of resting and post-exercise iron metabolism during acute and prolonged altitude exposure is unclear, despite iron's role in maintaining oxygen homeostasis and energy production. This review will thus focus on, 1) the haematological and non-haematological adaptations associated with different methods of hypoxic exposure, 2) the regulation of iron metabolism in normoxia 3) the regulation of iron metabolism in acute and prolonged hypoxia, and 4) strategies for maintaining iron balance in hypoxia.

2.2 Hypoxic Exposure Methods

Athletes use acute and prolonged hypoxic exposure methods to enhance their aerobic performance. Acute hypoxic exposure methods include intermittent hypoxic exposure (IHE); which involves short, episodic exposure to simulated hypoxia over several weeks, and intermittent hypoxic training (IHT); characterised by long- or short-duration interval training in hypoxia, interspersed by recovery periods in either normoxia or hypoxia (sometimes termed “live low: train high”). Athletes sometimes combine these two methods of hypoxic exposure, whereby IHE is interspersed by periods of IHT. In comparison, prolonged hypoxic exposure methods include LHTH, which involves athletes living and training at natural or simulated altitude, and LHTL, where athletes live in hypoxia but instead train in normoxia.

2.2.1 Acute Hypoxic Exposure Methods

2.2.1.1 Influence on Aerobic & Anaerobic Performance

The influence of IHE and IHT on aerobic and anaerobic performance in normoxia is equivocal [reviewed by (4, 35)]. To date, exercise in acute hypoxia has been shown to improve several indicators of aerobic exercise performance such as maximal oxygen uptake (VO_{2max}) (36-40), and maximal aerobic running velocity (vVO_{2max}), peak power output (41) and lactate threshold power (39). Furthermore, IHE/IHT also enhances exercise economy at submaximal workloads (40). Several authors have also observed no improvement in VO_{2max} following IHT/IHE (41-44). The equivocal benefits of IHT/IHE on aerobic performance may result from differences in the training status of participants assessed, the exercise protocol employed or the hypoxic stimulus. Consequently, it is difficult to conclude IHE/IHT methods clearly benefit aerobic performance in elite athletes.

Few studies to date have investigated the influence of IHT/IHE on anaerobic exercise performance. Morton & Cable (44) found intermittent hypoxic training (4 weeks of 10 × 1 min cycling at 80% of maximal power output, three times per week) did not improve Wingate anaerobic test performance compared with a normoxic control group. In contrast, Hendriksen & Meeuwsen (45) reported a 4.1% increase in mean power output during a Wingate anaerobic test following a 10-day hypoxic training protocol (2 h cycling per day at 60-70% of heart rate reserve at a simulated altitude of 2,500 m). Similarly, Hamlin et al. (46) found a 3% greater increase in mean power output on a 30 s Wingate anaerobic test performance relative to normoxic placebo group using the same protocol. More recently, Faiss et al. (35) demonstrated a repeated sprint training protocol (3 sets of 5 × 10 s maximal sprints) performed at a simulated altitude of 3,000 m (F_IO_2 ~14.6%) similarly improved 10 s and 30 s Wingate power output. The hypoxic training group, however, were able to perform more sprints before exhaustion relative to a normoxic training group. On balance, IHT/IHE may benefit aerobic and anaerobic exercise performance, although the total hypoxic dose (normally ~0.5-1.5 h), as well as the exercise intensity, work duration (47) and duration of the recovery interval (48) appear to moderate the magnitude of the benefits derived from these training methods.

2.2.1.2 Haematological Adaptations

Acute hypoxic exposure methods are insufficient to stimulate erythropoiesis. Although 90 min of IHT up-regulates serum EPO concentration (49), at least 3-5 days of continuous hypoxic exposure are required to stimulate the maturation of erythroid precursor cells to reticulocytes (50). Indeed, whilst some studies report no increase in haemoglobin concentration following acute hypoxic exposure (43, 44), others found have found small enhancements in haemoglobin concentration (36, 51). However, the

use of haemoglobin concentration to measure the erythropoietic benefits of altitude exposure is limited since plasma volume expansion and dehydration may influence the accuracy of this measure (52).

In contrast, measuring Hb_{mass} via the optimised 2 min carbon monoxide (CO) rebreathing technique instead provides a superior estimate of haemoglobin production in response to long-term acute hypoxic exposure since it is less sensitive to the effects of plasma volume expansion and dehydration (53). Currently, only Humberstone-Gough et al. (54) and Robertson et al. (55) have investigated the influence of long-term IHE on Hb_{mass} production using the CO rebreathing technique. In a placebo-controlled design, these authors found 17 days of IHE (60-90 min per day at a simulated altitude of 3,500-6,000 m) did not improve Hb_{mass}, VO_{2max}, *v*VO_{2max} or running economy elite triathletes relative to a control group. Robertson et al. (55) investigated the influence of combining IHT (four training sessions per week at ~2,200 m) with LHTL (3,000 m simulated altitude, 14 $h \cdot d^{-1}$) with IHT exposure alone (four session per week at 600 m natural altitude) on 3,000 m time trial performance, VO_{2max} and Hb_{mass} in 17 well-trained distance runners. Compared with the IHT group, the LHTL + IHT group improved 3,000 m run time trial performance (-0.9 ± 1.4%), VO_{2max} (2.6 ± 3.2%), Hb_{mass} (4.3 ± 3.2%). Whereas Hb_{mass} increased by 3.6 ± 3.3% in the LHTL+ IHT group, on average, Hb_{mass} did not increase relative to pre-exposure values (-0.7 ± 3.9%) in the IHT group. Collectively, the lack of change in Hb_{mass} suggests the possible benefits of acute hypoxic exposure do not appear to arise from enhanced erythropoiesis.

2.2.1.3 Non-haematological Adaptations

Acute hypoxic training methods may enhance aerobic and anaerobic performance independent of a change in VO_{2max} by up-regulating genes involved in both oxidative and glycolytic metabolism. Indeed, combining acute hypoxic exposure with exercise enhances HIF gene transcription beyond that observed following exercise or acute hypoxic exposure alone (56). To date, IHT has been shown to enhance oxidative (i.e. citrate synthase) (57, 58) and glycolytic (monocarboxylate transporter-1 & 4) enzyme concentrations (35), which are mostly regulated by HIF-1. Additionally, IHT induces several skeletal muscle adaptations, including enhanced capillary-to-muscle fibre ratio (57), mitochondrial density (59, 60) and myoglobin concentration (57, 61).

Adaptations to IHT depend on training intensity, with higher intensity exercise more likely to enhance glycolytic rather than oxidative enzyme concentration. For example, high intensity aerobic interval training (2 × 3 × 2 min at 90% peak power output) performed in hypoxia (3,000 m simulated altitude) did not enhance VO_{2max} or monocarboxylate transport-1 or 4 concentration compared with an equivalent training in normoxia, despite improving peak power output (62). In comparison, repeated sprint training in hypoxia (3 sets of 5 × 10 s sprints performed at 3,000 m simulated altitude) increased glycolytic (i.e. monocarboxylate transporter-4) but not oxidative (i.e. citrate synthase) enzyme concentrations relative to an equivalent training protocol in normoxia (61). Furthermore, these authors found repeated sprint training in hypoxia down-regulated genes involved in mitochondrial biogenesis such as peroxisome proliferator-activated receptor-gamma co-activator-1α, mitochondrial transcription factor A. Given these findings, repeated sprint training in hypoxia but not normoxia may support a greater shift towards anaerobic (glycolytic) metabolism (61) by enhancing by skeletal muscle glycolytic enzyme concentration.

2.2.2 Prolonged Hypoxic Exposure Methods

2.2.2.1 Live High: Train High

The classical LHTH model usually involves individuals living and training at either low-to-moderate (1,800-2,500 m) or high (>3,000 m) natural altitudes, for 3-6 weeks (3). The continuous hypoxic stimulus of LHTH readily induces the body's adaptive response to hypoxia, but decreased VO_{2max} and arterial oxygen saturation may limit training quality (63, 64), thus blunting the benefits normally derived from high intensity training (65).

The benefit of LHTH on endurance performance in elite athletes is difficult to interpret since much research has been conducted on well-trained, rather than elite athletes. In addition, blinding athletes to LHTH protocols is challenging, thus many studies cannot rule out the possibility of a placebo (athletes believing they will perform better because they undertook altitude exposure) or nocebo (athletes believe they are disadvantaged because they did not undertake altitude exposure) effect. For example, in a cross-over design, Adams et al. (66) split 12 highly-trained middle distance runners into two group (n = 6 in both groups) who ran 19.3 $km \cdot d^{-1}$ at 75% sea level VO_{2max} for three weeks either at sea level or 2,300 m, switched training conditions and then completed three more weeks of training. Two mile (3.2 km) run time did not improve in either group after altitude exposure. Later, Levine & Stray-Gundersen (67) compared the influence of LHTH with LHTL on 5,000 m run time performance in 39 competitive distance runners (27 males, 12 females). After two-weeks of familiarisation training and four weeks of supervised training, athletes were randomised to either LHTH (living and training at 2,800 m), LHTL (living at 2,500 m, training at 1,250 m natural altitude) or a sea level training group (living and

training at sea level). Following altitude exposure 5,000 m time trial performance was 13.4 ± 10.0 s faster in the LHTL group, but 3.3 ± 9.0 s and 26.7 ± 13.0 s slower in the LHTH and sea level groups respectively. These authors proposed athletes' ability to maintain high intensity training explained the superior performance benefits derived from LHTL compared with the LHTH protocol. Whilst the effect of LHTH on distance running performance is largely unclear, when considering the within-athlete variation in distance running performance (68), LHTH exposure may enhance endurance performance in some, but not all, endurance athletes.

2.2.2.2 Live High: Train Low

LHTL involves athletes living at a natural or simulated moderate altitude (2,000-3,000 m) but training at lower altitudes or near sea level (3). The LHTL model allows athletes to maintain the quality of high intensity training sessions by temporarily reducing the hypoxic stimulus. To some extent, the ability to maintain exercise intensity during LHTL may explain the enhancements in aerobic performance typically associated with LHTL protocols. Although some studies to date have demonstrated clear improvements in endurance performance following LHTL [(67, 69)], other researchers have reported variable improvements in distance running performance after LHTL (55, 70). Meta-analytic data suggests several weeks of LHTL improves sea level aerobic performance in elite endurance athletes by 4.0 ± 3.7% depending upon the magnitude of hypoxic stimulus applied and the training status of the athletes (i.e. sub-elite *versus* elite) (31).

2.2.2.3 Haematological Adaptations

Hypoxic stabilisation of HIF-2α at altitude increases kidney and liver EPO production, which stimulates increased erythropoiesis in the bone marrow.

Erythropoietin production increases after ~90 min of hypoxic exposure (71), with peak levels occurring within the first few days of prolonged altitude exposure (72). Erythropoietin declines thereafter and reaches baseline levels after approximately one week (73). Renal and hepatic EPO production increases in a dose-dependent manner based on the altitude elevation. For example, Robach et al. (74) observed a progressive increase in EPO in athletes who were exposed to increasingly higher altitudes (from 5,000-8,000 m); with a maximum 33.8 fold increase in EPO production at 8,000 m. Furthermore, Ge et al. (75) suggested a threshold altitude of 2,100-2,500 m is required to sustain EPO release. Considerable intra- (76) and inter-individual (75, 77, 78) variation exists, however, in the magnitude of the EPO response to prolonged hypoxic exposure.

An exercise-mediated decrease in EPO production during early altitude adaptation may contribute towards the inter-individual variation in the EPO response. Indeed, high intensity exercise markedly increases inflammatory cytokine and blood lactate production (79), which impair kidney EPO release (80, 81). Athletes may therefore benefit from performing low intensity exercise during the first few days of altitude acclimatisation to avoid inhibiting EPO release (3).

Prolonged altitude exposure induces a three-to-five-fold increase in erythropoiesis to maintain tissue oxygen homeostasis (82), although the magnitude of the haematological benefits resulting from prolonged altitude exposure is a current topic of debate. In a recent meta-analysis, Gore et al. (6) calculated moderate (i.e. 2,000-3,000 m) altitude increases Hb_{mass} by ~1% per 100 h of exposure, with an individual-subject standard deviation of ~2%. These authors concluded most athletes could expect a benefit in Hb_{mass} following at least two weeks of LHTL at a moderate altitude and suggested 300 h (typically ~3 weeks exposure) of LHTL likely enhances an

athletes' Hb_{mass} by 1.1-6.0%. In contrast to Gore et al. (6), Rasmussen et al. (83) used a Monte Carlo simulation to model the effects of altitude exposure on red cell volume in athletes and proposed both pre-altitude red cell volume levels and altitude elevation moderated the increase in red cell volume post-altitude. Furthermore, these authors concluded higher altitudes produced greater benefits in red cell volume and recommended athletes undertake more than two weeks of exposure to an altitude of at least 4,000 m to enhance red cell volume. However, Rasmussen et al. (83) based their conclusions on data derived from several red cell analysis methods (i.e. the optimised CO rebreathing method, Evan's Blue Dye, radioiodinated albumin, and brilliant red dilution). Gore et al. (6), however, only analysed changes in Hb_{mass} from studies using the optimised CO rebreathing technique, which has superior reliability than the Evan's Blue Dye method (84). Compared with Rasmussen et al. (83) the findings of Gore et al. (6) may provide a more accurate estimate of the increase in red cell production athletes should expect following LHTL at moderate altitude. Hence, prolonged moderate altitude exposure seems to enhance red blood cell production when the hypoxic dose is sufficient.

The increase in Hb_{mass} following altitude exposure should improve oxygen transport to the skeletal muscle at sea level since each gram of haemoglobin carries ~1.34 mL of oxygen (85). Specifically, a 1 g increase in Hb_{mass} enhances oxygen transport by ~4 $mL \cdot min^{-1}$ (86) and ~2.3 $mL \cdot min^{-1}$ (8) in elite and recreational athletes, respectively. Given the relationship between Hb_{mass} and VO_{2max}, increased Hb_{mass} post-altitude directly increases an athletes' VO_{2max}, which may translate into improved sea level aerobic performance.

Saunders et al. (87) recently calculated a moderate correlation between ΔHb_{mass} (%) and ΔVO_{2max} (%) (r = 0.57, 95% confidence limit [0.27, 0.87]) for athletes exposed to

altitude. These authors concluded athletes could expect a ~0.6-0.7% increase in VO_{2max} for each 1% increase in Hb_{mass}, although this model accounted for only ~15% of the variance in the VO_{2max} response to LHTL. Factors other than Hb_{mass}, such as daily exercise training may therefore influence the VO_{2max} response to altitude exposure and account for the between-athlete variability in the Hb_{mass} response to prolonged altitude exposure.

The influence of prolonged altitude exposure on VO_{2max} was meta-analysed by Bonetti & Hopkins (31). These authors estimated LHTH protocols increase VO_{2max} by 4.3 ± 2.6% in sub-elite athletes, but decreased VO_{2max} by 1.5 ± 2.0% in elite athletes. A reduction in overall training volume and the time spent training at high intensities may account for the decrease in VO_{2max} in elite athletes during LHTH (4). In comparison, LHTL at natural altitude increased VO_{2max} by 6.4 ± 11.2% in sub-elite athletes and by 6.4 ± 9.4% in elite athletes. Similarly, the superior increase in VO_{2max} with LHTL compared with LHTH protocols may result from athletes being able to maintain their training volume and the intensities of interval sessions (3).

Athletes are sometimes classified as altitude “responders” or “non-responders” based on the magnitude of their haematological response (77). The haematological response to moderate altitude exposure, however, is not a genetically fixed trait. For example, Robertson et al. (70) reported a moderate correlation (r = 0.47, n = 8) between the Hb_{mass} response of two LHTL blocks in distance runners who undertook two, three week blocks of LHTL altitude exposure (14 $h \cdot d^{-1}$ at 3,000 m simulated altitude) scheduled five weeks apart. Instead, ensuring athletes are in an adaptive state (i.e. well trained, free of illness and injury, and with adequate pre-altitude iron stores) before exposure (8) and focusing on low intensity training session during the first week of altitude exposure (3) may help to ensure they maximise their haematological

adaptations. As such, an athlete considered an altitude "non-responder" might gain a performance benefit from altitude exposure when the training environment is optimised (i.e. they are free of illness and injury, are training sufficiently and have sufficient pre-altitude iron stores) (88).

2.2.2.4 Non-Haematological Adaptations

Prolonged hypoxic exposure may improve aerobic performance independent of an increase in Hb_{mass} (89). Indeed, successful aerobic performance requires athletes to possess an excellent fractional utilisation of oxygen and movement economy (90). Hence, non-haematological adaptations to hypoxia may enhance aerobic performance in normoxia independent of an improvement in VO_{2max} (7). Non-haematological adaptations to prolonged hypoxia include increased blood lactate clearance (increased monocarboxylate transporter 1 & 4 production), skeletal muscle buffer capacity (via increased carbonic anhydrase production), aerobic (i.e. citrate synthase) and anaerobic enzyme production (i.e. phosphofructokinase, hexokinase, lactate dehydrogenase), and mitochondrial energy coupling [via increased nitric oxide synthase (NOS) production] (7). Garvican et al. (89) presented compelling evidence to suggest non-haematological factors contribute towards improved aerobic performance in endurance athletes following LHTL. In an elegant study, 11 highly trained female cyclists undertook 26 nights of simulated altitude exposure (16 $h \cdot d^{-1}$, 3,000 m simulated altitude). After 14 days of LHTL, athletes were pair-matched based on their Hb_{mass} response and divided into a response group, who were free to adapt, and a clamp group, where the weekly increase in Hb_{mass} from baseline was negated via phlebotomy. As expected, VO_{2peak} increased by the response group (3.5 ± 2.3%) but not in the clamp group (0.3 ± 2.6%). Interestingly, despite blocking an estimated 5% increase in Hb_{mass} in the clamp group, these athletes improved their 4 min maximal

mean power by ~3.6% *versus* 5.6% in the response group, although time to exhaustion was markedly worse in the clamp group compared with the response group (-37%, 90% confidence limit: [-58.9, -5.0]). These findings suggest enhanced Hb_{mass} may not be the sole mechanism by which LHTL benefits aerobic performance post-altitude exposure. The physiological benefits of LHTH and LHTL should be interpreted cautiously however since changes in physiological parameters such as Hb_{mass} and VO_{2max} do not imply prolonged exposure to hypoxia will enhance endurance exercise performance.

2.2.3 Summary

Athletes use both acute and prolonged altitude exposure methods to improve their athletic performance. The short hypoxic dose associated with acute hypoxic exposure may not be sufficient to stimulate erythropoiesis, but may enhance oxidative and glycolytic enzyme concentration. In comparison, prolonged hypoxic exposure induces several haematological and non-haematological adaptations, which may translate into improved aerobic and/or anaerobic performance. However, several factors such as a high training volume, injury, illness and poor iron availability may compromise some of the hypoxic-mediated physiological adaptations associated with prolonged hypoxic exposure. Furthermore, improvements in physiological parameters such as Hb_{mass} and VO_{2max} may not always translate into enhanced athletic performance upon the removal of the hypoxic stimulus.

2.3 The Regulation of Iron Metabolism in Normoxia

A review of resting and post-exercise iron metabolism in normoxia is first warranted before considering the regulation of iron metabolism in hypoxia.

2.3.1 Iron – Biological Functions & Distribution

Iron is a dietary nutrient necessary for the synthesis of several haem and non-haem proteins involved in oxygen transport, oxygen storage and energy production (33). Iron forms the core of the protoporphyrin ring of the haem molecule, which is essential for haemoglobin and myoglobin synthesis as well as the formation of several electron transport chain cytochromes (91). Iron is also essential to several non-haem proteins involved in oxidative metabolism, namely iron-sulphur proteins such as the metalloflavoprotein, xanthine oxidase, and electron transport chain proteins such as succinate dehydrogenase and nicotinamide adenine dinucleotide dehydrogenase (33). The remaining iron-dependent proteins are specifically involved in iron absorption [divalent metal transporter-1, (DMT-1)], storage (ferritin) and transport [transferrin, (Tf)].

Iron is a reactive metal with excellent redox potential, which exists in two ionic states, ferric (III) and ferrous (II). Free (unbound) iron can be toxic since iron readily combines with oxygen and nitric oxide, catalysing the formation of a highly reactive hydroxyl group (OH^-) and peroxynitrite ($ONOO^-$) resulting in oxidative and nitrosative damage to proteins, lipids and nucleic acids (24). Thus, ferrous iron is bound to glycoprotein ligands to prevent oxidative stress when stored or transported.

2.3.2 Regulation of Systemic Iron Balance

The maintenance of systemic iron balance involves three cells, 1) duodenal enterocytes, which control iron absorption, 2) reticuloendothelial macrophages, which regulate iron recycling and, 3) liver hepatocytes. The hepatic hormone, hepcidin, is the body's master regulator of systemic iron balance (14), regulates iron balance by

moderating iron absorption and recycling mechanisms. The regulation of iron import, export and recycling is reviewed below.

2.3.2.1 Iron Import

Dietary iron exists in two forms, haem iron (derived mostly from meat proteins) and non-haem iron (derived from plants, cereals, legumes). Whereas 15-35% of haem iron is absorbed from the diet, only 2-20% of non-haem iron is absorbed, with the amount of iron absorbed depending on an individual's sex, ferrokinetics, current iron status, and rate of erythropoiesis, as well as dietary factors such as protein and calcium intake (92).

Absorption of dietary non-haem and haem iron occurs across the apical surface of duodenal enterocytes in the small intestine (93). To minimise tissue oxidative stress, iron is delivered to the enterocyte bound to the glycoprotein, transferrin, in an insoluble ferric (III) form. Thus, ferric (III) iron is first reduced to ferrous (II) iron by the ferric reductase, duodenal cytochrome B (DcytB), assisted by the acidic environment of the gut lumen, before it can be transported into the cell cytoplasm (94). Ferrous (II) iron is then absorbed into the cell cytoplasm through the iron import channel, DMT-1 (93, 95). In comparison, haem iron crosses the enterocyte brush boarder membrane bound to an unknown haem transporter, and is then reduced by haem-oxygenase, which extracts iron from the haem subunit (96). Iron liberated from non-haem and haem into the cytoplasm forms a labile (or chelatable) iron pool (LIP) that is able to supply iron to mitochondria for both haem synthesis and for the assembly of iron-sulphur clusters involved in producing other iron-dependent proteins (97).

2.3.2.2 Iron Export

Prior to export, ferrous (II) iron in the cytoplasm is oxidised to the insoluble ferric (III) iron by multi-copper ferroxidases, such as hephaestin (enterocyte) and ceruloplasmin (hepatocyte) (98, 99). Thereafter, iron required for cellular functions is exported the blood plasma by ferroportin, an iron export channel located on the basolateral surface of iron storage cells such as enterocytes and reticuloendothelial macrophages. During iron export, iron in the LIP migrates across the basolateral surface of the enterocyte. Ferrous (II) iron then exits the cell via ferroportin, where it binds to apotransferrin and is transported to various cells such as erythroid precursor cells and hepatocytes via the blood plasma as holotransferrin. Alternatively, cytoplasmic iron derived from both haem and non-haem sources that is not immediately used for cellular functions combines with the glycoprotein ligand, apo-ferritin, and is then stored as ferritin or attaches to low molecular weight ligands in the cytosol and resides in the LIP until exported into the blood plasma (100).

2.3.2.3 Iron Recycling

Macrophages of the reticuloendothelial system (or mononuclear phagocyte system) control iron recycling by erythrophagocytosis (i.e. the destruction of senescent erythrocytes). Erythrophagocytosis is predominately conducted by splenic macrophages but also occurs in the liver and bone marrow (13). In this process, senescent red blood cells are engulfed by a phagolysosome, where they undergo proteolysis to release the haem subunit. The haem subunit is then transported from the lumen of the phagolysosome into the macrophage cytosol by the haem-responsive protein gene-1 (101). Thereafter, haem-oxygenase lyses iron from the haem subunit in the ribosome of the endoplasmic reticulum. Iron liberated from haem binds to

apoferritin and is stored in the cytosol as ferritin, before being exported from the macrophage into the blood via ferroportin and finally being delivered to erythroid precursor cells (98).

2.3.2.4 Hepcidin – The Hormonal Regulator of Systemic Iron Balance

Hepcidin is a 25 amino-acid peptide hormone produced by liver hepatocytes and is the master regulator of systemic iron balance. Hepcidin acts by inducing the internalisation and degradation of ferroportin, located on the basolateral surface of reticuloendothelial macrophages and hepatocytes, thus promoting iron sequestration and limiting iron efflux from these cells into the blood plasma (15). Consequently, less iron can be transported by holotransferrin to erythroid precursor cells to support erythropoiesis. Hepcidin also acts upon the apical surface of duodenal enterocytes to degrade DMT-1, thus reducing intestinal iron import (102). Hepcidin production is regulated by plasma iron levels and the size of the iron storage compartment (i.e. the stores regulator – both positive and negative regulator) (103), the rate of erythropoiesis (i.e. the erythroid regulator – negative regulator) (104) and host defence mechanisms (i.e. inflammatory cytokines/inflammatory regulator – positive regulator) (105). In addition, several novel regulators of hepcidin have recently been identified, including testosterone (negative regulator) (106, 107), estrogen (negative regulator) (108), growth factors (such as PDGF-BB – negative regulator) (109) and nutrient-dependent activation of the mammalian target of rapamycin (mTOR) (110) [see Arosio (111) for an excellent review of the current hepcidin regulation pathways] (Figure 2.1).

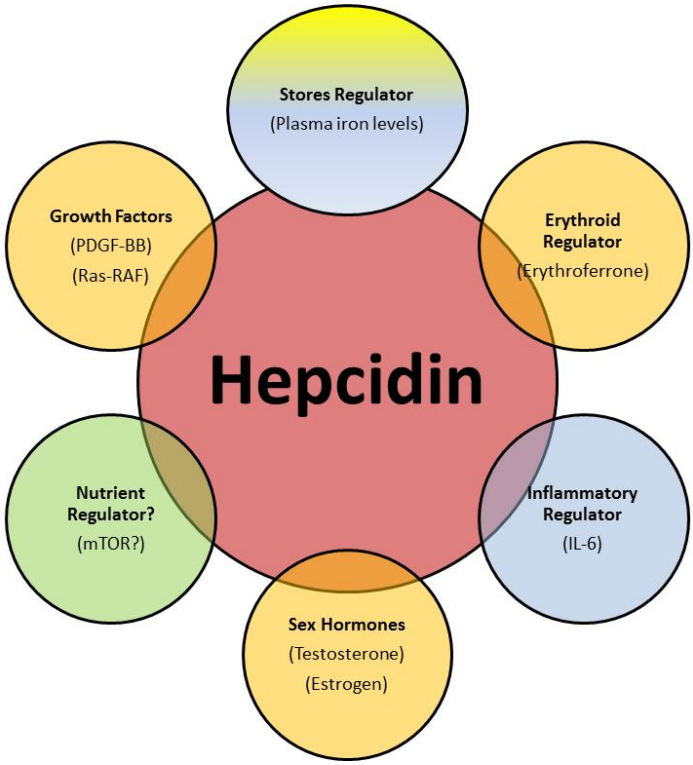


Figure 2.1: Known regulators of hepcidin expression. Yellow shapes indicate known suppressors of hepcidin expression. Blue shapes indicate known promoters of hepcidin expression. The body's iron stores (stores regulator) may either promote or suppress hepcidin expression depending on the size of the iron storage compartment and is thus shaded in both blue and yellow. Green shapes indicate emerging regulators of hepcidin expression. IL-6: Interleukin-6, PDGF-BB: Platelet derived growth factor-BB, Ras-RAF: Rat sarcoma-Rapidly accelerated fibrosarcoma pathway (part of the mitogen-activated protein kinase pathway), mTOR: Mammalian target of rapamycin.

