Muscle physiology and performance during intensified cycle training: Impact of carbohydrate-protein supplementation

Andrew Christian D’Lugos
James Madison University

Follow this and additional works at: https://commons.lib.jmu.edu/master201019
Part of the Kinesiology Commons

Recommended Citation
https://commons.libjmu.edu/master201019/188

This Thesis is brought to you for free and open access by the The Graduate School at JMU Scholarly Commons. It has been accepted for inclusion in Masters Theses by an authorized administrator of JMU Scholarly Commons. For more information, please contact dc_admin@jmu.edu.
Muscle Physiology and Performance During Intensified Endurance Cycle Training:
Impact of Carbohydrate-Protein Supplementation

Andrew C. D’Lugos

A thesis submitted to the Graduate Faculty of
JAMES MADISON UNIVERSITY
In
Partial Fulfillment of the Requirements
for the degree of
Master of Arts or Science

Kinesiology

August 2014
Acknowledgements

First and foremost, I would like to thank Dr. Nicholas Luden for serving as my thesis advisor and mentor. The lessons I have learned since first working with you roughly four years ago will never be forgotten. You are the reason I have made to where I am today.

Dr. Michael Saunders, thank you for the countless retelling of stories and sacks of coffee beans along the way. You have served a major role in my amazing experience at James Madison University.

Thank you Dr. Kent Todd for serving as a member of my thesis committee. I am truly honored you took time from building your wooden sanctuary to assist me through the perils of this project.

I would like to thank all of the subjects who endured this project; this would not have been possible without your remarkable dedication and effort throughout.

To all of the interns and practicum students who dedicated their early mornings and countless hours to ensuring this project ran as smoothly as possible.

Finally, I would like to thank my family for their undying support and patience throughout these past two years. You all have instilled a passion in me to continue pursuing what I really love.
# Table of Contents

Acknowledgments ...................................................................................................................... ii

List of Tables .......................................................................................................................... iv

List of Figures ........................................................................................................................... v

Abstract ..................................................................................................................................... vi

I. Introduction .............................................................................................................................. 1

II. Methodology .......................................................................................................................... 10

III. Manuscript ........................................................................................................................... 36

IV. References ............................................................................................................................. 77
List of Tables

Table 2.1: Staining Protocol for Immunofluorescence ............................... 63
Table 3.1: Subject Characteristics .......................................................... 64
Table 3.2: Macronutrient Content of Nutritional Treatments .................. 65
Table 3.3: Average Daily Dietary Intake .................................................. 66
Table 3.4: Peak Isokinetic Torque .......................................................... 67
Table 3.5: Whole Muscle Size and Muscle Fiber Size ............................ 68
Table 3.6: Body Weight ........................................................................ 69
List of Figures

Figure 2.1: Experimental Design ................................................................. 70
Figure 2.2: Study Schematic with Corresponding Data Collection .................. 71
Figure 3.1 A: Average Daily Training Duration ............................................. 72
Figure 3.1 B: Average Training Power .......................................................... 73
Figure 3.1 C: Average Training Heart Rate .................................................. 74
Figure 3.2: 30-km Time Trial Performance .................................................... 75
Figure 3.3: Rating of Perceived Muscle Soreness ......................................... 76
Abstract

Previous studies show that carbohydrate-protein (CP) ingestion can enhance short-term recovery following exercise, thereby benefiting subsequent exercise performance and compounding physiological parameters, when compared to carbohydrate (CHO) alone. Less is known about the influence that CP supplementation may have over a long-term intervention (several days/weeks). The purpose of this investigation was to determine whether CP supplementation was effective in improving tolerance to a period of intensified training (IT), compared to CHO. Additionally, the influence of CP on recovery/adaptation to a period of IT followed by a period of reduced volume training (RVT) was examined. Eight endurance-trained cyclists (age: 24.9±7.3 years, weight: 71.8±11.5 kg, VO\textsubscript{2max}: 63.4±7.9 ml/kg/min) completed two independent training phases, each with a 10 day period of IT, followed by a 10 day period of RVT. CHO supplementation was provided during (45 g/hr) and immediately following (1.2 g/kgBW) all exercise sessions in one phase, whereas CP treatments was provided throughout the other phase (45gCHO/hr + 15gPRO/hr during; 1.2gCHO/kgBW + 0.3gPRO/kgBW post). The impact of IT on cycling performance was ‘unclear’ (60 ± 210 sec), with an ‘unclear’ treatment effect. CP ‘likely’ preserved whole muscle function throughout IT, compared to CHO. Whole muscle size was ‘possibly’ maintained throughout IT with CP, compared to CHO. While CHO ‘likely’ increased MHC IIa CSA following IT, CP ‘likely’ increased MHC IIa CSA following RVT, compared to their respective treatments. Throughout a period of IT, CP supplementation may preserve whole muscle size and function, compared to CHO. However, CP minimally affected changes in cycling performance and muscle fiber size following IT.
Chapter One

Introduction

Condensed periods of high volume training combined with minimal rest (i.e. intensified training - IT) are often incorporated into training cycles of competitive athletes, with the fundamental goal of improving athletic performance. These training blocks can temporarily suppress physiological function and lead to short-term performance decrements (overreaching) (17, 18, 25, 26, 30, 40, 81). Among various other physiological changes (e.g. progressive decline in muscle glycogen concentration), IT is associated with declines in skeletal muscle strength and power production (17, 20, 54, 73), reduced muscle fiber size and contractile function (20, 28, 43), increased muscle soreness (1, 51, 58, 71, 72), and altered markers of muscle damage and physiological stress including higher creatine kinase (17, 27, 42, 60) and resting cortisol concentration (42, 74), respectively. These deleterious effects can generally be reversed with as little as a few days to a week of reduced training (22, 45). Importantly, there is strong evidence that reduced training (RVT) can enhance performance (compared to pre-taper) across a range of disciplines, including cycling (40, 52, 55–57), running (34, 35, 68) and swimming (13, 14, 78). So, IT is commonly followed by a short period of RVT (days) to minimally foster a return to pre-IT levels of function and to ideally lead to compensatory adaptive responses that improve performance. One of the chief risks of IT is that normal short-term decrements in function may persist beyond the period of planned recovery and consequently necessitate prolonged recovery and/or missed training. Because of the physical and psychological demands of this training approach, many athletes manipulate
their nutritional intake in an attempt to better manage the stresses of IT and to promote recovery.

The most widespread nutritional strategy used during endurance training is carbohydrate supplementation. It is widely accepted that depleted muscle glycogen stores can contribute to fatigue and impaired exercise performance (41). Acute carbohydrate supplementation is well known to improve endurance performance, which is partially accomplished by sparing endogenous carbohydrate stores (38). Successive days of IT can compromise muscle glycogen resynthesis (5, 15, 16, 69), and consequently impair performance. Logically, a high carbohydrate diet can result in better maintenance of exercise performance, compared to a low-carbohydrate diet (1, 26). Moreover, a carbohydrate-rich dietary strategy (>8 g/kgBW/day) that incorporates optimal timing of intake (<30 min post-exercise), can sustain muscle glycogen stores and prevent declines in carbohydrate/glycogen oxidation during periods of heavy training (1, 5, 69, 70). Carbohydrate supplementation not only helps athletes better tolerate the strains of IT, but may also enhance the adaptive response. Compared to a low carbohydrate supplement, a high carbohydrate treatment improved cycling performance from pre-intensified levels when administered throughout a post-IT taper (26). In addition to highlighting the potential for nutrition to enhance the benefits of a post-IT taper, these findings support the vital role carbohydrate intake posses in relation to endurance performance, especially during a period of IT.
Short Term Effects of Carbohydrate + Protein: <24 hours

Attention has recently been directed to the potential benefits of adding protein to carbohydrate feedings during and immediately after exercise. Little has been done to evaluate the efficacy of protein-enriched CHO supplementation (CP) during IT. Though the finding is not universal (4, 8, 53, 61, 64), there is plenty of support for short-term recovery advantages of protein supplementation (<24hrs), meaningful advantages that translate to improved subsequent performance (2, 3, 19, 49, 66, 80). Worth noting is that CP feedings can confer ‘next day’ performance benefits even when delivered isocalorically (replacing a small amount of CHO with PRO) and when following both primary forms of endurance exercise; running (2, 49) and cycling (3, 19). These data represent a growing consensus that CP ingestion can enhance recovery from heavy exercise and subsequent performance. Whether or not these benefits persist throughout IT is unknown.

Though the gains in performance with CP appear to be at least partially mediated through skeletal muscle, surprisingly few studies have assessed whole muscle function (peak force, peak torque) in response to different nutrient intake strategies following endurance exercise (7, 23, 24, 71, 79) and the results have been mixed. Protein does appear to have the capacity to better restore whole muscle function after heavy endurance exercise (24, 71, 79), but this is not evident in all studies (7, 23). The disparate findings may be a function of the extent of the initial muscle stress and the nuances of the nutritional intervention.

The documented improvements in performance and muscle function with protein intake may be related to aspects of muscle damage. Although speculative, CP
supplementation can potentially reduce muscle damage by maintaining branch chain amino acid levels in the muscle, resulting in a suppressed signal for proteolysis during and after prolonged exercise (76). CP ingestion has repeatedly been shown to reduce post-exercise creatine kinase levels compared to carbohydrate alone (10, 23, 24, 48, 61, 66, 71). In a recent study, CK levels and subsequent performance were measured following an initial ride to exhaustion to assess the effect of CP during and post-exercise on muscle damage (66). Subsequent performance was better with the CP treatment (66), and the authors later reported that the best performances in the subsequent exercise test occurred in the subjects who received the greatest attenuation of CK after the initial exercise (12). Post-exercise creatine kinase levels are commonly profiled in conjunction with perceived muscle soreness to better determine the magnitude of muscle damage. And similar to the attenuating impact that protein consumption has on creatine kinase, post-exercise muscle soreness is also typically lower with CP versus CHO alone (24, 48, 53, 61). Collectively, these data suggest that CP supplementation improves acute recovery from prolonged exercise compared to CHO alone (24).

Intermediate Term Effects of Carbohydrate + Protein: 1-7 days

Given that adding protein to a carbohydrate-treatment has short-term (<24hrs) benefits, it is logical to speculate that CP supplementation may also improve tolerance and adaptations to several days or weeks of heavy training. Although not systematically addressed, initial evidence indicates that protein-rich carbohydrate feedings alleviate the negative impact that consecutive heavy days of training can have on performance (11, 63, 71). CP supplementation maintained time-to-exhaustion performance during 3
consecutive days of cycling to fatigue compared to an isocaloric CHO supplement (71). Additionally, cyclists that consumed CP ad libitum throughout an 8-day mountain bike race performed better during the final 7 days compared to those that received the CHO only treatment (11). Lastly, when a protein-enriched diet (CP) was provided over a 4-day period of high-intensity cycling, recovery from prior exercise bouts was enhanced, resulting in superior performance on the fourth day compared to a control diet (CHO) (63). Studies that deviate from this conclusion were not conducted during heavy periods of training where the beneficial effects of CP ingestion are likely more pronounced (23, 48). Overall, CP intake appears to soften the detrimental effects that heavy cycle training has on subsequent performance, compared to CHO ingestion alone. As CP ingestion may sustain exercise performance, whole muscle function, an important feature in endurance performance, may also be maintained during a period of IT with CP intake compared to CHO.

