Towards an understanding of the interaction between exercise and dietary iron absorption: Refining treatment strategies for iron deficient athletes

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School of Human Sciences
(Exercise & Sport Sciences)
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Declaration

I, Rachel McCormick, certify that:

This thesis has been substantially accomplished during enrolment in the degree.

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Written consent has been received and archived for the research involving participant data reported in this thesis.

This thesis contains published work, which has been co-authored.

Rachel McCormick

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Statement of Contributors

This thesis contains published work that has been co-authored. The bibliographical details of the work and where it appears in the thesis are outlined below. A statement for each publication that clarifies the contribution of the student to the work is also provided.


Student Contribution 90%
(Appears in Chapter 3)


Student Contribution 90%
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Student Contribution 90%
(Appears in Chapter 5)
I, Peter Peeling, certify that the student’s statements regarding their contribution to each of the works listed above are correct.

As all co-authors’ signatures could not be obtained, I hereby authorise inclusion of the co-authored work in the thesis.

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Executive Summary

Iron deficiency (ID) continues to be a prevailing health issue amongst the athletic population, often manifesting in reduced work capacity, diminished training and performance outcomes, or a suppressed capacity to respond/adapt to training stress, primarily as the condition progresses in severity from a non-anaemic (IDNA) to anaemic (IDA) state. Athletes are more susceptible to ID because they contend with several avenues of iron loss during exercise, however, the body is absent of any process to endogenously generate its own iron supply. Accordingly, ample dietary iron intake and absorption is essential for athletes to replenish depleted stores and maintain a healthy iron balance. While seemingly simplistic, circumventing a negative iron balance is challenging for athletes on account of exercise-induced inflammation mediating a likely reduction in the (already low) bioavailability of dietary iron, by inducing a post-exercise increase in the primary iron regulatory hormone, hepcidin, which suppresses the absorption of dietary iron from the gut. This phenomenon suggests that there may exist a post-exercise period of reduced iron absorption; a likely mechanism of iron-regulation that contributes to the difficulty athletes have in fulfilling their daily iron needs.

Considering the challenge athletes have satisfying their iron requirements through diet alone, iron replacement therapy is commonly prescribed. Beyond nutritional intervention, daily oral iron supplementation is typically the ensuing treatment recommended by practitioners to address ID in athletes, unless an urgent increase in iron stores is required, in which case, parenteral iron therapy is considered. Despite the well-established efficacy of oral iron therapy, this treatment continues to be limited by the frequently reported gastrointestinal (GI) side-effects (e.g. pain, nausea, vomiting, abdominal distress, constipation and diarrhoea) that reduce compliance and potential benefits, likely rendering this treatment impractical for athletes with gut sensitivity. As a result, in addition to the aforementioned periods of potential limited iron absorption, it is clear that further work is also needed relevant to iron supplementation strategies, in order to improve the overall efficacy of treatment for ID athletes. Accordingly, the pervading aim of this thesis, entailing a comprehensive review of the relevant
literature (Chapter 2) and three original investigations (Chapters 3-5), was to explore and optimise iron supplementation strategies for ID athletic populations.

Initially, the first study (Chapter 3) of this thesis established the influence of the transient, exercise-induced hepcidin elevations on iron absorption during the post-exercise period. By extension, this study sought to identify the ideal time of day, relative to exercise, for athletes to consume iron to optimise its absorption during this period. Here, endurance-trained runners (n=16) with sub-optimal iron status (defined here as serum ferritin; sFer < 50 μg·L−1) completed a 90 min running protocol in the morning or in the afternoon, using a crossover study design. Here, iron absorption from both breakfast and dinner meals was measured via an iron-fortified fluid labelled with stable iron isotopes (⁵⁷Fe or ⁵⁸Fe). The outcomes of this investigation revealed that, despite a post-exercise increase in hepcidin levels, more iron was absorbed at breakfast following morning exercise, as compared with breakfast in a rested state, or when compared to the absorption from an evening meal. This indicates that, while the regulatory mechanism of hepcidin is instrumental in the control of iron absorption, other exercise-induced physiological changes potentially influence iron uptake in the post-exercise period, but are likely dependent upon the time of day that the iron is consumed. Of note, given that the post-exercise meals were consumed 30 minutes following exercise, it is likely that the iron within these meals reached the site of absorption prior to the well-known peak in hepcidin elevation at three to six hours post-exercise, presenting the suggestion that a good strategy for augmenting iron absorption might be for athletes to consume high iron-containing foods as close to finishing exercise as possible. Furthermore, we also noted a diurnal increase in hepcidin levels across the day, which may have implications on iron absorption in the afternoon, leading us to conclude that the morning intake of iron-containing foods/supplements may be a more effective strategic approach to enhance an athlete’s iron uptake.

The acute interaction between exercise, hepcidin and iron absorption explored in Chapter 3 impelled the work presented in this thesis to progress toward more chronic outcomes in athletes, with a specific aim to improve oral iron treatments commonly recommended for an athlete population. Recently, intermittent-day iron supplementation has been identified as a promising strategy capable of
circumventing the increase in hepcidin, and suppression of intestinal iron uptake, that occurs 24 h following the consumption of a high iron dose. Thus, the second study of this thesis (Chapter 4) translated an 8 week alternate-day oral iron supplementation protocol into an ID athlete cohort to establish whether this approach improved the efficacy and practicality of oral iron therapy for this population. The findings from this study suggest that the sFer response of endurance athletes to an alternate day oral iron supplementation protocol (n=15) is comparable to daily iron supplementation (n=15), despite a 50% lower total dosage of elemental iron consumed. However, despite the ~60% increase in sFer seen here, neither treatment had any effect on haemoglobin mass (Hbmass) over the 8 week intervention; although it is likely that such adaptation requires more than just a change in iron status alone for improvement. Of importance, the qualitative data captured from this investigation relevant to GI outcomes indicated that the frequency and severity of negative GI side-effects were lower following an alternate day iron supplementation protocol, as compared to the daily approach. As such, it was summarised that an alternate day iron supplementation protocol improves iron stores, whilst curtailing the occurrence of negative GI side-effects, lending support to a supplement strategy that might potentially improve the compliance and practicality of approach for athlete populations.

Beyond oral iron therapy, contemporary research pursues novel and alternate strategies of iron supplementation that bypass the gut to completely circumvent the GI side-effects and absorption issues of the more conventional approaches, but without the invasive procedure of intravenous administration. Transdermal iron delivery therefore presents as an encouraging method of iron provision for ID athletes, however, limited evidence currently exists to adequately assess the efficacy of such an approach. Therefore, the third study of this thesis (Chapter 5) sought to address the lack of empirical evidence surrounding transdermal iron supplementation by supplementing a cohort of ID athletes with either a daily oral iron supplement (n=15), or a transdermal iron patch (n=14), for 8 weeks, to establish whether transdermal iron supplementation is a viable method of iron therapy for this population. The results of this study suggested that daily oral iron supplementation for 8 weeks effectively increases sFer in athletes with sub-optimal iron stores by ~60%, although this treatment is accompanied with the well-documented GI side-effects. In contrast, the transdermal iron patch showed no beneficiary effects on
sFer, Hbmass or \( \dot{V}O_{2\text{max}} \), in athletes with sub-optimal iron stores. In this instance, the primary limiting factor to the transdermal iron patch was likely the capability of the passive delivery mechanism to penetrate the skin barrier, and consequently, we would advise athletes with compromised iron stores to continue to supplement with the conventional oral iron therapy, though on alternate days instead of daily.

Collectively, the outcomes of the three original investigations presented in this thesis have generated refined oral iron supplementation approaches for athletes, which lend themselves to improved efficacy of approach. Overall, this research establishes a contemporary strategy of oral iron therapy to benefit athletes, entailing morning supplementation, ideally within 30 minutes following morning exercise, on alternate days. Furthermore, this body of work identifies that further innovation is required before transdermal delivery of iron is a viable strategy of therapeutically delivering iron to athletes.
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<th>Description</th>
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<td>ALT</td>
<td>alternate day</td>
</tr>
<tr>
<td>AM</td>
<td>morning</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>B1-B6</td>
<td>blood sample 1-6</td>
</tr>
<tr>
<td>BLa</td>
<td>blood lactate</td>
</tr>
<tr>
<td>CHO</td>
<td>carbohydrate</td>
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<tr>
<td>CI</td>
<td>confidence interval</td>
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<tr>
<td>CO</td>
<td>carbon monoxide</td>
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<td>CO₂</td>
<td>carbon dioxide</td>
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<tr>
<td>CV</td>
<td>coefficient of variance</td>
</tr>
<tr>
<td>DAY</td>
<td>daily</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FPP</td>
<td>ferric pyrophosphate</td>
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<tr>
<td>GI</td>
<td>gastrointestinal</td>
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<tr>
<td>GXT</td>
<td>graded exercise test</td>
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<tr>
<td>Hb.mass</td>
<td>haemoglobin mass</td>
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<tr>
<td>ID</td>
<td>iron deficiency</td>
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<tr>
<td>IL-6</td>
<td>interleukin-6</td>
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<tr>
<td>IPC</td>
<td>polysaccharide-iron complexes</td>
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<tr>
<td>LEA</td>
<td>low energy availability</td>
</tr>
<tr>
<td>O₂</td>
<td>oxygen</td>
</tr>
<tr>
<td>PATCH</td>
<td>transdermal iron patch intervention</td>
</tr>
<tr>
<td>PILL</td>
<td>iron tablet intervention</td>
</tr>
<tr>
<td>PM</td>
<td>afternoon</td>
</tr>
<tr>
<td>RED-S</td>
<td>Relative Energy Deficiency in Sport</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RPE</td>
<td>rating of perceived exertion</td>
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<tr>
<td>sFer</td>
<td>serum ferritin</td>
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<tr>
<td>SST</td>
<td>serum separator tube</td>
</tr>
<tr>
<td>vVO₂max</td>
<td>velocity at maximal oxygen consumption</td>
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Introduction
1.1 Background

Iron deficiency (ID) continues to be a prevailing health issue amongst the athletic population, reported to affect ~15-35% of female and ~3-11% of male athletes [1]. Iron is fundamental for energy metabolism and oxygen transport, and accordingly, insufficient iron results in symptoms of lethargy and fatigue. In a sporting context, ID can manifest in reduced work capacity, diminished training and performance outcomes, or a suppressed capacity to respond/adapt to training stress, primarily as the condition progresses in severity from non-anaemic (IDNA) to anaemic (IDA) states [2-4]. Importantly, iron cannot be endogenously synthesised by the body, and thus, adequate iron intake and absorption is critical to replenishing depleted stores and maintaining a healthy iron balance. However, athletes are more vulnerable to ID than the general population, because they encounter additional avenues of iron loss during exercise from processes such as sweating, haematuria, gastrointestinal (GI) bleeding, and haemolysis [5]. Arguably, the fundamental challenge for athletes striving to achieve a healthy iron balance is replenishing these iron losses, which is not helped by the low bioavailability of iron consumed in the diet (2 - 35% of iron intake, dependent on source; haem vs. non-haem iron [6]). This predicament is further complicated on account of recent research indicating that exercise-induced inflammation may mediate a further reduction in iron bioavailability by inducing an increase in the circulating levels of the hormone, hepcidin [7]; the primary iron regulator which suppresses the absorption of dietary iron through duodenal enterocytes and iron recycling by macrophages [8, 9]. Research consistently shows a 2- to 4-fold increase in hepcidin levels at 3 h post-exercise, suggesting that there may exist a post-exercise period of reduced iron absorption [7, 10, 11]. Since athletes are encouraged to eat immediately following exercise to enhance the rate of glycogen restoration and protein synthesis [12], a possible disparity between mealtimes (i.e. breakfast or dinner) and optimal iron absorption may exist. This predicament is a likely mechanism of iron regulation that contributes to ID amongst athletes, presenting as a further challenge for athletes fulfilling their iron demands. However, the influence of this transient, exercise-induced, hepcidin elevation on iron absorption during the post-exercise period, remains to be completely understood.
Increasing dietary iron intake is typically the initial, and most conservative treatment for ID, though is often inadequate at replenishing taxed iron stores in athletes because of the interplay between exercise, hepcidin and iron absorption, combined with their high iron demand and the low bioavailability of dietary iron. Following a nutritional intervention, practitioners typically recommend daily oral iron supplementation to enhance the treatment of ID in athletes, unless a more urgent increase in iron stores is required, in which case, parenteral iron therapy is considered [1, 13]. When considering oral iron supplements, research consistently shows that a 40-80% increase in iron stores (serum ferritin; sFer) is a characteristic response of ID athlete cohorts following an 8-12 week period of daily oral iron supplementation (~100 mg daily) [14-19]. Nevertheless, high rates of GI side-effects (e.g. pain, nausea, vomiting, abdominal distress and diarrhoea) often lead to non-compliance, rendering this treatment less effective and potentially impractical for athletes with gut sensitivity [20]. Therefore, ongoing research endeavours to identify alternate strategies of oral iron therapy to increase fractional absorption, reduce gastric irritation, and ultimately, improve the efficacy of use.

With this in mind, a split-dose strategy of iron supplementation was recently investigated in endurance athletes, with the aim of optimising haematological adaptations during prolonged altitude exposure. Here, Hall et al. [21] established an increase in haemoglobin mass (Hbmass) during the 3 week intervention in both treatment groups, though identified a greater Hbmass response in athletes following single nightly doses of oral iron (200 mg elemental iron) when compared to a split-daily dose (2 x 100 mg elemental delivered morning and evening). These outcomes corroborated the findings of Stoffel and colleagues [22], who showed that both the total and fractional amount of iron absorbed by ID women following 14 d of single (120 mg) and split (2 x 60 mg) iron doses did not differ, and that twice-daily divided doses resulted in higher serum hepcidin concentration than once-daily dosing. However, despite these findings, supplementing with oral iron less frequently than once daily appears to be a more promising strategy to increase fractional iron absorption and reduce GI side-effects [21, 22]. In fact, Moretti and colleagues [23] recently established that hepcidin remained 2.2 times greater after 24 h following a 60 mg dose of iron, resulting in a 35-45% reduction in fractional absorption when a subsequent iron dose was consumed. This likely explains Stoffel et al.’s [22] outcomes, which revealed
~34% greater fractional and cumulative absorption of iron in women who supplemented with 60 mg of iron on alternate days for 28 d compared with women who supplemented with 60 mg of iron daily for 14 d. While this research is yet to be translated into an athletic population, alternate-day iron supplementation appears to be a favourable strategy to efficiently improve iron stores, whilst curtailing the risk of GI side-effects and potentially improving the compliance and practicality of iron therapy in athletes.

Aside from oral iron supplementation, parenteral iron delivery is a more contemporary approach of rectifying more severe cases of ID and treating athletes unresponsive to oral iron treatment. Parenteral iron therapy effectively bypasses the gut, improving iron status and circumventing the GI side-effects associated with conventional supplementation [16]. This treatment was also shown by Burden et al. [24] to significantly raise sFer in IDNA athletes, however, there were no ensuing improvements in aerobic capacity from this treatment. Nevertheless, parenteral iron therapy has been shown to have greater efficacy in athletes with lower iron stores [16], and thus, this approach is usually reserved for the more severe stage of ID due to the invasive nature of the procedure, the potential for severe side-effects (i.e., anaphylaxis), and the tainted perception surrounding the use of needles to treat athletes [1]. Given the limitations of parenteral iron delivery, contemporary approaches to leverage the advantage of bypassing the gut to deliver iron have recently evolved, with transdermal iron supplementation presenting as a potential strategy to therapeutically deliver iron across the skin barrier [25, 26]. While more empirical evidence is required to establish the efficacy of this approach, transdermal iron supplementation is theoretically capable of evading GI side-effects and invasive procedures; thereby warranting further research.

In summary, it is clear from the rationale presented above that there exists a clear need for further investigation in the area of iron supplementation for athlete populations, whereby various strategies require thought to improve the efficacy of approach, leading to revised, evidence-based iron supplementation strategies suitable for the athletic population.
1.2 Statement of the problem

The frequently reported GI side-effects associated with addressing ID via the conventional daily oral iron treatment are encumbering, often result in non-compliance, and ultimately renders the treatment impractical for ID athletes [20]. Iron deficiency continues to be a prevailing issue amongst the athletic population that yearns for innovative solutions to more effectively address it. Given that hepcidin suppresses the absorption and recycling of dietary iron, the transient increase in hepcidin 3 - 6 h post-exercise in response to exercise-induced inflammation, is a likely mechanism of iron-regulation contributing to ID in athletes [7]. Furthermore, since hepcidin and its presiding regulators inherently effect the efficacy of oral iron supplementation, the interaction of exercise, hepcidin and iron absorption could no doubt implicated the efficacy of this treatment in athletes. Furthermore, alternate day iron supplementation is a promising approach yet to be investigated in an athletic population, capable of increasing iron stores, whilst potentially reducing the occurrence of GI side-effects that often limit conventional daily oral iron treatment (e.g. pain, nausea, vomiting, abdominal distress and diarrhoea). Finally, there is currently insufficient evidence for the efficacy of more contemporary strategies of iron supplementation, such as transdermal iron delivery. Accordingly, research into alternate strategies of iron supplementation for the athletic population is warranted, and will therefore be considered throughout the ensuing thesis.

1.3 Thesis aims and hypotheses

The pervading aim of this thesis is to further explore potential strategies of iron supplementation for an ID athletic population. Chapter 1 (Introduction) provides a concise foundation for this research project and progresses into Chapter 2 (Literature Review); a critical evaluation and summary of the existing literature concerning the interplay of exercise, hepcidin and iron absorption, as well as current iron therapies to address ID in athlete cohorts. Subsequently, the key research objective is addressed using a series of three original investigations that constitute Chapters 3, 4 and 5. Firstly, Chapter 3 asserts the influence of the transient, exercise-induced hepcidin elevations on iron absorption during the post-exercise period, to identify the ideal time of day, relative to exercise, for athletes to consume iron to optimise its absorption during this period. In following, Chapter 4 endeavours to identify the optimal
oral iron supplement schedule over a more prolonged training period, translating the alternate-day oral iron supplementation protocol into an ID athlete cohort to establish whether the efficacy and practicality of oral iron therapy for this population can be improved. Thirdly, Chapter 5 looks beyond oral iron therapy, to establish whether transdermal iron supplementation is a viable strategy of therapeutically delivering iron to athletes, whilst evading GI side-effects and invasive procedures. Finally, Chapter 6 (Summary and Conclusions) integrates the outcomes from the three original investigations, and presents the primary conclusions, implications and recommendations for iron supplementation in athlete cohorts moving forward.

Outlined below are the specific aims and hypotheses of each original investigation:

**Chapter 3:** The Impact of Morning versus Afternoon Exercise on Iron Absorption in Athletes.

*Aim:* To examine the influence of an exercise bout on subsequent serum interleukin-6 (IL-6), hepcidin concentrations and iron absorption in endurance athletes, and to assess the impact of exercise timing (i.e., morning or afternoon exercise) on this relationship.

*Hypothesis:* Exercise will induce an acute increase in IL-6 and hepcidin, ensuing a reduction in iron absorption during the post-exercise period, relative to rested conditions.

**Chapter 4:** The Efficacy of Daily and Alternate Day Oral Iron Supplementation in Athletes with Sub-optimal Iron Status.

*Aim:* To compare the efficacy of alternate day oral iron supplementation with daily oral iron supplementation in endurance runners over an 8 week period of training.

*Hypothesis:* Alternate day oral iron supplementation will acquire similar sFer and Hbmass outcomes, compared to daily oral iron supplementation.

**Chapter 5:** The Efficacy of Transdermal Iron Patches in Athletes with Sub-optimal Iron Status.

*Aim:* To compare the efficacy of transdermal iron therapy via an iron patch, with daily oral iron supplementation, to elucidate the non-invasive treatment feasibility for ID athletes.
Hypothesis: Daily oral iron supplementation will procure better sFer outcomes compared to daily transdermal iron supplementation. Furthermore, daily transdermal iron supplementation will have no beneficial effects on Hb\textsubscript{mass} or VO\textsubscript{2max}.

1.4 Contribution of this research
Conventional oral iron therapy is often impractical in the context of athletes due to the high rate of GI side-effects. However, ID continues to be a major issue amongst athlete populations, and thus, the problem requires innovative solutions to more effectively address it. Accordingly, the research that follows provides athletes, coaches, and practitioners, with a more comprehensive understanding of the interaction between exercise, hepcidin and iron absorption that, in turn, will found the development of more athlete-specific recommendations to address ID. Specifically, this body of work identifies refined oral iron supplementation approaches, whilst exploring the efficacy of contemporary approaches as a viable strategy of therapeutically delivering iron.
1.5 References


Chapter 2

Literature Review
2.1 Introduction

Iron deficiency (ID) continues to be a prevalent nutritional issue among the athletic population, reported to affect ~15-35% of female and ~3-11% of male athletes [1]. Compromised iron status is typically associated with classic symptoms of lethargy and fatigue; however, in athletes, the issue may manifest in reduced work capacity, diminished training and performance outcomes, or a suppressed capacity to respond/adapt to training stress, particularly as the condition progresses in severity [2-4]. Athletes may be relatively more susceptible to ID because they encounter additional avenues of iron loss during exercise, such as sweating, haematuria, gastrointestinal (GI) bleeding and haemolysis [5, 6]. Since iron cannot be endogenously synthesised by the body, athletes are required to replace these increased iron losses through adequate iron intake and absorption, which is therefore critical to maintaining a healthy iron balance. However, replenishing taxed iron stores through diet alone is a significant challenge for athletes, partly due to the low bioavailability of haem (15-35%) and non-haem (2-20%) dietary iron [7]. This predicament is further complicated by the stimulating effect that exercise-induced inflammation has on the primary iron regulatory hormone, hepcidin, which when elevated, further suppresses the absorption of dietary iron by duodenal enterocytes and iron recycling by macrophages [8, 9]. To date, a large body of research has corroborated a 2- to 4-fold increase in hepcidin levels occurring at 3 h post-exercise, which likely leads to a period of impeded iron absorption that potentially contributes to the high rates ID among athletes.

Increasing dietary iron intake is typically the initial, and most conservative treatment for ID athletes, which should commence with a complete dietary analysis by an Accredited Sports Dietitian. However, considering the inhibitory influence of hepcidin on iron absorption, the proliferation of this hormone post-exercise promotes the prospect that scheduling dietary iron intake relative to exercise may benefit iron absorption outcomes. Furthermore, when evaluating the optimal time for athletes to ingest iron, account needs to be taken for exercise [9], food influences such as the inhibitory effect of calcium [10], polyphenols, tannins (contained in coffee and tea) and phytates (contained in legumes, nuts, wholegrains) [11, 12], and the diurnal rise in hepcidin into the afternoon [13]. Collectively, the timing of iron intake becomes logistically challenging for athletes, especially those undertaking multiple
training sessions per day. Thus, a high overall dietary iron intake is undoubtedly important for ID athletes, however, it is likely the strategic approach to iron ingestion (i.e. the timing of iron intake) could help to improve iron absorption outcomes. Nonetheless, further research is necessary to fully elucidate the optimal scheduling of iron ingestion in athlete populations.