2.3.3 Regulation of Intracellular Iron Balance

The majority of iron contained within cells is delivered to the mitochondria for haem biosynthesis via the Shemin pathway [see (112) for a review]. Intracellular iron balance is regulated both transcriptionally and post-transcriptionally by the interaction of iron responsive proteins (IRP) with iron responsive elements (IRE) [for

comprehensive reviews of this process, see (30, 113, 114)]. IRPs exist in two isoforms, IRP-1 and IRP-2, which sense the intracellular iron content and either stabilise mRNA expression or block IRP translation by interacting with IREs located on the 3' untranslated regions (i.e. transferrin-receptor-1 and DMT-1) or 5' untranslated regions (i.e. ferritin light-chain and ferritin heavy-chain). In turn, IRP/IRE binding alters intracellular iron storage and transport (113). When the intracellular iron pool is high, IRP-1 binds with iron-sulphur clusters, which converts IRP-1 into a cellular aconitase, thus inhibiting IRP-1/IRE interactions and stabilising ferritin mRNA.

2.3.4 Summary

Iron is involved in the synthesis of the oxygen transport and storage proteins, haemoglobin and myoglobin, as well as energy production via iron-dependent oxidative and glycolytic enzymes. The maintenance of systemic iron balance mostly involves three cells, hepatocytes, enterocytes and reticuloendothelial macrophages. The body's iron master regulatory hormone, hepcidin acts to coordinate systemic iron balance either directly or indirectly by interacting with these cells. Intracellular iron balance is controlled predominantly by the interaction of IRPs with their IREs, which alter intracellular iron content by moderating iron import and transport.

2.4 Daily Iron Turnover

The body must balance iron intake and absorption with daily iron losses to maintain iron homeostasis and support physiological functions (Figure 2.2). The majority of iron (90-95%) required for erythropoiesis is recycled from senescent erythrocytes by reticuloendothelial macrophages by the process of erythrophagocytosis (13). In addition, ~1-2 $mg \cdot d^{-1}$ of iron must be absorbed from the diet to replenish basal daily

iron losses resulting from the desquamation of epithelial cells and faecal blood losses of ~0.85 $mg \cdot d^{-1}$ in males and ~1.40 $mg \cdot d^{-1}$ in females (34). Daily iron losses are often higher in active individuals (~2-3 $mg \cdot d^{-1}$), owing to iron lost through mechanism such as foot-strike haemolysis, haematuria, gastrointestinal bleeding and, to a lesser extent, iron lost in sweat during exercise (34). Regardless, exercise-related iron losses are rather minimal and can often be compensated by an increase in intestinal iron absorption (34). Failure to absorb sufficient iron to replenish daily iron losses results in a negative iron balance and requires iron to be mobilised from storage sites to meet the body's iron needs. If not corrected, then a negative iron balance may deplete the body's iron stores leading to an iron deficiency and/or iron deficient erythropoiesis, potentially compromising athletic performance [see (115) and (116) for comprehensive reviews].

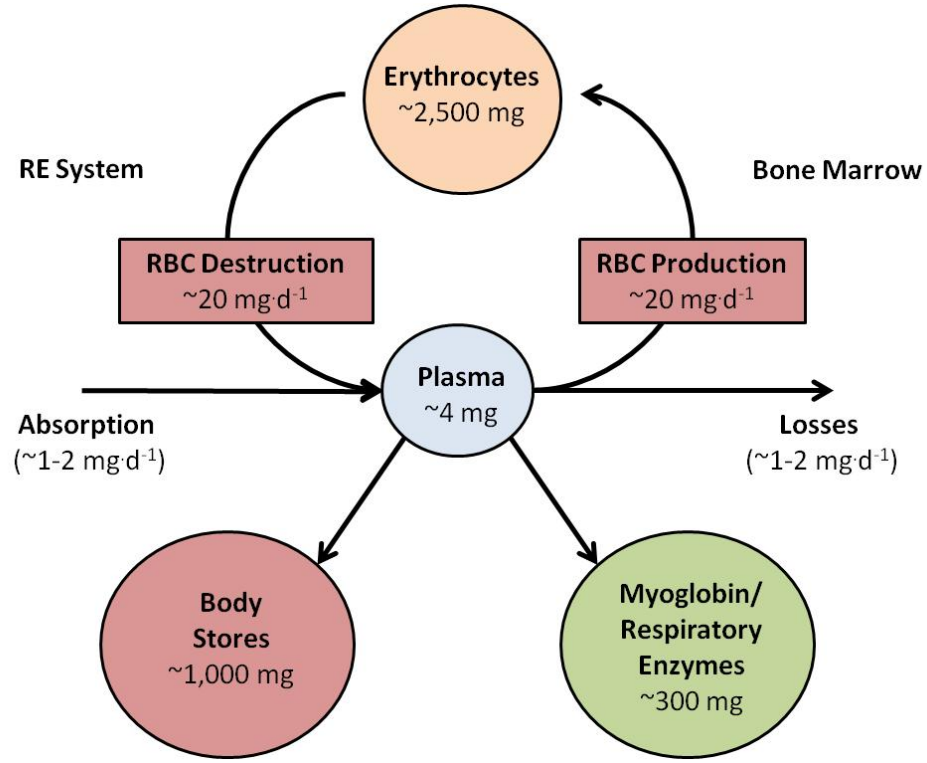


Figure 2.2: Daily iron turnover and iron balance in the body. RBC: Red blood cells; RE system: Reticuloendothelial system (or mononuclear phagocyte system).

2.5 Iron Deficiency in Athletes

2.5.1 Definition

Iron deficiency is a common nutrient disorder in athletes and classified by three stages, depending on its severity (Table 2.1). Iron deficiency first manifests as a decrease in serum ferritin levels. Mast et al. (117) calculated a serum ferritin threshold of <30 $\mu g \cdot L^{-1}$ provided superior diagnostic sensitivity in identifying iron deficiency in anaemic patients respectively compared with a serum ferritin threshold of 12 $\mu g \cdot L^{-1}$ (sensitivity: 92% *versus* 25%). The serum ferritin threshold used to diagnose iron deficiency without anaemia in athletes, however, varies between researchers. Indeed, several serum ferritin thresholds have been used to date: <12 $\mu g \cdot L^{-1}$ (118); <16 $\mu g \cdot L^{-1}$

(119), <20 $\mu g \cdot L^{-1}$, <30 $\mu g \cdot L^{-1}$ (117, Fallon, 2004 #629, 120), <35 $\mu g \cdot L^{-1}$ (121). Serum ferritin is a sensitive indicator of the body's iron stores (117), but is falsely elevated by inflammation (122) and may not always accurately represent the bone marrow iron content (123).

Table 2.1: The three stages of iron deficiency in athletes. Adapted from Peeling et al. (1).

Stage of Iron Deficiency	Haemoglobin ($g \cdot L^{-1}$)	Ferritin ($\mu g \cdot L^{-1}$)	Transferrin Saturation (%)
Stage 1 (Iron Deficiency)	≥115	≤35	>16
Stage 2 (Iron Deficient Erythropoiesis)	≤115	≤20	>16
Stage 3 (Iron Deficiency Anaemia)	<115	≤12	<16

The sTfR/ log ferritin index could also be useful in the diagnosis of iron deficiency in athletes since sTfR is largely unaffected by inflammation (124). The sTfR/log ferritin index has excellent sensitivity and specificity in diagnosing individuals with iron deficiency (125) and is sometimes used in conjunction with reticulocyte haemoglobin content to distinguish between individuals with anaemia of chronic disease and iron deficient anaemia in the Thomas diagnostic plot (126). Indeed, several studies (119, 127-130) to date have assessed iron status in athletes using the sTfR/log ferritin index.

Exhaustion of the body's iron stores reduces iron delivery to the erythron for haemoglobin synthesis (131), resulting in iron deficiency anaemia (IDA). The World Health Organisation define anaemia as a [Hb] <130 $g \cdot L^{-1}$ in males and <120 $g \cdot L^{-1}$ in females (132). In the clinical population, [Hb] is commonly used as the only

diagnostic measure of IDA, but since [Hb] is influenced by changes in plasma volume [which expands as a result of endurance training and contracts during altitude exposure (52)] athletes should not be diagnosed with IDA based on their [Hb] alone. [Hb] is also influenced by age, sex, ethnicity and residence at altitude (133). Whilst [Hb] thresholds can be adjusted to account for these factors, the considerable between-subject variation in [Hb] means some athletes may not be diagnosed as iron deficient, despite responding positively to iron supplementation. As mentioned above, the assessment of several iron parameters should therefore accompany [Hb] when diagnosing IDA in athletes.

Measuring the size of the red cell compartment in athletes via Hb_{mass} rather than [Hb] may assist the diagnosis of IDA in athletes. In contrast to [Hb], total Hb_{mass} is not influenced by plasma volume shifts and is a better correlated with mean cell volume and mean cell haemoglobin than [Hb] (134) and may be a more sensitive indicator of changes in red cell production in response to iron supplementation. For example, Garvican et al. (135) examined the changes in Hb_{mass} in an anaemic female endurance athlete (Hb_{mass}: 6.2 $g \cdot kg^{-1}$; [Hb]: 88 $g \cdot L^{-1}$; serum ferritin: 9.9 $\mu g \cdot L^{-1}$) following an iron injection (Ferrum H, iron polymaltose, 100 mg of iron) combined with 15 weeks of twice daily oral iron supplementation (325 mg ferrous sulphate, 105 mg elemental iron). In this athlete, Hb_{mass} continued to increase in the later stages of iron supplementation despite [Hb] remaining stable. A similar uncoupling between [Hb] and Hb_{mass} in response to iron supplementation was also noted by Wachsmuth et al. (134), who monitored the Hb_{mass} response to 10 weeks of oral iron supplementation (100 $mg \cdot d^{-1}$ elemental iron) in severely (serum ferritin: $\leq$12 $\mu g \cdot L^{-1}$) and moderately iron deficient (serum ferritin: 13-25 $\mu g \cdot L^{-1}$) recreational athletes. These authors reported that one iron deficient, non-anaemic athlete (serum ferritin: 14 $\mu g \cdot L^{-1}$; [Hb]:

140 $g \cdot L^{-1}$) increased their Hb_{mass} by 8.3% (52.7 g) over the oral iron supplementation period, indicating this athlete was suffering anaemia despite a clinically normal [Hb]. Given the potentially superior sensitivity of Hb_{mass} over [Hb] for assessing the red cell compartment and its stability despite plasma volume changes associated with exercise, the use of Hb_{mass} may provide a better marker of iron deficiency in athletes.

2.5.2 Prevalence

The estimated prevalence of iron deficiency in the athletic population varies depending upon the serum ferritin threshold used to define iron deficiency and the population assessed. In 576 elite Australian athletes, Fallon (120) defined iron deficiency as a serum ferritin level <30 $\mu g \cdot L^{-1}$. Iron deficiency affected ~3% of male athletes (10 out of 303) and ~17% (52 out of 273) of female athletes respectively. In comparison, a higher prevalence of iron deficiency was reported in a study of 71 female military recruits, where 77% met the criteria for iron depletion (serum ferritin: <20 $\mu g \cdot L^{-1}$) and 15% met the criteria for iron deficiency (serum ferritin: <12 $\mu g \cdot L^{-1}$, transferrin saturation: <15%) (136). Sinclair & Hinton (119) assessed the iron status of 121 recreationally active individuals (72 females, 49 males), reporting iron deficiency without anaemia (serum ferritin: <16 $\mu g \cdot L^{-1}$) in 29% of females and 4% of males. However, the prevalence of iron deficiency without anaemia increased to 36% in females and 6% in males when using sTfR/log ferritin index ≥4.5 as the diagnostic criteria. Based on the series of studies above and regardless of the exact criteria used to define iron deficiency, the prevalence of iron deficiency seems greater in athletes than in less active individuals (137). One reason for this athlete's lose more iron through mechanisms such as haemolysis, haematuria, gastrointestinal bleeding and iron lost in sweat (34). Additionally, daily iron losses may be higher in weight-bearing sports such as running and triathlon compared with non-weight bearing sports as a

result of greater iron lost through foot-strike haemolysis (138, 139) and more prevalent in females owing to increased iron lost through menstruation (140).

2.5.3 Influence on Athletic Performance

A suboptimal iron status may reduce endurance performance in athletes. Iron deficiency anaemia is characterised by a [Hb] <120 $g{\cdot}L^{-1}$ and impairs maximal aerobic and submaximal aerobic exercise performance by decreasing oxygen transport. Specifically, a 26 $g{\cdot}L^{-1}$ decrease in [Hb] is associated with an ~20% decrease in VO_{2max} (141). Furthermore, individuals with a [Hb] <120 $g{\cdot}L^{-1}$ show a clear reduction in physical work capacity, including greater heart rates at submaximal workloads, lower time to exhaustion and higher post-exercise blood lactate production relative to healthy controls (142).

The relationship between iron deficiency without anaemia (i.e. [Hb] >120 $g{\cdot}L^{-1}$) and exercise performance is less clear. Whereas [Hb] >120 $g.L^{-1}$ is considered clinically normal, decreased iron availability may impair athletic performance by compromising the function of iron-dependent oxidative enzymes and respiratory chain cytochromes (143, 144). Indeed, in addition to reducing oxygen transport via haemoglobin and oxygen storage in myoglobin, iron deficiency also inhibits the function of electron transport chain cytochromes a, b c oxidases and iron-sulphur containing dehydrogenases (such as NADH and succinate dehydrogenase) (33). Consequently, whereas iron deficient erythropoiesis (i.e. Stage 2 iron deficiency) inhibits oxygen transport by reducing haemoglobin synthesis and thus oxygen transport; the impaired function of iron-sulphur proteins contained in the skeletal muscle during Stage 1 iron deficiency (33). While a low [Hb] clearly impairs oxygen transport during exercise (141), the influence of iron deficiency without anaemia on athletic performance in

well-trained athletes is less clear. Indeed, iron deficiency without anaemia has been shown to impair submaximal (145) and maximal exercise performance (146), although the provision of oral iron supplements may restore aerobic exercise performance in some athletes (147, 148). More recently, Burden et al. (149) meta-analysed 17 studies for their effect of iron supplementation on iron status and VO_{2max} in iron deficient, non-anaemic athletes. These authors concluded iron supplementation had a large effect on serum ferritin (Hedges' *g*: 1.10, 95% CL: [0.91, 1.26]). A subsequent sub-group analysis determined serum ferritin levels in significantly improved in males and females and that both oral iron supplements and intramuscular iron injections were beneficial. Based on data extracted from 15 studies, iron supplementation had a moderate effect on VO_{2max} (Hedges' *g*: 0.61, 95% CL [0.40, 0.82]), with a sub-analysis revealing a significant effect for oral iron supplements. The improvement in serum ferritin levels and VO_{2max} following oral iron supplementation imply that iron deficiency without anaemia may impair aerobic power.

Low serum ferritin levels may not necessarily impair athletic performance, especially in female athletes. For example, in a case study of a female, iron deficient (serum ferritin: 24.5 ± 7.6 $\mu g \cdot L^{-1}$) Olympic level 1,500 m runner, Pedlar et al. (150) reported that this athlete's 1,500 m performance continued to improve over four years leading into the Olympics despite an apparent iron deficiency. Furthermore, [Hb], VO_{2max}, running economy and blood lactate threshold remained stable despite apparent an iron deficiency. These data suggest low iron stores may not always reduce athletic performance in elite athletes, or that the runner examined in the study by Pedlar et al. (150) was not iron deficient, despite her low serum ferritin levels. Theoretically, since iron deficiency without anaemia does not always seem to impair athletic performance, it is possible that some well-trained athletes will compensate for iron deficiency by

improving the metabolic efficiency of enzymes involved in oxygen-independent energy pathways. For example, improved lactate shuttling and generation of ATP via the Cori cycle in the liver may provide a viable alternative energy production method in iron deficiency, especially since the liver is less affected by iron deficiency than the skeletal muscle. Hence, increasing the reliance on energy production via glycolytic processes in iron deficient athletes involved in events lasting one to two minutes may ensure these athletes are able to maintain their athletic performance.

2.5.4 The Hepcidin Hypothesis

Some athletes may be at risk of developing an iron deficiency despite ingesting sufficient iron in their diet, owing to a transient, inflammatory-mediated increase in the iron regulatory hormone, hepcidin, 3-6 h post-exercise (16). Such an exercise-related increase in hepcidin during the post-exercise recovery phase may help to explain the high prevalence of iron deficiency in athletes (16). Briefly, elevated post-exercise hepcidin levels may transiently reduce iron availability by reducing iron recycling from senescent erythrocytes and blocking intestinal absorption (see Section 2.3.2 for an explanation of how hepcidin regulates systemic iron balance). Hypothetically, repeated post-exercise elevations in hepcidin throughout a competitive season may limit iron uptake from iron rich meals and/or oral iron supplements consumed during the post-exercise recovery phase. Consequently, the body must instead mobilise iron from storage sites to maintain iron balance. Over the course of a competitive season, this process may eventually exhaust an athlete's iron stores.

An increase in the release of the inflammatory myokine, IL-6, from the skeletal muscle during exercise is likely the main promoter of post-exercise hepcidin

expression (116). Factors influencing the magnitude of the post-exercise IL-6 response such as exercise intensity (17), duration (18), as well as the exercise modality (i.e. weight bearing *versus* non-weight bearing) (17) seem to moderate the post-exercise hepcidin response. In addition, glycogen depletion (151), and low glycogen availability during exercise (152) may exacerbate post-exercise hepcidin levels by augmenting skeletal muscle IL-6 production.

In addition to IL-6, elevated plasma iron levels and pre-exercise ferritin levels also regulate the magnitude of the post-exercise hepcidin response (153). Plasma iron levels increase post-exercise due to iron liberated from the mechanical destruction of erythrocytes (haemolysis) via skeletal muscle contraction and ground contacts. Free (unbound) iron is highly toxic since it catalyses the formation of reactive oxygen species via the Fenton Haber-Weiss reaction, increasing tissue oxidative stress (154). Thus, hepcidin production increases in response to elevated plasma iron levels immediately post-exercise and promotes the sequestration of iron into macrophages and reduces oxidative stress (155).

An athlete's pre-exercise iron stores may also moderate the magnitude of the post-exercise hepcidin response. Indeed, the post-exercise hepcidin response is often blunted in iron deficient athletes (16, 156), which may help to improve intestinal iron absorption and iron delivery to the bone marrow for erythropoiesis. For example, Peeling et al. (19) demonstrated post-exercise hepcidin production following high intensity interval exercise was greater in athletes with higher baseline serum ferritin levels and attenuated in athletes with serum ferritin <30 $\mu g \cdot L^{-1}$. Interestingly, hepcidin levels were attenuated regardless of a marked increase in IL-6 in athletes with low serum ferritin levels (i.e. < 30 $\mu g \cdot L^{-1}$), perhaps to ensure sufficient iron is available to support erythropoiesis. Hepcidin suppression in iron deficient athletes may therefore

be an adaptive response in an attempt to maintain iron balance and iron availability for erythropoiesis.

2.5.5 Summary

Iron deficiency is a common problem in athletes, owing to increased exercise-related iron losses. Whilst anaemia reduces oxygen transport resulting in impaired aerobic exercise performance, the relationship between low iron stores without anaemia is less clear. In addition to exercise-related iron losses, an IL-6-mediated elevation in hepcidin post-exercise may transiently reduce iron recycling from macrophages and intestinal iron absorption. Repeated post-exercise elevations in hepcidin may require athletes to mobilise iron from storage sites to maintain a healthy iron balance, consequently decreasing athletes' whole body iron stores throughout a competitive season.

2.6 The Regulation of Iron Metabolism in Hypoxia

Iron links oxygen sensing, transport and energy production pathways. Maintaining a healthy iron balance in hypoxia is therefore essential to oxygen homeostasis. The master regulator of the body's response to hypoxia is the hypoxia-inducible factor (HIF), which controls the expression of over 100 target genes involved in oxygen sensing and transport, erythropoiesis, oxidative and glycolytic enzyme production and iron metabolism (30). In normoxia, HIF is hydroxylated by the tissue oxygen sensor, prolpyl hydroxylase, which then binds to von Hippel-Lindau factor and targets the entire HIF complex for rapid degradation by E3 ubiquitin-ligase pathways (157). In comparison, exposure to hypoxia prolongs the half-life of the HIF-α subunit from <1 min to ~30 min, causing it to bind with the HIF-β subunit and enhance HIF gene transcription (158). The HIF gene exists in three isoforms, HIF-1, HIF-2 and HIF-3.

Each HIF isoform plays a distinct role in regulating the body's response to hypoxia. The HIF-2α isoform stimulates EPO expression from fibroblast-like cells in the renal cortex and, in severe hypoxia, liver hepatocytes (159). HIF-2α also controls the amount of iron available to support erythropoiesis by up-regulating the expression of several genes involved in intestinal iron absorption (e.g.. DMT-1, DCytB), and iron export (ferroportin) (160-162) (see Figure 2.3). In comparison, HIF-1α promotes angiogenesis (i.e. vascular endothelial growth factor) (163) and glycolytic enzyme production (i.e. phosphofructokinase, hexokinase, lactate dehydrogenase, glucose transporter-1 and monocarboxylate transporter-4) (30), and increased expression of the iron transport protein, transferrin receptor-1 (164). The functions of HIF-3α are less well known, although it seems to inactivate HIF-1α and 2α variants (165).

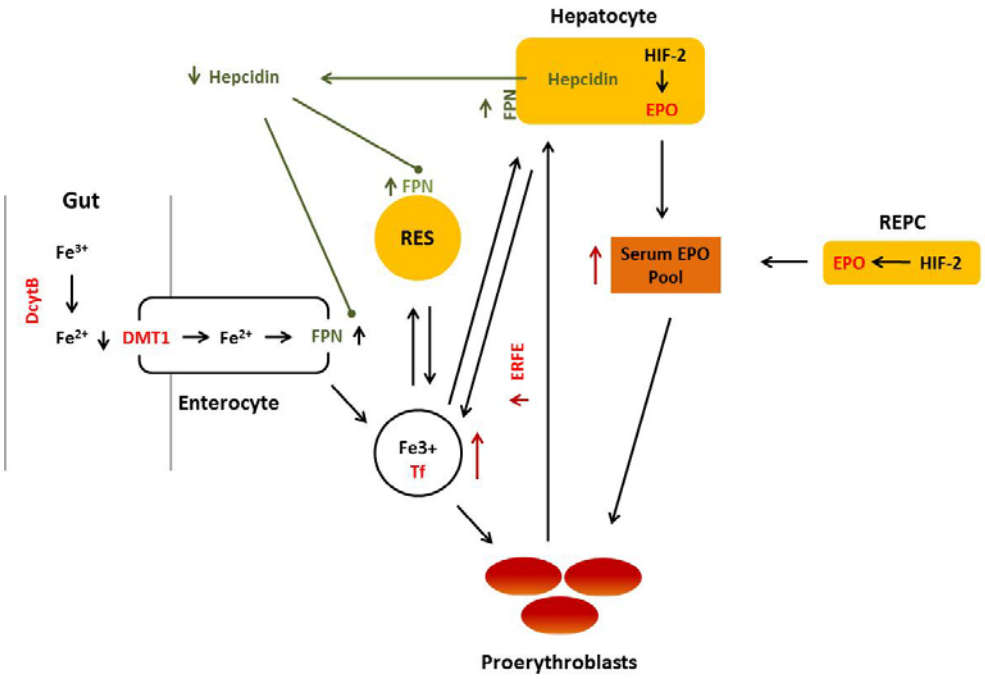


Figure 2.3: The regulation of iron metabolism by the hypoxia inducible factor (HIF) and erythropoietin (EPO) in response to hypoxia. The hypoxic-stabilisation of HIF-2α stimulates increased EPO production in the kidney (REPC: renal EPO producing cells) and liver, in turn up-regulating erythropoiesis. Iron availability increases to support accelerated erythropoiesis in hypoxia requiring several changes in iron protein expression. In the gut, duodenal cytochrome B (DctyB) reduces Fe^{3+} to Fe^{2+}, which is then transported to the enterocyte via divalent metal transporter-1 (DMT-1). Iron absorbed by the gut or contained in reticuloendothelial macrophages is exported into blood plasma via ferroportin (FPN), forms a complex with transferrin (Tf) and is then transported to erythron and other body organs. Simultaneously, EPO acts upon erythroid progenitor cells, stimulating the release of erythroferrone (ERFE), which suppresses hepcidin expression. Hepcidin suppression reduces FPN degradation, increasing iron export from reticuloendothelial macrophages, enterocytes and hepatocytes. DcytB, DMT-1, FPN expression are HIF-2α mediated, Tf is HIF-1α mediated. Figure adapted from Haase (2).

EPO promotes the survival, proliferation and differentiation of erythroid progenitor cells into mature erythrocytes. Specifically, EPO acts on the bone marrow to inhibit the apoptosis of erythroid precursor cells burst forming unit erythroid cells (BFUe), allowing them to mature into colony forming unit erythroid cells (CFUe). Thereafter, EPO promotes the maturation of CFUe into proerythroblasts and normoblasts within 48-72 h (12). These cells then mature into reticulocytes in the bone marrow over the next 24-48 h. Full maturation from a BFUe to erythrocyte normally occurs within ~3-5 days (50), but may occur in as little as one day in severe hypoxia. Furthermore, EPO stimulates the release of the hormone, erythroferrone from proerythroblasts. Erythroferrone is an erythroid regulator of hepcidin expression, acting to suppress hepcidin expression in hypoxia to increase the amount of iron available for erythropoiesis (104).