Very few studies have investigated the effect of CP supplementation on whole muscle function during consecutive days of heavy training. During three consecutive days of cycle training, whole muscle function as assessed by vertical jump height was better maintained with CP ingestion compared to CHO (71). Similar to the acute model, the degree of muscle damage may impact the ability to maintain whole muscle function during a period of IT. Attenuated muscle damage, as indicated by reduced post-exercise CK levels, has been observed with CP supplementation in cyclists (63, 71), runners (48), and soccer players (23) during training periods of 3 to 6 days. Additionally, CP intake evoked a more rapid recovery of post-exercise CK in swimmers during 4-weeks of IT, compared to CHO alone (10). Similarly, CP ingestion seems to attenuate perception of
muscle soreness during consecutive days of heavy training compared to CHO (21, 48, 71). Overall, the beneficial effects of CP intake observed in a short-term setting (<24hrs) seem to translate to longer durations of training.

**Prolonged Effects of Carbohydrate + Protein: >7 days**

To our knowledge only one prior study has investigated the effects of protein ingestion within the methodological framework proposed in the current study. Witard and colleagues recently investigated the efficacy of increased dietary protein (1.5 vs 3.0 gPRO/kgBW/day) on tolerance to a period of intensified training and adaptations during a week-long reduced volume training phase (81). The high protein diet ‘possibly attenuated’ performance decrements throughout intensified training, and ‘possibly enhanced’ performance restoration after a period of reduced-volume training (81). Importantly, Witard did not assess protein intake within the most nutrient sensitive time points, during and immediately after exercise. Additionally, the low-protein group received a fairly large amount of protein (1.5 gPRO/kgBW/day), potentially reducing any observed difference between treatment groups. Finally, the authors did not profile changes in skeletal muscle physiology (e.g. whole muscle function, muscle size, and fiber size). It is well established that periods of IT induce muscle fiber atrophy (20, 28, 43). And while a period of RVT has independently been shown to permit muscle fibers to grow (47, 56, 78), no one has assessed whole muscle size or fiber size in individuals subjected to both training periods. Likewise, the impact that protein ingestion may have on this response is unknown.
Therefore, the purpose of this study is to determine if CP supplementation during and following exercise throughout IT minimizes impairments in subsequent endurance performance, sustains skeletal muscle size and function, and attenuates markers of muscle damage, in comparison to CHO supplementation. The study was also designed to test the hypothesis that CP co-ingestion during and immediately following exercise during RVT improves subsequent performance, increases whole muscle size and fiber cross-sectional area, and improves skeletal muscle function, in comparison to CHO supplementation.
Aims and Hypotheses

Aim 1:
To determine whether CP ingestion during and following exercise enhances tolerance to 10 days of intensified cycling training in trained cyclists compared to CHO supplementation.

Hypothesis 1:
CP supplementation during and following exercise throughout intensified training will better maintain subsequent endurance performance, better sustain skeletal muscle function and size, attenuate biomarkers of muscle damage, and reduce ratings of perceived muscle soreness compared to CHO supplementation.

Aim 2:
To determine if CP ingestion during and immediately following exercise enhances skeletal muscle recovery/adaptations during 10 days of reduced volume training in trained cyclists compared to CHO supplementation.

Hypothesis 2:
CP ingestion during and immediately following exercise throughout a period of reduced volume training will facilitate larger improvements in subsequent performance, larger improvements in skeletal muscle function, and larger increases in whole muscle size and muscle fiber cross-sectional area compared to CHO supplementation alone.
**Significance**

There is a variety of evidence that CP ingestion can enhance short-term recovery following exercise, thereby benefiting subsequent exercise performance, when compared to CHO alone (3, 19, 49, 66, 80). Skeletal muscle function is also improved with CP intake (24, 66, 71, 79); possibly due to attenuations in muscle damage indicated by reduced post-exercise CK levels (10, 23, 24, 48, 61, 66, 71) and ratings of muscle soreness (24, 48, 53, 61).

Less is known about the influence that CP supplementation may have over a long-term intervention (several days/weeks). There are a few studies indicating CP ingestion may enhance subsequent exercise performance during periods of heavy endurance training ranging from 3 to 10 days compared to CHO alone (11, 63, 71, 81). Accordingly, skeletal muscle function may be maintained (71), while post-exercise CK levels (10, 23, 48, 71) and ratings of muscle soreness (21, 71) are attenuated with CP supplement. Moreover, these markers of improved recovery may occur as a consequence of the ability of CP to expedite post-exercise glycogen resynthesis (4, 39) and enhance protein balance during (37, 44) and following (9, 29, 36, 37, 49) endurance exercise. Surprisingly, little is known about the effects of during-or post-exercise nutrition on whole-muscle and fiber size. To date, no one has examined the effect of CP supplementation during and immediately after exercise on tolerance and subsequent recovery/adaptation to a period of IT followed by a period of RT. The results of this study, together with the comprehensive findings on the effects of supplemental protein in an acute setting, would provide insight into the potential that CP may possess in better supporting athletes undergoing periods of IT.
Chapter Two

Methods

SUBJECTS

Eight to twelve, male and female, endurance-trained cyclists between the ages of 18 and 45 years will be recruited from local cycling clubs, including the James Madison University Triathlon and Cycling Club teams. To be included, subjects must possess a VO$_{2\text{max}} \geq 50\text{ml/kg/min}$ or 4.0 L/min. Additionally, all subjects will have completed $\geq 7$ hr of cycle training each week for 2 months preceding participation. Study procedures have been approved by the James Madison University Institutional Review Board. Before participation, and after comprehensive verbal and written explanations of the study, all subjects will provide written, informed consent.

EXPERIMENTAL DESIGN

Subjects will perform two separate training blocks separated by a $\geq 2$-wk washout period (Figure 2.1). Each training block consists of distinct periods of normal training (NT), intensified cycle training (IT), and reduced-volume training (RVT), as described below. During each separate training block, subjects will receive one of two potential nutritional interventions. The two nutritional interventions incorporate feedings of either a carbohydrate (CHO) or protein-enriched carbohydrate (CP) supplement during and post-exercise. A crossover design will be utilized such that each subject receives both nutrition interventions, with order of nutritional treatment randomly counterbalanced.
among subjects. Subjects that meet the inclusion criteria will be familiarized with all testing procedures during the second week of NT.
Figure 2.1. Experimental Design
Training Quantification

Normal Training (NT)

Prior to any intervention, subjects will be instructed to perform their customary training habits for two weeks. The first week of NT will be used to quantify normal training volumes. The second week of normal training will be used for familiarization trials of all performance tests prior to nutritional intervention. Cycling during familiarization trials will be factored into the subject’s normal weekly training volume. Training details gathered during NT will be used to prescribe training volumes and intensities throughout the investigation. Each subject will be provided with a rear bicycle wheel equipped with an integrated PowerTap system (Saris Cycling Group Inc, Madison WI). These units are used to quantify power output, heart rate, total exercise duration and distance during all training sessions conducted outside the laboratory. Cycling power output and training heart rate will be used as indices of training intensity, whereas total training duration (minutes) will be used to quantify training volume.

Intensified Cycle Training (IT)

Immediately following NT, subjects will perform 10 days of IT, consisting of a 100% increase in average daily training volume. Cycling performance tests (preloaded Time Trial) will be conducted on days 1, 4, 7, and 10 and will contribute to total training load during IT (Figure 2.2). Training on days 2, 3, 5, 6, 8, and 9 will be executed outside of the laboratory. Subjects will be provided with individualized training guidelines for these days based on their training volume during NT. Power Tap units will be utilized
during all training days, and power output, heart rate, and training duration will be recorded to verify compliance to the training guidelines.

*Reduced Volume Training (RVT)*

Immediately following IT, subjects will perform 10 days of RVT in which average daily training volume is reduced by 60% relative to NT. A cycling performance test will be conducted on day 10 and will contribute to total training load during RVT (Figure 2.2). Similar to above, power output, heart rate, and training duration will be recorded during all sessions.

*Washout (WO)*

A washout phase of $\geq 2$ weeks will follow RVT. WO will be comprised of an individualized initial period of recovery, with the intent of restoring normal training loads by the end of the washout period. Following the washout period, subjects will participate in one week of NT (replicating training habits from the first NT period), before initiating a second phase of IT and RVT training.
**Figure 2.2. Study Schematic with Corresponding Data Collection**

| Days | -3 | -2 | -1 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | +1 |
|------|----|----|----|---|---|---|---|---|---|---|---|---|----|---|---|---|---|---|---|---|---|---|---|---|---|---|
| VO$_{2_{\text{max}}}$ | X |    |    | X |   |   |   |   |   |   |   |   | X |   |   |   |   |   |   |   |   |   |   |   |   |
| TT  |    | X |    | X | X | X |   |   |   |   |   |   | X |   |   |   |   |   |   |   |   |   |   |   |   |
| Bx  | X  |    |    |   |   |   |   |   |   |   |   |   | X |   |   |   |   |   |   |   |   |   |   |   |   |

VO$_{2_{\text{max}}}$ = VO$_{2_{\text{max}}}$, TT = preloaded time trial, Bx = skeletal muscle biopsy

Note: This design will be repeated twice (Figure 1). Therefore, days -1, -2, and -3 correspond to the final 3 days of normal training, whereas day +1 corresponds to day 1 of washout or study completion.
NUTRITIONAL TREATMENTS AND DIETARY CONTROLS

Treatment beverages will be administered during and immediately following all training sessions throughout IT and RVT (20 total CHO sessions and 20 CP sessions). During all laboratory TT sessions (Figure 2.2), subjects will ingest 250 ml of fluid every 20 minutes until TT completion (750 ml·hr⁻¹; details provided below). For all rides performed in the field (IT 2, 3, 5, 6, 8, 9 and RVT days 2-9), participants will be provided with 500 ml bottles filled with the appropriate beverage, and instructed to ingest 1 bottle during each 40-minute block of training (750 ml·hr⁻¹). Following each ride, participants will be supplied with bottles containing an individualized volume of fluid and instructed to finish the beverage within 30 minutes of terminating exercise. Participants will be instructed to avoid any other beverage or food intake for 2 hrs following the completion of each exercise session, with the exception of *ad libitum* water consumption. Additionally, subjects will be instructed to record any volume of unfinished post-exercise beverages.