The interaction between exercise, hepcidin, and iron absorption often makes it challenging for athletes to replenish their iron stores through diet alone [14]. Consequently, athletes often need to consider one of the numerous modes of iron replacement therapy available. Beyond dietary intervention, daily oral iron supplementation is the most widely used treatment of addressing ID, because oral iron supplements are relatively effective, inexpensive and low risk [15]. While ferrous sulphate preparations remain the established treatment for ID [16], there are several different oral iron preparations available. These supplements vary in salt, bioavailability, efficacy, side-effects and galenic form (fast or prolonged release), and are often available as either a tablet or liquid preparation. Nevertheless, a high rate of GI distress [17] often discourages athletes from fulfilling a full-term oral iron treatment plan, generally resulting in sub-optimal treatment efficacy; accordingly, exploration into more strategic approaches of oral iron supplementation is warranted.

With this in mind, parenteral iron delivery is a more contemporary method of rectifying more severe cases of ID, and is an increasingly more common method used for treating athletes unresponsive or intolerant to oral iron treatment. Such treatments effectively bypass the gut, rapidly improve iron status and circumventing the GI side-effects associated with conventional supplementation. Parenteral iron replacement, in the form of an intramuscular injection or intravenous (IV) infusion, is the most direct and effective method of iron replacement. An IV infusion characteristically yields 200-400% increases in iron stores (as measured by serum ferritin; sFer) resultant from a 300-550 mg dose of iron delivered in 1-4 administrations within 6 weeks [18, 19]. Nevertheless, this approach is usually reserved for the more severe stage of ID due to the invasive nature of the procedure, the potential for severe side-effects (i.e., anaphylaxis), a greater risk of iron overload, and the tainted perception surrounding the use of needles to treat athletes [1, 20]. Therefore, considering the current limitations of iron therapy, and the
unique challenge posed by the interaction between exercise, hepcidin and iron absorption, further development of athlete-specific iron treatment strategies is warranted through research.

2.2 Iron metabolism in athletes

What characterises an iron deficiency in athletes? A plethora of factors can contribute to a negative iron balance in athletes, including insufficient dietary intake, inflammatory conditions, exercise-induced iron losses, and in females, menstruation [5, 21]. Accordingly, athletes are vulnerable to a regression toward ID, ranging from an asymptomatic reduction in iron stores, to the cascading decrease in haemoglobin, yielding debilitating fatigue. The initial stage of ID is indicated by a reduction in sFer resulting from the depletion of total body iron stores [22, 23]. At this stage, normal levels of other iron indices and haemoglobin are maintained despite a fall in sFer. As ID progresses, a decrease in iron supply to erythroid marrow occurs, reflected by a decrease in transferrin saturation and an increase in total iron binding capacity. These earlier stages of ID are characterised by the conservation of healthy haemoglobin in the presence of inadequate iron indices, and are collectively referred to as iron deficiency non-anaemia (IDNA). Once iron stores and iron transport become suitably depleted and can no longer sustain the demand of haemoglobin synthesis, functional iron deficiency anaemia (IDA) develops [22, 23].

It is commonly agreed that ID is a spectrum that progresses as a result of negative iron balance, though clinically, there is some contention on the most suitable haematological measurements and their threshold values for diagnosing the stages of ID. However, it is advised that athletes have regular, standardised iron screening to ensure early detection [1]. Currently, due to the lack of clinical consensus related to this condition, IDNA has been defined inconsistently as sFer <12-40 μg·L⁻¹, with IDA developing once haemoglobin concentration falls below 11.5-12.0 g·dL⁻¹ [18, 23-31]. Furthermore, given their greater iron demands, athletes’ iron status have also been described as ‘sub-optimal’ when sFer is recorded at <50-65 μg·L⁻¹ [18, 32], reflecting the potential presence of a negative iron balance that should be monitored. Of interest, it is also worthwhile for clinicians to differentiate ostensive stages of ID from sports anaemia or pseudo anaemia [33], characterised by a transient reduction in
haemoglobin concentration that occurs in early phases of training. This phenomenon is induced by the relative delay between an increase in plasma volume and erythropoiesis, in the adaptive response to exercise, and will typically normalise without treatment during a steady-state phase of training provided all other iron markers are healthy [34, 35].

The ramifications of compromised iron stores in athletes depends on the severity of ID. The most debilitating symptoms arise from diminished erythropoiesis in cases of IDA, encompassing reduced oxygen carrying capacity (aerobic capacity) and relentless fatigue, which expectedly impairs physical and athletic performance [36-39]. In contrast, much of the research addressing the effect of IDNA on exercise performance is less-well understood, despite being the most prevalent diagnosis among athletes [1]. The evidence for detrimental performance effects in cases of IDNA is equivocal, with reports confirming and refuting the benefits of iron therapy in this population [18, 19, 23, 40-43]. This is likely due to a lack of accord as to what is rendered a healthy sFer level, the assortment of performance measures investigated (e.g. VO\textsubscript{2max}, submaximal exercise, time-trial performance), and the absence of standardised iron supplement protocols. A recent systematic review confirmed that the evidence is ambivalent for the benefits of iron treatment on performance in IDNA athletes (defined here as sFer \leq 20 \mu g\cdot L^{-1}, [Hb] >12.0 \text{g}\cdot \text{dL}^{-1}), though these authors suggested that commencement of iron supplementation in IDNA athletes may prevent future potential detriments to athletic performance [44]. Alternatively, it has been suggested that attenuated aerobic capacity may occur independent of reductions in haemoglobin and oxygen transport capacity, via compromised oxidative enzymes and respiratory protein activity, warranting iron therapy for performance in cases of IDNA [3, 24]. This sentiment was reflected in a meta-analysis conducted by Burden et al. [24] that determined iron treatment (encompassing both oral and parenteral) improved iron status and aerobic capacity (specifically VO\textsubscript{2max}) in IDNA endurance athletes (defined here as sFer \leq 35 \mu g\cdot L^{-1}, [Hb] >12.0 \text{g}\cdot \text{dL}^{-1}). However, these authors acknowledged that the studies with the largest effect sizes for VO\textsubscript{2max} had initial VO\textsubscript{2max} <40 \text{mL}\cdot \text{kg}^{-1}\cdot \text{min}^{-1}, compared with studies which showed no effect (>45 \text{mL}\cdot \text{kg}^{-1}\cdot \text{min}^{-1}), and therefore may have been a training response.
Nevertheless, although the evidence is equivocal as to whether IDNA affects athletic performance in the absence of compromised haematopoiesis, it is recognised that mitochondrial electron transport, protein synthesis and iron supply to erythroid marrow is compromised at this stage [25]. Furthermore, it has also been hypothesised that physiological adaptations typically encountered from endurance-based training may only be maximised in the presence of adequate iron supply [1, 43]. While the effects of IDNA on performance are inconclusive, a major issue with untreated IDNA in athletes is the progression of the issue into more severe states of IDA, making them liable to training and performance decrements over time. Therefore, precluding further declines in iron status should be prioritised, which supports the treatment of depleted iron stores in IDNA athletes, because such individuals are susceptible to continued and progressive states of negative iron balance. Since the repletion of exhausted iron stores typically takes 4-8 weeks using oral iron sources, the use of controlled iron supplementation [45] and/or structured dietary intervention is often recommended for IDNA athletes to reduce the foreseeable repercussions. Therefore, moving forward, further research is needed to clarify the thresholds for haematological demarcation of IDNA, its effect on performance, and the appropriate iron treatment for these athletes.

**How is dietary iron absorbed by the body?**

A preliminary understanding of iron metabolism, and specifically iron absorption, is fundamental to addressing ID in all populations. Iron is the most abundant trace element in the human body, amounting to ~50 mg·kg⁻¹ in adult males and ~40 mg·kg⁻¹ in adult females, of which 1-2 mg is lost daily [45, 46], excluding additional exercise losses. Several exercise-induced avenues of iron loss, including sweating, haematuria, GI bleeding, and haemolysis [5], likely account for an additional 3-5 mg of iron loss per day [47], which helps explain an athlete’s increased susceptibility to ID. This is compounded by the fact that the body has no innate process to replace the iron losses incurred, and therefore, exogenous iron sources are of key importance to iron balance.

Iron is primarily absorbed by duodenal enterocytes lining the absorptive villi near the gastroduodenal junction (Figure 1) [48]. The apical surface of these cells contain the enzymatic ferric reductase, known
as duodenal cytochrome b (DCYTB), which reduces ferric iron ($\text{Fe}^{3+}$) to its ferrous form ($\text{Fe}^{2+}$), enabling uptake of non-haem iron by the enterocyte via the protein divalent metal transporter 1 (DMT1) [46, 49-51]. Both DCYTB and DMT1 influence iron absorption, and appear to be down-regulated within 3 h, and for up to 72 h, after high oral doses of iron are consumed [54]. This is understood to be a protective mechanism of iron regulation known as the mucosal block, whereby iron transport proteins on the surface of enterocytes are reduced, likely impacting the efficacy of oral iron treatment [52]. Alternatively, haem iron is absorbed via the haem iron transporter (HCP) and undergoes endocytosis to liberate ferrous iron ($\text{Fe}^{2+}$) within the cell [53]. Following its uptake, iron is exported into the circulation from intestinal enterocytes, facilitated by the basolateral transport protein known as ferroportin, with the assistance of the membrane-bound ferroxidase, hephaestin; subsequently, the iron is transported to a variety of cells around the body, bound to the glycoprotein transferrin [54-56]. The majority of iron is then divided between three active sites; haemoglobin (~65%), myoglobin (~10%) and enzymes (~5%), which are collectively responsible for oxygen transport and energy metabolism [3], which clearly reinforces the importance of iron for athletes. Meanwhile, the remaining intracellular iron is converted into inactive storage depots, in the form of sFer, and its smaller counterpart, haemosiderin (~20%) [25], to inhibit the propensity of excess intracellular iron to catalyse the generation of toxic reactive oxygen species (ROS) [57]. The oxidative nature of excess intracellular iron also explains why the entire process of iron absorption is highly regulated by a variety of stimuli, including dietary iron intake, iron stores, inflammation and erythropoietic drive (erythropoietin production and subsequent erythroid proliferation in response to tissue hypoxia) [58].

**Figure 1.1:** Haem iron is absorbed via the haem iron transporter (HCP) on the apical surface of the enterocytes. Non-haem iron absorption requires the initial reduction of ferric iron ($\text{Fe}^{3+}$) to ferrous iron ($\text{Fe}^{2+}$) by duodenal cytochrome b (DCYTB). At the basolateral membrane, ferroportin mediates the transfer of iron to transferrin in the circulation. Figure obtained from [53].
How is iron regulated by the body?

The homeostatic regulation of iron depends on a reciprocal relationship between iron absorption and body iron stores. Considering there is no controlled process for the body to excrete iron, this ensures sufficient iron uptake whilst preventing toxic iron overload, a serious condition that can result in severe tissue damage and fibrosis [48]. It has long been established that following the ingestion of a large dose of iron, iron absorption is acutely reduced as a means of self-protection [52, 59]. As previously mentioned, this putative process is termed the mucosal block phenomenon, and was first reported in 1943 by Hahn et al., [60], describing the ability of a large oral iron dose to decrease the absorption of iron administered several hours later. This observation has since been elucidated as a rapid change in DMT and DCYTB expression at the brush border of enterocytes, but not ferroportin, in response to high intracellular iron content [52]. Nevertheless, the mucosal block is an acute transient response and it has since been acknowledged that iron homeostasis is primarily regulated by the liver produced, 25 amino-acid peptide, hepcidin, detectable in both urine and blood [13, 61-63].

The specific role of hepcidin is to suppress the absorption and recycling of dietary iron via its interaction with the body’s only known cellular iron exporter, ferroportin (Figure 2). Hepcidin expression stimulates the internalisation and degradation of ferroportin, thereby restricting the transfer of iron into the circulation. This influence can occur at the duodenum, on the cell surface of macrophages (involved in the recycling of iron from senescent red blood cells), and from iron-storing hepatocytes [58]. Typically, hepcidin follows a circadian rhythm characterised by a 2- to 6-fold increase between 06:00am and 15:00pm [13, 64-66], suggesting that iron absorption may vary across a day. Additionally, the synthesis and release of hepcidin is influenced in a feedback-controlled manner by a number of stimuli including dietary iron intake, iron stores, inflammation and erythropoietic activity (typically induced in athletes in response to hypoxia) [58].
Hepcidin expression is homeostatic in nature, whereby iron excess prompts an increase in hepcidin concentration, thus preventing further iron loading [68]. Conversely, hepcidin is suppressed during ID to allow more dietary iron to be absorbed in order to replenish iron stores [68]. This was reinforced in an athletic population by Peeling et al. [32] who identified lower baseline and post-exercise levels of hepcidin in ID athletes (i.e. those that need to absorb iron) compared to athletes with healthy iron stores. This work [32] suggests a progressive reduction in hepcidin activity associated with deteriorating iron status; a protective mechanism to increase dietary iron absorption and maintain iron homeostasis. Correspondingly, hepcidin levels are also attenuated in response to hypoxia or erythropoietic activity, to enable the release of stored iron from macrophages and hepatocytes, allowing the supply of iron for erythropoiesis [69]. This appears to be mediated by erythroferrone, a recently identified hormone that inhibits hepcidin transcription to increase iron availability. For instance McClung and colleagues [70] recently reported diminished hepcidin in association with increased haemoglobin and erythroferrone concentrations in non-acclimatised, healthy young men, following 19 days of high altitude exposure (4500m). Increased erythroferrone at altitude is associated with greater iron availability for erythropoiesis and is particularly relevant to athletes that live or travel to altitude seeking to acquire haematological adaptation. The underlying molecular mechanisms of hepcidin’s regulation via erythropoiesis have been completely described by Nemeth and Ganz [71].

In contrast to hypoxia, high dietary iron intake and inflammation stimulates hepcidin production to protect against toxic iron overload. Recently, Moretti and colleagues [59] characterised the acute
increases in hepcidin levels following iron ingestion, investigating the subsequent influence on iron absorption. It was found that hepcidin concentrations were ~2.2 times greater 24 h following the ingestion of 60 mg of iron, likely explaining the 35-45% reduction in fractional iron absorption observed following a second iron dose consumed the following day [59]. Furthermore, hepcidin has been identified as a positive acute phase peptide induced by inflammatory stimuli, and specifically in response to the upregulation of the cytokine interleukin-6 (IL-6) [72, 73]. Considering the inflammatory nature of exercise [74-77], this affiliation between IL-6 and hepcidin has become a key link between hepcidin and exercise. Accordingly, given the high prevalence of ID amongst the athletic population [2, 78-81], there has been a host of research conducted over the past decade that explores the post-exercise kinetics of hepcidin and its potential impact on the iron metabolism of athletes.

**How does exercise interact with hepcidin activity?**

One of the fundamental challenges encountered by athletes striving to replenish depleted iron stores is that hepcidin expression, and its ramifications, are innately influenced by exercise [59, 82]. The earliest hepcidin observations in an athletic context were described by Roecker and colleagues [83] who identified a 4- to 27-fold increase in urinary hepcidin across 14 female runners, 24 h following the 2004 Berlin Marathon. In this study, hepcidin levels returned to baseline at 72 h post-exercise. However, no hepcidin measurements were collected in the period between exercise completion and 24 h later, limiting the interpretation of hepcidin’s post-exercise kinetics. Furthermore, while no measures of inflammation accompanied, Roecker and colleagues [83] rationalised the observed elevations in hepcidin concentration as responsive to an increase in inflammatory cytokines induced by the run. These limitations prompted Peeling et al. [9] to collect measures of urinary hepcidin, inflammation and markers of iron status in 8 participants completing a 60 min treadmill based run, for 24 h post-exercise, which was compared to the outcomes of a non-exercise (control) trial. This study showed significant elevations in IL-6 and serum iron immediately post-exercise, in addition to raised levels of urinary hepcidin at 3, 6 and 24 h post-exercise (1.7-3.1 times greater than pre- and immediately post-exercise measurements) when compared to control values. Similar results were observed by Newlin and colleagues [84] who investigated the impact of exercise duration on 12 female athletes that completed
a 60 and 120 min treadmill running trial (approximately one month apart) at 65% VO\textsubscript{2peak}. Here, a significant increase in serum IL-6 was observed immediately post-exercise, again preceding peak serum hepcidin concentrations by 3 h. However, Newlin et al. [84] advanced the previous work in this space with evidence to suggest an impact of exercise duration on these responses, via a two-times greater change in hepcidin concentration following the 120 min run as compared to the 60 min run. Currently, it is well accepted that a transient rise in hepcidin occurs between 3 and 6 h post-exercise [9, 84, 85], and corroborates clinical research that shows peak hepcidin levels occur ~3 h following peak elevations in IL-6 [86].

Further investigation into the post-exercise kinetics of hepcidin later established that an athlete's iron status may dictate both the pre-exercise (resting) levels of hepcidin, and the magnitude of the post-exercise hepcidin response, such that the degree of hepcidin elevations following an exercise stimulus is inversely related to their sFer [32]. Iron deplete athletes (defined in the particular study mentioned here as sFer <30 μg·L\textsuperscript{-1}) appeared to have little to no post-exercise hepcidin elevation, prompting Peeling et al. [32] to suggest that, on the continuum of iron status, the post-exercise hepcidin response is of greatest concern to athletes with sub-optimal iron stores (30 ≤ sFer ≤ 50 μg·L\textsuperscript{-1}). This is because athletes with sub-optimal iron status are still vulnerable to a significant post-exercise elevation in hepcidin, potentially reducing their ability to recycle and absorb iron, thereby increasing the likelihood an ID could develop over time, which inevitably might also prevent (or make more difficult) the attainment of a healthy iron status (50 ≤ sFer ≤ 100 μg·L\textsuperscript{-1}). In following, Peeling et al. [32] proposed the prospective need to restore iron status beyond sub-optimal iron status in athletes, in an attempt to reduce the relatively high rate of relapse seen in athlete populations, thereby supporting longer-term or more contemporary (i.e. parenteral administration) iron supplementation protocols for IDNA athletes. Regardless, it is clear that post-exercise hepcidin elevations may have significant implications for athletes with sub-optimal iron stores endeavouring to achieve a healthy iron balance whilst training, since it is likely that iron absorption will be impeded during this post-exercise window. Furthermore,
this challenge may be exacerbated given that the post-exercise window often corresponds to the consumption of a high iron-containing meal or iron supplement (i.e. breakfast or dinner).

**How does exercise influence iron absorption?**

In an effort to better understand the interplay of exercise and iron absorption, Moretti and colleagues [87] assessed the chronic inflammatory and erythropoietic influences of exercise on plasma hepcidin concentration and iron absorption during a 3-week physical training period. This work sought to extend the work of McClung et al. [88], who originally reported significant elevations in resting IL-6 and hepcidin concentrations following 7 days of intensive military activity, by investigating the ramifications to iron absorption. Moretti et al. [87] found that the net effect of chronic exercise training was to decrease post-exercise hepcidin concentrations, and mildly increase iron absorption over time, in the 10 recreationally trained runners examined. These authors suggested a potential mechanism whereby, during the progression of training (additional 8 km run every second day for 21 days), the impact of inflammation and hepcidin elevations are offset by the erythropoietic stimuli, to chronically increase iron mobilisation and absorption for erythroid expansion; hence, the core prospect is that the body is attempting to maintain iron balance in these active individuals [87]. Moretti and colleagues’ [87] work was the first to describe the adaptive consequences of more chronic exercise-induced increases in inflammation and hepcidin on iron absorption. Their outcomes appear to contradict those of McClung and colleagues [88], though the 7 day exercise intervention investigated by McClung et al. [88] may not have been sufficient to observe the adaptive response observed by Moretti et al. [88]. Further work is still required to establish the acute influence of exercise on iron absorption, particularly during the post-exercise window, while hepcidin levels are transiently elevated. Such research would provide a better understanding of the ideal time for athletes to consume iron relative to their training, allowing practitioners new knowledge by which to optimise iron absorption outcomes.
2.3 Iron replacement strategies for athletes

What is an adequate intake of dietary iron for athletes?

The current recommended dietary intake (RDI) of iron is 8 mg·day⁻¹ for adult males and 18 mg·day⁻¹ for pre-menopausal adult women; the latter accounts for the additional iron loss resulting from menstruation [89, 90]. As established above, the body cannot endogenously synthesise iron, and as such, daily dietary intake and absorption is essential to acquire and counteract losses. There are two forms of dietary iron; haem iron, sourced from haemoglobin and myoglobin in animal-based food, and non-haem iron, present in both plant and animal tissue. Haem iron is more bioavailable than non-haem iron (15-35% and 2-20%, respectively [7, 91]) because it is more efficiently absorbed via specific, high affinity, mucosal brush-border haem-binding iron sites [7, 91, 92]. Thus, while haem iron typically constitutes ~10% of total dietary iron intake, this mineral form may provide up to one-third of absorbed dietary iron, elucidating a primary reason why vegetarians are at increased risk of ID [21, 91].

Given non-haem iron characteristically comprises the majority of total dietary iron intake, its already low bioavailability may be more problematic for athletes. Specifically, unlike haem iron, non-haem iron is profoundly influenced by the interaction of several iron-binding ligands commonly found in the diet; in some instances acting to promote iron absorption, though more frequently proceeding to inhibit iron absorption. For example, phenolic compounds, including polyphenols and tannins contained in tea, coffee and other plant foods, bind with non-haem iron and inhibit its absorption. Brune and colleagues [93] documented a 20% and 88% reduction in iron absorption from 5 mg and 100 mg of tannic acid respectively, suggesting tannins inhibit non-haem iron absorption in a dose-dependent manner. Additionally, tea and coffee beverages containing 20-50 mg of polyphenols were shown to reduce iron absorption from a bread meal by 50-70% [12]. Similarly, phytates, found in whole-grain cereals, legumes and nuts, are another collection of compounds known to reduce iron absorption in a dose-dependent manner, with 25 mg of phytate added to a wheat roll shown to reduce iron absorption by 64% [94]. Another significant inhibitor of iron absorption is calcium, with doses between 40 and 300 mg shown to reduce iron absorption by 39% and 74%, respectively [95]. This is especially problematic for athletes given bovine milk has been identified as an ideal post-exercise recovery drink, enhancing
post-exercise muscle protein synthesis, rehydration, and post-exercise glycogen resynthesis, while attenuating muscle soreness [96]. Overall, the plethora of non-haem iron interactions pose a substantial challenge for athletes who are typically recommended to consume a variety of foods, with the exception of ascorbic acid; the most powerful promoter of non-haem iron absorption. Ascorbic acid may help overcome some of the aforementioned inhibitory effects of other confounding nutrients, with ~75 mg ascorbic acid shown to increase iron absorption up to 3-fold [97, 98]. With a host dietary factors and homeostatic regulators influencing the bioavailability of dietary iron, it is plausible there would be daily and inter-individual variance in the dietary iron requirements of athletes, making a compelling argument that a ‘one size fits all’ approach would be hard to implement.

