

Acute dietary carbohydrate manipulation and the subsequent inflammatory and hepcidin responses to exercise

Claire E. Badenhorst¹ · Brian Dawson¹ · Gregory R. Cox² · Coby M. Laarakkers^{3,4} · Dorine W. Swinkels^{3,4} · Peter Peeling¹

Received: 8 December 2014 / Accepted: 28 July 2015 / Published online: 3 September 2015
© Springer-Verlag Berlin Heidelberg 2015

Abstract

Purpose To examine the effects of 24-h controlled carbohydrate intake on next day pre- and post-exercise inflammatory and hepcidin responses.

Methods In a crossover design, 12 well-trained endurance athletes (Ht 181.08 ± 7.68 cm; Wt 74.8 ± 11.5 kg, $\dot{V}O_{2peak}$ 68.9 ± 7.2 ml kg⁻¹ min⁻¹) completed two experimental (2-day) trials. On day 1, participants completed a glycogen depletion task, including a 16-km run (80 % $\dot{V}O_{2peak}$) and 5 × 1 min efforts (130 % $\dot{V}O_{2peak}$) separated by 2-min recovery. Subsequently, strict dietary control was enforced for 24 h, where low carbohydrate (LCHO 3 g kg⁻¹) or high carbohydrate (HCHO 10 g kg⁻¹) diets were provided. Twenty-four hours later, participants completed an 8 × 3 min interval running session at 85 % $\dot{V}O_{2peak}$ followed by 3-h monitored recovery. Venous blood samples were collected pre-, immediately post- and 3-h post-exercise, which were analyzed for interleukin-6, serum iron, ferritin and hepcidin.

Results Interleukin-6 was elevated ($p < 0.001$) immediately post-exercise compared to baseline in both conditions, but was lower in HCHO ($p = 0.015$). Hepcidin levels were also lower at baseline ($p = 0.049$) in HCHO, and a large effect ($d = 0.72$) indicated a trend for lower levels at 3-h post-exercise compared to LCHO. Serum iron was increased post-exercise for both trials ($p = 0.001$), whereas serum ferritin remained unchanged.

Conclusions Twenty-four hours of controlled low carbohydrate intake resulted in higher baseline hepcidin levels and post-exercise IL-6 responses than a high carbohydrate intake. Such hormone increases may be induced by gluconeogenic signaling of the liver, and may negatively impact an athlete's iron metabolism.

Keywords Carbohydrates · Iron metabolism · Inflammation · Athletes

Abbreviations

ANOVA	Analysis of variance
BLa	Blood lactate
cAMP	Cyclic adenosine monophosphate
CHO	Carbohydrate
CO ₂	Carbon dioxide
CREBH	cAMP response element-binding protein
CV	Coefficient of variation
Fpn	Ferroportin
GXT	Graded exercise test
HAMP	Hepcidin gene
Hb	Haemoglobin
HCHO	High carbohydrate trial
Hct	Haematocrit
Hp	Haptoglobin
HR	Heart rate
IL-6	Interleukin-6

Communicated by Fabio Fischetti.

✉ Claire E. Badenhorst
badenhorstclaire1@gmail.com;
claire.badenhorst@research.uwa.edu.au

¹ School of Sport Science, Exercise and Health, The University of Western Australia, M408, 35 Stirling Hwy, Crawley, WA 6009, Australia

² Sports Nutrition, Australian Institute of Sport, Gold Coast, QLD, Australia

³ Department of Laboratory Medicine (LGEM 830), Radboud University Medical Center, Nijmegen, The Netherlands

⁴ Hepcidinanalysis.com, Geert Grooteplein 10 (830), 6525 GA Nijmegen, The Netherlands

LCHO	Low carbohydrate trial
LSD	Least significant difference
O ₂	Oxygen
PPARGG1A	Peroxisome proliferator-activated receptor gamma coactivator 1 α
RPE	Rating of perceived exertion
SD	Standard deviation
TLCH	Train Low, Compete High
VO _{2peak}	Peak oxygen uptake
vVO _{2peak}	Velocity at peak oxygen uptake

Introduction

Physical activity has been associated with an intensified post-exercise inflammatory response, and in particular, marked increases in the signaling cytokine interleukin-6 (IL-6) (Steensberg et al. 2001). Produced by the contracting skeletal muscle, one of the primary roles of IL-6 is to serve in a counter-regulatory hormone-like manner, up-regulating glucose production at the site of the liver in an attempt to sustain energy output (Steensberg et al. 2000). Since prolonged exercise results in a progressive decline of muscle glycogen, it is suggested that exercise-induced IL-6 release is linked to intramuscular glycogen availability (Steensberg et al. 2000). Similarly, it has also been suggested that when exercise is performed with low muscle glycogen stores, the post-exercise IL-6 response is further augmented (Steensberg et al. 2001). However, an attenuation of the post-exercise increase of IL-6 can be achieved via the ingestion of a high carbohydrate (CHO) source during activity (Nieman et al. 1998). This effect has been demonstrated in athletes who were required to run for 3 h at 70 % VO_{2peak} while ingesting a 6 % (4 ml kg⁻¹/15 min) CHO beverage, as compared to a placebo control of sweetened water, controlled for taste, appearance and electrolyte content (Nieman et al. 1998). Keller et al. (2001) reported similar results when cyclists pedaled at 60 % VO_{2max} for 3 h when consuming fluids with 60 g of CHO per hour, compared to a placebo control. Therefore, it appears that variations in the rate of IL-6 release from contracting skeletal muscle are largely dependent on muscle glycogen status and ingested CHO intake.