2.6.1 Acute Hypoxic Exposure

The regulation of systemic and intracellular iron metabolism during acute hypoxic exposure is largely unknown. Short-term exposure to hypoxia increases HIF-1α and HIF-2α expression (166), which collectively regulate genes involved in iron import (160, 161), transport (164) and export (167), as well as several iron-dependent oxidative enzymes (57). However, acute exercise in hypoxia presents conflicting mechanisms for the regulation of systemic iron balance. Whereas hypoxia (153) and accelerated erythropoiesis (104) suppress hepcidin expression, exercise in acute hypoxia increases post-exercise IL-6 production relative to exercise in normoxia (27), which may in turn augment the post-exercise hepcidin response.

On the one hand, an augmented post-exercise hepcidin response following acute exercise in hypoxia could further reduce iron recycling and absorption during the

post-exercise recovery period compared with equivalent exercise in normoxia, thereby limiting iron delivery to the erythron during this time. On the other hand, low hepcidin levels post-exercise may transiently increase iron recycling and absorption, potentially enabling athletes to better deliver iron to bone marrow for erythropoiesis. However, hepcidin suppression is not directly mediated by increased HIF expression in hypoxia (168) and at least 6-15 h of continuous hypoxic exposure is required to stimulate EPO levels sufficient to increase the expression of erythroferrone from the proerythroblasts, which in turn acts to suppress hepcidin (104). Therefore, given the short duration of the hypoxic stimulus applied during IHT/IHE protocols (typically ~60-120 min); these protocols are unlikely to suppress post-exercise hepcidin expression relative to similar exercise performed in normoxia.

The regulation of systemic iron balance during the long-term (2-3 times per week for several weeks) use of IHT/IHE protocols is largely unknown. IHT/IHE methods seem unlikely to place a greater burden on the body's iron stores than exercise in normoxia since these methods do not seem to stimulate erythropoiesis. Accordingly, only a few studies have observed reductions in resting serum ferritin levels during the long-term use of IHT/IHE. In a randomised cross-over design, Bonetti et al. (51) compared the responses of red blood cell and iron parameters of ten male sub-elite kayakers to a three week IHE protocol (5 × 60 min per week: 5 min at S_aO_2 = 90-78%, alternating with 5 min normoxic recovery), and normoxic exposure (placebo condition), separated by a six week washout period. Serum ferritin levels in the IHE condition decreased by ~19.0% more than the placebo group three days post-intervention, an effect deemed "likely negative" using magnitude-based inferences. These authors suggested the decrease in ferritin levels paralleled with a 4% increase in [Hb] relative to the placebo condition provided evidence that IHE enhanced erythropoiesis.

Additionally, Ventura et al. (169) found a significant decrease in serum ferritin levels and a two-fold increase in reticulocytes (%) in seven endurance trained cyclists who performed six weeks of 30 min cycling at a rating of perceived exertion of 15, three times per week at a simulated altitude of 3,200 m. These authors noted a similar decrease in serum ferritin levels and increase in reticulocytes (%) between the control group and hypoxic groups. These authors concluded the hypoxic stimulus of the IHT protocol was insufficient to stimulate erythropoiesis and reasoned the changes in reticulocytes and serum ferritin instead resulted from normal exercise training.

Some authors have observed an increase in serum ferritin levels during the long-term use of IHT/IHE. Hinckson et al. (170) reported a "clearly trivial" 10.7% greater increase in serum ferritin levels following 14 consecutive days of IHE (36 min passive exposure to 10-15% O_2) relative to a normoxic placebo group (36 min of passive exposure to 21% O_2) in five male rugby players. Given the trivial changes in [Hb], the increase in serum ferritin levels following IHE may have resulted from an hypoxic-mediated increase in IL-6 production, since ferritin is a positive acute-phase protein (122). In comparison, Humberstone-Gough et al. (54) used magnitude-based inferences to calculate serum ferritin levels in seven national level triathletes were 31.0% higher than the placebo group by following 17 days of IHE at simulated altitude of 3,500-6,000 m. This effect was deemed "likely higher" relative to the "smallest worthwhile change" (0.2 × the between-subject standard deviation) of 8.2% calculated for serum ferritin. However, since pre-altitude ferritin values were 24% higher in the placebo group at baseline, it is also useful to express the change in serum ferritin levels in the IHE group relative to this group's pre-altitude values. When doing so, serum ferritin levels increased by a "trivial" 5.9% in the IHE group, which was lower than the smallest worthwhile change of 8.2% for serum levels. Thus, it

seems unlikely that long-term use of acute hypoxic exposure methods place an additional burden on the body's iron stores beyond that of normal exercise training, although no research to date has investigated the post-exercise hepcidin response during long-term IHT/IHE.

The control of intracellular iron balance following exercise in hypoxia is not well understood, but is thought to be coordinated by nitric oxide (NO) (171, 172). In addition to a reduction in oxygen-dependent proyl hydoxylases, HIF-gene transcription is also regulated by S-nitrosylation of HIF pathway components, thus increased NO expression can induce HIF-gene transcription. The regulation of HIF by NO will not be discussed here [see Ho et al. (173) for a review] owing to the complexity of this pathway. Furthermore, enhanced post-exercise NO production promotes iron redistribution and iron release from the cell cytosol following exercise (172, 174, 175). Specifically, NO encourages iron-sulphur cluster disassembly, resulting in increased IRP-1/IRE interactions. In turn, activation of IRP-1 down-regulates ferritin mRNA expression and concomitantly up-regulates TfR mRNA expression (176), thereby increasing iron uptake by erythroid cells to support erythropoiesis. Therefore, increased in NO production following exercise in acute hypoxia may help to regulate intracellular iron balance.

2.6.2 Prolonged Hypoxic Exposure

In contrast to acute hypoxic exposure, prolonged altitude exposure places a large demand on the body's iron stores since iron is required not only to support accelerated erythropoiesis (9), but is also required for the formation of iron-dependent non-haem proteins (7). Several changes in iron metabolism occur during prolonged altitude exposure to increase iron availability in hypoxia, including an increase in duodenal iron absorption, iron release from hepatocytes, iron recycling from senescent erythrocytes via reticuloendothelial macrophages and iron transport mechanisms. Collectively, these changes in iron metabolism in hypoxia help to ensure sufficient iron is available to facilitate the synthesis of haem and non-haem proteins (22).

Iron uptake by erythroid precursor cells approaches 100% of its maximal capacity during the first few days of altitude acclimatisation (9). The iron demands of erythroid precursor cells during early altitude exposure are first met by an increase in iron recycling from senescent erythrocytes and the mobilisation of iron from the labile iron pool (LIP) in reticuloendothelial cells (82). Indeed, iron mobilised from reticuloendothelial macrophages can support erythropoiesis two-to-three times above basal levels (177). Thus, if the iron storage compartment is sufficient, then ~15-40 $mg \cdot d^{-1}$ can be mobilised from the labile iron pool to meet erythroid iron requirements over three weeks of altitude exposure (177).

The mobilisation of iron from reticuloendothelial macrophages during altitude exposure is mediated simultaneously by the hypoxic stabilisation of HIF-2α transcription (167) and the suppression of hepcidin within 24-48 h of altitude exposure (22). In turn, these two mechanisms improve iron export from macrophages either by enhancing ferroportin expression (HIF-2α) or by reducing ferroportin

degradation (hepcidin). Hepcidin suppression at altitude is thought to be mediated by the release of a circulating erythroid factor, erythroferrone, by erythroblasts in response to increased kidney EPO production (104). In addition to erythroferrone, Sonnweber et al. (109) demonstrated in gene knock-out mice that PDGF-BB, a growth factor released platelets and macrophages, also plays a major role in regulating hepcidin suppression. However, the role of each of these candidate molecules is yet to be described in athletes undertaking prolonged altitude exposure.

Iron transport mechanisms are also up-regulated during early altitude acclimatisation resulting in improved iron delivery to the bone marrow. Indeed, plasma transferrin levels have been shown to increase after 16 h and 40 h of exposure to 3,400 m natural altitude (21), and after two days of high altitude exposure (4,559 m) and remain elevated even after four days of exposure (22). In contrast to other iron proteins, hypoxic exposure increases transferrin expression via a HIF-1α pathway (164). In addition, soluble transferrin receptor (sTfR) levels peak after ~5-7 days of altitude exposure following an increase in EPO during the first few days of altitude exposure, indicating increased iron uptake by erythroid precursor cells during this period (74, 89, 178).

As altitude exposure continues, duodenal iron absorption increases and stored iron is mobilised from hepatocytes to maintain iron delivery to the erythron (9, 179). These changes in iron metabolism are mediated by the hypoxic stabilisation of HIF-2α transcription and hepcidin suppression, which results in increased DMT-1 and DCytB expression on the enterocyte brush border after approximately 3-5 days of hypoxic exposure (180). Consequently, dietary iron absorption increases from 10-15% in normoxia to 20-25% at altitude to accommodate increased bone marrow requirements (9).

Similar to acute hypoxic exposure, prolonged altitude exposure increases the formation of reactive oxygen and nitrogen species (181), which may promote iron redistribution and turnover (182). In response to prolonged hypoxia, intracellular iron metabolism is regulated in two distinct phases, an early and a late phase (183). In the early phase (0-7 h), NO and H_2O_2 mediate the oxidation of iron-sulphur clusters, thereby inhibiting the aconitase activity of IRP-1, increasing the affinity of IRP-1 for its IRE (24). This is followed by an increased ferritin synthesis and TfR-1 and DMT-1 independent iron uptake. In the late phase (16-21 h), IRP-2 is the dominant regulator of IRP/IRE binding, decreasing TfR-1 independent iron uptake and ferritin protein synthesis. By this mechanism, iron uptake (via increased TfR-1 expression) and import (via DMT-1) are favoured over iron sequestration in hypoxia, facilitating increased iron availability for erythropoiesis. Consequently, an hypoxic-mediated increase in ROS and reactive nitrogen species formation after exercise may promote the mobilisation of iron from stores by reducing the translation of ferritin mRNA and increasing the translation of TfR-1, mRNA and DMT-1 (113, 172). Furthermore, a NO-mediated increase in TfR synthesis may increase TfR-Fe uptake by the bone marrow, potentially supporting erythropoiesis at altitude (171).

The control of IRP/IRE in hypoxia is regulated predominantly by an increase in HIF-2α transcription, which regulates erythropoiesis and intracellular iron balance in an organ-specific fashion (184). Whereas hypoxic activation of HIF-2α in the kidneys and liver increases EPO production, the up-regulation of HIF-2α transcription in the intestine increases DMT-1, DCytB and ferroportin gene expression, in turn improving intestinal iron absorption and plasma iron availability (2). However, if bone marrow iron uptake exceeds iron delivery, then the depletion of the intracellular iron storage pool may result in the formation of microcytic and/or hypochromic red cells. Since

erythropoiesis can only successfully proceed in iron replete conditions, IRP-1 acts to inhibit HIF-2α translation in hypoxia, thereby decreasing EPO production and erythropoiesis to conserve limited iron stores (185).

In general, iron is not mobilised from the skeletal muscle to support erythropoiesis under healthy conditions in normoxia (186). Although currently unclear, skeletal muscle iron may be sacrificed to maintain erythropoiesis during severe hypoxia in an autophagic manner (187, 188). Studies investigating the regulation of skeletal muscle iron metabolism in hypoxia have produced conflicting results (189, 190). For example, Robach et al. (191) reported a 35% down-regulation in myoglobin protein expression in nine healthy males exposed to high altitude (4,559 m) for 7-9 days. Altitude exposure also decreased skeletal muscle ferritin (35%), TfR-1 (50%) and total iron content (37%). Collectively, these findings suggest high altitude exposure is associated with a net loss of skeletal muscle iron content, perhaps indicating that iron is mobilised from the skeletal muscle to support erythropoiesis. In contrast, one month of low dose (~65 $IU \cdot kg^{-1}$) synthetic human EPO administration in eight healthy males induced skeletal muscle iron accumulation, with no change in myoglobin content despite the suppressed urinary hepcidin levels. Given these unexpected findings, these authors speculated lower ceruloplasmin expression during EPO administration, which is required for iron export via ferroportin, might explain the differences in skeletal muscle iron content they found here compared with their earlier study at high altitude. Finally, Robach et al. (190) compared the influence of high (~400 $IU \cdot kg^{-1}$) and low (~63 $IU \cdot kg^{-1}$) dose EPO injection on skeletal muscle iron metabolism in eight healthy males to elucidate a potential mechanism responsible for coordinating skeletal iron metabolism in hypoxia. Both high and low dose EPO suppressed urinary hepcidin expression within 24 h of administration, but putative mediators of hepcidin

suppression such as TSAT, sTfR, growth differentiation factor-15 were unchanged. Unlike altitude exposure, which stimulates EPO production secondary to the stabilisation of HIF, synthetic EPO injections bypass HIF stabilisation (50). Therefore, skeletal muscle iron metabolism may differ between recombinant human EPO injections and prolonged altitude exposure given the role of HIFs in regulating several iron-related proteins. Hence, further research is required to understand the regulation of skeletal muscle iron metabolism during prolonged hypoxic exposure.

2.6.2.1 Influence of Pre-Altitude Iron Status on Erythropoiesis

Sufficient pre-altitude iron stores are required to sustain accelerated erythropoiesis during prolonged altitude exposure. Iron mobilisation from hepatocytes during prolonged altitude exposure is characterised by a progressive decrease in serum ferritin levels, the magnitude of which is related to the hypoxic dose (74, 192), iron intake, iron losses and an individual's ferrokinetics (82, 179). However, the mobilisation of iron from hepatocytes does not occur until approximately four days after initial altitude exposure (9). Hence, if the labile iron pool is depleted before altitude exposure, then iron may not be mobilised from hepatocytes fast enough to support accelerated erythropoiesis during the first few days of altitude acclimatisation, resulting in iron deficient erythropoiesis (12).

An hypoxic-mediated increase in iron transport and intestinal iron absorption combined with the mobilisation of iron from storage sites may be sufficient to support accelerated erythropoiesis when the hypoxic dose is low, such as during short (less than one week) sojourns to altitudes less than 2,000 m. As the hypoxic dose increases, bone marrow iron demands may exceed iron delivery, resulting in iron deficient erythropoiesis and the development of a functional iron deficiency, even in athletes

with otherwise healthy iron stores (i.e. serum ferritin levels >35 $\mu g \cdot L^{-1}$) (177). In some athletes, low pre-altitude iron stores and/or impaired iron delivery to the erythron, may restrict erythropoiesis thereby limiting the haematological benefits of prolonged altitude exposure (10).

It is difficult to determine what constitutes a sufficient pre-altitude serum ferritin threshold to adequately support erythropoiesis during prolonged moderate altitude exposure, owing to inter-individual differences in the erythropoietic response (77, 78, 193) and iron uptake kinetics (92). For example, Stray-Gundersen et al. (10) found the increase in red cell volume in was blunted in iron deficient (pre-altitude serum ferritin: 15 ± 3 $\mu g \cdot L^{-1}$) compared with iron replete female distance runners (pre-altitude serum ferritin: 69 ± 10 $\mu g \cdot L^{-1}$) exposed to 2,800 m altitude for four weeks. Hence, the findings of Stray-Gundersen et al. (10) suggest low pre-altitude serum ferritin levels may blunt the haematological adaptations to prolonged altitude exposure. In contrast, Ryan et al. (194) found a weak, non-significant correlation (r = 0.34, p = 0.16) between the change in Hb_{mass} and pre-altitude serum ferritin levels in 19 recreational athletes exposed to 5,000 m altitude for 16 days. This weak relationship existed regardless of women with serum ferritin levels <20 $\mu g \cdot L^{-1}$ ingested 325 $mg \cdot d^{-1}$ ferrous sulphate, 2-3 times per day, for 2-3 weeks before altitude departure. Notably, two female athletes both increased their Hb_{mass} by >7% despite a severe iron deficiency (i.e. pre-altitude serum ferritin levels <12 $\mu g \cdot L^{-1}$). In light of these findings, pre-altitude serum ferritin may only weakly influence Hb_{mass} production during high altitude exposure, unless athletes are iron deficient before altitude exposure.

The size of the iron storage compartment before altitude exposure may influence iron metabolism during prolonged altitude exposure. For example, Peeling et al. (19)

reported lower basal hepcidin levels and an attenuated post-exercise hepcidin response in athletes with serum ferritin levels <30 $\mu g \cdot L^{-1}$ at sea-level. Since iron deficiency induces tissue hypoxia, it is possible that the post-exercise hepcidin response is suppressed in athletes with low iron stores during prolonged altitude exposure, although further research is required to confirm such a hypothesis. Furthermore, Mast et al. (195) recently demonstrated hepcidin expression was down-regulated in response to the hypoxic-stress of haemorrhage in non-active, healthy subjects with clinically healthy iron stores (serum ferritin: ≥26 $\mu g \cdot L^{-1}$). In comparison, iron deficient subjects (serum ferritin: ≤26 $\mu g \cdot L^{-1}$), already had low hepcidin levels before haemorrhage, and these subjects instead responded by markedly increasing erythropoietin production in an attempt to maintain oxygen homeostasis in response to hypoxia. Taken together, these two studies suggest iron metabolism during prolonged altitude exposure could differ between iron deplete and iron replete athletes, although further research is required to explore this hypothesis.

2.6.2.2 Influence on Post-Exercise Iron Metabolism

Performing exercise during prolonged altitude exposure places an additional demand on an athlete's iron stores since athletes must provide iron to support accelerated erythropoiesis as well as replenish iron lost through mechanisms such as foot-strike haemolysis, gastrointestinal bleeding, haematuria and sweating (116). Maintaining iron balance during prolonged altitude exposure may be difficult since altitude exposure presents opposing mechanisms for the regulation of systemic iron balance. On the one hand, altitude exposure suppresses hepcidin expression to improve iron availability and thus maintain oxygen homeostasis (21-23). Indeed, hypoxic hepcidin suppression and an increase in DMT-1 and DCytB enhances intestinal iron absorption (i.e. from ~10% at sea level to ~20% at altitude (179), may balance the ~2-3 $mg \cdot d^{-1}$ of

exercise-related iron losses in athletes (34). On the other hand, both prolonged altitude exposure and exercise increase systemic IL-6 production (196), which is a potent promoter of hepcidin expression (197), consequently compromising oxygen homeostasis by limiting iron delivery to the erythron during exercise recovery. One possibility is prolonged altitude exposure decreases the magnitude of the post-exercise hepcidin response relative to equivalent exercise performed at sea level. Furthermore, pre-altitude serum ferritin levels may influence the magnitude of the post-exercise hepcidin response (19) Thus, whilst increased erythropoiesis and reduced iron stores may blunt the post-exercise hepcidin response during prolonged altitude exposure, at the same time, altered iron availability exercise-induced inflammation increases the magnitude of the post-exercise hepcidin response. However, the dominant mechanism responsible for regulating post-exercise iron metabolism during prolonged altitude exposure is yet to be investigated.

2.6.3 Summary

In summary, acute hypoxic exposure is insufficient to stimulate erythropoiesis, but may enhance aerobic and anaerobic performance by stimulating oxidative and glycolytic enzyme production. Acute hypoxic exposure methods do not seem to place an additional burden on the body's iron stores compared with exercise in normoxia, although it is currently unclear whether inflammatory cytokines or EPO regulates post-exercise hepcidin production following exercise performed in acute hypoxia. Thus, further research is required to understand the control of systemic iron balance during the long-term use of IHT/IHE protocols. Finally, both exercise and hypoxic exposure may regulate intracellular iron balance by increasing post-exercise NO bioavailability, in turn promoting iron-sulphur cluster disassembly, increasing iron transport by Tf and enhancing the mobilisation of iron from ferritin.

Prolonged altitude exposure enhances renal EPO production within the first few days of altitude exposure and steadily declines to baseline after one week of exposure. EPO acts on the bone marrow to stimulate erythropoiesis, but a rise in blood lactate and inflammatory cytokines during early altitude adaptation may blunt kidney EPO release. Iron delivery to the bone marrow increases at altitude to service accelerated erythropoiesis, which places a large burden on the body's iron stores. To meet erythroid iron demands in hypoxia, the body enhances iron recycling, intestinal iron absorption and mobilises iron from storage sites. EPO, hepcidin and reactive oxygen and nitrogen species collectively regulate iron metabolism at altitude. Whereas an increase in post-exercise cytokine production following acute exercise in hypoxia may increase post-exercise hepcidin production; prolonged altitude exposure may suppress both resting and post-exercise hepcidin expression. However, the regulation of iron metabolism following exercise combined with acute and/or prolonged hypoxic exposure is currently unknown.

2.7 Maintaining Iron Balance at Altitude

2.7.1 Pre-Altitude Blood Screening

Athletes planning to undertake prolonged altitude exposure require a comprehensive blood pre-screening to ensure their iron stores are sufficient to support accelerated erythropoiesis. The exact amount of iron required to meet erythroid iron demands at altitude is highly individual and depends on the hypoxic dose (189), an individual's ferrokinetics (198) and the amount of iron lost through exercise-related processes (34). A pre-altitude blood screening should be obtained approximately 6-8 weeks before altitude exposure and include a full blood count and iron profile. Obtaining an iron profile 6-8 weeks before exposure may allow sufficient time for daily oral iron

supplements to restore an athlete's iron stores, if required. Typically, a [Hb] <120 $g \cdot L^{-1}$ in females and <130 $g \cdot L^{-1}$ in males indicates iron deficient erythropoiesis and is often associated with the exhaustion of the body's iron stores when serum ferritin levels are <12 $\mu g \cdot L^{-1}$ (115). A comprehensive examination of an athlete's iron status before altitude could include an estimation of an athlete's iron storage compartment (as indicated by serum ferritin), iron transport capacity (via transferrin and transferrin saturation) and the current rate of bone marrow iron uptake (via sTfR). Since ferritin and transferrin are positive and negative acute phase proteins respectively, these iron parameters may misrepresent the true iron storage pool in the presence of inflammation (122). At least two or three iron parameters in addition to serum ferritin should therefore be interpreted to determine an athlete's iron status and bone marrow function.

A positive haematological response to iron supplementation represents a true measure of the adequacy of an athlete's current iron storage pool. Recently, Hb_{mass} measured using the optimised 2 min CO rebreathing technique (199) has been proposed as a more stable measure of the red cell compartment than haemoglobin concentration since it is less sensitive to the potentially confounding effects of changes in plasma volume at altitude (52). An increase in Hb_{mass} may precede a rise in [Hb] in athletes ingesting iron supplements (134, 135), therefore, measuring Hb_{mass} under standardised conditions is useful to determine the size of the red cell compartment before altitude exposure. To this end, an increase in Hb_{mass} in response to oral iron supplementation *before* altitude exposure may indicate a pre-existing iron deficiency. For this reason, measuring Hb_{mass} before commencing oral iron supplementation and again immediately before altitude exposure, following a period of oral iron supplementation

may help to quantify whether an athlete has adequate iron stores to support accelerated Hb_{mass} production at altitude.

Given intestinal iron absorption approaches 20-25% in hypoxia (113), iron replete athletes (serum ferritin: >35 $\mu g \cdot L^{-1}$) may be able to maintain iron balance during short-term exposure at low to moderate altitudes by dietary sources alone, and may not require oral iron supplements. Vegetarian athletes or athletes with a low dietary iron intake may require oral iron supplementation to service accelerated erythropoiesis at altitude. Since prolonged exposure to moderate and/or high altitude exposure induces a three-to-five-fold increase in erythropoiesis (82), iron demands are unlikely to be met by dietary sources alone. In these instances, oral or intravenous iron supplements may be required to support erythropoiesis. Data from the clinical population suggest oral iron supplements are able to support up to a four-fold increase in erythropoiesis, but intravenous iron supplements are necessary to support a five-fold increase in erythropoiesis (200). Greater increases in erythropoiesis are unlikely in healthy subjects. Hence, Berglund (12) recommended providing oral iron supplements to all athletes before moderate-to-high altitude exposure to compensate for increase bone marrow iron uptake, but recommended providing IV iron supplements to athletes with pre-altitude serum ferritin level <40 $\mu g \cdot L^{-1}$. No clear serum ferritin threshold currently exists however to guide iron supplementation for athletes planning to undertake prolonged altitude exposure.

2.7.2 Dietary Considerations

A low dietary iron intake at altitude could place athletes at risk of an iron deficiency and limit the amount of iron available to support iron-dependent haem and non-haem protein synthesis (201). In hypoxia, dietary iron absorption increases from ~10-15%

in normoxic conditions to ~20-25% (202, 203). Despite this compensatory increase in iron absorption at altitude, a low dietary intake of protein (204) and/or a calorie restrictive diet may place athletes at risk of developing an iron deficiency. Non-vegetarian athletes are recommended to increase dietary intake of iron by ingesting food rich in haem iron (such as red meat), 2-3 weeks before altitude exposure and continue to consume red meat during altitude exposure where possible. Vegetarian athletes may need to increase the consumption of non-haem iron such as iron-fortified cereals, energy bars, green leafy vegetables, tofu and legumes (205). Ingesting foods and drinks high in vitamin C (i.e. orange juice, citrus fruits) at meal times assists dietary iron absorption (206). Athletes should avoid coffee, tea (207) and calcium (208) 30 min before and 60 min after meals since these food items inhibit iron absorption.

The influence of elevated post-exercise hepcidin levels on iron absorption and recycling at altitude is currently unknown. Altitude exposure suppresses resting hepcidin levels (21, 22), but an increase in post-exercise hepcidin levels may inhibit iron uptake during the post-exercise recovery period. Further research is required to determine how elevated post-exercise hepcidin levels influence intestinal iron absorption and bone marrow iron uptake at altitude. For example, the time course of the post-exercise hepcidin response at altitude is yet to be established. Such research may help to determine the optimal time to ingest iron rich compounds post-exercise at altitude to maximise their absorption.

2.7.3 Iron Supplementation

Iron supplements may ensure athletes have adequate iron stores to sustain accelerated erythropoiesis at altitude especially if dietary iron intake or the iron storages pool is

low. An appropriate iron supplementation strategy allows athletes to maintain their iron balance at altitude whilst also mitigating their risk of harm from oxidative stress. Two main iron supplementation options are available, oral iron supplements and intravenous iron preparations (including intravenous iron injections). The benefits and limitations of oral and intravenous iron supplements are discussed below.

2.7.3.1 Oral Iron Supplements

Oral iron supplementation is the most common method used to raise an athlete's iron stores in preparation for altitude exposure. Several oral iron salt preparations are available (e.g. ferrous sulphate, ferrous fumarate, ferrous gluconate) and are often combined with ascorbic acid to enhance their absorption in the intestine (e.g. Ferrogradumet C) (209). Ferrous sulphate, which supplies between 65-105 mg of elemental iron per 200 mg tablet, is the most widely used preparation available on the market (210) and is generally taken on an empty stomach to avoid interactions with compounds that inhibit iron absorption (121). Oral iron repletion therapy involves the supplementation of between 65-105 mg of elemental iron administered daily over 2-3 months (121) and its effectiveness is dependent on amount of iron absorbed via the intestine. An oral iron dose of 100 $mg \cdot d^{-1}$ elemental iron may increase serum ferritin levels by 30-50% over 8-weeks (147, 211).