Clearly, establishing an adequate intake of dietary iron is remarkably complex, since the amount of iron functionally absorbed from the GI tract is influenced by an abundance of factors. Given the additional avenues of iron loss associated with exercise (sweating, haematuria, GI bleeding and haemolysis [5, 6]), the athletic population is likely to have a greater iron demand compared to a sedentary population. This was recently highlighted when elite endurance athletes presented a 25-40% reduction in sFer following a 3 week intensified period of training, despite consuming 13-18 mg of dietary iron per day [14]. Such findings support the premise for more athlete-specific dietary iron recommendations to account for their increased iron demands, and potentially reduce the prevalence of ID within the athletic population [99]. In order to initiate this prospect effectively, it is recommended that all athletes liaise with an Accredited Sports Dietitian for nutritional analysis and counselling, because it may preclude the development of a negative iron balance. Furthermore, a dietitian may also recognise and address interrelated issues pertinent within the athletic population, including the identification of athletes in a state of low energy availability (LEA); a condition resultant of inadequate dietary energy intake to support normal physiological function [100]. Low energy availability is the underlying premise of Relative Energy Deficiency in Sport (RED-S), a contemporary framework encompassing a host of unfavourable health outcomes ensuing LEA, including menstrual dysfunction, poor bone health, suppressed metabolic rate, and of interest here, poor iron status [100, 101]. It is plausible that an inadequate energy intake would correspond with insufficient dietary intakes of several micronutrients,
including iron. Noteworthy, it has also been proposed that LEA may instigate metabolic disturbances to increase hepcidin and potentially reduce iron uptake [102, 103]. In any case, poor dietary practises may be a significant underlying factor for ID amongst athletes, and are therefore, addressable by trained dietetics personnel.

Increasing dietary iron intake is typically the initial, and most conservative treatment for ID. Under such circumstances, a sports dietitian will characteristically implement a dietary intervention focussed on increasing dietary iron intake to address an iron status that is sub-optimal or worse [21]. Understanding nuances in iron absorption ensuing from the effects of other dietary inhibitors/enhancers, in addition to the influence of exercise and the post-exercise elevation in hepcidin, may allow for more detailed and effective dietary interventions in the future. Currently, replenishing taxed iron stores through diet alone remains a challenge for many athletes because of the interaction between exercise, hepcidin and iron absorption, in combination with the low bioavailability of dietary iron. Consequently, athletes often consider iron supplementation in order to achieve a healthy iron balance.

**What is the role of oral iron supplementation treatments in athletes?**

Oral iron supplementation is typically the first avenue of iron replacement therapy beyond nutritional intervention, as it is cheap, non-invasive and shows positive efficacy of effect over time [16]. Previous research reports that an oral iron supplement schedule offering ~100 mg ferrous sulphate per day may increase an athletes iron stores 30-50% over a 6-8 week period [18, 27, 28, 30, 31, 104]. In accord, daily doses of 60-120 mg of elemental iron, continued over two months, are commonly recommended to ID athletes, varying based on the severity of ID and the individual tolerance from a GI perspective [25, 45, 105]. It has also been considered worthwhile to implement tolerable oral iron supplementation in cases of IDNA athletes, as primary prevention against a regression towards IDA [45]. While indiscriminate pharmacologic interventions contradict the nutritional behaviours promoted to athletic populations, it could be argued that, at specific phases of an endurance athletes training, oral iron supplements may be a more practical method, compared to a diet high in meat, when a high carbohydrate, low fat diet is being implemented [45]. Overall, oral iron supplementation is currently a pertinent treatment for ID.
amongst the athletic population, however a consensus is yet to be established regarding the optimal strategy of prescription.

**What compositions of oral iron supplement are available to athletes?**

There are many ‘off the shelf’ oral iron preparations available to athletes, varying widely in dosage, salt, chemical state (ferrous or ferric form) and galenic form (quick and prolonged release) [16]. Ferrous salts including ferrous sulphate (FeSO\(_4\)), ferrous gluconate (C\(_{12}\)H\(_{24}\)FeO\(_{14}\)) and ferrous fumarate (C\(_{4}\)H\(_{2}\)FeO\(_{4}\)) are the most extensively used iron preparation in clinical practice. The aforementioned preparations are cost-effective, have uniformly good bioavailability (between 10 and 15%), and are 3 to 4 times more bioavailable than ferric iron preparations (because ferric iron needs to be reduced into ferrous form to enter the mucosal cells) [16, 106, 107]. Oral iron supplements are available in both tablet and liquid form, each with comparable bioavailability [108]. While iron tablets are practical and most commonly used, unlike liquid iron, iron tablets are associated with rare cases of iron-induced gastric mucosal injury, an under-recognised, albeit serious condition [109-111]. Alternatively, liquid iron is thought to lack the concentration effect necessary to cause such damage [109], and may be better tolerated than iron tablets [112].

To date, the majority of studies exploring iron supplementation in athlete populations have used ferrous salts. Ferrous sulphate is considered the gold standard oral iron treatment and is thus the most commonly prescribed and studied oral iron therapy worldwide [17, 27, 30]. More recently, ferrous fumarate has also been investigated in athletes, showing positive outcomes on iron stores [113, 114], and in non-athletic populations, absorption kinetics appear similar between ferrous fumarate and ferrous sulphate [115, 116]. Of note, some oral iron supplements (e.g. FerroGrad® - C) also contain vitamin C (typically as ascorbic acid), since as previously mentioned, this vitamin is the most powerful promoter of iron absorption, and accordingly, acts to maximise iron bioavailability [97, 98]. However, while oral iron supplements composed of ferrous iron salts are currently the most common form of treatment for ID, their use is primarily limited by frequently reported GI side-effects, including symptoms such as pain, nausea, vomiting, abdominal distress, constipation and diarrhoea [17, 53, 117]. Since oral iron
supplementation requires relatively long-term commitment (4 to 12 weeks), such side-effects can lead to non-compliance, potentially rendering the treatment less effective [118]. A recent meta-analysis reported that daily supplementation with ferrous sulphate was associated with an odds ratio of 2.6 times the occurrence GI side-effects, compared to placebo or IV iron, and that adherence was reported as only 70-90% (in a cohort of pregnant women) as a consequence of the adverse effects [17]. Such iron supplement-related complications would undoubtedly interrupt exercise training consistency via the associated GI distress. Therefore, research continues to try and increase the efficacy of this form of treatment, identifying alternative strategies such as galenic formulations, and more recently, the timing and dosage of iron intake.

Beyond simple ferrous salts, various oral iron formulations have been developed to improve gut tolerability, without compromising bioavailability. Iron amino acid chelates are theoretically the most advantageous, because the chelates prevent iron from binding to dietary inhibitors within mixed meals [117]. Iron bound to a lipophilic chelate yields an inorganic structure that is able to be absorbed intact into the mucosal cells of the intestine, before being hydrolysed into its components, and is thought to protect against the negative GI side-effects [117, 119]. The ferrous bisglycinate chelate (Ferrochel®, 28 mg elemental iron) is highly stable and readily bioavailable, with recent studies demonstrating reduced GI distress in association with a 4-5 times greater absorption rate as compared with ferrous sulphate in the presence of phytates [117, 120, 121]. However, the use of ferrous bisglycinate as a treatment for ID may be limited by its relatively higher cost, though it may be advantageous to ID athletes who are inclined to supplement alongside nutritious mixed-meals containing iron inhibitors, and/or who experience GI side-effects [122].

Controlled release iron preparations, such as carbonyl iron and polysaccharide-iron complexes (IPC), have also been developed to improve the tolerability of oral iron therapy. These prolonged release formulations entail a polymeric complex that surrounds the Fe²⁺ ions to form a matrix that controls their iron availability to specific sections of the GI tract [123]. Kaltwasser et al. [123] induced ID in 18 otherwise healthy, sedentary, males to compare a prolonged-release ferrous sulphate supplement
(Tardyferon®, 80 mg elemental iron), with a quick release formulation (Eryfer®, 50 mg elemental iron). These authors reported comparable iron utilisation (measured via stable iron isotopes) and increases in haemoglobin between the two treatment groups [123]. The most recent formulation of controlled-release supplement is the IPC, which combines ferric iron and polysaccharide to create a stable structure that closely resembles endogenous carriers of iron, to minimise GI upset by delaying iron release in the intestine [124]. While IPC formulations (Maltofer®, 100 mg elemental iron) consistently indicate better tolerability than ferrous sulphate [125, 126], the therapeutic efficacy of IPC formulations has been debated, with some reports of non-responders and inferior bioavailability compared to ferrous salts [127, 128]. However, a meta-analysis conducted in adults with ID, comparing equivalent doses of IPC and ferrous sulphate, revealed both compounds attained comparable haemoglobin outcomes, with fewer adverse reactions reported in the IPC trials [126]. Interestingly, while both forms of iron are equally bioavailable for haemoglobin synthesis, it was shown that 12 weeks of ferrous sulphate supplementation was superior to ferric polymaltose at reconstituting sFer concentrations in ID, but otherwise healthy blood donors [129]. Similarly, Tuomainen et al. [130] conducted a 6 month controlled trial in 48 ID men, and found sFer increased 2.2-fold in the ferrous sulphate group (180 mg of iron daily) compared with a 1.3-fold change in the IPC group. Nevertheless, these authors reported that erythrocytic ferritin, considered a better marker of iron stores, increased equally in both treatment groups. Overall, this body of research would indicate a similar level of efficacy between IPC and ferrous sulphate, because the uptake and storage of iron in erythrocytes, and the production of haemoglobin, appear similar between supplement types [131]. However, there are no studies specifically investigating the efficacy of prolonged-release or IPC formulations in an ID athlete population, nor is there any research examining the influence prolonged-release iron may have on hepcidin activity. Therefore, at this point in time, ferrous sulphate preparations remain the established and standard oral iron treatment of addressing ID in athletes, however, in cases of poor tolerance, IPC formulations may be beneficial [1].
What is the optimal oral iron treatment protocol for athletes?

Iron absorption, and thus, the efficacy of oral iron supplementation, is inherently governed by hepcidin and its presiding regulators. Additionally, it has been established that iron absorption is suppressed for up to 24 h following the consumption of a high iron containing meal or supplement, likely to be a result of the mucosal block mechanism described above [52, 132] and the influence of homeostatic elevations in hepcidin on iron absorption [68]. As such, it is probable that a substantial proportion of the iron ingested during this 24 h period following a prior dose of iron, or during periods of elevated hepcidin, remains unabsorbed, and thus a potentially lost resource. Furthermore, the lack of absorption in the gut might underlie the negative GI side-effects associated to iron supplementation, given that the frequency of these adverse symptoms correlates with the concentration of ionised iron within the intestinal lumen [133]. Thus, in addition to the pharmaceutical formulations addressed earlier, contemporary research is currently investigating the optimal dosage and timing strategies of oral iron supplementation, with the goal of increasing fractional iron absorption, reducing gastric irritation, and ultimately improving the efficacy of oral iron therapy.

The relative effectiveness of various supplementation regimes (i.e. timing) was initially investigated in anaemic rats, where the effects of daily, alternate day and every third day supplementation, as well as the efficacy of split-daily doses, were investigated [134]. These authors reported that the rats receiving a supplement every 3rd day (consuming a total supplement dose of 12 mg iron) had a similar iron status to those receiving a supplement every day (consuming a total supplement dose of 28 mg), suggesting an equal efficacy of effect even though only ~43% of the daily supplement dose was consumed. Furthermore, no benefit of split-daily doses were found, acknowledging that this approach would likely reduce compliance in humans due to the greater attentiveness required to consistently recall multiple doses per day [134]. In humans, Moretti et al. [59] originally utilised stable iron isotopes in order to quantify the magnitude, and duration, of the acute iron-induced rise in hepcidin following increasing doses of iron; measuring the effect this had on consecutive-day iron absorption. These authors reported that hepcidin was 2.2 times greater than baseline, 24 h following a 60 mg dose of iron, and resulted in a 35-45% reduction in fractional absorption of a secondary dose at this time point. Interestingly,
absolute absorption still remained higher with larger iron doses, although a six-fold increase in dose (40-240 mg) only resulted in a three-fold increase in iron absorption (6.7-18.1 mg). Furthermore, the authors established that the total iron absorbed from a morning, evening and following morning dose of iron (to replicate a split-dose strategy) was not greater than two morning doses (replicating daily dosage), signifying that a split-daily dose strategy is likely inefficient [59].

Previously, a prolonged split-dose strategy was investigated by Stoffel et al. [135] who confirmed split-dosing is no more effective than supplementing with iron once-daily. In this investigation, both the total and fractional amount of iron absorbed following 14 d of single (120 mg) and split (2 x 60 mg) iron doses in ID women did not differ. However, twice-daily divided doses resulted in higher serum hepcidin concentration than once-daily, potentially inhibiting the absorption of additional sources of dietary iron to a greater extent [135]. Regardless, the split-dose strategy of iron supplementation was recently translated into an athletic population, with the aim of optimising haematological adaptations during a prolonged altitude exposure. Here, Hall et al. [113] established a greater haemoglobin mass (Hbmass) response in athletes following single nightly doses of oral iron (200 mg elemental iron) when compared to a split-daily dose of iron (2 x 100 mg elemental delivered morning and evening) provided over 21 days (although it should be considered that both groups improved Hbmass over the duration of the training camp). Interestingly, in this study by Hall et al. [113], the negative GI symptoms initially appeared less pronounced in the split dose group, supporting the idea that a lower total acute supplement dose could potentially improve GI tolerance. However, these side-effects were reported to subside by the third week of the intervention in single-dose group [113], and may indicate that a phased approach into daily iron supplementation may help to evade the initial negative GI consequences. Regardless, the iron absorption outcomes of this body of research reveal a conceivable ‘multiple daily dose’ redundancy, and current research has therefore shifted towards an intermittent-day iron supplementation approach.

Accordingly, there is an emerging body of research attesting to the efficacy of an intermittent-day oral iron supplementation protocols, revealing that this approach can effectively replete iron stores, increase fractional absorption, reduce gastric irritation and increase haemoglobin levels comparably to daily oral
The primary rationale for intermittent-day iron supplementation is to circumvent the marked local suppression of intestinal iron uptake by the epithelial cells 24 h following the consumption of a high iron dose [52, 132, 135]. Secondly, there are concerns that soluble oral iron may be destructive to colonic microbiota and that luminal iron may be a risk-factor for inflammatory signalling; accordingly, less frequent exposure may prevent these undesirable side-effects [137]. In a systematic review assessing the efficacy of intermittent oral iron supplementation (one, two, or three times per week on non-consecutive days) in menstruating women, it was concluded that women receiving intermittent oral iron supplementation had 4.58 g·L⁻¹ (95% CI: 2.56 < µ < 6.59) and 8.32 µg·L⁻¹ (4.97 < µ < 11.66 µg·L⁻¹) greater haemoglobin and sFer concentrations, respectively, when compared to a placebo control. However, the authors reported that, despite achieving similar haemoglobin outcomes at the end of the intervention, women receiving supplements intermittently were more likely to have a lower overall iron status at the conclusion of the intervention than those who received daily supplements [138]. Intermittent iron supplementation has also produced similar maternal and infant outcomes at birth (risk of anaemia and haemoglobin concentration) to daily supplementation, and was again associated with fewer side-effects [136]. More recently, Stoffel et al., [135] was the first to use iron isotopes to compare the iron absorption from oral iron supplements given on consecutive versus alternate days. Remarkably, these authors found ~34% greater fractional and cumulative absorption of iron in women who supplemented with 60 mg of iron on alternate days for 28 d compared with women who supplemented with 60 mg of iron daily for 14 d. While this body of research challenges the current practice of daily oral iron supplementation, it corroborates that intermittent-day iron supplementation is a promising strategy to address the GI side-effects associated with iron supplementation, however, this strategy remains to be investigated in an athletic population.

**What is the role of parenteral iron supplementation treatments in athletes?**

Beyond oral iron treatment, parenteral iron therapy, via intramuscular or IV administration, is an effective method of iron delivery because it bypasses the gut, circumventing the side-effects and absorption issues of the more orthodox approach. While both parenteral approaches effectively improve an athlete’s iron status [18, 104], IV iron administration has largely surpassed intramuscular injections
because of the soreness and site staining commonly associated with this approach, as well as the advancements in IV iron preparations increasing the safety, efficacy, and accessibility of this method [1, 20, 139]. Previous work has shown parenteral iron therapy to be significantly more effective (both in time and degree of increase) for improving sFer concentrations in athletes (sFer <40 μg·L⁻¹) than oral iron tablets [104]. While oral iron supplementation may be suitable when time afforded for substantial change is 4 - 12 weeks, IV supplementation is capable of providing a rapid source of iron to improve iron stores significantly within 7-15 days, and may facilitate physiological benefits (including \(Hb_{mass}\) and \(VO_{2\text{max}}\)) within 6 weeks [18, 104]. Intravenous iron therapy was shown to increase athletes’ iron stores (sFer) 200-400% from baseline following the administration of 300-550 mg of IV iron, delivered in 2-5 doses across a 10-42 day period [18, 140]. A further advantage of IV iron administration is that modern IV iron preparations enable large doses of iron to be delivered in a single bout [139]; such that Burden et al. [19] found comparable increases in sFer to Garvican et al. [18] following a single, rather than split, IV delivery of iron (500 mg) in an elite group of IDNA endurance runners. Both of these studies indicate that IV iron is safe for IDNA athletes and may counteract a rapid relapse into an ID state by providing an abundant magnitude of change in iron stores.

Regardless, it must be stated that while studies widely demonstrate vast improvements in athlete iron stores following IV iron supplementation, the majority of athlete studies report no significant changes in surrogate measures of endurance capacity or performance [19, 23, 40, 141, 142]. This likely relates to the research cohorts being primarily IDNA, rather than IDA, suggesting that their partial compromised iron stores do not limit erythropoiesis or aerobic capacity. Such events have led to some reservations towards IV iron treatment for IDNA athletes. Garvican et al. [18] is one of few studies to report an accompanying mean 2.7 g increase in \(Hb_{mass}\) and mean 3.3% improvement in \(VO_{2\text{max}}\) in IDNA athletes following IV iron. Furthermore, Woods et al. [140] reported improvements in perceived fatigue and mood in a group of healthy (sFer >30 μg·L⁻¹) distance runners after receiving 300 mg of IV iron over 4 weeks, despite no concurrent improvements in haemoglobin or performance. Typically however, IDNA athletes treated with IV iron present an initial increase in sFer that is sustained for only a number
of weeks, after which, iron stores begin to return to pre-treatment concentrations [143], with a lack of effect on haematological or performance indices reported [19, 23, 40, 141, 142]. Specifically, the improvements in an IDNA athlete’s sFer concentrations were shown to be transient and regress within weeks of parenteral iron treatment by Pedlar et al. [143], which may relate to the governing interaction between iron status and hepcidin. Burden and colleagues [19] reported that following a 500 mg dose of IV iron, IDNA endurance athletes exhibited an increase in sFer and an upregulation of hepcidin pre- and post-exercise, 24 h and 4 weeks post-treatment, independent of inflammation, suggesting that iron stores supersede inflammation in the regulation of hepcidin [19]. It appears the substantial increase in iron following IV administration increased iron availability, reminiscent of iron overload, stimulating a homeostatic increase in hepcidin to prevent further iron absorption [19]. This phenomenon may inadvertently result in a reduction of iron absorption during this period, explaining the rapid decline in sFer within weeks of IV iron administration, which may sabotage the prospect of improving an athlete’s iron stores in the long-term.

Regardless, parenteral iron supplementation has an important role in a sporting context when rapid improvements in iron stores are required, when GI complications render oral iron therapy impractical, or when iron status has been rendered severe and haemoglobin is compromised (i.e. IDA). In the case of a female, middle-distance, IDA athlete (sFer: 9.9 μg·L⁻¹, [Hb]: 88 g·L⁻¹), both athletic performance and oxygen transport capacity (measured via haemoglobin) were shown to be readily responsive to iron supplementation in the form of an intramuscular iron injection and continued oral iron supplementation [36, 142]. Likewise, clinical settings consistently reveal that IV iron prompts rapid sFer and haemoglobin responses and corrects anaemia more reliably than oral iron supplements [20, 144, 145]. Therefore, considering the lack of effect on haematological or performance indices in IDNA [19, 23, 40, 141, 142], parenteral iron therapy is normally reserved for cases of IDA, or when dietary and oral iron strategies have been exhausted. This principle is reinforced in light of the negative connotations associated with treating athletes with needles. Additionally, parenteral iron delivery does have related side-effects, which may manifest in symptoms ranging from a mild rash through to iron overload or anaphylaxis (in very rare cases) [146]. Therefore, parenteral iron supplementation may be less attractive
to some sporting organisations and sports physicians. Nevertheless, parenteral iron therapy highlights the significant benefits to iron uptake that emerge from bypassing the gut.

**What contemporary strategies of iron replacement are applicable to athletes?**

Given the high efficacy of parenteral iron supplementation, contemporary research is pursuing novel strategies of iron supplementation that bypass the gut, without the invasive procedure of IV administration. Transdermal iron supplementation has been identified as one such potential strategy, suggested to be capable of delivering iron across the skin, circumventing the side-effects of oral iron supplementation, making it a potential favourable prospect for ID athletes.

The primary challenge associated with the transdermal delivery of drugs is the barrier property of the skin, and therefore, to successfully develop a transdermal therapeutic system of iron delivery, it necessitates a safe, low molecular weight iron compound [147]. However, monomeric iron salts, such as those in oral iron supplements, are not suitable for parenteral administration because, should they permeate through the skin, they would liberate large amounts of free iron into the blood which can lead to oxidative stress and plasma protein denaturation [148]. Of late, there is an abundance of design patents for transdermal inventions to therapeutically deliver iron despite limited empirical evidence attesting to their efficacy. Nevertheless, commercially available transdermal iron patches do exist, with research required to investigate the efficacy of this novel approach to iron repletion.

Despite a limited number of studies investigating the efficacy of transdermal iron delivery mechanisms, ferric pyrophosphate (FPP) has been identified as an iron source suitable for transdermal administration [149]. Ferric pyrophosphate is appropriate for transdermal iron delivery because it does not liberate free iron, is able to directly transfer iron to transferrin, and is capable of triggering iron transfer between transferrin molecules and between transferrin and ferritin [149, 150]. Unfortunately however, it’s high molecular weight (745 Da) and hydrophilicity has resulted in poor passive permeation across the skin [151]. Notwithstanding, transdermal delivery of FPP was found to be enhanced by the electrically mediated technique, iontophoresis [151], and microporation via a soluble microneedle system [152].
the context of an exercise setting, the most encouraging study to date was conducted in rats by Modepalli et al. [152], who developed a soluble microneedle array to administer FPP, and confirmed the safety and feasibility of this approach using human dermal fibroblast cell lines to perform cell viability studies and ROS assays. The microneedles dissolved in the skin fluid within 3 hours and the dermal kinetics of FFP concentrations increased over 3-4 h (as the microneedles dissolved), followed by a slow decline until measurement ceased some 10 h later. This mechanism is yet to be confirmed in humans, although it clearly presents a conceivably convenient, minimally invasive and practical mode of iron supplementation that would avoid any GI side-effects and could be applicable to an athletic setting. Accordingly, much research is needed in the area of transdermal iron administration, and the methods associated; with a focus on human trials and athlete populations yet to be unearthed.

2.4 Conclusion
Hepcidin is the primary iron regulatory hormone and acts to suppress the absorption of dietary iron by duodenal enterocytes and iron recycling by macrophages [8, 9]. Copious research has corroborated a transient 2- to 4-fold increase in hepcidin levels 3 h following exercise, indicating there is a likely period of impeded post-exercise iron absorption. This interaction between exercise, hepcidin, and iron absorption is a unique, but fundamental challenge encountered by athletes, which likely influences their ability to replenish taxed iron stores and may elucidate the high rates of ID amongst this population. However, the relationship between iron absorption outcomes in conjunction with the transient elevation in hepcidin levels following exercise remain to be investigated.