Previously, elevations in IL-6 immediately post-exercise have been acknowledged as a primary up-regulator of the iron-regulatory hormone, hepcidin (Peeling et al. 2009a, b, c; Sim et al. 2012; Badenhorst et al. 2014). Hepcidin regulates iron metabolism via its interaction with the body's only known cellular iron exporter, ferroportin (Fpn) (Nemeth et al. 2004b). During periods of elevated hepcidin activity, Fpn channels are internalized and degraded, inhibiting the absorption of iron from the diet, and the recycling of iron from macrophage activity (Nemeth et al. 2004b). Research investigating the acute post-exercise hepcidin

response has established that peak elevations of the hormone occur approximately 3–6 h post-exercise (Peeling et al. 2009b), subsequent to the immediate post-exercise increases in IL-6. Additionally, in response to repeated bouts of strenuous physical activity (3 days continuous ski marching), hepcidin levels have been reported to increase by up to 57 % from baseline (McClung et al. 2013). With this in mind, it is both the acute and chronic hepcidin responses to strenuous activity that raise concern for endurance athletes with regard to their ability to absorb iron from the diet, or to recycle iron from macrophage activity during strenuous training periods, potentially increasing the likelihood of iron status degradation during such times. Based on the current knowledge of the effect of IL-6 on hepcidin, and that of CHO on IL-6, there exists the possibility that differing levels of pre-exercise muscle glycogen status and CHO intake strategies may impact on the hepcidin response post-exercise via IL-6 regulation.

A contemporary nutritional strategy that manipulates CHO availability during exercise is referred to as Train Low, Compete High (TLCH). This strategy requires athletes to purposefully train with low muscle glycogen levels in order to optimize training adaptations (i.e., increased fat metabolism and enzymatic responses: for review see (Hawley et al. 2006; Baar and McGee 2008), but to compete with high muscle glycogen stores to enhance performance (Halson et al. 2004). In addition, it is also likely that some athletes undertaking heavy daily training loads will 'accidentally' train in a state of low muscle glycogen as a result of poor nutritional practices and/or insufficient time to refuel muscle glycogen stores between training sessions. Nevertheless, regardless of whether an athlete has been advised to 'Train Low', or is 'accidentally training low', there has been minimal consideration in the current literature for the aforementioned association between glycogen stores, inflammation and hepcidin activity post-exercise. In such circumstances, it is possible that individuals training with sub-optimal muscle glycogen stores may significantly impact their ability to absorb dietary iron at the level of the gut as a result of augmented IL-6 and hepcidin responses, which over time may increase their likelihood of developing an iron deficiency. However, there is currently no existing research to quantify this premise, either acutely, or over a prolonged training period. Consequently, the aim of this study was to examine the acute impact of training with varied muscle glycogen stores, by manipulating the pre-exercise ingestion of CHO following a glycogen depletion protocol, and assessing the effect on the subsequent inflammatory and hepcidin responses post-exercise. It was hypothesized that IL-6 and hepcidin levels will be significantly increased post-exercise when a low CHO (3 g kg⁻¹ body mass) dietary intake is ingested for a 24-h period following a glycogen-depleting exercise task.

Methods

Participants

Twelve well-trained male recreational endurance runners and triathletes [age 27.5 ± 8.7 years; height 181.08 ± 7.68 cm; weight 74.8 ± 11.5 kg; peak oxygen uptake (VO_{2peak}) 68.9 ± 7.2 ml kg^{-1} min^{-1} , training volume 11.7 ± 2.5 h/week] participated in this study. Participants were excluded from the study if they presented with any food allergies (e.g., lactose intolerance, celiac disease) or iron deficiency [serum ferritin <30 μg l^{-1} (Peeling et al. 2014)]. Determination of the sample size was attained via a power analysis using customized computer software (GPOWER Version 3.1.5, Department of Psychology, Bonn University, Bonn, Germany), with data from previous investigations that measured similar variables (Peeling et al. 2009b, c). The power analysis suggested a sample size of 12, for an expected power of 0.8 with an alpha level of $p \leq 0.05$. The study protocol was approved by the University of Western Australia's Institutional Review Board, conforming to the Declaration of Helsinki on the use of human subjects. Participants were informed of the risks, protocols and requirements of the study before written consent was obtained. For participants under 18 years of age ($n = 1$), signed parental consent was obtained prior to the commencement of the study.

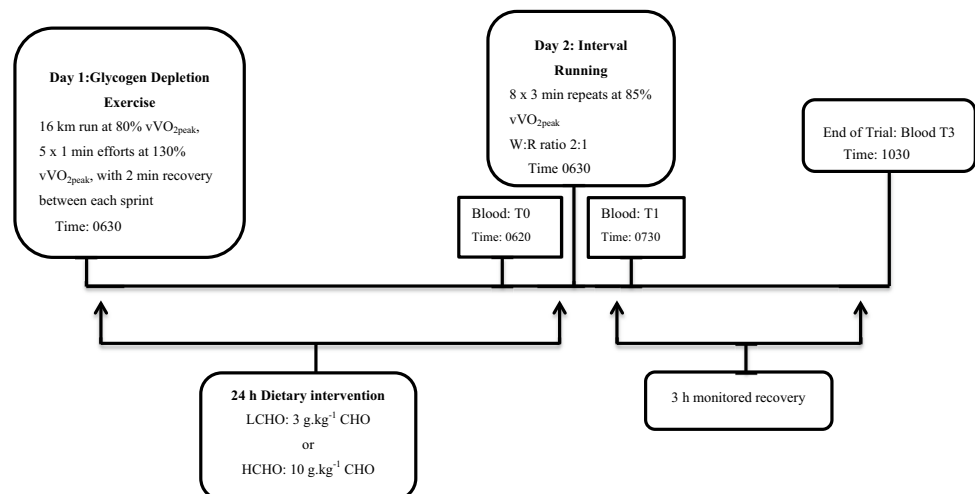
Experimental overview

Participants completed three separate running sessions at the exercise physiology laboratory of the School of Sport Science, Exercise and Health at the University of Western

Australia. These sessions were separated by a minimum of 7 days, with each being conducted at the same time of day (0630 ± 1 h) to minimize the influence of circadian variation. Diet and exercise were not controlled between the experimental testing sessions. However, participants were advised to maintain their usual exercise training programs and habitual food practices to maintain consistency for each trial. The initial testing session served as a familiarization trial (humidity 51.3 ± 9.1 %, temperature 24.3 ± 2.3 °C, pressure 1009 ± 5.7 hPa) for the participant to become accustomed to the laboratory environment and the equipment to be used during the experimental trials. This session concluded with a graded exercise test (GXT) to exhaustion, to determine each individual's peak oxygen uptake (VO_{2peak}). Subsequently, the velocities corresponding to 60, 80 and 85 % of their VO_{2peak} were used to establish the pace at which the two experimental running sessions were performed.

