Single versus Split Dose of Iron Optimizes Hemoglobin Mass Gains at 2106 m Altitude

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ABSTRACT

HALL, R., P. PEELING, E. NEMETH, D. BERGLAND, W. T. P. MCCLUSKEY, and T. STELLINGWERFF. Single versus Split Dose of Iron Optimizes Hemoglobin Mass Gains at 2106 m Altitude. *Med. Sci. Sports Exerc.*, Vol. 51, No. 4, pp. 751–759, 2019. **Purpose**: To determine if a single versus a split equivalent daily dose of elemental iron was superior for hemoglobin mass (Hbmass) gains at altitude while minimizing gastrointestinal (GI) discomfort. **Methods**: Twenty-four elite runners attended a 3.1 ± 0.3 wk training camp (Flagstaff, AZ; 2106 m). A two-group design, randomized and stratified to baseline Hbmass, sex, and ferritin (>30 $\mu \cdot L^{-1}$), was implemented daily as: 1) single dose of 1 × 200 mg (PM only, SINGLE) versus 2) split dose of 2 × 100 mg (AM and PM; SPLIT) elemental iron (ferrous fumarate). The Hbmass and venipuncture assessments were completed upon arrival and departure (±2 d) from camp for ferritin, hepcidin, and erythroferrone (ERFE) concentrations. Validated food frequency, GI distress, menstrual blood loss (MBL) and training questionnaires were implemented throughout. Univariate analysis was used to compare Hbmass, with baseline ferritin, dietary iron intake, MBL, and training volume used as covariates. **Results**: Both conditions increased Hbmass from baseline ($P < 0.05$), with SINGLE (867.3 ± 47.9 g) significantly higher than SPLIT (828.9 ± 48.9 g) ($P = 0.048$). The GI scores were worse in SINGLE for weeks 1 and 2 combined (SINGLE, 18.0 ± 6.7 points; SPLIT, 11.3 ± 6.9 points; $P = 0.025$); however, GI scores improved by week 3, resulting in no between-group differences ($P = 0.335$). Hepcidin significantly decreased over time ($P = 0.043$) in SINGLE, with a nonsignificant decrease evident in SPLIT (~22%). ERFE significantly decreased in both groups (~28.5%; $P < 0.05$). No between-group differences existed for ERFE, hepcidin, food frequency, MBL, or daily training outcomes ($P > 0.05$). **Conclusions**: A single nightly 200-mg dose of elemental iron was superior to a split dose for optimizing Hbmass changes at altitude in runners over an approximately 3-wk training camp. **Key Words:** SUPPLEMENTATION, ERYTHROPOIESIS, ELITE RUNNERS, MIDDLE-DISTANCE, LONG-DISTANCE

Iron is arguably the most important dietary mineral for endurance athletes, being a crucial constituent of hemoglobin and myoglobin, in addition to playing important roles in the production of adenotriphosphate and oxidative phosphorylation. Iron is of such importance in processes relevant to the aerobic capacity of humans, that left untreated, late-stage iron deficiency (i.e., anemia) has been associated with reduced maximal oxygen consumption ($\dot{V}O_{2max}$) and physical performance (1,2). As such, for endurance athletes, whose primary goal it is to maximize the aerobic phenotype through training interventions, optimizing iron status, despite its many challenges, is paramount. Despite this, the maintenance of sufficient iron stores is a well-known challenge for endurance athletes, likely a result of their regular encounters with established exercise-induced avenues of iron loss, such as sweating, gastrointestinal (GI) tract bleeding, hemolysis (from foot strike and muscle contraction), menstruation, injury, illness and the potential impact of poor energy availability (2–4). Accordingly, iron deficiency is thought to be as high as 15% to 25% in adolescent and adult female athletes (1,2). Additionally, the resolution of iron deficiency is further complicated by the iron regulatory hormone hepcidin, which responds in a homeostatic manner to regulate iron uptake. A rise in hepcidin levels degrades ferroportin exporters in the gut and on the cell surface of macrophages, thereby reducing iron absorption and iron recycling, respectively (5). Hepcidin has also been shown to increase with the acute inflammatory response to endurance exercise in a duration and intensity dependent manner, with elevated levels persistent for 3 to 6 h postexercise (6,7). Such factors should be considered when athletes are consuming iron as part of their diet and/or supplement regime.

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Optimizing iron status is especially paramount for those athletes undertaking altitude training in the pursuit of hemoglobin mass gains (8). Training at altitudes between 2000 and 2500 m is a strategic attempt to stimulate erythropoiesis and increase hemoglobin concentration (9–11), leading to enhanced $\dot{V}O_{2max}$ and likely competitive performance (12). It is well documented that athletes may achieve an approximate 1.1% increase in hemoglobin mass (Hbmass) for every 100 h of hypoxic exposure over a minimum of 2 wk (9,13), assuming adequate body iron stores are available to optimize the hematological and physiological adaptions to altitude (1,2,12). Interestingly, chronic altitude exposure has been shown to decrease baseline hepcidin levels in athletes, a homeostatic response initiated as a result of the increased need for iron in an attempt to adapt to the hypoxic environment (14). This may be mediated by erythroferrone (ERFE), a hormone produced by erythroid precursors, which functions as a suppressor of the hepatic hepcidin production (15).

