THE EFFECTS OF MACRO- AND MICRO- NUTRIENT TIMING ON POST-EXERCISE HEPcidIN RESPONSE IN ELITE AND PROFESSIONAL ATHLETES

by

DYLAN TIMOTHY DAHLQUIST

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Abstract

BACKGROUND INFORMATION

Iron deficiency (ID) has debilitating effects on athletic performance, causing significant reductions (-34%) in VO2max. Inflammation caused by exercise has been shown to impede iron absorption in the digestive tract by up-regulating the expression of the iron regulatory protein, hepcidin. To date, nutritional interventions to blunt hepcidin response have been few and equivocal. We investigated the effects of nutrient timing with essential macro- and micro- nutrients to potentially attenuate the post-exercise rise in hepcidin in highly trained athletes.

PURPOSE

To determine if a post-exercise drink consisting of whey protein isolate (25g) and carbohydrates (75g) with the addition of vitamins, D3 (5,000 IU) and K2 (1,000 mcg) (VPRO) or without D3 and K2 (PRO), following a bout of high-intensity interval exercise has an effect on the acute post-exercise hepcidin responses in athletes as compared to a non-caloric placebo drink (PLA). Our hypothesis was that both VPRO and PRO will significantly decrease hepcidin following a bout of high intensity exercise as compared to PLA, with VPRO supplementation having a greater effect on hepcidin versus PRO supplementation alone.

METHODS

Ten elite male cyclists (age: 26.9 ± 6.4 yrs; VO2max: 67.4 ± 4.4 ml/kg/min) partook in four cycling sessions. A randomized, placebo-controlled, single-blinded triple crossover design was utilized. Experimental days consisted of an 8-min warm-up at 50% pVO2max, followed by 8 x 3 min intervals at 85% pVO2max with, 1.5 min at 60% pVO2max between each interval. Blood samples were collected pre-exercise, post-exercise and three hours post-exercise. Three varying drinks (PRO, VPRO or PLA) were consumed immediately after the post-exercise blood sample.

RESULTS/CONCLUSIONS

The results from the investigation demonstrate that following a fatiguing interval-based cycling exercise in highly-trained athletes, subjects experienced a significant time-dependent increase in all biomarkers measured independent of post-exercise drink composition. In conclusion, the post-exercise drinks had no significant effect on any biomarker. The findings could potentially be related to the dosage of nutrients, the timing of blood samples, or the training status of individuals. The lack of an effect in either of the drinks on hepcidin and other biomarkers are contrary to our hypothesis.
Preface

Collaborators of this thesis study are as follows:

- Dr. Michael Koehle, MD, PhD, supervised the project, assisted in the ethics application, research design, funding application to Own The Podium (OTP) and provided guidance throughout the entire data collection and writing process.
- Dr. Trent Stellingwerff, PhD, helped in formulating the research design, aided with writing the proposal and obtaining funding via Own The Podium (OTP) grant in conjunction with the Canadian Sport Institute – Pacific (CSI-P), provided advice during the data collection and assisted in writing the final thesis.
- Dr. Don McKenzie, MD, PhD, assisted in trouble shooting potential issues with data collection, provided guidance for obtaining proper resources for sample analysis and aided in writing the final thesis.
- Dr. Brad Dieter, PhD, assisted with data collection, data analysis, troubleshooting, and provided ample amounts of feedback and ideas during the writing process.
- Dylan Dahlquist, BS, developed the research design, obtained funding from Own The Podium (OTP), the Canadian Sport Institute – Pacific (CSI-P) and the UBC Faculty of Education, created and completed the application for ethics approval, recruited subjects, performed all the data collected, purchased equipment for testing, assisted in assay analysis, analyzed the data and wrote the final thesis document.

No manuscripts resulting from the work presented in this thesis have been published to date. An abstract and poster will be submitted for a poster presentation at the SPort INnovation (SPIN) Summit organized by OTP in September 2016 held in: Calgary, Canada.

This study involved human subjects and was granted full board approval from the University of British Columbia Clinical Research Ethics Board (H15-00721).
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**List of Abbreviations**

ID: iron deficiency  
FPN1: ferroportin  
FECH: ferrochelatase  
RE: reticuloendothelial  
RES: reticuloendothelial system  
gp130: glycoprotein 130  
$\text{VO}_2\text{max}$: maximal oxygen uptake  
$p\text{VO}_2\text{max}$: power output (W) at maximal oxygen uptake ($\text{VO}_2\text{max}$)  
$v\text{VO}_2\text{max}$: velocity at maximal oxygen uptake ($\text{VO}_2\text{max}$)  
$\text{VO}_2$-kinetics: characteristics of oxygen uptake  
W: watts  
CBC: complete blood count  
ELISA: enzyme-linked immunosorbent assay  
EDTA: ethylenediaminetetraacetic acid  
SD: standard deviation  
RPE: rating of perceived exertion  
HR: heart rate  
HAMP: hepcidin antimicrobial peptide gene  
JAKs: janus kinases  
STAT: signal transducers and activators of transcription proteins  
HIF: hypoxic inducible factors  
ERFE: erythroferrone  
MPS: muscle protein synthesis  
mTORC1: mammalian target of rapamycin complex1  
PI3K-mTOR: phosphatidylinositol 3-kinase, mammalian target of rapamycin pathway  
IL-1B: interleukin-1B  
IL-6: interleukin-6  
IL-12: interleukin-12  
IL-10: interleukin-10  
TNF-α: tumor necrosis factor alpha  
Hbmass: hemoglobin (Hb) mass  
CKD: chronic kidney disease  
ALT: alanine  
AST: aspartate  
RANKL: receptor activator nuclear factor-κB ligand  
CRP: C-reactive protein  
PAR-Q: physically active readiness questionnaire  
LPS: lipopolysaccharide  
K$_1$: phylloquinone
K₂, MK-4: menaquinone-4
K₃: menadione
DMK: 2,3-dimethoxy-1,4-naphthoquinone
KCAT: 2-methyl, 3-(2’methyl)-hexanoic acid-1,4-naphtoquinone
LCHF: low-carbohydrate, high-fat
EPO: erythropoietin
RBCs: red blood cells
Hb: hemoglobin
ATP: adenosine triphosphate
GI: gastrointestinal tract
Fe³⁺: ferric
Fe²⁺: ferrous
dcytb: duodenal cytochrome B
STEAP: human 6-transmembrane epithelial antigen of prostate proteins
DMT1: divalent metal transporter 1
HPC1: heme protein carrier 1
25(OH)D: 25-hydroxyvitamin D
CHO: carbohydrate
PRO: protein and carbohydrate drink
VPRO: protein and carbohydrate with vitamin D₃ and K₂ drink
PLA: placebo drink
min: minute
g: gram
kg: kilogram
hr: hour
mL: milliliter
L: liter
UVB: ultraviolet B
EE: energy expenditure
Kcal: kilocalorie
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Introduction

Overview

Iron has central roles in oxygen delivery, heme structure, enzymatic transfers of electrons, cardiovascular health, oxidative and glucose metabolism, exercise-induced inflammation, neurological function, bone health and skeletal muscle tissue. All are critical components to athletic performance and animal and human studies have revealed that even without anemia, ID can decrease endurance performance and the oxygen carrying capacity of the blood up to ~30% (Brownlie, Utermohlen, Hinton, & Haas, 2004; Davies et al., 1984; Davies, Maguire, Brooks, Dallman, & Packer, 1982; Dellavalle & Haas, 2011). Despite this, iron deficiency (ID) is a global issue and the most prevalent nutrient deficiency in the world (Cogswell et al., 2009; Dellavalle & Haas, 2014), affecting roughly 20% of the world’s population (Schumann, Elsenhans, & Maurer, 1998) and athletes are even more susceptible to becoming ID (Koehler et al., 2012); with ~60 percent of all female athletes and ~4-50% suffering from some form of ID in a given year (Brandy, Christine, Robert, & Stephanie, 2003).

Although it seems plausible that ID can be prevented in athletes by meeting the recommended dietary intake of elemental iron (males: 8 mg/day; pre-menopausal females: 18 mg/day (Sim, Dawson, Landers, Trinder, & Peeling, 2014)), athletes need a higher intake of iron due to some of the deleterious outcomes of prolonged physical activity performed at very high intensities (Dopsaj et al., 2013); in addition to an increased iron turnover rate, gastrointestinal bleeding, foot strike hemolysis, iron malabsorption, sweat losses, inadequate hydration and, for women, menstruation (Brandy et al., 2003; Chatard, Mujika, Guy, & Lacour, 1999). Accordingly, recent data have shown that increases in inflammation caused by exercise (Varaumenti et al., 2013) down-regulates iron absorption in the human digestive tract (Przybyszewska & Zekanowska, 2014) by up-regulating the gene expression of the iron regulatory protein hepcidin (Roecker, Meier-Buttermilch, Brechtel, Nemeth, & Ganz, 2005). Hepcidin is an iron-regulatory protein (Roecker et al., 2005) that inhibits iron absorption, and has been shown to have both an acute and chronic phase in its increase post-exercise (Dellavalle & Haas, 2012). It has been reported that three hours post-exercise, hepcidin levels peak, and return to pre-exercise baseline by 24 hours (Wachsmuth, Aigner, Volzke, Zapf, & Schmidt, 2015), potentially as long as athletes have not subsequently supplemented with iron (Moretti et al., 2015). This response has been demonstrated both in urine collection (Peeling et al., 2009) and blood analysis (Sim et al., 2012). It has been
shown that baseline serum ferritin levels correlate with resting hepcidin concentrations (Peeling et al., 2014) and the rise in hepcidin post-exercise correlates with the increase in inflammation via anti-inflammatory cytokines, specifically interleukin-6 (IL-6) (Wachsmuth et al., 2015).

**Oxygen Delivery and Iron Metabolism**

An athlete’s maximal oxygen uptake (VO\textsubscript{2max}) is one of the best indicators, and most widely accepted measurements of an individual’s cardiorespiratory (aerobic) and fitness levels (Bassett & Howley, 1999). Trained individuals have been seen to have an enhanced ability to transport and utilize oxygen that is being carried throughout the bloodstream by the globular protein, hemoglobin (Hb), located on red blood cells (RBCs) (Hoffbrand, Moss, & Pettit, 2006). In order for Hb to properly function, it must have adequate supplies of macro- and micro- nutrients and minerals, which act as key regulators for a number of biochemical reactions in the body, including metabolic function and adenosine triphosphate (ATP) production. During strenuous exercise, metabolic adenosine triphosphate (ATP) production increases up to 1,000 times (Baker, McCormick, & Robergs, 2010), signifying the importance of and an increased nutrient demand for physically active individuals or athletes training at high intensities.

Although multiple physiological, biological and external factors can negatively affect the number of circulating Hb molecules in the bloodstream, and/or the quaternary protein structure of Hb (which disturbs the oxygen-hemoglobin dissociation curve) (Bottomley & Fleming, 2014), one of the most prevalent factors is dietary iron. It is well recognized that the trace mineral iron plays multiple roles in the human body. Dietary iron enables oxygen to properly bind to Hb at the alveoli, to transport it from the lungs through the blood and then unload it at the peripheral tissues (McLellan, 2004).

Iron derived from the diet goes through a complex absorption and exportation matrix that takes place in the human gastrointestinal (GI) tract, specifically, the proximal segment of the small intestine, (i.e. the jejunum and the duodenum) (Brie, Christopher, & Gregory, 2012; Okhee, 2011). There are two main membranes in the transportation process that the iron must cross to get from the GI into the enterocytes and then finally the blood (see Figure 1): the apical membrane and the basolateral membrane (Waldvogel-Abramowskia et al., 2014). Glycoproteins located in the duodenum called mucins (Przybyszewska & Zekanowska, 2014), are synthesized by GI mucosa and bind to iron ions (both ferric (Fe\textsuperscript{3+}) and ferrous (Fe\textsuperscript{2+}) forms), enabling absorption from the duodenum. Non-heme iron (commonly found in the Fe\textsuperscript{3+} state) is reduced to the more stable form
of iron, Fe\textsuperscript{2+} (Brie et al., 2012) by three different mechanisms: (1) the acidic environment of the gut, (2) reductase activities performed by duodenal cytochrome B (Dcytb) (Jeehyea et al., 2012) and STEAP proteins (Ohgami, 2006) located on the brush border (apical membrane) of the duodenum, and (3) reduction by ferrireductase (Ohgami et al., 2005). The reduced Fe\textsuperscript{2+} molecule interacts with a multitude of transporter proteins (Brie et al., 2012), mainly divalent metal transporter 1 (DMT1) (Przybyszewska & Zekanowska, 2014), to be transported into the enterocytes where it is stored as ferritin until later use.