Oral iron supplements are unfortunately associated with several negative side effects. For example, a recent meta-analysis found oral iron supplements such as ferrous sulphate were not well tolerated and were associated with nausea, blackened stools and gastrointestinal discomfort (212). Such side effects may result in non-compliance, therefore reducing the efficacy of this supplementation method. In the event of gastrointestinal discomfort, athlete may be advised to progressively increase their

dose of oral iron supplements, which may help athletes tolerate some of the gastrointestinal side effects. Alternatively, ingesting oral iron supplements every 2-3 days or in combination with food may alleviate some of the negative side effects (205).

Oral iron supplements have been shown to disrupt the bacteria of the gut mucosa, potentially promoting the formation of pathogenic microbacteria (213). Furthermore, unbound iron may enhance oxidative stress on the gut mucosa (24). Another potential concern is that the provision of oral iron supplements to athletes who already have filled iron stores before altitude exposure could place them at risk of developing an iron overload, since altitude exposure enhances intestinal iron absorption. Considering the short duration of iron supplementation and the increase in bone marrow iron uptake during altitude exposure, the risk of developing an iron overload at altitude is low unless individuals have a pre-existing iron overload disorder such as haemochromatosis. Developing altitude-specific serum ferritin thresholds to guide the prescription of oral iron supplements may assist to maintain iron balance during altitude sojourns.

Several studies to date have investigated the influence of oral iron supplementation on haematological and iron parameters in athletes planning to undertake or currently undertaking altitude exposure. Hannon et al. (214), found iron supplementing healthy females with 300 $mg \cdot d^{-1}$ of liquid ferrous sulphate for four weeks in preparation for high altitude exposure (4,300 m) significantly increased their baseline haematocrit levels, despite them having serum iron levels within the healthy physiological range. In contrast, a randomised, double blind, placebo control trial showed 10 weeks of oral iron supplements (105 $mg.d^{-1}$ elemental iron) failed to enhance total Hb_{mass} in 16 highly active female Air Force cadets undergoing basic combat training whilst living

at an altitude of 2,200 m (215). In another study, Friedmann et al. (216) investigated the influence of iron supplementation *versus* non-supplementation on total Hb_{mass} in non-iron deficient, national level male boxers after 18 d of moderate (1,800 m) altitude exposure. These authors concluded oral iron supplementation (200 $mg \cdot d^{-1}$ elemental iron) did not enhance red cell production compared with a non-iron supplemented placebo group despite signs of an increased erythrocyte iron turnover at altitude. Since serum ferritin levels were not significantly reduced from baseline measures in the placebo or iron supplementation group at any time during or after altitude exposure, pre-altitude iron stores were likely sufficient to cope with the additional iron uptake by the erythroid marrow. Moreover, the moderate altitude used in this study induced only a small increase in $Hb_{mass,}$ suggesting a low erythroid iron uptake. In comparison, the erythroid iron demand is greater at higher altitudes than those used by Friedmann et al. (216) (i.e. 2,000-3,000 m *versus* 1,800 m) (74, 192), thus exposure to higher altitudes likely places a greater burden on the body's iron stores. In support of this assertion, Robertson et al. (70) observed a significant decrease in serum ferritin and increase in sTfR from pre-altitude levels in eight distance runners exposed to 2 × 3 week LHTL camps (14 $h \cdot d^{-1}$ at 3,000 m simulated altitude, train at 600 m natural altitude) scheduled three-weeks apart. The decrease in serum ferritin occurred despite athletes ingesting 325 $mg \cdot d^{-1}$ of ferrous sulphate, one week before, and for the duration, altitude exposure. In comparisons, serum ferritin and sTfR levels were unchanged in either of the three-week training camps in the sea level control group (n = 8). These observations suggest increased iron mobilisation from storages cells (reduced serum ferritin) as well as increased erythroid iron uptake (increase in sTfR). Based on the series of studies above, it is important to consider the hypoxic dose as well as pre-altitude serum ferritin levels when deciding upon the

appropriate iron supplementation strategy to ensure athletes maintain an optimal iron balance at altitude.

In summary, oral iron supplementation may be required to sustain a three-fold increase in erythropoiesis associated with prolonged exposure to moderate altitudes (2,000-3,000 m). Unfortunately, oral iron supplements may be associated with several negative side effects and are often poorly tolerated. Oral iron supplementation may not be required for athletes with healthy pre-altitude iron stores (serum ferritin: >40 $\mu g \cdot L^{-1}$) or when the hypoxic dose is low, such as during short sojourns to altitudes lower than ~2,000 m, but this has yet to be determined.

2.7.3.2 Intravenous Iron Supplements

IV iron administration may be necessary to restore the iron storage compartment in anaemic athletes (serum ferritin <12 $\mu g \cdot L^{-1}$, TSAT <16%, Hb <115 $g \cdot L^{-1}$) who require a rapid increase in serum ferritin levels before altitude exposure or those who are unresponsive to oral iron supplementation (209). Currently, the infusion or injection of >50 mL of fluid over 6 h is a World Anti-Doping Agency prohibited practice (217). Moreover, one may question the ethics of providing IV iron supplements to athletes with otherwise healthy serum ferritin levels (i.e. >35 $\mu g \cdot L^{-1}$), who are able to tolerate oral iron supplements (209), on the basis that IV iron therapy may unnecessarily increase an athlete's risk of anaphylaxis, tissue oxidative and nitrosative damage and infection risk (24). Yet, many of the reservations about the use of IV iron are based on the risks associated with older, high molecular weight iron dextran formulations (218). In contrast, newer low molecular weight IV iron formulations contained in a carbohydrate shell (such as ferric carboxymaltose) seem to pose a smaller risk of harm

than older iron dextran formulations (219) and are associated with fewer adverse drug reactions (218).

IV iron administration is more efficacious than oral iron supplementation at restoring the iron storage compartment and improving Hb_{mass} in iron deficient athletes (220). IV iron preparations such as ferric carboxymaltose are associated with fewer gastrointestinal side effects since iron compounds are delivered directly into the blood stream, thereby bypassing the gut mucosa (212). Furthermore, these iron compounds can be delivered as a bolus injection of ~ 2-4 mL (delivering ~200-500 mg depending on the athlete's requirements), which does not breach the WADA anti-doping code and do not require a test dose (221). Garvican et al. (220) recently compared the efficacy of six weeks of oral (305 mg ferrous sulphate ingested daily for six weeks) or IV iron supplementation (2 injections of ferric carboxymaltose per week for six weeks) on iron status and Hb_{mass} in 27 highly trained endurance athletes (14 males, 13 females) with low (serum ferritin: <35 $\mu g \cdot L^{-1}$, TSAT: <20% or serum ferritin: <15 $\mu g \cdot L^{-1}$) or suboptimal (serum ferritin: <65 $\mu g \cdot L^{-1}$, TSAT: >20%) iron status. Although both iron supplementation methods improved athletes' serum ferritin levels, IV supplementation more effectively raised athletes' serum ferritin levels. The change in Hb_{mass} was 5.2%, (90% CL: [0.8, 9.8%]) and VO_{2max} 2.1% (90% CL: [-3.3, 7.7]) greater in IV low group compared with the oral low group, six weeks post-injection. In another study, six weeks of IV iron administration (3 × 2 mL ferric carboxymaltose injection) improved perceived fatigue and mood and enhanced 10 × 400 m performance in 14 iron replete (serum ferritin: 30-100 $\mu g \cdot L^{-1}$), trained athletes relative to a placebo injection group, despite no change in Hb_{mass}, [Hb] or 3,000 m time trial performance (222). In iron replete athletes, IV iron administration may therefore improve exercise performance, perception of fatigue and mood, without concurrent

improvements in Hb_{mass}. These authors concluded IV iron administration increased the amount iron available for energy production via glycolytic and oxidative pathways. In comparison, Burden et al. (223) found no improvement in measures of aerobic performance (VO_{2max}, vVO_{2max}, running economy) or total Hb_{mass} in seven well-trained distance runners (serum ferritin: <30 $\mu g \cdot L^{-1}$) 24 h or four weeks after an IV iron injection (500 mg ferric carboxymaltose). Collectively, the series studies discussed above suggests an IV iron injection does not seem to enhance Hb_{mass} or VO_{2max} unless athletes are at least Stage 2 iron deficient (i.e. Stage 2 iron deficient, serum ferritin < 20 $\mu g \cdot L^{-1}$).

IV iron administration could be a more viable option to increase the iron storage compartment in athletes who need to replenish their iron stores rapidly before altitude exposure, who are non-responsive to oral iron supplementation, or who are prone to gastrointestinal side effects with oral iron supplementation. Furthermore, IV iron supplementation may be required to sustain erythropoiesis in athletes who are living or training at altitudes greater than 3,000 m owing to a large increase in erythroid iron uptake (74). In future, a randomised placebo control trial is required to compare the effectiveness of IV *versus* oral iron supplements in improving iron status, aerobic and anaerobic performance in athletes undertaking prolonged altitude exposure to establish the efficacy of iron supplementation strategies used to prepare athletes for altitude exposure.

2.7.4 Summary

Maintaining a healthy iron balance at altitude ensures sufficient iron is available to support an hypoxic-mediated increase in bone marrow iron uptake, as well as increased oxidative and glycolytic enzyme production. Daily oral iron

supplementation may enhance iron availability during altitude exposure and thereby ensure athletes can sustain accelerated erythropoiesis at altitude. Unfortunately, the gastrointestinal side effects related to oral iron supplementation mean they are often poorly tolerated. Additionally, currently no serum ferritin thresholds exist to guide oral iron supplementation in athletes planning to undertake altitude exposure. Furthermore, the optimal time to ingest oral iron supplements to maximise their absorption at altitude is also unknown. Alternatively, IV iron supplementation may be indicated in athletes planning to undertake altitude exposure who are severely iron deficient or cannot tolerate oral iron supplements. When used under the supervision of a qualified sports physician, the use of low molecular weight, carbohydrate-based IV iron supplements are safe and effective method of replenishing iron stores.

2.8 Conclusion

Acute hypoxic exposure does not stimulate erythropoiesis, but may instead enhance anaerobic performance by increasing skeletal muscle glycolytic enzyme concentration. In comparison, Hb_{mass} increases by ~1% per 100 h exposure following moderate (>2,200 m) altitude exposure, which enhance aerobic performance. Low pre-altitude iron stores may however jeopardise both haematological and non-haematological adaptations to prolonged altitude exposure. Hence, oral iron supplementation may help iron deficient athletes to support accelerated erythropoiesis at altitude, but further research is required to develop altitude-specific oral iron supplementation guidelines to ensure athletes can maintain a healthy iron balance.

The regulation of iron metabolism during acute and prolonged hypoxic exposure is not well understood. On the one hand, both exercise and hypoxia increase IL-6 production, which is a potent promoter hepcidin production. On the other hand,

hepcidin is suppressed during prolonged hypoxic exposure (22). Hence, a better understanding of how iron metabolism is regulated in hypoxia may enable sport scientists to develop strategies for athletes to maintain iron balance during prolonged altitude exposure. Indeed, the outcomes of such research may help to optimise both the haematological and non-haematological adaptations associated with prolonged altitude exposure.

3 CHAPTER THREE

ACUTE HYPOXIC EXERCISE DOES NOT ALTER POST-EXERCISE IRON METABOLISM IN WELL-TRAINED ENDURANCE ATHLETES

Govus, A. D., Abbiss, C. R., Garvican-Lewis, L. A., Swinkels, D. W., Laarakkers, C. M., Gore, C. J., & Peeling, P. (2014). Acute hypoxic exercise does not alter post-exercise iron metabolism in moderately trained endurance athletes. *European Journal of Applied Physiology*, 114(10): 2183-2191. Impact factor: [2.3].

3.1 Abstract

Purpose: To measure the influence of acute hypoxic exercise on Interleukin-6 (IL-6), hepcidin and iron biomarkers in athletes. **Methods:** In a repeated measures design, thirteen well-trained endurance athletes performed 5 × 4 min intervals at 90% of their peak oxygen consumption velocity (vVO_{2peak}) in both normoxia (NORM, fraction of inspired oxygen (F_IO_2) = 0.2093, 15.3 ± 1.7 $km \cdot h^{-1}$) and normobaric hypoxia (HYP, F_IO_2 = 0.1450, 13.2 ± 1.5 $km \cdot h^{-1}$). Venous blood samples were obtained pre-, post-, and 3 h post-exercise, and analysed for serum hepcidin, IL-6, ferritin, iron, soluble transferrin receptor (sTfR) and transferrin saturation. **Results:** Peak heart rate was significantly lower in HYP compared with NORM (p = 0.01); however, the rating of perceived exertion was similar between trials (p = 0.24). Ferritin (p = 0.02), transferrin (p = 0.03) and IL-6 (p = 0.01) significantly increased immediately post-exercise in both conditions, but returned to baseline 3 h later. Hepcidin increased in both conditions 3 h post-exercise (p = 0.05), with no significant differences between trials. A significant treatment effect was observed between trials for sTfR (p = 0.01); but not iron and transferrin saturation. **Conclusion:** Acute exercise in hypoxia did not influence post-exercise IL-6 production, hepcidin or iron parameter responses compared with exercise at the same relative intensity in normoxia. Hence, acute exercise performed at the same relative intensity in hypoxia poses no further risk to an athlete's iron status compared with exercise in normoxia.

3.2 Introduction

Athletes commonly undertake acute (minutes to hours) and/or prolonged (weeks) exposure to hypoxia to enhance normoxic exercise performance. Prolonged hypoxia improves tissue oxygen transport and extraction by augmenting several haematological and non-haematological biomarkers (7). Maintaining a healthy iron status (serum ferritin >30 $\mu g \cdot L^{-1}$) ensures bone marrow iron delivery sufficiently supports accelerated erythropoiesis during hypoxic exposure, because iron is involved in haemoglobin and myoglobin synthesis whilst also playing a key role in energy metabolism and cellular respiration (143, 144). However, low iron stores have been shown to compromise haematological adaptations associated with prolonged hypoxic exposure, potentially reducing the effectiveness of this training modality (10).

Exercise may transiently compromise iron metabolism in athletes 3-6 h after exercise via an Interleukin-6 (IL-6)-mediated increase in the iron regulatory hormone, hepcidin (16-18, 139, 152, 224). Elevated hepcidin levels reduce iron recycling from senescent erythrocytes and intestinal iron absorption by degrading ferroportin iron export channels, consequently blocking the efflux of iron contained on iron storage cells (15) into the blood plasma, which in turn decreases iron delivery to the bone marrow for erythropoiesis. Increased post-exercise hepcidin levels may therefore place athletes at a heightened risk of iron deficient erythropoiesis, compared with the non-athletic population.

In addition to IL-6, serum iron concentration, hypoxia, and erythropoietic activity regulate hepcidin synthesis (153). However, the influence of exercise performed in hypoxia on post-exercise iron metabolism is currently unclear because IL-6 and hypoxia have opposite effects on hepcidin synthesis. Indeed, although acute hypoxic-

exercise increases IL-6 production (27), continuous hypoxic exposure and an increase in erythropoietic activity suppress hepcidin levels within 24 to 48 h (23, 225, 226). Several other mechanisms, including an enhanced transcription of HIF-1α and HIF-2α (159, 162), an increase in serum EPO concentration (190, 225), and an up-regulation of growth differentiation factor-15 (227) or twisted gastrulation factor-1 (228) may also suppress hepcidin expression.

Reduced hepcidin levels in hypoxic conditions enhances intestinal iron absorption and iron mobilisation from storage sites in the liver, macrophages, intestine and skeletal muscle (202, 203). Such mobilisation improves iron delivery to the bone marrow for erythropoiesis during times of limited iron bioavailability, such as during hypoxic exposure, or when an individual is iron deficient. With this in mind, a hypoxic-mediated reduction in hepcidin expression could improve iron absorption from iron rich meals consumed post-exercise, thus enabling the maintenance of iron status more effectively. However, whether hepcidin suppression occurs after acute exercise in hypoxia is currently unknown.

This study therefore compared the influence of high-intensity exercise performed in normoxia and normobaric hypoxia on post-exercise IL-6, hepcidin and iron responses. We hypothesised exercise in normobaric hypoxia would suppress post-exercise hepcidin production, independent of increased post-exercise IL-6 levels.

3.3 Methods

3.3.1 Participants

Thirteen (seven males, six females) well-trained endurance runners (n = 9) or triathletes (n = 4) participated in this study (mean ± standard deviation for age: 28.8 ±

5.3 y, body mass: 63.9 ± 11.8 kg, peak oxygen consumption (VO_{2peak}): 58.2 ± 6.4 $mL \cdot kg^{-1} \cdot min^{-1}$). Each participant provided written informed consent before testing. The Human Research Ethics Committee at Edith Cowan University granted approval for this study in accordance with the Declaration of Helsinki.

3.3.2 Experimental Design

Participants performed four exercise sessions, scheduled at least 7 d apart. The first two sessions were graded exercise tests (GXT) in either normoxia (NORM, fraction of inspired oxygen (F_IO_2) = 0.2093) or normobaric hypoxia (HYP, F_IO_2 = 0.1450). This test determined the athlete's VO_{2peak}, running velocity attained at VO_{2peak} (vVO_{2peak}), and peak heart rate (HR_{peak}) within each environment. The remaining two experimental sessions included a high-intensity interval run (INT) on a motorised treadmill in either NORM or HYP, in a counterbalanced order. These two interval sessions consisted of 5 × 4 min efforts at 90% vVO_{2peak} separated by 1.5 min of passive recovery. Before each INT session, a standardised 5 min treadmill-based running warm-up was conducted at 65% $vVO_{2peak,}$ making the total session duration 31 min. During each INT session, venous blood samples were collected from a forearm antecubital vein before, immediately after exercise and after 3 h of passive recovery in normoxia.

3.3.3 Experimental Procedures

3.3.3.1 Graded Exercise Test (GXT)

The GXT commenced at a speed of 10 $km \cdot h^{-1}$ (females) or 12 $km \cdot h^{-1}$ (males) in NORM, and at 8 $km \cdot h^{-1}$ (females) or 10 $km \cdot h^{-1}$ (males) in HYP at a 1% gradient throughout (229). Treadmill speed increased by 1 $km \cdot h^{-1}$ every 4 min, separated by 1

min of passive recovery until participants reached volitional exhaustion. During the GXT, VO_2 for each workload was calculated based upon expired gas fractions and volumes. Briefly, expired gas volumes were collected during the final 30 s of each workload into a Collins 120 L chain compensated gasometer (Warren E Collins, Braintree, MA, USA) as per (230). Thereafter, expired gas fractions were determined by drawing an expirate sample out the gasometer into a 2 L Douglas bag, which were analysed using a calibrated electrochemical oxygen analyser and an infrared carbon dioxide analyser (Ametek Gas Analyzers, Applied Electrochemistry, SOV S-3A/1 and COV CD-3A, Pittsburgh, PA).

3.3.3.2 High-Intensity Interval Running Session (INT)

Participants attended the laboratory having refrained from exercise and caffeine for 12 h. After collection of the pre-exercise venous blood sample, participants performed a standardised warm-up of 5 min treadmill running at 65% vVO_{2peak} followed by 5 min of dynamic stretching. Thereafter, participants ran 5 × 4 min intervals on a motorised treadmill in either NORM or HYP conditions. Additionally, during each session, heart rate (HR) was recorded continuously and a rating of perceived exertion (RPE) was obtained at the conclusion of each interval.

3.3.3.3 Simulated Altitude Environment

Altitude was simulated through nitrogen injection (flow rate 270 $L \cdot min^{-1}$, VPSA S325 V16, van Amerongen, Tiel, The Netherlands) in a 40 m^3 environmental chamber (b-Cat BV S879, Tiel, The Netherlands). To simulate 3,000 m altitude, the chamber was set at a F_IO_2 of 0.1450 because the chamber was located at sea level (Perth, Western Australia).

3.3.3.4 Venous Blood Collection

After 10 min of supine rest to control for postural shifts in plasma volume (231), a venous blood sample was collected from a forearm antecuitbal vein using a 22-gauge needle, filling two 8.5 mL blood collection tubes (SST II Gel; BD Vacutainer™, New Jersey, USA). These tubes then clotted at room temperature for 30 min before centrifugation at 10 °C and a speed of 1.1 rcf (3,000 g) for 10 min. After centrifugation, the serum supernatant was transferred into 4 × 1 mL aliquots and stored at -80 °C for batch analysis.

3.3.3.5 Hepcidin

Serum hepcidin measurements were performed (Testing lab: Hepcidinanalysis.com, Nijmegen, The Netherlands) by a combination of weak cation exchange chromatography and time-of-flight mass spectrometry (WCX-TOF MS) using an internal standard (synthetic hepcidin-24; custom made Peptide International Inc.) for quantification (232, 233). Peptide spectra were generated on a Microflex LT matrix-assisted laser desorption/ionisation TOF-MS platform (Bruker Daltonics). Serum hepcidin-25 concentrations were expressed as $nmol.L^{-1}$ [nM]. The lower limit of detection of this method was 0.5 nM. The median reference level of serum hepcidin-25 (Dutch population) is 4.5 nM for men, 2.0 nM for premenopausal women, and 4.9 nM for postmenopausal women. The reference levels for the WCX-TOF MS method are derived from those of our enzyme method (234), based on the regression line between the Enzyme Linked Immunosorbent Assay (ELISA) and WCX-TOF MS results obtained for the same samples from patients without hepcidin isoforms. Details on the WCX-TOF MS reference values for hepcidin in adults can be found at *www.hepcidinanalysis.com*.

3.3.3.6 Interleukin-6

Serum IL-6 was analysed using a commercially available ELISA kit (Quantikine, HS, R & D Systems, Minneapolis, USA) with an assay range of 0.447–9.960 $ng \cdot L^{-1}$.

3.3.3.7 Iron Studies

Serum ferritin concentration was analysed on an Architect i4000SR analyser (Abbott Diagnostics, Abbott Laboratories, Abbott Park, IL 60064 USA) using Chemoluminesence Microparticle Immunoassay (CMIA) technology. Serum iron and transferrin were measured on the Architect analyser (Abbott Diagnostics, Abbott Laboratories, Abbott Park, IL 60064, USA). Serum iron levels were measured using spectrophotometry after adding an iron reagent (Sentinel Diagnostics, Milan, Italy). Serum transferrin levels were measured using immunoturbidimetry after adding transferrin antibodies (Abbott Diagnostics, Abbott Laboratories, Abbott Park, IL 60064 USA). Additionally, transferrin saturation was calculated by dividing serum iron by the total iron binding capacity. Soluble transferrin receptor (sTfR) was measured by latex enhanced immunoturbidimetry via Tina Quant® (Roche Diagnostics GmbH, Mannheim, Germany) on a Cobas Integra 800 modular system (Roche Diagnostics GmbH) with absorbance measured at 583 nM.

3.3.4 Statistical Analysis

A 2 × 2 repeated measures analysis of variance (ANOVA) analysed the effects of Time (2 levels: pre-exercise, post-exercise) and Treatment (2 levels: NORM, HYP) on IL-6 and hepcidin levels. Additionally, a 3 × 2 ANOVA analysed the effects of Time (3 levels: pre-exercise, post-exercise, 3 h post-exercise), Treatment (2 levels: NORM, HYP) and their interaction on the abovementioned iron parameters. In the event of a

significant interaction effect, a post-hoc Bonferroni correction for multiple comparisons was applied to adjust the family-wise error rate. Significance was set at an alpha level of $p \leq 0.05$.

Data were also analysed via a contemporary statistical approach using standardised mean differences as a Cohen's *d* effect size (235). To calculate the standardised mean difference, the mean difference between pre-exercise and post-exercise values was divided by the standard deviation of the pre-exercise value. Effect sizes were then interpreted using Cohen's Scale for Effect Sizes (236) with the following qualitative descriptors; *trivial* (0.0-0.2), *small* (0.2-0.6), *moderate* (0.6-1.2), *large* (1.2-2.0), *very large* (2.0-4.0). The imprecision of the point estimate was quantified using 90% confidence limits. Situations where the confidence interval simultaneously overlapped positive and the negative thresholds were deemed unclear.

3.4 Results

3.4.1 Interval Running Session

There was no difference in ambient temperature (NORM: 22.2 ± 1.4 °C, HYP: 22.9 ± 1.3 °C, $p = 0.12$) or barometric pressure (NORM: 759.5 ± 9.41 mmHg, HYP: 758.1 ± 3.1 mmHg, $p = 0.52$) between the NORM or HYP sessions. The F_IO_2 was 0.2081 ± 0.0026 and 0.1450 ± 0.0005 for NORM and HYP, respectively. The relative running velocity (90% vVO_{2peak}) and HR_{peak} were lower during HYP as compared to NORM (vVO_{2peak}: $p = 0.00$, HR_{peak}: $p = 0.01$), but there was no difference ($p = 0.24$) in RPE between trials (Table 3.1).

Table 3.1: Response of common physiological variables to a 5 × 4 min interval running session at 90% maximal aerobic running velocity ($v\dot{V}O_{2peak}$) in normoxia (F_IO_2 = 0.2093) and normobaric hypoxia (F_IO_2 = 0.1450).

	Normoxia	Hypoxia
$v\dot{V}O_{2peak}$ (km·h^{-1})	17.0	14.7**
	(1.9)	(1.7)
90% $v\dot{V}O_{2peak}$ (km·h^{-1})	15.3	13.2**
	(1.7)	(1.5)
Peak Heart Rate (beats·min^{-1})	173	168**
	(9)	(11)
Rating of Perceived Exertion	15.0	15.0
	(1.5)	(2.0)

* Significantly different to normoxic condition ($p < 0.05$)

** Significantly difference different to normoxic condition ($p < 0.01$)

3.4.2 Interleukin-6

IL-6 increased from baseline immediately post-exercise in both conditions (p = 0.01); however, there were treatment (p = 0.60) or interaction effects (p = 0.48) (Table 3.2). The time effect was supported by moderate (d = 0.60) and large (d =1.49) effect sizes for NORM and HYP conditions, respectively (Table 3.2).

3.4.3 Hepcidin

Serum hepcidin increased from baseline levels at 3 h post-exercise in both conditions (p = 0.05). This time effect was supported by moderate effect sizes in both the NORM and HYP conditions (Table 3.2). No treatment (p = 0.66) or interaction effect (p = 0.39) was present in NORM and HYP, respectively (Table 3.2).

