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Kasper Degn Gejl[1], Line Thams[1], Mette Hansen[2], Torben Rokkedal-Lausch[3],
Peter Plomgaard[4,5], Lars Nybo[6], Filip J Larsen[7,8], Daniele A Cardinale[8,9], Kurt Jensen[1],
Hans-Christer Holmberg[10,11], Kristian Vissing[2], Niels Ørtenblad[1,10]

[1]Department of Sports Science and Clinical Biomechanics, University of Southern Denmark,
Odense, Denmark; [2]Section for Sport Science, Department of Public Health, Aarhus
University, Aarhus, Denmark; [3]SMI, Department of Health Science and Technology, Faculty
of Medicine, Aalborg University, Aalborg, Denmark; [4]Department of Clinical Biochemistry,
Rigshospitalet, Copenhagen, Denmark; [5]Department of Infectious Diseases, Center for
Physical Activity Research, Rigshospitalet, University of Copenhagen, Copenhagen,
Denmark; [6]Department of Nutrition, Exercise and Sports, University of Copenhagen,
Copenhagen Denmark; [7]Department of Physiology and Pharmacology, Karolinska Institute,
Stockholm, Sweden; [8]Swedish School of Sport and Health Sciences, Stockholm, Sweden;
[9]Elite Performance Centre, Swedish Sports Confederation, Stockholm, Sweden; [10]Swedish
Winter Sports Research Centre, Department of Health Sciences, Mid Sweden University,
Östersund, Sweden; [11]Swedish Olympic Committee, Stockholm, Sweden

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[1]Department of Sports Science and Clinical Biomechanics, University of Southern Denmark, Odense, Denmark; [2]Section for Sport Science, Department of Public Health, Aarhus University, Aarhus, Denmark; [3]SMI, Department of Health Science and Technology, Faculty of Medicine, Aalborg University, Aalborg, Denmark; [4]Department of Clinical Biochemistry, Rigshospitalet, Copenhagen, Denmark; [5]Department of Infectious Diseases, Center for Physical Activity Research, Rigshospitalet, University of Copenhagen, Copenhagen, Denmark; [6]Department of Nutrition, Exercise and Sports, University of Copenhagen, Copenhagen Denmark; [7]Department of Physiology and Pharmacology, Karolinska Institute, Stockholm, Sweden; [8]Swedish School of Sport and Health Sciences, Stockholm, Sweden; [9]Elite Performance Centre, Swedish Sports Confederation, Stockholm, Sweden; [10]Swedish Winter Sports Research Centre, Department of Health Sciences, Mid Sweden University, Östersund, Sweden; [11]Swedish Olympic Committee, Stockholm, Sweden

Address for correspondence

Kasper Gejl

Department of Sports Science and Clinical Biomechanics

University of Southern Denmark

Campusvej 55

5230 Odense M

Denmark

kgejl@health.sdu.dk

+45 29284155

Abstract

Purpose: The present study investigated the effects of periodic CHO restriction on endurance performance and metabolic markers in elite endurance athletes. **Methods:** Twenty-six male elite endurance athletes (VO_{2max}: 65.0 ml $O_2 \cdot kg^{-1} \cdot min^{-1}$) completed 4 weeks of regular endurance training, while matched and randomized into two groups training with (Low) or without (High) carbohydrate (CHO) manipulation three days a week. The CHO manipulation days consisted of a 1-hr high intensity bike session in the morning, recovery for 7 hrs while consuming isocaloric diets containing either high CHO (414±2.4 g) or low CHO (79.5±1.0 g), and a 2-hr moderate bike session in the afternoon with or without CHO. VO_{2max}, maximal fat oxidation and power output during a 30-min time trial (TT) were determined before and after the training period. The TT was undertaken after 90 mins of intermittent exercise with CHO provision before the training period and both CHO and placebo after the training period. Muscle biopsies were analyzed for glycogen, citrate synthase (CS) and β-hydroxyacyl-coenzyme A dehydrogenase (HAD) activity, carnitine palmitoyltransferase (CPT1b) and phosphorylated acetyl-CoA carboxylase (pACC). **Results:** The training effects were similar in both groups for all parameters. On average, VO_{2max} and power output during the 30-min TT increased by $5 \pm 1\%$ ($P<0.05$) and TT performance was similar after CHO and placebo during the preload phase. Training promoted overall increases in glycogen content ($18 \pm 5\%$), CS activity ($11 \pm 5\%$) and pACC ($38 \pm 19\%$) ($P<0.05$) with no differences between groups. HAD activity and CPT1b protein content remained unchanged. **Conclusion:** Superimposing periodic CHO restriction to 4 weeks of regular endurance training had no superior effects on performance and muscle adaptations in elite endurance athletes.

