Canadian Science Publishing
NRC Research Press journals | Publishing Services

Applied Physiology, Nutrition, and Metabolism
Physiologie appliquée, nutrition et métabolisme

Effects of Macro- and Micro- Nutrients on Exercise Induced Hepcidin Response in Highly-Trained Endurance Athletes

Journal:	*Applied Physiology, Nutrition, and Metabolism*
Manuscript ID	apnm-2017-0207.R1
Manuscript Type:	Article
Date Submitted by the Author:	22-May-2017
Complete List of Authors:	Dahlquist, Dylan; University of British Columbia, School of Kinesiology; Canadian Sport Institute - Pacific Stellingwerff, Trent; Canadian Sport Institute - Pacific; University of British Columbia, School of Kinesiology Dieter, Brad; Providence Health Care, Providence Sacred Heart Medical Center and Children's Hospital McKenzie, Donald; University of British Columbia, Division of Sports Medicine; University of British Columbia, School of Kinesiology Koehle, Michael; University of British Columbia, School of Kinesiology; University of British Columbia, Division of Sports Medicine
Is the invited manuscript for consideration in a Special Issue? :	
Keyword:	cycling < sports, inflammation < muscle damage, Iron Metabolism, Protein and Carbohydrates, Vitamin D

Effects of Macro- and Micro- Nutrients on Exercise Induced Hepcidin Response in Highly-Trained Endurance Athletes

Running Title: Nutrient Composition and Hepcidin in Highly-Trained Athletes

Author(s): Dylan T. Dahlquist[1,2], Trent Stellingwerff[1,2], Brad P. Dieter[4], Donald C. McKenzie[1,3] and Michael S. Koehle[1,3]

Location:

[1]UBC Environmental Physiology Laboratory, School of Kinesiology, University of British Columbia, Vancouver, British Columbia, Canada

[2]Canadian Sport Institute - Pacific, Victoria, British Columbia, Canada

[3]Division of Sports Medicine, University of British Columbia, Vancouver, British Columbia, Canada

[4]Providence Health Care, Providence Sacred Heart Medical Center and Children's Hospital

Corresponding Author:

Name: Dylan Timothy Dahlquist, MSc, CSCS

Location: University of British Columbia, School of Kinesiology

UBC Environmental Physiology Laboratory – Vancouver, BC V6T 1Z4

Phone: (604) 710-4800

Fax: (604) 822-9058

Email: dyl.dahlquist@gmail.com

Co-Authors Email Addresses:

TS: tstellingwerff@csipacific.ca
BPD: brad.dieter@providence.org
DCM: don.mckenzie@ubc.ca
MSK: michael.koehle@ubc.ca

Word Count: 8,911 words (including full manuscript, table titles and notes).

Abstract

Iron deficiency (ID) has ergolytic effects on athletic performance. Exercise induced inflammation impedes iron absorption in the digestive tract by up-regulating the expression of the iron regulatory protein, hepcidin. Limited research indicates the potential of specific macro- and micro- nutrients on blunting exercise induced hepcidin. Therefore, we investigated the effects of post-exercise supplementation with protein and carbohydrate (CHO) and vitamins D_3 and K_2 on the post-exercise hepcidin response. Ten highly-trained male cyclists (age: 26.9±6.4 years; VO_2max: 67.4±4.4 ml/kg/min) completed four cycling sessions in a randomized, placebo-controlled, single-blinded triple crossover study. Experimental days consisted of an 8-min warm-up at 50% power output at VO_2max ($p$$VO_2$max), followed by 8x3 min intervals at 85% $p$$VO_2$max with 1.5 min at 60% $p$$VO_2$max between each interval. Blood samples were collected pre- and post- exercise, and 3-hr post-exercise. Three different drinks consisting of CHO (75g) and protein (25g) with (VPRO) or without (PRO) vitamins D_3 (5,000 IU) and K_2 (1,000 mcg), or a zero-calorie control drink (PLA) were consumed immediately after the post-exercise blood sample. Results showed that the post-exercise drinks had no significant ($p \geq 0.05$) effect on any biomarker measured. There was a significant ($p < 0.05$) increase in hepcidin and IL-6 following intense cycling intervals in the participants. Hepcidin increased significantly ($p < 0.05$) from baseline (nmol/L: 9.94±8.93, 14.18±14.90, 10.44±14.62) to 3-hr post-exercise (nmol/L: 22.27±13.41, 25.44±11.91, 22.57±15.57) in VPRO, PRO and PLA respectively. Contrary to our hypothesis, the drink compositions used did not blunt the post-exercise hepcidin response in highly-trained athletes.

Key Words

Cycling; Inflammation; Iron metabolism; Protein and Carbohydrates; Vitamin D

Introduction

Iron deficiency (ID) is the most prevalent nutrient deficiency in the world (Cogswell et al. 2009; DellaValle and Haas 2014), affecting billions worldwide (McLean et al. 2009), with even higher susceptibility to this deficiency in endurance athletes (Koehler et al. 2012). Twenty to 60% of all female athletes and 4 to 50% of all male athletes (Brandy et al. 2003; Hinton 2014) suffer from ID in a given year. This deficiency can lead to deleterious effects on endurance performance and reduce the oxygen carrying capacity of the blood by up to ~30% (Woodson et al. 1978; Davies et al. 1982; Davies et al. 1984; Brownlie et al. 2004; Dellavalle and Haas 2011). Iron repletion studies in ID athletes have shown reductions in fat mass and increases in lactate threshold, improved exercise economy, and a 7.4% increase in VO_2max (Hinton and Sinclair 2007; DellaValle and Haas 2014; Wachsmuth et al. 2015).

One of the mechanisms that may be responsible for the high rates of ID in endurance athletes could be, in part, due to the acute inflammation response caused by intense exercise (Varamenti et al. 2013). It has been shown that exercise (Auersperger et al. 2013), injury/illness (Auersperger et al. 2013) and/or hypoxia (Haase 2010; Liu et al. 2012) increase the inflammatory cytokine interleukin-6 (IL-6) (Nicolas et al. 2002; Wachsmuth et al. 2015). High IL-6 concentrations are correlated with the down-regulation of iron absorption in the human digestive tract (Przybyszewska and Zekanowska 2014) by up-regulating the gene expression of the iron regulatory protein hepcidin (Roecker et al. 2005). Hepcidin inhibits iron absorption (Przybyszewska and Zekanowska 2014), and has been shown to increase post-exercise, peak three to six hours later, and then slowly return to pre-exercise baseline concentrations 24 hours later (Peeling et al. 2009; Peeling et al. 2014). Furthermore**,** it has been shown that the fate of the stored form of iron, ferritin, is multifaceted and is tightly regulated by the size of the iron pool and demands of iron within the body (Aisen 2001). Primarily, ferritin levels below 30 mcg/L

(Auersperger et al. 2013; Peeling et al. 2014) decrease baseline and exercise-induced hepcidin levels (Nemeth and Ganz 2006) while simultaneously impairing performance in elite rowers (Dellavalle and Haas 2011). Lastly, there is a greater hepcidin release occurring with increased exercise duration and intensity (Peeling et al. 2009; Newlin et al. 2012; Peeling et al. 2014), potentially leading to decreased post-exercise iron absorption (Collins et al. 2008; Kwapisz et al. 2009; Dellavalle and Haas 2012; Ganz and Nemeth 2012; Domínguez et al. 2014).

Attempting to attenuate post-exercise hepcidin release, preliminary research has focused on different nutritional interventions (Robson-Ansley et al. 2011; Sim et al. 2012; Badenhorst et al. 2014), to try to increase iron absorption and recycling by minimizing the exercise-induced inflammatory response (Ganz and Nemeth 2012). Protein ingested during exercise (Schroer et al. 2014), and protein combined with carbohydrate (CHO) after exercise (Kerasioti et al. 2013), have both been shown to downregulate IL-6 concentrations and systemic inflammatory responses immediately after and four hours post-exercise. Furthermore, large doses of carbohydrate (CHO) solution (6%, 250 mL every 15 minutes [4 mL kg^{-1}]) during a 2.5-hour run can decrease exercise-induced inflammation, specifically IL-6, by 40% (Nehlsen-Cannarella et al. 1997; Nieman et al. 1998). Despite this, four recent studies in trained endurance athletes have shown intra- or post- CHO supplementation has no effect on hepcidin following high intensity runs (Robson-Ansley et al. 2011; Sim et al. 2012; Ihalainen et al. 2014; Badenhorst et al. 2015b); and restricting CHO intake over a 24-hour period can increase susceptibility to ID by elevating baseline hepcidin concentration (Badenhorst et al. 2015a).