Iron supplementation is often required to optimize the individual's iron storage pool and to enhance hematological adaptation to altitude training. However, iron supplementation adds further challenges, as iron acutely increases hepcidin levels as a feed-forward mechanism (16). A recent study by Moretti et al. (16) conducted at sea level in sedentary iron-depleted, nonanemic females showed that an increase from 40 to 240 mg of elemental iron, given in a single supplemental dose, decreased the fractional percentage of iron absorbed from 19.6% to 11.8%, respectively, which coincided with a concurrent increase in circulating hepcidin levels. Of note, the absolute amount of iron absorbed in this study remained much greater when the 240-mg dose was consumed (~28.2 mg vs ~7.9 mg in the 40-mg dose). However, it is important to consider the potential practical implications of high-dose iron supplementation, because higher doses have been associated with negative side effects, such as nausea, increased gastric discomfort, constipation and/or diarrhea, which may negatively impact on iron supplementation compliance and ultimately iron status (16).

Optimal iron supplementation dose and its impact on moderate altitude adaptation (1300–3000 m) has been previously explored via the compilation of longitudinal data, across a multitude of athlete cohorts (11). Here, greater gains in Hbmass at altitude with a single daily dose of 210 mg of elemental iron (+4.0% Hbmass) versus 105 mg (+3.3% Hbmass) versus no iron (+1.1% Hbmass) were reported (11). However, no intervention-based studies have examined the impact of daily periodization of iron supplementation at altitude, and whether single or multiple daily doses of the same absolute amount is superior. To this end, the current study implemented a randomized group design to investigate the impact of two different approaches to iron supplementation on Hbmass, GI tolerance and hepcidin responses. Here, a split dose (2 × 100 mg daily) versus a single dose (1 × 200 mg daily) of elemental iron provided over a 3-wk camp at 2106 m natural altitude was explored. Our *a priori* hypotheses were as follows: 1) the single iron dose would produce greater gains in Hbmass, 2) that hepcidin levels would decrease across the intervention as a result of altitude exposure and despite iron supplementation, and 3) the single dose would have lesser reported GI symptoms at each recorded time point.

METHODS

Participants

Twenty-six (n = 8 male and n = 16 female) elite middle- and long-distance runners volunteered to take part in this study taking place at Flagstaff, AZ (3.1 ± 0.3 wk at 2106 m in April 2017; Table 1). Flagstaff sits at 2106 m above sea level and when combined with length of altitude exposure can be used to calculate kilometers per hour (9). This group had an average International Association of Athletics Federation (IAAF) score of approximately 1100 points, which translates to 13:30 and 15:32 for men's and women's 5000-m times, respectively (six Olympian and/or World Championship team members). Athletes were eligible if attending the altitude camp for at least 15 d. Exclusion criteria included athletes that presented ill or injured at study commencement and or baseline serum ferritin <30 $\mu g \cdot L^{-1}$, of which two female athletes were excluded due to low serum ferritin (N = 24 for final analysis). After both written and verbal instruction and information on the study, participants signed informed consent to participate. Ethics approval for this study was obtained through the Human Research Ethics Board of the University of Victoria, BC, Canada (protocol number 17-109) in accordance with the Declaration of Helsinki.

Experimental Overview

A two-group design, randomized and stratified to baseline Hbmass, sex, and ferritin concentration was implemented as:

1. Single dose of 1 × 200 mg elemental iron (between 9:00 and 10:00 PM only, SINGLE, n = 11) versus;
2. Split dose of 2 × 100 mg elemental iron daily (100-mg dose between 7:00 and 8:00 AM and 100-mg dose between 9:00 and 10:00 PM; SPLIT, n = 13).

Randomization and stratification of groups was completed by a blinded computer script. Hbmass and venipuncture

TABLE 1. Baseline athlete characteristics and FFQ questionnaire scores (N = 24).

Group	SPLIT (n = 13; 9 F, 4 M)	SINGLE (n = 11; 7 F, 4 M)
Athlete characteristics		
Age (yr)	24.6 ± 2.5	24.3 ± 1.7
Stature (cm)	170 ± 10	170 ± 1 0
Mass (kg)	60.6 ± 7 .8	62.7 ± 5.9
IAAF score	1102.5 ± 48.3	1116.2 ± 49.8
Serum ferritin ($\mu g \cdot L^{-1}$)	82.9 ± 42.9	95.4 ± 47.9
Hepcidin ($ng \cdot mL^{-1}$)	25.3 ± 23.2	21.6 ± 9.0
Erythroferrone ($ng \cdot mL^{-1}$)	20.3 ± 4.9	25.8 ± 8.5
FFQ questionnaire		
Dietary iron intake (mg)	22.4 ± 6.0	28.5 ± 11.3

Dietary iron intake refers to iron intake from habitual food intake only in the 3 months before camp as reported in a baseline FFQ questionnaire.
Data are presented as geometric mean (±95% CI) for Hepcidin, and as mean (± SD) for all other variables.
F, female; M, male.