![Image](image.png)

**Fig 1:** The effects of exercise and post-exercise hepcidin response on iron metabolism.

Heme iron absorption is less well understood, but enters the enterocyte via heme protein carrier 1 (HPC1) and is then cleaved of its hemoproteins to Fe\textsuperscript{2+} by heme oxygenase to then be added to the same pool of iron within enterocytes (Krishnamurthy, Xie, & Schuetz, 2007; Solange Le, Michael, & Miguel, 2012). Once in the enterocytes, the fate of the stored ferritin is multifaceted and is tightly regulated by the size of the iron pool and demands of iron within the body (Aisen,
2001). Primarily, if iron is required by the body for erythropoiesis, ferritin will be converted into Fe^{2+} and will exit the enterocyte via the main protein transporter, ferroportin (FPN1) (Ward & Kaplan, 2012), located on the basolateral membrane to reach the circulation.

Once in the bloodstream Fe^{2+} must reach the mitochondria. To do so it is transported there via two main proteins, transferrin (which contains roughly 90% of iron in the body) and transferrin receptor 1 (TfR1) (Chatard et al., 1999; Hoffbrand et al., 2006). One or two iron molecules bind to transferrin and are transported through the blood to interact with TfR1 located on cell membranes of erythroblasts in bone marrow (Punnonen, Irjala, & Rajamaki, 1997). Here is where iron is incorporated to synthesize a mature Hb protein (Hoffbrand et al., 2006). The protein Hb is largely synthesized in the mitochondria of a cell and is comprised of four polypeptide (globin) chains, α_1β_1 and α_2β_2, and a heme group. The polypeptide chains are derived from amino acids and the heme group is synthesized after protoporphyrin (derived from bone marrow) combines with iron in the Fe^{2+} state by the rate-limiting enzyme, ferrochelatase (FECH) (Bottomley & Fleming, 2014). However, without enough circulating iron, Hb synthesis is attenuated which will subsequently decrease oxidative metabolism, lactate threshold and aerobic performance (Dellavalle & Haas, 2012). Physical activity performed at very high intensities and/or other illnesses producing an inflammatory state impairs iron absorption from the gut (Przybyszewska & Zekanowska, 2014) by up-regulating the gene expression of the iron regulatory protein hepcidin (Roecker et al., 2005).

**Hepcidin, IL-6 & Iron Absorption**

Hepcidin is an iron-regulatory protein (Roecker et al., 2005) that inhibits iron absorption, and has been shown to have both an acute and chronic phase in the post-exercise window (Dellavalle & Haas, 2012). The 25 amino acid cysteine-rich peptide is largely synthesized in hepatocytes (Munoz, Villar, & Garcia-Erce, 2009) and secreted into the blood from the liver (Edina et al., 2013). It is expressed in response to various stimuli (Schmidt & Prommer, 2010); such as liver iron levels, inflammation due to injury/illness (Auersperger et al., 2013), hypoxia (Haase, 2010; Liu, Davidoff, Niss, & Haase, 2012) and exercise (Auersperger et al., 2013). Upon stimulation, the hepcidin antimicrobial peptide (HAMP) gene encodes hepcidin production after the prohormone version of hepcidin, pro-hepcidin, binds to and transcribes the expression of HAMP (Edina et al., 2013). This suggests the hepcidin gene expression acts as an autoregulatory
loop, regulating its own production (Edina et al., 2013). Hepcidin then acts as an antimicrobial protein and controls the rate of iron exportation from the enterocytes and macrophages by binding to the main protein transporter located on the cell membranes, FPN1 (Ward & Kaplan, 2012).

It has been reported that three hours post-exercise, hepcidin levels peak, and return to baseline 24 hours later (Wachsmuth et al., 2015). This time course has been in both urine and blood analysis (Peeling et al., 2009; Sim, Dawson, Landers, Trinder, et al., 2014). The increase in hepcidin concentration happens secondary to the rise of the anti-inflammatory cytokine, IL-6 (Wachsmuth et al., 2015). IL-6 is stimulated by acute inflammation and binds to IL-6 receptor alpha and gp130 (Heinrich et al., 2003). Once bound to the complex, IL-6 activates Janus kinases (JAKs) and in turn stimulates the proliferation of signal transducers and activators of transcription (STAT) proteins. Out of the STAT family, STAT3 has been shown to bind to the hepcidin promoter gene and in turn upregulates hepcidin expression (Wrighting & Andrews, 2006). Furthermore, hepcidin levels are also increased during periods of iron overload (Xinggang et al., 2012) and reversed in cases of anemia or ID (Nemeth & Ganz, 2006). Both mechanisms result in an attenuation of stored ferritin being released from enterocytes, macrophages and the liver (Munoz et al., 2009); inhibiting the body’s ability to facilitate erythropoiesis (Gammella et al., 2015). In addition to the above, hepcidin concentrations express a diurnal rhythm and are innately affected by periods of prolonged fasting (Troutt et al., 2012) and iron supplementation (Moretti et al., 2015). Multiple studies have shown that hepcidin levels are the highest in the morning, progressively decline throughout the day and then increase again in the late evening (Dale, Burritt, & Zinsmeister, 2002; Sinniah, Doggar, & Neil, 1969; Wiltink, Kruithof, Mol, Bos, & Van Eijk, 1973). Additionally, supplementation with oral iron increases hepcidin concentrations within a 24-hour period and thus further impedes iron absorption the following day (Moretti et al., 2015).

Recent work has focused on different recovery manipulations in order to elucidate ways to mitigate the acute post-exercise hepcidin response in the athletic population. The use of carbohydrate nutrient partitioning on the transient shift of hepcidin following a training session will be the first part of the following section. We then elaborate on two areas that warrant further research and are the focus of this proposal, the specific nutrient timing with protein and carbohydrates, and vitamins D₃ and K₂.
Carbohydrate Feeding

Supplementation with carbohydrates (CHO) to prolong exercise performance has been an established practice in events lasting at least one hour in duration (Stellingwerff & Cox, 2014). Recent work has shown that not only does CHO intake have an effect on sparing muscle glycogen (Stellingwerff et al., 2007) leading to an increase in time to exhaustion performance, but large doses of 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., 1985; Nieman et al., 1998). It has been shown that depleted intramuscular glycogen content drives IL-6 response (Keller et al., 2001) which subsequently influences hepatic glucose metabolism (Steensberg et al., 2000), and the consumption of exogenous CHO in and around a bout of exercise prevents muscle glycogen depletion (Steensberg et al., 2001; Stellingwerff et al., 2007; Tsintzas, Williams, Boobis, & Greenhaff, 1995, 1996). It has therefore been postulated that CHO intake could blunt the post-exercise hepcidin response by modulating the IL-6 response.

Robson-Ansley and colleagues (2011) utilized a much lower dosage of CHO, to prevent potential GI discomfort (Rehrer, van Kemenade, Meester, Brouns, & Saris, 1992; Smith et al., 2013; Stellingwerff et al., 2007), to see the effects of both pre- and intra- CHO ingestion on circulating hepcidin post-exercise. In a randomized double-blind crossover design, participants consumed a beverage immediately before and every 20 minutes during a 120-minute run at 60% VO\(_2\)max followed by a five-km time trial. The beverages were either an 8% CHO (2 mL kg\(^{-1}\)) solution or a matched placebo (PLA) (Robson-Ansley, Walshe, & Ward, 2011). Results showed CHO supplementation significantly reduced post-exercise IL-6 concentrations, but had no effect on serum hepcidin concentrations when compared to the PLA group, (Robson-Ansley et al., 2011). This finding could be due to the fact that the authors only measured hepcidin immediately and 24 hours after cessation of the 120-minute run and five-km time trial, and not three hours post-exercise when levels have been shown to peak (Dellavalle & Haas, 2011). Furthermore, hepcidin levels return to baseline measurements 24 hours thereafter.

However, recent work by Sim and colleagues (2012) further built on the model that CHO supplementation during exercise could blunt the post-exercise hepcidin response, and strengthened the design by looking at hepcidin responses both immediately, three hours and 24 hours post-exercise. The researchers concluded that ingestion of a 6% CHO solution at 3 mL kg\(^{-1}\) every 20 minutes during the endurance run (90 minutes at 75% peak oxygen uptake velocity (\(v\)VO\(_2\)peak))
had no effect on inflammatory biomarkers (e.g., IL-6) or serum hepcidin levels when compared to consuming water alone (Sim et al., 2012). Ihalainen and colleagues (2014) supported the above findings, reporting that post-exercise CHO supplementation at various doses (0.0%, 1.5% and 7.0%) had no effect on IL-6 concentrations following a continuous 18- to 20- km run at 75% of VO2\textsubscript{max} in seven recreational runners (4 men and 3 women) (Ihalainen, Vuorimaa, Puurtinen, Hamalainen, & Mero, 2014). Badenhorst and colleagues (2015) showed in 11 well-trained endurance athletes that early (immediately post-exercise and two hours post-exercise) or late (two hours post-exercise and four hours post-exercise) post-exercise feeding with 1.2 g kg\(^{-1}\) of CHO supplementation did not significantly alter post-exercise IL-6 and hepcidin responses in either condition after 8 x 3 minute high intensity interval runs at 85% vVO2\textsubscript{max}. The authors did show a time-dependent increase in hepcidin concentrations both three and five hours post-exercise compared to baseline, but there was no significant difference between the early or late feeding strategies (Badenhorst et al., 2015b).

Recently, low-carbohydrate, high-fat (LCHF) diets have gained in popularity in the athletic population. Although controversy exists whether one can actually increase performance by adapting to a LCHF diet (Burke, 2015), recent finding from Badenhorst and colleagues (2015) showed that over the course of 24 hours, a moderate CHO diet (3 g kg/day\(^{-1}\)) in well-trained endurance athletes (VO2\textsubscript{peak}: 68.9 ± 7.2 mL kg\(^{-1}\) min\(^{-1}\)) resulted in significantly higher pre- and post- exercise measurements of hepcidin when compared to a high CHO diet (10 g kg\(^{-1}\)). The authors report that the reason for the increase hepcidin levels are due to the upregulation of gluconeogenic signaling of the liver caused by the impaired glycogen stores from the LCHF diet (Badenhorst et al., 2015a).

**Protein and Inflammation**

The common practice of elite athletes training multiple times per day, is to consume some form of high-quality protein in order to maximize training adaptations and initiate the remodeling of skeletal muscle tissue (Phillips & Van Loon, 2011). Moreover, an athlete’s ability to recover from strenuous exercise relies on the ability to upregulate net muscle protein synthesis (MPS). Multiple physiological, biological and morphological factors can negatively affect that rate of protein synthesis in skeletal muscle tissue (Schoenfeld, 2010). The mammalian target of rapamycin complex 1 (mTORC1) is commonly accepted as one of the master regulators for cellular growth
and regeneration (Knight, Schmidt, Birsoy, Tan, & Friedman, 2014). Inhibition of mTORC1 will lead to a potential decrease in hypertrophic and strength adaptations to resistance training (Atherton & Smith, 2012). More specifically, the PI3K-mTOR pathway has been shown to modulate the expression of pro- and anti-inflammatory cytokines involved in the acute phase response of exercise (Weichhart & Saemann, 2009). Mutations in PI3K-mTOR leads to an increase inflammatory state and atrophy to muscle tissue (Weichhart & Saemann, 2009), which could be mediated by chronic elevations IL-6 (Haddad, Zaldivar, Cooper, & Adams, 2005). However, the initial release of IL-6 from working skeletal muscle plays a pinnacle role in the growth and regeneration of muscle tissue via the stimulation of myosatellite cells and the incorporation of new myonuclei into pre-existing muscle fibers (Morgan & Partridge, 2003; Serrano, Baeza-Raja, Perdiguer, Jardi, & Munoz-Canoves, 2008). Additionally, the JAK/STAT pathway described earlier, has a dual role in proliferation and differentiation of muscle cells (Sun et al., 2007; Wang, Wang, Xiao, Wang, & Wu, 2008). First, the JAK1/STAT1/STAT3 pathway prevents premature myoblast differentiation and fusion by blocking the expression of MyoD and MEF2 (Fernando, Kelly, Balazsi, Slack, & Megeney, 2002; Hunt, Upadhyay, Jazayeri, Tudor, & White, 2011). Alternatively, the JAK2/STAT2/STAT3 pathways augments the expression of the above proteins, in addition to increasing growth factors related to hypertrophy, such as insulin-like growth factor (IGF) (Glass, 2010; Jacquemin, Furling, Bigot, Butler-Browne, & Mouly, 2004).