In addition to macronutrients, vitamin supplementation could also affect the inflammatory response to exercise. Supraphysiological doses of 50,000 IU (7,143 IU/day for three months) and 100,000 IU (single dose) of vitamin D significantly reduce hepcidin levels

(28% and 34%, respectively) in healthy individuals and patients suffering from chronic kidney disease (Bacchetta et al. 2014; Zughaier et al. 2014). At much lower dosages, 4,000 IU/day of vitamin D over 28 days in physically-active males demonstrated marked reductions in exercise induced inflammatory cytokines (Barker et al. 2013). Despite the positive findings, post-exercise inflammatory response has been reported to be different in trained versus untrained individuals (Evans et al. 1986; Schild et al. 2016), and results may be different in highly-trained athletes. No study to our knowledge has investigated this discrepancy. Additionally, vitamin K has been shown to prevent vitamin D toxicity (Dahlquist et al. 2015) and decrease IL-6 concentrations (Reddi et al. 1995; Ohsaki et al. 2006), yet a long term study using vitamin K_1 supplementation at dosages of 500 mcg/day did not demonstrate a decrease in IL-6 activity (Shea et al. 2008b). Therefore, the authors chose a vitamin K variant (vitamin K_2) that is more potent at blunting IL-6 production compared to vitamin K_1 (Reddi et al. 1995) and utilized a dose (1,000 mcg) postulated to be effective at preventing vitamin D toxicity (Dahlquist et al. 2015) for the purpose of this study.

With limited evidence that macro- and micro-nutrients may help address ID via attenuating post-exercise inflammation, we investigated the effects of a CHO and protein-rich post-exercise drink with the addition of vitamins D_3 and K_2 (VPRO) or without addition vitamins (PRO) versus a non-caloric placebo drink (PLA) on exercise-induced hepcidin response. We hypothesized that (1) both VPRO and PRO would significantly attenuate the rise of hepcidin following a bout of high intensity exercise as compared to PLA and (2) that VPRO supplementation would have a greater blunting effect on hepcidin concentration versus PRO supplementation alone.

Methods

Ethical Approval

Subjects completed a Physical Activity Readiness Questionnaire (PAR-Q) and provided written consent following a comprehensive written and explanation of the study. The University of British Columbia Clinical Research Ethics Board granted approval for the study (H15-00721).

Participants

Ten highly-trained male cyclists (age: 26.9±6.4 years; weight: 72.0±6.4 kg) with a VO_2max of 67.4±4.4 mL/kg/min, classified as Performance Level (PL) 4 and 5 (De Pauw et al. 2013) partook in four separate cycling sessions after passing and adhering to the following inclusion criteria: endurance athletes who were healthy, were not consuming anti-inflammatories on a regular basis, 18-45 years of age, and training 5-6 times a week with at least one year of training experience, with baseline serum ferritin levels of ≥ 30 mcg/L. Subjects also refrained from taking vitamin D, C and K, multi-vitamins 24 hours prior to experimental days and fish oil, krill oil, turmeric and serrapeptidase supplements at least 48 hours prior to experimental days. Female participants were excluded from the study due to the effects of the menstrual cycle and estradiol on hepcidin, since high estrogen and/or progesterone levels can attenuate the elevation in hepcidin levels (Yang et al. 2012; Sim et al. 2014).

Sample Size Determination

A sample size of n=10 was determined using data from (Badenhorst et al. 2014). In this study, 10 well-trained male endurance athletes completed two 8x3 minute interval running sessions at 85% of their maximal aerobic velocity ($v$$VO_2$max) on a motorized treadmill before being randomly allocated to a three hour recovery period in a hypoxic (~2,900 m above sea level) or

normoxic environment. Based on the differences of mean and standard deviations between baseline measurements and three hours post-exercise for the hypoxic intervention on hepcidin-25 (2.18±1.26 nM), an effect size of (dz=1.73) and a minimum sample size of n=9 subjects were needed.

Research Design

A randomized, placebo-controlled, single-blinded triple crossover design was used. A 48-hour washout period was used and each session took place at the same time of day. Subjects recorded their diet and activity the day prior to the experimental visits and fasted for 10 hours overnight before reporting to the laboratory. To maximize ecological validity, subjects consumed a standardized meal 60 minutes prior to arrival and adhered to a supplement protocol 48- and 24-hours prior to experimental days, described earlier. The standardized meal consisted of: 3 packs of granola bars (Nature Valley, Crunchy – Honey & Oats; Minneapolis, MN, US) with one pack containing 191 kcal, 7g of fat, 29g of CHO, 3g of protein and 1.44 mg of iron (Total for 3 pack: 573 kcal, 21g of fat, 87g of CHO, 9g of protein and 4.32 mg of iron). Subjects chose how much of the bars to consume, but were required to replicate their intake for subsequent visits.

Screening Day

Prior to familiarization (Visit 1) subjects were assessed for baseline serum ferritin and serum vitamin D, in order to ensure that their ferritin levels were ≥ 30 mcg/L as baseline hepcidin, and subsequent release, is reported to be lower in ID subjects (Auersperger et al. 2013; Peeling et al. 2014). Furthermore, it has been suggested that ferritin levels below 30 mcg/L in athletes are low (or ID) and should be the standard cut off going forth in this specific population (Clenin et al. 2015).

Visit 1, Familiarization Day

Visit 1 consisted of equipment familiarization and an incremental cycle exercise test (Wilkie et al. 2015) on an electronically-braked ergometer (Wahoo KICKR, Wahoo Fitness, Atlanta, GA, US). These results were used to determine the cycling power output (W) at percent (%) VO_2max (pVO_2max) for subsequent visits. Subjects then performed a familiarization to the exercise challenge, consisting of an 8-minute warm-up (cycle) at 50% power output (Watts) at VO_2max (pVO_2max), followed by 2 x 3 minute intervals at 85% pVO_2max. Each interval was separated by a 1.5 minute of active recovery at 60% pVO_2max.

Incremental Exercise Test (IET)

Participants performed a 10-minute self-selected warm-up protocol on their own bike on a cycle trainer (Wahoo KICKR, Wahoo Fitness, Atlanta, GA, US). Upon completion of the warm-up, the IET began immediately. Subjects were required to pedal at a cadence of ≥ 60 revolutions per minute (rpm) and work rate began at 0 Watts (W) (Wilkie et al. 2015). Work rate increased by 30 W every minute until volitional exhaustion. Subjects were fitted with a heart rate monitor (Wahoo TICKR, Wahoo Fitness, Atlanta, GA, US) and a face-mask (Oro-Nasal 74550 V2 Mask, Hans Rudolph, Shawnee, KS) connected to a two-way non-rebreathing valve (2700 T-shape, Hans Rudolph; Shawnee, KS) attached to a metabolic cart (TrueOne, Parvo Medics; Sandy, UT) in order to assess VO_2kinetics throughout the IET. The subjects' VO_2max was then utilized to determine cycling power output (W) at percent (%) VO_2max (pVO_2max) for subsequent cycling tests.

Visits 2 to 4

Venous blood samples were collected upon arrival, immediately post-exercise, and three hours post-exercise. Body mass (kg) was recorded both pre- and post- exercise (599KL Digital Scale,

Health O Meter ® Professional Scales; McCook IL, US). Subjects were allowed to drink water *ad libitum* during all trials. HR was collected throughout (Wahoo TICKR, Wahoo Fitness, Atlanta, GA, US) and rating of perceived exertion (RPE) was collected at 3-minute intervals using the Borg perceptual scale (Borg 1981; Borg 1998). After an 8-minute cycle warm-up at 50% pVO_2max, subjects began the cycle test of 8x3 minute intervals at 85% pVO_2max with a 1.5-minute active recovery (60% pVO_2max) separating the bouts. Following the final interval a 8-minute cool-down at 50% pVO_2max was undertaken and followed by a blood sample. Immediately following the blood sample, subjects consumed one of the three randomized experimental recovery. Participants then rested in the laboratory for three hours, a venous blood sample was collected, and then subjects departed.