Keywords: diet manipulation**,** glycogen, enzyme activity, triathletes, endurance performance

Introduction

The availability of muscle glycogen and other forms of carbohydrate (CHO) exerts an impact on performance during prolonged submaximal or high-intensity intermittent exercise (19,27). Accordingly, nutritionists have focused on various dietary manipulation strategies designed to maximize pre-exercise carbohydrate availability and post-exercise glycogen resynthesis. At the same time, the availability of CHO and glycogen may be involved in the regulation of muscle cell metabolism, including central cell signaling pathways (29). Thus, research developments indicate that periodic restriction of CHO availability during or following exercise ("train-low") by endurance athletes may result in acute improvements of parameters associated with muscle oxidative capacity, mitochondrial biogenesis and lipid oxidation (9,23,30,37).

The translation of these "train-low" adaptations into persistent alterations in performance have been investigated by different strategies, involving training in a fasting state (8.11,36), training twice a day (10,18,22,25,38) and high intensity training in the evening followed by overnight fasting and training in the morning ("sleep-low") (8,24). Training studies lasting three weeks or more have reported superior effects of "train-low" on muscle cell adaptations related to mitochondrial function and lipid oxidation (4,18,22,25,36,38). However, in endurance trained individuals, the translation of cellular adaptations into performance enhancement is crucial and unfortunately, performance improvements have generally been lacking or have been marginal, despite the promising findings on the muscle cell level (8,22,38). Yet, a recent 3-week study by Marquet and colleagues (24) in moderately trained triathletes did reveal superior effects on endurance performance by using the "sleep-low" strategy. Nevertheless, it has to be clarified whether these positive results are related to low CHO availability or the combination of low energy and low CHO availability. By using a variety of "train-low" strategies involving both

CHO and overall energy restriction in elite race walkers, Burke and colleagues showed that performance was enhanced by 5.3%, and to the same extent as a high CHO diet (8), thus indicating that persistent effects of the "train-low" are dependent on the training status. Although elite athletes anecdotally use "train-low" strategies, little is known about the real effects of this training strategy (33). Only a small number of training studies have evaluated "train-low" strategies in highly trained endurance athletes (8, 22) and to construct future recommendations there is a need for further research of both regulatory events and performance effects induced by periodic CHO restriction.

Since the superior acute regulatory effects of CHO restriction are particularly important to endurance performance, one explanation for the lack of persistent performance benefits after using "train-low" strategies in highly trained athletes may be the use of relatively short performance test protocols (≤ 1 hr) (8,10,18,25,36). Only three "train-low" training studies have evaluated endurance performance by tests lasting more than 75 mins (22,24,38). However, these studies either involved moderately trained individuals (24), group differences in training intensity induced by different glycogen levels (38), or have determined endurance performance in a fasting state (22). Also, unintentional losses of body mass and deviations from customary training routines have been some of the consequences in previous "train-low" studies (8,24,38). Conversely, the long-term effects of periodic "train-low" on endurance performance are not well described in elite endurance athletes under conditions with optimal nutrition and applicable training volumes, durations and intensities. In order to determine potential performance-enhancing effects of CHO restriction in elite athletes, it should logically be investigated in a matched and controlled design that properly reflects customary training routines and circumvent detrimental effects of glycogen depletion on training quality. Thus, a practical approach,

combining the best of high-intensity exercise and CHO restriction, would be to commence the high intensity training sessions with ample glycogen available, but then with periodic CHO restriction during some of the less intense training sessions.