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procedures were completed upon arrival and departure from the training camp for ferritin, hepcidin and ERFE concentrations. Validated food frequency (FFQ), GI comfort, menstrual blood loss (MBL), and training questionnaires were implemented throughout.

Iron Supplementation Protocol

Upon immediate arrival to altitude, and following baseline blood tests, all athletes were provided with ferrous fumarate (Palafer™) with the instructions to consume a 1 × 100 mg elemental iron dose at 9:00 to 10:00 PM until group stratification and randomization could occur (baseline blood ferritin and Hbmass values could be assessed). Athletes were assigned a group and commenced their group-specific iron dosing protocol within 2.5 ± 0.9 d of baseline blood collection. All athletes were asked to avoid caffeine, calcium supplements or calcium-rich dairy foods within an hour of scheduled supplementation to maximization iron absorption. To ensure supplement compliance, athletes were sent reminders for every supplemental dose and required to confirm back via phone text message throughout the entire study (reported compliance was 98.9%).

Blood Collection and Analysis

Within 1.2 ± 0.4 d of arrival to altitude, athletes had baseline bloods drawn in an overnight fasted and rested state, without iron supplementation in the 24 h prior. Additionally, Hbmass was assessed using the optimized 2 min CO rebreathing technique (17,18). In brief, subjects rebreathed with a nose clip via closed circuit spirometry a dose of CO based on body mass (BM) (males: 1.25 mL·kg^{-1} BM; females: 1.00 mL·kg^{-1} BM) coupled with ~4 L pure oxygen for 2 min. Detection of possible leaks was conducted by a portable CO meter (FLUKE CO-220, Everett, WA). Determination of %HbCO was measured at baseline, plus 6 and 8 min after rebreathing, from capillary fingertip blood samples tested with OSM3 hemoximeter (Radiometer, Copenhagen, Denmark). Hbmass was calculated from the mean change in %HbCO before and after CO rebreathing. All Hbmass measurements were conducted by the same technician in Flagstaff, AZ, with typical laboratory error for Hbmass being 1.9%. The postcamp blood testing was 22 ± 1.9 d later, featuring venipuncture bloods taken, again in the fasted and rested state with the same blood analysis procedures completed.

The venipuncture blood draws were completed by trained phlebotomists for the measurement of ferritin, total iron, iron saturation, transferrin, total iron binding capacity (TIBC), unsaturated iron binding capacity (UIBC), red blood cell (RBC) count, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean cell hemoglobin concentration (MCHC), hemoglobin, hematocrit, hepcidin, ERFE, and C-reactive protein (CRP). All whole blood samples were centrifuged for 10 min at 3000 rpm onsite and then transported on dry ice for analysis. The ferritin and total iron were measured via commercial medical laboratory analysis in Flagstaff using standard procedures (Laboratory Corporation of America, Phoenix, AZ). Blood serum samples collected for ERFE and Hepcidin (only measured on n = 14 participants; 5 mL) were stored in 1-mL aliquots at −80°C before batch analysis. Hepcidin was measured using Intrinsic Hepcidin IDx™ ELISA Kit (Intrinsic LifeSciences LLC, La Jolla, CA) according to the manufacturer's instructions. Erythroferrone was measured using sandwich ELISA as described previously (19).

Questionnaires

Upon arrival, and throughout the intervention, athletes were asked to complete a series of questionnaires. These included:

FFQ questionnaire. A Canadian validated FFQ to qualify iron intake (20) was used to ascertain habitual dietary iron intake from both dietary and supplemental means. The FFQ was completed within the first few days of arrival. Athletes were asked to reflect on habitual intake in the 3 months before camp, as reflective of the lifespan of a red blood cell. The FFQ also asked athletes to list all sports foods and supplements used.

GI questionnaire. The athlete-specific GI questionnaire, used extensively in endurance-trained subjects and explained elsewhere (21), was instructed at baseline to be completed as a reflection of GI symptoms (not occurring in or immediately posttraining) in the week before arrival to camp. The questionnaire asked athletes to rate 10 different upper and lower GI symptoms and three non–GI-specific symptoms as possible dehydration, on degree of severity on a 10-point scale. After baseline GI assessment, follow-up questionnaires were completed at the conclusion of each week spent at altitude (i.e., end of week 1, week 2, and week 3; specifically reflecting on GI symptoms over the prior 7 d). Weeks 1 and 2 GI questionnaire scores were combined (summed) due to the differing arrival and departure dates of athletes on individual schedules.

MBL questionnaire. The MBL questionnaire, previously validated for ages 18 to 35 yr, was administered at baseline with instructions for completion (22). The questionnaire equates a score based on recorded use of different female sanitary items during the heaviest days of bleeding as it relates to the number of total days of menstruation. Female participants were asked to complete the MBL questionnaire on conclusion of their period while on camp or the day of postcamp venipuncture blood draws (whichever came prior). If no menses occurred, female athletes were to return the form stating they did not menstruate during the camp.