The PI3K-mTOR pathway is activated in response to a favorable energy status (eucaloric or hypocloric), growth factors (e.g., IGF), amino acids and cellular stress (Howell & Manning, 2011; Sengupta, Peterson, & Sabatini, 2010). Consequently, mTORC1 located in erythrocytes is down-regulated in response to low iron (both in vivo and in vitro), low energy availability and deoxyribonucleic acid (DNA) damage (Dunlop & Tee, 2009; Soliman, 2005); which indicates that mTORC1 is, in part, mediated by the availability of iron in the blood (Knight et al., 2014). Furthermore, it has been demonstrated that mTOR plays a part in regulating the expression of hepcidin by direct inhibition of HAMP gene expression (Mleczko-Sanecka et al., 2014). Thus, being ID and having chronically elevated levels of IL-6 could be deleterious to the human body. By upregulating mTORC1 by maximizing MPS could potentially lead to the suppression of hepcidin either indirectly (e.g., blunting chronic elevations of IL-6) or directly (e.g., inhibiting HAMP gene expression).
Three grams of the essential amino acid, leucine, maximizes muscle protein synthesis (Pennings et al., 2011; Tang, Moore, Kujbida, Tarnopolsky, & Phillips, 2009). Whey protein has been shown to have a greater effect on net protein synthesis in the muscle when compared to casein and soy (Pennings et al., 2011; Tang et al., 2009), due to in part the high leucine content (Churchward-Venne et al., 2014; Rowlands et al., 2015). Evidence shows that when ample amounts of carbohydrates (CHO) are added to a protein-rich meal post-exercise, it further potentiates muscle glycogen re-synthesis and recovery (Berardi, Price, Noreen, & Lemon, 2006; Howarth, Moreau, Phillips, & Gibala, 2009; Ivy et al., 2002). Thus, it could be postulated that combining ample amounts of CHO and protein around a bout of exercise, could blunt hepcidin by putting an individual in an energy positive state while supplying ample amounts of amino acids to stimulate mTOR.

Kerasioti et al (2013) tested this hypothesis by taking nine physically-active men (age: 28 ± 2 years, VO\textsubscript{2}max: 4.1 ± 0.2 L/min) and subjecting them to a two-hour exhaustive cycling bout. Immediately post-exercise, subjects either consumed a special calorically matched CHO-whey cake with high protein (23.92 ± 0.40 g) or low protein (9.20 ± 0.40 g). Compared to the low protein cake, the high protein cake significantly (\(p < 0.05\)) reduced levels of IL-6 (-50%) and CRP (-46%) four hours post-exercise (Kerasioti et al., 2013). Furthermore, a recent intra-exercise supplementation study by Schroer et al (2013) showed a significant decrease in IL-6 activity following a 120-minute constant load cycle (55% of peak power) when subjects supplemented with 90 g of whey protein hydrolysate (45 g hour\(^{-1}\)) compared to a noncaloric sweetened (Splenda™) placebo drink (Schroer, Saunders, Baur, Womack, & Luden, 2014). The authors concluded that the differences in IL-6 activity following the session were likely due to the protein ingestion and a reduction of metabolic damage.

Given the associations with hepcidin and inflammation, one can theorize that an ingestion of a protein-rich drink following a bout of fatiguing exercise may be able to blunt the hepcidin rise three hours following cessation. This hypothesis has yet to be tested in the current literature.

**Vitamin D\textsubscript{3} and K\textsubscript{2} on Inflammation**

Vitamin D, a fat-soluble vitamin (McCollum, Simmonds, Becker, & Shipley, 1922), is an essential vitamin acting as a precursor steroid to a host of metabolic and biological processes in the human body. Insufficient levels in the major circulating metabolite of vitamin D, 25-
hydroxyvitamin D [25(OH)D] and the active hormonal form, 1,25-dihydroxyvitamin D have been shown to significantly ($p < 0.001$) decrease hemoglobin concentrations in a linear fashion (Patel et al., 2010). Patel and colleagues (2010) reported that insufficient levels were associated with a higher prevalence of anemia (2-3 fold increase) when compared to subjects with higher levels (Patel et al., 2010). Recent research has focused on the benefits of vitamin D supplementation to decrease inflammatory cytokines (Nonn, Peng, Feldman, & Peehl, 2006), when induced by exercise (Barker, Schneider, Dixon, Henrik sen, & Weaver, 2013), in healthy individuals (Bacchetta et al., 2014) and in chronically ill patients with chronic kidney disease (CKD) (Zughaier, Alvarez, Sloan, Konrad, & Tangpricha, 2014). More explicitly, vitamin D supplementation has been shown to blunt hepcidin via the direct reduction of HAMP expression and up-regulating FPN1 (Bacchetta et al., 2014).

Two recent studies have shown that dosages of 50,000 IU (7,143 IU/day for three months) and 100,000 IU (single dose) of vitamin D can significantly reduce hepcidin levels (28% and 34%, respectively) in healthy individuals and patients suffering from CKD (which affects over 40% of adults ages 65 and older) (Bacchetta et al., 2014; Zughaier et al., 2014). Zughaier and colleagues (2014) further demonstrated a dose-dependent response in lipopolysaccharide (LPS) stimulated THP-1 cells treated with 1,25-dihydroxyvitamin D from 5 nm to 40 nm lead to a subsequent decrease in pro-hepcidin cytokines (Zughaier et al., 2014).

Furthermore, at a much lower dosage and in physically-active healthy male subjects, Barker and his colleagues (2013) showed an inverse relationship with vitamin D supplementation and exercise-induced inflammation in a randomized, double-blind, placebo-controlled study. The authors demonstrated that 4,000 IU/day of vitamin D over 28 days, significantly ($p < 0.05$) decreases the inflammatory biomarkers alanine (ALT) and aspartate (AST) after 10 sets of 10 repetitions of peak isometric force eccentric-concentric jumps 24, 48, 72 and 168 hours later.

The literature regarding toxicity levels of vitamin D remain inconclusive (Heaney, 2008; Holick, 2005). In a recent review by our lab, a relationship has been seen with vitamin D acting synergistically with vitamin K in order to prevent toxicity and calcification (Dahlquist, Dieter, & Koehle, 2015). Specifically, a vitamin K$_2$ variant, MK-4, has been shown to regulate osteoclastogenesis. Vitamin K$_2$ prevents vascular calcification and hypervitaminosis by carboxylating osteocalcin proteins formed in mature bone cells stimulated by receptor activator nuclear factor-kB ligand (RANKL) (Gundberg, Lian, & Booth, 2012). RANKL is produced via
the stimulation of vitamin D. If vitamin K is not supplemented with vitamin D, osteocalcin proteins remain un-carboxylated, which potentially causes vascular calcification and toxicity (Koul et al., 2011). Additionally, un-carboxylated osteocalcin cells has been shown to increase IL-6 and C-reactive protein (CRP) concentrations in the elderly from poor plasma levels of vitamin K (Shea, Dallal, et al., 2008). This suggests that increasing vitamin K stores may reduce inflammatory cytokine activity, in addition to preventing vitamin D toxicity.

Redi and colleagues (1995) demonstrated that various vitamin K variants inhibit the production of IL-6 via lipopolysaccharide (LPS) stimulated human fibroblasts, in vitro (Reddi et al., 1995). Each compound tested, phylloquinone (K₁), menaquinone-4 (K₂, MK-4), menadione (K₃), 2,3-dimethoxy-1,4-naphthoquinone (DMK), and 2-methyl, 3-(2’methyl)-hexanoic acid-1,4-naphtoquinone (KCAT) [synthetically derived] were capable of inhibiting IL-6 production at varying degrees. Out of the commonly found vitamin K variants in supplements and food, K₁ and K₂, MK-4 decreased IL-6 production to a greater extent than K₁ (~195 pg/mL and ~95 pg/mL, respectively) (Reddi et al., 1995).

Rat models have demonstrated that vitamin K-rich diets can decrease LPS-induced inflammation when compared to vitamin K-deficient diets (Koul et al., 2011; Ohsaki et al., 2006). Furthermore, higher concentrations of circulating vitamin K are significantly (p < 0.05) associated with lower concentrations of inflammatory markers, specifically CRP (Shea, Booth, et al., 2008). In a study of 510 elderly patients with type II diabetes mellitus evaluated after a one-year follow-up, individuals consuming a vitamin K-enriched (70.5 to 767.5 mcg/day) diet had lower concentrations of IL-6 (-27.9%) and other inflammatory cytokines when compared to those with insufficient (≤ 69.4 mcg/day) vitamin K diets (Juanola-Falgarona et al., 2013). However, it is uncertain whether vitamin K₁ supplementation at dosages of 500 mcg/day are sufficient enough to decrease IL-6 activity over a 3-year time period in obese individuals (Shea, Dallal, et al., 2008) and standard American diets have been shown to have far less than the RDA (Booth, Webb, & Peters, 1999), such as 12-24 mcg/day in all age groups (2-70+ years of age) (Booth, Pennington, & Sadowski, 1996a) with the exception of infants fed baby formula (Booth, Pennington, & Sadowski, 1996b; Shearer, 2009; von Kries & Gobel, 1992). The above findings suggest the need for further studies with higher dosages of vitamin K and different vitamin K variants.

Approximately 4,000 to 5,000 IU/day of vitamin D₃ in combination with 50 to 1,000 mcg/day of vitamin K₁ and K₂ seems to be a safe dose and has the potential to aid athletic
performance. Additionally, based on the above literature, dosages in this range can potentially blunt the post-exercise hepcidin response if timed appropriately.

**Summary & Potential Significance**

Athletes, both female and male, are susceptible (~60% and ~4 to 50%, respectively) to the adverse effects of ID. In individuals with ID, VO\(_2\)max can drop by up to 34% (Booth et al., 1996a; Woodson, Wills, & Lenfant, 1978). When it comes to the elite few, marginal gains of a mere 1% can dictate whether or not an Olympic Athlete will place on the podium (10,000 m Final, London Olympics 2012, 3\(^{rd}\) and 4\(^{th}\)), this 34% reduction is problematic. There are currently many strategies to correct ID non-anemic and ID anemic athletes (DellaValle & Haas, 2014; Hinton & Sinclair, 2007; Wachsmuth et al., 2015); which have all shown to augment performance measurements when compared to a placebo. In contrast, research shows inconsistent results when trying to prevent ID from occurring by mitigating the rise of hepcidin following training.

As previously mentioned, both macro- and micro- nutrients have potent effects on post-exercise inflammatory biomarkers which are associated with hepcidin. Intervention trials manipulating CHO alone have had equivocal findings (Badenhorst et al., 2015b; Ihalainen et al., 2014; Nehlsen-Cannarella et al., 1985; Nieman et al., 1998; Robson-Ansley et al., 2011; Sim et al., 2012), and more so, restricting CHO intake over a 24-hour period can increase one’s susceptibility to ID by elevating baseline hepcidin concentrations (Badenhorst et al., 2015a). Protein ingested during (Schroer et al., 2014) and after with additional CHO (Kerasioti et al., 2013) has been shown to down regulate IL-6 activity both immediately after exercise and four hours after exercise. Furthermore, recent research has focused on the benefits of vitamin D supplementation to decrease inflammatory cytokines (Nonn et al., 2006), when induced by exercise (Barker et al., 2013), in chronically ill patients with CKD (Zughaier et al., 2014) and healthy individuals (Bacchetta et al., 2014). Lastly, vitamin K has been shown to decrease IL-6 concentrations (Ohsaki et al., 2006; Reddi et al., 1995) and prevent vitamin D toxicity (Dahlquist et al., 2015).