**During Exercise**

*CP*

The during-exercise CP treatment will be Gatorade® with additional hydrolyzed whey protein isolate, obtained from American Casein Company (AMCO, Burlington NJ). During all training sessions, beverages (6% carbohydrate, 2% protein) will be ingested at a rate of 750 ml·hr⁻¹, which provides subjects with 45 g CHO·hr⁻¹ and 15 g PRO·hr⁻¹.
The during-exercise CHO treatment will be Gatorade® (without additional protein). During all training sessions, beverages (6% carbohydrate) will be ingested at a rate of 45 g CHO·hr$^{-1}$ (750 ml of fluid·hr$^{-1}$), providing equal carbohydrate content to CP, and a similar flavor of either lemon-lime or fruit-punch.

**Post-Exercise**

**CP**

Immediately following each training session, a non-fat chocolate milk beverage will be provided. Each serving consists of 9.93 ml·kg BW$^{-1}$, and provides 1.2 g CHO·kg BW$^{-1}$ and 0.4 g PRO·kg BW$^{-1}$.

**CHO**

Immediately following each training session, an isocarbohydrate beverage, relative to CP, will be provided. Each serving consists of 9.93 ml·kg BW$^{-1}$, and provides 1.2 g CHO·kg BW$^{-1}$. The beverage will be created by mixing the appropriate amount of commercially available chocolate flavored carbohydrate gels (Clif Shots) with water, providing a similar taste/color compared to non-fat chocolate milk.

**Dietary Controls**

During the time period between the onset of each training session and 2 hrs following each training session, participants will not receive any nutrients other than the CHO or CP beverages. Prior to any nutritional intervention, subjects will complete a
nutritional consultation with a registered dietician to review acceptable dietary choices during training periods and instructions on completing dietary records. 24-hour dietary records will be gathered during NT, whereupon individualized feedback will be provided about total caloric- and macronutrient intake; participants with inadequate daily carbohydrate intake (<6.5 g·kgBW\(^{-1}\)·day\(^{-1}\)) will be encouraged to increase their dietary carbohydrate levels prior to the training intervention. Dietary intake will also be recorded throughout IT (10 days) and RVT (10 days). Using copies of dietary records obtained from the first intervention phase, subjects will then be instructed to replicate their dietary habits during the second phase of the cross-over design. Subjects will submit their dietary intake forms every three days during the IT and RVT periods. During the second phase, subjects will be provided with forms listing their exact diet from the first phase, as well as two possible substitutions for each corresponding meal. Researchers will contact the subjects with specific dietary suggestions if dietary intake intakes vary appreciably from the prescribed dietary guidelines.

All laboratory testing (i.e. skeletal muscle biopsies, VO\(_{2\text{max}}\) tests, and TT) will be performed after an 8-10 hr overnight fast (ad libitum water consumption). In addition, subjects will be provided with a standardized boxed-lunch at all laboratory sessions. The standardized boxed-lunch will include two sandwiches from a selected menu, a bag of potato chips, and a beverage of choice. Subjects will be provided with several choices of lunch contents, provided by JMU dining services (ARAMARK) and are required to repeat the selection throughout the duration of the study (phase 1 and phase 2). Subjects will be instructed to consume all contents of the boxed-lunch and to record all unconsumed boxed-lunch contents during the 2-6 hr post-exercise period with only ad
libitum water consumption permitted during this time period. Subjects will be provided with several choices of lunch contents, provided by JMU dining services (ARAMARK) and are required to repeat the selection throughout the duration of the study (phase 1 and phase 2). This will allow for the standardization of dietary intake for approximately 6 hours after each laboratory time trial session.

MEASUREMENTS

Endurance Performance

$VO_{2\text{max}}$ test

Subjects that meet the inclusion criteria will complete an incremental exercise test to determine $VO_{2\text{max}}$. Height and weight will be recorded prior to the incremental exercise test, which will take place prior to the initial phase of NT. The test will be conducted on a computerized cycle ergometer (Velotron, Racermate Inc, Seattle WA). Subjects will warm-up for 5 mins at an individualized workload based on a light perceived exertion. The workload will be increased by 25 W every 2 min until subjects voluntarily request to stop due to fatigue or are unable to maintain a cadence of >50 rpm. Oxygen uptake will be assessed during each stage in 30-s intervals using indirect calorimetry via an automated Moxus Modular Metabolic System (AEI Technologies, Bostrop TX) and SensorMedics VMax 229 metabolic cart (Yorba Linda, CA). The initial $VO_{2\text{max}}$ test will be used for inclusion/exclusion, and as a familiarization for subsequent $VO_{2\text{max}}$ testing. Test duration (time to fatigue) will be used as a measure of performance.
Preloaded Time-trial (TT)

Subjects will perform TT’s on a computerized cycle ergometer (Velotron, Racermate Inc, Seattle WA) on IT days 1, 4, 7, 10 and RVT day 10, as illustrated in Figure 2.2. The TT will include an initial period of 120 minutes of cycling at 50% $W_{\text{max}}$ (obtained from VO$_{2\text{max}}$ test) followed immediately by a simulated 30-km time trial. 30-km TT finishing times and average power output will be recorded and used as performance measures. Each TT will contribute to the training load during IT and RVT, and therefore the same nutritional guidelines as field training sessions will be utilized.

Skeletal Muscle Physiology

Peak Isokinetic Force (Bdx)

Peak isokinetic concentric muscle force will be assessed following a standardized 5-min warm-up on a cycle ergometer. This test will be conducted using a Biodex isokinetic dynamometer (Biodex Medical System Inc., Shirley NY). Peak unilateral isokinetic force (power) will be assessed by having subjects push as hard as possible against a shin pad connected to an electronic device that controls the speed of movement through the leg-extension. Subjects will perform a maximal effort on four occasions for each speed, with each repetition separated by 30s of rest. Selected rotational speeds are 240 degrees/sec and 120 degrees/second. The faster speed will be performed first in order to avoid premature muscular fatigue before conducting the later speed. This assessment will be performed immediately following each skeletal muscle biopsy (using the non biopsied leg).
**Maximal Voluntary Contraction (MVC)**

Skeletal muscle function will be assessed immediately before each VO$_{2\text{max}}$ test using a custom-built leg extension device to determine peak unilateral isometric contraction force. Following a standardized 5-min warm-up (3mph treadmill walk), subjects will be positioned in the leg extension device and prompted to exert maximum force against a shin bar for three seconds on four occasions, with each repetition separated by 1 min of rest. Peak force will be recorded in Newtons, using the right leg.

**Serum Creatine Kinase (CK) and Cortisol**

Fasting venous blood samples will be obtained from an antecubital vein prior to TT1, TT4, TT5 and following the 2-hr fixed intensity phase of the TT protocol (6 total). Upon entering the lab, subjects will rest in an upright phlebotomy cathedra for 5 min prior to receiving blood draw. Following the 2-hr fixed intensity phase of the TT protocol, subjects will be instructed to return to the phlebotomy cathedra from the cycle ergometer as quickly as possible to receive their second blood draw. Transition time from cycle ergometer to initiation of blood draw will be recorded and standardized for subsequent trials. Approximately 10 ml of whole blood will be obtained at each blood draw and centrifuged at 10000 rpm following 30 min of coagulation. Serum samples will be stored at -80° for later analysis. CK will be subsequently analyzed using an automated table-top analyzer (Johnson and Johnson Vitro DT 6011), whereas cortisol will be analyzed using standard enzyme-linked immunosorbent assay (ELISA) procedures. CK
and cortisol levels will be measured to indicate levels of cellular membrane damage and inflammation respectively.

**Muscle Soreness**

Soreness ratings will be obtained prior to each lab visit (VO_{2max}, TT, Biopsy) using a 100mm visual analog scale, with 0 indicating no muscle soreness and 100 indicating impaired movement due to muscle soreness.

**Skeletal Muscle Size**

Vastus Lateralis (VL) thickness will be measured using a Shenzen Mindray DC-6 ultrasound device (Nanshan, Shenzen, China) in B-mode with a 10MHz capacity linear array transducer. During the initial visit, mid-muscle belly of the VL will be identified and recorded for subsequent visits, using a technique adapted from Kumagai et al. (46). The distance between the bony protuberance of the greater trochanter of the femur to the prominence of the lateral femoral condyle will be determined. This point will then be intersected with a perpendicular line drawn from the midway point through a vertical line drawn from the lateral border of the patella past the midway point of the greater trochanter and femoral condyle. This point is where the ultrasound images will be captured. Subjects will be positioned sitting upright, using a custom-built device to standardize the selected leg position. Subject will rest in this position for 15min prior to obtaining the ultrasound measurement. Using the 7.5MHz probe frequency setting, the transducer head will be positioned until the aponeuroses of the VL can be clearly delineated. Upon capturing images, the outline of the transducer head will be outlined
with a surgical skin marker denoting the location for future imaging. Further, the subjects will be provided with personal skin markers and instructed to maintain the initial marking until the subsequent trial. This measurement will be made prior to each VO2max test.

**Muscle Fiber Size**

**Skeletal Muscle Biopsies**

Vastus Lateralis (VL) muscle biopsies will be obtained on the dates indicated in Figure 2.2 (6 total). At each time point, percutaneous needle biopsies will be obtained under local anesthetic (2-3 ml Lidocaine) (6). Muscle samples will be dissected free of any visible connective and adipose tissue and frozen in isopentane, cooled in a liquid nitrogen bath. Samples will be stored at -80°C for later analysis.

**Immunohistochemistry**

Serial cross-sections (5 um) will be cut at -25°C (Minotome Plus; Triangle Biomedical Sciences, Durham, NC) from biopsy samples and will be arranged on an uncoated glass slide. The details of the combined stain of glycogen (PAS) with immunofluorescence are summarized in Table 2.1. Sections will be fixed for 1 h at 4°C with 3.7% formaldehyde (Fisher Scientific, Fair Lawn, NJ) in PBS immediately after removal from the freezer. Next, the slides will be rinsed for 10 min in PBS and treated with 0.1% Triton X-100 (Acros Organics, Geel, Germany) for 5 minutes. After the fixation and permeability steps, the PAS staining will begin by rinsing the slides in PBS for 15 min and deionized water (D.I. H2O) for 30 s. Then slides will be treated with 1% periodic acid (Sigma-Aldrich, St. Louis, MO) in D.I. H2O followed by a wash in D.I. H2O for 1 min. Slides will then undergo incubation in Schiff’s reagent (Sigma-Aldrich,
St. Louis, MO) for 15 min at room temperature followed by washing steps in D.I. H$_2$O for 5 s and running tap water for 10 min. Thereafter, slides will be rinsed with PBS for 15 min and incubated for 30 min with blocking buffer (0.05% Triton X-100, Normal Goat Serum). Different concentrations of primary antibodies in 0.05% Triton X-100 will be applied to the slides for 2 h at room temperature. Slides will be rinsed for 30 min with 0.05% Triton X-100 in PBS and incubated with appropriate conjugated secondary antibodies for 2 h at room temperature, in the dark. After incubation, the slides will be rinsed in the dark for 30 min with 0.05% Triton X-100 in PBS. After a final rinse in the dark for 15 min in PBS, coverslips will be mounted on the slides with Fluormount G mounting medium (Southern Biotechnology Associates, Birmingham, AL) and stored in the dark at 4°C for later analysis.