Furthermore, hepcidin and its presiding regulators inherently affect the efficacy of oral iron supplementation, currently the first avenue of iron replacement therapy beyond nutritional intervention. A sound body of research corroborates that oral iron treatment characteristically increases athlete iron stores 30-50% over a 6-8 week period [18, 27, 28, 30, 31, 104]. Nevertheless, high rates of GI side-effects (e.g. pain, nausea, vomiting, abdominal distress and diarrhoea [17, 53, 117]) are problematic for sporting activities, often instigating non-compliance, generating substandard outcomes [118]. Furthermore, hepcidin elevations following a dose of iron were recently established, highlighting that
hepcidin remained significantly higher 24 h following its ingestion, resulting in a considerable reduction in fractional absorption [59]. These observations likely explain the conclusions that a split-dose strategy of iron supplementation (a morning and evening dose, daily) is no more effective than once-daily supplementation [59, 113, 135]. This principle has governed a shift in research toward intermittent-day iron supplementation, with the aim of increasing fractional iron absorption, reducing gastric irritation, and ultimately improving the efficacy of oral iron therapy. Remarkably, Stoffel et al. [135] recently found ~34% greater fractional and cumulative absorption of iron, and less GI discomfort, in women who supplemented with 60 mg of iron on alternate days for 28 d when compared with women who supplemented with 60 mg of iron daily for 14 d. Such research demonstrates that alternate-day supplementation is a promising strategy to address the GI side-effects associated with iron supplementation, and thus, research should investigate its efficacy in an athletic population.

Finally, ID is a prevailing issue amongst the athletic population that yearns for innovative solutions to address it more effectively. Following on from the well-established efficacy of parenteral iron supplementation in athletes [18, 104], transdermal iron delivery has recently been a topical potential strategy of iron supplementation that bypasses the gut, without the invasive procedure of IV iron administration. Despite an abundance of design patents for transdermal inventions to therapeutically deliver iron across the skin, to date, limited empirical evidence supports the efficacy. Nevertheless, commercially available transdermal iron patches do exist, thereby presenting a conceivably convenient treatment worthy of further exploration. Overall, existing research observations would arguably render the current practice of daily oral iron supplementation inefficient or impractical for many athletes, and warrants the forthcoming research (of the ensuing doctoral thesis) into alternative, athlete-specific, strategies of iron therapy.
2.5 References


Chapter 3

The Impact of Morning versus Afternoon Exercise on Iron Absorption in Athletes

This chapter is based on a paper published in *Medicine and Science in Sports and Exercise*:

3.1 Abstract

**Purpose:** This study examined post-exercise inflammatory, hepcidin and iron absorption responses to endurance exercise performed in the morning versus the afternoon. **Methods:** Sixteen endurance-trained runners (10 male, 6 female) with serum ferritin (sFer) < 50 μg·L⁻¹ completed a 90 min running protocol (65% vVO₂max) in the morning (AM), or the afternoon (PM), in a crossover design. An iron-fortified fluid labelled with stable iron isotopes (₅⁷Fe or ₅⁸Fe) was administered with a standardised meal 30 min following the exercise and control conditions during each trial, serving as a breakfast and dinner meal. Venous blood samples were collected pre-, immediately post-, and 3 h post- the exercise and control conditions to measure sFer, serum interleukin-6 (IL-6), and serum hepcidin-25. A final venous blood sample was collected 14 d after each trial to determine the erythrocyte iron incorporation, which was used to calculate iron absorption. Linear mixed-modelling was used to analyse the data. **Results:** Overall, exercise significantly increased the concentrations of IL-6 (4.938 pg·mL⁻¹; p=0.006), and Hepcidin-25 concentrations significantly increased 3 h following exercise by 0.380 nM (p<0.001). During the PM trial, hepcidin concentrations exhibited diurnal tendency, increasing 0.55 nM at rest (p=0.007), before further increasing 0.68 nM (p<0.001) from pre- to 3 h post-run. Fractional iron absorption was significantly greater at breakfast following the AM run, compared with both the rested condition (0.778%; p=0.020) and dinner in the AM run trial (0.672%; p=0.011). **Conclusion:** While exercise resulted in increased concentrations of IL-6 and hepcidin, iron was best absorbed in the morning following exercise, indicating there may be a transient mechanism during the acute post-exercise window to promote iron absorption opposing the homeostatic regulation by serum hepcidin elevations.

**Keywords**

Hepcidin, iron deficiency, running, nutrient timing
3.2 Introduction

Iron deficiency (ID) is the most prevalent nutritional disorder worldwide and continues to be a prevailing health issue in athletes [1]. Existing literature reports the incidence of ID to be up to 17% in male and 50% in female endurance athletes across various cohort studies [2-6]. It has been established that ID impairs an individual’s oxygen transport and energy metabolism, with severe cases (i.e. anaemia) linked to decreases in work capacity and maximal oxygen consumption ($\overline{V}O_{2\text{max}}$) [7]. The high prevalence of ID in athletes is likely due to a combination of insufficient iron intake and mechanisms of iron loss that are exacerbated by activity. These include sweating, haematuria, gastrointestinal (GI) bleeding, and haemolysis; and in female athletes, menstrual losses [8]. Moreover, the low bioavailability of dietary heme (15-35%) and non-heme (2-20%) iron [9], may substantiate the difficulty athletes experience in replenishing their daily iron losses from food. Recently, exercise-induced inflammation has also been implicated in the elevation of the primary iron regulatory hormone, hepcidin, which suppresses the absorption of dietary iron by duodenal enterocytes and iron recycling by macrophages [10, 11]. To date, numerous research papers have explored the post-exercise response of hepcidin and its impact on iron metabolism in athletes. This work has consistently shown a 2- to 4-fold increases in hepcidin levels at 3 h post-exercise, with the magnitude of this response mediated by exercise duration, and by the athletes’ pre-exercise serum ferritin (sFer) level [11-13]. Of note, both Peeling et al. [11] and Newlin et al. [12] observed a significant increase in interleukin-6 (IL-6) immediately following exercise, which preceded the peak in serum hepcidin concentrations by 3-6 h post-exercise.

With the time-course profile of hepcidin response (3-6 h post-exercise) established [11], it is likely that there is a period of impeded iron absorption in the gastrointestinal tract following exercise, which may (negatively) affect an athlete’s iron status. Since athletes are encouraged to eat immediately following exercise to enhance the rate of glycogen restoration and protein synthesis [14], a possible disparity between mealtimes (i.e. breakfast or dinner) and optimal iron absorption may exist. Consequently, exercise-induced elevations in serum hepcidin concentration, and the prospect of subsequent post-exercise reductions in iron absorption, is a likely mechanism of iron-regulation that potentially
contributes to ID in athletes; however, this hypothesis is yet to be confirmed. When considering iron absorption, Moretti and colleagues [16] recently explored the inflammatory and erythropoietic influences of exercise on plasma hepcidin concentration and iron absorption during a 3-week training period using recreationally trained runners. In this study, the net effect of exercise was to decrease hepcidin concentrations and mildly increase iron absorption over time. These authors suggested a potential mechanism whereby, during the progression of training (additional 8 km run every second day), the impact of inflammation and hepcidin elevations are offset by the erythropoietic stimuli, to chronically increase iron mobilisation and absorption for erythroid expansion, in an attempt to maintain iron balance in active individuals [16]. However, the effect of acute elevations in serum hepcidin concentration following exercise on measurements of iron absorption remains to be investigated. Therefore, the aim of this study was to examine the influence of an exercise bout on subsequent serum IL-6, hepcidin concentrations and iron absorption in endurance athletes, and to assess the impact of exercise timing (i.e. morning or afternoon exercise) on this relationship.

3.3 Materials and Methods

Participants

Sixteen endurance-trained runners (10 male and 6 female) with sub-optimal iron status [17] (Table 3.1) were recruited for this study. Participants could not be supplementing with iron within 3 weeks of participating in the study. Participants were informed of the purpose, requirements and risks associated with their involvement. Written informed consent was obtained prior to study commencement. Ethics approval for this study was obtained from the Human Research Ethics Committee of The University of Western Australia.
Table 3.1: Mean ± SD participant characteristics.

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<th></th>
<th>Age (years)</th>
<th>Mass (kg)</th>
<th>Height (cm)</th>
<th>Serum ferritin (µg·L⁻¹)</th>
<th>Haemoglobin (g·L⁻¹)</th>
<th>VO₂max (mL·(kg·min)⁻¹)</th>
<th>65% vVO₂max (km·h⁻¹)</th>
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<td>27.8 ± 6.9</td>
<td>65.4 ± 6.9</td>
<td>176.4 ± 8.3</td>
<td>27.13 ± 10.66</td>
<td>139 ± 12</td>
<td>59.2 ± 11.6</td>
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<td>Males</td>
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<td>25.1 ± 4.8</td>
<td>71.0 ± 4.2</td>
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<td>146 ± 6</td>
<td>67.0 ± 5.6</td>
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<td>Females</td>
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<td>32.2 ± 7.6</td>
<td>56.0 ± 7.6*</td>
<td>168.3 ± 7.9*</td>
<td>25.05 ± 11.86</td>
<td>126 ± 6*</td>
<td>48.7 ± 9.1*</td>
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* Indicates a significant difference between male and female participants (p < 0.05).
Experimental Overview

This study adopted a randomized, repeated measures crossover design. Participants attended one introductory, and two laboratory-based testing sessions during the experimental period. Sessions were separated by 14 days for males, and 28 days for females (to control for menstrual cycle phase). The introductory trial familiarised participants to the laboratory equipment and was concluded with a graded exercise test (GXT) to determine the individual’s $\dot{V}O_2\text{max}$. Following the introductory trial, participants undertook two separate experimental trials, inclusive of:

(i) A 90 min morning running trial, performed at 65% $\dot{V}O_2\text{max}$ velocity ($v\dot{V}O_2\text{max}$), with a stable iron isotope consumed at 30 min and 10 h post-exercise to replicate a breakfast and evening dinner meal following morning exercise (AM).

(ii) A 90 min afternoon running trial, performed at 65% $v\dot{V}O_2\text{max}$, with a stable iron isotope consumed 7.5 h pre-exercise and at 30 min post-exercise to replicate a breakfast and evening dinner meal on a day where exercise was conducted in the afternoon (PM).

Participants did not exercise for 24 h prior to each experimental trial and were provided with a standardised low iron, high carbohydrate (CHO) diet to consume during this period. Similarly, during each experimental trial, all food and water consumption was standardised. On the day of each experimental trial (Figure 3.1), participants arrived at the laboratory at 0600 h, having fasted overnight. Basic anthropometric measures were taken, and a cannula was placed inside the participant’s forearm vein before a baseline venous blood sample was drawn (B1). Participants consumed 300 mL of sports drink (340kJ, 21g CHO) immediately following the baseline blood sample. During the AM trial, participants commenced the exercise intervention at 0630 h, alternatively, when undertaking the PM trial, participants remained in a rested state at the exercise physiology laboratory. Blood lactate (BLa) was sampled at the beginning and conclusion of the 90 min running task. Additionally, heart rate (HR) measurements were continuously monitored, and documented every 30 min of the run, and a rating of perceived exertion (RPE) was recorded immediately following the run. At the immediate conclusion of the run/rest task, a second venous blood sample was taken from the participant’s forearm (B2), and 30
min later, a low iron-containing meal was consumed with 200 mL of standardised water containing 5 mg of $^{57}$Fe as ferrous sulphate (FeSO$_4$). At 3 h post-exercise/rest, participants provided a third venous blood sample (B3), and at 1530 h, a fourth venous blood sample was collected (B4), which served as a pre-exercise blood sample for participants during the PM trial. During the PM trial, participants commenced the exercise intervention (as described above) at 1600 h, and a post-exercise venous blood sample was collected immediately after finishing the run (B5). At 1800 h, participants consumed the same low iron-containing meal with 200 mL of standardised water containing 5 mg of $^{58}$Fe as FeSO$_4$. A final venous blood sample was collected at 2030 h (3 h post-exercise in the PM trial; B6), before the cannula was removed and the participant was free to leave the laboratory. Fourteen days following each administration of the iron labels, a final venous blood sample was collected to determine the erythrocyte incorporation of the absorbed stable iron isotopes [15].

**Figure 3.1:** Diagrammatic representation of experimental overview.

**Experimental Procedures**

**Graded Exercise Test (GXT):** The running GXT was conducted on a motorized treadmill (h/p/Cosmos Venus 200/100r, Germany) utilizing 3 min work and 1 min rest periods. The initial work velocity was set to $11.3 \pm 1.4$ km·h$^{-1}$ with subsequent 1 km·h$^{-1}$ increments over each work period until volitional
exhaustion. During the GXT, ventilation and expired air was analysed for concentrations of O₂ and CO₂ using a TrueOne 2400 Metabolic Measurement System (ParvoMedics, UT, United states). This system was calibrated pre-test according to the manufacturer’s specifications. The VO₂max was determined as highest 30 s VO₂ reached during the final 3 min of the GXT. Participant’s vVO₂max was determined as the weighted average of the velocity in the final 3 min of the test, and 65% of the associated vVO₂max was calculated for the experimental trials.

**Diet:** The day prior to, and throughout both trials, participants adhered to a standardised diet (Table 3.2) that was devised in collaboration with a sports dietitian using Foodworks software (version 8.0.3553, AusBrands 2015 and AusFoods 2015 databases). The standardised iron-labelled breakfast and dinner meals were identical and consisted of eggs and toasted bread (Table 2). In addition to the iron-labelled breakfast and dinner, participants were provided with supplementary low iron containing meals and snacks.
Table 3.2: Mean ± SD breakdown of standardised diet.

<table>
<thead>
<tr>
<th></th>
<th>Energy (kJ·kg⁻¹)</th>
<th>Carbohydrate (g·kg⁻¹)</th>
<th>Iron (mg)</th>
<th>Vitamin C (µg)</th>
<th>Calcium (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Male</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day before</td>
<td>223 ± 9</td>
<td>7.8 ± 0.3</td>
<td>14.4 ± 1.4</td>
<td>148 ± 0</td>
<td>2885 ± 125</td>
</tr>
<tr>
<td>Day of trial</td>
<td>192 ± 3</td>
<td>6.7 ± 0.1</td>
<td>13.6 ± 0.7</td>
<td>23 ± 0</td>
<td>877 ± 14</td>
</tr>
<tr>
<td>Iron-labelled meal</td>
<td>29 ± 2</td>
<td>0.9 ± 0.1</td>
<td>2.4</td>
<td>0</td>
<td>92</td>
</tr>
<tr>
<td><strong>Female</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day before</td>
<td>181± 25</td>
<td>5.2 ± 0.5</td>
<td>10.0 ± 0.9</td>
<td>150 ± 3</td>
<td>1996 ± 223</td>
</tr>
<tr>
<td>Day of trial</td>
<td>164 ± 16</td>
<td>4.6 ± 0.2</td>
<td>9.2 ± 0.3</td>
<td>23 ± 2</td>
<td>1027 ± 8</td>
</tr>
<tr>
<td>Iron-labelled meal</td>
<td>28 ± 5</td>
<td>0.8 ± 0.1</td>
<td>1.6</td>
<td>0</td>
<td>64</td>
</tr>
</tbody>
</table>
**Blood Lactate:** Blood lactate concentration was assessed via a capillary sample taken from the fingertip of the participant. The site of collection was cleaned with a sterilized alcohol swab, before a small incision was made into the fingertip. The first blood droplet was discarded to ensure the integrity of the sample, and the blood sample was collected into a lactate pro strip to be analysed by a Lactate Pro II Analyser (Arkray Inc., Kyoto, Japan).

**Blood Collection:** Venous blood was collected via a cannula inserted into a forearm vein by a trained phlebotomist with the athlete lying down for 5 min beforehand to standardise postural shifts in plasma volume. Blood was collected into a 5 mL EDTA and 8.5 mL SST II Gel vacutainer tubes. Subsequently, the sample in the SST II Gel vacutainer tube was allowed to clot for 30 min at room temperature before being centrifuged at 10°C and 3000 rpm for 10 min. Serum was divided into 1 mL aliquots and stored at -80°C until further analysis. Frozen serum samples were analysed for circulating concentrations of sFer, IL-6 and hepcidin-25 and whole blood samples were analysed for haemoglobin concentration and erythrocyte incorporation of the absorbed stable iron isotopes. Concentrations of sFer were determined using a sandwich immunoradiometric assay (Roche Diagnostics). Serum IL-6 samples were measured in duplicate using a commercially available ELISA (Quantikine HS, R&D Systems, Minneapolis, MN). The analytical coefficient of variation (CV%) for IL-6 was 3.3%. Serum hepcidin-25 concentration was measured by weak cation exchange enrichment of hepcidin coupled to time-of-flight mass spectrometry (WCX-TOF MS) as described previously [18, 19].

**Measurement of Iron Bioavailability:** An iron-fortified fluid labelled with stable isotopes was administered under the supervision of trained research personnel, together with a standardised meal consisting of eggs and toasted bread. Meals and fluid were provided 30 min post-exercise/rest and contained 5 mg of $^{57}$Fe or $^{58}$Fe as FeSO$_4$ in 200 mL of water. The labelled solutions were prepared by dissolving isotopically enriched elemental iron ($^{57}$Fe: 97.9%, $^{58}$Fe: 99.8%, Chemgas, Boulogne-Billancourt) in diluted sulphuric acid (0.1 mol·L$^{-1}$) and by flushing the containers with argon to keep Fe in the (II) oxidation state [21]. The stable iron isotope solution (5 mg·mL$^{-1}$) was added to the water just before consumption. Erythrocyte incorporation of the oral iron dose was determined from the amount
of the respective iron isotope in the red blood cells 14 d following administration. Blood samples were prepared and analysed using procedures previously described by Hotz et al. [22] with a high resolution, multicollector ICP-MS instrument at ETH Zürich, Switzerland. Iron absorption was calculated assuming that 80% of the absorbed iron is incorporated into erythrocytes. Total circulating iron in erythrocytes was calculated based on the measured haemoglobin concentration, the iron content of haemoglobin (3.47 mg Fe·g⁻¹ haemoglobin) and the individual blood volume, which was estimated using previously established formulas for females [23] and males [24].

**Data Analysis:** All data was initially checked for normal distribution. Iron absorption and hepcidin-25 were log-transformed before analysis to stabilise the variance and back transformed. Iron absorption relative to run condition (2 levels: AM, PM) and meal time was analysed using linear mixed-effects models with a random intercept for each participant. Covariates considered were gender, age, mass, meal time (2 levels: breakfast, dinner), hepcidin-25 measures (4 levels: B1, B3, B4, B6), sFer measures, IL-6 measures (4 levels: B1, B2, B4, B5), run velocity and HR. Linear mixed-effects models with a random intercept for each participant were also used to analyse the effect of exercise on IL-6 and hepcidin-25. Covariates considered were gender, age, mass, meal time (2 levels: breakfast, dinner), sFer measures, exercise (2 levels: yes, no), run velocity, HR and concentrations of IL-6 (4 levels: B1, B2, B4, B5) or hepcidin-25 (4 levels: B1, B3, B4, B6) (in their respective models). All covariates were initially included in the linear mixed-effects models, and were subsequently removed in a stepwise fashion based on their p-value (retained if p<0.05). Results from all linear mixed-effects models have been expressed using Beta coefficients and residual standard deviation (residual SD). Subsequent paired-samples t tests were then used to analyse any within-condition effects over time for IL-6 and hepcidin-25 and have been expressed as mean differences and 95% confidence intervals (95% CI). Paired-samples t tests were also used to explore any condition effects between AM and PM trials for sFer, dietary analysis and the physiological markers measured during the two running trials (HR, RPE and BLa) and expressed as mean ± SD. The alpha level was defined as \( \alpha \leq 0.05 \).
3.4 Results

Group characteristics

Participant demographics are shown in Table 1. Baseline sFer was 27.88 ± 11.46 μg·L⁻¹ and 26.38 ± 9.37 μg·L⁻¹ in the AM and PM run trials, respectively. There were no significant differences between trials (p=0.511). Furthermore, there were no significant differences in sFer between males and females in the AM or PM trial (p=0.847 and p=0.292, respectively). However, female participants had significantly lower baseline haemoglobin concentrations than male participants (p<0.001).

Dietary analysis

Table 3.2 presents the nutrient analysis of the standardised diet. There were no significant differences (all p>0.05) in relative energy intake, carbohydrate, iron, vitamin C or calcium intake between AM and PM run trials.

Run

The average HR during the AM and PM run trials was 149 ± 13 b·min⁻¹ and 149 ± 11 b·min⁻¹, respectively. The corresponding RPE for both runs was 13 ± 1. Concentrations of BLα pre- and post-run were 2.1 ± 0.7 mmol·L⁻¹ and 1.6 ± 0.8 mmol·L⁻¹ in the AM trial, and 2.1 ± 0.9 mmol·L⁻¹ and 1.8 ± 1 mmol·L⁻¹ in the PM trial. There was insufficient evidence to reject the null hypothesis for a difference in HR, RPE or BLα between the AM and PM run trials (all p>0.05).

Serum interleukin-6 concentrations

Serum IL-6 concentrations are illustrated in Figure 3.2. Exercise significantly increased the concentration of IL-6 (p=0.006). The IL-6 concentration was 4.938 pg·mL⁻¹ (residual SD = 6.82 pg·mL⁻¹) higher following exercise. No other covariates were found to have a significant effect. The AM trial showed IL-6 concentrations increased 4.482 pg·mL⁻¹ from B1 to B2 (p<0.001, 95% CI: 3.144 < μ < 5.820 pg·mL⁻¹), remaining elevated at B4 (6.981 pg·mL⁻¹ increase from B1, p<0.001, 95% CI: 3.701 < μ < 10.262 pg·mL⁻¹) and at B5 (9.274 pg·mL⁻¹ increase from B1, p=0.003, 95% CI: 3.702 < μ < 14.846
The PM trial showed IL-6 concentrations were increased $4.699 \text{ pg} \cdot \text{mL}^{-1}$ from B1 to B4 ($p < 0.001$, 95% CI: $2.389 < \mu < 7.008 \text{ pg} \cdot \text{mL}^{-1}$), and rose $7.752 \text{ pg} \cdot \text{mL}^{-1}$ further from pre- to post-run (B4 to B5; $p = 0.004$, 95% CI: $2.895 < \mu < 12.610 \text{ pg} \cdot \text{mL}^{-1}$).

**Figure 3.2:** Interleukin-6 concentrations recorded across the day, corresponding to a pre- (0600 h and 1530 h) and immediately post-exercise (0800 h and 1730 h) measure (and the analogous rested measures) for both the morning (AM) and afternoon (PM) running trials. Note: Time points on the x-axis correspond with B1 (0600 h), B2 (0800 h), B4 (1530 h) and B5 (1730 h). * indicates a significant difference between pre- and post-exercise in each trial ($p < 0.05$). † indicates a significant increase from baseline ($p < 0.05$).
Serum hepcidin-25 concentrations

Serum hepcidin-25 concentrations are illustrated in Figure 3.3. Exercise had a significant effect on the change in hepcidin-25 from B1 to B3 and B4 to B6. Overall, the change in hepcidin-25 concentration was 0.80 nM (residual SD = 0.65 nM) higher post-exercise (p<0.001). No other covariates were found to have a significant effect. The AM trial showed hepcidin-25 increased 0.86 nM from B1 to B3 (p<0.001, 95% CI: 0.62 < μ < 1.18 nM), remaining elevated (0.80 nM increase from B1) at B4 (p<0.001, 95% CI: 0.55 < μ < 1.13 nM), before returning to 1.05 ± 0.82 nM at B6, which was not different to baseline concentrations (p=0.091, 95% CI: 0.32 < μ < 0.59 nM). The PM trial showed hepcidin-25 increased 0.55 nM from B1 to B3 (p=0.007, 95% CI: 0.39 < μ < 0.78 nM) and continued to increase a further 0.54 nM from B3 to B4 (p=0.005, 95% CI: 0.40 < μ < 0.73 nM) and 0.68 nM from B4 to B6 (p<0.001, 95% CI: 0.52 < μ < 0.89 nM).
**Figure 3.3:** Hepcidin-25 concentrations recorded across the day, corresponding to a pre- (0600 h and 1530 h) and 3 h post-exercise (1100 h and 2030 h) measure (and the analogous rested measures) for both the morning (AM) and afternoon (PM) running trials. Note: Time points on the x-axis correspond with B1 (0600 h), B3 (1100 h), B4 (1530 h) and B6 (2030 h). * indicates a significant difference between pre- and post-exercise in each trial (p < 0.05). † indicates a significant increase from baseline (p < 0.05).