The experimental sessions were conducted using a repeated measures, crossover design, where the 24-h controlled CHO intake (i.e., high CHO or low CHO) was randomly assigned. Each experimental session (Fig. 1) was completed over a 2-day period under typical laboratory conditions (humidity 51.7 ± 6.4 %, temperature 22.2 ± 1.6 °C, pressure 1012 ± 3.4 hPa). On the first day, participants were required to complete a glycogen depletion running task on a motorized treadmill (VR3000, NuryTech Inc., Korea) with the aim of significantly reducing their muscle glycogen stores. In brief, this task comprised a 5-min warm-up run at 60 % vVO_{2peak} , 5 min of structured dynamic stretching, a 16-km run at 80 % of their vVO_{2peak} , immediately followed by 5×1 min efforts at 130 % vVO_{2peak} with a 2-min recovery between each

Fig. 1 Diagrammatic representation of the two-day experimental trial



Key:

T0: Pre-exercise

T1: Post-exercise

T3: 3 h post-exercise

Table 1 Percentage contribution of macronutrients and total calorie intake of the high and low carbohydrate diets provided to participants

	Low CHO (3 g kg ⁻¹ body mass)	High CHO (10 g kg ⁻¹ body mass)
Carbohydrates (%)	22	69
Carbohydrates (g)	279.9 (49.2)	767.6 (123.2)*
Protein (%)	24	13
Protein (g)	241.6 (20.5)	134.9 (16.1)*
Fat (%)	49	15
Fat (g)	209.4 (50.5)	86.4 (10.8)*
Iron (mg)	17.8 (2.7)	18.1 (2.8)
Energy intake (Cal)	4093 (751)	4499 (641)

* Significantly different ($p < 0.05$) from LCHO

repetition, then a 5-min cool-down run at 60 % $v\dot{V}O_{2peak}$. A similar exercise protocol has previously been reported to reduce muscle glycogen levels by ~50 % (Costill et al. 1981). Since we were unable to measure muscle glycogen content in our participants, a similar depletion effect was assumed here. During this protocol, heart rate (HR) and ratings of perceived exertion (RPE) were monitored at 15-min intervals during the continuous run, and again after the final 1-min effort. Capillary blood samples for lactate (BLa) analysis were collected after the continuous run and the final 1-min effort. Participants were then provided with either a low carbohydrate (LCHO 3 g kg⁻¹ CHO) or a high carbohydrate (HCHO 10 g kg⁻¹ CHO) for the following 24-h period (Table 1), with analysis of diets being completed on FoodWorks7 software. The high CHO intake was based on previous literature demonstrating that 10 g kg⁻¹ CHO intake for 1 day was an effective CHO-loading protocol to achieve maximal muscle glycogen levels in type I, IIa and type IIb muscle fibers (Bussau et al. 2002). In addition, 10 g kg⁻¹ CHO is recommended as the daily CHO intake for fueling and recovery needs for athletes undertaking strenuous exercise programs (Burke 2010). All meals and snacks were supplied to participants, with diets individualized for body mass and food preferences. Dietary compliance was assessed by having the participants return all empty food containers and bags on the following morning, prior to the subsequent interval running session.

The interval running session commenced 24 h after the glycogen depletion protocol, with participants returning to the laboratory following a 12-h overnight fast. This session commenced with a 5-min warm-up at 60 % $v\dot{V}O_{2peak}$, followed by 5 min of dynamic stretching, and 8 × 3 min running repeats at 85 % $v\dot{V}O_{2peak}$ with 90-s active recovery (60 % $v\dot{V}O_{2peak}$) between repetitions (i.e., utilizing a 2:1 work:rest ratio). During this task, HR and RPE were monitored at the completion of each 3-min workload and capillary blood (30 µl) for BLa analysis was collected after the fourth and eighth repetitions. A 5-min cool-down run at 60 % $v\dot{V}O_{2peak}$ was completed after the final repeat, before participants rested in the laboratory for the next 3 h. This running session has been used previously in research

investigating post-exercise hepcidin responses (Sim et al. 2014; Badenhorst et al. 2014), and therefore was used as the running session on day 2 of this experimental design.

On the second day of each experimental trial (i.e., HCHO or LCHO), venous blood samples were collected on arrival (baseline), after the interval running session (post-exercise) and after 3 h of recovery (3-h post-exercise). Participants were provided with a minimum of 400 ml of water to drink ad libitum during each interval running session, to minimize any hemoconcentration effects. In addition, due to exercise being completed in a fasted state, a standardized snack of ~300 ml of strawberry jelly (Aeroplane Jelly, Australia 99.5 Cal, 18.3 g carbohydrates, 1.4 g of protein and 0.0 g of fat) was provided at 90-min post-exercise. Water was also provided ad libitum during experimental trial 1 for both the glycogen depletion exercise task and during the 3-h post-exercise recovery period that followed the next day interval efforts, with the recorded intake matched during trial 2.

Experimental procedures

Graded exercise test (GXT)

The GXT was conducted on a motorized treadmill (VR3000, NuryTech Inc., Korea) using 3-min exercise and 1-min rest periods, as per previously established methods (Peeling et al. 2009a; Sim et al. 2012, 2014). The initial speed was 12 km h⁻¹, with subsequent increments of 1 km h⁻¹ until voluntary exhaustion. The treadmill was set to a 1 % gradient to simulate conditions commonly encountered outdoors (Jones and Doust 1996). Throughout the GXT, expired air was analyzed for O₂ and CO₂ concentrations using Ametek Gas Analyzers (Applied Electrochemistry, SOV S-3A/1 and COV CD-3A, Pittsburgh, PA, USA). These analyzers were calibrated pre-test and verified post-test with certified gravimetric gas mixtures (BOC Gases, Chatswood, Australia). Ventilation was recorded at 15-s intervals via a turbine ventilometer (Vacu-Med Ventura, California, USA), also calibrated prior to the test and verified after exercise using a 1-l syringe in accordance with

the manufacturer's specifications. The $VO_{2\text{peak}}$ was determined by summing the four highest consecutive 15-s VO_2 values obtained throughout the test.