Post-exercise Recovery Drink Consumption

The beverages looked, smelled, and tasted similarly based on subject feedback while consuming. They were mixed in identical opaque bottles and consisted of the following ingredients:

1) **PLA:** non-nitrogenous, zero calorie control drink (artificial flavour, sweetener [Kraft Foods, Crystal Light; Northfield, IL, US] and water [550 mL]).
2) **VPRO:** 75g of CHO (Cytocarb^2, CytoMax - CytoSport; Benicia, California, US), 25g of protein (Whey Protein Isolate – Vanilla - Restore, EXOS Fuel; Sandpoint, ID, US), 10 droplets of a vitamin D complex (Vitamin D + K Complex, EXOS Fuel; Sandpoint, Idaho, US) containing 5,000 IU of vitamin D_3 and 1,000 mcg of vitamin K_2 (as menatetrenone), artificial flavour, sweetener (same as PLA) and water (550 mL).
3) **PRO:** 75g of CHO (Cytocarb^2, CytoMax - CytoSport; Benicia, California, US), 25g of protein (Whey Protein Isolate – Vanilla - Restore, EXOS Fuel; Sandpoint, ID, US), artificial flavour, sweetener (same as PLA) and water (550 mL).

Laboratory Procedures

Blood Collection

Blood sampling procedures mimicked that of previous literature (Badenhorst et al. 2014; Peeling et al. 2014; Badenhorst et al. 2015b; Badenhorst et al. 2015a). All venous blood samples were taken after the subject rested in a seated position for five minutes with a 21-gauge needle into two 5 mL SST Gel separator tubes, a 3 mL SST Gel separator tube, a 4.5 mL PST Gel separator tube, and a 3 mL EDTA tube. Immediately following blood collection, the fresh samples were taken to the University of British Columbia Hospital laboratory to perform a complete blood count (CBC), serum iron and serum ferritin within a 24 hour period. The remaining samples were allowed to clot for 60 minutes at room temperature and then centrifuged at 10^{o} C and 1,500 G for 10 minutes. The centrifuged samples were separated into 1 mL aliquots and stored at -80^{o} C.

Blood Analysis

The remaining frozen serum samples were used to measure the bioactive form of hepcidin, hepcidin-25 via ELISA, and interleukin-6 (IL-6). Serum IL-6 was analyzed using a commercially-available ELISA (Quantikine HS, R&D Systems; Minneapolis, Minnesota, USA) with a sensitivity of 0.11 pg/mL and range of 0.2-10 pg/mL. Hepcidin-25 was assessed using a commercially-available ELISA (Quantikine HS, R&D Systems; Minneapolis, Minnesota, USA) with a sensitivity of 3.81 pg/mL and a detection range of 15-1,000 pg/mL. Coefficient of variation (CV) for IL-6 and hepcidin-25 were <5%.

Statistical Analysis

Results were analyzed with SPSS (IBM North America, New York, NY). Data and results were reported as mean ± SD. A 3 X 3 repeated measures analysis of variance (supplement type X

time) for each of hepcidin-25, Hb, IL-6, serum ferritin and serum iron was performed. Paired sample *t*-tests were performed to determine significance between time points within-treatments. A multivariate general linear model was performed in order to determine if starting baseline vitamin D measurements had a significant effect on individual post-exercise hepcidin responses to the VPRO drink only. The data that did not pass normality (hepcidin-25, IL-6, and serum ferritin) were transformed using the natural logarithm. Data that were statistically analyzed utilizing log-transformed data are presented as mean ± SD of non-transformed data within tables and results. *F*-ratios will be found significant at $p \leq 0.05$.

Results

Subject Characteristics

Subject characteristics and VO_2max results are presented above in the methods. Subjects had serum ferritin levels of 109±77 mcg/L and serum vitamin D levels of 75±28 nmol/L at baseline.

Physiological Responses

Mean HR, RPE, energy expenditure (Vogt et al. 2005; Haakonssen et al. 2013), pre- and post-exercise body mass and power output for the interval cycling trials are presented in Table 1.

*****Insert Table 1 Here*****

Serum Interleukin-6

There was no significant difference in baseline measurements of IL-6 across conditions. There was a significant effect over time, but no significant condition effect on IL-6 (see Table 2).

Hepcidin-25 & Baseline Vitamin D

There was a significant effect of time, but not treatment (drink) on hepcidin-25 (see Table 2). There was a significant increase from baseline to post-exercise in VPRO and PRO, but no

significant increase in PLA. There was a significant increase in hepcidin-25 from baseline and post-exercise to three hours post-exercise for all conditions. Furthermore, there was no order effect for hepcidin values based on visit day. Starting vitamin D levels (74±28 nmol/L, MIN = 34 nmol/L, MAX = 132 nmol/L) did not affect hepcidin-25 response in the VPRO condition.

Iron Parameters

There was a significant time effect on serum iron and serum ferritin for all conditions (see Table 2). There was no condition effect on serum iron or log transformed serum ferritin.

Hemoglobin & Hematocrit

Significant time effects were recorded for Hb and hematocrit. No significant difference was observed for either Hb or hematocrit between VPRO, PRO and PLA (see Table 2).

*****Insert Table 2 Here [Landscape or Own Page if Possible]*****

Discussion

The present study examined the effects of a post-exercise drink consisting of whey protein isolate and carbohydrates (CHO) with vitamins D_3 and K_2 (VPRO) or without vitamins (PRO) on the acute post-exercise hepcidin response in highly-trained athletes. The results from the investigation demonstrate that following a fatiguing interval-based cycle, subjects experienced a significant time-dependent increase in hepcidin, IL-6, Hb, hematocrit and iron biomarkers independent of the post-exercise drink composition (Table 2). Contrary to our hypothesis, VPRO, PRO or PLA post-exercise drink compositions had no significant effect on any biomarker measured and is not likely to impact iron malabsorption due to post-exercise hepcidin release.

The hepcidin levels in the current study mimic those of previous literature, where the highest levels were seen concurrent with peak levels of IL-6 (Kemna et al. 2005). IL-6 has been reported to drastically increase five- to 100-fold after exercise (Ostrowski et al. 1999; Peeling et al. 2009), and peak levels are attained immediately following the cessation of exercise (Pedersen 2000). Our results demonstrated that IL-6 significantly increased from baseline to post-exercise in each condition (see Table 2), with no significant differences between the drinks. These findings could suggest that the exercise intensity and muscle glycogen content were similar between trials since both can alter IL-6 concentration (Ivy et al. 1988; Keller et al. 2001; Steensberg et al. 2001).

Furthermore, a limited number of studies on the effects of peri-workout consumption of both protein and CHO on inflammatory markers (such as IL-6) remain equivocal. Participants given either a 6% CHO solution or a 4:1 CHO-protein solution during a cycling session saw no significant differences in IL-6 concentrations between conditions (Cosio-Lima et al. 2012). Likewise, the ingestion of a high protein (0.7 g kg^{-1} $hour^{-1}$), moderate CHO (0.26 g kg^{-1} $hour^{-1}$) or a low protein (0.1 g kg^{-1} $hour^{-1}$), high CHO (2.1 g kg^{-1} $hour^{-1}$) beverage had no meaningful effect on IL-6 (Rowlands et al. 2008). Our results correspond with the findings of these two studies, since the PRO and VPRO drinks had no significant effect on IL-6 or hepcidin when compared to the PLA drink. However, Kerasioti et al (2013) demonstrated a significant 50% decrease in IL-6 concentrations when subjects consumed a high protein, moderate CHO cake (Total Intake, Protein: 23.92 g and CHO: 82.8 g, Calories: 426.9) compared to a low protein, high CHO (Total Intake, Protein: 9.2 g and CHO: 101.2 g, Calories: 441.6) cake during a four-hour recovery period after an exhaustive cycling session. Furthermore, it has been demonstrated that the consumption of 45g $hour^{-1}$ of protein significantly decreases IL-6 concentrations immediately post-exercise, when compared to a placebo drink in individuals cycling at a constant

load for 120 minutes (Schroer et al. 2014). Contrary to Kerasioti et al (2013) and Schroer et al (2014), our results are in line with findings by Corsio-Lima et al (2012) and Rowlands et al (2008), where the protein and CHO dosages of the PRO and VPRO drinks had no significant effect on IL-6 levels when compared to the PLA drink.