Given the associations of hepcidin release with inflammation (e.g., IL-6), an ingestion of a CHO plus protein rich drink with or without the addition of vitamin D\(_3\) and K\(_2\) following a bout of fatiguing exercise may be able to blunt the post-exercise hepcidin response. This hypothesis has yet to be tested.
Effect of a Post-exercise Drink with or without Additional Vitamins on Hepcidin

Introduction
Iron deficiency (ID) is disturbingly prevalent in elite endurance athletes (~60% of all female athletes (Brandy et al., 2003) and ~4 to 50% of all male athletes (Hinton, 2014) suffer from some form of ID in a given year), with significant potential for deleterious effects on performance. Iron-repletion studies in ID and ID non-anemic athletes have shown significant ($p < 0.05$) reductions in fat mass and increases in lactate threshold, energetic efficiency and expenditure, ventilatory threshold, and in severe ID cases when an athlete takes an iron supplement, studies have shown a 7.4% increase in maximal oxygen uptake ($VO_2^{max}$) and Hbmass (DellaValle & Haas, 2014; Hinton & Sinclair, 2007; Wachsmuth et al., 2015). Hbmass is an indicator of aerobic capacity in elite field hockey players (Hinrichs et al., 2010), and every one gram increase in Hbmass results in a 2.3 mL/min increase in $VO_2^{max}$ (Wachsmuth et al., 2015).

Some initial and preliminary research has focused on different recovery modalities and nutritional interventions in order to try and mitigate the increase in acute post-exercise hepcidin response in athletic populations (Badenhorst et al., 2014; Robson-Ansley et al., 2011; Sim et al., 2012), which would mechanistically increase the bioavailability of iron absorption and recycling (Ganz & Nemeth, 2012). 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., 1985; 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 (Badenhorst et al., 2015b; Ihalainen et al., 2014; Robson-Ansley et al., 2011; Sim et al., 2012). Furthermore, low CHO availability over a 24 hour period has been shown to increase baseline and post-exercise hepcidin levels in trained men (Badenhorst et al., 2015a).

A recent intra-exercise protein supplementation study by Schroer et al. (2014) showed a significant ($p < 0.05$) decrease in IL-6 activity following a 120 minute constant load cycle (55% of peak power) performance when subjects supplemented with 90 g of whey protein hydrolysate over two hours (45 g hour$^{-1}$) compared to a noncaloric sweetened (Splenda) placebo drink (Schroer et al., 2014). The differences in IL-6 activity in this study design were postulated to be contributed to the protein ingestion (Schroer et al., 2014). Furthermore, consumption of a special CHO-whey protein cake has shown a favorable effect on inflammation following an exhaustive bout of cycling
(two hours) (Kerasioti et al., 2013). Compared to the low protein cake (Total Intake: 101.20 ± 3.40 g of CHO and 9.20 ± 0.4 g of protein), the high protein cake (Total Intake: 82.80 ± 3.40 g of CHO and 23.92 ± 0.40 g of protein) significantly (p < 0.05) reduced the inflammatory levels of IL-6 (-50%) and C-reactive protein (CRP) (-46%) four hours post-exercise (Kerasioti et al., 2013). Given the associations of hepcidin release with inflammation (and IL-6), an ingestion of a CHO plus protein rich drink following a bout of fatiguing exercise may be able to blunt the post-exercise hepcidin response. Furthermore, this mimics that of a more common practice of elite athletes post-training, which is to co-ingest CHO and protein in order to maximize training adaptations and initiate the remodeling of skeletal muscle tissue (Phillips & Van Loon, 2011).

Lastly, recent research has focused on the benefits of vitamin D supplementation to decrease inflammatory cytokines (Nonn et al., 2006), when induced by exercise (Barker et al., 2013), in chronically ill patients with chronic kidney disease (CKD) (Zughaier et al., 2014) and healthy individuals (Bacchetta et al., 2014). Additionally, vitamin K has been shown to decrease IL-6 (Ohsaki et al., 2006; Reddi et al., 1995) and prevent vitamin D toxicity (Dahlquist et al., 2015).

Therefore, instead of focusing on the iron supplementation side of therapy which in essence, is repairing something that is already broken, the main objective of the study is to reverse the hepcidin mediated interference with iron absorption through specific nutrient timing with essential macro- and micro- nutrients.

**Purpose, Objectives and Hypothesis**

Based on the literature presented above, the primary objective of the project was to employ a novel yet practical method to address iron malabsorption and ID by down-regulating the exercise-induced protein hepcidin following a training session with a CHO and protein-rich post-exercise drink with or without the addition of vitamins D₃ and K₂. Decreasing the activity of hepcidin via this method could help increase iron absorption in athletes and prevent issues of ID. Lastly, we aimed to investigate whether there was a further benefit from adding additional vitamins (D₃ and K₂) to the post-exercise drink to determine whether hepcidin was not further blunted when compared to the consumption of protein and CHO alone.
The Aims and Objectives of This Study are Listed Below

Research Question(s)
To determine if a post-exercise drink consisting of whey protein isolate (~25g) and CHO (~75g) with the addition of vitamins, D₃ (5,000 IU) and K₂ (1,000 mcg) (VPRO) or without vitamin addition (PRO) following a bout of exercise has an effect on the acute post-exercise hepcidin response in athletes as compared to a placebo drink (zero calorie; PLA).

Objective(s)
1) To investigate the effects of PLA vs. VPRO vs. PRO drink following a high intensity exercise bout on inflammation and hepcidin, 2) To test a novel recovery modality that can be utilized by athletes to mitigate iron malabsorption.

Primary Research Outcomes
The specific aim of this project was to address ways athletes can manipulate their recovery period from training in order to optimize iron absorption and distribution by blunting the hepcidin response.

Secondary Research Outcomes
To determine if a post-exercise drink consisting of high CHO and protein content derived from whey protein immediately following a bout of an exhaustive training session had an effect on the acute post-exercise inflammatory markers.

Hypothesis
(1) That both VPRO and PRO will significantly decrease hepcidin following a bout of high intensity exercise as compared to PLA; and

(2) VPRO supplementation will have a greater effect on hepcidin versus PRO supplementation alone.
Experimental Design / Description of Project

Subject Recruitment

Ten male elite endurance athletes were recruited for the study with the following inclusion criteria: endurance or endurance-power sport athletes who were healthy, physically active, currently or previously competing at a high level (e.g., Elite, Professional or CAT 1/2/3 for Cycling, International or National Level, etc.), were not consuming anti-inflammatories on a regular basis, 18-45 years of age, and training 5-6 times a week with at least ≥1 year of training experience. Furthermore, athletes were required to have baseline serum ferritin levels of ≥ 30 mcg/L in order to partake in the study (Auersperger et al., 2013; Peeling et al., 2014), and subjects refrained from taking vitamin D, vitamin C, vitamin K, and multi-vitamins 24 hours prior to experimental days were. Furthermore, subjects refrained from using fish oil, krill oil, turmeric and serrapeptidase supplements for 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 (Sim, Dawson, Landers, Trinder, et al., 2014; Yang, Jian, Katz, Abramson, & Huang, 2012). Once the criteria were met, subjects completed a Physically Active Readiness Questionnaire (PAR-Q) and provided written consent. The University of British Columbia Clinical Research Ethics Board granted full approval for the study (H15-00721).

Sample Size Determination

A conservative 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 8 x 3 minute intervals running sessions at 85% of their maximal aerobic velocity (vVO₂max) 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. There was a significant time (p = 0.01) and interaction effect (p = 0.049) between hypoxic and normoxic conditions on hepcidin-25. Based on the differences of mean and standard deviations between baseline measurements and three hours post-exercise for the hypoxic intervention (2.18 and 1.26, respectively) on the biomarker, hepcidin-25, an effect size of (dz = 1.730159) and a minimum sample size of n = 9 subjects were needed.
Methods

Study Protocol
A randomized, placebo-controlled, single-blinded triple crossover design was used. Ten elite and professional male cyclists (age: 26.9 ± 6.4) with a VO\(_2\)max of 67.4 ± 4.4 mL/kg/min partook in four separate cycling sessions. A 48-hour washout was used and each session took place at the same time of day. Subjects recorded their standard diet and daily activity the day prior to the experimental visits and fasted for 10 hours overnight before reporting to the laboratory. Subjects were provided a standardized meal, which they consumed 60 minutes prior to arrival. Each meal was provided to the subjects on the previous visit (e.g., meal for visit 2 was given to the subjects after visit 1). The standardized meal consisted of a choice of the following food items: 3 packs of granola bars (Natures Valley, Crunchy – Honey & Oats; Minneapolis, MN, US). Subjects had the option to consume as much or as little as they wanted, and were required to repeat the exact selection on subsequent visits (3 and 4) (e.g. if they ate only 1 granola bar on visit 2, they had to eat only 1 granola bar on visits 3 and 4).

Screening Day
Prior to the familiarization phase (visit 1), subjects reported to a local laboratory in order to measure baseline serum ferritin and serum vitamin D, in order to rule out ID and ID anemia (Figure 2). Subjects with ferritin levels of ≥ 30 mcg/L were excluded from the study.

Visit 1, Familiarization Day
Visit 1 lasted two hours and consisted of equipment familiarization and VO\(_2\)max testing using an incremental cycle test (IET). Subjects then performed a familiarization to the exercise challenge, consisting of an 8-minute warm-up (cycle) at 50% power output (Watts) at VO\(_2\)max (pVO\(_2\)max), followed by 2 x 3 minute intervals at 85% pVO\(_2\)max. Each interval was separated by a 1.5 minute of active recovery at 60% pVO\(_2\)max (Figure 2).

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, Dominelli, Sporer, Koehle, & Sheel, 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$_2$ kinetics throughout the IET. The subjects’ VO$_2$max was then utilized to determine cycling power output (W) at percent (%) VO$_2$max ($p$VO$_2$max) for subsequent cycling tests.

**Fig 2**: Timeline of data collection (pre-screening and visit 1). After subjects passed pre-screening, they arrived to the laboratory to take part in visit 1. Visit 1 consisted of a self-selected warm-up directly into an incremental exercise test (IET) to exhaustion to determine $p$VO$_2$max for experimental visits (visits 2-4). Following the IET, subjects commenced a familiarization protocol. After familiarization, subjects began a self-selected cool-down and left the laboratory.
Visits 2 to 4 Experimental Days
During the three experimental days, 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. Subjects were allowed to drink water *ad libitum* during the trial. They were positioned on their own bike (used in visit 1) attached to the ergometer and fitted with a heart rate monitor. After an 8-minute warm-up at 50% \( p\text{VO}_2\text{max} \), subjects began the exercise test of 8 x 3 minute intervals at 85% \( p\text{VO}_2\text{max} \) with a 1.5 minute active recovery (60% \( p\text{VO}_2\text{max} \)) separating the bouts. Heart rate (HR) was collected throughout and rating of perceived exertion (RPE) was collected after each 3-minute interval. Following the final interval and 8-minute cool-down cycle at 50% \( p\text{VO}_2\text{max} \), a blood sample was taken. Immediately following the blood sample, subjects consumed one of the three experimental recovery drinks in a randomized single-blind triple crossover fashion (see below). Participants then rested in the laboratory for three hours, a venous blood sample was collected, and then subjects departed (see Figure 3). Total time for experimental days was approximately 6 hours.

A randomized, placebo-controlled, single-blinded triple crossover design
(\( \text{VPRO} \) vs. \( \text{PRO} \) vs. \( \text{PLA} \))
**Fig 3:** Timeline of data collection (experimental visits 2-4). After **baseline blood sampling** and **anthropometrics** were taken, subjects were set up on their own bicycle on the cycle trainer and fitted with a heart rate monitor. They then commenced the **warm-up** followed by the **high intensity cycling task** and subsequently the **cool-down** immediately following the final interval. Once subjects were off the bicycle, **body mass (kg)** was recorded and a **second blood sample** was taken. They then consumed one of the three **post-exercise drinks** (VPRO, PRO, or PLA) within 10 minutes following the end of the cool-down, then rested in the laboratory for three hours (**recovery period**). Following the recovery period, a **final blood sample** was taken and then participants departed.

**Post-exercise Recovery Drink Consumption**

One of three post-exercise drinks was immediately consumed within 10 minutes after the cessation of the cool-down in a randomized single-blind triple crossover fashion consisting of the following ingredients:

1) **PLA**: non-nitrogenous, zero calorie control drink (artificial flavour, sweetener and water).