Elite athletes anecdotally use "train-low" strategies although only few studies have investigated the effect of "train-low"-strategies under "real-life" conditions and despite a lack of scientific evidence of performance benefits in elite endurance athletes. Accordingly, in order to help formulate evidence-based guidelines for highly trained endurance athletes, the purpose of the present study was to determine the effects of 4 weeks of routine endurance training, supplemented with periodic CHO restriction on performance, oxidative capacity and metabolism of elite endurance athletes. In order to understand the role of carbohydrate availability *per se*, the athletes consumed isocaloric meals at the same time of the experimental training days. We hypothesized that periodic CHO restriction would improve oxidative capacity and endurance performance to a greater extent than ingestion of a high CHO diet in endurance athletes performing identical training interventions.

Methods

Subjects, matching and ethical approval

Twenty-six highly trained male triathletes (n = 22) and road cyclists (n = 4) were enrolled in the study (Table 1). The subjects trained at least 10 hours per week, demonstrated a VO_{2max} greater than 60 ml $\cdot$ kg^{-1} $\cdot$ min^{-1} and had at least 2 years of experience in their discipline. Among the triathletes, eight were current members of the Danish National Team competing at international Olympic and sprint distances (Olympic Games, World Triathlon Series, World Cups and

Continental Cups), 10 participated in national elite competitions (Olympic, ½ ironman and ironman distances), while the remaining four competed at a lower level (½ ironman and ironman distances). All four cyclists had A-licenses and competed at the national elite level.

The participants were fully informed of any potential risk associated with the experiments before verbal and written consents were obtained. The Ethics Committee of Southern Denmark approved the study protocol (Project-ID S-20150034) and the experiments adhered to the standards of the *Declaration of Helsinki*.

Experimental Overview

In order to obtain equivalent groups for comparison of the effects of periodic CHO restriction, the participants were paired off on the basis of their main sport discipline (road cycling, Olympic-distance triathlon or long-distance triathlon), training history and VO_{2max}. The matched subjects in each pair were required to conduct all training sessions together in an identical manner. One athlete from each of the matched pairs was randomly assigned to perform 4 weeks of endurance training, including a normal carbohydrate-enriched diet (High CHO), while the other followed the same training protocol, but occasionally with an isocaloric low CHO diet three times per week (Low CHO) (Fig. 1). The paired design ensured identical average training volumes and intensities of training during the intervention between the two groups.

During the 4-week intervention period, the subjects accomplished three training blocks per week with carbohydrate manipulation (Monday, Wednesday and Friday) (Fig. 1). Accordingly, 11 carbohydrate manipulation days were distributed across the training period. On these days, each subject completed one hour of high intensity intermittent cycling in the morning, in order to

deplete muscle glycogen (34). Then the athletes recovered for approximately 7 hrs and performed 2 hrs of moderate-intensity cycling thereafter. During the recovery period, the athletes in each pair consumed either a high CHO diet or an isocaloric low CHO diet and during the afternoon session the low CHO group consumed water and the high group a CHO bevrage. On the other days, each athlete trained once to three times daily in accordance with a supervised program and consumed a CHO-enriched diet of their own choice.

Before the 4-week intervention, subjects reported to the laboratory on two separate days to perform submaximal incremental cycling and VO2max tests (test day 1) and an endurance performance test with provision of CHO (test day 2). Following the intervention, the participants underwent similar procedures, as well as the same performance test twice, with provision of CHO or placebo in a randomized order (test days 3 to 5; see further below). Within subjects, all tests were carried out at the same time of the day.

The training intervention

Based on retrospective training diaries and with the advice of exercise researchers and the Danish national triathlon coach, training schedules were designed for each pair of athletes (Fig. 1). Weekly customized training plans were similar within pairs but different between pairs throughout the training period. On average, the matched pairs carried out 16 hrs [12 to 20 hrs] of training per week (triathletes avg. 17 hrs [14 to 20 hrs]; road cyclists avg. 14 hrs [12 to 17 hrs]). The three weekly 2h "train-low" sessions comprised 30-50% of the total training volume. For the purposes of recovery, one (triathletes) or two (cyclists) weekly training days involved a maximum of 45 mins of easy training. The remaining training days included a total of two easy-to-moderate bike sessions in road cyclists (1.5 to 3.5hrs at 65-75 % of HR_{max}) and 2 to 4 easy-to-

moderate swim sessions and 4 to 6 easy-to-moderate run sessions (65-85% HR_{max}) in triathletes (Fig. 1).