These findings could potentially be related to the timing of the blood sample and/or the timing of nutrient intake. Kerasioti and colleagues (2013) reported that IL-6 was significantly different four hours after the cessation of exercise, which also corresponds with a potential peak in hepcidin concentration identified afterwards (Peeling et al. 2009; Peeling et al. 2014). Thus, we may have seen a significant difference between conditions if we had measured IL-6 and hepcidin levels four hours and not three hours post-exercise. Additionally, Schroer et al (2014) utilized a peri-workout feeding protocol, where subjects consumed 45 g of protein per hour over a two-hour cycling session. Although they saw a marked reduction in metabolic and oxidative stress, performance in the 30km time trial was impaired by 2% (Schroer et al. 2014). This finding of a performance decrement warrants caution with this strategy if attempting to optimize performance.

In addition, muscle glycogen depletion can significantly augment IL-6 concentration produced by skeletal muscle tissue (Keller et al. 2001; Steensberg et al. 2001), and both intensity and duration of exercise are important factors to determine the rate of muscle glycogen utilization (Ivy et al. 1988). It may take upwards to 2-3 hours of exercise at 70-80% VO_2max in order to fully deplete muscle glycogen stores (Coyle et al. 1983) which can lead to a ~40 to ~60- fold increase in IL-6 gene activation (Keller et al. 2001). The meal containing ~1.2 g CHO kg^{-1} consumed one hour prior to our high-intensity exercise trial could have served to maximize carbohydrate stores and minimized muscle and liver glycogen depletion (Ivy 2004; Burke 2010).

Furthermore, elite endurance athletes commonly consume diets rich in CHO (8 to 11 g/kg = 576.24±51.52 to 792.33±70.84) (Burke 2001; Jeukendrup et al. 2005; Vogt et al. 2005; Robins 2007; Jeukendrup 2011). Thus, the pre-exercise high CHO meal and habitually CHO heavy diets could have potentially attenuated the magnitude of IL-6 responses from post-exercise to three hours post-exercise and negated the effect of VPRO and PRO beverages. Additionally, with the highly-trained athletes used in the study, the chronic adaptations they acquire from both resistance (Calle and Fernandez 2010) and endurance (Farney et al. 2012) training leads to a marked reduction in reactive oxygen species and a subsequent decrease anti- and pro-inflammatory cytokines following a bout of high intensity exercise. Lastly, the final blood sample at three hours post-exercise may have been too soon, thus missing a peak in hepcidin and/or inflammation occurring 1-3 hours later (Peeling et al. 2009; Kerasioti et al. 2013; Peeling et al. 2014; Schroer et al. 2014). This combination of factors could possibly explain the current findings.

Significant increases in serum ferritin and serum iron were reported from baseline to post-exercise in the current study (Table 2), which has been commonly observed in prior studies (Peeling et al. 2008; Peeling et al. 2009; Sim et al. 2012; Marc et al. 2013; Badenhorst et al. 2014; Peeling et al. 2014; Badenhorst et al. 2015a). Exercise-induced inflammation has a robust effect on the reticuloendothelial system, which is comprised of monocytes, macrophages and precursor cells (Knutson and Wessling-Resnick 2003) contributing to iron recycling and storage. As erythrocytes become damaged, the cell membrane containing stored ferritin becomes impaired thus causing a leakage into circulating plasma (Pattini et al. 1990). This leads to an increase in ferritin reuptake and reticuloendothelial cells then recycle ferritin via the spleen, liver and bone marrow (Knutson and Wessling-Resnick 2003). In addition to the above, exercise-

induced dehydration has been shown to decrease plasma volume which subsequently increases the concentration of circulating proteins (Jimenez et al. 1999; Reljic et al. 2013). Body mass decreased by 0.6±0.4 kg (~500-600 mL of body water or 1.0±1.0%) during each experimental visit, indicating that there may have been some hemo-concentration. However, since we did not monitor fluid intake during and after the bouts, we cannot fully quantify the effects of hydration on the biomarkers (Maughan and Shirreffs 2010).

There was a significant decrease in serum ferritin, Hb and hematocrit seen three hours post-exercise (Table 2) which could be attributed to the reticuloendothelial system clearance mechanism of both serum ferritin and free Hb from the circulating plasma, and thus, increasing ferritin stores in the liver (Nemeth et al. 2004; Nemeth and Ganz 2006) or the impact of hemo-dilution / rehydration. Furthermore, elevated levels of hepcidin have been shown to prevent iron recycling (and ferritin release) by macrophages (Nemeth et al. 2004), which could also contribute to the marked decrease in serum iron and serum ferritin levels three hours post-exercise in the current study for VPRO, PRO and PLA.

Vitamin D supplementation in healthy individuals (Bacchetta et al. 2014; Smith et al. 2016) and individuals suffering from chronic kidney disease (Zughaier et al. 2014) has led to a marked decrease in hepcidin response following acute and chronic dosing. Doses 20- and 50- fold higher than that used in our study have shown a 34% and 73% reduction in hepcidin concentrations 24 hours after supplementation, respectively (Bacchetta et al. 2014; Smith et al. 2016). In chronic kidney disease patients, 7,143 IU/day of vitamin D_3 for three months decreased hepcidin concentrations by 28% (Zughaier et al. 2014). Furthermore, Barker and his colleagues (2013) showed that 4,000 IU/day of vitamin D over 28 days significantly decreased inflammatory biomarkers in moderately active adult males at 24, 48, 72 and 168 hours following the exercise

bouts. The current study looked at the acute effects of vitamin D_3 and K_2 supplementation within a three-hour time frame and at a much lower dose. No differences in IL-6 or hepcidin concentrations were found for VPRO drink when compared to PRO or PLA. Thus, lower dose or the acute time frame could have accounted for the lack of effect of these supplements in the current study. A longer time course, or chronic supplementation might have been necessary to detect a significant effect.

In the current study, baseline values of vitamin D had no significant effect on post-exercise hepcidin response in the VPRO condition. However, only 10% of our subjects were deficient in 25(OH)D, whereas 41% of Canadian males in this age group are deficient (Canada 2015). These more favourable values in the study sample could be related to dietary factors or to the time of year when data collection occurred (the end of the outdoor cycling season). Thus, these higher 25(OH)D concentrations seen in our study population could have mitigated the effects of supplemental vitamin D.

Lastly, vitamin K supplementation has been shown to blunt the increase inflammatory cytokine IL-6 in *in vitro* studies in rat models, isolated cell cultures and obese humans (Reddi et al. 1995; Ohsaki et al. 2006; Shea et al. 2008a; Shea et al. 2008b). Our results did not show any significant difference in IL-6 or hepcidin when vitamin K_2 was combined with vitamin D_3 supplementation. Thus, extrapolation to healthy and highly fit males may not be warranted based on the findings of the study (Tappy et al. 1994; Reagan-Shaw et al. 2008; Sharma and McNeill 2009).

Strengths and Limitations

The current study has multiple strengths and limitations. The triple cross-over nature allowed us to mitigate the discrepancies commonly seen between individuals. Additionally, the study was

adequately powered *a priori,* substantiating the negative finding of the current study. Furthermore, the athletic profile of the subjects makes the findings of this study highly applicable to the highly-trained population, indicating that the timing and constituents of the post-exercise drinks may not help athletes enhance the absorption of iron zero to three hours following the cessation of intense training sessions. A limitation of this study was that it was only performed on male participants, since the menstrual cycle and estradiol affect the post-exercise hepcidin response (Sim et al. 2014). Secondly, greater control over diet and daily activity during the 24-hour period prior to the visits could have reduced confounding variables in our data. Instead, our subjects might have started the study with slightly depleted or fully replenished glycogen stores, especially due to the CHO-dense pre-workout meal, subsequently affecting the post-exercise inflammatory response. Lastly, although subjective reports indicated that the subjects were blinded to the beverage they consumed at each trial, this was not systematically measured, and thus they may not have been fully blinded.