**Fractional iron absorption (erythrocyte iron incorporation)**

Fractional iron absorption is illustrated in Figure 3.4. The linear mixed-effects model found that significantly more iron (0.67%, residual SD = 1.47%) was absorbed from the breakfast meal compared to the dinner meal (p=0.011) when participants ran in the AM. The covariates age (-0.93%; p=0.001), mass (-0.97%; p=0.032) and sFer (-0.96%; p=0.002) were also found to have a significant effect on iron absorption. The linear mixed-effects model found there was no significant difference in the amount of
iron absorbed from the breakfast meal compared to the dinner meal (p>0.05) when participants ran in the PM. It was found that pre-PM run hepcidin-25 (B4) was the only significant covariate to explain iron absorption (-0.33%, residual SD = 1.39%; p=0.01) when participants ran in the PM. The linear mixed-effects model also found that iron absorption at breakfast was significantly higher (0.78%, residual SD = 1.17%; p=0.02) when they ran in the AM compared to when they ran in the PM. The covariate run velocity (1.15%; p=0.032), was also found to have a significant effect. The linear mixed-effects model estimates that iron absorption at dinner was higher (0.46%, residual SD = 1.12%; p<0.001) when they ran in the AM compared to when they ran in the PM. Within this model, the covariates age (0.94%; 0.005), B1 hepcidin (0.41%; p=0.001), B3 hepcidin (0.58%; p=0.009), B6 hepcidin (2.98%; p<0.001) and sFer (0.98%; p=0.009) were also found to have a significant effect on iron absorption at dinner.
3.5 Discussion

Our results reveal that the interaction of exercise and time-of-day influences the amount of iron absorbed by athletes with sub-optimal (sFer ≤ 50 μg·L⁻¹) [17] iron stores. We have shown that despite a post-exercise increase in hepcidin concentrations, more iron was absorbed at breakfast following morning exercise, as compared with breakfast in a rested state, or when compared to the absorption from an evening meal. This outcome suggests that, while the regulatory mechanism of hepcidin is at
the forefront of iron absorption, there are other exercise-induced physiological changes that influence iron uptake.

Given exercise has consistently been shown to induce an increase in hepcidin concentrations [11-13], the impact on subsequent iron absorption is of interest. In agreement with prior research, which consistently report hepcidin concentrations to increase 2- to 4-fold at 3 h after exercise [11, 12], the 90 min running protocol employed in the current study elicited a ~3-fold increase in hepcidin at 3 h following the AM run, and a ~2-fold increase at 3 h following the PM run. Similarly, the magnitude of the hepcidin increase (~4 nM) was consistent with those previously reported in athletes with sFe in the range of 30-50 μg.L⁻¹ [17]. The present data also illustrates a 2- to 4-fold increase in serum IL-6 concentration following the 90 min running protocol, confirming that an inflammatory response was induced by this exercise protocol, and suggests that IL-6 was the likely mechanism responsible for the increase in hepcidin concentration 3 h thereafter. Of note, this elevation in serum IL-6 concentration persisted across the day following the AM run, an outcome likely linked to the circadian rhythm of IL-6, which has previously been shown to increase 3- to 5-fold between the hours of 0800 h and 1900 h, and is exacerbated by sleep deprivation and fatigue [25]; both factors to consider here.

Similar to IL-6, serum hepcidin concentrations (in a non-exercise setting) also exhibit a diurnal variation in healthy adults [26, 27]. The kinetics of serum hepcidin’s circadian rhythm were shown by Kemna et al. [27], who reported a 2- to 6-fold rise in hepcidin from 0600 h to 1500 h, which subsided into the evening. While there was no completely rested condition in this study, an analogous diurnal increase in hepcidin was observed in the PM run trial in the present study, with resting concentrations of hepcidin increasing ~2.7-times from 0600 h to 1530 h in the absence of exercise. Our results suggest that the greater hepcidin response seen following the PM (compared with the AM) run trial is an aggregate effect of elevated hepcidin concentrations in response to the combined effects of the exercise-induced inflammation and the diurnal nature of hepcidin. Here, hepcidin was found to be elevated approximately 2-fold greater following exercise in the afternoon (as compared to in the morning), indicating a potentially larger inhibitory effect on iron absorption, thereby helping to explain the (comparatively)
reduced iron absorption post-run in the afternoon. Of note, however, despite a significantly greater serum hepcidin concentration at 1100 h, the iron absorption post-exercise in the morning was greater than at rest, whilst the amount of iron absorbed post-exercise in the afternoon not substantially different to the quantity of iron absorbed from breakfast consumed at rest. Such findings support the premise that iron absorption during the post-exercise period may be a net effect of one or more exercise-induced mechanisms that promote iron absorption, opposing the inhibitory effects of hepcidin activity, to influence the overall iron absorption. In the context of the present investigation, the net effect of the interaction between exercise-induced mechanisms and post-exercise hepcidin concentration is an overall positive influence on iron absorption in the morning, whereas in the afternoon, greater increases in hepcidin concentrations appear to negate these potential exercise driven mechanisms. Recently, only one study has explored iron absorption in an exercise setting [16]. These authors found that over a 3-week period of increased exercise intensity and erythropoiesis, hepcidin concentrations decreased, and iron absorption increased by 24% compared to a control period of less intense exercise activity. Such outcomes corroborate that there is an exercise-induced mechanism that regulates iron balance, which is likely yet to be completely understood.

It is well-known that exercise prompts a number of transient physiological changes to the cardiovascular, metabolic and hormonal function of an individual; one or more of which may influence iron absorption. Interestingly, the statistical modelling of the present investigation identified age, mass and run velocity (but not sex) as contributing covariates to iron absorption. Intuitively, however, each of these variables are likely associated to sex, and as such, more research is required to specifically establish the potential sex-differences in iron absorption after exercise. In the context of exercise, haemolysis is one avenue of exercise-induced iron-loss [28] that may prompt changes in iron metabolism via an upregulation of intestinal iron transport proteins, as shown in mice induced with haemolytic anaemia [29]; however, this concept is yet to be confirmed in humans. Alternatively, exercise has been shown to create a transient increase in the permeability of the small intestine (‘leaky gut’) as a result of splanchnic hypoperfusion and repetitive mechanical movement during exercise; such outcomes are thought to increase the transfer of iron across the intestinal border [30, 31]. This may also
be reflected in the outcomes of Nachtigall et al. [15] who reported both a 3-fold increase GI blood loss in periods of intensive training, compared to periods of rest, and an upregulation of iron absorption in ID (serum ferritin [sFer] <35 µg-L⁻¹), male distance runners. However, no measures of inflammation were made during this study, and therefore, it is also possible that exercise-induced inflammation is at the origin of these changes in iron absorption.

In consideration of this, is might also be possible that there may exist an exercise-induced upregulation of iron transport proteins in the intestine, similar to those found in mice and human small-bowel cultures, in response to inflammatory cytokines, particularly tumour necrosis factor alpha (TNFα) [32, 33]. Of interest, Sharma et al. [33] demonstrated that TNFα transiently increases the expression and localisation of enterocyte iron transport proteins, divalent metal transporter 1 (DMT1) and ferroportin in a human intestinal cell line and ex vivo small bowel cultures. One hour following TNFα treatment, ferroportin and DMT1-mRNA expression were increased, resulting in significant increases to iron import and export [33]. As TNFα is a pro-inflammatory cytokine related to IL-6, cytokine mediated changes to iron transport proteins may stimulate iron absorption immediately post-exercise in the morning. However, this effect appears to be an acute response to inflammation, and in fact, prolonged treatment with TNFα appeared to block iron transport and increase iron storage [33]. As a result, it is possible that the acute benefits to iron absorption may exist more after morning exercise, and that the prolonged diurnal increase in IL-6 concentration prior to exercise in the PM trial may explain the lack of similar effect in the afternoon.

While the specific mechanisms stimulating iron absorption following exercise are currently unknown, similar physiological phenomena have already founded contemporary nutritional strategies. Athletes will consume combinations of nutrients during and around exercise to optimise performance and recovery, with a specific aim to maximise muscular adaptation and to facilitate the repair of damaged tissue [34]. For example, the post-exercise period, often termed the ‘anabolic window of opportunity’ [34], is a limited time (after exercise) of super-compensated rebuilding of damaged tissue and
restoration of energy reserves [34]. Within this paradigm, the highest rates of glycogen resynthesis appear to occur when large amounts of carbohydrates (>1 g·(kg·h)) are consumed within 2 h of exercise cessation, due to the activation of glycogen synthase and increased permeability of the muscle cell membrane to glucose [14, 35]. In parallel, based on the present findings, athletes with sub-optimal iron status might be advised to consume/supplement with iron during this post-exercise window in the morning to achieve optimal iron absorption outcomes. However, further research should aim to establish the best time to ingest iron during and around morning exercise, since pre-exercise iron ingestion and subsequent rates of absorption remain to be explored. Furthermore, the impact of extending the time post-exercise to feeding should also be quantified, since there may be a critical point at which the post-exercise elevation in hepcidin concentration begins to impact on the rate of iron absorption if the food is consumed at a time closer to the peak in hormone response.

In line with this thinking, the rate of gastric emptying and the exact time the test iron arrived at the site of absorption (relative to both exercise-induced changes in iron transport and hepcidin activity) should be considered. Previously, Siegel and colleagues [36] measured the gastric emptying rate of a comparable test meal consisting of eggs and bread, in both the solid and liquid state, recording a half-emptying time of 77.6 min for the solid meal. In the context of our investigation, the post-exercise meals would have reached the absorption site in just under 2 h post-exercise, and over an hour before the established [11] peak post-exercise hepcidin concentration (i.e. B3 and B6). Additionally, the iron isotope we provided was consumed immediately prior to the solid test meal in the liquid state, formerly shown to bypass an initial lag-phase in the stomach accompanying solid meals [36]. Moreover, rates of gastric emptying have been shown to be equivalent, or even enhanced, during moderate intensity exercise up to 70% \( \dot{V}O_2\text{max} \), such as the 90 min protocol used here [37, 38]. Consequently, the test-iron dose provided in the current study may have reached the site of absorption at a time foregoing hepcidin’s peak obstructive influence. Lastly, gastric emptying half-time of an evening meal (2000 h) has previously been shown to be 54% longer compared with morning emptying half-times (0800 h) [39]. Therefore, it is possible that with faster gastric emptying and lower overall hepcidin concentrations in
the morning, the aforementioned mechanisms, such as the post-exercise anabolic window, may be more influential, and thus summate to overall greater iron absorption after morning exercise.

3.6 Conclusion
In summary, these findings reveal that, despite a post-exercise increase in hepcidin concentration, more iron is absorbed at breakfast following morning exercise, as compared with breakfast in a rested state, or when compared to iron absorption from an evening meal. While the physiological mechanisms at play promoting iron absorption post-exercise remains elusive, the current investigation demonstrates that overall iron absorption is impacted by inflammation and a cumulative effect of hepcidin, in addition to one or more transient post-exercise physiological changes. Such transient mechanisms may likely include increased gut permeability or upregulation of intestinal iron transport proteins post-exercise; however, further research is required to elucidate these prospects. The practical outcomes of this study would be to advise active individuals to consume/supplement with iron shortly after morning exercise for optimal iron absorption. Moving forward, future research should aim to find the optimal time-frame of morning iron consumption to maximize iron uptake.
3.7 References


Chapter 4

The Effectiveness of Daily and Alternate Day Oral Iron Supplementation in Athletes with Sub-Optimal Iron Status

This chapter is based on a paper published in the *International Journal of Sports Nutrition and Exercise Metabolism*:

4.1 Abstract

We compared the effectiveness of daily (DAY) versus alternate day (ALT) oral iron supplementation in athletes with sub-optimal iron. Endurance-trained runners (9 male and 22 female), with serum ferritin (sFer) concentrations <50 μg·L⁻¹, supplemented with oral iron either DAY or ALT for 8 weeks. Serum ferritin was measured at baseline, and at fortnightly intervals. Haemoglobin mass (Hbmass) was measured pre- and post-intervention in a participant subset (n=10). Linear mixed effects models were used to assess the effectiveness of the two strategies on sFer and Hbmass. There were no sFer treatment (p=0.928) or interaction (p=0.877) effects; however, sFer did increase (19.7 μg·L⁻¹; p<0.001) over the 8-week intervention in both groups. Additionally, sFer was 21.2 μg·L⁻¹ higher (p<0.001) in males than females. No Hbmass treatment (p=0.146) or interaction (p=0.249) effects existed, however, a significant effect for sex indicated that Hbmass was 140.85 g higher (p=0.004) in males compared to females. Training load (p=0.001) and dietary iron intake (p=0.015) also affected Hbmass. Finally, there were six complaints of severe gastrointestinal (GI) side-effects in DAY, but only one in ALT. In summary, both supplement strategies increased sFer in athletes with sub-optimal iron status, however, the ALT approach was associated with lower incidence of GI upset.

Keywords

Supplementation strategy, oral iron therapy, nutrition
4.2 Introduction

Replenishing iron stores through dietary intake is challenging due to the low bioavailability of this mineral (2-35% [1]). This becomes more problematic for athletes, since exercise is a known stimulus for increasing the iron regulatory hormone, hepcidin, in the 3-6 h post-exercise period [2]. Hepcidin elevations are known to suppress the absorption and recycling of dietary iron by duodenal enterocytes and macrophages, respectively [3, 4]. Additionally, athletes must also contend with alternate avenues of exercise-induced iron loss, such as sweating, haematuria, gastrointestinal (GI) bleeding and haemolysis [5]. Therefore, athletes commonly require iron supplementation in order to maintain healthy iron stores; but although common, the strategies to optimise iron uptake from supplementation are varied, and limited evidence exists to support best practice protocols in this population.

The interaction of exercise, hepcidin and iron absorption could no doubt implicate the effectiveness of oral iron therapy, which is typically the first strategy for iron replacement beyond nutritional intervention [6]. Daily oral iron supplementation (~100 mg of elemental iron) is known to increase serum ferritin (sFer) by 40-80% over an 8-12 week period [7, 8]. However, oral iron treatment requires prolonged adherence, and is often limited by negative gastro-intestinal (GI) side-effects, which can lead to non-compliance, rendering the treatment ineffective [9].

In order to combat these negative GI effects, a split-dose supplement strategy was recently investigated in iron replete athletes, with the aim of optimising haematological adaptations during prolonged altitude exposure; when iron requirements may be increased [10]. Hall et al.’s [10] research established a greater haemoglobin mass (Hbmass) response in athletes following single nightly doses of oral iron (200 mg elemental iron) as compared to that of a split-daily dose (2 x 100 mg elemental iron delivered morning and evening). However, the negative GI symptoms appeared less pronounced in the split dose group, supporting the idea that a lower total acute supplement dose could potentially improve GI tolerance.

In support of the potential for reducing the acute iron load, Stoffel et al., [11] reported ~34% greater fractional and cumulative absorption of iron in iron deficient women who supplemented with 60 mg of
iron on alternate days for 28 d compared with women who supplemented with 60 mg of iron daily for 14 d. Alternate day iron supplementation is a promising strategy that may be able to circumvent the marked local suppression of intestinal iron uptake 24 h following a high dose of iron [11-13]. However, this strategy is yet to be investigated in an athletic population, where exercise may influence the hormonal regulation of hepcidin. Therefore, the aim of this study was to compare the effectiveness of daily and alternate day iron supplementation in endurance runners.

4.3 Materials and Methods

Participants

Thirty one endurance-trained runners (9 male and 22 female) with sub-optimal iron status (sFer levels <50 μg·L⁻¹; [14]) were recruited. Of note, 6 males and 9 females were recruited from the daily oral iron intervention from our companion paper (Part 1; Chapter 5). Inclusion criteria also required participants not to supplement with iron within 3 weeks of the study. Written informed consent was obtained prior to participant commencement. Ethics approval was obtained from the host institution Human Research Ethics Committee.

Experimental Overview

This study was an 8-week intervention (Figure 4.1), employing a parallel-group study design. Participants were required to have a rested pre-investigation blood test to confirm their baseline sFer concentration. Following pre-investigation blood measurements, participants were assigned to one of two groups supplemented with an oral iron supplement; (1) daily (DAY) for 8 weeks; or (2) on alternate days (ALT) for 8 weeks.

Figure 4.1: Diagrammatic representation of experimental overview. Note: Haemoglobin mass was only measured in 10 of the 31 total participants.
Participant groups were matched by sFer concentration. As per our companion paper (Part 1; Chapter 5), the effectiveness of each iron supplementation intervention was assessed via measurements of sFer concentration at the commencement, and at fortnightly intervals, throughout the 8-week training period (5 blood samples overall). Participants acted as their own control via comparisons in sFer response to baseline. Venous blood samples were collected and analysed by a commercial pathology laboratory (Clinipath Pathology, Western Australia). Haemoglobin mass was also measured prior to, and after the 8-week supplementation period in a subset of participants (n=10) to further our understanding of the effectiveness of each iron supplementation regimen.

During the supplementation period, participants were required to consume their iron supplement upon waking to optimise iron absorption [15], documenting the time-of-day of consumption in a daily journal. Participants were asked not to consume dairy-based food, tea or coffee within 60 min of taking the iron supplement and were encouraged to report any supplementation side-effects they experienced in an open-ended comments box on each day of their journal. Participants also completed a 4-day food diary in the initial, mid and final fortnight of the intervention period. No additional supplements were permitted throughout the intervention.

**Experimental Procedures**

**Iron supplementation:** During the intervention period, participants supplemented with one Ferro Grad C tablet (Mylan Health Pty Ltd, Millers Point, NSW, Australia) upon waking, either daily (DAY) or on alternate days (ALT). Each tablet contained 325 mg of ferrous sulphate and 500 mg of ascorbic acid, equating to 105 mg of elemental iron.

**Blood collection:** Venous blood was collected and analysed for sFer by a commercial pathology laboratory (Clinipath Pathology, Western Australia) at baseline and fortnightly intervals during the intervention. Participants were instructed to visit the phlebotomy practice for a morning blood sample
in a rested, non-fasting state (i.e., no morning exercise prior to blood collection). Participants still consumed their oral iron supplement on the morning blood was collected.

**Haemoglobin mass:** Haemoglobin mass was assessed using the optimised 2 min CO rebreathing technique [16]. Determination of carboxyhaemoglobin (%HbCO) was measured at baseline, plus 7 min after rebreathing, from capillary fingertip blood samples tested with an ABL80 blood gas analyser (Radiometer, Copenhagen, Denmark). Haemoglobin mass was calculated from the mean change in %HbCO before and after CO rebreathing. All Hb\textsubscript{mass} measurements were conducted by the same technician.

**Training load, dietary and menstrual monitoring:** Participants were required to document their daily exercise, necessitating duration (min), distance (km), and a rating of perceived exertion [17] with anchors 6 (no exertion) and 20 (maximal exertion). This data was used as a measure of training load, calculated as daily training impulse (TRIMP) [18]. The training journal also included a section for female athletes to record their menstruation. All participants also completed a handwritten 4-day food diary in the initial, mid and final fortnight of the intervention period. Participants attended an information session with a sports dietitian, prior to the commencement of the study, to familiarise themselves with portion measurements. This information was analysed using the nutritional analysis software, Foodworks (version 9.0.3871, AusBrands 2017 and AusFoods 2017 databases).

**Statistical analysis:** All diet and training data was initially analysed using a two-way, repeated-measures ANOVA to establish any differences between the DAY and ALT groups. Linear mixed effects models were subsequently used to assess the effectiveness of DAY and ALT iron supplementation on sFer and Hb\textsubscript{mass}. Both sFer and Hb\textsubscript{mass} were independently analysed relative to time, treatment (DAY or ALT), and sex, using linear mixed-effects models with a random intercept for each participant. Covariates included; training load, energy intake, and (dietary) iron consumption. In the case of Hb\textsubscript{mass}, sFer was included as a covariate in the model to account for its requirement for haemoglobin synthesis. Initial models included all possible interactions, but non-significant interactions, except time*group,
were removed for ease of interpretation. Outcomes of the models have been expressed as mean and 95% confidence intervals (95% CI). The alpha level was defined as $$\alpha \leq 0.05$$.

### 4.4 Results

**Group demographics:**

Group demographics are shown in Table 4.1. The DAY group consisted of 15 participants (6 male and 9 female) and consumed 55 ± 3 iron supplements (98.7% compliance: 5824 ± 348 mg of elemental iron) throughout the 8-week intervention. The ALT group consisted of 16 participants (3 male and 13 female) and consumed 28 ± 1 iron supplements (99.1% compliance: 2914 ± 105 mg of elemental iron) throughout the 8-week intervention. Total consumed iron from supplements was significantly different between groups ($p<0.001$). There were no significant differences in age, body mass, baseline sFer or baseline Hbmass between groups (all $p>0.05$). Furthermore, two female athletes did not report menstruating during the 8-week intervention, though one of these instances was a secondary effect of contraception.

**Table 4.1:** Baseline characteristics for the daily and alternate day iron supplement treatment groups.

Data presented as mean ± standard deviation.

<table>
<thead>
<tr>
<th></th>
<th>Age (years)</th>
<th>Body mass (kg)</th>
<th>Serum ferritin (µg·L⁻¹)</th>
<th>Haemoglobin mass (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DAY</strong> Female (n=9)</td>
<td>27 ± 6</td>
<td>61.7 ± 7.4</td>
<td>23 ± 12</td>
<td>633.9 ± 30.7 (n=3)</td>
</tr>
<tr>
<td>Male (n=6)</td>
<td>24 ± 5</td>
<td>69.3 ± 2.5</td>
<td>41 ± 6</td>
<td>831.5 ± 35.5 (n=2)</td>
</tr>
<tr>
<td>Overall</td>
<td>26 ± 6</td>
<td>64.8 ± 6.9</td>
<td>31 ± 13</td>
<td>712.9 ± 111.8 (n=5)</td>
</tr>
<tr>
<td><strong>ALT</strong> Female (n=13)</td>
<td>27 ± 6</td>
<td>59.5 ± 6.4</td>
<td>30 ± 11</td>
<td>574.0 ± 109.6 (n=5)</td>
</tr>
<tr>
<td>Male (n=3)</td>
<td>24 ± 4</td>
<td>68.6 ± 8.5</td>
<td>42 ± 8</td>
<td>-</td>
</tr>
<tr>
<td>Overall</td>
<td>26 ± 5</td>
<td>61.2 ± 6.3</td>
<td>32 ± 11</td>
<td>574.0 ± 109.6 (n=5)</td>
</tr>
</tbody>
</table>
Training and dietary analysis:

Table 4.2 presents the training load and nutrient intake of the DAY and ALT treatment groups during the 8-week intervention. There was no group effect, time effect or time*group interaction for fortnightly training load, energy, protein, fat, carbohydrate, or dietary iron intake (all p>0.05).