Blood sampling and analysis

During the GXT, glycogen depletion run and interval running task, capillary blood samples were collected for the analysis of BLa using a Lactate Pro II analyzer (ARKRAY, Japan). Venous blood samples were collected from a forearm antecubital vein, with the participant lying down for 10 min to control for postural shifts in plasma volume. These samples were collected using a 22-gauge needle into two 8.5-ml SST Gel separator tubes and one 4-ml EDTA collection tube (BD Vacutainer, Australia). Immediately after collection, the EDTA tubes were taken to the Royal Perth Hospital pathology laboratory for hemoglobin (Hb) concentration and hematocrit (Hct) measurement. This datum was used for the calculation of percentage changes in plasma volume resulting from the exercise task (Dill and Costill 1974). The SST samples were allowed to clot for 60 min at room temperature before subsequently being centrifuged at 10 °C and 3000 rpm (1.4 rcf) for 10 min. The serum supernatant was divided into 1-ml aliquots and stored at -80 °C until further analysis. Once all blood samples were collected, the frozen serum was transported to Royal Perth Hospital pathology laboratory for analysis of IL-6, serum iron and ferritin. In addition, the remaining frozen serum samples were sent to Radboud University Medical Centre (Nijmegen, The Netherlands) for serum hepcidin-25 analysis.

Serum IL-6 was analyzed using a commercially available ELISA (Quantikine HS, R&D Systems, Minneapolis, USA) with an assay range of 0.38–10 ng l^{-1} . The coefficient of variation (CV) for IL-6 determination at 0.49 and 2.78 ng l^{-1} was 9.6 and 7.2 %, respectively. Serum iron was measured using the Architect (c1600210) analyzer. Serum iron levels were determined using an iron reagent (Sentinel Diagnostics, Milano, Italy) and an Architect analyzer (c1600210). The CV for iron determination at 10.50 and 42.96 $\mu\text{mol } l^{-1}$ was 3.15 and 1.00 %, respectively. Serum ferritin levels were determined using an Architect analyzer (1SR06055) and a ferritin reagent (Abbott Diagnostics, Illinois, USA). The CV for ferritin determination at 5.48, 34.46, 187.52 and 403.87 $\mu\text{g } l^{-1}$ was 5.05, 4.36, 4.44 and 3.91 %, respectively. Serum hepcidin-25 measurements were performed (<http://www.hepcidinanalysis.com>, Nijmegen, The Netherlands) by a combination of weak cation exchange chromatography and time-of-flight mass spectrometry (WCX-TOF-MS) (Kroot et al. 2010). An internal standard (synthetic hepcidin-24; custom-made Peptide International Inc.) was used for quantification (Laarakkers et al. 2013). Peptide spectra were generated

on a Microflex LT matrix-enhanced laser desorption/ionization TOF-MS platform (Bruker Daltonics). Serum hepcidin-25 concentrations were expressed as nmol l^{-1} (nM). The median reference level of serum hepcidin-25 (Dutch population) is 4.5 nM for men, 2.0 nM for premenopausal women and 4.9 nM for postmenopausal women.

Heart rate and perceived exertion

In all trials, HR was measured continuously via a Garmin HR monitor (Garmin 210, USA), and RPE was recorded using the Borg perceptual scale (Borg 1982) encompassing the anchor points 6 (very, very light) through to 20 (maximal exertion).

Statistical analysis

Results are presented as mean and standard deviation (\pm SD). The IBM Statistical Package for Social Sciences (IBM SPSS version 19.0) was used. A repeated measures ANOVA was used to analyze the time, condition and interaction (time \times condition) effects of LCHO vs. HCHO on the subsequent inflammatory, hepcidin and iron-related responses. Post hoc and least significant differences (LSD) paired samples t tests were used to determine where specific trial differences existed. Significance was accepted at $p \leq 0.05$. Where appropriate, Cohen's d effect sizes were calculated to suggest data trends and were interpreted as $d < 0.39$ (small); $d = 0.40$ – 0.69 (moderate); $d > 0.7$ (large) (Hopkins 2005). Only moderate and large effect sizes are presented below.

Results

Physiological responses

Mean HR, RPE and BLa data for the interval running trials are presented in Table 2. Significant time ($p = 0.001$) and condition ($p = 0.017$) effects were recorded for RPE between trials. Significantly, lower RPE for the interval running task was recorded for HCHO, as compared to LCHO ($p = 0.034$). Significant condition ($p = 0.005$) and interaction ($p = 0.016$) effects were reported for the average HR of the running tasks. Average HR for the interval running task was significantly lower in HCHO compared to LCHO ($p = 0.012$). Significant time ($p = 0.011$) but not condition ($p = 0.357$) or interaction (0.802) effects were recorded for BLa within the HCHO and LCHO trials. The percentage change in plasma volume recorded immediately post-exercise approached significance between the HCHO and LCHO trials ($p = 0.061$) (Table 2).

Table 2 Mean (SD) heart rate (HR), rating of perceived exertion (RPE) and blood lactate (BLa) responses during the interval running trials, and the percentage change in plasma volume from baseline to immediately post-exercise in the low carbohydrate (LCHO) and high carbohydrate (HCHO) conditions

	Heart rate (bpm)	Rating of perceived exertion	Blood lactate (mmol l ⁻¹)	Changes in plasma volume (%)
Low CHO (3 g kg ⁻¹ BM) interval running task	171 (8)	15 (1.9)	2.4 (0.9)	3.53 (1.70)
High CHO (10 g kg ⁻¹ BM) interval running task	166 (7)*	13 (1.2)*	2.6 (0.8)	2.03 (1.26)

* Significantly different ($p < 0.05$) from LCHO

Table 3 Mean (SD) interleukin-6 (IL-6), hepcidin-25, serum iron and ferritin levels at baseline, post-run and 3-h post-run in the low carbohydrate (LCHO) and high carbohydrate (HCHO) conditions