Conclusions

The consumption of a CHO and protein-rich drink with or without the addition of vitamin D_3 and K_2 had no significant effects on hepcidin-25, IL-6, Hb, hematocrit, serum ferritin or serum iron in the present study. This may be due to a combination of the very high level of fitness of the subjects or the fact that they were fed a high CHO meal prior to each trial. Nevertheless, hepcidin and IL-6 response in the current study were characteristic of normal post-exercise inflammatory responses in trained individuals (Ward and Kaplan 2012; Wachsmuth et al. 2015). Thus, regardless of the interventions used in the current study, individuals could still be susceptible to iron malabsorption post-exercise. Future research needs to revisit the effectiveness

of peri-workout consumption, the constituents and dose of macro- and micro- nutrient protocols from both an acute and chronic standpoint.

Conflict of Interest

All authors declare no conflict of interest.

Funding

This study was funded by the Innovations 4 Gold (I4G) grant through Own The Podium and the Canadian Sport Institute – Pacific.

Acknowledgments

The authors would like to thank the subjects who partook in this study. We would also like to thank Dr. Ted Steiner and the impeccable work performed by May Wong who ran the ELISA's for hepcidin-25 and interleukin-6.

Compliance with Ethical Standards

References

Aisen, P. 2001. Chemistry and biology of biology of eukaryotic iron metabolism. Int. J. Biochem. Cell. Biol. **33:** 940-959. PMID:11470229.

Auersperger, I., Skof, B., Leskosek, B., Knap, B., Jerin, A., and Lainscak, M. 2013. Exercise-induced changes in iron status and hepcidin response in female runners. PLoS One. **8**: e58090. doi:10.1371/journal.pone.0058090.

Bacchetta, J., Zaritsky, J.J., Sea, J.L., Chun, R.F., Lisse, T.S., Zavala, K., Nayak, A., Wesseling-Perry, K., Westerman, M., Hollis, B.W., Salusky, I.B., and Hewison, M. 2014. Suppression of iron-regulatory hepcidin by vitamin D. J. Am. Soc. Nephrol. **25**: 564-72. doi:10.1681/ASN.2013040355.

Badenhorst, C.E., Dawson, B., Cox, G.R., Laarakkers, C.M., Swinkels, D.W., and Peeling, P. 2015a. Acute dietary carbohydrate manipulation and the subsequent inflammatory and

hepcidin responses to exercise. Eur. J. Appl. Physiol. **115**: 2521-30. doi:10.1007/s00421-015-3202-0.

Badenhorst, C.E., Dawson, B., Cox, G.R., Laarakkers, C.M., Swinkels, D.W., and Peeling, P. 2015b. Timing of post-exercise carbohydrate ingestion: influence on IL-6 and hepcidin responses. Eur. J. Appl. Physiol. **115**: 2215-22. doi:10.1007/s00421-015-3252-3.

Badenhorst, C.E., Dawson, B., Goodman, C., Sim, M., Cox, G.R., Gore, C.J., Tjalsma, H., Swinkels, D.W., and Peeling, P. 2014. Influence of post-exercise hypoxic exposure on hepcidin response in athletes. Eur. J. Appl. Physiol. **114**: 951-9. doi:10.1007/s00421-014-2829-6.

Barker, T., Schneider, E.D., Dixon, B.M., Henriksen, V.T., and Weaver, L.K. 2013. Supplemental vitamin D enhances the recovery in peak isometric force shortly after intense exercise. Nutr. Metab. **10**: 69. doi:10.1186/1743-7075-10-69.

Borg, G.A. 1998. Borg's perceived exertion and pain scales. Champaign, IL, US: Human Kinetics.

Borg, G.A. 1981. Psychophysical bases of perceived exertion. Med. Sci. Sports. Ex. **14**: 377-381. PMID:7154893.

Brandy, C., Christine, R., Robert, S., and Stephanie, S. 2003. Policies on screening female athletes for iron deficiency in NCAA division I-A institutions. Int. J. Sports. Nutr. Exerc. Metab. **13**: 277-285. doi:10.1123/ijsnem.13.3.277.

Brownlie, T., Utermohlen, V., Hinton, P., and Haas, J. 2004. Tissue iron deficiency without anemia impairs adaptation in endurance capacity after aerobic training in previously untrained women. Am. J. Clin. Nutr. **79**: 437-443. PMID:14985219.

Burke, L.M. 2001. Nutritional practices of male and female endurance cyclists. Sports. Med. **31**: 521-32. PMID:11428689.

Burke, L.M. 2010. Fueling strategies to optimize performance: training high or training low? Scand. J. Med. Sci. Sports. **20** (Suppl) 2: 48-58. doi:10.1111/j.1600-0838.2010.01185.x

Calle, M.C. and Fernandez, M.L. 2010. Effects of resistance training on the inflammatory response. Nutr. Res. Pract. **4**: 259-69. doi:10.4162/nrp.2010.4.4.259.

Canada, S. 2015. Vitamin D Levels of Canadians.

Clenin, G., Cordes, M., Huber, A., Schumacher, Y.O., Noack, P., Scales, J., and Kriemler, S. 2015. Iron deficiency in sports - definition, influence on performance and therapy. Swiss. Med. Wkly. **145**: w14196. doi:10.4414/smw.2015.14196.

Cogswell, M.E., Looker, A.C., Pfeiffer, C.M., Cook, J.D., Lacher, D.A., Beard, J.L., Lynch, S.R., and Grummer-Strawn, L.M. 2009. Assessment of iron deficiency in US preschool children and nonpregnant females of childbearing age: National Health and Nutrition Examination Survey 2003-2006. Am. J. Clin. Nutr. **89**: 1334-42. doi:10.3945/ajcn.2008.27151.

Collins, J.F., Wessling-Resnick, M., and Knutson, M.D. 2008. Hepcidin regulation of iron transport. J. Nutr. **138**: 2284-8. doi:10.3945/jn.108.096347.

Cosio-Lima, L.M., Desai, B., Stelzer, J.W., and Schuler, P.B. 2012. Effects of 4:1 carbohydrate/protein solution versus a carbohydrate-alone solution on IL-6, TNF-alpha, and cortisol during prolonged cycling in hot environmental conditions. Open. Access. J. Sports. Med. **3**: 21-6. doi:10.2147/OAJSM.S28176.

Coyle, E., Hagberg, J., Hurley, B., Martin, W., Ehsani, A., and Holloszy, J. 1983. Carbohydrate feeding during prolonged strenuous exercise can delay fatigue. Amer. Physiol. Soc. PMID:6350247.

Dahlquist, D.T., Dieter, B.P., and Koehle, M.S. 2015. Plausible ergogenic effects of vitamin D on athletic performance and recovery. J. Int. Soc. Sports. Nutr. **12**: 33. doi:10.1186/s12970-015-0093-8.

Davies, K.J., Donovan, C.M., Refino, C.J., Brooks, G.A., Packer, L., and Dallman, P.R. 1984. Distinguishing effects of anemia and muscle iron deficiency on exercise bioenergetics in the rat. Am. J. Physiol. **246**: E535-43. PMID:6742115.

Davies, K.J., Maguire, J.J., Brooks, G.A., Dallman, P.R., and Packer, L. 1982. Muscle mitochondrial bioenergetics, oxygen supply, and work capacity during dietary iron deficiency and repletion. Am. J. Physiol. **242**: E418-27. PMID:7091311.

De Pauw, K., Roelands, B., Cheung, S.S., de Geus, B., Rietjens, G., and Meeusen, R. 2013. Guidelines to classify subject groups in sport-science research. Int. J. Sports. Physiol. Perform. **8**: 111-22. PMID:23428482.