2) **VPRO**: 75g of CHO (maltodextrin), 25g of protein (whey protein supplement), 10 droplets of a vitamin D complex containing 5,000 IU of vitamin D₃ and 1,000 mcg of vitamin K₂ (as menatetrenone), artificial flavour, sweetener and water.

3) **PRO**: 75g of CHO (maltodextrin), 25g of protein (whey protein supplement), artificial flavour, sweetener and water.

**Experimental Drink Contents**

Experimental drinks consisted of a whey protein supplement (Whey Protein Isolate – Vanilla - Restore, EXOS Fuel; Sandpoint, ID, US) containing whey protein isolate, sunflower lecithin, evaporated cane juice sugar, carboxymethylcellulose gum, xanthan gum, and stevia extract (leaf). 32g of dry protein powder (~1 scoop), equating to ~25g of protein, was weighed using a digital food scale and mixed with 550 mL of water for both **PRO** and **VPRO** conditions. Unflavoured maltodextrin (Cytocarb², CytoMax - CytoSport; Benicia, California, US) was used as the carbohydrate (CHO) source for each drink (1g of dry powder = 1g of CHO), and was sweetened with a commercially-available artificial flavour and sweetener (Kraft Foods, Crystal Light; Northfield, IL, US). Additionally, 10 droplets of a vitamin D + K complex (Vitamin D + K Complex, EXOS Fuel; Sandpoint, Idaho, US) containing 5,000 IU of vitamin D₃ and 1,000 mcg of vitamin K₂ was placed in the **VPRO** drink only. The placebo (**PLA**) drink consisted of 550 mL of water and the same artificial flavour and sweetener utilized for **PRO** and **VPRO**. All drinks...
looked, smelled, and tasted similar and were mixed in identical opaque bottles by a designated team member to blind the subject.

**Laboratory Procedures**

**Blood Collection**

Venous blood samples were taken at three different time periods, prior to exercise, pre-supplementation (post-exercise), and three hours post-exercise to measure serum ferritin, serum iron, serum interleukin-6 (IL-6), serum hemoglobin (Hb), hematocrit and hepcidin-25. All venous blood samples were taken after the subject rested in a seated position for five minutes in order to minimize the confounding effects of plasma volume changes due to posture. Blood was sampled 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 collection tube for blood sampling. 20.5 mL of blood was collected with each sample, for a total blood collection of 61.5 mL (~1% of total blood volume). Immediately following blood collection, the fresh samples (3 mL SST, 4.5 mL PST, and 3 mL EDTA) were taken to the University of British Columbia Hospital laboratory to perform a complete blood count (CBC), serum iron and serum ferritin. The two 5.0 mL SST samples were allowed to clot for 60 minutes at room temperature and then centrifuged at 10 °C and 1,500 G for 10 minutes. The centrifuged samples were separated into 1 mL aliquots and stored at -80 °C until later use. Once the blood samples were ready to be analyzed, they were transferred to a paid service lab where they carried out enzyme-linked immunosorbent assay (ELISA) on the blood biomarkers described above.

**Blood Analysis**

Blood was analyzed within a 24-hour period for Hb (CBC), serum iron and serum ferritin. The remaining frozen serum samples were used to measure the bioactive form of hepcidin, hepcidin-25 via c-ELISA, and 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 ng/mL.
Statistical Analysis
Results were analyzed utilizing Microsoft Excel (Microsoft, Office – Excel; Redmond, WA, US) and SPSS software (IBM North America, New York, NY). Data and results were reported as mean ± standard deviation (SD). A 3x3 repeated measures analysis of variance (supplement type X time) for each of hepcidin-25, Hb, IL-6, serum ferritin and serum iron was performed to assess the differences between the three conditions. Both IL-6 and hepcidin-25 were tested for order effect. Paired sample t-tests were performed to determine significance between 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 were transformed using the natural logarithm. Data that were statistically analyzed utilizing log transformed data are presented as mean ± standard deviation (SD) of non-transformed data within tables and results. Data presented in figures are either transformed or non-transformed (indicated on figures). F-ratios will be found significant at $p \leq 0.05$. 
Results

Subject Characteristics

Ten healthy, highly-trained male elite and professional athletes volunteered for the study. The subject pool consisted of Category 1 and 2 road and track cyclists, a national level track cyclist, two professional mountain bikers and one professional triathlete. Subject characteristics, baseline measurements, and VO\(_2\)max results are shown in Table 1.

<table>
<thead>
<tr>
<th>Subject (#)</th>
<th>Age (years)</th>
<th>Weight (kg)</th>
<th>Ferritin (ug/L)</th>
<th>Vitamin D (nmol/L)</th>
<th>VO(_2)max (mL/kg/min)</th>
<th>W(_{\text{max}}) (WATTS) @ VO(_2) Watts / kg</th>
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<tr>
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<td>101</td>
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<td>132</td>
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<td>61.7</td>
<td>94</td>
<td>54</td>
<td>72.5</td>
<td>410</td>
</tr>
</tbody>
</table>

| Mean       | 26.9        | 72.0        | 109             | 75                 | 67.4                   | 452                             |
| SD         | 6.4         | 6.4         | 77              | 28                 | 4.4                    | 35                              |
| Min        | 21.0        | 61.7        | 44              | 34                 | 61.3                   | 410                             |
| Max        | 38.0        | 83.5        | 313             | 132                | 72.5                   | 527                             |

Table 1: Subject characteristics, baseline measurements and VO\(_2\)max results.

Physiological Responses

Mean ± standard deviations (SD) of HR (bpm), RPE [1-10 Borg scale] (G. Borg, 1998; G. A. Borg, 1981), energy expenditure (Kcal) (Haakonssen, Martin, Burke, & Jenkins, 2013; Vogt et al., 2005), pre- and post-exercise body mass (kg) and mean power output (Watts) for the interval cycling trials are presented in Table 2. There were no significant differences between HR, RPE, pre- and post-exercise mass and mean power output between conditions. There was a significant difference between pre- and post-exercise body mass for PRO (\(p = 0.001\)), VPRO (\(p = 0.001\)) and PLA (\(p < 0.001\)), where mass decreased from pre- to post-exercise.

Serum Interleukin-6

There was a significant time effect (F[1.901, 15.205] = 53.638, \(p < 0.001\)), for PLA, VPRO and PRO (see Figure 4). However, there was no significant condition (F[1.241, 9.929] = 0.012, \(p = 0.948\)) effect on IL-6 (Table 3). There was no significant difference in baseline measurements of IL-6 for VPRO v PRO (t[-1.586], \(p = 0.147\)), VPRO v PLA (t[-0.407], \(p = 0.694\)), or PRO v PLA
(t[0.733], \( p = 0.482 \)). There was a significant increase from baseline to post-exercise for VPRO (2.869 ± 1.367 pg/mL, \( p = 0.001 \)), PRO (2.304 ± 1.649 pg/mL, \( p = 0.001 \)) and PLA (2.060 ± 1.604 pg/mL, \( p = 0.001 \)), and then a significant decrease from post-exercise to three hours post-exercise for VPRO (-2.153 ± 1.432 pg/mL, \( p = 0.001 \)), PRO (-1.832 ± 1.309 pg/mL, \( p = 0.001 \)) and PLA (-1.396 ± 1.467 pg/mL, \( p = 0.001 \)). IL-6 was significantly higher at three hours post-exercise than at baseline for each VPRO (\( p = 0.002 \)), PRO (\( p = 0.042 \)) and PLA (\( p = 0.004 \)) (0.716 ± 0.623 pg/mL, 0.472 ± 0.707 pg/mL and 0.664 ± 0.540 pg/mL, respectively). VPRO was significantly (\( p = 0.034 \)) higher than PRO immediately post-exercise. There was no order effect for IL-6 Values based on visit day.

**Serum Interleukin-6**

*Fig 4:* Mean ± SD of serum interleukin-6 levels at baseline, post-exercise and three hours post-exercise for VPRO, PRO and PLA conditions. § = 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. n=10 except for PLA post-exercise where n=9.
**Hepcidin-25 & Baseline Vitamin D**

There was a significant effect of time (F[1.234, 9.874] = 33.237, p < 0.001), but not type of drink (F[1.987, 15.897] = 0.911, p = 0.421) on hepcidin-25 for all conditions (see Figure 5). 3x3 repeated measures ANOVA revealed a significant increase from baseline to post-exercise (11.07 ± 41.72 ng/mL), baseline to three hours post-exercise (89.16 ± 57.40 ng/mL) and post-exercise to three hours post-exercise (78.82 ± 74.16 ng/mL) on hepcidin-25 levels in each condition (see Figure 2). There were no significant differences observed in the rate of change from baseline to post-exercise or post-exercise to three hours post-exercise or baseline to three hours post-exercise in VPRO vs PRO (p = 0.912, 0.961, and 0.894), VPRO vs PLA (p = 0.753, 0.615, and 0.589), or PRO vs PLA (p = 0.955, 0.902, and 0.542). Paired sample t-tests indicated a significant increase from baseline to post-exercise in VPRO and PRO (p = 0.031, 0.021, respectively) but no significant increase in PLA (p = 0.185). There was a significant increase from post-exercise to three hours post-exercise for VPRO, PRO and PLA (p = 0.110, 0.002, 0.000, respectively). Hepcidin-25 concentrations were significantly higher at three hours post-exercise than at baseline for VPRO, PRO and PLA (p = 0.110, 0.002, < 0.001, respectively). Furthermore, there was no order effect for hepcidin values based on visit day. A multivariate ANOVA revealed starting vitamin D levels (74.50 ± 28.00 nmol/L, MIN = 34 nmol/L, MAX = 132 nmol/L) did not affect hepcidin-25 response in the VPRO condition (p = 0.225).
**Fig 5:** Mean ± SD of hepcidin-25 levels at baseline, post-exercise and three hours post-exercise for VPRO, PRO and PLA conditions. § = 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. n=10 except for PLA post-exercise where n=9.

**Iron Parameters**

Repeated measures ANOVA revealed a significant time ($p = 0.001$) effect on serum iron and serum ferritin for all conditions (see Table 3 and Figure 6-7). There was no effect of drink on iron ($F[2, 14] = 1.518, p = 0.253$). After log transforming the non-normally distributed data of serum ferritin, results indicated no significant difference between VPRO, PRO and PLA for serum ferritin ($F[1.187, 9.494] = 4.733, p = 0.051$). Follow-up paired sample $t$-tests revealed a significant difference between VPRO when compared to PRO and PLA for serum ferritin at all time periods ($p < 0.05$). Paired sample $t$-tests demonstrated a significant increase from baseline to post-exercise for both serum ferritin and serum iron in VPRO ($p = 0.001$ [ferritin], 0.017 [iron]) and PRO ($p = 0.001$ [ferritin], 0.028 [iron]) and PLA ($p = 0.001$ [ferritin], 0.004 [iron])). There was a significant
decrease from post-exercise to three hours post-exercise for serum ferritin and serum iron in VPRO ($p = 0.004$ [ferritin], 0.001 [iron]) and PRO ($p = 0.002$ [ferritin], 0.002 [iron]). There was no significant difference from post-exercise to three hours post exercise in serum ferritin for PLA ($p = 0.090$). However, there was a significant decrease from post-exercise to three hours post-exercise in serum iron for PLA ($p = 0.004$). Serum ferritin levels were significantly higher for baseline ($p = 0.001, 0.037$), post-exercise ($p = 0.002, 0.035$) and three hours post-exercise ($p = 0.001, 0.016$) in VPRO when compared to PRO and PLA, respectively. Serum ferritin was significantly higher three hours post-exercise compared to baseline for PRO ($p = 0.003$), VPRO ($p = 0.001$) and PLA ($p = 0.014$). Serum iron was significantly lower three hours post-exercise compared to baseline in PRO ($p = 0.019$) and VPRO ($p = 0.032$) but not PLA ($p = 0.065$).

**Serum Ferritin**

![Graph showing serum ferritin levels](image)

**Fig 6:** Mean ± SD of serum ferritin levels at baseline, post-exercise and three hours post-exercise for VPRO, PRO and PLA conditions. § = Significant difference ($p < 0.05$) from Baseline to Post-exercise. * = Significant difference ($p < 0.05$) from Post-exercise to 3 hr
Post-exercise. \( \delta \) = Significant difference \((p < 0.05)\) from Baseline to 3 hr Post-exercise. 
n=10 except for PLA post-exercise where n=9.