Blood parameter	LCHO (3 g kg ⁻¹ body mass)			HCHO (10 g kg ⁻¹ body mass)		
	Baseline	Post-exercise	3-h post-exercise	Baseline	Post-exercise	3-h post-exercise
Serum IL-6 (pg ml ⁻¹)	0.98 (0.39)	3.02 (1.37) [†]	–	1.01 (0.35)	2.04 (0.82) ^{†,*}	–
Hepcidin-25 (nM)	4.2 (3.6)	–	6.4 [†] (5.1)	2.2 (1.1)*	–	4.1 (3.2) [†]
Serum iron (μmol l ⁻¹)	19.5 (4.8)	21.4 (4.7) [†]	20.6 (4.7)	17.8 (5.5)	21.1 (6.3) [†]	23.1 (6.4) ^{†,♦}
Serum ferritin (μg l ⁻¹)	108 (66.4)	112.0 (68.3)	108.0 (65.9)	101.6 (70.3)	102.3 (69.9)	103.1 (70.8)

[†] Significantly different ($p < 0.05$) to baseline

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

♦ Significantly different ($p < 0.05$) to immediately post-exercise

Interleukin-6

Significant time ($p = 0.001$), condition ($p = 0.049$) and interaction ($p = 0.003$) effects were recorded for IL-6 (Table 3). Within both LCHO and HCHO, there was a significant increase ($p = 0.001$) in IL-6 from baseline to immediately post-exercise during the interval running task. However, the immediate post-exercise IL-6 levels in HCHO were significantly lower than those recorded at the same time point in LCHO ($p = 0.015$).

Hepcidin

Significant time ($p = 0.002$) and interaction ($p = 0.037$) effects were observed for hepcidin levels (Table 3). For both trials, there was a significant increase in hepcidin levels from baseline to 3-h post-exercise (LCHO: $p = 0.002$, HCHO: $p = 0.014$). However, baseline hepcidin levels in LCHO were significantly greater than in HCHO ($p = 0.049$). Finally, although not significantly different ($p = 0.120$), a large effect size was evident between the 3-h post-exercise hepcidin levels of each condition ($d = 0.72$), showing a trend for lower hepcidin levels in HCHO compared to LCHO.

Iron parameters

There was no significant time ($p = 0.331$), condition ($p = 0.217$) or interaction ($p = 0.073$) effects recorded for serum ferritin between LCHO and HCHO at any of the

measured time points (Table 3). Furthermore, there was also no condition effect ($p = 0.920$) in serum iron between the two conditions. However, a significant time ($p = 0.001$) effect indicated that higher serum iron levels were present immediately post-exercise compared to baseline and 3-h post-exercise.

Discussion

The findings of this investigation demonstrated that following an exercise task designed to compromise muscle glycogen stores, consuming a controlled LCHO dietary intake for 24 h resulted in a greater next day post-exercise IL-6 response after an intense interval running session, as compared to a HCHO equivalent. In addition, there were significantly reduced hepcidin levels pre-exercise and a large effect for lower hepcidin levels at 3-h post-exercise when a HCHO dietary intake was followed for 24 h, as compared to the LCHO approach. Finally, the intensity- and duration-matched exercise, evident from the lack of difference in the BLa responses between the trials, was reported to be significantly harder under conditions of the LCHO trial, evident from the higher HR and RPE recorded during this interval running session.

The elevated post-exercise IL-6 responses reported here in both conditions support the outcomes of previous research findings (Nieman et al. 1998; Peeling et al. 2009a,

b, c). However, significant differences in post-exercise IL-6 after the interval running session were evident between the HCHO and LCHO trials. Previously, the magnitude of the IL-6 response following an acute exercise bout has been related to exercise intensity, mode, duration and the starting muscle glycogen status of the individual (Steensberg et al. 2001; Helge et al. 2003; Fischer 2006). However, exercise mode, duration and intensity in this investigation were matched between trials, and therefore, it is likely that the observed differences in the post-exercise IL-6 response are a result of the differences in dietary intake consumed after performing a glycogen depletion exercise protocol 24 h prior. It is well established that compromised muscle glycogen stores are associated with increases in IL-6 production from contracting skeletal muscles (Steensberg et al. 2001; Keller et al. 2001). Steensberg et al. (2001) demonstrated that in a muscle glycogen-depleted leg (~40 % lower muscle glycogen levels compared to control leg), significantly greater intramuscular levels of IL-6 (compared to control) were recorded during two-legged dynamic knee extensor exercise, despite no differences in IL-6 levels prior to exercise. Thus, the exaggerated IL-6 levels seen immediately post-exercise in the LCHO trial may have been a result of the reduced levels of muscle glycogen created from the exercise and restricted carbohydrate intake 24 h prior.

The biological significance of reduced IL-6 is not fully understood; however, previous research has suggested that IL-6 has a significant influence on hepatic glucose metabolism (Steensberg et al. 2001). Previously, the injection of recombinant human IL-6 into participants has been shown to increase hepatic glucose production (Stouthard et al. 1995). Furthermore, in rodent hepatocytes, increases in IL-6 appear to inhibit glycogen synthase, while accelerating glycogen phosphorylase activity (Kanemaki et al. 1998). Such outcomes suggest IL-6 may mediate the hepatic glucose output necessary to maintain blood glucose homeostasis when uptake by skeletal muscle is increased during prolonged or intense exercise (Steensberg et al. 2001). To this end, it is likely that the compromised muscle glycogen state invoked here (i.e., LCHO) resulted in significantly greater levels of IL-6 to serve in a hormone-like manner, increasing hepatic glucose production to help sustain the skeletal muscle activity during the intense interval running session performed 24 h after the glycogen depletion run.