Training Analysis

Athletes were required to submit their training plans to the researchers, reporting any significant differences from planned to actual training. These submitted training plans were then analyzed, coded, and split into the number of training minutes in zone 1 (recovery/easy), zone 2 (threshold/steady-state), or

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zone 3 ($\dot{V}O_{2max}$ or faster) for each individual athlete. The number of prescribed running kilometers and track interval distances were also used to back-estimate total training volumes in distance as well as in minutes.

Statistical Analysis

Normal distribution of the data was initially assessed via Shapiro–Wilk tests. Hepcidin data showed nonnormal distribution and were therefore log-transformed before analysis. Hepcidin data were then back-transformed postanalysis via antilog calculation, allowing presentation of geometric mean ± 95% confidence interval (23). All other data are presented as mean ± standard deviation. A univariate analysis was conducted to explore the impact of the supplement protocol and altitude exposure on postexercise Hbmass; postcamp results are reported as model estimates. The model explored group and sex interactions, with the covariates of BM, baseline ferritin, baseline Hbmass, training metrics, MBL, and iron supplement days accounted for. Additionally, repeated-measures ANOVA for within-group (time) effects were conducted on all variables measured preexercise to postexercise and on the questionnaire data. *Post hoc* paired samples *t* tests were conducted in the event of significant time effects. A fixed factor of condition (SPLIT vs SINGLE) was used to differentiate between groups, with independent samples *t* tests used to explore significant group effects. Spearman's correlation was used to assess the relationship between serum ferritin and resting hepcidin. Because untransformed hepcidin data was used here, the data of one male athlete with (relatively) high serum ferritin and hepcidin levels was removed to avoid spurious correlations; therefore, correlation analysis is conducted on $n = 13$. The alpha level was set at $P < 0.05$ for all analyses.

RESULTS

Total altitude exposure over the camp equated to 520.8 ± 5.0 h or 1096.8 km·h.

There were no significant differences in baseline Hbmass between the two groups ($P = 0.613$; Fig. 1A). Both the SPLIT ($P = 0.001$) and SINGLE ($P = 0.007$) groups experienced a significant increase in Hbmass across the 3-wk camp; however, postcamp measures were significantly greater in SINGLE versus SPLIT ($P = 0.048$; Fig. 1A). Percentage change scores were calculated to demonstrate individual Hbmass response (Fig. 1B).

No between-group differences at baseline (PRE; Table 1) or postcamp (POST) time points were evident for serum ferritin (PRE, $P = 0.510$; POST, $P = 0.750$; Table 2), hepcidin (PRE, $P = 0.718$; POST, $P = 0.384$, Fig. 2A) or ERFE (PRE, $P = 0.157$; POST, $P = 0.714$, Fig. 2B). Further, no between-group differences were evident at baseline for FFQ scores (dietary iron, $P = 0.106$; Table 1). Serum ferritin within the SPLIT group was significantly higher at the POST camp time point ($P = 0.013$; Table 2).

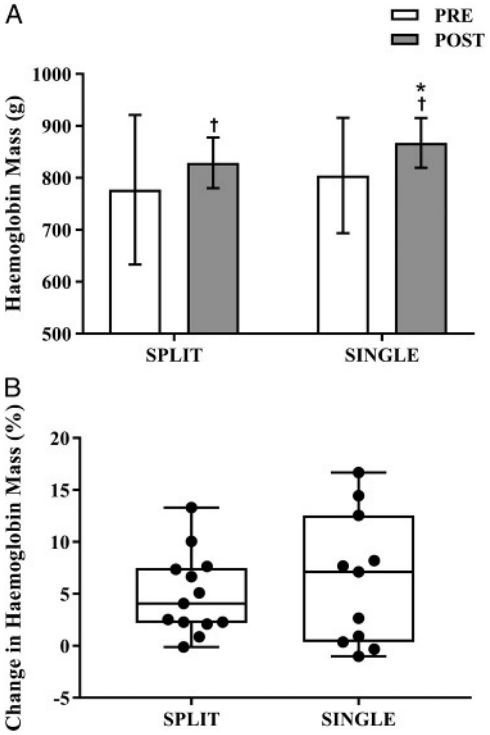

FIGURE 1—Hemoglobin mass (Hbmass) measures collected prealtitude and postaltitude camp in groups supplemented with either 1 × 200 mg iron supplement per day (SINGLE) or 2 × 100 mg iron supplement per day (SPLIT). (A) Hbmass model estimate values are presented as mean (±SD). Hbmass individual responses from PRE to POST are presented as percentage change scores (B). *Statistically significant between-group difference at postcamp time point ($P = 0.048$). †Statistically significant within-group difference at postcamp time point (SPLIT $P = 0.001$, SINGLE $P = 0.007$).