Dellavalle, D.M. and Haas, J.D. 2011. Impact of iron depletion without anemia on performance in trained endurance athletes at the beginning of a training season: A study of female collegiate rowers. Int. J. Sport. Nutr. Exerc. Metab. **21**: 501-506. PMID:22089308.

Dellavalle, D.M. and Haas, J.D. 2012. Iron status is associated with endurance performance and training in female rowers. Med. Sci. Sports. Exerc. **44**: 1552-9. doi:10.1249/MSS.0b013e3182517ceb.

DellaValle, D.M. and Haas, J.D. 2014. Iron supplementation improves energetic efficiency in iron-depleted female rowers. Med. Sci. Sports. Exerc. **46**: 1204-15. doi:10.1249/MSS.0000000000000208.

Domínguez, R., Vicente-Campos, D., Vicente-Campos, D., and Chicharro, J. 2014. Hepcidin response to exercise: A review. Turkish J. Endo. Met. **18**: 84-91. doi:10.4274/tjem.2438.

Evans, W.J., Meredith, C.N., Cannon, J.G., Dinarello, C.A., Frontera, W.R., Hughes, V.A., Jones, B.H., and Knuttgen, H.G. 1986. Metabolic changes following eccentric exercise in trained and untrained men. J. Appl. Physiol. **61**: 1864-8. PMID:3491061.

Farney, T.M., McCarthy, C.G., Canale, R.E., Schilling, B.K., Whitehead, P.N., and Bloomer, R.J. 2012. Absence of blood oxidative stress in trained men after strenuous exercise. Med. Sci. Sports. Exerc. **44**: 1855-63. doi:10.1249/MSS.0b013e3182592575.

Ganz, T. and Nemeth, E. 2012. Hepcidin and iron homeostasis. Biochim. Biophys. Acta. **1823**: 1434-43. doi:10.1016/j.bbamcr.2012.01.014.

Haakonssen, E.C., Martin, D.T., Burke, L.M., and Jenkins, D.G. 2013. Energy expenditure of constant- and variable-intensity cycling: power meter estimates. Med. Sci. Sports. Exerc. **45**: 1833-40. doi:10.1249/MSS.0b013e31828e18e6.

Haase, V.H. 2010. Hypoxic regulation of erythropoiesis and iron metabolism. Am. J. Physiol. Renal. Physiol. **299**: F1-13. doi:10.1152/ajprenal.00174.2010.

Hinton, P.S. 2014. Iron and the endurance athlete. Appl. Physiol. Nutr. Metab. **39**: 1012-8. doi:10.1139/apnm-2014-0147.

Hinton, P.S. and Sinclair, L.M. 2007. Iron supplementation maintains ventilatory threshold and improves energetic efficiency in iron-deficient nonanemic athletes. Eur. J. Clin. Nutr. **61**: 30-9. doi:10.1038/sj.ejcn.1602479.

Ihalainen, J.K., Vuorimaa, T., Puurtinen, R., Hamalainen, I., and Mero, A.A. 2014. Effects of carbohydrate ingestion on acute leukocyte, cortisol, and interleukin-6 response in high-intensity long-distance running. J. Strength. Cond. Res. **28**: 2786-92. doi:10.1519/JSC.0000000000000470.

Ivy, J.L. 2004. Regulation of muscle glycogen repletion, muscle protein synthesis and repair following exercise. J. Sports. Sci. Med. **3**: 131-8. PMID:24482590.

Ivy, J.L., Katz, A.L., Cutler, C.L., Sherman, W.M., and Coyle, E.F. 1988. Muscle glycogen synthesis after exercise: effect of time of carbohydrate ingestion. J. Appl. Physiol. **64**: 1480-5. PMID:3132449.

Jeukendrup, A.E. 2011. Nutrition for endurance sports: marathon, triathlon, and road cycling. J. Sports. Sci. **29** (Suppl) 1: S91-9. doi:10.1080/02640414.2011.610348.

Jeukendrup, A.E., Jentjens, R.L., and Moseley, L. 2005. Nutritional considerations in triathlon. Sports. Med. **35**: 163-81. PMID:15707379.

Jimenez, C., Melin, B., Koulmann, N., Allevard, A.M., Launay, J.C., and Savourey, G. 1999. Plasma volume changes during and after acute variations of body hydration level in humans. Eur. J. Appl. Physiol. Occup. Physiol. **80**: 1-8. doi:10.1007/s004210050550.

Keller, C., Steensberg, A., Pilegaard, H., Osada, T., Saltin, B., Pedersen, B.K., and Neufer, P.D. 2001. Transcriptional activation of the IL-6 gene in human contracting skeletal muscle: influence of muscle glycogen content. FASEB. J. **15**: 2748-50. doi:10.1096/fj.01-0507fje.

Kemna, E., Pickkers, P., Nemeth, E., van der Hoeven, H., and Swinkels, D. 2005. Time-course analysis of hepcidin, serum iron, and plasma cytokine levels in humans injected with LPS. Blood, **106**: 1864-6. doi:10.1182/blood-2005-03-1159.

Kerasioti, E., Stagos, D., Jamurtas, A., Kiskini, A., Koutedakis, Y., Goutzourelas, N., Pournaras, S., Tsatsakis, A.M., and Kouretas, D. 2013. Anti-inflammatory effects of a special carbohydrate-whey protein cake after exhaustive cycling in humans. Food. Chem. Toxicol. **61**: 42-6. doi:10.1016/j.fct.2013.01.023.

Knutson, M. and Wessling-Resnick, M. 2003. Iron metabolism in the reticuloendothelial system. Crit. Rev. Biochem. Mol. Biol. **38**: 61-88. doi:10.1080/713609210.

Koehler, K., Braun, H., Achtzehn, S., Hildebrand, U., Predel, H.G., Mester, J., and Schanzer, W. 2012. Iron status in elite young athletes: gender-dependent influences of diet and exercise. Eur. J. Appl. Physiol. **112**: 513-23. doi:10.1007/s00421-011-2002-4.

Kwapisz, J., Slomka, A., and Zekanowska, E. 2009. Hepcidin and its role in iron homeostasis. EJIFCC. **20**: 124-8. PMID:27683336.

Liu, Q., Davidoff, O., Niss, K., and Haase, V.H. 2012. Hypoxia-inducible factor regulates hepcidin via erythropoietin-induced erythropoiesis. J. Clin. Invest. **122**: 4635-44. doi:10.1172/JCI63924.

Marc, S., Brian, D., Grant, L., Dorine, S., Harold, T., Debbie, T., and Peter, P. 2013. Effect of exercise modality and intensity on postexercise interleukin-6 and hepcidin levels. Int. J. Sport. Nutr. Exerc. Metab. **23**: 178-186. doi:10.1123/ijsnem.23.2.178.

Maughan, R.J. and Shirreffs, S.M. 2010. Dehydration and rehydration in competative sport. Scand. J. Med. Sci. Sports. 20 (Suppl) **3**: 40-7. doi:10.1111/j.1600-0838.2010.01207.x.

McLean, E., Cogswell, M., Egli, I., Wojdyla, D., de Benoist, B. 2009. Worldwide prevalence of anaemia, WHO vitamin and mineral nutrition information system, 1993–2005. Public. Health. Nutr. **12**(4): 444–454. doi:10.1017/S1368980008002401.

Nehlsen-Cannarella, S.L., Fagoaga, O.R., Nieman, D.C., Henson, D.A., Butterworth, D.E., Schmitt, R.L., Bailey, E.M., Warren, B.J., Utter, A., and Davis, J.M. 1997. Carbohydrate and the cytokine response to 2.5 h of running. J. Appl. Physiol. **82**: 1662-1667. PMID:9134917.

Nemeth, E. and Ganz, T. 2006. Hepcidin and iron-loading anemias. Haematologica, 91: 727-32. PIMD:16769573.

Nemeth, E., Tuttle, M.S., Powelson, J., Vaughn, M.B., Donovan, A., Ward, D.M., Ganz, T., and Kaplan, J. 2004. Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. Science, **306**: 2090-3. doi:10.1126/science.1104742.