**Primary and Secondary Antibodies**

Muscle fiber type and cross-sectional area will be assessed through application of the following monoclonal primary antibodies: A4.840 supernatant, a mouse monoclonal IgM antibody directed against human myosin heavy chain I (MHC I) (Developmental Studies Hybridoma Bank, Iowa City, Iowa); SC-71, supernatant, a mouse monoclonal IgG1 antibody directed against human myosin heavy chain IIa (MHC IIa) (Developmental Studies Hybridoma Bank, Iowa City, Iowa); and 2E8 supernatant, a mouse monoclonal IgG2a antibody directed at human laminin, a basement membrane protein (Developmental Studies Hybridoma Bank, Iowa City, Iowa). Each primary antibody will be paired with its appropriate conjugated secondary antibodies: MHC I, Alexa Fluor 555 Goat anti-mouse IgM; MHC IIa, MHCIIa: Alexa Fluor 647 Goat anti-
mouse IgG; Laminin, Alexa Fluor 488 Goat anti-mouse IgG₂a (Molecular Probes, Leiden, The Netherlands).

**Imaging and Quantification**

Slides will be examined using fluorescent microscopy (Nikon Eclipse TE2000-E, Tokyo Japan). Within each sample, the largest possible area will be imaged. Epifluorescence signal will be recorded using Texas Red excitation filter (534-556 nm) and Cy5 excitation filter (628-672 nm) for MHCⅠ and MHCⅡa muscle fibers, respectively. Additionally, FITC excitation filter (450-490 nm) will be used to record epifluorescence signal for laminin. Images will be captured in each filter and overlaid using NIS Elements software (Nikon, Tokyo, Japan). Cross-sectional area will be determined by measuring the laminin-positive stain outlining each muscle fiber; peripheral muscle fibers that have irregular edge staining patterns or disrupted cell membranes will be marked and excluded from analyses. This quantification will provide a fiber-type specific measure of cross-sectional area.
Table 2.1. *Staining Protocol for periodic acid-Schiff (PAS) staining with immunofluorescence (IF)*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PAS with IF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fixation</td>
<td>3.7% Formaldehyde in PBS for 60min @ 4°C</td>
</tr>
<tr>
<td>Rinsing</td>
<td>PBS for 10min (2 x 5min)</td>
</tr>
<tr>
<td>Permeabilisation</td>
<td>0.1% Triton X-100 in PBS for 5min</td>
</tr>
<tr>
<td>Rinsing</td>
<td>PBS for 15min (3 x 5min)</td>
</tr>
<tr>
<td>Rinsing</td>
<td>Deionized (D.I.) H₂O for 30sec</td>
</tr>
<tr>
<td>Pretreatment</td>
<td>1% Periodic acid in D.I. H₂O for 5min</td>
</tr>
<tr>
<td>Rinsing</td>
<td>D.I. H₂O for 1 min</td>
</tr>
<tr>
<td>Staining</td>
<td>Schiff’s Reagent for 15min @ room temp.</td>
</tr>
<tr>
<td>Rinsing</td>
<td>D.I. H₂O for 5sec and tap water for 10min</td>
</tr>
<tr>
<td>Rinsing</td>
<td>PBS for 15min (3 x 5min)</td>
</tr>
<tr>
<td>Incubation</td>
<td>10% NGS in 0.05% Triton X-100 for 30min @ room temp.</td>
</tr>
<tr>
<td>Incubation</td>
<td>Primary antibodies in 0.05% Triton X-100 for 2hr @ room temp.</td>
</tr>
<tr>
<td>Rinsing</td>
<td>0.05% Triton X-100 in PBS for 30min (5 x 6min)</td>
</tr>
<tr>
<td>Incubation</td>
<td>Secondary antibodies in 0.05% Triton X-100 for 2hr @ room temp. (in dark)</td>
</tr>
<tr>
<td>Rinsing</td>
<td>0.05% Triton X-100 in PBS for 30min (5 x 6min) (in dark)</td>
</tr>
<tr>
<td>Rinsing</td>
<td>PBS for 15min (3 x 5min) (in dark)</td>
</tr>
<tr>
<td>Mounting</td>
<td>Fluormount G (in dark)</td>
</tr>
</tbody>
</table>
STATISTICAL ANALYSIS

All data will be log transformed to diminish the effects of nonuniformity. For each measurement variable, change scores between the three measurement time points, within each treatment, will be calculated. Dependent T-tests comparing the change scores between treatments will be applied to generate p-values for subsequent analysis, as described below. Statistical analyses will be performed using IBM Statistical Package for Social Sciences (SPSS) 21 for Macintosh (SPSS Inc., Chicago, IL, USA).

Magnitude-based inferences about the data will be derived using methods described by Hopkins and colleagues (31). A standardized difference in means (mean difference between treatments divided by the between-subject SD under CHO conditions: SD units) will be calculated for each variable whereby observed values equivalent to or exceeding 0.2 SD units will qualify as a substantial treatment effect (i.e. threshold for substantial effect) (31). The 0.2 SD unit threshold is used for all variables with the exception of time trial performance, for which a previously established ‘smallest worthwhile change’ in performance is used as the threshold (32). The smallest worthwhile change in performance has been defined as 0.3 x the within subject variability across repeated time trials, which translates to a ~1% change in performance (0.3 x 3.4%), 0.13 SD units, or ~35 seconds for the current data (32).

A published spreadsheet (33) will be used to determine the likelihoods of the true treatment effect (of the population) reaching the substantial change threshold (0.2 SD); these were classified as <1% almost certainly no chance, 1-5% = very unlikely, 5-25% = unlikely, 25-75% = possible, 75-95% = likely, 95-99% = very likely, and >99% = almost certain. If the percent chance of the effect reaching the substantial change threshold is
<25% and the effect was clear, it is classified as a ‘trivial’ effect. If 90% confidence intervals included values that exceeded the substantial change threshold for both a positive and negative effect, effects will be classified as ‘unclear’ (>5% chance of reaching the substantial threshold for both a positive and negative effect). For ease of interpretation data will be displayed as raw means ± SD and/or percent difference between treatments ± CL (90% confidence limit; to illustrate uncertainty in treatment effects).
References


Chapter Three

Manuscript
Introduction

Condensed periods of high volume training with minimal rest (i.e. intensified training, IT) are often incorporated into the training cycles of competitive athletes, with a fundamental goal of improving athletic performance. These training blocks can suppress physiological function, leading to short-term performance decrements (17, 18, 25, 27, 30, 40, 81) and various other physiological changes that include declines in skeletal muscle strength/power (17, 20, 54, 73), and reduced muscle fiber size and contractile function (20, 28, 43). Periods of IT are generally followed by a short period (i.e. several days) of reduced volume training (RVT). This strategy is widely observed to promote a return to pre-IT levels of function and often lead to compensatory adaptations, thus improving performance (13, 34, 40, 45). Many athletes also manipulate their nutritional intake during heavy training in an attempt to better manage the stresses of IT, and improve recovery.

Carbohydrate (CHO) supplementation is the most widespread nutritional strategy used during endurance training and has demonstrated the ability to maintain exercise performance during heavy training, potentially via preserved muscle glycogen stores and sustained carbohydrate oxidation rates during periods of IT (1, 5, 26). In addition to helping athletes better tolerate the strains of IT, CHO may also enhance the adaptive response throughout a period of RVT (26). While CHO has been widely shown to be effective, little has been done to evaluate the efficacy of protein-enriched CHO (CP) supplementation during IT.

The effects of acute (<24hrs) CP supplementation during and immediately after exercise have received considerable attention over the past decade. Though the finding is
not universal (8, 61, 64), CP feedings can translate to improved subsequent performance (19, 49, 65, 66) versus CHO alone, even when delivered isocalorically (19, 49). The gains in performance with CP appear to be partially mediated through skeletal muscle, where whole muscle function (strength/power), an important component in endurance performance, appears to be restored after IT when CP is administered (24, 71, 79). Further, these documented improvements in performance and muscle function with CP may result from the attenuation of muscle damage. CP can potentially reduce signs of muscle damage, compared to CHO (10, 23, 48, 61, 66). Given that adding protein to a carbohydrate-beverage has short-term benefits, it is logical to speculate its effectiveness during longer blocks of heavy training.

Overall, the existing body of literature appears to demonstrate that CP ingestion attenuates the detrimental effects of heavy endurance training on subsequent performance (11, 63, 71). Although relatively few multi-day studies have been completed, CP supplementation seems to better preserve whole muscle function (71) and attenuate muscle damage compared to CHO (21, 48, 63, 71). A recent study (81) reported increased dietary protein (1.5 vs. 3.0 gPRO/kgBW/day) to ‘possibly attenuate’ performance decrements during one week of IT, and ‘possibly enhance’ performance restoration after one week of RVT (81). However, this study did not examine changes in skeletal muscle physiology (i.e. whole muscle function, muscle size, and fiber size), nor has anyone assessed whole muscle or fiber size in individuals subjected to both IT and RVT.

The purpose of this study was to examine the effect of protein-enriched carbohydrate supplementation on tolerance to 10 days of IT and subsequent adaptation
following 10 days of RVT. We hypothesized that 1) CP supplementation throughout IT would minimize impairments in performance, sustain skeletal muscle size and function, and attenuate markers of muscle damage compared to CHO and 2) CP ingestion throughout RVT would improve subsequent performance, increase whole muscle size and fiber size, and improve muscle function compared to CHO alone.
MATERIALS AND METHOD

Subjects

Ten male (n=8) and female (n=2) endurance-trained cyclists from James Madison University and the Harrisonburg, VA area were recruited to participate in this study. One subject was excluded due to noncompliance to standardization procedures, while another withdrew due to circumstances unrelated to the study, resulting in a total of 8 subjects (6 males; 2 females). Intervention timing was standardized relative to the menstrual phase of both female subjects. Subjects were required to have competed ≥ 7 hrs of weekly cycling for two months prior to participation in the study. Subjects were provided written and oral information about experimental procedures and potential risks prior to giving informed consent. All procedures were approved by the James Madison University Institutional Review Board prior to testing. Subject characteristics are displayed in Table 3.1.

Experimental Design

Subjects completed two separate training blocks, each consisting of three distinct periods of training: Normal training (NT), intensified cycle training (IT), and reduced-volume training (RVT) (Figure 2.1). A double blind, partially counterbalanced, crossover design was implemented whereby subjects received one of two potential nutritional interventions during IT and RVT of each training block. The nutritional interventions were during- and post-exercise feeding of either carbohydrate (CHO) or protein-enriched carbohydrate (CP) supplement. Training blocks were separated by ≥ 2-wk washout
period. Subjects were familiarized with all testing procedures during the second week of NT during the initial training block.