Within-group hepcidin levels showed a significant decrease in the SINGLE group (~32%; $P = 0.043$), but only a nonsignificant decrease was evident in the SPLIT group (~22%; $P = 0.214$; Fig. 2A). Within groups, ERFE significantly decreased across the camp in both the SPLIT (~29%; $P = 0.018$) and SINGLE (~35%; $P = 0.047$) supplement groups (Fig. 2B).

Hemoglobin, RBC, and hematocrit significantly increased in both groups from PRE to POST camp measures (hemoglobin SPLIT, $P < 0.001$; SINGLE, $P < 0.001$; RBC SPLIT, $P < 0.001$; SINGLE, $P = 0.002$; hematocrit SPLIT, $P < 0.001$; SINGLE, $P = 0.002$). There were no significant differences within or between groups for TIBC, UIBC, serum iron, iron saturation, serum transferrin, MCV, MCH, or MCHC. A moderate positive correlation ($r = 0.593$, $P = 0.033$) was evident between baseline serum ferritin and resting hepcidin PRE camp, and a weak positive correlation ($r = 0.294$; $P = 0.329$) was evident POST camp.

In addition to blood variables, the GI distress questionnaire scores (Fig. 3) were greater in SINGLE compared with SPLIT at weeks 1 and 2 combined ($P = 0.025$); however, SINGLE GI distress scores significantly decreased by week 3 ($P = 0.004$), with no difference between SPLIT and SINGLE groups evident at week 3 ($P = 0.335$).

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TABLE 2. Group means for iron markers, complete blood count and CRP.

	SPLIT		SINGLE	
	Prealtitude	Postaltitude	Prealtitude	Postaltitude
Iron markers				
TIBC ($\mu g \cdot dL^{-1}$)	298.5 ± 44.2	306.9 ± 36.8	312.7 ± 31.2	307.2 ± 38.2
UIBC ($\mu g \cdot dL^{-1}$)	215.8 ± 62.4	206.1 ± 64.0	210.4 ± 52.1	211.0 ± 34.5
Serum iron ($\mu g \cdot dL^{-1}$)	82.7 ± 38.0	100.8 ± 33.8	101.5 ± 36.8	96.2 ± 24.6
Iron saturation (%)	28.5 ± 14.2	33.9 ± 13.4	33.2 ± 13.4	31.4 ± 7.0
Hemoglobin ($g \cdot dL^{-1}$)	14.5 ± 0.7	15.6 ± 0.8[a]	14.4 ± 0.9	15.4 ± 1.2[a]
Serum ferritin ($ng \cdot mL^{-1}$)	82.9 ± 42.9	94.6 ± 45.4[a]	95.4 ± 47.9	100.2 ± 37.2
Serum transferrin ($mg \cdot dL^{-1}$)	252.6 ± 39.2	253.2 ± 29.4	262.5 ± 30.7	251.4 ± 29.9
Complete blood count				
RBC count (×10E6/μL)	4.68 ± 0.33	4.98 ± 0.37[a]	4.66 ± 0.36	4.92 ± 0.38[a]
Hematocrit (%)	43.7 ± 1.9	46.6 ± 2.2[a]	43.4 ± 2.8	46.1 ± 3.0[a]
MCV (fL)	93.8 ± 4.1	93.9 ± 3.7	93.4 ± 2.5	93.8 ± 3.5
MCH (pg)	31.2 ± 1.2	31.4 ± 1.3	30.9 ± 1.2	31.4 ± 1.1
MCHC ($g \cdot dL^{-1}$)	33.1 ± 0.7	33.5 ± 0.5	33.9 ± 2.7	33.5 ± 0.7
Other				
CRP ($mg \cdot L^{-1}$)	2.7 ± 6.6	3.1 ± 8.1	0.9 ± 2.1	0.3 ± 0.1

[a]Statistically significant within-group difference at postcamp time point from baseline.
Data reported as mean (± SD).

Finally, the collated training data (minutes per day $P = 0.572$, kilometers per week $P = 0.611$ or intensity $P = 0.864$) and MBL ($P = 0.169$) showed no differences between groups (Table 3).

DISCUSSION

To our knowledge, this is the first study to examine the effects of within day iron supplement periodization on Hbmass changes and GI disturbance over a 3-wk altitude camp in elite-level endurance athletes. Our findings show that both SPLIT (2 × 100 mg, AM and PM) and SINGLE (1 × 200 mg, PM) groups had significantly increased hemoglobin concentrations and Hbmass at the conclusion of the camp. However, the SINGLE nightly dose group observed a greater Hbmass increase (+6.7% ± 6.3%; $P = 0.048$) compared to SPLIT (+4.6% ± 3.9%). Of note, there was a 37% increase in the overall GI distress score associated with the SINGLE versus SPLIT dose over weeks 1 and 2 combined. This difference suggests the athletes may have gut-adapted to the acute SINGLE dose iron protocol over the initial 2-wk period as no difference between groups was observed at week 3.