Table 3.2: Interleukin-6 and hepcidin response to a 5 × 4 min interval running session at 90% maximal aerobic running velocity performed in normoxic (F_IO_2 = 0.2093) and hypoxic (F_IO_2 = 0.1450) conditions. Cohen's *d* effect sizes are expressed with 90% confidence limits.

	Interleukin-6 ($ng \cdot mL^{-1}$)			**Hepcidin** ($nmol \cdot L^{-1}$)		
Condition	*Pre*	*Post*	*Effect Size*	*Pre*	*3 h Post*	*Effect Size*
Normoxia	0.50 (0.34)	1.11** (0.43)	0.61 [0.39, 0.89]	3.32 (2.48)	4.17* (2.94)	0.84 [0.13, 1.56]
Hypoxia	0.56 (0.46)	1.24** (0.56)	1.49 [1.15, 1.82]	2.85 (2.01)	4.33* (3.29)	0.74 [0.19, 1.29]

** Significantly different to baseline ($p < 0.01$)

* Significantly different to baseline ($p < 0.05$)

3.4.4 Iron Parameters

Iron parameters are presented in Table 3.3. No time (p = 0.27), treatment (p = 0.49) or interaction effect (p = 0.62) was present for serum iron concentration. Serum ferritin and transferrin concentrations increased from baseline levels immediately post-exercise (serum ferritin: p = 0.04, serum transferrin: p = 0.08) but returned to baseline levels by 3 h post-exercise (serum ferritin: p = 0.05, serum transferrin: p = 0.02). Serum sTfR trivially increased (p = 0.01) in the NORM (d = 0.08) and HYP conditions (d = 0.14) (Table 3.3), but no time (p = 0.07) or interaction effect (p = 0.06) existed. No time (serum iron: p = 0.27, transferrin saturation: p = 0.65), treatment (serum iron: p = 0.49, transferrin saturation: p = 0.62) or interaction effect (serum iron: p = 0.62, transferrin saturation: p = 0.72) existed for serum iron or transferrin saturation.

Table 3.3: Response of iron parameters to a 5 × 4 min interval running session at 90% of maximal aerobic running velocity in normoxia and normobaric hypoxia. Cohen's *d* effect sizes are expressed with 90% confidence limits.

	Normoxia (*n* = 13)					Hypoxia (*n* = 13)				
Serum Iron Parameters	Pre	Post	3 h	Effect Size (Pre *vs.* Post)	Effect Size (Pre *vs.* 3 h)	Pre	Post	3 h Post	Effect Size (Pre *vs.* Post)	Effect Size (Pre *vs.* 3 h)
Iron	16.75	17.14	16.21	0.38	-0.55	15.48	16.24	15.59	0.22	0.03
($\mu mol \cdot L^{-1}$)	(3.80)	3.97	4.43	[-0.03, 0.80]	[-1.66, 0.56]	(3.43)	(3.58)	-5.23	[0.09, 0.35]	[-0.49, 0.56]
Ferritin	75.67	81.72*	74.42†	0.1	-0.02	73.88	80.35*	74.20†	0.11	0.01
($\mu g \cdot L^{-1}$)	(61.87)	66.93	61.05	[0.04, 0.15]	[-0.15, 0.10]	-57.08	-60.51	-58.54	[0.04, 0.18]	[-0.06, 0.07]
Transferrin	31.57	32.34*	31.13†	0.15	-0.25	30.91	31.51*	30.65†	0.01	-0.05
($mg \cdot L^{-1}$)	(5.00)	(4.90)	(4.68)	[0.06, 0.25]	[0.08, 0.17]	-5.59	-5.37	-5.49	[0.04, 0.17]	[-0.13, 0.04]
Transferrin Saturation	26.89	26.89	26.33	0	-0.56	25.3	26.02	25.34	0.14	0.01
(%)	(6.57)	-7.02	-8.04	[0.00, 0.00]	[-2.55, 1.99]	-5.13	-5.1	-6.5	[0.00, 0.28]	[-0.58, 0.59]
Soluble Transferrin Receptor	2.94^	2.97	2.88	0.08	-0.19	2.74^	2.79	2.7	0.14	-0.11
($mg \cdot L^{-1}$)	(0.35)	-0.37	-0.3	[-0.04, 0.20]	[-0.64, 0.25]	-0.37	-0.4	-0.39	[0.02, 0.25]	[-0.27, 0.05]
Haemoglobin Concentration	139	141*	138	0.20	-0.02	136	140*	135	0.34	-0.04
($g \cdot L^{-1}$)	(108)	(102)	(95)	[0.01, 0.39]	[-0.31, 0.26]	141	127	113	[0.16, 0.51]	[-0.25, 0.17]
Haematocrit	0.40	0.41	0.40	0.15	-0.02	0.39	0.40*	0.38	0.19	-0.19
($L \cdot L^{-1}$)	(0.00)	(0.00)	(0.00)	[-0.02, 0.32]	[-0.32, 0.27]	0.00	0.00	0.00	[0.04, 0.34]	[0.41, 0.03]

^ Significant difference pre-exercise ($p < 0.05$)

* Significant difference post-exercise ($p < 0.05$)

3.5 Discussion

To our knowledge, this is the first study to examine the relationship between IL-6, hepcidin and iron metabolism after exercise conducted in acute hypoxia. We hypothesised acute exercise in hypoxia performed at the same relative intensity as exercise in normoxia, would attenuate hepcidin levels 3 h after passive recovery in normoxia. Our findings did not support this hypothesis, with no differences in the response of IL-6 and hepcidin following interval exercise in normoxic and hypoxic conditions. Furthermore, no differences in iron biomarkers were observed between the two conditions post-exercise, indicting a similar iron response between trials.

Exercise intensity and duration are key moderators of the IL-6 response, which has recently been proposed as the main mechanism responsible for elevated hepcidin levels 3-6 h after exercise in normoxia (16-18, 139, 152, 224). As such, the matched relative exercise intensity may account for the similarity of the post-exercise biomarker responses between the two conditions observed in the present study. Indeed, exercise intensity, rather than hypoxic exposure itself, may be a greater mediator of IL-6 production after an acute exercise session in hypoxia. Similar to our findings, Lundby et al. (27) reported comparable IL-6 production immediately after 60 min of cycling in both a normoxic (F_IO_2 = 0.2093) and hypoxic environment (F_IO_2 = 0.1240) at a matched relative intensity of ~45% VO_{2max} (27), although cycling at the same absolute intensity (154 W, ~54% VO_{2max}) in hypoxia augmented IL-6 production post-exercise. However, further research is required to determine whether a similar hepcidin and iron biomarker response occurs when participants exercise at the same absolute rather than relative intensity in hypoxia compared with normoxia.

The length of hypoxic exposure in this study (~ 31 min) may have been insufficient to up-regulate EPO, suppress hepcidin synthesis or affect iron uptake kinetics. Hepcidin suppression occurs within 24-48 h when induced by low and high dose EPO administration (190, 225, 226, 237), blood removal (226), or prolonged hypoxic exposure (21, 23). Although EPO concentration was not measured in the current study, Rodriguez et al. (49) previously observed a significant increase in EPO after 90 min hypoxic exposure at a simulated altitude of 5,500 m (540 hPa). Furthermore, Mackenzie et al. (238) reported a significant increase in EPO levels associated with 90 min hypoxic exposure (F_IO_2 = 0.1480) preceding a 30 min treadmill run at 50% VO_{2max}, although Knaupp et al. (239) observed no increase in EPO after ~120 min of hypoxic exposure (F_IO_2 = 0.1050). Considering the duration of the hypoxic exposure used in these studies was 3 to 4 times greater than ours, but no clear or consistent increase in EPO levels were observed, the length of hypoxic exposure in the current study was likely insufficient to up-regulate EPO synthesis, thus having little influence on hepcidin synthesis and iron metabolism. However, further studies are required to determine the response of these biomarkers to prolonged, continuous or intermittent hypoxic exposure both at resting conditions and during recovery from acute exercise.

Whilst athletes do not typically recover in hypoxic conditions after intermittent hypoxic training (IHT) protocols (54), it is unlikely recovering in normoxia affected the iron biomarker responses seen here. Recovering in hypoxia would have extended the total hypoxic dose to ~3.5 h, which is still considerably less than the 24-48 h reportedly required to suppress hepcidin (21, 23, 190, 226), and lower than experienced by athletes undertaking prolonged hypoxic exposure (21 d of 14 $h \cdot d^{-1}$ hypoxic exposure). Nevertheless, Badenhorst et al. (240) observed ~27% and ~34% lower hepcidin levels after 3 h and 24 h after interval exercise (8 × 3 min at 85%

vVO_{2peak}) respectively, when athletes recovered for 3 h in hypoxia (F_IO_2 = 0.1508) compared with normoxia (F_IO_2 = 0.2093). However, a similar iron biomarker response existed 3 h and 24 h post-exercise between these two recovery conditions. Hence, athletes who recover in hypoxia may experience an attenuated rise in hepcidin levels post-exercise, which may promote iron mobilisation from storage sites and intestinal iron absorption.

Athletes may recover in hypoxia after exercise during simulated and natural live high: train low (LHTL) and live high: train high (LHTH) protocols. The post-exercise hepcidin response in these protocols is likely to be lower compared with acute IHT, owing to a larger total time spent in hypoxia. Indeed, from a practical perspective, Saunders et al. (3) recommended athletes avoid interval exercise upon initial exposure to moderate (2,000-3,000 m) and high altitudes (> 3,500 m) to reduce the effect of an exercise-induced increase in haemolysis or inflammatory cytokines, and an impairment in EPO production resulting from the acidic nature of high-intensity exercise. Thus, given high-intensity exercise is unlikely to be performed in the first 24-48 h of hypoxic exposure during LHTL and LHTH protocols, hepcidin levels would already be suppressed if exercise were performed on the third or fourth day of prolonged hypoxic exposure. If so, then recovering in hypoxic conditions may lower hepcidin levels during recovery from exercise, thus improving iron absorption from post-exercise meals and its mobilisation from storage sites, consequently providing the bone marrow with sufficient iron to cope with accelerated erythropoiesis in hypoxia. However, whether or not the cumulative hypoxic exposure experienced during long-term intermittent hypoxic training protocols similarly influences post-exercise hepcidin expression, is currently unknown.

3.5.1 Iron Parameters

In the current investigation, serum ferritin and transferrin concentrations significantly increased from baseline levels post-exercise in both trials, but returned to baseline levels within 3 h of recovery. Serum iron, transferrin saturation and soluble transferrin receptor concentrations however did not increase above baseline levels at any time point in either exercise trial. The rise in IL-6 and serum ferritin immediately post-exercise in the current study are analogous to an acute phase response (241), which reduces iron availability to invading pathogens in a process known as iron withholding. Serum iron, ferritin and transferrin saturation are positive acute phase reactants (122), although an increase in serum iron and transferrin saturation may also result from exercise-related haemolysis. Our findings are comparable to Sim et al. (152) who also reported an increase in serum ferritin and transferrin in male endurance athletes immediately after a 90 min treadmill run at 75% $\dot{V}O_{2peak}$. Whilst transferrin is considered a negative acute phase protein (122), a small increase (152) or no change in this biomarker (242) has been reported after acute exercise. Indeed, as Giminez et al. (243) proposed, haemoconcentration could explain the elevation in transferrin after high-intensity exercise seen in the current study. In contrast to Sim et al. (152), sTfR was not significantly elevated post-exercise in either condition. An elevation in sTfR reflects increased bone marrow iron requirements associated with accelerated erythropoiesis (124) such as occurs during prolonged hypoxic exposure (178, 244). However, in this study, an exercise-induced increase in inflammatory cytokines and/or haemolysis rather than hypoxic exposure was likely the key driver of changes in these markers of iron status after exercise.

3.6 Conclusion

Acute interval exercise performed at the same relative intensity in hypoxic and normoxic conditions did not attenuate hepcidin production or alter iron protein responses post-exercise, which was most likely because of the short duration of the hypoxic exposure. These results indicate an acute hypoxic exercise session of a similar duration and relative intensity, followed by recovery in normoxia, poses no greater risk to athletes' iron status than exercise performed in normoxia. However, future research is required to determine whether exercise performed at a similar absolute intensity elicits a similar hepcidin and iron biomarker response. Additionally, investigating the influence of long-term (several weeks) intermittent hypoxic training on hepcidin production and iron-related protein responses could provide a better understanding how iron balance is maintained throughout such hypoxic training methods.

4 CHAPTER FOUR

LIVE HIGH: TRAIN LOW - INFLUENCE ON RESTING AND POST-EXERCISE HEPCIDIN LEVELS

4.1 Abstract

Purpose: To investigate the influence of 14 days live high: train low (LHTL) (14 $h \cdot d^{-1}$ at 3,000 m simulated altitude) on resting and post-exercise hepcidin levels. **Methods:** Ten well-trained distance runners performed 6 × 1,000 m intervals at 90% maximal aerobic running velocity (vVO_{2max}) in both normoxia (NORM1) and normobaric hypoxia (3,000 m simulated altitude) (HYP1) before, and on the 11th (NORM2) and 14th day (HYP2) of LHTL. Venous blood was collected after two nights of LHTL, before and 3 h after each interval running session; and analysed for hepcidin and ferritin. Haemoglobin mass (Hb_{mass}) was measured via CO rebreathing one week before and two days after LHTL. **Results:** Two nights of LHTL decreased resting hepcidin by 2.8 nM [95% confidence limit: -3.7, -5.9] from 4.0 ± 1.2 nM to 1.2 ± 0.2 nM ($p < 0.01$). Interval running elevated hepcidin 3 h post-exercise in NORM1 (39.3% [8.0, 79.6], $p = 0.02$) and HYP1 (64.5% [16.9, 131.6], $p < 0.01$). The 3 h post-exercise hepcidin response was not different from equivalent exercise performed at baseline following NORM2 ($p = 0.10$) and HYP2 ($p = 0.37$). Hepcidin was 56.6% [-77.0, -18.1] lower 3 h post-exercise at HYP2 compared with HYPO1 ($p < 0.01$). Resting ferritin decreased by 27.6% [-37.9, -15.5] and 29.9% [36.2, 22.9] following 11 and 14 days of LHTL, respectively. LHTL increased Hb_{mass} by 2.2% [1.0, 3.4] ($p < 0.01$). **Conclusion:** 14 days of LHTL suppresses resting hepcidin levels after two nights of LHTL, but does not alter the post-exercise hepcidin response to interval exercise in normoxia and hypoxia, compared with equivalent exercise pre-LHTL.

4.2 Introduction

Exposure to moderate (2,000-3,000 m) or high (>3,000 m) altitude enhances erythropoiesis some three-fold during the first few days of exposure (82). Since iron is required for haem synthesis, iron uptake by erythroid precursor cells approaches almost 100% of their maximal capacity during early adaptation to altitude, placing a large burden on the body's iron stores. However, low pre-altitude iron stores (serum ferritin levels <40 $\mu g \cdot L^{-1}$), or poor mobilisation of iron from storage sites during the first few days of hypoxic exposure may compromise erythropoiesis. For example, red cell volume was blunted in iron deplete (serum ferritin: 15 ± 3 $\mu g \cdot L^{-1}$) but not iron replete (serum ferritin: 69 ± 10 $\mu g \cdot L^{-1}$) athletes exposed to 2,500 m natural altitude for four weeks (10). In addition to sufficient pre-altitude iron stores, iron must be readily available to support accelerated erythropoiesis during prolonged hypoxic exposure.

The majority of iron used for erythropoiesis is obtained from the labile iron pool, which consists mostly of iron contained on reticuloendothelial macrophages. Iron export from macrophages occurs via the iron export channel, ferroportin, and is regulated by the hepatic hormone, hepcidin (15). Specifically, hepcidin controls ferroportin degradation on the cellular surface of iron storage cells in response to increased intracellular iron content and inflammation. As such, high hepcidin levels promote iron sequestration in reticuloendothelial macrophages, effectively reducing iron efflux into the blood plasma (15). Reduced plasma iron availability in response to increased hepcidin may compromise iron delivery to erythroid precursor cells, thus limiting their maturation into erythrocytes.

Several changes in iron metabolism occur during prolonged exposure to hypoxia to improve iron availability. A key change in iron metabolism in hypoxia is the

suppression of hepcidin within 24-48 h of exposure (21-23). Hepcidin suppression is a favourable early response to prolonged hypoxia, which promotes intestinal iron absorption and iron export from reticuloendothelial macrophages into the blood plasma. Furthermore, the stabilisation of the hypoxia-inducible factor in hypoxia up-regulate proteins involved in iron absorption (DMT-1, Dcyt B), transport (TfR), uptake (sTfR) and export (ferroportin) (161), consequently improving iron delivery to the erythron.

In contrast to prolonged hypoxic exposure, exercise in normoxia and acute normobaric hypoxia (31 min at 3,000 m simulated altitude) both elevate Inteleukin-6 (IL-6) immediately post-exercise (245), increasing hepcidin levels 3 h thereafter. Hypothetically, elevated post-exercise hepcidin production during prolonged hypoxic exposure could inhibit iron export from reticuloendothelial macrophages and intestinal iron absorption during exercise recovery, thus reducing iron available for erythropoiesis and iron-dependent enzyme production. Decreased intestinal iron absorption post-exercise may also limit iron uptake from dietary sources, which are important to replenish daily iron losses. The post-exercise hepcidin response to interval exercise performed during prolonged hypoxic exposure, however, is currently unknown.

Given the conflicting effects of acute and prolonged hypoxic exposure on hepcidin production, we investigated the post-exercise hepcidin and iron parameter responses during prolonged hypoxic exposure. Specifically, we hypothesised two weeks of live high: train low (LHTL) in normobaric hypoxia (3,000 m simulated altitude) would: 1) suppress resting hepcidin levels, 2) decrease the post-exercise hepcidin production regardless of whether exercise is conducted in normoxia or hypoxia, 3) decrease resting plasma ferritin levels, and 4) increase haemoglobin mass (Hb_{mass}) post-LHTL.

4.3 Methods

4.3.1 Participants

Ten well-trained middle- or long-distance runners (six males, four females) were recruited from the local running community (mean ± standard deviation; age: 28.6 ± 6.7 y; body mass: 62.6 ± 7.6 kg; maximal oxygen consumption (VO_{2max}): 65.6 ± 8.1 $mL \cdot kg^{-1} \cdot min^{-1}$). Participants were pre-screened for an existing iron deficiency and those deemed Stage 2 iron deficient (plasma ferritin <20 $\mu g \cdot L^{-1}$) were excluded (1). The Human Research Ethics Committee at Edith Cowan University approved this study in accordance with the Declaration of Helsinki. Each participant provided written informed consent before testing.

4.3.2 Experimental Design

An overview of the study design is shown in Figure 1. Participants then undertook 14 days of continuous normobaric hypoxic exposure [simulated altitude of 3,000 m, fraction of inspired oxygen (F_IO_2): ~15.5%] in a purpose-built, normobaric altitude facility (89). Throughout the two-week intervention, participants were instructed to accumulate a minimum of 14 $h \cdot d^{-1}$ of hypoxic exposure. With the exception of experimental sessions, participants continued their normal training programme at ~600 m natural altitude.

One week before LHTL, athletes' iron parameters, Hb_{mass}, VO_{2max} and running velocity at maximal oxygen consumption (vVO_{2max}) were measured. The graded exercise protocol used has been described previously (246). Additionally, athletes completed a standardised interval exercise trial in normoxia and, two days later, in normobaric hypoxia (3,000 m simulated altitude). In this session, hepcidin and iron

parameter responses were measured in normoxia pre- and 3 h post-exercise. An equivalent interval running session was performed on the 11th (NORM2) and 14th (HYP2) day of LHTL, respectively. Participants refrained from exercise for 12 h and caffeine for 6 h before each interval exercise session.

One week before, and for the duration of LHTL, athletes with pre-altitude ferritin levels <100 $\mu g \cdot L^{-1}$ (four females and one male; pre-altitude plasma ferritin: 44.2 ± 19.8 $\mu g \cdot L^{-1}$) ingested an oral iron supplement [one tablet of FerroGrad C, 325 mg ferrous sulphate (105 mg elemental iron) and 1,000 mg vitamin C; Abbott Australia, Botany, Australia] daily. Iron supplementation was provided as per the recommendation of a qualified sports physician and in accordance with the iron supplementation recommendations of Berglund (12).

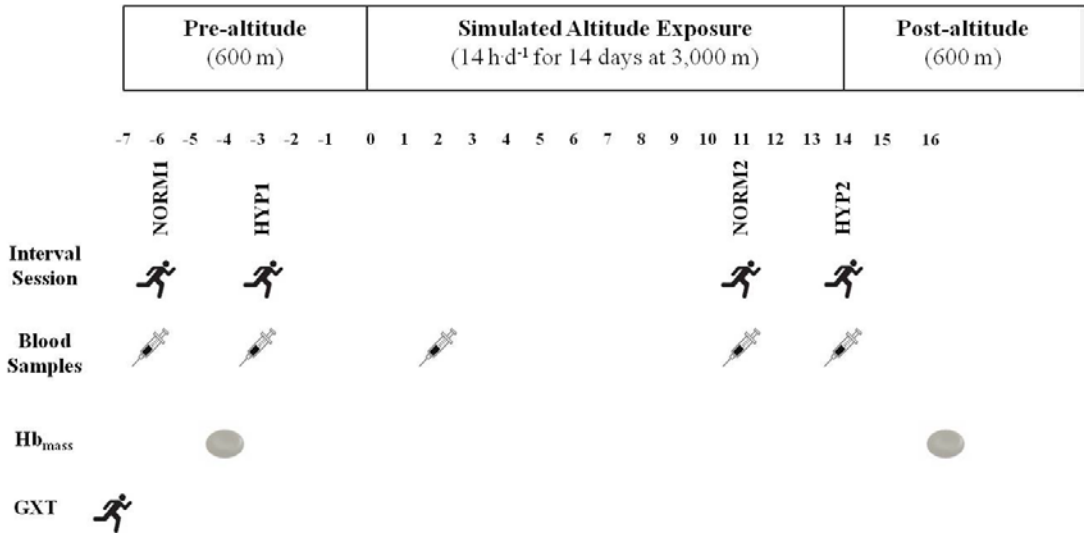


Figure 4.1: Diagram of the study design. Haemoglobin mass, iron parameters and maximal oxygen consumption [via a graded exercise test (GXT)] were measured before 14 days of live high: train low (LHTL) at a simulated altitude of 3,000 m. Interval exercise sessions (6 × 1,000 m at 90% maximal aerobic running velocity) were performed one week before (NORM1 & HYP1), and on the 11th (NORM2) and 14th day (HYP2) of LHTL. Haemoglobin mass was measured again two days after LHTL.

4.3.3 Interval Running Sessions

Participants performed 15 min of a self-selected running warm-up followed by 10 min of dynamic stretching. Following this, participants ran 6 × 1,000 m at 90% *v*VO2max separated by 1.5 min passive recovery, which was performed either on a synthetic running track in normoxia (600 m natural altitude) or on a motorised treadmill in hypoxia (3,000 m simulated altitude). Heart rate was measured continuously throughout each session using a heart rate monitor (Polar RS600, Polar Electro, Finland) and rate of perceived exertion was measured at the conclusion of each interval using the Borg 6-20 visual analogue scale (247). The intensity of the hypoxic interval session was based upon participants' estimated vVO_{2max} at 3,000 m altitude,

which we showed in Study One to be ~15-20% less than their normoxic vVO_{2max}. The running velocity for the normoxic and hypoxic sessions were 17.0 ± 2.0 $km \cdot h^{-1}$ and 13.3 ± 4.9 $km \cdot h^{-1}$ respectively. One athlete did not perform the second hypoxic interval exercise session due to injury and was omitted from the data set. Barometric pressure, relative humidity and ambient temperature were measured using a calibrated weather meter (Kestrel 4500 Weather Meter, Kestrel, Michigan, USA) before each of the four interval sessions. At the conclusion of each interval session, athletes recovered in normoxia and did not engage in any recovery modalities for 3 h post-exercise.

4.3.4 Haemoglobin Mass

A modified version of the two-minute optimised rebreathing method measures athletes' Hb_{mass} (248). Briefly, the participants rebreathed a bolus of ~1.2 $mL \cdot kg^{-1}$ carbon monoxide (CO) through a closed system for 2 min. The percentage carboxyhaemoglobin contained in capillary blood was measured at baseline and 7 min after rebreathing using an OSM3 haemoximeter (OSM3, Radiometer, Copenhagen, Denmark). To minimise the measurement error, at least five replicates of each blood sample were measured. The typical error of measurement (with 90% confidence limits) expressed as the coefficient of variation was 1.8% [1.3, 2.2] (248).

4.3.5 Venous Blood Samples

Venous blood samples were collected by a trained phlebotomist, immediately before and 3 h after the four interval running sessions. Blood samples were collected at the same time for each session to counteract the effects of diurnal variation on iron parameters (249) and hepcidin (250). A venous blood sample was also collected under resting conditions after two nights of LHTL and analysed for hepcidin and EPO.

Before any blood sample collection, participants rested in a supine position for 10 min to control for postural shifts in plasma volume (231). Venous blood samples were collected from a forearm antecubital vein into four 2 mL lithium heparin blood collection tubes (BD Vacutainer™, New Jersey, USA), and then centrifuged at 4 °C and a speed of 2,200 g for 10 min. After centrifugation, the plasma supernatant was stored at –80 °C for later batch analysis.

4.3.6 Laboratory Analyses

Plasma hepcidin-25 measurements were performed (*www.hepcidinanalysis.com*, Nijmegen, The Netherlands) by a combination of weak cation exchange chromatography and time-of-flight mass spectrometry (232). An internal standard (synthetic hepcidin-24; custom made, Peptide International Inc.) was used for quantification (251). Peptide spectra were generated on a Microflex LT matrix-enhanced laser desorption/ionisation time-of-flight mass spectrometry platform (Bruker Daltonics). Plasma hepcidin-25 concentrations were expressed as $nmol \cdot L^{-1}$ (nM). The median reference level of plasma hepcidin-25 (Dutch population) is 4.5 nM for men and 2.0 nM for pre-menopausal women (*www.hepcidinanalysis.com*, accessed April 26, 2015).

Plasma blood samples were analysed for iron, transferrin and ferritin, using a COBAS Integra 400 automated clinical chemistry analyser (Roche Diagnostics, Switzerland). Transferrin saturation was calculated as iron/total iron binding content (252). Whole blood was analysed using a Sysmex XT-2000i (Sysmex Corporation, Japan) with key markers being haemoglobin concentration [Hb] and haematocrit (HCT). Plasma EPO was measured using an EPO immunoassay on an Immulite 1000 automated immunoassay analyser (Siemens Diagnostic Products, Los Angeles, California, USA).