**Training Procedures**

**Training quantification**

In order to monitor training as accurately as possible and standardize training stimuli between phases, subjects were provided with a rear bicycle wheel equipped with an integrated PowerTap system (Saris Cycling Group Inc, Madison WI). Power output, heart rate, exercise duration, and distance were recorded during all training sessions outside of the laboratory.

Training details gathered in the first week of NT, at the onset of the study, were used to prescribe training duration and power output throughout the investigation. All familiarization trials were performed during the second week of NT. Immediately following NT, subjects performed 10 days of IT, which consisted of a 100% increase in average daily training volume, relative to the first week of NT. Based on the training volume during NT, individualized training guidelines, incorporating all experimental testing, were provided to the subjects for IT and RVT. During IT, subjects cycled daily, performing preloaded time trials (detailed below) on IT days 1, 4, 7, and 10, which contributed to total training load. During RVT, average daily training volume was reduced by 60% relative to NT. A cycling time trial was performed on day 10 and contributed to total training load (Figure 2.2). Each training block was separated by a washout phase of ≥ 2 weeks following RVT. The washout phase allowed subjects as much time as needed to fully recover from phase one. Upon full recovery, subjects
progressed training to then replicate the second week of NT from phase one prior to initiating the second training phase. Training details were replicated from one training phase to the next.

**Nutritional Treatments and Dietary Control**

Prior to any nutritional intervention, subjects completed a nutritional consultation with a registered dietician to overview acceptable dietary choices and instruction on completing dietary records. Twenty-four hr dietary records were gathered during the last two days of NT and everyday throughout IT and RVT. Subjects were provided with copies of their dietary records from the first phase and instructed to replicate dietary habits during the second intervention phase. Caloric and macronutrient intake were similar between the interventions (Table 3.3).

All laboratory testing was performed after an 8-10 hr overnight fast. Additionally, subjects were provided with a standardized boxed-lunch, prepared by JMU dining services (ARAMARK), following all laboratory sessions. Treatment beverages were administered during and following all training sessions throughout IT and RVT. During all training sessions, subjects ingested either a CHO or CP supplement at a rate of 750 ml·hr⁻¹. Immediately following each training session, subjects consumed an individualized amount (9.93 ml·kg BW⁻¹) of either a CHO or CP supplement (Table 3.2). In an attempt to better control nutritional intake in close proximity to exercise, subjects abstained from any other caloric intake for 2 hrs following the completion of each training session.
Experimental Trials

**VO$_{2\text{max}}$ test**

Subjects performed an incremental exercise test to fatigue on an electromagnetically braked cycle ergometer (Velotron, Racermate Inc, Seattle WA) to determine $W_{\text{max}}$, and VO$_{2\text{max}}$, as previously described (67). Following a standardized 5 min warm-up, subjects began the test at a self-selected workload estimated as “a moderate pace for a 60 min ride.” Power was increased by 25 W every two min until the subject reached volitional exhaustion. Metabolic measurements were assessed throughout the test using a Moxus Modular Metabolic System (AEI Technologies, Bostrop TX) and SensorMedics VMax 229 metabolic cart (Yorba Linda, CA). VO$_{2\text{max}}$ was determined by the highest 30 sec mean oxygen uptake value. $W_{\text{max}}$ was defined by the power corresponding to the final successful stage, and was used to prescribe workload for the 120 min constant-load period of the subsequent time trials. Prior to NT, a preliminary VO$_{2\text{max}}$ was performed to confirm inclusion criteria was met, and also serve as a familiarization to testing procedures. Three VO$_{2\text{max}}$ tests were performed during each training phase (Figure 2.2).

**Time trial (TT)**

Subjects performed five preloaded time-trials on IT days 1, 4, 7, 10, and RVT day 10, as illustrated in Figure 2.2. Additionally, a familiarization time trial was performed during the 7 days prior to IT to acquaint subjects with testing procedures. The TT consisted of 120 min of constant-load cycling at 50% $W_{\text{max}}$ (164 W ± 29 W) followed
immediately by a simulated 30-km TT, as previously described (67). A pedestal fan was placed ~2 m from the handlebars and utilized on high speed setting to provide uniform cooling during each trial. Transition time from the constant-load cycling period to the simulated 30-km was standardized for all trials. Subjects were instructed to treat the 30-km portion of each trial as a competition and provide a maximal effort. No feedback was provided during the 30-km other than elapsed distance.

**Peak isokinetic force (Bdx)**

Peak isokinetic concentric muscle force of the knee flexors and extensors was assessed using a Biodex isokinetic dynamometer (Biodex Medical System Inc., Shirley NY) following a standardized 5 min warm-up. Two rotational speeds were used for testing, 240 degrees·sec⁻¹ and 120 degrees·sec⁻¹. Four trials, of one maximal repetition each, were performed at each speed, with each trial separated by 30 sec of rest. Testing was performed immediately following each skeletal muscle biopsy, using the non-biopsied leg. Within a training phase, biopsies were performed on the same leg, with the contralateral leg biopsied during the opposite phase. Furthermore, opposite legs were used for peak force assessments between training phases.

**Maximal voluntary contraction (MVC)**

Peak unilateral isometric force of the knee extensors was assessed using a custom-built leg extension device. Following a standardized 5 min warm-up subjects provided a maximal contraction against a stationary shin bar for 3 sec on four occasions, with each repetition separated by 1 min of rest.
**Blood analysis**

Fasting venous blood samples were obtained from an antecubital vein before TT1, TT4, and TT5. Upon entering the lab, subjects rested in an seated position for 5 min. Approximately 4 ml of whole blood was then obtained [5 ml BD Vacutainer Serum Separation (SST) tube with Polymer gel, Silica activator (Becton Dickinson & Company, Franklin Lakes, NJ, USA)] and centrifuged at 3000 rpm for 10 min at 4°C following 30 min of coagulation. Serum was extracted and stored at -80°C for later analysis. Plasma CK was analyzed using an automated biochemical assay instrument (ChemWell-T 4600, Awareness Technolgy Inc., Palm City, FL). Cortisol was analyzed using quantikine high sensitivity enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, MN, USA).

**Muscle soreness**

Soreness rating were obtained prior to each lab visit using a 100 mm visual analog scale, with 0 indicating no muscle soreness and 100 indicating extreme soreness.

**Skeletal muscle size**

Vastus Lateralis (VL) thickness was measured using a Shenzen Mindray DC-6 ultrasound device (Nanshan, Shenzen, China) in B-mode with a 10MHz capacity linear array transducer. During the initial visit, mid-muscle belly of the VL was identified and recorded for subsequent visits, using a technique adapted from Kumagi et al. (46). Subjects were positioned sitting upright, using a custom-built device to standardize leg
position, where they rested for 15 min prior to obtaining ultrasound measurement. Ultrasound imaging was performed using the 7.5MHz probe frequency at the aforementioned site on the VL. Probe position relative to the VL was recorded for subsequent measurements using a surgical skin marker.

**Skeletal muscle biopsies (Bx)**

A total of six muscle biopsies were collected from the VL (6) (3 from each leg), as indicated in Figure 2.2. Muscle samples were quickly dissected free of any visible connective tissue and adipose tissue, frozen in liquid nitrogen-cooled isopentane, and stores at -80°C for later analysis.

**Immunohistochemistry**

Serial cross-sections (5 um) were cut at -25°C (Minotome Plus; Triangle Biomedical Sciences, Durham, NC) and arranged on an uncoated glass slide. Details of the combined stain glycogen (PAS) with immunofluorescence are summarized in Table 2.1. Following fixation in 3.7% formaldehyde (Fisher Scientific, Fair Lawn, NJ) for 60min at 4°C, sections were rinsed in PBS for 10 min and treated with 0.1% Triton X-100 (Acros Organics, Geel, Germany) for 5 min. After fixation and permeabilisation, PAS staining was performed by treating sections with 1% periodic acid (Sigma-Aldrich, St. Louis, MO) followed by a wash in D.I. H$_2$O for 1 min. Sections were then incubated in Schiff’s reagent (Sigma-Aldrich, St. Louis, MO) for 15 min, followed by washing steps in D.I. H$_2$O for 5 sec and running tap water for 10 min. Thereafter, slides were rinsed in PBS for 15 min and incubated in blocking buffer (10% Normal Goat Serum in 0.05%
Triton X-100). Sections were incubated in primary antibodies for 1 hr at room temperature, rinsed for 30 minutes in 0.05% Triton X-100, and treated with appropriate secondary antibodies for 1 hr at room temperature, in the dark. After a final 30 min rinse in 0.05% Triton X-100 and 15 min rinse in PBS, coverslips were mounted on slides with Fluormount G mounting medium (Southern Biotechnology Associates, Birmingham, AL) and stored in the dark at 4°C for later analysis.

**Primary and secondary antibodies**

Fiber type and cross-sectional area are shown through application of the following monoclonal primary antibodies (Developmental Studies Hybridoma Bank, Iowa City, IA): A4.840 supernatant, a mouse monoclonal IgM antibody directed against human myosin heavy chain I (MHC I); SC-71 supernatant, a mouse monoclonal IgG1 antibody directed against human myosin heavy chain IIa (MHC IIa); and 2E8 supernatant, a mouse monoclonal IgG2a antibody directed at human laminin, a basement membrane protein. Each primary antibody was paired with an appropriate conjugated secondary antibody (Molecular Probes, Leiden, The Netherlands): MHC I, Alexa Fluor 555 Goat anti-mouse IgM; MHC IIa, Alexa Fluor 647 Goat anti-mouse IgG1; Laminin, Alexa Fluor 488 Goat anti-mouse IgG2a.

**Imaging and quantification**

Slides were examined using fluorescent microscopy (Nikon Eclipse TE2000-E, Tokyo, Japan). Within each sample, the largest possible area was imaged (captured at 4x magnification). Epifluorescence signal was recorded using Texas Red excitation filter
(534-556 nm) and Cy5 excitation filter (628-672 nm) for MHC and MHCIIa muscle fibers, respectively. The FITC excitation filter (450-490 nm) was used to record epifluorescence signal for laminin. Images were captured in each filter and overlaid using NIS Elements software (Nikon, Tokyo, Japan). Images were analyzed using Image J (NIH, Bethesda, MD) after converting post-hoc to 8-bit. Cross-sectional area was determined by measuring outline of each muscle fiber, determined by laminin-positive staining; peripheral fibers with irregular staining patterns or disrupted cell membranes were marked and excluded from analysis.

**Statistical analysis**

All raw data were log transformed to diminish the effects of nonuniformity. Dependent t-tests were applied to generate p-values for subsequent analyses described below. The effects of the training phases on all measurement variables during both CHO and CP (NT vs. IT, IT vs. RVT, NT vs. RVT) were analyzed. Likewise, change scores in the measurement variables during each training phase were compared to determine the influence of CP compared to CHO (i.e. Δ 30-km TT time NT-IT with CHO vs. Δ 30-km TT time NT-IT with CP). Statistical analyses were performed using IBM Statistical Package for Social Sciences (SPSS) 21 for Macintosh (SPSS Inc., Chicago, IL).