Hematological changes. Regardless of the iron supplement protocol, there was a significant increase in Hbmass postaltitude camp (collapsed group outcome: +5.6% ± 5.1%; Fig. 1), which aligns with previous altitude literature in elite runners (13,24,25). In terms of collated group results, this study compares very well to the predictive value of 5.7% derived from the equation by Gore et al. (13) based on a 1.1% increase in Hbmass for every 100 h spent at altitude, as

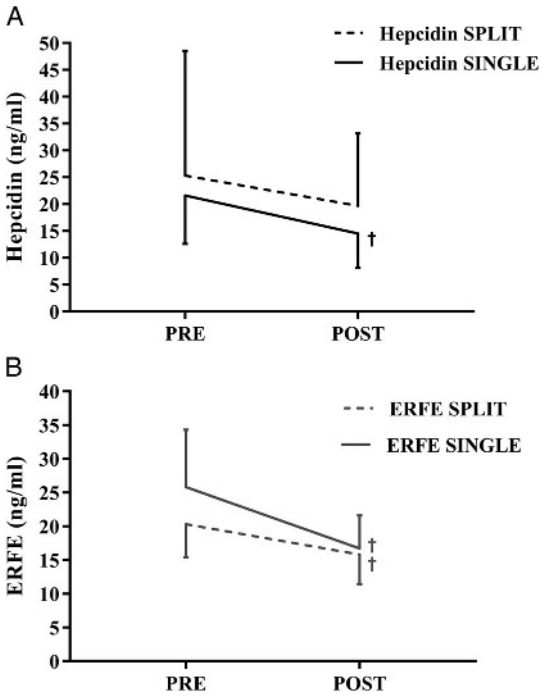

FIGURE 2—Hepcidin (A) and ERFE (B); measures from PRE to POST camp in SPLIT (2 × 100 mg) group compared to SINGLE (1 × 200 mg) group. Data are presented as geometric mean (±95% CI) for Hepcidin, and as mean (± SD) for ERFE. *Statistically significant within-group difference at postcamp time point for hepcidin SINGLE ($P = 0.043$). †Statistically significant within-group difference at postcamp time point from baseline for ERFE (SPLIT $P = 0.018$, SINGLE $P = 0.047$).

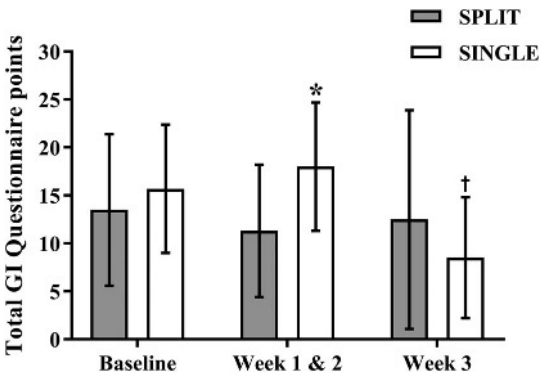

FIGURE 3—Mean GI distress questionnaire scores at baseline, weeks 1 and 2 combined and week 3. All data presented as mean (± SD). SPLIT represented as filled bars and SINGLE represented as open bars. *Statistically significant between-group difference at weeks 1 and 2 combined ($P = 0.025$). †Statistically significant within-group difference at week 3 from weeks 1 and 2 combined for SINGLE ($P = 0.004$).

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TABLE 3. Group means for training data and MBL scores across 3-wk camp period.

	SPLIT	SINGLE
Training data		
Minutes per day	54.9 ± 15.8	51.1 ± 17.5
Kilometers per day	11.8 ± 3.6	11.0 ± 4.3
Intensity	4.7 ± 0.5	4.7 ± 0.5
Kilometers per week	82.6 ± 25.1	76.8 ± 29.9
MBL score		
MBL score, units	0.3 ± 0.6	4.5 ± 10.7

Data reported as mean (± SD).

compared to the predicted 4.1% increase by the kilometers per hour metric proposed by Garvican-Lewis et al. (9). However, it is important to note the dangers of using such predictive equations, because individual variation to the altitude stimulus has been well documented (Fig. 1B), and the equations themselves have their limitations (26). Both equations were also developed utilizing moderate altitude doses over ~3 to 6 wk hypoxic durations, and thus would probably not be adequate for extreme altitudes and durations.