Table 4.2: Average fortnightly training load and average dietary intake for the daily and alternate day iron supplement treatment groups. Data presented as mean ± standard deviation.

<table>
<thead>
<tr>
<th></th>
<th>DAY</th>
<th>ALT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fortnightly training load (AU)</td>
<td>8537 ± 4292</td>
<td>7756 ± 3921</td>
</tr>
<tr>
<td>Energy intake (kJ/day)</td>
<td>10765 ± 3254</td>
<td>9175 ± 2452</td>
</tr>
<tr>
<td>Carbohydrate intake (g/day)</td>
<td>279 ± 83</td>
<td>248 ± 78</td>
</tr>
<tr>
<td>Protein intake (g/day)</td>
<td>107 ± 28</td>
<td>88 ± 23</td>
</tr>
<tr>
<td>Fat intake (g/day)</td>
<td>104 ± 41</td>
<td>86 ± 27</td>
</tr>
<tr>
<td>Alcohol intake (g/day)</td>
<td>3.59 ± 7.69</td>
<td>2.68 ± 4.24</td>
</tr>
<tr>
<td>Iron intake (mg/day)</td>
<td>15 ± 9</td>
<td>13 ± 4</td>
</tr>
</tbody>
</table>

Gastrointestinal outcomes:

One participant from DAY withdrew from the study due to debilitating GI side-effects. There were six further complaints of severe GI side-effects in DAY, encompassing nausea (n=1), stomach cramps (n=2) and constipation (n=3). For ALT, one report of ongoing constipation was recorded.

Serum ferritin:

Fortnightly sFer concentrations are summarised in Figure 4.2 alongside a more comprehensive depiction of individuals' pre- and post-intervention outcomes in Figure 4.3. While there was no group*time interaction (p=0.877) for sFer, significant time (p<0.001) and sex effects (p<0.001) were recorded. Overall, the model revealed that sFer significantly increased 13.5 µg·L⁻¹ in the first fortnight
(p<0.001; 95% CI: 9.9 < µ < 17.1 µg·L⁻¹), and increased further 6.5 µg·L⁻¹ in the third fortnight (p=0.023; 95% CI: 1.0 < µ < 12.0 µg·L⁻¹). Serum ferritin was also 21.2 µg·L⁻¹ (p<0.001; 95% CI: 12.5 < µ < 30.0 µg·L⁻¹) higher overall in males compared to females. No other covariates appeared to affect sFer.

**Figure 4.2**: Fortnightly serum ferritin concentrations for the daily and alternate day iron supplement treatment groups. Data presented as mean ± standard deviation. * indicates a significant increase from the preceding fortnight (p<0.05).
Figure 4.3: Pre- and post-intervention serum ferritin concentrations for males and females in the daily and alternate day iron supplement treatment groups. Group data presented as mean ± standard deviation. * indicates a significant increase from baseline (p<0.05).

Haemoglobin mass:
There was no time effect (p=0.500) or time*group interaction (p=0.249) for Hb\text{mass}. Baseline and post-intervention Hb\text{mass} measures were 712.9 ± 111.8 g and 748.5 ± 107.9 g in the DAY group, and 574.0 ± 109.6 g and 565.9 ± 68.6 g in the ALT group. There was a significant sex effect wherein Hb\text{mass} was 140.85 g higher overall (p=0.004; 95% CI: 69.13 < µ < 212.56 g) in males, compared to females. Of note, training load (p=0.001) and dietary iron intake (p=0.015) were significantly associated with Hb\text{mass}.

4.5 Discussion
Recent research indicates that adhering to an alternate day iron supplementation protocol, rather than daily, is a more effective strategy [11], yet this prospect remained to be translated into an athletic population. With the unfavourable GI side-effects associated with daily oral iron supplements, combined with the greater iron demands of athletes, we wanted to determine if an ALT iron supplement schedule would improve endurance athletes’ sFer, an important determinant of iron status. Our findings
suggest that the sFer response of endurance athletes to an ALT oral iron supplementation protocol is comparable to DAY iron supplementation, despite 50% lower total dosage of elemental iron over 8 weeks (2914 mg vs. 5824 mg, respectively). Additionally, the 60% increase in sFer observed in both groups is a characteristic response of ID athlete cohorts, who typically present 40-80% increases in sFer in response to ~100 mg of supplemented iron per day for 8-12 weeks [7, 8]. These outcomes corroborate the effectiveness of alternate day iron supplementation, establishing its applicability to an athletic population with sub-optimal iron status (defined as sFer <50 μg·L⁻¹ [14].

Traditionally, practitioners advise daily oral iron supplementation to treat ID. However, ingestion of a large quantity of iron provokes an acute reduction in the amount of iron absorbed from subsequent daily doses (Moretti et al. 2015). This phenomenon is the primary rationale for intermittent iron supplementation. Moretti and colleagues [19] characterised the acute increases in hepcidin following iron ingestion and investigated the influence it had on consecutive day iron absorption. These authors found that 24 h following a 60 mg dose of iron, hepcidin remained elevated, and quantified a 35-45% reduction in fractional iron absorption from a secondary 60 mg dose. Fractional iron absorption also declined with increasing dosage. Such findings may explain our results, especially since participants were supplemented with >1.5 times the quantity of iron provided by Moretti and colleagues [19]. Although not measured here, the acute rise in hepcidin following 105 mg of iron may have been of greater magnitude and/or persisted longer than 24 h, and consequently could have reduced the bioavailability of consecutive daily doses in the DAY group, to produce a similar overall outcome to the ALT group. Our outcomes also suggest that the short-term effects described by Moretti et al. [19] persist during a prolonged period of iron supplementation typically prescribed by practitioners.

The acute reduction in fractional iron absorption 24 h following a dose of iron [19] warranted subsequent research into chronic outcomes of an ALT iron supplement schedule. Stoffel et al. [11] recently compared DAY and ALT iron supplement schedules in non-active ID women (n=40, median age 22 y). Results indicated that fractional and total iron absorbed was greater in women who supplemented with 60 mg of iron on alternate days for 28 d compared with 60 mg of iron daily for 14
(the same total iron dose). Our research aimed to translate a comparable protocol into an athletic setting; although, we elected to standardise the intervention duration, thereby halving the total iron dose consumed in the ALT group to better reflect the practical nature of how such supplement protocols would be implemented in athletes. While direct measures of iron absorption were not taken here, our sFer data suggests that, akin to Stoffel et al.’s [11] findings, fractional iron absorption was greater in the ALT strategy, but that overall iron absorption was not substantially different to the DAY regime. The inconsistency between the greater total iron absorbed in Stoffel et al.’s (2017) ALT group and the comparable time points, namely the 14 d and 28 d sFer measurements in the DAY (46 ± 11 µg·L⁻¹) and ALT (44 ± 19 µg·L⁻¹) group, respectively, may be because sFer is an indirect measure of iron absorption.

Beyond sFer, this study also sought to explore the effect of an ALT iron protocol on Hb_{mass}, often considered the functional beneficiary of iron to an athlete. It was suggested that Hb_{mass} is a desirable supplementary marker of determining an athlete’s iron status since it is not affected by shifts in plasma volume incurred during athletic training [20]. We acknowledge that Hb_{mass} was conducted in a subset of participants in an effort to elucidate our understanding of the outcomes of each iron supplementation protocol. Nonetheless, our data suggests that neither the ALT nor DAY strategy influenced Hb_{mass} outcomes over 8 weeks. The sex effect likely arose because our sample consisted of two males (average baseline Hb_{mass} = 831.5 ± 35.5 g) and eight females (average baseline Hb_{mass} = 596.5 ± 90.0 g). This probably accounts for training load and dietary iron intake emerging as significant covariates in the Hb_{mass} model, since the two males had substantially greater training loads and overall dietary iron intakes than the females.

Alongside haematological outcomes, the lower reported rates of GI side-effects in the ALT condition further justifies the use of this iron supplementation strategy. Non-athletic populations have reported less frequent, and less severe GI distress while supplementing on alternate days, compared with daily, which may in turn improve the long-term compliance and effectiveness of oral iron therapy [21]. A recent meta-analysis reported daily supplementation with ferrous sulphate was associated with an odds
ratio of 2.6 times the occurrence GI side-effects, compared to placebo or intravenous iron, and that adherence was reported as only 70-90% in pregnant women as a consequence of the adverse effects [9]. While we only captured qualitative data on GI outcomes, the relative severity of GI side-effects, and drop-out rate, was less in the ALT group, compared to the DAY group. Aside from one complaint of ongoing and debilitating constipation from the ALT group, the three other reported GI symptoms from the ALT group were short-lived and mild (including bloating and mild stomach discomfort). In contrast, there were six reports of ongoing GI distress in the DAY group, encompassing nausea, stomach cramps, diarrhoea and constipation, with one participant unable to endure the full term of supplementation. Some of these athletes (n=4) also reported the symptoms to have affected their training, overwhelmingly advocating that the ALT iron supplement regime may be better suited to an athletic population, as it effectively improves iron status and is less likely to interfere with their athletic pursuits.

Finally, the sex effect identified in this investigation reiterates that ID is more common and/or severe in females compared to males. Males represented 29% of participants (Figure 3) and there was a clear difference in baseline sFer between males (41 ± 6 µg L⁻¹) and females (27 ± 11 µg L⁻¹). This distinction is generally explained as menstrual losses of iron, though there may be more underlying this sex difference. For example, only 10% of female athletes here achieved the recommended dietary intake of ≥18 mg/day, while all male athletes consumed ≥8 mg/day. Nevertheless, recently, international caliber endurance athletes presented 25-40% reductions in sFer following 3 weeks of intensified training, despite consuming the recommended daily intake of iron (13-18 mg; [22]). This suggests that the current recommendations for iron intake may not be adequate for athletic populations, especially female athletes subject to menstrual losses. Hence, it is likely that female athletes require more than the recommended 18 mg of iron daily, yet only two female participants here achieved ≥18 mg of iron daily. Consequently, females may be less likely than males to achieve a daily iron intake (and/or energy intake) adequate to sustain both training and reproductive demands, increasing their vulnerability to ID. Furthermore, plenty remains unknown regarding the influence of sex hormones (including menstrual cycle phase) on iron metabolism [23]. This should be addressed in future research to ascertain any sex
differences in iron metabolism and to inform specific iron intake recommendations that account for sex and training loads.

4.6 Conclusion
In summary, this investigation reveals that alternate day iron supplementation is as effective as daily iron supplementation at increasing sFer in athletes with sub-optimal iron status over 8 weeks. Our findings may be explained by the transient increase in hepcidin following an iron supplement, which likely reduces the bioavailability and fractional absorption of a secondary iron supplement 24 h later [19]. Thus, despite ingesting half the total amount of iron, it is likely that the ALT group procured similar increases in sFer to the DAY group from greater fractional absorption across the intervention. Importantly, the ALT protocol was associated with less GI distress, typically a major barrier to oral iron therapy, and is more cost effective in practice. Collectively, we have established that, in practice, athletes with sub-optimal iron status can effectively improve their sFer levels (60%; a marker of iron stores) supplementing with 105 mg of iron in the morning on alternate days in a relatively short time frame of 8 weeks.
4.7 References

19. Moretti, D., Goede, J. S., Zeder, C., Jiskra, M., Chatzinakou, V., Tjalsma, H., et al. (2015). Oral iron supplements increase hepcidin and decrease iron absorption from daily or twice-


The Effectiveness of Transdermal Iron Patches in Athletes with Sub-Optimal Iron Status

This chapter is based on a paper published in the *International Journal of Sports Nutrition and Exercise Metabolism*:

5.1 Abstract

We compared the effectiveness of two modes of daily iron supplementation in athletes with sub-optimal iron stores; oral iron (PILL) versus transdermal iron (PATCH). Endurance-trained runners (9 male and 20 female), with serum ferritin (sFer) concentrations <50 µg·L⁻¹, supplemented with oral iron or iron patches for 8 weeks, in a parallel group study design. Serum ferritin was measured at baseline and fortnightly intervals. Haemoglobin mass (Hb_mass) and maximal oxygen consumption (VO₂max) were measured pre- and post-intervention in PATCH. A linear mixed effects model was used to assess the effectiveness of each mode of supplementation on sFer. A repeated-measures ANOVA was used to assess Hb_mass and VO₂max outcomes in PATCH. There was a significant time effect (p<0.001), sex effect (p=0.013) and time*group interaction (p=0.009) for sFer. At week 6, PILL had significantly greater sFer compared to PATCH (15.27 µg·L⁻¹ greater in PILL; p=0.019). Serum ferritin was 15.53 µg·L⁻¹ greater overall in males compared to females (p=0.013). There were no significant differences in Hb_mass (p=0.727) or VO₂max (p=0.929) pre- to post-intervention in PATCH. Finally, there were six complaints of severe gastrointestinal (GI) side-effects in PILL, and none in PATCH. In summary, PILL effectively increased sFer in athletes with sub-optimal iron stores, whilst PATCH showed no beneficial effects.

Keywords

Supplementation mode, oral iron therapy, gastrointestinal side-effects
5.2 Introduction

Dietary intake alone is often insufficient to fulfil the iron demands of an athlete. This issue has manifested in the ongoing high rates of iron deficiency (ID) amongst male (~3-11%) and female (~15-35%) athletes [1]. Symptoms of ID include lethargy, fatigue, and in more severe cases, reduced work capacity [2], which may impede training and performance outcomes in athletes. Iron balance is a challenge for athletes because they encounter additional mechanisms of iron loss during exercise, including sweating, haematuria, gastrointestinal (GI) bleeding and haemolysis [3]. Negative iron balance ensues when athletes consume a sub-optimal amount of dietary iron to counteract these losses. Additionally, these losses can be difficult to restore due to the low bioavailability of dietary iron (15-35% for heme and 2-20% for non-heme [4]) and the inability to endogenously replenish taxed iron stores [1]. Furthermore, recent research has identified a link between exercise-induced inflammation and an increase in the primary iron regulatory hormone, hepcidin. Hepcidin is reported to peak at 3 h and remain elevated for 6 h post-exercise, and is linked to suppressed dietary iron absorption and recycling by duodenal enterocytes and macrophages, respectively [5, 6]. Therefore, athletes will often need to consider supplemental sources of iron, beyond dietary sources, to achieve healthy iron status.

Typically, oral iron supplementation is the first approach to iron replacement therapy beyond a nutritional intervention, with ferrous sulphate the most commonly prescribed oral iron therapy [7, 8]. Characteristically, athletes’ serum ferritin (sFer) will increase 40-80% following an 8-12 week supplementation period, consisting of ~100 mg of elemental iron daily [8, 9]. However, adverse side-effects associated with oral iron therapy, predominantly gastrointestinal (GI) distress, are frequently reported [8, 11, 12], often provoking non-adherence and ultimately treatment failures. Furthermore, there are concerns that soluble oral iron may be destructive to colonic microbiota and that luminal iron may be a risk-factor for inflammatory signalling [13]. As such, contemporary research endeavours to ascertain strategies of iron supplementation which optimise iron absorption, and reduce adverse side-effects. Beyond oral iron therapy, iron delivery bypassing the gut is a promising prospect because it circumvents the side-effects and absorption issues of the conventional therapy.
Parenteral iron therapy has been shown to be very effective at improving athletes’ iron status, with 200-400% increases in sFer reported from 300-550 mg of intravenous (IV) iron delivered across 6 weeks [10]. In following, recent research pursues novel and alternate strategies of iron supplementation that bypass the gut, without the invasive procedure of IV administration, and has identified transdermal iron delivery as a potential strategy. The primary barriers of transdermal iron delivery include the reactive nature of free systemic iron and the low lipophilic permeability of the skin [14], however ferric pyrophosphate (FPP) was recently identified as an iron source suitable for transdermal administration [15]. Currently, a commercially available transdermal iron patch is being advertised as an alternative mode of iron supplementation, despite no existing data to support the efficacy of such an approach. Therefore, this investigation sought to be the first to compare the effectiveness of transdermal iron therapy via an iron patch, with oral iron supplementation, to elucidate the non-invasive treatment feasibility for ID athletes.

5.3 Materials and Methods

Participants: Twenty nine endurance-trained runners (9 male and 20 female) with sub-optimal iron status (defined as sFer levels <50 μg.L⁻¹ [16]) were recruited for this study. Pre-participation conditions required participants not to be supplementing with iron within 3 weeks of commencing the study. Participants were informed of the purpose, requirements and risks associated with their involvement. Written informed consent was obtained prior to study commencement. Ethics approval was obtained from the Human Research Ethics Committee of The University of Western Australia (RA/4/1/9030).

Experimental overview: The experimental approach used in this study required athletes to supplement with iron for 8 weeks, in a parallel group study design (Figure 5.1). Potential candidates undertook a rested pre-investigation blood measurement to confirm sub-optimal iron status. Subsequently, eligible participants were assigned to one of two groups; (1) a treatment group that supplemented with a daily oral iron supplement (PILL) for 8 weeks, or (2) a treatment group that supplemented with a transdermal iron supplement (PATCH) for 8 weeks. Participant groups were matched by sFer concentration. The
impact of the iron supplementation intervention on iron status was assessed via measurements of sFer concentration at the commencement, and at fortnightly intervals, throughout the 8-week training period (total of 5 blood samples). Participants acted as their own control via comparisons in sFer response to baseline.

**Figure 5.1:** Diagrammatic representation of experimental overview.

During the supplementation period, the PILL group were required to consume their iron supplement upon waking to optimise iron absorption [17], documenting the time-of-day of consumption in a daily supplement and training log. These participants were asked not to consume dairy-based food, tea or coffee within 60 min of ingesting the iron supplement. The PATCH group were required to apply one patch overnight (for 8 hours) to bare skin superficial of the upper trapezius muscle, according to the manufacturer’s directions (Iron Plus, PatchMD, Las Vegas, USA). All participants were encouraged to report any supplementation side-effects they may have experienced in an open-ended comments box on each day of the supplement log and were also required to complete a 4-day food diary in the initial, mid and final fortnight of the intervention period. In addition to the fortnightly sFer measures, a graded exercise test (GXT) and haemoglobin mass (Hbmass) measurement was undertaken prior to, and after the 8-week supplementation period in the PATCH group to further explore the effectiveness of the iron patches.

**Experimental Procedures:**

**Iron supplementation:** During the intervention period, PILL participants supplemented with one Ferro Grad C tablet (Mylan Health Pty Ltd, Millers Point, NSW, Australia) upon waking daily. Each tablet
contained 325 mg of ferrous sulphate and 500 mg of ascorbic acid, equating to a dose of 105 mg of elemental iron. The PATCH group supplemented with one Iron Plus supplement patch (PatchMD, Las Vegas, USA), for 8 hours every night of the intervention. Each patch contains 45 mg of iron in the form of iron bispiglycinate.

**Blood collection:** Venous blood was collected and analysed for sFer by a commercial pathology laboratory (Clinipath Pathology, Western Australia) at baseline and fortnightly intervals during the intervention. Participants were instructed to visit the phlebotomy practice for a morning blood sample in a rested, non-fasting state (i.e., no morning exercise prior to blood collection). Participants still consumed their oral iron supplement on the morning blood was collected.

**Haemoglobin mass:** Haemoglobin mass was assessed using the optimised 2 min CO rebreathing technique as outlined by Schmidt and Prommer (Schmidt and Prommer 2005). Determination of carboxyhaemoglobin (%HbCO) was measured at baseline, plus 7 min after rebreathing, from capillary fingertip blood samples tested with an ABL80 blood gas analyser (Radiometer, Copenhagen, Denmark). Haemoglobin mass was calculated from the mean change in %HbCO before and after CO rebreathing. All Hbmass measurements were conducted by the same technician.

**Graded Exercise Test (GXT):** The running GXT was conducted on a motorized treadmill (h/p/Cosmos Venus 200/100r, Germany) utilizing 3 min work and 1 min rest periods. The initial work velocity was set to 11.5 ± 1.3 km·h⁻¹ with subsequent 1 km·h⁻¹ increments over each work period until volitional exhaustion. During the GXT, ventilation and expired air was analysed for concentrations of O₂ and CO₂ using a TrueOne 2400 Metabolic Measurement System (ParvoMedics, UT, United states). This system was calibrated pre-test according to the manufacturer’s specifications. The VO₂max was determined as highest 30 s \( \dot{V}O_2 \) reached during the final 3 min of the GXT.

**Training load, dietary and menstrual monitoring:** Participants were required to document their daily exercise, necessitating a measure of duration (min), distance (km), and a rating of perceived exertion
(19) with anchors 6 (no exertion) and 20 (maximal exertion). This data was used to calculate a daily training impulse (TRIMP) (20) as a measure of training load. The training journal also included a section for female athletes to record their menstruation. All participants were also required to complete a 4-day food diary in the initial, mid and final fortnight of the intervention period, using the mobile diet-tracking app, Easy Diet Diary (version 5.0.22, Xyris Software, Australia). Participants attended an information session with a sports dietitian, prior to the commencement of the study, to familiarise themselves with portion measurements and the Easy Diet Diary app. This information was then analysed using the nutritional analysis software, Foodworks (version 9.0.3871, AusBrands 2017 and AusFoods 2017 databases).

**Statistical analysis:** All diet and training data was initially analysed using a two-way, repeated-measures ANOVA to check for any differences between the PILL and PATCH groups. Linear mixed effects models were then used to assess the effectiveness of PILL and PATCH iron supplementation treatments on sFer. Serum ferritin was analysed relative to time, treatment (PILL or PATCH), and sex using linear mixed-effects models with a random intercept for each participant. Covariates considered were training load, energy intake, and (dietary) iron consumption. Initial models included all possible interactions, but non-significant interactions, except time*group, were dropped from the models for ease of interpretation. A repeated measures ANOVA was also used to assess the within group differences in Hb\(_{\text{mass}}\) and VO\(_{\text{2max}}\) measures over the intervention in the PATCH group. Results are expressed as mean and 95% confidence intervals (95% CI). The alpha level was defined as \(\alpha \leq 0.05\).

### 5.4 Results

**Group demographics:** Treatment group demographics are shown in Table 5.1. The PILL group consisted of 14 participants (6 male and 8 female) and consumed 55 ± 3 iron supplements (98.7% compliance: 5824 ± 348 mg of elemental iron) throughout the 8-week intervention period. The PATCH group consisted of 14 participants (3 male and 11 female) and applied 56 iron patches (99.5% compliance: 2507 ± 21 mg of elemental iron) throughout the 8-week intervention period. There were
no significant differences in age or baseline sFer between groups; however, there was a significant difference in body mass between groups (p=0.043). Of note, two female athletes did not report menstruating during the 8-week intervention, though one of these instances was a secondary effect of contraception.

Table 5.1: Group characteristics for PILL and PATCH treatments. Data presented as mean ± standard deviation. * indicates a significant difference between groups (p>0.05).