In addition to hepatic glucose regulation, IL-6 is also reported to be a primary up-regulator of the iron-regulatory hormone, hepcidin (Nemeth et al. 2004a). Exercise-based studies have demonstrated that hepcidin levels peak approximately 3-h post-exercise, subsequent to the well-established increases in IL-6 seen immediately post-exercise (Peeling et al. 2009a, b, c). Increased hepcidin levels result in the degradation and internalization of Fpn export

channels located on cell surfaces of intestinal enterocytes and macrophages (Nemeth et al. 2004b), and as such, may be a potential mechanism for altered iron metabolism in athletes (Peeling et al. 2009a), possibly explaining the high incidence of iron deficiency seen in this population. The outcomes of the current study show that the hepcidin levels at baseline (i.e., 24 h after the glycogen depletion run) in the HCHO trial were significantly lower than LCHO, with a trend also suggesting this attenuation persisted at 3-h post-exercise. These outcomes are likely explained by the CHO content of the diets provided following the glycogen depletion exercise task. However, of interest, the delta change of the post-exercise increase in the hepcidin response for both the LCHO and HCHO trials was not significantly different ($p = 0.713$). This may suggest that the exercise-induced increase in IL-6 resulted in a similar magnitude of post-exercise hepcidin increase in both trials, but that the absolute post-exercise response was ultimately lower in the HCHO, likely due to the lower pre-exercise hepcidin levels recorded on Day 2 as a result of the higher CHO dietary intake from the previous 24 h.

Iron abnormalities have been reported in diseases that appear to have disordered gluconeogenic signaling pathways, such as glycogen storage disease type I (GSD1) (Weinstein et al. 2002), obesity (Bekri et al. 2006) and diabetes (Jiang et al. 2011), thus suggesting that iron status is in part regulated by gluconeogenic signals. Recently, Vecchi et al. (2014) showed that hepcidin was transcriptionally regulated by gluconeogenic signals when rodents were fasted for a 24- to 48-h period. In the fasted state, gluconeogenesis at the site of the liver was initially signaled by an increase in cyclic adenosine monophosphate (cAMP) response element-binding proteins (CREBH), which was shown to promote hepcidin gene (*HAMP*) expression within 5 h of the starvation, resulting in an increase in serum hepcidin levels. Subsequently, it was shown that increases in peroxisome proliferator-activated receptor gamma coactivator 1- α (PPARGC1A) sustained this increased transcription of the *HAMP* gene, by stabilizing the CREBH proteins that were still bound to the *HAMP* gene promoter (Vecchi et al. 2014). These responses were shown to occur independent of any inflammatory stimuli, such as IL-6. Vecchi et al. (2014) concluded that during metabolic disturbances, such as starvation or reduced food intake, the up-regulation of hepcidin via gluconeogenic signals is a protective mechanism that assists in the preservation of tissue iron, the maintenance of energy balance, and supports gluconeogenesis in the liver. This recently established mechanism of iron regulation through gluconeogenic signaling may help to explain the significantly elevated baseline hepcidin levels seen in the LCHO trial 24 h subsequent to the glycogen depletion run, in the absence of any increases in baseline IL-6. Although not fasted here,

the glycogen depletion exercise task, in addition to a low carbohydrate intake, would invoke gluconeogenesis in the liver, possibly indicating that energy intake and muscle glycogen content are essential to this process. In addition, the increased IL-6 levels at the completion of the intervals run in the LCHO trial potentially resulted in the trend for continued elevated hepcidin levels subsequent to the interval running task. Therefore, it is possible that a reduced carbohydrate intake following strenuous exercise may influence the hormonal regulation of iron metabolism in athletes, providing some evidence that training with low muscle glycogen (as a result of insufficient carbohydrate intake) may lead to tissue iron retention, hypoferremia and iron-restricted erythropoiesis.

As previously suggested, athletes who fail to adequately replenish their post-exercise muscle glycogen stores through a diet high in carbohydrates may be adopting the nutritional strategy of TLCH, whereby they undergo training with low muscle glycogen stores, before restoring their glycogen levels for competition. This TLCH strategy is based on research suggesting that intracellular signaling pathways underpinning adaptations to endurance training are enhanced when exercise is completed with low muscle glycogen stores (Hawley et al. 2006; Baar and McGee 2008). Alternatively, athletes may be unintentionally training in a state of low muscle glycogen or hypoglycemia as a result of poor nutritional preparation, or due to time constraints between training sessions. Regardless, in these instances, elevated gluconeogenic signals in the post-exercise period may be sustained in an attempt to increase hepatic glucose production. Our results suggest that this may lead to elevated hepcidin levels prior to exercise completed up to 24 h later. It is possible that over a prolonged training period, these circumstances could result in cumulative elevations to hepcidin levels, which could be detrimental to an athlete's iron status by reducing iron absorption and recycling, thus increasing their risk of developing iron deficiency. Previously, both McClung et al. (2013) and Sim et al. (2014) have shown that hepcidin levels are cumulatively elevated over the course of a 7-day training intervention. Consequently, it is possible that a well-planned diet which contains adequate carbohydrate to refuel muscle glycogen stores may reduce any such effect, while training with compromised muscle glycogen may exacerbate it. Furthermore, Peeling et al. (2014) recently suggested that the post-exercise hepcidin response in athletes with sub-optimal iron stores (defined by these authors as a serum ferritin of 30–50 µg/l) were similar to those presenting with healthy iron stores, and that this magnitude of hepcidin response in borderline iron depletion individuals could potentially lead to iron deficiency. As such, utilizing the concept of TLCH, or inadvertently training in a glycogen-depleted state may have particular implications for borderline iron-depleted

athletes, due to the potential reduction in iron absorption during the post-exercise period when hepcidin levels are elevated. However, the impact of TLCH on iron metabolism over a prolonged training period and the aforementioned statements remain to be investigated.

It must also be considered that significant increases in post-exercise serum iron were reported for both trials in the current investigation. Such increases have previously been proposed to be the result of exercise-induced hemolysis (Peeling et al. 2009c). Hemolysis causes red blood cells to rupture, spilling its hemoglobin (Hb) and associated iron into the extracellular fluid (Buchman et al. 1998), possibly helping to explain the increases in serum iron seen here. Hemolysis stimulates serum haptoglobin (Hp) to bind with free Hb (Giblett 1968), forming a Hb–Hp complex that is cleared from the circulation by binding to CD163 hemoglobin scavenger receptors on macrophages (Kristiansen et al. 2001). Due to the hepcidin response being linked to reductions in macrophage iron recycling, it is possible that an interaction exists between the amount of hemolysis incurred and iron deficiency in athletes; however, such an association requires further investigation. In addition, no significant differences were reported for serum ferritin levels between conditions or over time, similar to the results reported by Schumacher et al. (2002). However, other research has demonstrated elevated serum ferritin levels after endurance exercise (Fallon 2001; Peeling et al. 2009b, c), likely to be an acute-phase response to the exercise task.