Although iron supplementation is not the cause of the observed Hbmass increases at altitude, optimized iron availability at altitude is crucial for erythropoiesis. A retrospective study by Govus et al. (11) on 178 athletes attending altitude camps at varying moderate altitudes (1300–3000 m) demonstrated significantly greater Hbmass gains with daily 210 mg (+4.0%), compared to both 105 mg (+3.3%) and 0 mg (+1.1%) of elemental iron supplementation, supporting the notion of greater Hbmass changes over the same period when sufficient iron is available for adaptation (11). Our data demonstrate greater Hbmass gains with 200 mg of elemental iron compared with Govus et al. (11), which may be explained by a greater around-the-clock hypoxic stimulus in this study compared with the majority of athletes in the Govus study only spending 14 h of 24 $h \cdot d^{-1}$ in a simulated altitude house. Alternate suggestions include better supplement compliance (we demonstrated a 98.9% compliance rate) compared with no reported compliance record in the Govus study, and a greater proportion of females in our study; as females tend to show a greater percent change in Hbmass due to lower relative baseline measures. Furthermore, it is well established in the literature that a threshold elevation of >2000 m is required to see increases in erythropoietin (11,26). Despite variations in elevations across the iron groups, Govus and colleagues (11) demonstrated similar serum ferritin responses as those seen in the current study, reporting increases in serum ferritin when 210 mg of elemental iron was consumed (+36.8%) versus a decline in both the 105 mg (−13.8%) and 0 mg (−33.2%) groups. In the current study, both the SPLIT and SINGLE groups, consuming 200 mg of elemental iron per day, had increases in serum ferritin at the POST camp time point (SPLIT, +14.1%; SINGLE, +5.0%), although only the SPLIT group's increase reached significance ($P = 0.013$; Table 2), which suggests iron was not limiting to Hbmass increases. Taken collectively, the prealtitude to postaltitude camp ferritin outcomes of both Govus et al. (11) and the current study suggest that supplementing athletes with 200 mg of elemental iron per day (when exposed to ~2000 m of altitude) will likely optimize hematological adaptation to the stimulus, as measured by Hbmass outcomes (11,27).

Iron and hepcidin outcomes. Despite the fact that both groups received 200 mg of elemental iron daily, the distribution of iron provision was different throughout the day. Therefore, within-day iron availability may be the key in explaining the between-group differences in Hbmass change. At both sea level and altitude, the iron regulatory hormone, hepcidin, has been shown to increase in response to iron supplementation (16,28), exercise stress (6,7,29), inflammation (30), and/or underlying iron stores (31). An acute rise in hepcidin levels are known to result in decreased iron bioavailability through degradation of ferroportin; an iron exporter found in enterocytes in the gut and expressed on macrophages and hepatocytes (32,33). Accordingly, the frequency of iron supplementation, and when that iron is taken in relation to the daily training session, might have profound effects on subsequent iron bioavailability. At sea level, studies by Moretti et al. (16) and Stoffel et al. (34) have shown that multiple daily doses of elemental iron results in a prolonged elevation of hepcidin and a greater period of decreased iron bioavailability. Indeed, this acute mechanism may help to explain the attenuated Hbmass increases seen in the SPLIT group of the current investigation, such that the daily hepcidin levels may have been greater in SPLIT versus SINGLE due to the greater frequency of iron intake (independent of dose). Stoffel et al. (34) observed exactly this at sea level with twice daily supplementation of 60-mg elemental iron resulting in a significant elevation in hepcidin levels ($P = 0.013$) over three consecutive days compared to a single dose of 120 mg daily. In support of this supposition, research by Schaap et al. (35) found that the diurnal rhythms of hepcidin increased across the day from 8:00 AM to 4:00 PM and that iron supplementation further magnified this observed diurnal increase (35). In another arm of the Stoffel et al. (34) study, alternate day versus consecutive single daily dosing with 60 mg of iron resulted in both greater fractional and total iron absorption, suggesting alternate day dosing may offer greater iron uptake and iron availability. To this end, the complex interactions between diurnal fluctuations, iron supplementation, exercise timing, and hypoxic stress, which all have an impact on hepcidin, require further insight for a greater understanding of the underlying mechanisms and the flow on effect on overall iron availability. Obviously, for mechanistic insights, it would have been ideal to have a time course (4 to 6 measures) of hepcidin response to iron supplementation throughout a single day in our subjects at the start and the end of the 3-wk altitude camp. However, given that our subject pool was elite-level athletes preparing for Olympic and National level competitions, such an approach was deemed too invasive for implementation here.

Although within-day hepcidin changes were not quantified here, resting hepcidin levels observed at the PRE and POST camp timepoints showed a decline in both groups by

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~22% to 33% (Fig. 3), concurring with other hypoxic-based published literature (14,36). Worthy of note, is the large hepcidin variability in the SPLIT group, which is due to one athlete with extremely elevated levels at both the PRE and POST camp measures; however, this athlete also had correspondingly high ferritin levels at the same time points. Interestingly, baseline hepcidin, measured in the fasted and rested state, has been shown to be a moderate predictor of iron bioavailability (32). Furthermore, a positive correlation has also been reported between baseline serum ferritin and the acute hepcidin response to exercise at sea level (31). In agreeance, the current study found a moderate positive correlation between serum ferritin and resting hepcidin levels at the PRE camp time point; however, only a weak correlation was observed in the POST camp measures. This outcome is interesting, because we also observed an overall increase in serum ferritin levels across the camp, a unique finding in combination with a decrease in hepcidin levels to our study. Contrary to our outcomes, Govus et al. (14) showed that the decrease in resting hepcidin levels was coupled with a small decrease in serum ferritin over a 14-d period when athletes were supplemented with (up to) 105 mg of elemental iron daily at 3000 m simulated hypoxia. The increase in serum ferritin seen in our investigation may be a result of the greater overall iron intake (200 mg), and the weak correlation between serum ferritin and hepcidin levels seen in the POST camp period may suggest that other signaling mechanisms are possibly acting as a stronger influence on hepcidin levels than that exerted initially by serum ferritin. Regardless, it is clear that further work is likely required to identify the driving mechanisms suggested here.