<table>
<thead>
<tr>
<th></th>
<th>Age (years)</th>
<th>Body mass (kg)</th>
<th>Serum ferritin (µg L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PILL</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female (n=9)</td>
<td>27 ± 6</td>
<td>61.7 ± 7.4</td>
<td>23 ± 12</td>
</tr>
<tr>
<td>Male (n=6)</td>
<td>24 ± 5</td>
<td>69.3 ± 2.5</td>
<td>41 ± 6</td>
</tr>
<tr>
<td>Overall</td>
<td>26 ± 6</td>
<td>64.8 ± 6.9</td>
<td>31 ± 13</td>
</tr>
<tr>
<td><strong>PATCH</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female (n=11)</td>
<td>27 ± 5</td>
<td>57.7 ± 6.0</td>
<td>33 ± 10</td>
</tr>
<tr>
<td>Male (n=3)</td>
<td>26 ± 5</td>
<td>65.8 ± 4.0</td>
<td>37 ± 14</td>
</tr>
<tr>
<td>Overall</td>
<td>27 ± 5</td>
<td>59.5 ± 6.5*</td>
<td>34 ± 11</td>
</tr>
</tbody>
</table>
Training and dietary analysis: Table 5.2 presents the training load and nutrient intake of the PILL and PATCH treatment groups during the 8-week intervention. There was no time, group or time*group effect for fortnightly training load, energy, protein, fat, CHO or dietary iron intake (all p>0.05).

Table 5.2: Average fortnightly training load and average dietary intake for the PILL and PATCH treatment. Data presented as mean ± standard deviation.

<table>
<thead>
<tr>
<th></th>
<th>PILL</th>
<th>PATCH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fortnightly training load (AU)</td>
<td>8537 ± 4292</td>
<td>7915 ± 3821</td>
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<tr>
<td>Energy intake (kJ/day)</td>
<td>10765 ± 3254</td>
<td>9396 ± 2573</td>
</tr>
<tr>
<td>Carbohydrate intake (g/day)</td>
<td>279 ± 83</td>
<td>248 ± 79</td>
</tr>
<tr>
<td>Protein intake (g/day)</td>
<td>107 ± 28</td>
<td>98 ± 27</td>
</tr>
<tr>
<td>Fat intake (g/day)</td>
<td>104 ± 41</td>
<td>85 ± 23</td>
</tr>
<tr>
<td>Alcohol intake (g/day)</td>
<td>3.59 ± 7.69</td>
<td>4.86 ± 5.93</td>
</tr>
<tr>
<td>Dietary iron intake (mg/day)</td>
<td>15 ± 9</td>
<td>12 ± 5</td>
</tr>
</tbody>
</table>

Serum ferritin: Fortnightly sFer concentrations are depicted in Figure 5.2 alongside a more comprehensive depiction of individuals’ pre- and post-intervention outcomes in Figure 5.3. There was a significant time effect (p<0.001), sex effect (p=0.013) and time*group interaction (p=0.009) for sFer. Our model indicates that by 6 weeks, the PILL group had a significantly greater sFer compared to the PATCH group (15.27 μg·L⁻¹ greater in PILL; p=0.019; 95% CI: 2.83 < μ < 19.78 μg·L⁻¹). Serum ferritin was also 15.53 μg·L⁻¹ greater overall in males compared to females (p=0.013; 3.55 < μ < 27.52 μg·L⁻¹). No other covariates appeared to effect sFer.
Figure 5.2: Fortnightly serum ferritin concentrations for the oral (PILL) and transdermal (PATCH) iron supplement treatment groups. Data presented as mean ± standard deviation. * indicates a significant increase from the preceding fortnight (p<0.05). † indicates a significant difference between groups (p<0.05).
Figure 5.3: Pre- and post-intervention serum ferritin concentrations for males and females in the oral (PILL) and transdermal (PATCH) iron supplement treatment groups. Group data presented as mean ± standard deviation.* indicates a significant increase from baseline (p<0.05). † indicates a significant difference between groups (p<0.05).

Haemoglobin mass: There was no significant difference in Hb\text{mass} pre- to post-intervention (652 ± 125 g and 650 ± 134 g, respectively) in the PATCH group (p=0.727).

Maximal oxygen consumption: There was no significant difference in VO\text{2max} pre- to post-intervention (56.06 ± 9.09 mL·min⁻¹·kg⁻¹ and 55.97 ± 9.73 mL·min⁻¹·kg⁻¹, respectively) in the PATCH group (p=0.929).

Adverse side-effects outcomes: One participant from PILL withdrew from the study due to debilitating GI side-effects. There were six further complaints of severe GI side-effects in PILL encompassing nausea (n=1), stomach cramps (n=2) and constipation (n=3). There were no reported adverse effects in the PATCH group.
5.5 Discussion
Parenteral iron delivery techniques, such as IV administration, effectively increase athletes’ sFer [10], and bypass the gut, circumventing the GI side-effects associated with oral iron supplementation that often limit compliance. However, IV iron delivery is highly invasive and is typically reserved for more severe cases of ID in athletes [1]. Hence, the alternative strategy of transdermal delivery of iron is appealing and applicable to the athletic population because of its purported capacity to bypass the gut, and is non-invasive. Iron patches are currently being advertised as an alternate mode of iron supplementation despite very little data existing on their efficacy. To our knowledge, this is the first study to investigate the effects of iron patches and the potential for transdermal iron supplementation in athletes with sub-optimal iron stores. Nevertheless, the results of our study suggest that, unlike daily oral iron supplementation, daily use of a commercial iron patch for 8 weeks (as per manufacturer’s recommendation) does not increase an athlete’s iron stores.

Beyond diet alone, oral iron supplementation is typically the first approach to iron replacement therapy because it is relatively cheap, safe and effective [21]. The 60% increase in sFer observed in the PILL group of this study attests to the 40-80% increase in sFer that is characteristic of ID athlete cohorts following 8-12 weeks of daily iron supplementation (~100 mg of elemental iron per day) [9, 10]. Ferrous sulphate is currently the most commonly prescribed oral iron therapeutic, however, ferrous sulphate supplementation is renowned for its associated GI side-effects that can lead to non-compliance and treatment failure [8]. Correspondingly, there were six reports of ongoing GI distress in the PILL group. Side effects included nausea, stomach cramps and constipation, with one participant even unable to endure the full term of supplementation. This highlights the incentive for developing alternate modes of iron supplementation. In contrast, despite showing no beneficiary effects on sFer, Hbmax or VO2max in our athlete population, there were no complaints of any adverse side-effects in the PATCH group. While it is not yet clear that this method of iron delivery is effective, the absence of adverse side-effects warrants further research into transdermal iron supplements.
Contemporary research is endeavoring to devise a viable transdermal iron therapy due to the high rates of non-compliance and GI toxicity associated with oral iron therapy [15]. Ferric pyrophosphate (FPP) was recently identified as an iron source suitable for transdermal administration [15] because it does not liberate free iron, is able to directly transfer iron to transferrin, and is capable of triggering iron transfer between transferrin molecules and between transferrin and ferritin [22, 23]. Unfortunately, its high molecular weight (745 Da) and hydrophilicity resulted in poor passive permeation across the skin [24]. Nevertheless, the transdermal delivery of FPP was found to be enhanced by the electrically mediated technique, iontophoresis [24], and microporation via a soluble microneedle system [25]. Of note, the commercially available iron patch used in the present study is a small topical patch containing iron bisglycinate (molecular weight; 204 Da) that is alleged to absorb passively over 8 hours; however, our sFer and Hbmass data did not support this supposition. Iron bisglycinate is an amino acid chelate that has been identified as an ideal food fortificant because of its high bioavailability and low reactivity [26], but like FPP, may require penetration enhancement to successfully be delivered transdermally. Our study outcomes suggest that the assumption of passive transdermal iron absorption cannot be relied upon and that future research needs to establish an effective transdermal iron transport mechanism before it can be marketed as an effective iron supplementation strategy.

The capability of the passive delivery mechanism to penetrate the skin barrier is likely the primary limiting factor to the outcomes of the PATCH group; however, there was also a clear discrepancy in the dosage of iron between the PILL and PATCH group (105 mg and 45 mg, respectively). Nevertheless, while the PILL group ingested 105 mg of elemental iron daily, the bioavailability of iron is low, and Moretti and colleagues [27] previously demonstrated that fractional absorption decreases as oral iron dosage increases. In fact, Moretti et al. [27] revealed that ~19% of an 80 mg oral dose of iron is functionally absorbed, which in the context of our study, would equate to, at best, ~20 mg of iron being functionally absorbed daily in the PILL group. In comparison, the PATCH group received 45 mg delivered transdermally, and while the bioavailability of this mechanism remains unknown, our data would suggest it is very low and ineffective for athletes with sub-optimal iron.
While our work appears to be the first study to investigate the effectiveness of iron patches in athletes, Saurabh et al. [28] recently assessed the multivitamin patch in gastric bypass patients. Here, these authors found that patients using the multivitamin patch had twice the frequency of vitamin deficiencies after 12 months, and concluded that the transdermal multivitamins were not as effective as oral vitamin supplements. In parallel, the data from the present study indicates that it is currently advantageous for athletes with sub-optimal iron status to supplement with oral iron. However, a recent review describing the latest advances and novel approaches to transdermal vitamin delivery techniques highlights promising theoretical formulations but a lack of sufficient clinical and preclinical assessments (Rejinold et al. 2019). Therefore, future research should endeavour to improve therapeutic delivery or iron, and other vitamins, because the non-invasive procedure and potential for reductions to GI sensitivity may be applicable to several populations.

Finally, this investigation highlights the relatively greater prevalence and severity of ID in female, compared to male, athletes (Figure 3). Males represented 31% of participants and there was a clear distinction in baseline sFer between females (29 ± 12 µg·L⁻¹) and males (39 ± 8 µg·L⁻¹). However, this may be elucidated, in part, by the dietary iron intakes captured in the present study, with only 10% of female athletes consuming the recommended dietary iron intake of ≥18 mg/day, while all male athletes consumed ≥8 mg/day. This would suggest that females may be less likely than males to achieve a daily iron intake (and/or energy intake) adequate to sustain both training and reproductive demands, increasing their vulnerability to ID.

5.6 Conclusion
In summary, this study demonstrates that daily oral iron supplementation for 8 weeks effectively increases sFer in athletes with sub-optimal iron stores, although this treatment is accompanied with the well-documented GI side-effects. In contrast, the transdermal iron patch showed no beneficiary effects on sFer, Hbmax or $\dot{V}O_{2max}$ in athletes with sub-optimal iron stores. Consequently we would advise athletes with compromised iron stores, who have initially addressed any dietary iron intake deficits, to
continue to supplement with the conventional oral iron therapy, or in severe cases, to consider parenteral iron approaches under the direction of their physician. In consideration of our companion paper (Part 2), athletes with sub-optimal iron may consider alternate day oral iron supplementation, as this was shown to increase sFer comparably with daily oral iron, and was associated with less GI side-effects. Nevertheless future research should continue to pursue the potential for effective transdermal iron supplementation to eliminate the limiting GI side-effects.
5.7 References


Chapter 6

Summary and Conclusions
6.1 Summary

Compromised iron status in athletes is counterproductive to their sporting pursuits, manifesting in symptoms of lethargy and fatigue capable of adversely affecting their optimal work capacity, training and performance outcomes, or capacity to respond/adapt to training stress, especially as the condition progresses in severity from non-anaemic (IDNA) to anaemic (IDA) states [1-3]. Nevertheless, ID continues to affect ~15-35% of female and ~3-11% of male athletes, and therefore, innovative solutions to more effectively address this issue are warranted [4]. This predicament likely develops because athletes contend with several additional avenues of iron loss during exercise, including sweating, haematuria, gastrointestinal (GI) bleeding, and haemolysis [5]. These phenomena are thought to, in part, account for the high iron demands of athletes, and their increased susceptibility to ID [4]. In order to counteract a negative iron balance, it is essential that depleted iron stores are replenished via adequate dietary iron intake and absorption, since the body is unable to endogenously synthesise iron. However, the bioavailability of dietary iron is generally low (2-35% [6]), and in the context of athletes, exercise-induced inflammation may mediate a further reduction in iron bioavailability by inducing an increase in the levels of the master iron regulator, hepcidin, ~3-6 h post-exercise [7]. Given that hepcidin suppresses the absorption and recycling of dietary iron [8, 9], this transient increase in hepcidin concentrations following exercise is likely an imminent period of reduced iron absorption [7, 10, 11]. Accordingly, the interaction between exercise, hepcidin and iron absorption likely contributes to the high rates of ID amongst athlete populations. Therefore, a primary aim of this thesis, addressed in Chapter 3, was to assert the influence of this transient, exercise-induced, hepcidin elevation on iron absorption during the post-exercise period.

Accordingly, the first study of this thesis (Chapter 3) specifically examined the post-exercise inflammatory, hepcidin and iron absorption response to endurance exercise performed in the morning versus the afternoon, in athletes with sub-optimal iron status (considered here as serum ferritin; sFer ≤ 50 µg·L⁻¹ [12]). The outcomes of this investigation revealed that, despite a post-exercise increase in hepcidin concentrations, more iron was absorbed at breakfast following morning exercise, as compared with breakfast in a rested state, or when compared to the absorption from an evening meal. This result
challenged our initial hypothesis, and indicates that, while the regulatory mechanism of hepcidin is instrumental in regulating iron absorption, other exercise-induced physiological changes appear to influence iron uptake too, yielding the notion of a potential post-exercise ‘open window’ of enhanced iron absorption. In this instance, given the post-exercise meals were consumed 30 minutes following exercise, it is likely that the iron reached the site of absorption prior to the peak in hepcidin elevation at 3-6 hours post-exercise, indicating a potential window of opportunity for greater iron absorption, provided the iron is consumed within this acute post-exercise time-frame (within 30 minutes). Furthermore, it was identified that both the inflammatory and hepcidin responses to exercise were greater in the afternoon, compared to the morning, thought to reflect the innate diurnal increase of both interleukin-6 and hepcidin across the day, potentially producing an additive effect to the exercise-induced elevations of these peptides. Consequently, it was concluded that the consumption of iron containing foods/supplements in the morning might be a more optimal strategic approach to the timing strategies relevant to iron intake. Overall, it would appear the net effect of the interaction between any transient exercise-induced mechanisms that may promote iron absorption, and the opposing post-exercise hepcidin concentration, is an overall positive influence on iron absorption in the morning, likely because the iron reaches the site of absorption prior to peak hepcidin activity; whereas in the afternoon, diurnal increases in hepcidin concentrations across the day appear to negate these potential exercise-driven mechanisms. While further research is required to clarify the exercise-driven mechanisms and their time-of-effect, in practice, athletes with sub-optimal iron status would be advised to consume/supplement with iron in the morning (to avoid the diurnal increase in hepcidin activity); and, if training in the morning, they should consume/supplement with iron during this transient window within 30 minutes post-exercise, to achieve optimal absorption outcomes.

With the aforementioned acute interactions between exercise, hepcidin and iron absorption explored, the focus of this thesis then turned to more chronic outcomes, with a focus on the oral iron treatment strategies commonly recommended to athlete populations. Oral iron supplementation is typically the first strategy of iron replacement therapy beyond nutritional intervention [13]. In ID athletes, daily oral iron supplements (in the form of ferrous sulphate containing ~100 mg elemental iron) across 8-12
weeks, characteristically increases sFer by 40-80% [14-19]. However, this treatment continues to be limited by the frequently reported gastrointestinal (GI) side-effects (e.g. pain, nausea, vomiting, abdominal distress, constipation and diarrhoea) that reduce compliance and potential benefits, likely rendering this treatment impractical for athletes with gut sensitivity [20]. This predicament has been a catalyst for a proliferation of iron supplementation research seeking to improve iron absorption outcomes and tolerability of supplements. Most recently, there has been a shift toward intermittent iron supplementation to improve bioavailability and tolerability, after Moretti and colleagues [21] established that hepcidin remained 2.2 times greater 24 h following a 60 mg dose of iron, resulting in a 35-45% reduction in fractional absorption from a subsequent dose. Moreover, in an applied setting, Stoffel et al., [22] found ~34% greater fractional and cumulative absorption of iron in women who supplemented with 60 mg of iron on alternate days for 28 d compared with women who supplemented with 60 mg of iron daily for 14 d. Thus, with the unfavourable GI side-effects associated with daily oral iron supplements, combined with the greater iron demands of athletes, the second study of this thesis (Chapter 4) ensues that of Stoffel et al. [22], seeking to determine if an alternate day iron supplement schedule would improve the iron status of a group of endurance-trained athletes, in the same manner as shown to impact a more general (non-exercising) population.

Accordingly, Chapter 4 compared the efficacy of alternate-day oral iron supplementation with a conventional daily oral iron supplement protocol, in endurance runners with sub-optimal iron status. The findings from this investigation suggest that the sFer response of endurance athletes to an alternate day oral iron supplementation protocol is comparable to daily iron supplementation, despite a 50% lower total dosage of elemental iron consumed. However, despite the ~60% increase in sFer, neither treatment had any effect on haemoglobin mass (Hbmass) over the 8-week intervention, although it is likely that such adaptation requires more than a change in iron status alone for improvement. Moreover, the qualitative data captured on GI outcomes indicates that the relative severity and frequency of GI side-effects (e.g. nausea, stomach cramps and constipation) are lower following an alternate day iron supplementation protocol, as compared to the daily approach. The similarity in sFer responses between the two treatment groups suggest that fractional iron absorption was greater in the alternate day oral
iron strategy, akin with Stoffel et al.’s previous study [22]. This observation is likely elucidated by the findings of Moretti et al. [21], who ascertained a reduction in fractional absorption (35-45% less) from a subsequent iron dose consumed 24 h after the initial 60 mg intake. Moretti and colleagues attributed the reduced iron absorption in their study to the elevation of circulating hepcidin levels, whereas an alternate day iron supplement protocol may circumvent the suppression of intestinal iron uptake by epithelial cells 24 h following the consumption of a high iron dose [22-24]. Overall, the results of Chapter 4 advocate that an alternate day iron supplement regime may be more practical to an athletic population (despite their daily training load), compared to the conventional daily oral iron therapy typically prescribed by practitioners, given that it effectively improves iron status to a similar level and is less likely to interfere with their athletic pursuits through negative GI interactions.

While the outcomes of Chapter 4 are promising, beyond oral iron therapy, iron delivery bypassing the gut is a promising prospect for athlete iron replacement because it is capable of completely circumventing the GI side-effects and absorption issues of the more conventional therapy. Accordingly, parenteral iron therapy has been shown to efficiently improve athlete iron stores, though is typically reserved for the more severe stages of ID (i.e. IDA) due to the invasive nature of the procedure, the potential for severe side-effects (i.e., anaphylaxis), and the tainted perception surrounding the use of needles to treat athletes [4]. Considering the limitations of parenteral iron delivery, contemporary research has identified an alternate strategy to bypass the gut by therapeutically delivering iron across the skin via transdermal iron supplementation [25, 26]. Therefore, the final investigation of this thesis (Chapter 5) sought to compare the efficacy of transdermal iron therapy via a novel iron patch, in comparison to the outcomes established from a standard daily oral iron supplement regime. Ultimately, our goal was to elucidate the feasibility and efficacy of this non-invasive treatment for ID athletes.

The results of Chapter 5 indicate that daily use of a commercial iron patch for 8 weeks had no beneficial effects on sFer, Hbmass or \( \dot{V}O_2_{max} \), in athletes with sub-optimal iron stores. In contrast, daily oral iron supplementation for 8 weeks effectively increased sFer by \(-60\%\), although this treatment is accompanied with the well-documented GI side-effects. In this instance, the primary limiting factor to
the outcomes of the transdermal patch was likely the capability of the passive delivery mechanism to penetrate the skin barrier. Despite a recent review describing the latest advances and novel approaches to transdermal vitamin delivery techniques highlighting promising theoretical formulations, there is a current lack of sufficient clinical and preclinical assessments to conclusively recommend their use [27]. Accordingly, we would advise athletes with compromised iron stores to continue to supplement with the conventional oral iron therapy, though on alternate days instead of daily. Future research should endeavour to establish the possibility of therapeutic delivery of transdermal iron supplementation, because the non-invasive procedure and potential for reductions to GI sensitivity may be applicable to several populations, including ID athletes.

6.2 Conclusions
Collectively, the outcomes of the three studies encompassing this thesis have generated refined oral iron supplementation approaches for athletes, which lend themselves to an improved efficacy of approach. Furthermore, this body of research identifies that more work is needed to establish the prospect of transdermal iron delivery, and that further innovation would be required before this approach is considered a viable strategy of therapeutically delivering iron to athletes. The interaction between exercise, hepcidin and iron absorption identified and explored throughout this thesis is a unique challenge contended by athletes striving to maintain a healthy iron balance. Accordingly, this thesis has examined the interplay between these three factors, and has determined several practical strategies that may enhance the efficacy of iron supplementation, with the goal of improving the advice given for oral iron therapies to athletes with sub-optimal iron status.
6.3 Limitations

The outcomes of this thesis undoubtedly have valuable applications; however, the following limitations should be acknowledged:

- The findings stemming from this series of investigations are specifically related to endurance runners with sub-optimal iron status (defined here as sFer <50 µg·L⁻¹, based on [12]) and may not translate equivalently into alternate populations.

- Serum ferritin was the primary variable used to evaluate iron status throughout this thesis because it is the most widely used and clinically accepted parameter for assessing iron status [28]. While control conditions were implemented and additional measures of Hb_mass were taken where possible, it is recognised that sFer measurements have limitations [28].

**Study 1**

- Post-exercise iron absorption was measured from meals provided 30 minutes after exercise completion. Thus, the study cannot determine how long the post-exercise outcomes would persist considering maximal hepcidin concentrations occur 3-6 hours following exercise.

- This investigation did not consider how exercise may affect iron absorption from a pre-exercise meal/supplement.

- The standardised diet employed in this study excluded some iron absorption inhibitors (tea and coffee) that are typical of a breakfast meal, and therefore, it remains unknown how these common ‘real world’ confounding variables would affect the outcomes.

**Study 2 and 3**

- Only one of numerous available formulations of oral iron supplement, namely Ferro Grad C tablets (Abbott Laboratories, Botany Bay, Australia), was used throughout this thesis. Consequently, the outcomes are specifically relevant to a dosage of 325 mg oral ferrous sulphate (equivalent to 105 mg elemental iron). Likewise, only one commercial transdermal iron patch (Iron Plus, PatchMD, Las Vegas, USA) containing 45 mg of elemental iron as iron bisglycinate, was investigated.

- Both investigations entailed an 8-week period of training that relied on participants accurately recording their training and food intake, in addition to accurately recording when they
consumed/applied the iron supplement. Furthermore, athletes were assumed to have abided by the specific study instructions; namely the 60 min dairy/caffeine restriction implemented prior to consuming oral iron supplements, and the hair-removal (location and duration) process of the patch application.

- Both studies quantified dietary intake via three 4-day food diaries which are associated with potential reactivity, specifically related to more conscious food choices. Furthermore, this method of dietary assessment relies on the athletes’ ability to estimate portion sizes accurately. Finally, there is also the underlying assumption that this data was representative of the 8-week interventions in their entirety.

- There was a total iron dosage discrepancy between the daily and alternate day iron supplementation protocols (105 mg daily vs 105 mg on alternate days, respectively) in Chapter 4, and between the oral iron and transdermal iron treatments (105 mg daily vs 45 mg daily, respectively) in Chapter 5. As such, it remains unknown how alternate dosages of iron supplementation (such as smaller/larger doses, or a mixed/periodized approach) would influence these outcomes.