Limitations

It should be acknowledged that the dietary intake of the participants was only controlled for 24 h in this investigation, and that no record was made of the diet consumed prior to this point. However, it should also be noted that the investigators tried to match each individual's 24-h diet to their habitual food choices to remove any wild variance from their usual food practices. In addition, the controlled 24-h dietary intake was based primarily on manipulation of CHO content, while being mindful of keeping the intake isocaloric. As a result, there are observable differences in the fat and protein content between the LCHO and HCHO diets, since strict control of all three macronutrients without large changes in energy intake confounding the outcomes is not possible. It should also be acknowledged that the muscle glycogen content was not quantified during this investigation. However, previous research has demonstrated an ~50 % depletion in muscle glycogen stores after completing the exercise task employed here (Costill et al. 1981), and therefore, we are confident that a similar magnitude of response was present. Notwithstanding, no blood measures were taken immediately before, or immediately after the glycogen depletion task of day 1. As a

result, the time course of elevated IL-6 levels and hepcidin, and the influence of the diet on the recovery of such levels cannot be quantified at this time. Finally, the age range of the cohort investigated here was relatively wide (i.e., 16–44 years old). However, our primary inclusion criteria were based on running ability rather than age. In addition, the repeated measures nature of the analysis conducted here allows for a between trial comparison of each subject directly to themselves, thus ensuring participant homogeneity between trials. Regardless, future investigations may wish to examine any age-related differences that may occur with respect to post-exercise iron-regulatory hormone activity, since to date there are no investigations that specifically address this consideration.

Conclusion

In summary, after the completion of a glycogen-depleting exercise task that has previously been established to reduce muscle glycogen content by ~50 % (Costill et al. 1981), a high (compared to low) carbohydrate diet consumed for a 24-h period was able to attenuate the post-exercise inflammatory response (as shown by reduced IL-6 levels), in addition to the hepcidin response before exercise, with a strong effect to suggest this difference remained at 3-h post-exercise. Possibly, an increased post-exercise gluconeogenic signaling of the liver was required in the LCHO trial to sustain the second (next day) exercise task. Such signaling may have contributed to the significant increase in hepcidin levels at baseline and the large effect for greater hepcidin levels at 3-h post-exercise on day 2 of the trial. Possibly, the lower hepcidin levels in the HCHO trial could assist the bioavailability of iron, reducing the risk of developing an iron deficiency over a prolonged training period. As a result, athletes who struggle to maintain a healthy iron balance should be advised that adopting a ‘Train Low, Compete High’ nutritional strategy may increase their risk of developing an iron deficiency. Furthermore, it is suggested that athletes at risk of developing an iron deficiency (i.e., endurance-trained females) should adjust their dietary intake and meal planning to ensure adequate recovery of muscle glycogen during the post-exercise period. Future research should consider examining the impact of a high carbohydrate nutritional strategy over a prolonged training period, by assessing the impact this may have on hepcidin and iron parameters within athletes.

Acknowledgments The authors wish to acknowledge the High Performance Sports Research Grant funding received from the Australian Sports Commission.

Compliance with ethical standards

Conflict of interest The authors report no conflict of interest.

References

- Baar K, McGee S (2008) Optimizing training adaptations by manipulating glycogen. *Eur J Sport Sci* 8:97–106. doi:10.1080/17461390801919094
- Badenhorst CE, Dawson B, Goodman C et al (2014) Influence of post-exercise hypoxic exposure on hepcidin response in athletes. *Eur J Appl Physiol* 114:951–959. doi:10.1007/s00421-014-2829-6
- Bekri S, Gual P, Anty R et al (2006) Increased adipose tissue expression of hepcidin in severe obesity is independent from diabetes and NASH. *Gastroenterology* 131:788–796. doi:10.1053/j.gastro.2006.07.007
- Borg GA (1982) Psychophysical bases of perceived exertion. *Med Sci Sports Exerc* 14:377–381
- Buchman AL, Keen C, Comisso J et al (1998) The effect of a marathon run on plasma and urine mineral and metal concentrations. *J Am Coll Nutr* 17:124–127
- Burke LM (2010) Fueling strategies to optimize performance: training high or training low? *Scand J Med Sci Sports* 20(Suppl 2):48–58. doi:10.1111/j.1600-0838.2010.01185.x
- Bussau VA, Fairchild TJ, Rao A et al (2002) Carbohydrate loading in human muscle: an improved 1 day protocol. *Eur J Appl Physiol* 87:290–295. doi:10.1007/s00421-002-0621-5
- Costill DL, Sherman WM, Fink WJ et al (1981) The role of dietary carbohydrates in muscle glycogen resynthesis after strenuous running. *Am J Clin Nutr* 34:1831–1836
- Dill DB, Costill DL (1974) Calculation of percentage changes in volumes of blood, plasma, and red cells in dehydration. *J Appl Physiol* 37:247–248
- Fallon KE (2001) The acute phase response and exercise: the ultramarathon as prototype exercise. *Clin J Sport Med* 11:38–43
- Fischer CP (2006) Interleukin-6 in acute exercise and training: what is the biological relevance? *Exerc Immunol Rev* 12:6–33
- Giblett E (1968) The haptoglobin system. *Ser Haematol* 1:3–20
- Halson SL, Lancaster GI, Achten J et al (2004) Effects of carbohydrate supplementation on performance and carbohydrate oxidation after intensified cycling training. *J Appl Physiol* 97:1245–1253. doi:10.1152/jappphysiol.01368.2003
- Hawley JA, Tipton KD, Millard-Stafford ML (2006) Promoting training adaptations through nutritional interventions. *J Sports Sci* 24:709–721. doi:10.1080/02640410500482727
- Helge JW, Stallknecht B, Pedersen BK et al (2003) The effect of graded exercise on IL-6 release and glucose uptake in human skeletal muscle. *J Physiol* 546:299–305
- Hopkins W (2005) A spreadsheet for fully controlled crossovers. *Sport Sci* 9:3
- Jiang F, Sun Z-Z, Tang Y-T et al (2011) Hepcidin expression and iron parameters change in Type 2 diabetic patients. *Diabetes Res Clin Pract* 93:43–48. doi:10.1016/j.diabres.2011.03.028
- Jones AM, Doust JH (1996) A 1% treadmill grade most accurately reflects the energetic cost of outdoor running. *J Sports Sci* 14:321–327. doi:10.1080/02640419608727717
- Kanemaki T, Kitade H, Kaibori M et al (1998) Interleukin 1beta and interleukin 6, but not tumor necrosis factor alpha, inhibit insulin-stimulated glycogen synthesis in rat hepatocytes. *Hepatology* 27:1296–1303. doi:10.1002/hep.510270515
- Keller C, Steensberg A, Pilegaard H et al (2001) Transcriptional activation of the IL-6 gene in human contracting skeletal muscle: influence of muscle glycogen content. *FASEB J* 15:2748–2750. doi:10.1096/fj.01-0507fje
- Kristiansen M, Graversen JH, Jacobsen C et al (2001) Identification of the haemoglobin scavenger receptor. *Nature* 409:198–201. doi:10.1038/35051594
- Kroot JJC, Laarakkers CMM, Geurts-Moespot AJ et al (2010) Immunochemical and mass-spectrometry-based serum hepcidin