ERFE. A secondary finding of this study was identified from looking at an upstream iron-regulatory hormone, ERFE. An increase in ERFE reflects an increased need for iron by the erythron, and acts as a counterregulator of hepcidin by suppressing *HAMP* gene transcription in the liver (15,37). Though previously not explored in athletes at altitude, rodent data demonstrate that increases in ERFE can be used as a marker of erythropoiesis and subsequent suppression of the hepcidin response (15,37). Although it may be anticipated that with adaptation to the hypoxic environment erythropoiesis would continue across the 3-wk camp, thereby increasing ERFE over time, the results of this study observed a significant ERFE decrease in both SPLIT and SINGLE groups between the PRE and POST camp time points (SPLIT, $P = 0.018$; SINGLE, $P = 0.047$; Fig. 2B). Like hepcidin, we were unable to test for the acute within-day time course of ERFE before or immediately upon arrival, or departure, to altitude. However, it is possible that the 1.2 ± 0.4 d at altitude before the baseline bloods may have also meant that an initial acute ERFE response to the hypoxic stimulus was missed, because ERFE has been shown to increase acutely within hours in mice and humans injected with erythropoietin (19,38), followed by return to baseline levels after 1 d in mice or several days in humans. Erythroferrone has been observed to be greater in one study of pediatric patients with iron deficiency versus controls at sea level (39). The same study also demonstrated a negative correlation between ERFE and Hbmass; when Hbmass was observed to increase, ERFE declined ($P < 0.05$) (39). This would support the findings in our study and that of Kautz and Nemeth (5). However, there is a clear need for further research in this space, to understand the kinetics of ERFE changes during athlete adaptation to hypoxia at high altitude.

GI outcomes. The reported severity of GI symptoms was significantly greater in the SINGLE dose for weeks 1 and 2 combined, when compared to the SPLIT dose (Fig. 3). Despite higher GI-distress scores in the SINGLE group at weeks 1 and 2, it is important to note that even the worst scores were only 18 of a possible 126 points. Furthermore, we have consistently noticed that negative GI side effects associated with iron supplementation appear to be minimized during altitude training camps with athletes when compared with sea level, despite taking larger doses of 200 mg of elemental iron (*unpublished observations*), which might have a link to the body's increased need for iron to support erythropoiesis. Interestingly, results of this study also showed GI distress symptoms decreased in the SINGLE dose group by week 3, resulting in no between-group differences at this point in time. It has been reported in the literature that GI symptom onset to iron supplementation tends to present within days, potentially as a result of changes to gut microflora and/or free radical damage to the gut lumen from unabsorbed iron (40). The results of this study suggest a period of approximately 2 wk for adaptation to the SINGLE nightly supplementation protocol. It may also be practical to fashion a hybrid of the two protocols for those athletes who wish to maximize gut comfort yet still optimize Hbmass gains.

Limitations. There are always challenges in conducting research in elite athletes whose first priority was focusing on their altitude training camp. As such, every effort was made to reduce the research demand for the athletes, which inevitably results in some study limitations. These may include: no control group or cross-over trial; only single Hbmass measures collected at the PRE and POST timepoints; no comparison of pre to during camp training load; and, the use of questionnaires that rely on recall for completion. Due to athletes traveling from various home training locations it was also not possible to collect any additional data points that may have provided valuable further insight, such as blood samples at sea level before or on return from the camp. A desire to minimize disruption to the athletes' training meant additional acute measures during the camp (especially multiple intraday measures for both hepcidin and ERFE) were also not possible.

CONCLUSIONS

A significant increase in Hbmass was observed in both SPLIT and SINGLE iron supplement dosing protocol groups over an approximate ~3-wk training camp at 2106 m altitude. Though both groups received equivalent elemental iron daily (200 mg), the SINGLE nightly dose group observed greater Hbmass gains when compared to the SPLIT group.

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PRE to POST camp Hbmass measures can lead us only to cautiously speculate that the unobserved intraday hepcidin response may be one possible mechanism behind the observed difference between groups. A secondary finding of this study was the unexpected decline in resting ERFE across the camp in both groups when erythropoiesis was expected to be increasing. Although greater GI distress scores were associated with the SINGLE nightly dose over weeks 1 and 2, this research suggests some level of gut adaptation with no difference between groups at week 3. For athletes and coaches, our data suggest the implementation of a SINGLE 200 mg nightly dose of elemental iron for superior Hbmass outcomes at altitude compared with a SPLIT equivalent daily dose. However, it does not discount the use of the SPLIT protocol in those athletes with heightened GI sensitivity, as significant increases in Hbmass were observed in both supplemented groups.

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Conflicts of Interest: E. N. is a stockholder and consultant for Intrinsic LifeSciences and Silarus Therapeutics. The remaining authors have no conflicts of interest to declare.

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