The p-values derived from the comparisons outlined above were used to formulate magnitude-based inferences about the data using methods described by Hopkins and colleagues (31). A standardized difference in means (mean difference between treatments divided by the between-subject SD under CHO conditions: SD units) was calculated for each variable whereby observed values equivalent to or exceeding 0.2
SD units qualified as a substantial treatment effect (i.e. threshold for substantial effect) (31). The 0.2 SD unit threshold was used for all variables with the exception of time trial performance, for which a previously established ‘smallest worthwhile change’ in performance was used as the threshold (32). The smallest worthwhile change in performance has been defined as 0.3 x the within subject variability across repeated time trials, which translates to a ~1% change in performance (0.3 x 3.4%), 0.13 SD units, or ~35 seconds for the current data (32).

A published spreadsheet (33) was used to determine the likelihoods of the true treatment effect (of the population) reaching the substantial change threshold (0.2 SD); these were classified as <1% almost certainly no chance, 1-5% = very unlikely, 5-25% = unlikely, 25-75% = possible, 75-95% = likely, 95-99% = very likely, and >99% = almost certain. If the percent chance of the effect reaching the substantial change threshold was <25% and the effect was clear, it was classified as a ‘trivial’ effect. If 90% confidence intervals included values that exceeded the substantial change threshold for both a positive and negative effect, effects were classified as unclear (>5% chance of reaching the substantial threshold for both a positive and negative effect). For ease of interpretation data was displayed as raw means ± SD and/or percent difference between treatments ± CL (90% confidence limit; to illustrate uncertainty in treatment effects).
RESULTS

Training Load

Training data throughout NT, IT, and RVT in CP and CHO conditions are displayed in Figure 3.1. Average daily training volume increased from NT (63 ± 7 min/day) to IT (136 ± 16 min/day) with no significant difference between CP and CHO conditions (p = 0.401). Average daily training volume decreased from IT (136 ± 16 min/day) to RVT (41 ± 5 min/day) with no significant difference between CP and CHO conditions (p = 0.380). Average training power decreased from NT (187 ± 28 W) to IT (170 ± 29 W), with no significant difference between treatment conditions (p = 0.203). Average training power increased from IT (170 ± 29 W) to RVT (198 ± 37), with no significant difference between treatment conditions (p = 0.255).

Dietary Intake

Total caloric and macronutrient intake throughout NT, IT, and RVT in CHO and CP conditions are displayed in Table 3.3. Two subjects were omitted from dietary analysis due to incomplete dietary records; therefore six subjects are included in the dietary analysis. Average caloric intake during NT was 3218 ± 735 kcal with the following carbohydrate (454 ± 150 g), protein (123 ± 23 g), and fat (98 ± 23 g) intake. Daily caloric and macronutrient intake during IT were similar between CHO (kcal: 3255 ± 611 kcal, cho: 471 ± 293 g, pro: 136 ± 27 g, fat: 127 ± 23 g) and CP (kcal: 2609 ± 1443 kcal, cho: 360 ± 106 g, pro: 157 ± 70 g, fat: 123 ± 106 g) conditions. Likewise, daily caloric and macronutrient intake during RVT were similar between CHO (kcal: 2793 ± 459 kcal, cho: 335 ± 74 g, pro: 117 ± 16 g, fat: 104 ± 21 g) and CP (kcal: 2584 ± 497 kcal).
kcal, cho: 344 ± 142 g, pro: 116 ± 16 g, fat: 96 ± 14 g) conditions. The caloric and macronutrient data do not include treatment beverages.

30-km Time Trial Performance

Time trial performance after NT, IT, and RVT in CP and CHO conditions are displayed in Figure 3.2. The impact of IT with both CHO and CP on 30-km TT time was ‘unclear’. Relative to IT, RVT ‘likely’ improved TT time with CHO (-114 ± 180 sec) and ‘possibly’ improved TT time with CP (-78 ± 132 sec). Relative to NT, RVT resulted in an ‘unclear’ improvement in TT time with CHO and a ‘very likely trivial’ improvement in TT performance with CP. Overall, the effects of the nutritional treatments on changes in performance were ‘unclear’.

Whole Muscle Function

Assessment of whole muscle function was conducted at two different assessment speeds, 120 deg·sec⁻¹ and 240 deg·sec⁻¹. Peak isokinetic torque of the knee flexors and extensors at both assessment speeds is displayed in Table 3.4.

120 deg·sec⁻¹

IT ‘likely’ reduced peak isokinetic torque of the knee extensors at 120 deg·sec⁻¹ with CHO (-11.4 ± 14.7 ft·lbs⁻¹) whereas IT had a ‘possibly trivial’ effect on peak torque with CP (2.3 ± 10.9 ft·lbs⁻¹). Overall CP ‘likely’ preserved peak knee extensor strength compared to CHO.
The influence of IT on peak knee flexor torque with CHO was ‘likely trivial’ (-0.8 ± 5.6 ft·lbs⁻¹), while IT ‘possibly’ increased peak knee flexor torque with CP (2.8 ± 4.1 ft·lbs⁻¹). CP ‘likely’ preserved peak knee flexor strength from NT to IT compared to CHO.

RVT had an ‘unclear’ effect on leg extension torque with both CHO and CP, resulting in an ‘unclear’ treatment effect. The effect of RVT on leg flexion was ‘likely trivial’ with CHO and ‘possibly trivial’ with CP. Overall there was an ‘unclear’ treatment effect on knee flexion with RVT.

Comparing NT to RVT, there was a ‘possibly trivial’ reduction in leg flexion torque with CHO (-2.1 ± 7.8 ft·lbs⁻¹) and a ‘possible’ increase in leg extension torque with CP (4.7 ± 7.4 ft·lbs⁻¹). However, the treatment effect was ‘unclear’.

### 240 deg·sec⁻¹

IT ‘likely’ impaired peak knee extension torque at 240 deg·sec⁻¹ with CHO (-9.5 ± 16.7 ft·lbs⁻¹) but had a ‘possibly trivial’ effect with CP (-4.1 ± 13.0 ft·lbs⁻¹). Overall there was an ‘unclear’ treatment effect. IT ‘possibly’ improved peak knee flexion torque with CHO (2.8 ± 6.4 ft·lbs⁻¹) and ‘likely’ improved knee flexion torque with CP (5.6 ± 5.7 ft·lbs⁻¹), resulting in a ‘likely’ benefit of CP vs. CHO.

RVT had an ‘unclear’ effect on knee extension strength with CHO and a ‘likely trivial’ effect with CP, resulting in an ‘unclear’ treatment effect.

RVT had a ‘possibly trivial’ effect on peak knee flexion strength with both CHO and CP, with an ‘unclear’ difference between treatments. Comparing NT to RVT, there was an ‘unclear’ change in knee extension torque with CHO and a ‘possibly trivial’ effect with CP, resulting in an ‘unclear’ difference between treatments.
Comparing NT to RVT, leg flexor torque was ‘likely’ improved with CHO (5.2 ± 8.6 ft·lbs⁻¹) and ‘possibly’ improved with CP (3.8 ± 5.4 ft·lbs⁻¹). However, there was an ‘unclear’ treatment effect.

**Muscle Soreness**

Muscle soreness ratings throughout NT, IT, and RVT in CP and CHO conditions are displayed in Figure 3.3. With CHO, IT ‘likely’ increased ratings of muscle soreness (18.6 ± 28.6 mm), while with CP, IT ‘very likely’ increased rating of muscle soreness (17.9 ± 16.9 mm). Overall, there was an ‘unclear’ treatment effect.

RVT ‘very likely’ reduced muscle soreness with CHO (-21.7 ± 23.0 mm) and ‘almost certainly’ reduced soreness with CP (-21.4 ± 10.8 mm). There was an ‘unclear’ treatment effect.

Comparing RVT to NT, there was an ‘unclear’ change in muscle soreness, along with an ‘unclear’ treatment effect.

**Serum CK and Cortisol**

IT ‘possibly’ reduced CK levels with CHO (-15.6 ± 34.8 U·L⁻¹), but had a ‘likely trivial’ effect with CP (3.9 ± 23.8 U·L⁻¹). Overall, CK ‘likely’ increase from NT to IT with CP compared to CHO.

The effect of RVT on CK was ‘unclear’ with both treatments. Consequently, there was an ‘unclear’ treatment effect.

Compared to NT, RVT had an ‘unclear’ effect on CK with both treatments, thereby leading to an ‘unclear’ treatment effect.
IT had an ‘unclear’ effect on cortisol with CHO (-6.2 ± 37.1 mmol·l⁻¹) but ‘possibly’ reduced cortisol with CP (-12.4 ± 26.1 mmol·l⁻¹). Overall, there was an ‘unclear’ difference between treatments.

Changes in cortisol from IT to RVT and from NT to RVT were ‘unclear’ with CHO and CP, resulting in ‘unclear’ treatment effects.

Whole Muscle Size

Changes in whole muscle size are displayed in Table 3.5. The change in whole muscle size was ‘unclear’ following IT with both CHO and CP, yet CP ‘possibly’ maintained muscle size compared to CHO (CP: 0.05 ± 0.24 cm, CHO: -0.05 ± 0.38). RVT had an ‘unclear’ effect on muscle size with CHO and a ‘possibly trivial’ effect with CP. Therefore, changes in whole muscle size from IT to RVT were ‘possibly’ reduced with CP compared to CHO (CP: 0.06 ± 0.21, CHO: 0.15 ± 0.36).

Comparing RVT to NT, whole muscle size ‘likely’ increased with CHO (0.18 ± .28 cm), with an ‘unclear change’ with CP (0.11 ± .31 cm). Overall, there an ‘unclear’ treatment effect on whole muscle size.

Muscle Fiber Cross Sectional Area

Two subjects were omitted from these analyses. One subject was omitted from analysis on account of impaired tissue samples from biopsies, whereas the other was omitted from all RVT analysis due to an insufficient tissue yield during the biopsy procedure.
IT had an ‘unclear’ impact on MHC I CSA with both treatments, resulting in an ‘unclear’ treatment effect. MHC IIa CSA ‘likely’ increased following IT with CHO (474.97 ± 770.46 um²) whereas there was an ‘unclear’ change in MHC IIa CSA with CP. Therefore, CHO ‘likely’ increased CSA compared to CP.

RVT ‘likely’ decreased MHC I CSA with CHO (-574.64 ± 601.40 um²) but had an ‘unclear’ effect on CSA with CP, resulting in an ‘unclear’ treatment effect. MHC IIa CSA ‘very likely’ decreased with CHO (-853.46 ± 570.62 um²) but ‘likely’ increased (165.05 ± 1822.98 um²) with CP, resulting in a treatment effect that was ‘very likely’.