Newlin, M.K., Williams, S., McNamara, T., Tjalsma, H., Swinkels, D.W., and Haymes, E.M. 2012. The effects of acute exercise bouts on hepcidin in women. Int. J. Sport. Nutr. Exerc. Metab. **22**: 79-88. doi:10.1123/ijsnem.22.2.79.

Nicolas, G., Chauvet, C., Viatte, L., Danan, J.L., Bigard, X., Devaux, I., Beaumont, C., Kahn, A., and Vaulont, S. 2002. The gene encoding the iron regulatory peptide hepcidin is regulated by anemia, hypoxia, and inflammation. J. Clin. Invest. **110**: 1037-44. doi:10.1172/JCI15686.

Nieman, D.C., Nehlsen-Cannarella, S.L., Fagoaga, O.R., Henson, D.A., Utter, A., Davis, J.M., Williams, F., and Butterworth, D.E. 1998. Influence of mode and carbohydrate on the cytokine response to heavy exertion. Med. Sci. Sports. Exerc. **30**: 671-8. doi:10.1097/00005768-199805000-00005.

Ohsaki, Y., Shirakawa, H., Hiwatashi, K., Furukawa, Y., Mizutani, T., and Komai, M. 2006. Vitamin K suppresses lipopolysaccharide-induced inflammation in the rat. Biosci. Biotechnol. Biochem. **70**: 926-32. doi:10.1271/bbb.70.926.

Ostrowski, K., Rohde, T., Asp, S., Schjerling, P., and Pedersen, B. 1999. Pro- and anti-inflammatory cytokine balance in strenous exercise in humans. J. Physiol. **55.1**: 287-291. PMID:9925898.

Pattini, A., Schena, F., and Guidi, G.C. 1990. Serum ferritin and serum iron changes after cross-country and roller ski endurance races. Eur. J. Appl. Physiol. Occup. Physiol. **61**: 55-60. doi:10.1007/BF00236694.

Pedersen, B. 2000. Exercise and cytokines. Immuno. Cell. Biol. **78**: 532-535. doi:10.1111/j.1440-1711.2000.t01-11-.x.

Peeling, P., Dawson, B., Goodman, C., Landers, G., and Trinder, D. 2008. Athletic induced iron deficiency: new insights into the role of inflammation, cytokines and hormones. Eur. J. Appl. Physiol. **103**: 381-91. doi:10.1007/s00421-008-0726-6.

Peeling, P., Dawson, B., Goodman, C., Landers, G., Wiegerinck, E.T., Swinkels, D.W., and Trinder, D. 2009. Effects of exercise on hepcidin response and iron metabolism during recovery. Int. J. Sport. Nutr. Exerc. Metab. **19**: 583-97. PMID:20175428.

Peeling, P., Sim, M., Badenhorst, C.E., Dawson, B., Govus, A.D., Abbiss, C.R., Swinkels, D.W., and Trinder, D. 2014. Iron status and the acute post-exercise hepcidin response in athletes. PLoS One. **9**: e93002. doi:10.1371/journal.pone.0093002.

Przybyszewska, J. and Zekanowska, E. 2014. The role of hepcidin, ferroportin, HCP1, and DMT1 protein in iron absorption in the human digestive tract. Prz. Gastroenterol. **9**: 208-13. doi:10.5114/pg.2014.45102.

Reagan-Shaw, S., Nihal, M., and Ahmad, N. 2008. Dose translation from animal to human studies revisited. FASEB. J. **22**: 659-61. doi:10.1096/fj.07-9574LSF.

Reddi, K., Henderson, B., Meghji, S., Wilson, M., Poole, S., Hopper, C., Harris, M., and Hodges, S.J. 1995. Interleukin 6 production by lipopolysaccharide-stimulated human fibroblasts is potently inhibited by naphthoquinone (vitamin K) compounds. Cytokine, **7**: 287-290. doi:*10.1006/cyto.1995.0034*.

Reljic, D., Hassler, E., Jost, J., and Friedmann-Bette, B. 2013. Rapid weight loss and the body fluid balance and hemoglobin mass of elite amateur boxers. J. Athl. Train. **48**: 109-17. doi:10.4085/1062-6050-48.1.05.

Robins, A. 2007. Nutritional recommendations for competiting in the ironman triathlon. Current. Sports. Med. Rep. **6**: 241-248. PMID:17618000.

Robson-Ansley, P., Walshe, I., and Ward, D. 2011. The effect of carbohydrate ingestion on plasma interleukin-6, hepcidin and iron concentrations following prolonged exercise. Cytokine, **53**: 196-200. doi:10.1016/j.cyto.2010.10.001.

Roecker, L., Meier-Buttermilch, R., Brechtel, L., Nemeth, E., and Ganz, T. 2005. Iron-regulatory protein hepcidin is increased in female athletes after a marathon. Eur. J. Appl. Physiol. **95**: 569-71. doi:10.1007/s00421-005-0055-y.

Rowlands, D.S., Rossler, K., Thorp, R.M., Graham, D.F., Timmons, B.W., Stannard, S.R., and Tarnopolsky, M.A. 2008. Effect of dietary protein content during recovery from high-intensity cycling on subsequent performance and markers of stress, inflammation, and muscle damage in well-trained men. Appl. Physiol. Nutr. Metab. **33**: 39-51. doi:10.1139/H07-136.

Schild, M., Eichner, G., Beiter, T., Zugel, M., Krumholz-Wagner, I., Hudemann, J., Pilat, C., Kruger, K., Niess, A.M., Steinacker, J.M., and Mooren, F.C. 2016. Effects of acute

endurance exercise on plasma protein profiles of endurance-trained and untrained individuals over time. Mediators. Inflamm. **2016**: 4851935. doi:10.1155/2016/4851935.

Schroer, A.B., Saunders, M.J., Baur, D.A., Womack, C.J., and Luden, N.D. 2014. Cycling time trial performance may be impaired by whey protein and L-alanine intake during prolonged exercise. Int. J. Sport. Nutr. Exerc. Metab. **24**: 507-15. doi:10.1123/ijsnem.2013-0173.

Sharma, V. and McNeill, J.H. 2009. To scale or not to scale: the principles of dose extrapolation. Br. J. Pharmacol. **157**: 907-21. doi:10.1111/j.1476-5381.2009.00267.x.

Shea, M.K., Booth, S.L., Massaro, J.M., Jacques, P.F., D'Agostino, R.B., Sr., Dawson-Hughes, B., Ordovas, J.M., O'Donnell, C.J., Kathiresan, S., Keaney, J.F., Jr., Vasan, R.S., and Benjamin, E.J. 2008a. Vitamin K and vitamin D status: associations with inflammatory markers in the Framingham Offspring Study. Am. J. Epidemiol. **167**: 313-20. doi:10.1093/aje/kwm306.

Shea, M.K., Dallal, G.E., Dawson-Hughes, B., Ordovas, J.M., O'Donnell, C.J., Gundberg, C.M., Peterson, J., and Booth, S.L. 2008b. Vitamin K, ciculating cytokines, and bone mineral density in older men and women. Am. J. Clin. Nutr. **88**: 356-63. PMID:18689371.

Sim, M., Dawson, B., Landers, G., Trinder, D., and Peeling, P. 2014. Iron regulation in athletes: exploring the menstrual cycle and effects of different exercise modalities on hepcidin production. Int. J. Sport. Nutr. Exerc. Metab. **24**: 177-87. doi:10.1123/ijsnem.2013-0067.

Sim, M., Dawson, B., Landers, G., Wiegerinck, E.T., Swinkels, D.W., Townsend, M.A., Trinder, D., and Peeling, P. 2012. The effects of carbohydrate ingestion during endurance running on post-exercise inflammation and hepcidin levels. Eur. J. Appl. Physiol. **112**: 1889-98. doi:10.1007/s00421-011-2156-0.

Smith, E., Alvarez, J., Kearns, M., Hao, L., Sloan, J., Konrad, R., Ziegler, T., Zughaier, S., and Tangpricha, V. 2016. High-dose vitamin D3 reduces circulating hepcidin concentrations: A pilot, randomized, double-blind, placebo-controlled trial in healthy adults. Clin. Nutr. Journal. **s0261-5614**(16): 30148-0. doi: 10.1016/j.clnu.2016.06.015.