4.3.7 Statistical Analysis

Differences in heart rate and perceptual responses to interval exercise performed in normoxia and hypoxia before and after altitude exposure were analysed using a two-way ANOVA with repeated measures [Time (2 levels): pre-exercise, 3 h post-exercise) × Condition (2 levels): pre-LHTL, post-LHTL]. Additionally, two-tailed, paired samples *t*-tests analysed the change in red cell parameters from baseline levels after altitude exposure and the change in hepcidin levels from baseline after two nights of LHTL altitude exposure. The change in plasma EPO from baseline were analysed using a one-way repeated measures ANOVA [Time (3 levels): baseline, 48 h, post-altitude).

The influence of prolonged normobaric hypoxic exposure on iron metabolism interval exercise in normoxia and hypoxia was examined using linear mixed models in the *nlme* package (253) in the R statistics programme (254). Since no three-way interaction effect existed for any iron parameter, independent models were fit for the normoxic and hypoxic interval exercise conditions. All data were log-transformed before analysis, then back transformed and converted to percentages to improve interpretation. Results from each model are presented as non-standardised regression coefficients ($\hat{\beta}$) as a percentage change with 95% confidence limits (CL) to denote the imprecision of the point estimate.

4.4 Results

4.4.1 Environmental Conditions & Physiological Responses to Interval Exercise

Participants accumulated 196.2 ± 25.6 h of altitude exposure over 14 days. The environmental conditions as well as participants' heart rate and perceptual responses to interval exercise are presented in Table 4.1. Barometric pressure was 8.7 mmHg higher ($p < 0.01$) and relative humidity was 13.2% higher ($p < 0.01$) in NORM2 compared to NORM1. Barometric pressure was 22.5 mmHg higher ($p < 0.01$) and temperature was 1.8 °C lower ($p = 0.03$) in HYP1 compared with HYP2. Heart rate was lower in HYP compared with NORM ($p = 0.05$). Additionally, RPE was similar between the HYP and NORM trials ($p = 0.26$).

Table 4.1: Environmental conditions, heart rate and perceptual response to 6 × 1,000 m interval session at 90% maximal aerobic running velocity performed in normoxia (NORM, $n = 10$) and hypoxia (simulated, normobaric altitude of 3,000 m, HYP, $n = 9$) before and after 14 days of live-high: train-low.

	NORM1	NORM2	HYP1	HYP2
Barometric Pressure (mmHg)	706.7 ± 1.0	715.4 ± 0.7*	694.9 ± 2.3	717.5 ± 0.9*
Temperature (°C)	15.5 ± 3.3	13.1 ± 3.7	20.3 ± 1.4	18.5 ± 1.5*
Relative Humidity (%)	58.2 ± 8.5	71.4 ± 14.3*	45.6 ± 13.6	39.4 ± 3.3
Heart Rate (beats·min^{-1})	174 ± 7	173 ± 7	168 ± 6	165 ± 5^
Rate of Perceived Exertion	14 ± 2	14 ± 2	14 ± 2	13 ± 2

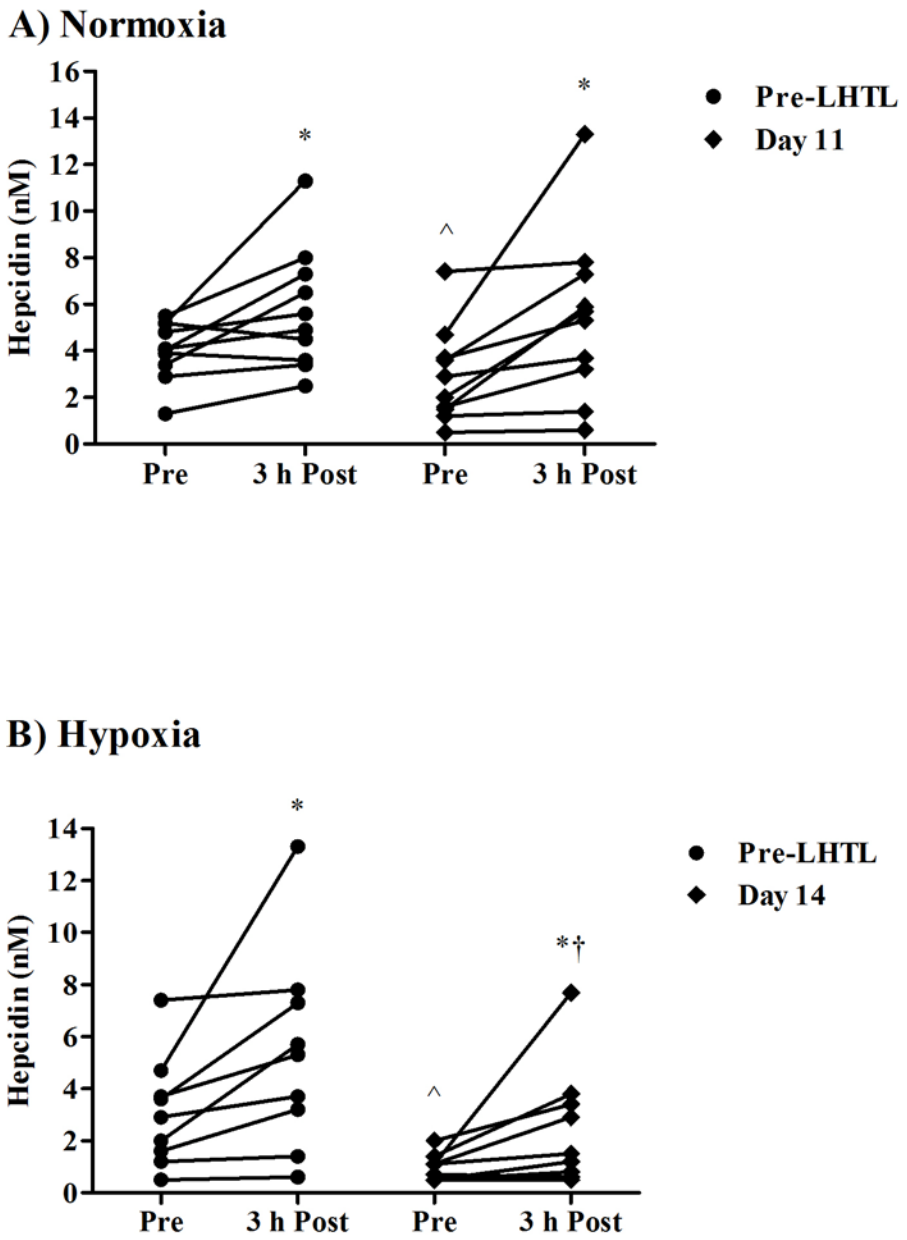
* Significantly different from pre-altitude ($p < 0.05$)

^ Significantly different between conditions ($p < 0.05$)

4.4.2 Hepcidin Response

Two nights of LHTL decreased resting hepcidin levels from 4.0 ± 1.3 nM to 1.2 ± 0.2 nM ($p < 0.01$). Resting hepcidin levels were 58.6% [-77.0, -25.6] lower at NORM2 (day 11) compared with NORM1 and 63.1% [-77.5, -39.6] lower at HYP2 (day 14) compared with HYP1 (both $p < 0.01$).

Figure 4.2 highlights the hepcidin response to interval exercise performed in normoxia and normobaric hypoxia respectively during LHTL. Interval exercise in normoxia elevated hepcidin from baseline, 3 h post-exercise by 39.3% [2.5, 89.4] and 80.5% [32.8, 145.4] at NORM1 and NORM2 (day 11) respectively (both $p < 0.01$). The magnitude of post-exercise hepcidin response was not different at NORM1 compared with NORM2 ($p = 0.10$). In comparison, interval exercise in normobaric hypoxia elevated hepcidin from baseline, 3 h post-exercise by 64.5% [8.5, 149.5] and 93.6% [27.6, 193.7] at HYP1 and HYP2 (day 14), respectively (both $p < 0.01$). The magnitude of the post-exercise hepcidin response was not different between HYP1 and HYP2 ($p = 0.37$).



* Significantly different from pre-exercise ($p < 0.05$), ^ Significantly different from pre-LHTL ($p < 0.01$), † Significantly different from pre-LHTL ($p < 0.01$).

Figure 4.2: Pre- and 3 h post-exercise hepcidin response to a standardised 6 × 1,000 m interval session at 90% maximal aerobic running velocity performed in (A) normoxia (600 m natural altitude) before and on the 11th day of LHTL and, (B) hypoxia (simulated altitude of 3,000 m) before and on the 14th day of live high: train low (LHTL).

4.4.3 Iron Parameter Responses

The iron parameter responses to LHTL and interval exercise are presented in Table 4.2. Hypoxic exposure decreased resting ferritin levels by 27.6% [-37.9, -15.5] and 29.9% [-36.2, -22.9] at NORM2 (day 11) and HYP2 (day 14) compared with NORM1 and HYP1 respectively (all $p < 0.01$). Transferrin levels were 5.1% [-8.6, -1.4] lower at NORM1 than NORM2 ($p = 0.01$).

In the normoxic trial, iron and TSAT levels decreased by 33.7% [-44.7, -20.6] and 31.3% [-42.0, -18.5] from baseline levels, 3 h post-exercise at NORM1 (both $p < 0.01$), but these parameters were not different from baseline levels 3 h post-exercise at NORM2 (both $p = 1.00$). Interval exercise in normoxia did not alter any other iron parameters, 3 h post-exercise relative to baseline. The magnitude of the post-exercise iron and TSAT responses were 51.4% [17.8, 94.6] and 42.9% [12.8, 81.0] higher at NORM2 (day 11) and compared with NORM1 (both $p < 0.01$). No iron parameters were altered from baseline levels 3 h post-exercise in normobaric hypoxia. The magnitude of the post-exercise iron and TSAT responses were 43.6% [3.2, 99.9] and 44.8% [5.1, 99.4] higher at HYP2 (day 14) compared with HYP1 (iron: $p = 0.04$; TSAT: $p = 0.03$).

Table 4.2: Iron parameters response to interval exercise (6 × 1,000 m at 90% maximal aerobic running velocity) performed in normoxia (600 m natural altitude, *n* = 10) and normobaric hypoxia (3,000 m simulated normobaric altitude, *n* = 9) both before and during 14 days of live high: train low at 3,000 m simulated, normobaric altitude.

	NORM1		NORM2		HYP1		HYP2	
Iron Parameters	**Pre**	**3 h Post**	**Pre**	**3 h Post**	**Pre**	**3 h Post**	**Pre**	**3 h Post**
Iron ($\mu mol \cdot L^{-1}$)	21.5 ± 4.9	14.5 ± 4.1*	20.1 ± 5.6	20.8 ± 8.2	19.8 ± 5.2	18.0 ± 4.9	17.1 ± 4.7	22.2 ± 6.5
Ferritin ($\mu g \cdot L^{-1}$)	97.3 ± 66.0	100.2 ± 65.6	66.0 ± 41.0^	65.5 ± 41.3	105.2 ± 69.2	98.7 ± 61.8	72.1 ± 44.0^	67.5 ± 10.3
Transferrin ($mg \cdot L^{-1}$)	2.7 ± 0.2	2.6 ± 0.2	2.6 ± 0.2^	2.6 ± 0.3	2.5 ± 0.2	2.6 ± 0.2	2.4 ± 0.2	2.5 ± 0.3
Transferrin Saturation (%)	35.3 ± 8.6	24.5 ± 6.9*	34.8 ± 10	35.2 ± 14.9	34.4 ± 9.1	30.7 ± 10.2	31.5 ± 9.1	39.2 ± 10.9

† Significantly interaction effect ($p < 0.05$)

* Significantly different post-exercise ($p < 0.05$)

^ Significantly different post-hypoxic exposure ($p < 0.01$)

4.4.4 Red Cell Parameter & Haemoglobin Mass Response

Table 4.3 presents the red cell parameter responses to 14 days of LHTL. Erythropoietin levels did not increase from baseline after two nights (18.4% [-4.7, 47.3]) or 14 days of LHTL (17.9% [-31.3, 102.2]) (p = 0.61). Fourteen days of LHTL increased [Hb] by 2.7% [1.5, 3.9] and HCT by 3.2% [0.9, 5.7] from baseline values (both p < 0.01). Hb_{mass} increased by 2.2% [0.8, 3.6] (p = 0.01) from baseline following 14 days of LHTL.

Table 4.3: Red blood cell parameters before and after 14 days of live high: train low at 3,000 m simulated, normobaric altitude (n = 10).

	Pre-Altitude	**Post-Altitude**
Haemoglobin Mass (g)	783.7 ± 200.4	801.6 ± 198.1*
Haemoglobin Concentration ($g \cdot L^{-1}$)	144 ± 13.2	148 ± 14.5*
Haematocrit ($L \cdot L^{-1}$)	39.9 ± 3.6	41.2 ± 3.9*
Mean Cell Volume (*f*L)	84.7 ± 3.9	85.2 ± 3.7
Mean Cell Haemoglobin (*p*g)	30.5 ± 1.3	30.5 ± 1.2
Mean Cell Haemoglobin Concentration ($g \cdot L^{-1}$)	36.0 ± 1.3	35.8 ± 1.2
Erythropoietin ($mU \cdot mL^{-1}$)	13.6 ± 5.2	17.4 ± 9.3

*Significantly different post-altitude (p < 0.01)

4.5 Discussion

The main findings of this study are 14 days of LHTL at 3,000 m simulated normobaric altitude suppressed resting hepcidin levels after two nights of LHTL, but did not attenuate post-exercise hepcidin production after interval exercise performed in normoxia and simulated normobaric hypoxia, compared with an equivalent session performed before LHTL. However, 3 h post-exercise hepcidin levels were lower in the HYP condition after LHTL compared with before LHTL. Post-exercise iron and TSAT levels were higher 3 h after interval exercise in both NORM and HYP conditions following LHTL compared with equivalent exercise performed before LHTL. Finally, Hb_{mass} increased by 2.2%, in association with a decrease in resting plasma ferritin levels post-LHTL.

4.5.1 Hepcidin Response

Resting hepcidin levels were suppressed after two nights, 11 and 14 days of LHTL. Our findings are in line with previous studies that have observed hepcidin suppression within 48 h of short-term high (> 3,000 m) natural altitude exposure (21-23). Such a reduction in resting hepcidin expression during the first few days of altitude exposure is proposed to improve intestinal iron absorption and iron export from reticuloendothelial macrophages, thereby increasing plasma iron availability to support accelerated erythropoiesis (22). Hepcidin suppression in hypoxia is thought to be mediated by the erythroid regulator of hepcidin, erythroferrone, a hormone released by erythroblasts in response to EPO (104). Alternatively, Platelet Derived Growth Factor-BB, released from platelets, macrophages and endothelial cells has also been shown to suppress hepcidin during early exposure to hypoxia (109).

However, the extent to which these mechanisms are affected by combining altitude exposure with exercise during the first few days of altitude exposure is unknown.

To our knowledge, this is the first study to report the influence of LHTL on the post-exercise hepcidin response in well-trained endurance athletes. Here, the interval exercise performed in both normoxia and simulated hypoxia during LHTL increased hepcidin levels 3 h post-exercise. Furthermore, hepcidin levels increased post-exercise despite suppressed resting hepcidin levels. Our decision to measure hepcidin level 3 h post-exercise was based on the findings of Peeling et al. (16) who demonstrated hepcidin peaks 3-6 h post-exercise in normoxia. We acknowledge hepcidin levels could have continued to rise beyond the 3 h time point and prolonged hypoxic exposure may indeed alter the time course of post-exercise hepcidin production. Indeed, further research to establish the time course of hepcidin production post-exercise during prolonged hypoxia is warranted. Such research may assist sport scientists to determine the optimal time to ingest iron rich compounds during prolonged hypoxic exposure to avoid peak post-exercise hepcidin levels and optimise intestinal iron absorption.

Contrary to our hypothesis, LHTL did not attenuate post-exercise hepcidin levels relative to pre-LHTL values in either NORM or HYP. In the absence of other known promoters of hepcidin post-exercise, the most likely mechanism responsible for the post-exercise rise in hepcidin levels observed here is an exercise-induced increase in IL-6. Although we did not measure IL-6 in the current study, Lundby & Steensberg (27) showed IL-6 increased after cycling exercise in hypoxia. Hence, an exercise-induced increase in IL-6 seems the most likely mechanism responsible for increased hepcidin levels 3 h post-exercise during prolonged hypoxic exposure, regardless of

the strong inhibitory stimulus of hypoxia and increased erythropoiesis on hepcidin expression.

Given the post-exercise hepcidin response during prolonged hypoxia exposure we observed here, we suggest athletes avoid ingesting iron rich meals and/or oral iron supplements after exercise when hepcidin levels are elevated. Indeed, iron ingested during the post-exercise recovery phase is unlikely to be absorbed by the intestine, and is therefore unlikely to be delivered to the erythron for erythropoiesis. Future research should therefore focus on modelling the ferrokinetics of iron ingested during the post-exercise recovery phase at altitude to discern the optimal time after exercise to ingest an oral iron supplement and/or to consume iron rich meals to maximise its absorption.

4.5.2 Iron Parameter Responses

Resting ferritin levels decreased after 11 and 14 days of LHTL, which is a common response to prolonged hypoxic exposure. For example, Robertson et al. (70) observed a decrease in serum ferritin levels in athletes undertaking three weeks of LHTL (3,000 m simulated, normobaric hypoxia). The decrease in ferritin levels during prolonged hypoxic exposure likely results from an increase in iron export from reticuloendothelial macrophages to support accelerated erythropoiesis at altitude (255), since iron uptake by erythroid precursor cells reaches almost 100% of its maximal capacity during the first few days of high altitude exposure (9). In further support of this assertion, the reduction in ferritin we observed in the current study was accompanied by an ~2% increase in Hb_{mass} post-LHTL.

4.5.3 Red Cell Parameter & Haemoglobin Mass Responses

The 2.2% increase in Hb_{mass} we observed after 14 days of LHTL (for a total of 196 h) is line in with the recent meta-analysis by Gore et al. (6), who calculated Hb_{mass} increases by ~1% per 100 h of moderate altitude exposure (>2,000 m). However, the Hb_{mass} response to hypoxia is subject to substantial inter-individual variability and is affected by factors such as illness, injury, caloric deficit and iron deficiency with some athletes classified as altitude non-responders (8). Indeed, five (2 males, 3 females) of our ten participants did not increase their Hb_{mass} following LHTL beyond the coefficient of variation for the CO rebreathing method within our laboratory (1.8%) (248).

Erythropoietin production normally increases after ~90 min of hypoxic exposure, reaching peak levels during the first week of altitude exposure, and declines towards baseline levels thereafter (72). However, the EPO response to prolonged, moderate altitude exposure varies somewhat between athletes (76, 78). In the current study, EPO did not increase significantly from baseline after two nights or 14 days of LHTL. Furthermore, five participants (3 Hb_{mass} responders, 2 Hb_{mass} non-responders) increased their serum EPO by ~72% from baseline values after 14 days of LHTL. Previously, Chapman et al. (77) found athletes who increased their red cell volume following 30 h of moderate altitude exposure (i.e. responders) had a significantly greater 3% increase in serum EPO during the first 30 h of moderate altitude exposure compared with altitude non-responders. Since kidney EPO production depends on the severity of the hypoxic stimulus applied (74), some of the athletes we studied may have required a greater hypoxic dose (i.e. either a higher altitude or longer exposure duration) to elicit a sufficient EPO levels to enhance their Hb_{mass} production. Thus, we believe the failure of EPO to increase despite an increase in Hb_{mass} results from the

high variation in our small sample population, rather than a true physiological response.

Impaired kidney EPO production during early altitude exposure may also explain the inter-individual differences in the Hb_{mass} response we observed here in some individuals following LHTL. One possible cause of impaired kidney EPO production at altitude is an exercise-induced rise in IL-6 and/or blood lactate during early exposure (80). Furthermore, elevated post-exercise IL-6 levels promote hepatic hepcidin production 3-6 h post-exercise (16). Consequently, we speculate a large rise in IL-6 during early altitude exposure may limit the differentiation of erythroid precursor cells by blunting kidney EPO production, indirectly compromising their maturation into erythrocytes by reducing iron delivery to the erythron. An investigation of the relationship between the intensity of exercise performed during the first few days of continuous hypoxic exposure and the post-exercise IL-6, hepcidin and EPO response may help establish what factors limit red cell maturation in athletes undertaking prolonged hypoxic exposure.

4.6 Limitations

Athletes were instructed to maintain their normal diet throughout the hypoxic exposure, therefore differences in total dietary iron intake may account for some of the between-athlete variation we observed in hepcidin, Hb_{mass} and iron parameter responses. Furthermore, we provided oral iron supplements to athletes with serum ferritin levels <100 $\mu g \cdot L^{-1}$ based on the current medical guidelines at the Australian Institute of Sport. The provision of iron supplements to some athletes may have therefore confounded the post-exercise hepcidin response we observed following altitude exposure. Future studies should directly examine the influence of oral iron

supplementation on the post-exercise hepcidin response in athletes undertaking prolonged hypoxic exposure compared with a non-iron supplemented matched for pre-exposure serum ferritin levels.

Whilst we did not measure post-exercise IL-6 levels in the current study, alterations in resting IL-6 levels during prolonged altitude exposure are reported elsewhere (21, 22, 196). Furthermore, Lundby & Steensberg reported cycling exercise during prolonged altitude exposure elevated post-exercise IL-6 production.

For logistical reasons, the interval exercise trials were performed on a synthetic running track (NORM) and treadmill (HYP) respectively. Such differences in the running surface could have altered the amount of iron lost through foot-strike haemolysis during running potentially altering the post-exercise hepcidin response. Nevertheless, Peeling et al. (224) found a similar post-exercise hepcidin and iron parameter response to an interval running protocol (10 × 1,000 m at 90% $v\dot{V}O_{2max}$) performed on grass and road respectively. Thus, training surface is unlikely to have influenced the post-exercise hepcidin response in the current study.

4.7 Conclusion

The novel finding of this study is that 14 days of LHTL at 3,000 m suppressed resting hepcidin levels but did not attenuate the post-exercise hepcidin response to interval exercise performed in normoxia and normobaric hypoxia, compared with an equivalent running session performed before LHTL. Hepcidin levels were however lower 3 h after interval exercise performed in normobaric hypoxia post-LHTL compared with pre-LHTL. Given hepcidin production increases post-exercise during prolonged hypoxic exposure, athletes may therefore benefit from ingesting iron rich meals or oral iron supplements away from exercise during LHTL to ensure optimal

iron absorption. The optimal timing of such oral iron consumption post-exercise, however, remains elusive.

5 CHAPTER FIVE

PRE-ALTITUDE SERUM FERRITIN LEVELS AND DAILY ORAL IRON SUPPLEMENT DOSE MEDIATE IRON PARAMETER AND HEMOGLOBIN MASS RESPONSES TO ALTITUDE EXPOSURE

Govus, A. D., Garvican-Lewis, L. A., Abbiss, C. R., Peeling, P., Gore, C.J. (2015) Pre-altitude serum ferritin levels and daily oral iron supplement dose mediate iron parameter and hemoglobin mass responses to altitude exposure. *PLoS One.* 10(8):e0135120. Impact factor: [3.5]

5.1 Abstract

Purpose: To investigate the influence of daily oral iron supplementation on changes in haemoglobin mass (Hb_{mass}) and iron parameters after 2-4 weeks of moderate altitude exposure. **Methods:** Haematological data collected from 178 athletes (98 males, 80 females) exposed to moderate altitude (1,350-3,000 m) were analysed using linear regression to determine how altitude exposure combined with oral iron supplementation influenced Hb_{mass}, total iron incorporation (TII) and blood iron parameters [ferritin and transferrin saturation (TSAT)]. **Results:** Altitude exposure (mean ± *s*: 21 ± 3 days) increased Hb_{mass} by 1.1% [-0.4, 2.6], 3.3% [1.7, 4.8], and 4.0% [2.0, 6.1] from pre-altitude levels in athletes who ingested nil, 105 $mg{\cdot}d^{-1}$ and 210 $mg{\cdot}d^{-1}$ respectively, of oral iron supplement. Serum ferritin levels decreased by -33.2% [-46.9, -15.9] and 13.8% [-32.2, 9.7] from pre-altitude levels in athletes who supplemented with nil and 105 $mg{\cdot}d^{-1}$ of oral iron, but increased by 36.8% [1.3, 84.8] in athletes supplemented with 210 $mg{\cdot}d^{-1}$ of oral iron. Finally, athletes who ingested either 105 $mg{\cdot}d^{-1}$ or 210 $mg{\cdot}d^{-1}$ of oral iron supplement had a greater TII compared with non-supplemented athletes (0 *versus* 105 $mg{\cdot}d^{-1}$: effect size (*d*) = -1.88 [-2.56, -1.17]; 0 *versus* 210 $mg{\cdot}d^{-1}$: effect size (*d*) = -2.87 [-3.88, -1.66]). **Conclusion:** Oral iron supplementation during 2-4 weeks of moderate altitude exposure may enhance Hb_{mass} production and assist the maintenance of iron balance in some athletes with low pre-altitude iron stores.

5.2 Introduction

Prolonged moderate altitude exposure (i.e. several weeks at >2,000 m) enhances oxygen transport and utilization by stimulating haematological [i.e. increased haemoglobin mass (Hb_{mass})] and non-haematological (i.e. increased skeletal muscle buffer capacity, mitochondrial density, glycolytic and oxidative enzyme concentration) adaptations (7). Substantial inter- and intra-individual variability exists in the magnitude of the Hb_{mass} response to prolonged moderate altitude exposure (193), owing to several factors including the hypoxic dose (5), variations in kidney erythropoietin production during early adaptation (78), as well as injury and illness (256).

Sufficient iron stores are required to support an hypoxic-mediated increase in haem synthesis and iron-dependent enzyme production during prolonged altitude exposure (143, 144). Indeed, altitude exposure is associated with a three- to five-fold increase in erythropoiesis, with erythroid iron uptake approaching 100% of its maximal capacity during the first few days of adaptation (179). However, low pre-altitude iron stores, or an inability to rapidly mobilise iron from reticuloendothelial macrophages may reduce the amount of iron available to support haematological and non-haematological adaptations during initial altitude exposure. For example, red cell volume did not improve following four weeks of moderate altitude (2,500 m) exposure in nine, non-iron supplemented, iron deficient runners (pre-altitude serum ferritin: 15 ± 3 $\mu g \cdot L^{-1}$), suggesting athletes require sufficient pre-altitude serum ferritin levels to support accelerated erythropoiesis at altitude (10). In contrast, Ryan et al. (194) reported 7 of 9 non-iron supplemented female subjects exposed to 5,000 m for 16 days increased their Hb_{mass} despite low pre-altitude serum ferritin levels (28.9 ± 15.5 $\mu g \cdot L^{-1}$). Moreover, a weak correlation (r = 0.33, p = 0.16) existed between pre-

altitude serum ferritin levels and the percentage change in Hb_{mass} after 16 days of high altitude exposure. The influence of pre-altitude ferritin levels on the Hb_{mass} response to prolonged altitude exposure, however, is currently unclear.