Compared to NT, MHC I CSA was ‘likely’ smaller after RVT with CHO (432.13 ± 510.02 um²) but ‘unclear’ with CP, resulting in an ‘unclear’ treatment effect. MHC IIa CSA was ‘likely’ smaller after RVT compared to NT with CHO (378.49 ± 751.56 um²) whereas there was ‘unclear’ difference in CSA with CP. There was an ‘unclear’ treatment effect. Muscle fiber cross-sectional areas following NT, IT, and RVT in CP and CHO conditions are displayed in Table 3.5.

**Body Weight**

The impact of IT on body weight was ‘most likely trivial’. However, CP ‘likely’ maintained body weight better than CHO (CP: 0.3 ± 1.1 kg, CHO: -0.6 ± 0.9 kg).

RVT had a ‘most likely trivial’ influence on body weight. Yet, body weight following RVT was ‘likely’ decreased with CP compared to CHO (CP: -0.2 ± 0.5 kg, CHO: 0.5 ± 0.9 kg).
Compared to NT, there was a ‘most likely trivial’ difference in body weight after RVT. There was an ‘unclear’ treatment effect. Raw means ± SD are displayed in Table 3.6.
DISCUSSION

This study was designed to assess the impact of carbohydrate and protein supplementation (CP) on cycling performance and various skeletal muscle parameters following a period of intensified cycle training (IT), compared to carbohydrate alone (CHO). Additionally, we examined the effect of CP supplementation on cycling performance and skeletal muscle adaptations following a period of reduced volume training (RVT). Although CP supplementation did not impact the ‘unclear’ changes in cycling performance that occurred with IT, it positively influenced some aspects of skeletal muscle function and size, compared to CHO alone. Furthermore, CP did not impact the ‘unclear’ effect on cycling performance or any skeletal muscle parameters following RVT. This is the first evidence that during- and post-exercise CP supplementation can better sustain whole muscle function and size during 10 days of IT. However, we did not observe any clear evidence that continued CP supplementation throughout 10 days of RVT enhanced the adaptive response to IT. These findings also provide initial insight into fiber-type specific responses and adaptations to a period of IT followed by a period of RVT.

Surprisingly, cycling performance was not clearly impaired following IT, regardless of treatment- a finding contrary to much of the existing literature (17, 18, 25, 27, 30, 40, 81). IT was immediately followed by RVT, a training strategy reported to facilitate recovery from IT and to possibly enhance performance compared to pre-IT levels (40, 52, 55–57). RVT induced only a ‘possibly trivial’ effect on cycling performance. An explanation for the lack of any performance change with such dramatic adjustments in training is unclear, especially considering that the duration and intensity of
IT were well within the range (6-14 days with a 50-100% increase in weekly training duration) of training overload programs shown to impair performance in prior studies (25, 27, 30, 40, 81). This may be at least partially related to the marked range of racing and training backgrounds, thereby contributing to substantive variability in the response to the different training phases. Although all eight subjects were trained endurance cyclists (VO$_{2\text{max}}$ ≥ 50 ml·kg$^{-1}$·min$^{-1}$, ≥ 7 hr cycling/week), three subjects had minimal competitive racing experience prior the initiation of the study. Additionally, the wide age range (19 – 42 yrs.) and mixed sex (2 female, 6 male) of the cohort presumably contributed to increased variability in TT performances.

Independent of cycling background, it is plausible that the subjects’ high dietary protein intake, (CHO- 1.8 gPRO·kg$^{-1}$BW·day$^{-1}$, CP- 2.6 gPRO·kg$^{-1}$BW·day$^{-1}$, including supplementation from the treatments) helped them tolerate the heavy training. Additionally, this high dietary protein intake may have reduced the physiological stress from IT that is necessary for a super-compensatory response of RVT. Indeed, daily protein intake during both conditions exceed the recommended daily intake for endurance athletes of 1.2 - 1.7 gPRO·kg$^{-1}$BW·day$^{-1}$ (75, 77). In a recent study (81), cyclists better sustained cycling performance after 7 days of IT with 3.0 gPRO·kg$^{-1}$BW·day$^{-1}$ compared to a control diet of 1.5 gPRO·kg$^{-1}$BW·day$^{-1}$. These findings in combination with the current study suggest that dietary protein levels near/above the upper limit of the current recommendations are appropriate for endurance athletes during heavy training. In addition to high dietary protein intake, subjects’ daily CHO intake may have contributed to the unclear effect of CP supplementation. Average CHO intake during both treatments (CHO - 7.7 g·kg$^{-1}$BW·day$^{-1}$, CP - 7.0 g·kg$^{-1}$BW·day$^{-1}$) failed to meet current
recommendations for heavy endurance training (< 8-10 g·kg⁻¹·BW·day⁻¹) (15, 16, 59, 69). As discussed above, Witard et al. (81) reported that high levels of dietary protein intake attenuates the detrimental effects of IT on cycling performance; there subjects consumed 6.0 gCHO·kg⁻¹·BW·day⁻¹, an average intake nearly 1.5 gCHO·kg⁻¹·BW·day⁻¹ less than the current study. Therefore it is possible that the positive effect of protein observed by Witard (81) were due to the comparatively low CHO availability of their subjects. Also worth mentioning, is the caloric difference during IT between the CHO and CP conditions (CHO: 3255 ± 611 kcal; CP: 2609 ± 1443 kcal). Although statistically similar, the comparatively reduced caloric intake during IT with CP (which was almost exclusively due to lower carbohydrate intake) may have blunted any difference in treatment.

Though cycling performance was not influenced by training or treatment, there was evidence of changes in skeletal muscle function. At 120 deg·sec⁻¹, CP ‘likely’ enhanced whole muscle function of both the knee flexors and extensors following IT, compared to CHO. CP also ‘likely’ amplified the increase in function of the knee flexors at 240 deg·sec⁻¹, while knee extensor strength decreased regardless of treatment. The more apparent benefit of CP on whole muscle function is shown at the slower contractile velocity. The slower rotational velocity elicits comparatively higher torque values than the faster assessment speed, and therefore could be a more sensitive measure of peak muscle function. This notion is supported by the findings of Coutts et al. (17) who observed clear effects of IT on peak isokinetic torque at 1.05 rad·sec⁻¹, while no clear effect existed at 5.25 rad·sec⁻¹. Only one other study (71) has assessed the influence of CP on whole muscle function following IT, reporting a benefit of CP compared to CHO,
similar to the current findings. To our knowledge, no other study has observed the changes in whole muscle function throughout a period of IT, followed by a period of RVT. Although the influence of training on these assessments of whole muscle function was mixed, CP seemed to preserve several indices of muscle function throughout IT. However, the practical applications of these changes in muscle function are not apparent, as they did not translate to improved cycling performance. This observed disconnect from cycling performance is somewhat explained by the variable changes in muscle function at 240 deg·sec$^{-1}$ throughout training, as this angular velocity has been shown to closely relate to cycling specific velocities (62). Further research is needed to delineate how changes in peak isokinetic torque translate to cycling specific performance.

Creatine kinase (CK) levels and perceived muscle soreness, together offer valuable insight into possible mechanisms involved in changes in muscle function with training and nutrition, as they are generally indicative of muscle damage. Although ratings of muscle soreness increased as a result of IT, as expected (1, 50, 58, 72), the unclear effect of CP contradicts previous literature (21, 24, 48, 71). Interestingly, changes in CK levels following IT did not mirror those of perceived muscle soreness. CHO ‘likely’ reduced CK levels during IT compared to CP, although the magnitude of change in CK was functionally negligible. Accordingly, RVT resulted in no clear effect on the negligible changes in CK levels with either treatment. The ‘unclear’ influence of CP on CK response opposes earlier findings of CP attenuating CK levels after heavy training (48, 63, 71). Given that IT induced no clear effect on cycling performance, and mixed results with whole muscle function, it is possible that the increased muscle soreness following IT was a function of psychological stress, rather than cellular
damage/inflammation. This notion is supported by the lack of clear increases in CK levels following IT and recent evidence displaying a worsened psychological state following IT in a similar training model (81).

To further explain the influence of training and nutrition on skeletal muscle function we examined changes in whole muscle size. To our knowledge, we are the first to examine whole muscle size and fiber size throughout a block of IT followed by RVT. Likewise, the influence of CP supplementation on this response is also original. Whole muscle size was ‘possibly’ better maintained with CP throughout IT, while no clear treatment effect persisted through RVT. Changes in MHC I CSA were unclear throughout IT and RVT, agreeing with negligible changes observed in previous studies (20, 43, 78). Contrary to existing literature demonstrating MHC IIa vulnerability to heavy training (20, 28, 43), we observed a ‘likely’ increase in CSA among MHC IIa fibers with CHO, while CP had no clear influence. Following RVT, CHO had the opposite effect, reducing MHC IIa CSA while CP ‘likely’ increased MHC IIa fiber size. However, MHC IIa fiber size was no different from NT under either treatment. Given the physiological variables measured in the current study, it is difficult to expose the mechanisms behind the independent effects of CP on whole muscle size, together with the influence of CHO on fiber size. The overall change in whole muscle size observed with CP may be due to changes other than fiber size, including systemic inflammation and fluid dynamics. The unclear influence of CP on muscle fiber size throughout IT and RVT is novel, and mirrors the responses in cycling performance. Changes in muscle function and thus cycling performance are dependent not only on structural changes, but metabolic and neurological changes as well.
The current findings provide preliminary evidence for the potential of during- and post-exercise CP supplementation throughout IT followed by RVT. During a period of IT, CP may better sustain skeletal muscle function and whole muscle size compared to CHO. However, it is important to emphasize that endurance performance, markers of muscle damage, and muscle fiber size were all minimally effected by CP supplementation, perhaps as a result of the relatively high dietary protein intake under both treatment conditions (CHO- 1.8 gPRO·kg⁻¹BW·day⁻¹, CP- 2.6 gPRO·kg⁻¹BW·day⁻¹). Future research should be conducted to determine if the timing of CP supplementation is of greater importance for athletes consuming lower quantities of dietary carbohydrate and protein.