- assays for iron metabolism disorders. *Clin Chem* 56:1570–1579. doi:[10.1373/clinchem.2010.149187](https://doi.org/10.1373/clinchem.2010.149187)
- Laarakkers CMM, Wiegerinck ET, Klaver S et al (2013) Improved mass spectrometry assay for plasma hepcidin: detection and characterization of a novel hepcidin isoform. *PLoS One* 8:e75518. doi:[10.1371/journal.pone.0075518](https://doi.org/10.1371/journal.pone.0075518)
- McClung JP, Martini S, Murphy NE et al (2013) Effects of a 7-day military training exercise on inflammatory biomarkers, serum hepcidin, and iron status. *Nutr J* 12:141. doi:[10.1186/1475-2891-12-141](https://doi.org/10.1186/1475-2891-12-141)
- Nemeth E, Rivera S, Gabayan V et al (2004a) IL-6 mediates hypoferrremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin. *J Clin Invest* 113:1271–1276. doi:[10.1172/JCI20945](https://doi.org/10.1172/JCI20945)
- Nemeth E, Tuttle MS, Powelson J et al (2004b) Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. *Science* 306:2090–2093. doi:[10.1126/science.1104742](https://doi.org/10.1126/science.1104742)
- Nieman DC, Nehlsen-Cannarella SL, Fagoaga OR et al (1998) Influence of mode and carbohydrate on the cytokine response to heavy exertion. *Med Sci Sports Exerc* 30:671–678
- Peeling P, Dawson B, Goodman C et al (2009a) Cumulative effects of consecutive running sessions on hemolysis, inflammation and hepcidin activity. *Eur J Appl Physiol* 106:51–59. doi:[10.1007/s00421-009-0988-7](https://doi.org/10.1007/s00421-009-0988-7)
- Peeling P, Dawson B, Goodman C et al (2009b) Effects of exercise on hepcidin response and iron metabolism during recovery. *Int J Sport Nutr Exerc Metab* 19:583–597
- Peeling P, Dawson B, Goodman C et al (2009c) Training surface and intensity: inflammation, hemolysis, and hepcidin expression. *Med Sci Sports Exerc* 41:1138–1145. doi:[10.1249/MSS.0b013e318192ce58](https://doi.org/10.1249/MSS.0b013e318192ce58)
- Peeling P, Sim M, Badenhorst CE et al (2014) Iron status and the acute post-exercise hepcidin response in athletes. *PLoS One* 9:e93002. doi:[10.1371/journal.pone.0093002](https://doi.org/10.1371/journal.pone.0093002)
- Schumacher YO, Schmid A, König D, Berg A (2002) Effects of exercise on soluble transferrin receptor and other variables of the iron status. *Br J Sports Med* 36:195–199
- Sim M, Dawson B, Landers G et al (2012) The effects of carbohydrate ingestion during endurance running on post-exercise inflammation and hepcidin levels. *Eur J Appl Physiol* 112:1889–1898. doi:[10.1007/s00421-011-2156-0](https://doi.org/10.1007/s00421-011-2156-0)
- Sim M, Dawson B, Landers GJ et al (2014) A seven day running training period increases basal urinary hepcidin levels as compared to cycling. *J Int Soc Sports Nutr* 11:14. doi:[10.1186/1550-2783-11-14](https://doi.org/10.1186/1550-2783-11-14)
- Steensberg A, van Hall G, Osada T et al (2000) Production of interleukin-6 in contracting human skeletal muscles can account for the exercise-induced increase in plasma interleukin-6. *J Physiol* 529(Pt 1):237–242
- Steensberg A, Febbraio MA, Osada T et al (2001) Interleukin-6 production in contracting human skeletal muscle is influenced by pre-exercise muscle glycogen content. *J Physiol* 537(Pt 2):633–639
- Stouthard JM, Romijn JA, Van der Poll T et al (1995) Endocrinologic and metabolic effects of interleukin-6 in humans. *Am J Physiol* 268:E813–E819
- Vecchi C, Montosi G, Garuti C et al (2014) Gluconeogenic signals regulate iron homeostasis via hepcidin in mice. *Gastroenterology* 146:1060–1069. doi:[10.1053/j.gastro.2013.12.016](https://doi.org/10.1053/j.gastro.2013.12.016)
- Weinstein DA, Roy CN, Fleming MD et al (2002) Inappropriate expression of hepcidin is associated with iron refractory anemia: implications for the anemia of chronic disease. *Blood* 100:3776–3781. doi:[10.1182/blood-2002-04-1260](https://doi.org/10.1182/blood-2002-04-1260)