Steensberg, A., Febbraio, M., Osada, T., Schjerling, P., van Hall, G., Saltin, B., and Pedersen, B. 2001. Interleukin-6 production in contracting human skeletal muscle is influenced by pre-exercise muscle glycogen content. J. Physiol. **537**: 633-639. PMID:11731593.

Tappy, L., Acheson, K., Curchod, B., Schneiter, P., Normand, S., Pachiaudi, C., Temler, E., Riou, J.P., and Jequier, E. 1994. Overnight glucose metabolism in obese non-insulin-dependent diabetic patients and in healthy lean individuals. Clin. Physiol. **14**: 251-65. doi:10.1111/j.1475-097X.1994.tb00383.x.

Varamenti, E.I., Kyparos, A., Veskoukis, A.S., Bakou, M., Kalaboka, S., Jamurtas, A.Z., Koutedakis, Y., and Kouretas, D. 2013. Oxidative stress, inflammation and angiogenesis markers in elite female water polo athletes throughout a season. Food. Chem. Toxicol. **61**: 3-8. doi:10.1016/j.fct.2012.12.001.

Vogt, S., Heinrich, L., Schumacher, Y.O., Grosshauser, M., Blum, A., Konig, D., Berg, A., and Schmid, A. 2005. Energy intake and energy expenditure of elite cyclists during preseason training. Int. J. Sports. Med. **26**: 701-6. doi:10.1055/s-2004-830438.

Wachsmuth, N.B., Aigner, T., Volzke, C., Zapf, J., and Schmidt, W.F. 2015. Monitoring recovery from iron deficiency using total hemoglobin mass. Med. Sci. Sports. Exerc. **47**: 419-27. doi:10.1249/MSS.0000000000000420.

Ward, D.M. and Kaplan, J. 2012. Ferroportin-mediated iron transport: expression and regulation. Biochem. Biophys. Acta. **1823**: 1426-33. doi:10.1016/j.bbamcr.2012.03.004.

Wilkie, S.S., Dominelli, P.B., Sporer, B.C., Koehle, M.S., and Sheel, A.W. 2015. Heliox breathing equally influences respiratory mechanics and cycling performance in trained males and females. J. Appl. Physiol. **118**: 255-64. doi:10.1152/japplphysiol.00400.2014.

Woodson, R., Wills, R., and Lenfant, C. 1978. Effect of acute and established anemia on O2 transport at rest, submaximal and maximal work. J. Appl. Physiol. Respir. Environ. Exerc. Physiol. doi:10.1016/j.jcte.2014.01.003.

Yang, Q., Jian, J., Katz, S., Abramson, S.B., and Huang, X. 2012. 17beta-Estradiol inhibits iron hormone hepcidin through an estrogen responsive element half-site. Endocrinology, **153**: 3170-8. doi: 10.1210/en.2011-2045.

Zughaier, S.M., Alvarez, J.A., Sloan, J.H., Konrad, R.J., and Tangpricha, V. 2014. The role of vitamin D in regulating the iron-hepcidin-ferroportin axis in monocytes. J. Clin. Transl. Endocrinology, **1**: 19-25. doi:10.1016/j.jcte.2014.01.003.

Table 1. Mean ± SD heart rate (HR), energy expenditure (Kcal), rating of perceived exertion (RPE), power output (Watts) and pre- and post-exercise mass in the whey protein isolate + carbohydrates drink (PRO), whey protein isolate + carbohydrates + vitamins D_3 and K_2 drink (VPRO) and non-nitrogenous zero calorie control drink (PLA) conditions.

Drink	HR (BPM)	Average Power (Watts)	Watts/kg	Energy Expenditure (Kcal)	MAX Power (Watts)	Rating of Perceived Exertion (1-10)	Mass (kg) Pre	Mass (kg) Post	Mass (kg) Change
PRO	169 ± 9	269 ± 33	3.7 ± 0.3	942 ± 117	427 ± 43	8.7 ± 1.3	72.2 ± 6.2	71.7 ± 6.2 §	-0.58 ± 0.38
VPRO	170 ± 11	278 ± 31	3.8 ± 0.3	971 ± 107	430 ± 46	8.6 ± 0.9	72.3 ± 6.6	71.7 ± 6.4 §	-0.60 ± 0.36
PLA	169 ± 10	270 ± 31	3.8 ± 0.3	946 ± 109	427 ± 55	9.0 ± 1.3	72.1 ± 6.4	71.6 ± 6.4 §	-0.51 ± 0.29

Note: Results are presented as (mean ± SD). § = Significant difference ($p < 0.05$) from Baseline to Post-exercise. SD, standard deviation; BPM, beats per minute; kg, kilograms; Kcal, kilocalories; VPRO, whey protein isolate + carbohydrates + vitamins D_3 and K_2 drink; PRO whey protein isolate + carbohydrates drink, PLA, non-nitrogenous zero calorie control drink.

Table 2. Mean ± SD for interleukin-6, hepcidin-25, serum iron, serum ferritin, hemoglobin and hematocrit levels at baseline, post-exercise, and three hours post-exercise in the whey protein isolate + carbohydrates drink (PRO), whey protein isolate + carbohydrates + vitamins D_3 and K_2 drink (VPRO) and non-nitrogenous zero calorie control drink (PLA) conditions.

Blood Biomarker	PRO			**VPRO**			**PLA**		
	Baseline	Post-exercise	3 hr Post-exercise	Baseline	Post-exercise	3 hr Post-exercise	Baseline	Post-exercise	3 hr Post-exercise
Serum Iron (mcg mol L-1)	21.30 ± 5.08	23.00 ± 5.39 §	18.80 ± 5.22 *δ	20.70 ± 7.51	23.00 ± 8.82 §	17.90 ± 5.88 *δ	23.80 ± 8.90	25.44 ± 9.32 §	21.10 ± 6.79 *δ
Serum Ferritin (mcg L-1)	101.10 ± 85.17	108.30 ± 85.38 §	104.20 ± 84.27 *δ	88.20 ± 68.91	87.50 ± 77.86 §	91.90 ± 70.51 *δ	87.00 ± 81.74	84.60 ± 89.19 §	90.70 ± 79.52
Serum IL-6 (pg mL-1)	0.92 ± 1.70	3.79 ± 2.94 §	1.64 ± 1.98 *δ	0.81 ± 0.74	3.12 ± 2.32 §	1.29 ± 1.31 *δ	0.73 ± 0.66	3.10 ± 1.99 §	1.40 ± 0.89 *δ
Hemoglobin (mg L-1)	151.50 ± 8.18	154.40 ± 7.68 §	150.44 ± 10.36 *	150.20 ± 10.37	155.56 ± 10.73 §	147.40 ± 10.02 *δ	149.10 ± 7.91	153.22 ± 9.68	146.30 ± 9.88 *
Hematocrit	0.43 ± 0.02	0.44 ± 0.02 §	0.43 ± 0.02 *	0.43 ± 0.02	0.45 ± 0.02 §	0.42 ± 0.03 *δ	0.43 ± 0.01	0.44 ± 0.02	0.42 ± 0.02 *δ
Hepcidin-25 (nmol L-1)	14.18 ± 14.90	17.84 ± 19.84 §	25.44 ± 11.91 *δ	9.94 ± 8.93	11.78 ± 10.24 §	22.27 ± 13.41 *δ	10.44 ± 14.62	10.09 ± 7.68	22.57 ± 15.57 *δ

Note: Results are presented as (mean ± SD). § = Significant difference ($p < 0.05$) from Baseline to Post-exercise, * = Significant difference ($p < 0.05$) from Post-exercise to 3 hr Post-exercise, δ = Significant difference ($p < 0.05$) from Baseline to 3 hr Post-exercise. SD, standard deviation; hr, hour; mcg, micrograms, mol, mole; L, liter; mL, milliliter; pg, pictograms; VPRO, whey protein isolate + carbohydrates + vitamins D_3 and K_2 drink; PRO; whey protein isolate + carbohydrates drink; PLA, non-nitrogenous zero calorie control drink.