Our inability to delineate any effect of training or CP within the current experimental model may have arisen from the inclusion both male and female cyclists with a wide range of competitive cycling experience, thus leading to further variance in any performance outcomes. Across all subjects, dietary protein intake was relatively high, which possibly attenuated any measureable difference between treatments. Overall, it is not clear if CP is effective in improving tolerance to intensified training. Nor is it clear that CP supplementation can augment the adaptive response to IT, following a period of reduced training. A moderate-CHO, high-PRO diet appears sufficient to tolerate a block of intense endurance training, in comparison to when supplemental protein is provided. Future research is needed to elucidate the underlying mechanisms behind the acute benefit of CP and whether this is an effective strategy to promote adaptations to longer durations of heavy training.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>PAS with IF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fixation</td>
<td>3.7% Formaldehyde in PBS for 60min @ 4°C</td>
</tr>
<tr>
<td>Rinsing</td>
<td>PBS for 10min (2 x 5min)</td>
</tr>
<tr>
<td>Permeabilisation</td>
<td>0.1% Triton X-100 in PBS for 5min</td>
</tr>
<tr>
<td>Rinsing</td>
<td>PBS for 15min (3 x 5min)</td>
</tr>
<tr>
<td>Rinsing</td>
<td>Deionized (D.I.) H₂O for 30sec</td>
</tr>
<tr>
<td>Pretreatment</td>
<td>1% Periodic acid in D.I. H₂O for 5min</td>
</tr>
<tr>
<td>Rinsing</td>
<td>D.I. H₂O for 1 min</td>
</tr>
<tr>
<td>Staining</td>
<td>Schiff’s Reagent for 15min @ room temp.</td>
</tr>
<tr>
<td>Rinsing</td>
<td>D.I. H₂O for 5sec and tap water for 10min</td>
</tr>
<tr>
<td>Rinsing</td>
<td>PBS for 15min (3 x 5min)</td>
</tr>
<tr>
<td>Incubation</td>
<td>10% NGS in 0.05% Triton X-100 for 30min @ room temp.</td>
</tr>
<tr>
<td>Incubation</td>
<td>Primary antibodies in 0.05% Triton X-100 for 2hr @ room temp.</td>
</tr>
<tr>
<td>Rinsing</td>
<td>0.05% Triton X-100 in PBS for 30min (5 x 6min)</td>
</tr>
<tr>
<td>Incubation</td>
<td>Secondary antibodies in 0.05% Triton X-100 for 2hr @ room temp. (in dark)</td>
</tr>
<tr>
<td>Rinsing</td>
<td>0.05% Triton X-100 in PBS for 30min (5 x 6min) (in dark)</td>
</tr>
<tr>
<td>Rinsing</td>
<td>PBS for 15min (3 x 5min) (in dark)</td>
</tr>
<tr>
<td>Mounting</td>
<td>Fluormount G (in dark)</td>
</tr>
<tr>
<td>Subjects, n</td>
<td>Age, yr</td>
</tr>
<tr>
<td>------------</td>
<td>-----------</td>
</tr>
<tr>
<td>8</td>
<td>24.9 ± 7.3</td>
</tr>
</tbody>
</table>

**Table 3.1:** Subject Characteristics
Table 3.2: Macronutrient Content of Nutritional Treatments

<table>
<thead>
<tr>
<th></th>
<th>CHO</th>
<th>PRO</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>During Exercise</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHO</td>
<td>45 g·hr⁻¹</td>
<td>0 g·hr⁻¹</td>
</tr>
<tr>
<td>CP</td>
<td>45 g·hr⁻¹</td>
<td>15 g·hr⁻¹</td>
</tr>
<tr>
<td><strong>Post Exercise</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHO</td>
<td>1.2 g·kg BW⁻¹</td>
<td>0 g·kg BW⁻¹</td>
</tr>
<tr>
<td>CP</td>
<td>1.2 g·kg BW⁻¹</td>
<td>0.4 g·kg BW⁻¹</td>
</tr>
</tbody>
</table>

CHO = carbohydrate; CP = carbohydrate-protein.
Table 3.3: Average Daily Dietary Intake

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>NT</th>
<th>CHO IT</th>
<th>CHO RVT</th>
<th>CP IT</th>
<th>CP RVT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (kcal)</td>
<td>3218 ± 735</td>
<td>3255 ± 611</td>
<td>2793 ± 459</td>
<td>2609 ± 1443</td>
<td>2584 ± 497</td>
</tr>
<tr>
<td>Carbohydrate (g)</td>
<td>454 ± 150</td>
<td>471 ± 293</td>
<td>335 ± 74</td>
<td>360 ± 106</td>
<td>344 ± 142</td>
</tr>
<tr>
<td>Pro (g)</td>
<td>123 ± 23</td>
<td>136 ± 27</td>
<td>117 ± 16</td>
<td>157 ± 70</td>
<td>116 ± 16</td>
</tr>
<tr>
<td>Fat (g)</td>
<td>98 ± 23</td>
<td>127 ± 23</td>
<td>104 ± 21</td>
<td>123 ± 106</td>
<td>96 ± 14</td>
</tr>
</tbody>
</table>

CHO = carbohydrate; CP = carbohydrate-protein. Values are expressed as mean ± SD, not including nutrients from treatment beverages.
<table>
<thead>
<tr>
<th></th>
<th>CHO</th>
<th></th>
<th></th>
<th>CP</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NT</td>
<td>IT</td>
<td>RVT</td>
<td>NT</td>
<td>IT</td>
<td>RVT</td>
</tr>
<tr>
<td><strong>120 deg·sec⁻¹</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Knee Extension</td>
<td>112.3 ± 28.1</td>
<td>100.9 ± 21.5</td>
<td>100.2 ± 30.0</td>
<td>98.6 ± 25.1</td>
<td>100.8 ± 21.8</td>
<td>98.4 ± 20.0</td>
</tr>
<tr>
<td>Knee Flexion</td>
<td>72.7 ± 16.0</td>
<td>71.8 ± 15.8</td>
<td>70.6 ± 16.6</td>
<td>71.2 ± 14.4</td>
<td>74.0 ± 15.2</td>
<td>75.8 ± 15.5</td>
</tr>
<tr>
<td><strong>240 deg·sec⁻¹</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Knee Extension</td>
<td>80.1 ± 24.0</td>
<td>70.6 ± 18.5</td>
<td>76.5 ± 27.5</td>
<td>75.1 ± 21.6</td>
<td>71.0 ± 11.3</td>
<td>71.6 ± 16.7</td>
</tr>
<tr>
<td>Knee Flexion</td>
<td>54.6 ± 12.9</td>
<td>57.3 ± 12.8</td>
<td>59.8 ± 15.9</td>
<td>55.8 ± 14.0</td>
<td>61.5 ± 14.4</td>
<td>59.7 ± 13.7</td>
</tr>
</tbody>
</table>

CHO = carbohydrate; CP = carbohydrate-protein. Values are expressed as mean ± SD.
**Table 3.5: Whole Muscle Size and Muscle Fiber Size**

<table>
<thead>
<tr>
<th></th>
<th>CHO</th>
<th></th>
<th></th>
<th>CP</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NT</td>
<td>IT</td>
<td>RVT</td>
<td>NT</td>
<td>IT</td>
<td>RVT</td>
</tr>
<tr>
<td>VL Thickness (cm)</td>
<td>2.79 ± 0.45</td>
<td>2.74 ± 0.34</td>
<td>2.93 ± 0.53</td>
<td>2.82 ± 0.51</td>
<td>2.89 ± 0.53</td>
<td>2.98 ± 0.63</td>
</tr>
<tr>
<td>MHC I (um²)</td>
<td>4394 ± 1174</td>
<td>4536 ± 1172</td>
<td>3961 ± 997</td>
<td>4065 ± 1617</td>
<td>3965 ± 1319</td>
<td>4348 ± 1319</td>
</tr>
<tr>
<td>MHC IIa (um²)</td>
<td>4250 ± 851</td>
<td>4725 ± 990</td>
<td>3871 ± 766</td>
<td>4504 ± 1925</td>
<td>4070 ± 1508</td>
<td>4940 ± 1851</td>
</tr>
<tr>
<td>Δ VL Thickness (%)</td>
<td>-1.8</td>
<td>5.0</td>
<td></td>
<td>2.5</td>
<td>5.7</td>
<td></td>
</tr>
<tr>
<td>Δ MHC I (%)</td>
<td>3.2</td>
<td>-9.8</td>
<td></td>
<td>-2.5</td>
<td>7.0</td>
<td></td>
</tr>
<tr>
<td>Δ MHC IIa (%)</td>
<td>11.2</td>
<td>-8.9</td>
<td></td>
<td>-9.7</td>
<td>9.7</td>
<td></td>
</tr>
</tbody>
</table>

CHO = carbohydrate; CP = carbohydrate-protein. Values are expressed as mean ± SD, where *italics* are a percentage change from NT.
### Table 3.6: Body Weight

<table>
<thead>
<tr>
<th></th>
<th>CHO</th>
<th></th>
<th>CP</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NT</td>
<td>IT</td>
<td>RVT</td>
<td>NT</td>
</tr>
<tr>
<td>Body Weight (kg)</td>
<td>72.1 ± 11.5</td>
<td>71.5 ± 11.1</td>
<td>71.9 ± 11.1</td>
<td>72.1 ± 11.3</td>
</tr>
</tbody>
</table>

CHO = carbohydrate; CP = carbohydrate-protein. Values are expressed as mean ± SD.
Figure 2.1: Experimental Design
**Figure 2.2: Study Schematic with Corresponding Data Collection**

<table>
<thead>
<tr>
<th>Days</th>
<th>-3</th>
<th>-2</th>
<th>-1</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>+1</th>
</tr>
</thead>
<tbody>
<tr>
<td>VO₂max</td>
<td>X</td>
<td></td>
<td></td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td></td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Bx</td>
<td></td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>

VO₂max = VO₂max, TT = preloaded time trial, Bx = skeletal muscle biopsy

Note: This design will be repeated twice (Figure 1). Therefore, days -1, -2, and -3 correspond to the final 3 days of normal training, whereas day +1 corresponds to day 1 of washout or study completion.
Figure 3.1: A, Average daily training duration during normal (NT), intensified (IT), and reduced volume (RVT) training in CHO and CP conditions. B, Average daily training power during NT, IT, and RVT in CHO and CP conditions. C, Average daily training heart rate during NT, IT, and RVT in CHO and CP conditions. CHO = carbohydrate; CP = carbohydrate-protein. Data are displayed as means ± SD.
Figure 3.1: A, Average daily training duration during normal (NT), intensified (IT), and reduced volume (RVT) training in CHO and CP conditions. B, Average daily training power during NT, IT, and RVT in CHO and CP conditions. C, Average daily training heart rate during NT, IT, and RVT in CHO and CP conditions. CHO = carbohydrate; CP = carbohydrate-protein. Data are displayed as means ± SD.
**Figure 3.1:** A, Average daily training duration during normal (NT), intensified (IT), and reduced volume (RVT) training in CHO and CP conditions. B, Average daily training power during NT, IT, and RVT in CHO and CP conditions. C, Average daily training heart rate during NT, IT, and RVT in CHO and CP conditions. CHO = carbohydrate; CP = carbohydrate-protein. Data are displayed as means ± SD.
Figure 3.2: A, 30-km time trial finishing time after normal (NT), intensified (IT), and reduced volume (RVT) training in carbohydrate (CHO) and carbohydrate+protein (CP) conditions. B, 30-km time trial power after NT, IT, and RVT in CHO and CP conditions. Data are displayed as means ± SD.
Figure 3.3: Rating of perceived muscle soreness following normal (NT), intensified (IT), and reduced volume (RVT) training. CHO = carbohydrate; CP = carbohydrate-protein. Data are displayed as means ± SD.
References