**Serum Iron**

![Serum Iron Graph]

**Fig 7:** Mean ± SD of serum iron levels at baseline, post-exercise and three hours post-exercise for VPRO, PRO and PLA conditions. \( \$ \) = Significant difference \((p < 0.05)\) from Baseline to Post-exercise. \( * \) = Significant difference \((p < 0.05)\) from Post-exercise to 3 hr Post-exercise. \( \delta \) = Significant difference \((p < 0.05)\) from Baseline to 3 hr Post-exercise. 
n=10 except for PLA post-exercise where n=9.

**Hemoglobin & Hematocrit**

Significant time \((p = 0.001)\) effects were recorded for Hb and hematocrit. No significant difference was observed for either Hb \(F[2, 14] = 0.777, p = 0.479\) or hematocrit \(F[2, 14] = 0.424, p = 0.663\) between VPRO, PRO and PLA (see Table 3 and Figure 8-9). Paired sample \(t\)-tests demonstrated a significant increase from baseline to post-exercise for both Hb and hematocrit in VPRO \((p = 0.022 \text{ [Hb], 0.019 [hematocrit]}\)) and PRO \((p = 0.005 \text{ [Hb], 0.007 [hematocrit]}\)). There was no significant difference from baseline to post-exercise for Hb \((p = 0.066)\) or hematocrit \((p = 0.154)\) in PLA. There was a significant decrease from post-exercise to three hours post-exercise in all conditions.
for both Hb ($p = 0.004$ [VPRO], 0.002 [PRO], 0.001 [PLA]) and hematocrit ($p = 0.001$ [VPRO], 0.002 [PRO], 0.001 [PLA]). Hb was significantly lower three hours post-exercise compared to baseline in PRO ($p = 0.003$) only. Hematocrit was significantly lower three hours post-exercise compared to baseline in PRO ($p = 0.001$) and PLA ($p = 0.041$).

**Fig 8:** Mean ± SD of serum hemoglobin levels at baseline, post-exercise and three hours post-exercise for VPRO, PRO and PLA conditions. § = 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. n=10 except for PLA post-exercise where n=9.
**Fig 9:** Mean ± SD of hematocrit percent levels at baseline, post-exercise and three hours post-exercise for VPRO, PRO and PLA conditions. § = 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. n=10 except for PLA post-exercise where n=9.
Table 2: Summary for heart rate (HR), power output (Watts), energy expenditure (Kcal), rating of perceived exertion (RPE), pre- and post-exercise mass for VPRO, PRO, and PLA conditions. Expressed in mean ± SD. § = Significant difference (p < 0.05) from Baseline.

Table 3: Mean ± SD for serum interleukin-6 (IL-6), hepcidin-25, serum iron, serum ferritin, hemoglobin and hematocrit levels at baseline, post-exercise, and three hours post-exercise in VPRO, PRO, and PLA conditions. § = 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. n=10 except for PLA post-exercise where n=9.
Discussion
The present study examined the effects of a post-exercise drink consisting of whey protein isolate and carbohydrates (CHO) with vitamins D₃ and K₂ (VPRO) or without (PRO) as compared to a no-calorie placebo (PLA) following a bout of exercise on the acute post-exercise hepcidin response in highly-trained athletes. The results from the investigation demonstrate that following a fatiguing interval cycling exercise (8 x 3 min intervals at 85% pVO₂max), subjects experienced a significant time-dependent increase in hepcidin, IL-6, Hb, hematocrit and iron biomarkers independent of the post-exercise drink composition (see Table 3). Contrary to our hypothesis, VPRO, PRO or PLA post-exercise drink compositions had no significant (p > 0.05) effect on any biomarker measured.

The hepcidin levels in the current study mimic that of previous literature where peak levels were seen three hours post-exercise for all conditions (see Figure 5). This response is secondary to that of peak activity of IL-6 (Kemna, Pickkers, Nemeth, van der Hoeven, & Swinkels, 2005). There was no significant difference between either of the conditions from post-exercise to three hours post-exercise in the current study. This peak level in hepcidin three hours post-exercise has been correlated with altered iron metabolism within the athletic population (Wachsmuth et al., 2015), partly due to hepcidin interference with the main exporter of iron from the enterocyte and macrophage, FPN1 (Ward & Kaplan, 2012). Thus, individuals may be more susceptible to iron malabsorption near and around three hours post-exercise when hepcidin levels are at their highest. Furthermore, baseline measurements of hepcidin were not significantly different in any of the conditions, indicating that individuals did not come into the testing day in an already inflamed state. Hepcidin response in the current study is therefore characteristic of normal post-exercise inflammatory responses in combination with the rise of IL-6.

Following a bout of exercise, IL-6 has been reported to drastically increase five- to 100-fold (Ostrowski, Rohde, Asp, Schjerling, & Pedersen, 1999; Peeling et al., 2009), and peak levels are attained immediately following the cessation of exercise (Pedersen, 2000). IL-6 acts as both an anti-inflammatory (during exercise) and pro-inflammatory (post-exercise) cytokine (Wachsmuth et al., 2015), while also regulating glucose uptake into the cell as a glucoregulatory hormone (Steensberg et al., 2001). Our results demonstrated IL-6 significantly increasing from baseline to post-exercise in each condition (see Figure 4). The timing and quantity of CHO ingestion in and around a bout of exercise in relation to IL-6 changes has been well-studied, but the impact is
equivocal. The consumption of large quantities of CHO-rich beverages before and during exercise can blunt plasma IL-6 concentrations (Nehlsen-Cannarella et al., 1985; Nieman et al., 1998; Robson-Ansley et al., 2011). However, dosages more practical to prevent GI issues have yet to show a significant effect on IL-6 (Ihalainen et al., 2014; Sim et al., 2012).

There are a limited number of studies showing the effects of both protein and CHO consumption in and around a bout of exercise on inflammatory markers, and the results remain equivocal. Corsio-lima et al (2012) took six well trained (VO₂max: 66 ± 2.7 mL/kg/min) cyclists and subjected them to two separate 2.5-hour cycling sessions at 75% VO₂max in a hot environment (35 degrees Celsius and 60% relative humidity). Participants were given either a 6% CHO solution (4 mL kg⁻¹ bodyweight) or a 4:1 CHO-protein solution (4 mL kg⁻¹ bodyweight) in a randomized, crossover design every 15 minutes during the cycling sessions (Cosio-Lima, Desai, Stelzer, & Schuler, 2012). Total caloric ingestion over the 2.5-hour cycle was 1,040 calories or 416 calories/hour (CHO: 210 g and Protein: 50 g) for the 4:1 CHO-protein solution and 1,120 calories or 448 calories/hour (CHO: 280 g and Protein: 0 g) for the CHO only solution. There was a significant increase in IL-6 concentrations post-exercise, but no significant difference (p > 0.05) between conditions (Cosio-Lima et al., 2012). Another study took 12 well-trained cyclists and subjected them to a battery of high-intensity cycling tests on two different visits (Rowlands et al., 2008). Following the tests, subjects were given either a high protein (0.7 g kg⁻¹ hour⁻¹), moderate CHO (0.26 g kg⁻¹ hour⁻¹) beverage or a low protein (0.1 g kg⁻¹ hour⁻¹), high CHO (2.1 g kg⁻¹ hour⁻¹) beverage with equal fat content during a four hour recovery period. There was only a trivial effect on TNF-α and IL-6 despite the drink composition (Rowlands et al., 2008). These findings are in contrast of a more recent study in nine physically active men (age: 28 ± 2, VO₂max: 4.2 ± 0.2 L/min) who completed a two-hour cycle at 60-65% VO₂max followed by a four-hour recovery period. During the recovery period, both immediately after and two hours into the recovery period, subjects ingested either a high protein, moderate CHO cake (Total Intake, Protein: 23.92 g and CHO: 82.8 g, Calories: 426.9) or a low protein, high CHO cake (Total Intake, Protein: 9.2 g and CHO: 101.2 g, Calories: 441.6) (Kerasioti et al., 2013). There was a significant decrease in IL-6 concentrations by ~50% when subjects consumed the high protein, moderate CHO cake compared to the low protein, high CHO cake. Furthermore, it has been demonstrated in individuals cycling at a constant load (55% peak power) for 120 minutes that the consumption of 45 g of protein per hour significantly decreases IL-6 concentrations immediately post-exercise, when compared to a
non-caloric sweetened (Splenda™) placebo drink (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.

Our 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. 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. The protein source utilized by Schorer and colleagues (2014) was high in BCAA. Branched chain amino acids supplemented pre- and intra-workout have been shown to attenuate muscle soreness, muscle damage and inflammatory markers related to metabolic stress. Only water was utilized during the cycling intervals for the study at hand. Thus, metabolic and oxidative stress may have been attenuated if we chose to utilize a different feeding strategy (Schroer et al., 2014).

In addition to the above, the artificial (sucralose) and natural (Stevia) sweeteners utilized in the drinks may have had a small influence on post-exercise inflammation. Sucralose, commonly known as Splenda™, has been shown to have a immunosuppressant effect and decreases IL-6 and IL-10 (Rahiman & Pool, 2014). Rats fed 100, 300, 500, or 1,000 mg/kg of Splenda™ for 12 weeks demonstrated altered gut microflora an increased gut permeability in a dose dependent manor (Abou-Donia, El-Masry, Abdel-Rahman, McLendon, & Schiffman, 2008). Furthermore, Stevia has antioxidant, anti-inflammatory and antimicrobial effects (Muanda, Soulmani, Diop, & Dicko, 2011). Cultured LPS-activated macrophage cells treated with Stevia suppressed LPS-induced nuclear factor-κB and IL-6 in a dose:dependent fashion (6.25, 12.5, 25, and 50 mcg/mL) (Jeong et al., 2010). These finding are further supported by Kim and colleagues (2013), showing Stevia inhibited nuclear factor-κB gene expression in LPS-stimulated cells. The authors concluded that Stevia may have anti-inflammatory effects (Kim et al., 2013). Collectively, it could be assumed that both the sucralose and Stevia used in our study may have affected the inflammatory biomarkers three hours post-exercise. Despite the above findings to suggest this, neither the VPRO or PRO drinks which contained both sucralose and Stevia, nor the PLA drink which contained...
only sucralose, had any significant effect on IL-6 or hepcidin-25. Additionally, there was no significant difference between any of the conditions three hours post-exercise. This suggests that the artificial and natural sweeteners used in our study may not have had a large enough effect to show discrepancies between treatments; despite there being significant effects in rat models and \textit{in vitro} (Abou-Donia et al., 2008; Jeong et al., 2010; Kim et al., 2013; Rahiman & Pool, 2014).

Significant increases in serum ferritin and serum iron were reported from baseline to post-exercise in the current study (see Figure 6 and 7). This response mimics that of previous literature showing significant increases in serum ferritin levels and serum iron levels following the cessation of exercise (Badenhorst et al., 2015a; Badenhorst et al., 2014; Marc et al., 2013; Peeling, Dawson, Goodman, Landers, & Trinder, 2008; Peeling et al., 2009; Peeling et al., 2014; Sim et al., 2012). Exercise-induced inflammation has a robust effect on the reticuloendothelial system (RES), which is comprised of monocytes, macrophages and precursor cells (Knutson & 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, Schena, & Guidi, 1990). This leads to an increase in ferritin reuptake and reticuloendothelial (RE) cells then recycle ferritin via the spleen, liver and bone marrow (Knutson & 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, Hassler, Jost, & Friedmann-Bette, 2013). Our subjects lost a total of 0.6 ± 0.4, 0.6 ± 0.4 and 0.5 ± 0.3 kg for the VPRO, PRO and PLA visits, respectively. Every one g of body mass lost equates to roughly 1 mL of water (Cheuvront, Haymes, & Sawka, 2002; Sawka, Cheuvront, & Kenefick, 2012). Based on that assumption, our subjects would have lost approximately ~500-600 mL of water during each cycling session, and could have altered plasma volume and further explain why we saw a significant increase in serum Hb, hematocrit, serum ferritin post-exercise in all conditions. However, because we did not monitor fluid intake pre-exercise, intra-exercise and/or during the three hour recovery period, we cannot fully assess the effects of fluid intake and rehydration on the blood biomarkers measured in the current study; due to the fact the hydration status of the individual can greatly affect hematological responses to exercise (Maughan & Shirreffs, 2010).
Following post-exercise, there was a significant decrease in serum ferritin, Hb and hematocrit seen three hours post-exercise (see Figure 6, 8 and 9) could be attributed to this clearance mechanism of both serum ferritin and free Hb from the circulating plasma, and thus, increasing ferritin stores in the liver (Nemeth & Ganz, 2006; Nemeth et al., 2004). Furthermore, elevated levels of hepcidin have been shown to prevent iron recycling by macrophages (Nemeth et al., 2004), which could also further 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, due to ferritin being unable to be released from the macrophages.