### 6.4 Directions for future research

Moving forward, the findings of this thesis provide a foundation for further research to improve iron replacement therapies for the athletic population, and ultimately, to reduce the prevalence of ID amongst athletes. Future research should endeavour to:

- Evolve the outcomes of Chapter 3 to encompass further times of the morning, including pre-exercise and 2-6 h post-exercise, to clarify the optimal time to ingest iron relative to physical activity. In turn, this work might identify any exercise-induced mechanisms that may increase iron absorption following morning exercise, their time-of-effect, and whether they may counteract the influence of hepcidin in the 3-6 hours post-exercise window.

- Determine whether the efficacy of the iron supplement strategies identified in this series of investigations pertain to alternate athletic populations, such as team sport athletes and/or non-weight-bearing sports.
- Investigate the alternate day iron supplement strategy using alternate dosage protocols using smaller and larger doses of iron. For instance, larger iron doses are particularly relevant in the context of athletes training at moderate altitude (1300-3000m), wherein iron supplementation is implemented to optimise haematological adaptations [29, 30]. Alternatively, smaller iron supplement doses may increase fractional iron absorption and reduce GI side-effects, and therefore, might be an important consideration for athletes with gut sensitivity. Finally, investigation of a mixed or periodised (relative to training load) iron dosages may allow athletes to gain benefits from both strategies.

- Investigate the efficacy of more contemporary formulations of oral iron supplement, such as prolonged release and polysaccharide-iron complexes, that may be better tolerated by the gut.

- Refine and further develop a viable system of transdermal iron delivery (such as the transdermal, soluble microneddle design [25]), able to provide a therapeutic dose of iron to athletes.

- Further investigate the sex effect identified in Chapters 4 and 5 to elucidate the greater frequency/severity of ID in female, compared to male athletes, and identify whether there may be underlying sex differences in iron metabolism.

- Further explore the observations made within Chapters 4, suggesting that females may be less likely than males to achieve an adequate daily iron intake (and/or energy intake) to sustain both training and reproductive demands, making them more vulnerable to ID. This would entail a chronic, comprehensive assessment of dietary intake, training load and reproductive health to increase our understanding of female athlete demands, and further elucidate the link between ID and Relative Energy Deficiency in Sport (RED-S).

- Address the lack of athlete-specific daily iron intake recommendations to account for their increased iron demands, and better establish the optimal dosage and protocol for treatment of ID athletes with and without anaemia.
Ultimately, ID continues to be a vast issue amongst the athletic population, and plenty remains to be investigated in order to address the shortcomings of existing iron therapy. Clearly, further innovation is required to enhance the strategies of iron supplementation for the ID athletic population.

6.5 Practical Applications
The outcomes of this research are readily applicable to athletes endeavouring to maintain or increase their iron stores. The findings comprised within this thesis establish that athletes ought to consume the majority of their daily iron intake (from food or oral supplements) in the morning (to avoid diurnal increases in hepcidin), and if training, ideally within 30 minutes of exercise completion, in order to maximise the amount of iron absorbed. Furthermore, this body of work confirms the efficacy, and improved feasibility, of an alternate day oral iron supplementation protocol for the athletic population, relative to the conventional daily oral iron approach, with evidence of comparable sFer responses, reduced occurrence of GI side-effects, and the inherent improvement in cost/resource efficiency. Finally, this thesis perpetuates oral iron therapy to treat ID in athletes (unless severe cases require parenteral iron delivery), since further advancements in alternative non-invasive treatments are still required before the transdermal delivery of iron is a viable strategy for recommendation. Overall, this research establishes a contemporary strategy of oral iron therapy to benefit athletes, entailing morning supplementation, ideally within the 30 minutes following morning exercise, and in athletes with gut sensitivity, on alternate days.
6.6 References


Appendix A

Ethics and Information Sheets
A.1 Ethics Approval Letters

**Human Ethics**

Office of Research Enterprise
The University of Western Australia
MBS, 35 Stirling Highway
Crawley WA 6009 Australia
T  +61 6 6488 3703 / 4703
F  +61 6 6488 8775
E  humanethics@uwa.edu.au

Our Ref: RA/4/1/9030

17 May 2017

Dr Peter Peeling
School of Human Sciences
MJD/P 4/08

Dear Dr Peter Peeling

**HUMAN RESEARCH ETHICS APPROVAL - THE UNIVERSITY OF WESTERN AUSTRALIA**

**Athlete Iron Consumption: Timing is everything – but when is best?**

Ethics approval for the above project has been granted in accordance with the requirements of the National Statement on Ethical Conduct in Human Research (National Statement) and the policies and procedures of The University of Western Australia. Please note that the period of ethics approval for this project is five (5) years from the date of this notification. However, ethics approval is conditional upon the submission of satisfactory progress reports by the designated renewal date. Therefore initial approval has been granted from 17 May 2017 to 16 May 2018.

You are reminded of the following requirements:

1. The application and all supporting documentation form the basis of the ethics approval and you must not depart from the research protocol that has been approved.
2. The Human Ethics office must be approached for approval in advance for any requested amendments to the approved research protocol.
3. The Chief Investigator is required to report immediately to the Human Ethics office any adverse or unexpected event or any other event that may impact on the ethics approval for the project.
4. The Chief Investigator must submit a final report upon project completion, even if a research project is discontinued before the anticipated date of completion.

Any conditions of ethics approval that have been imposed are listed below:

**Special Conditions**

None specified

The University of Western Australia is bound by the National Statement to monitor the progress of all approved projects until completion to ensure continued compliance with ethical principles.

The Human Ethics office will forward a request for a Progress Report approximately 30 days before the due date.

If you have any queries please contact the Human Ethics office at humanethics@uwa.edu.au.

Please ensure that you quote the file reference – RA/4/1/9030 – and the associated project title in all future correspondence.
Our Ref: RA/4/20/4236

26 February 2018

Dr Peter Peeling
School of Human Sciences
MBDP: M408

Dear Dr Peter Peeling,

HUMAN RESEARCH ETHICS APPROVAL - THE UNIVERSITY OF WESTERN AUSTRALIA

The efficacy of daily and alternate day oral iron supplementation in iron deficient athletes.

Ethics approval for the above project has been granted in accordance with the requirements of the National Statement on Ethical Conduct in Human Research (National Statement) and the policies and procedures of The University of Western Australia. Please note that the period of ethics approval for this project is five (5) years from the date of this notification. However, ethics approval is conditional upon the submission of satisfactory progress reports by the designated renewal date. Therefore initial approval has been granted from 26 February 2018 to 25 February 2019.

You are reminded of the following requirements:

1. The application and all supporting documentation form the basis of the ethics approval and you must not depart from the research protocol that has been approved.
2. The Human Ethics office must be approached for approval in advance for any requested amendments to the approved research protocol.
3. The Chief Investigator is required to report immediately to the Human Ethics office any adverse or unexpected event or any other event that may impact on the ethics approval for the project.
4. The Chief Investigator must submit a final report upon project completion, even if a research project is discontinued before the anticipated date of completion.

Any conditions of ethics approval that have been imposed are listed below:

Special Conditions
None specified

The University of Western Australia is bound by the National Statement to monitor the progress of all approved projects until completion to ensure continued compliance with ethical principles.

The Human Ethics office will forward a request for a Progress Report approximately 30 days before the due date.

If you have any queries please contact the Human Ethics office at humanethics@uwa.edu.au.

Please ensure that you quote the file reference – RA/4/20/4236 – and the associated project title in all future correspondence.

Yours sincerely,

[Signature]

[Human Ethics]
Office of Research Enterprise
The University of Western Australia
M405, 35 Stirling Highway,
 Crawley WA 6009 Australia
T +61 8 6488 3703 / 4703
F +61 8 6488 8775
E humanethics@uwa.edu.au
CREDO Provider Code: 022360.
A.2 Participant Information Sheets

Athlete Iron Consumption: Timing is everything – but when is best?

Participant Information Form

Purpose
Hepcidin is a hormone that regulates the absorption of dietary iron. Recent studies have shown that hepcidin levels increase following exercise. This project aims to measure the influence of post-exercise hepcidin activity on iron absorption, and to determine the best timing for iron consumption to optimise absorption.

Methods
This investigation involves two laboratory-based testing sessions (in addition to a familiarisation session), separated by 14 days. Each trial will take place at the Western Australian Institute of Sport.

After we have gained your consent to participate in the study, you will need to have a blood screening to assess your iron status, and you will complete a Physical Activity Readiness Questionnaire (PAR-Q). If your iron levels fall within a certain range, and if you meet the standards set on the PAR-Q, you will become eligible for this study.

If eligible, within 7-days you will be required to attend the laboratory for a familiarisation session, allowing you to become accustomed to the laboratory equipment that will be used in future testing sessions. This session will include a graded exercise test to determine your maximal oxygen uptake (VO2max). Following the familiarisation session, you will be required to attend two experimental trials.

Experimental Trials
The day before you come to the laboratory for testing, we will provide you with all food to consume throughout the day. You will need to eat all of the food that we provide (nothing more, nothing less). You are strictly not allowed to exercise during this period.

When you arrive at the laboratory (fasted from 10 pm the night before) at 0515am the following day, a cannula (small indwelling needle) will be placed in a forearm vein by a trained phlebotomist. We will then collect a baseline blood sample from the cannula which will remain in position for the day to draw future blood samples. At 0630 am, if you are undertaking Trial 1 (Figure 1), you will be required to complete a 90 min run at 65% VO2max [meanwhile Trial 2 (Figure 2) participants will remain resting]. During this task, we will record a measure of heart rate and collect capillary blood samples (a drop of blood from your earlobe) to measure blood lactate levels before and after exercise. At the end of the run/test task, a second venous blood sample will be drawn from the cannula in your forearm. You will then be provided with a breakfast meal that you must eat in full. This meal will contain a stable iron
isotope that we can subsequently trace in your blood. At 11:00am, another blood sample will be collected and you will be provided with a lunch meal, followed by a snack at 14:30pm. At 15:30pm, another blood sample will be collected. At 16:00pm, if you are undertaking Trial 2, you will be required to complete the 90 min run task at 60% VO_{2peak} (meanwhile Trial 1 participants will remain resting), with the same measurements taken throughout, as explained above. At the end of the run/rest task, another blood sample will be drawn from the cannula. You will then be provided with a dinner meal, which will again contain a stable iron isotope. You must finish this meal in its entirety. A penultimate blood sample will be collected at 20:30pm (with snack provided in the meantime). The cannula will then be removed from your forearm and you will be free to leave the facility. You will be required to return fourteen days later to provide a final venous blood sample – this will occur after each of the two testing sessions.

![Figure 1: Trial 1](image1.png)

![Figure 2: Trial 2](image2.png)

**What are stable iron isotopes?**

Stable iron isotopes have been used regularly in nutrition studies to measure the absorption (bioavailability) of iron in adults, infants and pregnant women. Stable iron isotopes are a natural component of the human body and are safe for human consumption. The present study will use stable iron isotopes to label some of your test meals so that we can measure the iron you absorb from the meal.

**Iron isotopic labelling**

The present study is a nutrition investigation study with measurements of iron bioavailability by using iron isotopic tracers at meal times. It is highly unlikely there will be any adverse effects from the administration of iron in the form of stable isotopes since these isotopes occur naturally and are already part of the iron pool of the human body. The form of iron, (FeSO₄) used in this study is widely used to fortify commercial foods and is generally recognised as safe. In the present study, the total iron intake
from one test meal will be about 5 mg. This amount of iron is below the Australian guidelines of 8-18 mg of iron, defined as the required daily iron intake for males and females 19 to 50 years of age, and well within the normal dietary iron content of a single meal.

Risks and Side Effects
There are no significant risks associated with this study. There is a minor risk of bruising and infection associated with all blood collection; however, cannula insertion will be performed by a trained phlebotomist and all aseptic techniques will be implemented throughout the day to maintain the sterility of the cannula in an effort to reduce the risk. The cannula can also be easily removed if it causes you undue distress. Secondly, the endurance-nature of the exercise testing sessions may create a degree of musculoskeletal stress and fatigue. Although, it should be noted that the exertion required throughout testing is no more demanding than your normal training or competition load. However, adverse events can occur irrespective of study treatment, and therefore you will be continuously monitored whilst in the laboratory and all tests will be terminated at your request, or if you appear to be excessively distressed.

Benefits
Individual: By participating in this study, you may gain insight into the ideal timing of iron ingestion to optimise absorption and develop an awareness of your body’s iron status and how it may influence performance. Furthermore, you will be exposed to the new facilities at WAIS and will receive a free VO2max test and will be provided with 4 days’ worth of food.

Community: Although the impact of this project is targeted primarily at endurance runners, it is highly likely the outcomes will be applicable to the majority of the athletic population. The outcomes of this project may assist to establish a simple nutritional strategy that could promote healthy iron status.

Confidentiality
Personal details and results from this testing program will be treated confidentially at all times. Individual data will not be identified, but collective results may be published in peer-reviewed scientific journals and other media outlets. No data will be stored on public computers. All data will be stored on the chief investigator’s computer in a secure location until the completion of this research program for a period of 7 years. Additionally; all collected blood samples will be stored at Sports Science, Exercise and Health, the School of Human Sciences, until the completion of the study. These samples will then be sent for analysis at various pathology laboratories; however personal details will remain confidential.

Participant Rights
Participation in this research is voluntary and you are free to withdraw at any time without prejudice. You can withdraw for any reason and you do not need to justify your decision. If you withdraw from the study and you are an employee or student at UWA, or you are a scholarship holder at WAIS, this will not prejudice your status and rights as employee or student of UWA, or as an athlete of WAIS. If you do withdraw, we may wish to retain the data that we have recorded from you, but only if you agree, otherwise your records will be destroyed.
If you have any questions concerning this research, please feel free to contact the researchers listed below:

**PhD Candidate:**
Miss Rachel McCormick. Sport Science, Exercise and Health. School of Human Sciences. The University of Western Australia. P: (+61) 424 811 850, E: 21137148@student.uwa.edu.au

**Primary Student Research Supervisor:**
Associate Professor Peter Peeling. Sport Science, Exercise and Health. School of Human Sciences. The University of Western Australia. P: (+61) 410 667 532, E: peter.peeling@uwa.edu.au

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Daily vs. Alternate day oral iron supplementation for athletes.

Participant Information Form

Purpose
Oral iron supplementation is typically the first avenue of iron replacement therapy, beyond diet. Recent studies have shown that alternate day iron supplementation may be as effective as supplementing every day at increasing iron levels with a lower incidence of gastrointestinal side-effects. This project aims to assess the efficacy of daily and alternate day iron supplementation in iron deficient athletes to determine the best strategy to restore and maintain an athlete’s iron stores.

Methods
This investigation will involve an eight-week supplementation period. After we have gained your consent to participate in the study, you will need to have a blood screening to assess your iron status, and you will complete a Physical Activity Readiness Questionnaire (PAR-Q). If your iron levels fall within our range, and if you meet the standards set on the PAR-Q, you will become eligible for this study.

Eligible participants will be assigned to one of two groups: (1) a treatment group that will supplement daily or (2) a second treatment group that supplements on alternate days, for 8 weeks. Following group assignment, each participant will be required to attend a familiarisation session where you will be supplied with oral iron supplements and a training/nutrition journal for the subsequent 8-week training period. Afterwards, and prior to the supplementation period, you will be asked to attend the laboratory on two separate occasions to undertake a sub-maximal running economy test on a motorised treadmill and a haemoglobin mass test.

During the supplementation period, participants will be reminded to take their supplement via an automated reminder text in the morning or evening, and will be required to record the time-of-day they supplement in the diet and exercise journal that will be provided and completed daily. No inhibitors of iron absorption (specifically calcium, tea and coffee) are to be consumed for 60 minutes before and after taking each supplement. Participants will be required to record a measure of duration, distance, HR and a rating of perceived exertion (RPE) in their journal at the conclusion of each training session during the intervention period. Furthermore, at the beginning, middle and end of the intervention, you will also be asked to complete a comprehensive 4-day food diary (included in the journal that will be provided).
At the conclusion of every fortnight during the intervention, participants will provide a venous blood sample to obtain and track measures of iron status. At the conclusion of the intervention, participants will return to the laboratory to re-perform the running economy test, conducted as per pre-intervention.

**Risks and Side Effects**
There are no significant risks associated with this study. There is a minor risk of bruising and infection associated with all blood collection; however, venepuncture will be performed by a trained phlebotomist and all aseptic techniques will be implemented to maintain the sterility of the procedure to reduce any risk. Secondly, oral iron supplements may cause mild gastrointestinal discomfort for some individuals. The exercise-nature of the running economy tests may create a degree of musculoskeletal stress and fatigue however it should be noted that the exertion required throughout this test is no more demanding than your normal training or competition load. Additionally, there is a minor risk associated with the very low dose of carbon monoxide you will inhale for 2 minutes during the hemoglobin mass test, however the gas is not harmful in the quantities used for this procedure.

**Benefits**
*Individual:* By participating in this study, you may gain insight into the ideal strategy of iron supplementation to optimise iron stores and develop an awareness of your body’s iron status and how it may influence performance. Furthermore, you will gain free iron status blood screenings, hemoglobin mass values and be supplied with 8 weeks’ worth of oral iron supplements.

*Community:* Although the impact of this project is targeted primarily at endurance runners, it is highly likely the outcomes will be applicable to the majority of the athletic population. The outcomes of this project may assist to establish a simple supplementation strategy that could promote healthy iron status.

**Confidentiality**
Personal details and results from this testing program will be treated confidentially at all times. Individual data will not be identified, but collective results may be published in peer-reviewed scientific journals and other media outlets. No data will be stored on public computers. All data will be stored on the chief investigator’s computer in a secure location until the completion of this research program for a period of 7 years. Additionally; all collected blood samples will be stored at Sports Science, Exercise and Health, the School of Human Sciences, until the completion of the study. These samples will then be sent for analysis at various pathology laboratories; however personal details will remain confidential.

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All research participants are entitled to retain a copy of any Participant Information Form and/or Participant Consent Form relating to this research project.
Daily vs. Alternate day oral iron supplementation for athletes.

Participant Information Form

Purpose
Oral iron supplementation is typically the first avenue of iron replacement therapy, beyond diet. A transdermal iron supplement has recently been developed to avoid the gastrointestinal side-effects associated with oral iron supplements. This project aims to assess the efficacy of a transdermal iron patch relative to daily and alternate day oral iron supplementation in iron deficient athletes to determine the best strategy to restore and maintain an athlete’s iron stores.

Methods
This investigation will involve an eight-week supplementation period. After we have gained your consent to participate in the study, you will need to have a blood screening to assess your iron status, and you will complete a Physical Activity Readiness Questionnaire (PAR-Q). If your iron levels fall within our range, and if you meet the standards set on the PAR-Q, you will become eligible for this study.

Eligible participants will be assigned to one of three groups; (1) a treatment group that will supplement daily with oral iron supplements, (2) a second treatment group that supplements with oral iron supplements on alternate days or (3) a third treatment group that supplements with a transdermal patch daily, for 8 weeks. Following group assignment, each participant will be required to attend a familiarisation session where you will be supplied with oral or transdermal iron supplements and a training/nutrition journal for the subsequent 8-week training period. Afterwards, and prior to the supplementation period, you will be asked to attend the laboratory on two separate occasions to undertake a graded exercise test on a motorised treadmill and a haemoglobin mass test.

During the supplementation period, participants will be reminded to take/wear their supplement via an automated reminder text in the morning or evening, and will be required to record the time-of-day they supplement in the diet and exercise journal that will be provided and completed daily. No inhibitors of iron absorption (specifically calcium, tea and coffee) are to consumed for 60 minutes before and after taking each supplement. Participants will be required to record a measure of duration, distance, HR and a rating of perceived exertion (RPE) in their journal at the conclusion of each training session during the intervention period. Furthermore, at the beginning, middle and end of the intervention, you will also
be asked to complete a comprehensive 4-day food diary (included in the journal that will be provided).

At the conclusion of every fortnight during the intervention, participants will provide a venous blood sample to obtain and track measures of iron status. At the conclusion of the intervention, participants will return to the laboratory to re-perform the running economy test, conducted as per pre-intervention.

Risks and Side Effects
There are no significant risks associated with this study. There is a minor risk of bruising and infection associated with all blood collection; however, venepuncture will be performed by a trained phlebotomist and all aseptic techniques will be implemented to maintain the sterility of the procedure to reduce any risk. Secondly, oral iron supplements may cause mild gastrointestinal discomfort for some individuals. There is also a minor risk of skin irritation from the transdermal patches, however they have been specifically designed to stay on your skin for up to 12 hours without causing discomfort. The exercise-nature of the running economy tests may create a degree of musculoskeletal stress and fatigue however it should be noted that the exertion required throughout this test is no more demanding than your normal training or competition load. Additionally, there is a minor risk associated with the very low dose of carbon monoxide you will inhale for 2 minutes during the hemoglobin mass test, however the gas is not harmful in the quantities used for this procedure.

Benefits
Individual: By participating in this study, you may gain insight into the ideal strategy of iron supplementation to optimise iron stores and develop an awareness of your body’s iron status and how it may influence performance. Furthermore, you will gain free iron status blood screenings, hemoglobin mass values and be supplied with 8 weeks’ worth of oral iron supplements.

Community: Although the impact of this project is targeted primarily at endurance runners, it is highly likely the outcomes will be applicable to the majority of the athletic population. The outcomes of this project may assist to establish a simple supplementation strategy that could promote healthy iron status.

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A.3 Participant Consent Forms

Athlete Iron Consumption: Timing is everything – but when is best?

Participant Consent Form

I __________________________________________ (the participant), have read the information provided and any questions I have asked have been answered to my satisfaction. I agree to participate in this investigation, realizing that I may withdraw at any time without reason and without prejudice.

I understand that all identifiable (attributable) information that I provide is treated as strictly confidential and will not be released by the investigator in any form that may identify me. The only exception to this principle of confidentiality is if documents are required by law.

I have been advised as to what data is being collected, the purpose for collecting the data, and what will be done with the data upon completion of the research.

I agree that research data gathered for the study may be published provided my name or other identifying information is not used.

Participant Signature __________________________ Date ________________

Approval to conduct this research has been provided by the University of Western Australia, in accordance with its ethics review and approval procedures. Any person considering participation in this research project, or agreeing to participate, may raise any questions or issues with the researchers at any time. In addition, any person not satisfied with the response of researchers may raise ethics issues or concerns, and may make any complaints about this research project by contacting the Human Ethics Office at the University of Western Australia on (08) 6488 3703 or by emailing to humanethics@uwa.edu.au. All research participants are entitled to retain a copy of any Participant Information Form and/or Participant Consent Form relating to this research project.
Daily vs. Alternate day oral iron supplementation for athletes.

Participant Consent Form

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Transdermal vs. oral iron supplementation for athletes.

Participant Consent Form

I ___________________________ (the participant), have read the information provided and any questions I have asked have been answered to my satisfaction. I agree to participate in this investigation, realizing that I may withdraw at any time without reason and without prejudice.

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Participant Signature ___________________________ Date ___________________________

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Appendix B

Raw Data
B.1 Raw Data Sets

All raw data collected and analysed throughout this thesis is available via the University of Western Australia’s open access Research Repository, under the heading “Towards an understanding of the interaction between exercise and dietary iron absorption: Refining treatment strategies for iron deficient athletes.”

Link: https://research-repository.uwa.edu.au

Thank you to anyone who has made it to this point 😊