No clear iron supplementation guidelines currently exist for athletes planning to undertake moderate altitude exposure. Raising an athlete's pre-altitude iron stores via oral iron supplementation in the weeks before altitude exposure may assist them to maintain a healthy iron balance when training at altitude (12). Furthermore, although oral iron supplementation guidelines at sea level recommend providing oral iron supplements to athletes with serum ferritin levels <35 $\mu g \cdot L^{-1}$ (121), athletes with otherwise healthy serum ferritin levels (i.e. >35 $\mu g \cdot L^{-1}$) planning to undertake a period of altitude exposure may need to ingest oral iron supplements to provide sufficient iron for erythropoiesis and iron-dependent enzyme production. Additionally, iron supplementation may alleviate the symptoms of altitude-related illnesses such as acute mountain sickness (29), and help to maintain cognitive function in hypoxia (28).

Few studies have investigated the influence of oral iron supplementation on Hb_{mass} production in elite athletes undertaking moderate altitude exposure. In one study, oral iron supplementation (200 $mg \cdot d^{-1}$ elemental iron) did not enhance the Hb_{mass} response relative to a placebo in 17 National level boxers exposed to moderate altitude (1,800 m) for 18 days (216). Oral iron supplementation may have been unnecessary in this group since their pre-altitude serum ferritin levels were clinically normal (serum ferritin: 70 ± 23 $\mu g \cdot L^{-1}$) and the overall hypoxic dose was lower than typically used during LHTL protocols (i.e. 21 days exposure to 3,000 m). However, male and female endurance athletes with serum ferritin levels <40 $\mu g \cdot L^{-1}$, or who are training for long periods at moderate (2,000-3,000 m) or high altitudes (>3,000 m) may require oral iron supplementation to sustain accelerated erythropoiesis (12).

To this end, we modelled the Hb_{mass} and blood iron parameters [serum ferritin and TSAT] response to prolonged, moderate altitude exposure in a large sample of well-trained athletes. We also investigated how different oral iron supplement doses moderated the Hb_{mass} and iron parameter responses in well-trained athletes to understand the importance of iron supplementation to Hb_{mass} production during moderate altitude exposure.

5.3 Methods

5.3.1 Ethics Statement

The Human Ethics Committee at the Australian Institute of Sport granted ethical approval for each altitude exposure. The Human Research Ethics Committee at Edith Cowan University granted ethical approval for data analysis.

5.3.2 Participants

De-identified haematological data (iron parameters and Hb_{mass}) from 178 athletes (males = 98, females = 80) who undertook altitude exposure at the Australian Institute of Sport (AIS) from 2006 to 2014 were extracted from the AIS medical record database. This sample represents the entire population of endurance athletes who undertook altitude exposure at the AIS during this time. Athletes were from one of seven sports, including; cycling (n = 60), rowing (n = 9), swimming (n = 2), triathlon (n = 5), distance running (n = 43), race walking (n = 39) and water polo (n = 20). Athletes were AIS scholarship holders and/or national team members who undertook altitude exposure as part of a training camp, or who attended the AIS for research purposes. Hence, some aspects of the combined data set presented here have been published previously (54, 55, 70, 178, 244, 257).

Three females were diagnosed by a sports physician as having hereditary hemochromatosis and were removed at the data extraction phase and not included in the final data analysis. Athletes' iron status was classified based on their pre-altitude exposure serum ferritin levels. Iron deficiency was categorised into three stages: Stage 1 (iron deficiency): serum ferritin <35 $\mu g \cdot L^{-1}$, TSAT >16%; Stage 2 (iron deficient erythropoiesis): serum ferritin <20 $\mu g \cdot L^{-1}$, TSAT >16%; Stage 3: serum ferritin <12 $\mu g \cdot L^{-1}$, TSAT <16%. Stage 3 iron deficiency is normally associated with anemia (1).

Prior to altitude exposure, 21 athletes (5 males, serum ferritin: 30.1 ± 5.3 $\mu g \cdot L^{-1}$; 16 females, serum ferritin: 29.7 ± 3.4 $\mu g \cdot L^{-1}$) met the criteria for Stage 1 iron deficiency. Six athletes met the criteria for Stage 2 iron deficiency (2 males, serum ferritin: 17.4 ± 0.7 $\mu g \cdot L^{-1}$; 4 females, serum ferritin: 15.2 ± 3.7 $\mu g \cdot L^{-1}$) and one female athlete met the criteria for Stage 3 iron deficiency (serum ferritin: 11.8 $\mu g \cdot L^{-1}$).

5.3.3 Altitude Exposure

Four altitude training camps employed a classic (LHTH) training approach and were conducted at either Stelvio Pass, Italy [~2,700 m, *n* = 5; (244)], St. Moritz, Switzerland (~1,850 m, *n* = 5), Perisher Valley, Australia (~1,720 m, *n* = 8) or Thredbo, Australia (~1,350 m, *n* = 13). The remaining altitude camps used a LHTL approach (*n* = 147) and were conducted in normobaric, altitude-training facility at the AIS (257), designed to simulate 3,000 m altitude. During LHTL camps, athletes were required to spend a minimum of 14 $h \cdot d^{-1}$ at altitude, whilst they trained near sea level (~600 m, Canberra, Australia). One LHTL group lived at 3,000 m but performed three training sessions per week at 2,200 m (55).

5.3.4 Iron Supplementation

Iron supplements (Ferro Grad C, 325 mg ferrous sulphate & 1,000 mg ascorbic acid, delivering 105 mg elemental iron, Abbott Laboratories, Botany Bay, Australia) were administered to athletes one week prior to, and for the duration of, altitude exposure. The iron supplement dose administered during altitude exposure was based on athletes' pre-altitude ferritin levels. Fifteen athletes (13 males, 2 females) were not iron supplemented because of high (i.e. >100 $\mu g \cdot L^{-1}$) pre-altitude serum ferritin levels [serum ferritin: 164 ± 35 $\mu g \cdot L^{-1}$]. Additionally, 144 athletes (82 males, 62 females) ingested one iron tablet per day (105 $mg \cdot d^{-1}$ elemental iron; serum ferritin: 76 ± 32 $\mu g \cdot L^{-1}$). Finally, 19 athletes (3 males, 16 females) ingested two iron tablets per day (210 $mg \cdot d^{-1}$ elemental iron; serum ferritin: 25 ± 7 $\mu g \cdot L^{-1}$).

5.3.5 Haemoglobin Mass

Haemoglobin mass was measured before and after altitude exposure via the optimised 2 min carbon monoxide (CO) rebreathing technique (199). Briefly, this technique involved the rebreathing of a bolus of ~1.2 $mL \cdot kg^{-1}$ CO for 2 min. Capillary blood samples were taken before and 7 min after rebreathing CO, and measured five times using an OSM3 Hemoximeter (Radiometer, Copenhagen, Denmark) to determine the percentage change in carboxyhemoglobin (HbCO). Haemoglobin mass was then calculated from the change in %HbCO before and after CO rebreathing. The typical error of measurement for Hb_{mass} in our laboratory is ~1.8%, 90% CI [1.3, 2.2] (248).

5.3.6 Iron Parameters

Blood samples were collected at rest from a forearm antecubital vein by a trained phlebotomist. Pre- and post-altitude iron parameters (serum ferritin and TSAT) were measured at the AIS using either a Hitachi 911 (Boehringer, GmbH, Mannaheim, Ingelheim, Germany) or a COBAS Integra 400 (Roche Diagnostics, Switzerland) via

immunoturbidimetry (258), with the same analyser always used for each pre-post comparison.

5.3.7 Total Iron Incorporation

Total Iron Incorporation (TII) was calculated to determine total erythrocyte iron uptake (HII) and iron stored as ferritin during altitude exposure (SII) (134). Here, TII = [(Δ Hb_{mass} (g) × 3.38 mg) + (Δ Ferritin ($\mu g \cdot L^{-1}$) × 8 mg)], where, 3.38 mg represents the typical iron content of Hb (259) and 8 mg represents the iron content of ferritin (260).

5.3.8 Statistics

5.3.8.1 Linear Regression

The influence of altitude exposure on Hb_{mass} and iron parameters was modelled using a multiple regression in the R statistics programme (254). Hb_{mass}, serum ferritin, TSAT and were log-transformed before analysis, back transformed, and then expressed as a percentage difference from the specified reference level to improve interpretability. Data were first modelled to investigate the influence of the following covariates on the change in Hb_{mass} in response to prolonged moderate altitude exposure: altitude category (2 levels: natural, simulated), altitude duration (<2 weeks, 2-3 weeks, >3 weeks), altitude elevation (3 levels: 1,350-1,850 m, 2.600-2,800 m, 3,000 m) and sport (7 levels: race walking, distance running, road cycling, triathlon, rowing, swimming and waterpolo). There were no significant differences between the changes in Hb_{mass} or iron parameters following altitude exposure for sex and altitude category, thus these data were pooled during the final analysis. Furthermore, since the

interaction between sport and oral iron supplement yielded a small sample size, we chose to omit these covariates from the final model.

The percentage change in Hb_{mass} and serum iron parameters during altitude exposure was analysed via linear regression with daily oral iron supplement dose (3 levels: none, 105 $mg \cdot d^{-1}$, 210 $mg \cdot d^{-1}$) as the only model covariate. Results are reported as the percentage change from pre-altitude values, with 95% confidence limits to denote the imprecision of the point-estimate.

5.3.8.2 Effect Sizes

The magnitudes of the change in pre-altitude *versus* post-altitude values for TII, HII and SII are expressed as a Cohen's *d* effect size (236). Effect sizes were interpreted using Cohen's Scale for Effect Sizes (236) with the following qualitative descriptors: *trivial (0.0-0.2), small (0.2-0.6), moderate (0.6-1.2), large (1.2-2.0), very large (2.0-4.0)*. The imprecision of the point estimates were quantified using 95% confidence limits. Since TII and SII included negative values, non-normality was addressed by adding 1,000 to these values before applying a Box-Cox (power) transformation (261).

5.4 Results

5.4.1 Haemoglobin Mass and Iron Parameter Responses

The Hb_{mass} and iron parameter responses to moderate altitude exposure adjusted for oral iron supplement doses are presented in Table 5.1. Mean and standard deviation for iron parameter and Hb_{mass} responses are available in Appendix A (Chapter 8). Hb_{mass} increased from pre-altitude levels in athletes who ingested 105 $mg \cdot d^{-1}$ and 210 $mg \cdot d^{-1}$ of oral iron supplements (both $p < 0.01$). In comparison, Hb_{mass} did not

increase from pre-altitude levels following moderate altitude exposure in non-iron supplemented athletes (p = 0.14). ΔHb_{mass} was 2.1% [0.3, 4.0] and 2.9% [0.5, 5.3] higher in athletes who ingested 105 $mg \cdot d^{-1}$ and 210 $mg \cdot d^{-1}$ of oral iron compared with non-iron supplemented athletes. We also classified athletes as haematological responders based on a change in Hb_{mass} greater than the typical error of measurement for the CO rebreathing technique (i.e. 1.8%) (248). Based on this criterion, 120 athletes (57 males, 63 females) athletes were classified as haematological responders and 58 athletes (35 males, 23 females) as non-responders.

Serum ferritin decreased following moderate altitude exposure in non-iron supplemented athletes and those athletes who ingested 105 $mg \cdot d^{-1}$ of iron (both p < 0.01), but increased relative to pre-altitude levels in those athletes who ingested 210 $mg \cdot d^{-1}$ of oral iron (p < 0.01). Finally, oral iron supplementation did not influence the ΔTSAT following moderate altitude exposure (p > 0.05).

Table 5.1: Parameter estimates (Est.) with 95% confidence limits (CL) for the changes (Δ) in Hb_{mass}, ferritin and transferrin saturation (TSAT) during prolonged moderate altitude exposure, when controlled for oral iron supplement dose.

Effect		**Δ Hb_{mass}**			**Δ Ferritin**			**Δ TSAT**	
Supplement Dose	**Est.**	**95% CL**	***n***	**Est.**	**95% CL**	***n***	**Est.**	**95% CL**	***n***
None	1.1	[-0.4, 2.6]	15	-33.2	[-46.9, -15.9]	10	-22.3	[-48.7, 17.8]	4
105 $mg \cdot d^{-1}$	3.3	[1.7, 4.8]	144	-13.8	[-32.2, 9.7]	97	-6.8	[-6.8, 42.6]	75
210 $mg \cdot d^{-1}$	4.0	[2.0, 6.1]	19	36.8	[1.3, 84.8]	15	10.9	[-31.6, 80.0]	11

5.4.2 Total Iron Incorporation

Figure 5.1 compares the TII after altitude exposure in iron supplemented and non-iron supplemented athletes. Overall, TII was higher in athletes who were supplemented with either 105 $mg \cdot d^{-1}$ or 210 $mg \cdot d^{-1}$ of iron orally during altitude exposure compared

with non-iron supplemented athletes. Specifically, iron supplementation of either 105 $mg \cdot d^{-1}$ or 210 $mg \cdot d^{-1}$ of oral iron during altitude exposure induced a large and very large increase in TII (0 *versus* 105 $mg \cdot d^{-1}$: d = 1.52 [0.95, 2.07]; 0 *versus* 210 $mg \cdot d^{-1}$: d = 2.13 [1.24, 2.92]).

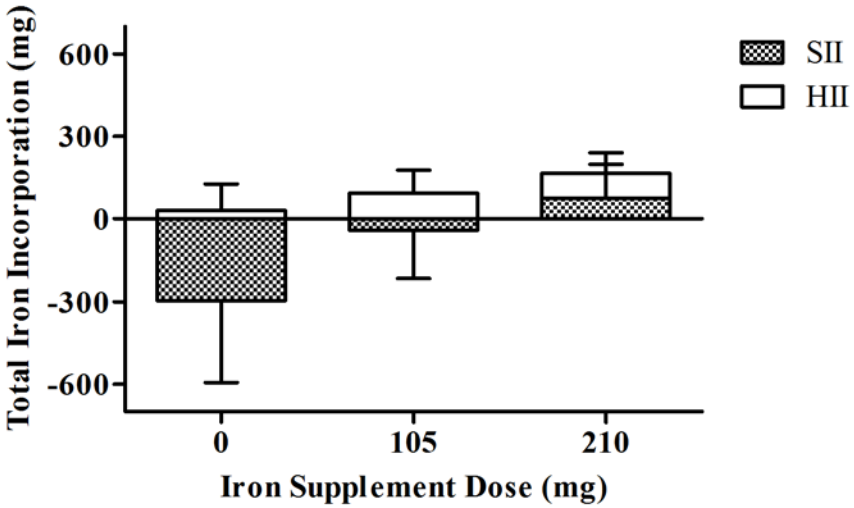


Figure 5.1: Iron incorporation into the iron storage (SII) and red cell compartment (HII) for each category of iron supplement dose [0 mg (n = 10), 105 $mg \cdot d^{-1}$ (n = 97), 210 $mg \cdot d^{-1}$ (n = 15)]. Overall, total iron incorporation (TII) was higher in iron-supplemented athletes compared with non-iron supplemented athletes.

5.5 Discussion

This study found prolonged, moderate altitude exposure (median elevation: 3,000 m; median duration: 21 days) enhanced erythroid iron incorporation and Hb_{mass} in iron supplemented athletes. Moreover, iron supplementation attenuated the reduction in serum ferritin levels following altitude exposure compared with non-iron supplemented athletes. We conclude daily oral iron supplementation may support Hb_{mass} production and maintain iron balance in athletes with low pre-altitude serum ferritin levels.

5.5.1 Haemoglobin Mass Response to Moderate Altitude

In the current study, oral iron supplemented athletes increased their Hb_{mass} by ~3-4% after moderate altitude exposure. These findings support Gore et al. (6), who calculated an individual athlete exposed to moderate altitude >2,200 m for 300 h might expect a mean increase in Hb_{mass} of ~3.2%. In contrast, non-iron supplemented athletes in the current study did not enhance their Hb_{mass} beyond the typical error of measurement of the CO rebreathing technique (~1.8%) (6). We speculate oral iron supplementation may have supported further Hb_{mass} production in these non-iron supplemented athletes. However, poor intestinal iron absorption and/or erythroid iron uptake also limited iron incorporation by erythroid precursor cells, thereby compromising Hb_{mass} production in these athletes. Although our findings largely suggest oral iron supplementation supports Hb_{mass} production in athletes undertaking moderate altitude exposure, further research is required to establish a clear serum ferritin threshold to guide oral iron supplementation in athletes planning to undertake altitude exposure.

Our findings that moderate altitude exposure enhances Hb_{mass} by 3-4% in iron-supplemented athletes contrast those of the Monte Carlo simulation by Rasmussen et al. (83). These authors concluded altitude exposure must exceed two weeks at an altitude of >4,000 m to increase red cell volume. However, Garvican-Lewis et al. (262) recently demonstrated a 3.1% increase in Hb_{mass} in distance runners following three weeks exposure to 1,800 m. We speculate that differences in oral iron supplementation practices could explain some of the discrepancies between our findings and those of Rasmussen et al. (83). Specifically, whereas 72% of the athletes analysed by Rasmussen et al. (83) were not iron supplemented, 92% of athletes in our study ingested oral iron supplements. Routine oral iron supplementation may

therefore ensure athletes are able to support erythropoiesis when exposed to moderate altitude exposure for two weeks or more.

5.5.2 Total Iron Incorporation & Iron Parameter Responses

We estimated the amount of the iron mobilised from the iron storage compartment and directed to the erythron based on the change in Hb_{mass} and serum ferritin levels following moderate altitude exposure. In general, oral iron supplemented athletes incorporated more iron into the erythron and mobilised less iron from the iron storage compartment compared with non-iron supplemented athletes. Therefore, oral iron supplementation may assist athletes to maintain a healthy iron balance at altitude, in turn ensuring sufficient iron is available to support accelerated erythropoiesis at altitude.

Athletes supplemented with 210 $mg \cdot d^{-1}$ of oral iron increased their serum ferritin levels following moderate altitude exposure, despite a large Hb_{mass} response. Such an observation indicates the oral iron supplement dose exceeded bone marrow iron uptake in these athletes resulting in the storage of excess iron. Up-regulated iron absorption mechanisms in iron deficient athletes before altitude exposure may explain why these athletes increased their iron stores post-altitude exposure. Iron deficiency and altitude exposure both result in systemic hypoxia and stimulate similar changes in iron metabolism. Two such changes in iron metabolism are an increase in HIF-2α transcription (167, 195) and hepcidin suppression (21-23). Hepcidin suppression is a favourable response to hypoxia that improves iron export from storage cells by reducing the degradation of ferroportin iron export channels located on the cellular surface of iron storage cells. Simultaneously, HIF-2α increases the expression of ferroportin (167) and proteins involved in iron absorption (i.e. divalent-metal

transporter-1, duodenal cytochrome B) (263). Finally, the hypoxic stabilisation of HIF-1α enhances the expression of iron transport proteins (i.e. TfR-1) (164). These changes in iron absorption and transport mechanisms in iron deficient athletes during altitude exposure may explain why oral iron supplementation effectively replenished the iron storage compartment in athletes who were supplemented with 210 $mg \cdot d^{-1}$.

In contrast to iron-supplemented athletes, non-iron supplemented athletes mobilised a large amount of iron from storage sites during altitude exposure, but incorporated less iron into the erythron. Such a response may indicate impaired erythroid iron delivery in athletes who had otherwise healthy serum ferritin levels before altitude exposure. Although not measured in the current study, an exercise-induced increase in hepcidin during exercise recovery in non-supplemented athletes may have temporarily inhibited iron export from reticuloendothelial macrophages, thus limiting its delivery to the erythron during the post-exercise recovery period. In support of this hypothesis, Peeling et al. (19) showed athletes with high pre-exercise serum ferritin levels (>100 $\mu g \cdot L^{-1}$) at sea level exhibit a significantly greater post-exercise hepcidin response 3 h following interval exercise than athletes with low serum ferritin levels (serum ferritin <30 $\mu g \cdot L^{-1}$). Such a response may also occur during LHTL, despite hepcidin suppression at altitude (21), thus temporarily decreasing iron delivery to the bone marrow during the exercise recovery. However, the post-exercise hepcidin response during LHTL is yet to be investigated.

5.5.3 Serum Ferritin Threshold for Iron-Supplementation at Moderate Altitude

To date, no clear serum ferritin threshold exists to guide oral iron supplementation for athletes planning an altitude sojourn. At sea level, athletes with serum ferritin levels <35 $\mu g \cdot L^{-1}$ are often prescribed oral iron supplements to restore and maintain iron

balance (121). A higher serum ferritin threshold is likely required for athletes undertaking altitude exposure to compensate for an hypoxic-mediated increase in erythroid iron uptake at altitude. In the absence of clear criteria, we supplemented both males and females with serum ferritin levels <100 $\mu g \cdot L^{-1}$. We based our rationale on the findings of Brugnara et al. (264), who showed healthy subjects with serum ferritin levels <100 $\mu g \cdot L^{-1}$ developed a functional iron deficiency when undergoing synthetic EPO administration. We acknowledge that synthetic EPO administration bypasses an hypoxic-mediated increase in HIF-1α and HIF-2α transcription during prolonged altitude exposure. Therefore, some athletes may have sufficient iron stores to support erythropoiesis during moderate altitude exposure despite serum ferritin levels <100 $\mu g \cdot L^{-1}$, since HIF activation stimulates the expression of proteins involved in intestinal iron absorption and iron export from reticuloendothelial macrophages. Based on our results, we are unable to define a specific serum ferritin threshold at which to commence oral iron supplementation in athletes undertaking prolonged altitude exposure. Thus, further research is required to establish altitude-specific serum ferritin thresholds to guide oral iron supplementation during moderate altitude exposure.

Some disadvantages of oral iron supplementation include negative side effects such as nausea, flatulence and gastrointestinal discomfort. Furthermore, oral iron supplements transiently increases oxidative stress on the gut mucosa and may disrupt the gut microbacteria (212). Hence, if oral iron supplements are not well-tolerated, then parenteral iron supplementation may be indicated to increase an athlete's iron stores in preparation for altitude exposure (12). However, parenteral iron supplementation should be considered in consultation with a trained sports physician. In light of our findings, the development of altitude-specific, iron supplementation guidelines could

help to optimise athletes' haematological adaptations to prolonged altitude exposure. Furthermore, refinement of iron supplement guidelines for athletes undertaking altitude exposure may also help to avoid the health risks associated with unnecessary oral iron supplementation in athletes with otherwise healthy iron stores.

5.6 Limitations

This study was an observational, retrospective data analysis and unfortunately has several limitations. Firstly, we were unable to analyse athletes' training volume or dietary iron intake as covariates in our statistical model. We recommend future studies account for these factors to determine their influence on the Hb_{mass} and iron parameter response to altitude exposure. Secondly, exercise and/or illness may have increased inflammatory cytokines at altitude, in turn increasing serum ferritin levels owing to its role as a positive acute phase protein (265). In the current study, venous blood samples were collected before exercise under standardised conditions. We therefore believe an exercise-related increase in inflammatory cytokines unlikely influenced the blood iron parameter levels reported here. However, an IL-6 mediated increase in hepcidin during post-exercise recovery may have compromised intestinal iron absorption and/or iron recycling from reticuloendothelial macrophages. Thirdly, the low sample size of the non-iron supplemented and 210 $mg \cdot d^{-1}$ oral iron supplement group meant we were unable to extend our model to consider whether sport discipline and altitude duration moderated the Hb_{mass} response to altitude exposure. Thus, these factors may also have influenced the Hb_{mass} response to moderate altitude exposure reported here. We direct the reader to Gore et al. (6) who analysed the effect of altitude duration on the Hb_{mass} response to moderate altitude. Finally, given prolonged altitude exposure enhances several iron-dependent oxidative enzymes, such as cytochrome C oxidase and citrate synthase; some of the iron derived from oral iron

supplements was likely directed to the mitochondria to support the synthesis of these iron dependent non-haem proteins.

5.7 Conclusion

Two to four weeks of low-to-moderate altitude exposure (1,350-3,000 m) increased Hb_{mass} in well-trained iron supplemented athletes. Daily oral iron supplementation improved erythroid iron incorporation and resulted in higher serum ferritin levels in athletes who ingested oral iron at a dose of 105 $mg \cdot d^{-1}$ and 210 $mg \cdot d^{-1}$, relative to non-supplemented athletes. We conclude oral iron supplementation likely supports Hb_{mass} production during moderate altitude exposure, and may help to replenish the iron storage compartment in some iron deficient athletes.

6 CHAPTER SIX

SUMMARY, CONCLUSIONS & RECOMMENDATIONS

6.1 Thesis Summary

This thesis examined how high-intensity interval exercise influences post-exercise iron metabolism (i.e. hepcidin and iron parameters responses) in endurance athletes undertaking acute (~ 30 min) and prolonged (two-to-four weeks) altitude exposure. This thesis also examined how daily oral iron supplement dose influenced the Hb_{mass} response to prolonged moderate altitude exposure in a large cohort of well-trained athletes.

6.1.1 Iron Regulation during Acute and Prolonged Hypoxic Exposure

The outcomes of this thesis provide new insights about the regulation of post-exercise iron metabolism in well-trained athletes using acute and prolonged hypoxic exposure protocols. Specifically, interval exercise stimulates hepcidin production 3 h after a post-exercise increase in IL-6, regardless of whether exercise in performed in normoxia or acute hypoxia. Furthermore, prolonged hypoxic exposure for 14 days does not seem to attenuate the magnitude of the post-exercise hepcidin response. However, the results of Study Two suggest that in the absence of an inflammatory stimulus, resting hepcidin levels are suppressed during two weeks exposure to LHTL. Hence, athletes can safely perform a single interval session in hypoxia without further compromising post-exercise iron metabolism relative to an equivalent training session in normoxia. Accordingly, athletes may not need to alter their dietary iron intake when undertaking acute hypoxic exposure protocols such as IHT since these protocols likely place no additional demand on an athlete's iron stores compared with exercise in normoxia. In support of this assertion, few studies to date have reported reduced iron stores during IHT/IHE protocols (reviewed in Chapter Two, section 6.1). In contrast, given the increase in Hb_{mass} observed in Study Two and Three and large

mobilisation of iron from storage sites in non-iron supplemented athletes in Study Three, athletes undertaking prolonged hypoxic exposure may benefit from ingesting oral iron supplements to ensure they have sufficient iron stores to support their accelerated erythropoiesis.