Additionally, different modes of exercise can have a greater effect on hemolysis. High impact forces and exercise-induced hemolysis lead to an increase in serum ferritin and serum iron (Peeling et al., 2008). Running has been shown to have the greater impact on hemolytic responses when compared to cycling (Sim, Dawson, Landers, Swinkels, et al., 2014; Telford et al., 2003). Despite this, Sim et al (2012) reported that there are no significant differences on IL-6 or hepcidin concentrations if intensity and duration are matched between running and cycling. However, it has been shown that muscle glycogen depletion can significantly augment IL-6 activity 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, Katz, Cutler, Sherman, & Coyle, 1988). It may take upwards to 2-3 hours of exercise at 70-80% VO2max in order to fully deplete muscle glycogen stores (Coyle et al., 1983). Keller and colleagues (2001) demonstrated that depleted muscle glycogen stores leads to a ~40- and ~60- fold increase in IL-6 gene activation after 90 and 180 minutes of dynamic knee extensor exercises performed at 50-60% maximal workload when compared to a controlled (adequate supply) muscle glycogen content group. IL-6 was two-fold higher in the glycogen-depleted group then in the control group two hours post-exercise (Keller et al., 2001). With that in mind, the pre-workout meal containing roughly ~1.2 g CHO kg⁻¹ consumed one hour prior to the beginning of the high-intensity exercise trial, which has been shown to be the optimal range of CHO consumption to replete muscle glycogen stores (Burke, 2010; Ivy, 2004), and the nature of elite endurance athletes normal diet being high CHO (8 to 11 g/kg = 576.24 ± 51.52 to 792.33 ± 70.84) (Burke, 2001; Jeukendrup, 2011; Jeukendrup, Jentjens, & Moseley, 2005; Robins, 2007; Vogt et al., 2005) could have fully replenished muscle glycogen stores which subsequently decreases the post-exercise IL-6 response. Additionally, the nature of the exercise task at hand (8 x 3 min intervals at 85% pVO2max = ~900
Kcal), and the study population being elite and professional athletes who have been shown to have a greater capacity to store glycogen and replenish it at accelerated rates when compared to untrained individuals (Gollnick, Armstrong, Saubert, Piehl, & Saltin, 1972; Piehl, Adolfsson, & Nazar, 1973), muscle glycogen stores may not have been sufficiently taxed (depleted) in the PLA condition to begin to significantly influence IL-6 activity when compared to the VPRO and PRO conditions. Lastly, the post-exercise inflammatory response has been reported to be different in trained versus untrained individuals (Evans et al., 1986; Schild et al., 2016). Chronic adaptations to both resistance (Calle & 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. Thus, the findings of the study could be in part because our population was highly-trained athletes (VO2max of 67.4 ± 4.4 mL/kg/min). This combination of factors could possibly explain the current findings, where there was no significant differences in IL-6 or hepcidin activity for any condition immediately post-exercise and three hours post-exercise.

Vitamin D supplementation in healthy individuals (Bacchetta et al., 2014) and individuals suffering from CKD (Zughaier et al., 2014) have led to a marked decrease in hepcidin response following acute and chronic dosing. Bacchetta et al (2014) utilized a dosage 20-fold higher (100,000 IU) than that of the single dose used in our study. Furthermore, hepcidin measurements were monitored immediately before and 24 hours after supplementation (Bacchetta et al., 2014), and showed a 34% reduction in hepcidin concentrations. Results from Zughaier et al (2014) were obtained after a three-month follow up, with a dosing protocol of 7,143 IU/day of vitamin D3. The authors reported a 28% decrease in hepcidin concentrations in CKD patients (Zughaier et al., 2014). Furthermore, Barker and his colleagues (2013) showed that 4,000 IU/day of vitamin D supplementation over the course of 28 days significantly (p < 0.05) attenuated the inflammatory biomarkers alanine (ALT) and aspartate (AST) after 10 sets of 10 repetitions of peak isometric force eccentric-concentric jumps in modestly active and healthy adult males 24, 48, 72 and 168 hours following the exercise bouts. The study at hand looked at the acute effects of vitamin D3 and K2 supplementation within a three-hour time frame and at a much lower dose. No differences in IL-6 or hepcidin concentrations for our VPRO drink when compared to PRO or PLA. Thus, the supplements may not have been able to take effect within the small time frame and results could
have varied if IL-6 and hepcidin levels were re-assessed 24 hours after the ingestion of the drinks or if we utilized a chronic supplementation intervention.

Baseline measurements were taken for serum vitamin D in order to determine if starting levels had an effect on VPRO drink response (see Table 1). This measurement was taken since it has been shown that individuals with insufficient vitamin D stores have a higher risk of anemia (Patel et al., 2010) and low levels of ferritin blunts the post-exercise hepcidin response (Auersperger et al., 2013; Peeling et al., 2014). Thus, the authors made the assumption that baseline values of vitamin D may also effect the post-exercise hepcidin response in a similar fashion. Results from the study showed that baseline starting values of vitamin D had no significant effect on post-exercise hepcidin response three hours after the completion of the cycling protocol in the VPRO condition. Although a few of our subjects were classified as deficient (< 50 nmol/L, n=1) and insufficient (51-74 nmol/L, n=5) stores of 25(OH)D (Dahlquist et al., 2015), and Patel and colleagues (2010) reported that insufficient levels of 25(OH)D are associated with a higher prevalence of anemia (2-3 fold increase), none of our participants with low levels of 25(OH)D were classified with ID (see Table 1). Furthermore, only 10% of our subjects fell below 25(OH)D cut off set by Statistics Canada as compared with the mean value for Canadians in this age group (41%) (Canada, 2015). The higher than normal values seen with 25(OH)D could possibly be related to when the study commenced, which was right after summer cycling season for the athletes. It has been shown that 15 min of adequate sun exposure (290-315 nm of ultraviolet B [UVB] radiation) (Lim et al., 2005; Wolpowitz & Gilchrest, 2006) during the summer months produces 10,000 to 20,000 IU of vitamin D₃ (Heaney, 2008). It could be that sustained outdoor training and its concomitant increase in synthesis of vitamin D₃ (Zughaier et al., 2014), could have potentially elevated 25(OH)D concentrations in our study population and subsequently affected the blood biomarkers in the current study.

Lastly, vitamin K supplementation has been shown to blunt the inflammatory cytokine IL-6 (Ohsaki et al., 2006; Reddi et al., 1995; Shea, Booth, et al., 2008; Shea, Dallal, et al., 2008). Thus, it was postulated that hepcidin could be down-regulated by vitamin K supplementation. Unfortunately, our results did not show any significant difference in IL-6 or hepcidin when vitamin K₂ was combined with vitamin D₃ supplementation. In vitro studies in rat models, isolated cell cultures and obese humans demonstrate a marked reduction in IL-6 after vitamin K
supplementation with various dosages (Ohsaki et al., 2006; Reddi et al., 1995; Shea, Booth, et al., 2008; Shea, Dallal, et al., 2008). Ohaski and colleagues (2006) showed a marked reduction in LPS and a subsequent decrease in IL-6 when rats were fed a vitamin K-rich diet (75 mg/kg/day) compared to a vitamin K-deficient diet. Furthermore, 0.5 mL of vitamin K$_2$ supplementation to cell cultures treated with LPS have shown to decrease IL-6 (-190%) concentrations compared to cell cultures not treated with vitamin K (Reddi et al., 1995). Lastly, two studies conducted in elderly and obese individuals, have reported that those who had higher circulating concentrations of vitamin K and vitamin D had lower levels of inflammatory markers related to disease (Shea, Booth, et al., 2008; Shea, Dallal, et al., 2008). However, one of the studies indicated that further supplementation of 500 mcg/day of vitamin K showed no further impact on inflammatory markers, and the authors reported that the causation of lower levels of inflammation cannot solely be linked to vitamin K or vitamin D (Shea, Dallal, et al., 2008); because it could be assumed that diets rich in vitamin K are associated with healthier eating patterns. Thus, extrapolation to healthy and highly fit males may not be warranted based on the findings of the study (Reagan-Shaw, Nihal, & Ahmad, 2008; Sharma & McNeill, 2009; Tappy et al., 1994).
**Conclusions**

**Strengths, Limitations, Future Directions**

This is the first study to compare the effects of carbohydrates and protein with or without the addition of vitamin D$_3$ and K$_2$ against a placebo drink on the exercise-induced hepcidin response in elite and professional athletes in a randomized, placebo-controlled, single-blinded triple crossover design. The protein, CHO, and vitamin D + K supplements are all commercially-available and the acute intervention protocol utilized in the study is a practical method to be utilized for athletes training or competing. For the design of the experiment, safe dosages of vitamin D$_3$, K$_2$, CHO and protein were utilized for the intervention drinks to restore muscle glycogen, maximize MPS and prevent GI upset (Rehrer et al., 1992; Smith et al., 2013; Stellingwerff et al., 2007).

Although we did not report any significant effect on hepcidin, IL-6, Hb, hematocrit or iron biomarkers in any condition, other studies have utilized different nutrient timing protocols with similar CHO and protein dosages with varying results. Thus, future studies should revisit the effectiveness of pre- and peri-workout consumption of both CHO and protein with or without the addition of vitamin D$_3$ and K$_2$ on the exercise-induced hepcidin response. Other supplements with known effects on pro- and anti-inflammatory properties in the acute phase response also warrant further investigation (e.g., fish oil) (Li, Huang, Zheng, Wu, & Li, 2014).

Limitations to the study included: (1) the exclusion of females, (2) he lack of measurements of testosterone, (3) that subject diets were uncontrolled 24 hours prior to experimental visits, (4) that daily activity prior to experimental visits was not tightly controlled, (5) the lack of hematological and iron parameters 24 hours after cessation of exercise, and (6) the pre-workout meal. Sixty percent of all female athletes suffer from some form of ID within a given year. With such staggering statistics, research needs to emphasize ways to mitigate this issue. Since the menstrual cycle and estradiol affect the post-exercise hepcidin response (Sim, Dawson, Landers, Trinder, et al., 2014), the limited time frame in which the researchers had to complete the study made it impractical to include female participants. Future research needs to include females and consider the phase of the menstrual cycle during the time of testing in order to control for confounding variables. In response to heavy training loads, male elite endurance athletes have been shown to have significantly lower levels of circulating reproductive hormones (Banfi, Marinelli, Roi, & Agape, 1993; Cumming, Wheeler, & McColl, 1989; Ishigaki et al., 2005; Lin & Chang, 2008;
Lucia et al., 2001; MacConnie, Barkan, Lampman, Schork, & Bieitins, 1986; Weimann, 2002). Of the main reproductive hormones, testosterone has an intricate role in regulating hepcidin (Bachman et al., 2010; Bachman et al., 2014; Guo et al., 2013). Low testosterone levels act in a similar dose:response manner to high estrogen and/or progesterone levels to blunt hepcidin concentrations (Sim, Dawson, Landers, Trinder, et al., 2014; Yang et al., 2012). Since our subjects were highly trained elite and professional endurance athletes, testosterone levels could have potentially been low which would have inflated the hepcidin response of our study. Future studies looking at the effects of testosterone on hepcidin concentrations three hours post exercise in this population is warranted. Twenty-four-hour diet control is also suggested for future research. Although our results indicated that baseline IL-6 and hepcidin concentrations were not significantly different between testing days, it has been shown that low glycogen availability can increase these markers at baseline. Since diet was not controlled for within a 24-hour period leading into experimental visits, subjects might have started the study in a slightly depleted state, confounding the results. Additionally, the reverse could have happened, and muscle glycogen stores could have been topped out, especially due to the CHO heavy pre-workout meal consumed 60 minutes prior to the cycling test, subsequently effecting the post-exercise inflammatory response. Furthermore, daily activity was not monitored; elite and professional athletes train multiple times per week (Knechtle, Rust, Rosemann, & Martin, 2014; Stellingwerff, 2012, 2016; Storen, Bratland-Sanda, Haave, & Helgerud, 2012), thus in order to make the study and adherence protocol mimic that of real life training, athletes were allowed to continue their normal training throughout the experimental visits. Lastly, we did not monitor any hematological changes four or 24 hours after the ingestion of the post-workout drink, significant effects on IL-6 activity have been demonstrated four hours after the cessation of exercise following the consumption of a CHO-protein mixture (Rowlands et al., 2008).

**Conclusions Regarding Thesis Hypotheses**

**Hypothesis 1:** I reject our hypothesis that VPRO and PRO would significantly decrease hepcidin following a bout of high intensity cycle as compared to PLA. There was no significant difference between all three conditions.
**Hypothesis 2:** I reject our hypothesis that VPRO supplementation would have a greater effect on decreasing the post-exercise hepcidin response when compared to PRO supplementation. There was no significant difference between VPRO and PRO and hepcidin concentrations post-exercise and three hours post-exercise.

**Conclusion & Practical Application**
Although previous research has demonstrated that consuming a CHO and protein-rich beverage post-exercise will help to accelerate recovery, increase subsequent performances in the same day and enhance muscle repair mechanisms, the consumption of a CHO and protein-rich drink with or without the addition of vitamin D₃ and K₂ had no significant effects on hepcidin, IL-6, Hb, hematocrit, serum ferritin or serum iron in the present study. Athletes, nutritionists and coaches should take this into consideration when structuring nutrient partitioning, and may consider avoiding the three-hour post-exercise window when supplementing iron in order to enhance iron absorption. The effects of different recovery modalities, nutrient timing strategies and exercise interventions on the post-exercise hepcidin and IL-6 response in the athletic population has continued to gain interest among researchers and sport medical practitioners, as we try to determine the optimal paradigm to maximize iron absorption in athletes. Future research needs to focus on the timing, the constituents and dose of nutrition and supplement protocols from both an acute and chronic standpoint.
References


cycling exercise spares muscle glycogen but does not affect intramyocellular lipid use, 454(4). doi:10.1007/s00424-007-0236-0


Appendix A

Participant Information Checklist

Research Study: The effects of post-workout macro- and micro-nutrient timing to maximize iron bioavailability by minimizing exercise-induced hepcidin release.

The following information will summarize important aspects that you should keep in mind before visiting our lab for testing days. Please feel free to contact me, Dylan Dahliquist, either by email: [email] or by my cell number [#]: Thanks!

Location of Testing Site (Laboratory):
Room #3031, Biological Sciences
6270 University Blvd.
Vancouver, BC, Canada V6T 1Z4

Special Considerations on Accessing the Lab: If the door is shut, please knock very loudly or call the lab number (#) at or my [Dylan’s] cell phone number listed above.

What to Bring With You

For The Experiment (Exercise)
- Cycling Clothing
- Cycling Shoes
- Cycling Socks
- Cycling Pedals + Cleats
- Water Bottle
- Your Personal Bike

After The Experiment (Recovery Period)
- Change of Clothes
  o You’ll be sweaty and will probably want to get out of your wet cycling clothes during the 3-hr Recovery Period
- Towel and Personal Care Products
  o There is a shower near our lab where you will be able to take one during the 3-hr Recovery Period if you choose to. Thus, we recommend you bring a towel to dry off and if you do not want to use the shampoo, conditioner and body soap that we will have onsite, you are more than welcome to bring your own as well.
- Something to keep you occupied during the 3-hr Recovery Period
  o Laptop
  o School Work
  o Reading Material
  o Video Games [e.g., Gameboy]
  o Knitting Tools
  o Etc.

If anything comes up and you would like to reschedule your testing days, please feel free to contact me either by email or my cell [#] as soon as possible. Thanks for all your help!

Best regards,

Dylan Dahliquist
Appendix B

SUBJECT CONSENT TO PARTICIPATE

Research Study: The effects of post-workout macro- and micro-nutrient timing to maximize iron bioavailability by minimizing exercise-induced hepcidin release.

By signing the form you indicate that you have read and understood the information regarding this study.

➢ My signature on this consent form means:
➢ I have read and understood the subject information and consent form.
➢ I have had the opportunity to ask questions and have had satisfactory responses to my questions.
➢ I have had sufficient time to consider the information provided and to ask for advice if necessary.
➢ I understand that all of the information collected will be kept confidential and that the results will only be used for scientific objectives.
➢ I understand that my participation in this study is voluntary and that I am completely free to refuse to participate or to withdraw from this study at any time without any consequence.
➢ I understand that I am not waiving any of my legal rights as a result of signing this consent form.
➢ I understand that there is no guarantee that this study will provide any benefits to me.
➢ I have been told that I will receive a signed copy of this consent form for my own records.

I consent to participate in this study.

[Subject’s Signature] [Printed Name] [Date]

[Signature of Person Obtaining Consent] [Printed Name] [Date]

[Investigator Signature] [Printed Name] [Date]
NUTRITION & TRAINING
To be filled out each day prior to testing

Today's Date (mm/dd/yyyy): ___________ Time of Day (hh:mm): _______________
ID (8): ___________________________ Sex: ________________________________

Food Intake
Please report food item, rough amounts (cups, tablespoons, grams, etc.), and time of consumption

Morning (Breakfast / Snacks / Brunch)

Mid-day (Lunch / Snacks)

Evening (Dinner / Snacks)
**Caffeine Intake:**
Do you consume caffeine on a regular basis?

- [ ] Yes
- [ ] No

If yes, please list the sources of caffeine and time of consumption of caffeine:

________________________________________________________________________

**Supplements:**
Do you consume any supplements on a regular basis? Examples: Vitamin D, Iron, Vitamin C, Multivitamins, Fish Oil, Creatine, etc.

- [ ] Yes
- [ ] No

If yes, please list the types of supplements, dosages and time of consumption:

________________________________________________________________________

________________________________________________________________________

**Anti-Inflammatories:**
Do you consume anti-inflammatories on a regular basis?

- [ ] Yes
- [ ] No

If yes, please list the how often and for what reason:

________________________________________________________________________

________________________________________________________________________

**Physical Activity:**
Please list out type, duration and intensity.

________________________________________________________________________

________________________________________________________________________

________________________________________________________________________

________________________________________________________________________

**Hours of Sleep:**

________________________________________________________________________

Description: Nutrient Timing Study – Training + Nutrition Questionnaire
Version: 3.0 (July 22, 2015)
TRAINING & HEALTH HISTORY
To be filled out prior to any testing

Today’s Date (mm/dd/yyyy): ____________ Time of Day (hh:mm): ____________
ID (#): ____________________________ Sex: ____________________________

Average Weekly Training Log
Please report all sport or activities you partake in, training volume (minutes per session, sessions per week, etc.), and intensity of each session.

Primary Sport (or Activity)

Secondary (or Additional) Sport (or Activity)

Health History
Have you ever had an extreme medical emergency, illness or biomechanical injury that prevented you from training and competing for a prolonged duration?

☐ Yes  ☐ No

If yes, please describe/list when and how it occurred, and when you returned to sport:

________________________________________________________________________________________

Description: Nutrient Timing Study – Training + Nutrition Questionnaire
Version: 3.0 (July 22, 2015)
**Competition History:**
Do you compete competitively?

- [ ] Yes
- [ ] No

If yes, please list how long you have been a competitor and when was your last race:


What is the highest level you competed at?


**Average Cycling Volume:**
How many hours/week do you cycle: __________________________

How many km/week do you cycle: __________________________

**Training Season & Phase**
What season are you in for your training (check one)?

- [ ] In-Season
- [ ] Off-Season

What type of phase are you in your training season?

- [ ] Strength
- [ ] Power
- [ ] Speed & Agility
- [ ] Endurance/Base

**Previous VO2max Testing**
If you know your VO2max, what was it: __________________________

If you answered the above question, please list when (mm-dd-yyyy), the rough time of day (hh:mm) and what modality was utilized (e.g., Bike, Treadmill, etc.): __________________________


Thanks for Taking the Time to Fill Out This Questionnaire!
UNIVERSITY OF BRITISH COLUMBIA
School of Kinesiology
Faculty of Education
Rm. 3031, Biological Sciences
6270 University Blvd
Vancouver, BC, Canada V6T 1Z4

EMERGENCY MEDICAL PLAN

Research Study: The effects of post-workout macro- and micro-nutrient timing to maximize iron bioavailability by minimizing exercise-induced haptoglobin release.

All personnel involved with exercise testing and supervision will be trained in cardiopulmonary resuscitation (CPR) and use of the automated external defibrillator (AED).

The following emergency equipment will be functioning and readily available at the testing site. All personnel will be notified of the location of the emergency equipment prior to initiation of exercise testing:

- Automated external defibrillator
- Oxygen tank
- Sphygmomanometer, including aneroid cuff and stethoscope
- AMBU bag (manual resuscitator) with pressure release valve
- Oxygen mask
- Oral airways
- Adhesive tape
- Alcohol preparation pads
- Gauze pads
- Gloves
- Emergency documentation forms

There will be at least one CPR and AED trained person immediately available at all times when maximal exercise testing is performed.

Telephone numbers for emergency assistance will be posted clearly on all telephones. Emergency communication devices will be functioning and readily available.

UBC Hospital Urgent Care Center (604-822-7222) the paramedics will be advised as to the exercise testing laboratory location as well as the usual times of operation. The UBC Hospital is approximately 250 meters from the laboratory.

If a problem occurs during exercise testing, the supervising physician or paramedic or code team will be summoned immediately. The physician will decide whether to call for evacuation to the nearest hospital. If a physician is not available and any questions exist as to the status of the patient, then emergency transportation to the closest hospital will be summoned immediately.

Description: Nutrient Timing Study – Emergency Protocol
Version: 1.0 (June 11, 2015)
Appendix E

PAR-Q & YOU

(A Questionnaire for People Aged 15 to 69)

Regular physical activity is fun and healthy and increasingly more people are starting to become more active every day. Being more active is very safe for most people. However, some people should check with their doctor before they start becoming much more physically active.

If you are planning to become much more physically active than you are now, start by answering the seven questions in the box below. If you are between the ages of 15 and 69, the PAR-Q will tell you if you should check with your doctor before you start. If you are over 69 years of age, and you are not used to being very active, check with your doctor.

Common sense is your best guide when you answer these questions. Please read the questions carefully and answer each one honestly: check YES or NO.

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If you answered YES to one or more questions

Talk with your doctor by telephone or in person BEFORE you start becoming much more physically active or BEFORE you have a fitness appraisal. Tell your doctor about the PAR-Q and which questions you answered YES.

* You may be able to do any activity you want — as long as you start slowly and build up gradually. Or, you may need to restrict your activities to those which are safe for you. Talk with your doctor about the kinds of activities you wish to participate in and follow his/her advice.
* Find out which community programs are safe and helpful for you.

If you answered NO honesty to all PAR-Q questions, you can be reasonably sure that you can:

* start becoming much more physically active — begin slowly and build up gradually. This is the safest and easiest way to go.
* take part in a fitness appraisal — this is an excellent way to determine your basic fitness so that you can plan the best way for you to be active. It is also highly recommended that you have your blood pressure evaluated. If your reading is over 144/94, talk with your doctor before you start becoming much more physically active.

DELAY BECOMING MUCH MORE ACTIVE:

* If you are not feeling well because of a temporary illness such as a cold or a fever — wait until you feel better; or
* If you are, or may be pregnant — talk to your doctor before you start becoming more active.

PLEASE NOTE: If your health changes so that you then answer YES to any of the above questions, tell your fitness or health professional. Ask whether you should change your physical activity plan.

No changes permitted. You are encouraged to photocopy the PAR-Q but only if you use the entire form.

NOTE: If the PAR-Q is being given to a person before he/she participates in a physical activity program or a fitness appraisal, this section may be used for legal or administrative purposes.

"I have read, understood and completed this questionnaire. Any questions I had were answered to my full satisfaction."

NAME

SIGNATURE OF PARENT
or GUARDIAN (for participants under the age of 18 months)

DATE

WITNESS

Note: This physical activity clearance is valid for a maximum of 12 months from the date it is completed and becomes invalid if your condition changes so that you would answer YES to any of the seven questions.

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