Despite the widespread use of hypoxic exposure methods by athletes, it was previously unknown what mechanisms regulate hepcidin expression following exercise during either acute or prolonged hypoxic exposure. Indeed, exercise in acute hypoxia involves two opposing hepcidin regulatory mechanisms, an exercise-induced increase in IL-6 production (16) (a hepcidin promoter) and hypoxia (153) (a hepcidin suppressor). On the one hand, an IL-6-mediated increase in hepcidin production post-exercise could transiently decrease plasma iron availability by blocking iron efflux from reticuloendothelial macrophages as well as impairing intestinal iron absorption (266). Long-term, repeated post-exercise increases in hepcidin could deplete the body's iron stores, consequently iron must be mobilised from storage sites to maintain iron balance. On the other hand, hepcidin suppression post-exercise may increase iron export into the blood plasma, potentially increasing iron available for erythropoiesis. The increase in hepcidin 3 h post-exercise observed in Study One and elevation in hepcidin 3 h post-exercise despite a large hypoxic dose in Study Two, however, suggests IL-6 is the dominant regulator of hepcidin expression following exercise in acute hypoxia.

The regulation of hepcidin in athletes combining exercise with either acute or prolonged hypoxic exposure most likely depends on the magnitude of post-exercise IL-6 response and hypoxic dose. For example, although Huang et al. (267) found accelerated erythropoietic drive inhibited the inflammatory control of hepcidin expression in mice injected with EPO and lipopolysaccharide, concluding the

individual strength of each regulator determines an individual's hepcidin levels in the presence of competing hepcidin regulators. Furthermore, increased erythropoietic drive in response to hypoxia, rather than hypoxia itself, is the mechanisms responsible for inducing hepcidin suppression (168). Hepcidin suppression requires at least 4 h of continuous hypoxic exposure and is mediated either by the release of erythroferrone by proerythroblast in response to increased EPO production or by increased PDGF-BB production by platelets and macrophages (109). Consequently, the typical durations of hypoxic exposure used in IHT protocols (~30-90 min) are likely insufficient to suppress hepcidin. In contrast, Study Two demonstrated two nights of LHTL (14 $h \cdot d^{-1}$) suppressed hepcidin under resting conditions, which is a comparatively larger hypoxic dose than used in Study One. Additionally, the lower post-exercise hepcidin levels observed in Study Two following the 14^{th} compared with the 11^{th} day of LHTL may have resulted from the accumulation of greater hypoxic dose by the 14^{th} day of LHTL. Thus, in presence of competing hepcidin regulatory mechanisms, the strength of each individual stimulus may determine which mechanism prevails (Figure 6.1).

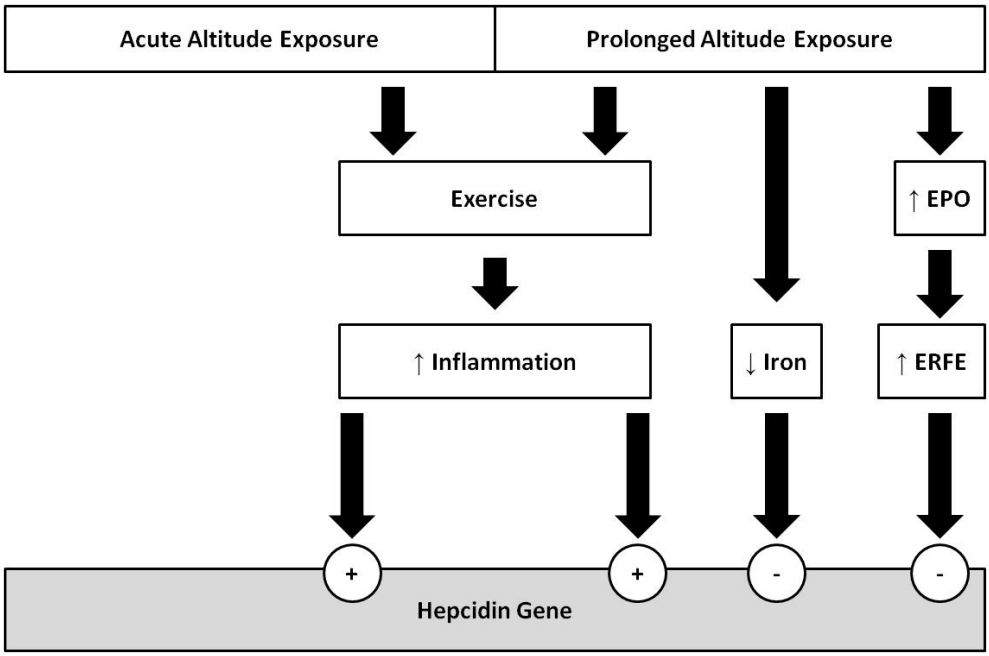


Figure 6.1: The interplay between hepcidin regulation pathways during acute and prolonged altitude exposure combined with exercise. **Acute altitude exposure:** The hypoxic stimulus is insufficient to suppress hepcidin production in acute hypoxia; therefore, an exercise-induced increase in inflammation promotes hepcidin prodcution post-exercise. **Prolonged altitude exposure:** Continuous hypoxic exposure stimulates erythropoietin (EPO) release from the kidney and liver, in turn acting on proerythroblasts to release the erythroid hepcidin regulator, erythroferrone (ERFE), suppressing resting hepcidin activity. A reduction in whole body iron stores during prolonged altitude exposure also suppresses resting hepcidin levels. A post-exercise increase in inflammation may override hypoxic hepcidin suppression during prolonged hypoxic exposure, regardless of the hypoxic stimulus.

Increased hepcidin production 3 h post-exercise during prolonged hypoxic exposure could impair iron recycling and absorption during exercise recovery, in turn limiting iron delivery to erythroid precursor cells. Further studies should therefore investigate how the post-exercise hepcidin response influences dietary iron uptake and/or the

absorption of oral iron supplements at altitude. Such research may allow the refinement of dietary iron guidelines for athletes planning to undertake prolonged altitude exposure; thereby ensuring athletes have sufficient iron available to support hypoxic-mediated adaptations in both iron-dependent haematological and non-haematological mechanisms.

6.1.2 Maintaining Iron Balance during Prolonged Hypoxic Exposure

Sufficient iron stores are required to support accelerated erythropoiesis at altitude. The increase in Hb_{mass} observed in Study Two and Three respectively, are consistent with the ~1% increase in Hb_{mass} per 100 h of moderate altitude exposure (>2,200 m) proposed by Gore et al. (6). However, the 3-4% increase in Hb_{mass} in oral iron supplemented athletes reported in Study Three contrast the conclusions of Rasmussen et al. (83), who suggest two weeks of continuous exposure to at least 4,000 m is required to increased red cell production. The discrepancy between our findings and Rasmussen et al. (83) may result from differences in iron supplementation protocols between these two studies. Whereas 92% of athletes in Study Three ingested oral iron supplements, only 72% of athletes included in the Monte Carlo simulation by Rasmussen et al. (83) ingested oral iron supplements. Although speculative, iron availability may have been lower in the athletes analysed by Rasmussen et al. (83), owing to a lack of iron supplementation. Such differences potentially explain why these authors concluded prolonged altitude exposure stimulates a lower haematological response than we observed in Studies Two and Three.

Low pre-altitude iron stores and/or poor mobilisation of iron from stores during early altitude exposure may blunt the haematological response to altitude (10) since iron is unavailable to support accelerated erythropoiesis. Indeed, Study Three showed non-

iron supplemented athletes mobilised more stored iron and had a lower Hb_{mass} response post-altitude than those athletes supplemented with 105 $mg \cdot d^{-1}$ or 210 $mg \cdot d^{-1}$ of elemental iron. In comparison, athletes supplemented with 210 $mg \cdot d^{-1}$ of elemental iron markedly increased their Hb_{mass} and iron stores post-altitude despite low pre-altitude iron stores. Hypothetically, increased intestinal iron absorption resulting from a HIF-2α-mediated up-regulation of iron import proteins, DCytB and DMT-1, combined with the suppression of hepcidin at altitude could enhance iron uptake by erythroid precursor cells (30), potentially explaining the large increase in Hb_{mass} and iron stores athletes observed in athletes who ingested a dose of 210 $mg \cdot d^{-1}$ elemental iron. Hence, oral iron supplementation may ensure athletes with low pre-altitude iron stores are able to maintain a healthy iron balance and provide sufficient iron to support erythropoiesis at altitude.

No specific iron supplementation guidelines are currently in place to ensure athletes can maintain a healthy iron balance during prolonged altitude exposure. Whereas short-term exposure to moderate altitude may not require oral iron supplements, prolonged (two-to-four weeks) exposure to moderate or high altitude places a large burden on the body's iron stores (9). Given iron uptake approaches almost 100% of its maximal capacity during the first few day of altitude exposure (179), the outcomes of Study Three suggest athletes may benefit from ingesting a minimum of 105 $mg \cdot d^{-1}$ of elemental iron to ensure they can sustain accelerated erythropoiesis at altitude. Future research should focus on developing altitude-specific serum ferritin thresholds to guide oral iron supplementation during prolonged altitude exposure.

6.2 Practical Implications

The outcomes of this thesis enhance our current understanding of how iron metabolism is regulated during acute and prolonged altitude exposure and provide some insights about how athletes can maintain a healthy iron balance in hypoxia.

Specifically, Study One demonstrated that exercise performed in acute hypoxia exposure seems to place no further risk to an athlete's iron status than exercise in performed in normoxia. Indeed, post-exercise iron metabolism was similar regardless of whether exercise was performed in hypoxia and normoxia. Thus, given these findings, athletes may not need to alter their dietary iron intake or post-exercise feeding habits following exercise in acute hypoxia. However, further research is required to determine whether the long-term use of acute hypoxic exposure protocols place an additional demand on athletes iron stores, or alter post-exercise iron metabolism, compared with long-term exercise in normoxia.

In contrast to Study One, the outcomes of Study Two and Three demonstrated prolonged, moderate altitude exposure alters both resting and post-exercise iron metabolism. Furthermore, oral iron supplementation during altitude exposure improved erythroid iron incorporation and raised the iron storage compartment in athletes with low pre-altitude iron stores who ingested 105 $mg \cdot d^{-1}$ or 210 $mg \cdot d^{-1}$ of elemental iron, relative to non-iron supplemented athletes. Consequently, oral iron supplementation likely assists athletes to maintain a healthy iron balance at altitude, so long as the iron supplement dose is appropriate for their needs. Indeed, the efficacy of oral iron supplementation may be improved at altitude relative to sea level owing to improved intestinal iron absorption and the suppression of hepcidin. Given the outcomes of this thesis, it is clear athletes with low pre-altitude serum ferritin levels

(i.e. <40 $\mu g \cdot L^{-1}$) should ingest oral iron supplements both before and during prolonged exposure to moderate altitude to support accelerated erythropoiesis. Feasibly, some athletes with serum ferritin levels >40 $\mu g \cdot L^{-1}$ might also benefit from ingesting oral iron supplements at altitude. Unless contraindicated, athletes should therefore ingest a minimum of 105 $mg \cdot d^{-1}$ of elemental iron to maintain a healthy iron balance during prolonged altitude exposure. Further research is required to establish clear serum ferritin thresholds for each altitude exposure method (LHTH, LHTL and IHT). An algorithm for oral iron supplementation is proposed in Figure 6.2.

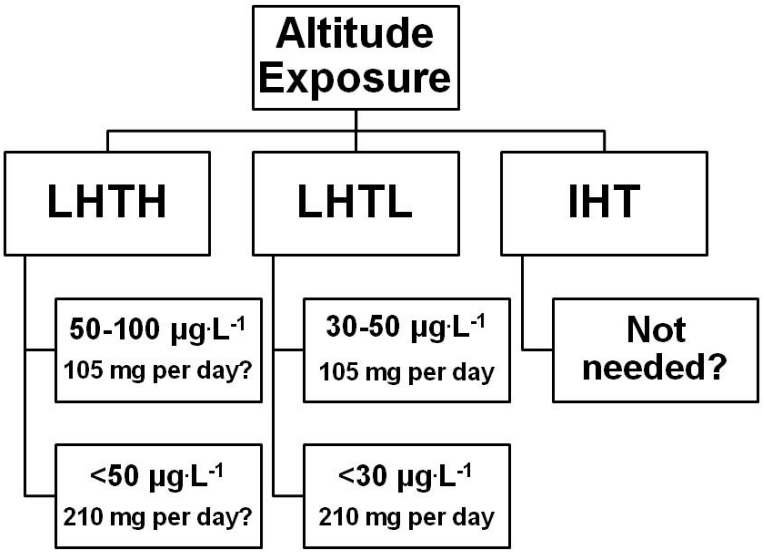


Figure 6.2: Recommendations for oral iron supplementation based on athletes' pre-altitude serum ferritin levels during live high: train high (LHTH), live high: train low (LHTL) and intermittent hypoxic training (IHT). However, further research is neccessary to establish serum ferritin thresholds to guide oral iron supplementation for each altitude exposure method.

6.3 Limitations

The outcomes of this thesis have meaningful practical and theoretical applications, but some limitations apply.

Study One matched the relative exercise intensity (90% of the $v\dot{V}O_{2max}$) between the normoxic and hypoxic interval exercise sessions. Previously, Lundby & Steensberg (27) demonstrated cycling exercise performed at the same absolute intensity (154 W, 54-59% $\dot{V}O_{2max}$) in normoxia and hypoxia (4,100 m natural altitude) respectively, augmented IL-6 production post-exercise in the hypoxic trial. Thus, post-exercise IL-6 production following hypoxic exercise in Study One may have been greater had the exercise protocol been performed at matched absolute, rather than relative exercise intensity, which in turn could have increased the post-exercise hepcidin response.

Study Two did not measure resting or post-exercise IL-6 or dietary iron intake during LHTL. Regardless, in the absence of an infection or a large elevation in plasma iron levels, an exercise-induced elevation IL-6 most likely stimulated the increase in hepcidin during the post-exercise recovery period. Indeed, the results of Study One reported IL-6 increased following acute exercise in hypoxia. Furthermore, Mazzeo et al. (196) found IL-6 increased following exercise performed during prolonged altitude exposure. Taken together, these studies suggest an exercise-mediated increase in IL-6 was the main stimulus responsible for the increase in hepcidin 3 h post-exercise.

Study Three did not analyse the influence of training volume and/or inflammatory cytokines on the iron parameter or Hb_{mass} response to LHTL due to a lack of available data. Furthermore, owing to a low sample size for an appropriate sub-analysis, we were unable to consider how altitude duration, elevation and sport discipline moderated the Hb_{mass} and iron parameter response in iron-supplemented athletes

exposed to moderate altitude. Accordingly, differences in training volume between athletes during LHTL could have confounded the iron parameter and Hb_{mass} response.

6.4 Directions for Future Research

Despite the findings contained in this thesis, several practical and theoretical questions related to iron metabolism at altitude remain.

6.4.1 Practical Directions

Intermittent hypoxic training has recently gained popularity as a cost-effective alternative to LHTL. Study One examined the post-exercise IL-6, hepcidin and iron parameter response to a single interval exercise session and compared the response to that of an interval session performed at matched relative exercise intensity in normoxic conditions. However, the post-exercise hepcidin and iron parameter response to interval exercise performed at matched absolute exercise intensities is currently unknown. Previously, cycling at the same absolute intensity (154 W, 54-59% of VO_{2max}) in hypoxia (4,100 m) has been shown to augment post-exercise IL-6 production (27). Therefore, a single IHT session performed at a matched absolute intensity may augment the post-exercise hepcidin response owing to a larger increase in IL-6 post-exercise, resulting in a greater reduction in iron metabolism during exercise recovery than exercise performed in normoxic conditions.

Typical IHT protocols often involve athletes performing multiple, high-intensity exercise sessions in hypoxic conditions over several weeks. Long-term exercise training in normoxic conditions could progressively decrease resting serum ferritin levels in endurance athletes owing to elevated resting hepcidin levels. Paradoxically, low pre-exercise iron stores would then lead to the suppression of the post-exercise

hepcidin response (19). Ma et al. (268), however, found no elevation in resting hepcidin levels in 20 female college distance runners during the competitive season. Unfortunately, these authors did not measure serum ferritin levels longitudinally throughout the competitive season in conjunction with hepcidin levels. Thus, the relationship between serum ferritin and resting hepcidin levels could not be determined. The effects of long-term IHT on resting and post-exercise hepcidin and iron parameter responses, however, are unknown and require further investigation to evaluate how athletes can effectively maintain iron balance when using IHT/IHE.

No specific iron supplementation guidelines currently exist to ensure athletes maintain a healthy iron balance at altitude. To this end, future research is required to establish altitude-specific serum ferritin thresholds to guide the provision of oral iron supplements to athletes who plan to undertake LHTL. Potential studies could focus on establishing the optimal time to ingest oral iron supplements during altitude exposure to maximise intestinal iron absorption. Ideally, a placebo controlled trial is required to discern whether moderate altitude exposure improves the efficacy of oral iron supplementation in athletes with otherwise healthy serum ferritin levels (serum ferritin: >35 $\mu g \cdot L^{-1}$) and low serum ferritin levels (serum ferritin: <35 $\mu g \cdot L^{-1}$) relative to a similar supplementation protocol at sea level.

To our knowledge, no study has directly compared the efficacy of daily oral iron supplementation with an IV iron injection to maintain iron status in endurance athletes undertaking prolonged altitude exposure. IV iron therapy (delivered as a bolus injection of ~2-4 mL to comply with the WADA anti-doping code) may more effectively prepare athletes for altitude exposure by raising pre-altitude serum ferritin levels more quickly than oral iron supplements. In some circumstances, IV iron therapy might be required to restore the iron storage compartment in anaemic athletes

or those who do not respond to oral iron supplements. Furthermore, IV iron supplementation may be associated with fewer side effects than oral iron supplementation since oral iron supplementation may cause negative side effects such as a disruption of the gut microbacteria and nausea (212). A future study comparing the efficacy of oral *versus* IV iron supplementation may therefore lead to new guidelines that improve athletes' preparedness for altitude exposure, potentially maximising both the haematological and non-haematological benefits of prolonged altitude exposure.

6.4.2 Theoretical Directions

The respective mechanisms responsible for controlling systemic and skeletal muscle iron metabolism in hypoxia are not well understood. Two candidates for the hypoxic regulator of hepcidin were discovered during the course of this thesis; erythroferrone, a hormone release by erythroblasts (104), and PDGF-BB, a growth factor release by platelets and macrophages (109). To date, the activity of these two candidate molecules has not been investigated in athletes undertaking altitude exposure. Measuring the activity of these molecules during prolonged exposure in future studies could provide useful insights into their role in iron metabolism in hypoxia.

The post-exercise hepcidin response may not follow the same time course following exercise performed during prolonged hypoxic exposure compared with normoxia. We chose to measure the hepcidin levels 3 h post-exercise in Study Two based on data from Peeling et al. (16), who demonstrated hepcidin levels peaks between 3-6 h post-exercise, but could still be elevated for up to 24 h post-exercise. Study Two showed hepcidin increased from baseline 3 h post-exercise following exercise in normoxia and acute normobaric hypoxia (3,000 m simulated altitude) during prolonged hypoxic

exposure. Similarly, Study One showed 3 h post-exercise hepcidin levels increased from baseline after acute exercise in normobaric hypoxia (3,000 m simulated altitude). We acknowledge post-exercise hepcidin could have continued to rise beyond the 3 h post-exercise time point in both Study One and Two. To our knowledge, no studies to date have established the time course of the post-exercise hepcidin response following performed during prolonged hypoxic exposure. Further research is therefore required to establish the time course of the post-exercise hepcidin response during prolonged altitude exposure. In addition, since athletes in Study One and Study Two recovered in normoxia post-exercise, another project could investigate whether recovering in hypoxia post-exercise during prolonged hypoxic exposure speeds the recovery of hepcidin to baseline levels. We envision research of this nature would promote a better understanding of the temporal relationship between hepcidin elevation and altered post-exercise iron metabolism. Accordingly, such research may provide information about the optimal time to ingest iron rich compounds during prolonged hypoxic exposure to avoid the post-exercise increase in hepcidin and thus maximise intestinal iron absorption.

The regulation of skeletal muscle iron balance at altitude also remains unclear. Currently, the three studies conducted by Robach et al. (190, 191, 225) investigating the influence of hypoxia (both natural altitude exposure and synthetic EPO administration) on skeletal muscle iron metabolism have yielded conflicting results. Whilst seven-to-nine days of high altitude exposure (4,559 m) mobilised iron from skeletal muscle (i.e. myoglobin), iron accumulated in skeletal muscle despite hepcidin suppression following low and high dose recombinant human EPO administration. Accordingly, iron mobilisation from skeletal muscle may require hypoxic stabilisation of HIF-1α and HIF-2α, a mechanism that is bypassed during EPO administration,

which could explain the different iron regulatory response between these two conditions. Recently, these authors hypothesised the existence of a master regulator of skeletal muscle iron metabolism (190), yet to date, no such molecule has been discovered. One possibility is iron is mobilised from the skeletal muscle to service erythropoiesis during severe hypoxia (i.e. >4,000 m). The breakdown of myoglobin to mobilise iron during hypoxia may be regulated by the interaction of HIF-1α and mTOR. The activation of mTOR in low stress, energy rich states promotes protein synthesis and cell survival (269). In contrast, stressful states, such as starvation or prolonged high altitude exposure are associated with a down-regulation of muscle protein synthesis, due to reduced mTOR expression as part of an autophagic response (269). mTOR may also be involved in coordinating skeletal muscle iron metabolism in hypoxia since mTOR has recently been shown to play a key role in regulating cellular iron balance by altering the expression of iron regulatory proteins such as TfR-1, FPN and IRP-1/IRP-2 (270) and suppresses hepcidin expression (110). However, further research is required to explore the role played by mTOR in the regulation of skeletal muscle iron metabolism at both high and moderate altitudes.

In conclusion, an improved understanding of iron regulation in acute and prolonged hypoxia may enable the development of best practice guidelines to ensure athletes are able to maintain iron balance when using these complementary training techniques.

6.5 Conclusion

In summary, this thesis examined how acute (~30 min) and prolonged (~2 weeks) altitude exposure influences resting and post-exercise iron metabolism in endurance athletes, aiming to improve our understanding of how athletes can maintain iron balance when using such training methods. This thesis concludes the following:

1) The post-exercise IL-6, hepcidin and iron parameter response to interval exercise at a matched relative intensity is similar regardless of whether exercise is performed in normoxia or acute hypoxia. Hence, IL-6 and not hypoxia, is likely the main regulator of the post-exercise hepcidin response following exercise in acute hypoxia.
2) Resting hepcidin levels are suppressed after two nights of LHTL and remain suppressed after 14 days of LHTL.
3) Interval exercise performed in normoxia and hypoxia during LHTL elicits a similar post-exercise hepcidin and iron parameter response to equivalent exercise performed before LHTL. Furthermore, the post-exercise hepcidin response was lower after 14 days of LHTL compared with the pre-altitude response. Thus, the hypoxic dose may moderate the magnitude of the post-exercise response during LHTL.
4) Supplementation of 105 $mg \cdot d^{-1}$ or 210 $mg \cdot d^{-1}$ of elemental iron in athletes with serum ferritin levels <100 $\mu g \cdot L^{-1}$ during moderate altitude exposure improves erythroid iron incorporation and may maintain iron balance compared with no iron supplementation. This may in turn support Hb_{mass} production during moderate altitude exposure. Therefore, endurance athletes who ingest daily oral iron supplements may be better able to provide sufficient iron to support erythropoiesis compared with non-iron supplemented athletes.

Overall, the findings of this thesis suggest high-intensity interval exercise performed during acute and/or prolonged hypoxia results in a similar post-exercise iron metabolism compared to exercise in performed in normoxia. The magnitude of the post-exercise hepcidin response may be moderated by the hypoxic dose applied, although further research is necessary to confirm this hypothesis. Daily oral iron

supplementation during prolonged, moderate altitude exposure may assist athletes to maintain a healthy iron balance and sustain accelerated erythropoiesis, as well as assist the synthesis of several iron-dependent oxidative and glycolytic enzymes. Indeed, suppressed hepcidin levels during prolonged altitude exposure may improve intestinal iron absorption and iron mobilisation from storage sites. Finally, further research should focus on investigating the optimal time to ingest iron rich meals and/or oral iron supplements to maximise iron absorption at altitude.

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8 APPENDICES

Appendix A: Haemoglobin mass and iron parameter response to prolonged (two-to-four weeks) exposure to moderate altitude (1,350-3,000 m) altitude. Effect sizes (ES) are expressed as Cohen's *d* with 95% confidence limits to denote the imprecision of the point estimate.

		None			105 mg			210 mg		
		Pre	**Post**	**ES (*d*)**	**Pre**	**Post**	**ES (*d*)**	**Pre**	**Post**	**ES (*d*)**
Ferritin	*n*	10	10	10	97	97	97	15	15	15
($\mu g \cdot L^{-1}$)	Mean	174.4	119.0	-1.67	77.4	69.4	-0.23	23.7	34.5	0.87
	SD	33.5	32.8	[-2.61, -0.59]	33.1	35.5	[-0.51, 0.05]	7.4	15.9	[0.10, 1.59]
Iron	*n*	4	4	4	75	75	75	11	11	11
($mmol \cdot L^{-1}$)	Mean	20.5	17.3	-0.48	19.3	19.2	-0.01	12.70	17.60	0.68
	SD	4.3	8.4	[-1.82, 0.98]	8.2	8.5	[-0.33, 0.31]	5.90	8.40	[-0.21, 1.51]
Transferrin	*n*	4	4	4	75	75	75	11	11	11
($mg \cdot L^{-1}$)	Mean	2.8	2.8	0.00	2.7	2.7	0.00	3.1	3.2	0.16
	SD	0.2	0.2	[-1.39, 1.39]	0.5	0.4	[-0.32, 0.32]	0.7	0.5	[-0.68, 1.00]
Transferrin Saturation	*n*	4	4	4	75	75	75	11	11	11
(%)	Mean	30.5	24.8	-0.57	28.5	25.9	-0.24	20.1	22.3	0.29
	SD	6.4	10.5	[-1.98, 0.84]	11.9	9.7	[-0.56, 0.08]	7.5	7.6	[-0.56, 1.12]
Haemoglobin Mass	*n*	15	15	15	144	144	144	19	19	19
(g)	Mean	1014.3	1023.3	0.04	849.0	876.6	0.15	677.6	704.5	0.23
	SD	236.4	225.9	[-0.68, 0.75]	186.6	191.6	[-0.09, 0.38]	114.8	118.8	[-0.41, 0.86]