This study was conducted in pre-season and the intensity in the mornings of the days involving CHO manipulation was greater than normal for this period, whereas the total volume was maintained and normal.

Training involving CHO manipulation

The training intensity on the three weekly days involving CHO manipulation was based on continuously measured heart rate (Polar Team 2, Polar Electro Oy, Kempele, Finland), thereby comprising a gradual increase in absolute training load during the intervention period. Both morning and afternoon sessions were carried out on personal bikes by use of turbo trainers (Tacx Bushido Smart T2780, Wassenaar, Netherlands).

The morning session consisted of eight 5-min cycling intervals interspersed with 2 mins active recovery. The first six intervals were conducted with a target intensity of 85% HR_{max}, while the final two 5-min blocks consisted of five 15s maximal sprints to recruit type II fibers, separated by 45s of easy spinning. This is a modified version of the 8 x 5-min protocol employed by Stepto et al., which was shown to reduce muscle glycogen content by 50% in well-trained athletes (34). The afternoon session entailed 2 hrs of moderate cycling exercise with a target intensity of 65% of HR_{max}. with CHO intake in the high group only (see below).

Power output during the morning and afternoon training sessions on days involving CHO manipulation was estimated on the basis of the W-HR relationship derived from the submaximal tests performed before and after the training intervention.

Dietary recording and meals in relation to tests

Both prior to and during the second week of the intervention period, all of the participants recorded individual food and fluid intake for 4 days, for subsequent analysis of caloric and macronutrient content (Madlog, Kolding, Denmark). A person with extensive food log experience informed the participants about how to perform the dietary recording. Of particular interest in this context was the detection of athletes with a daily CHO intake lower than recommended prior to the intervention. The mid-intervention dietary recording included two days with CHO manipulation and two days without CHO manipulation, and was used to evaluate the compliance with the dietary recommendations and maintenance of a mixed CHO-enriched diet on days with routine training.

Both energy intake and dietary composition was identical between groups before the intervention. The average total energy intake was 44 ± 2 kcal· kg bm^{-1}·day^{-1} in the Low CHO group (50% derived from CHO; 5.4 g·kg bm^{-1}·day^{-1}), while the High CHO group consumed 43 ± 3 kcal·kg bm^{-1}·day^{-1} (54% derived from CHO; 5.7 g·kg bm^{-1}·day^{-1}). Based on the pre-intervention dietary log, a few were asked to increase energy intake if total energy intake or CHO intake was less than recommended (1.8 PAL·(0.0669·bw + 2.28) MJ·day^{-1} or 5 g CHO·kg bm^{-1}·day^{-1}) (7,20).

On training days without CHO manipulation, athletes were asked to maintain their usual diet and to consume CHO enriched meals in the morning, for lunch and for dinner. To ensure a matched timing of meal consumption, all subjects received a time schedule informing about the consumption of each meal on all training days and the research group was in almost daily contact with the participants to make sure that they complied with the diet plan. Macronutrient intake on

training days without CHO manipulation was investigated from the mid-intervention diet log and identical between groups for both energy intake and macronutrient composition. Energy intake was 45 ± 3 kcal·kg bm^{-1} · day^{-1} (CHO: 5.9 ± 0.5 g· kg bm^{-1}·day^{-1}; Protein: 1.9 ± 0.1 g·kg bm^{-1}·day^{-1}; Fat: 1.4 ± 0.1 g·kg bm^{-1}·day^{-1}) in the Low group and 48 ± 2 kcal·kg bm^{-1}·day^{-1} in the High group (CHO: 6.3 ± 0.4 g·kg bm^{-1}·day^{-1}; Protein: 1.9 ± 0.1 g·kg bm^{-1}·day^{-1}; Fat: 1.7 ± 0.1 g·kg bm^{-1}·day^{-1}). Thus, 53% of the energy was derived from CHO in both groups on days without CHO manipulation.

To ensure dietary conformity, all subjects received food and beverages during the last 24 hrs prior to all tests. The day before, they received 5 g CHO·kg bm^{-1}·day^{-1} with a total energy intake of 34.8 kcal·kg bm^{-1}·day^{-1} (56% CHO, 16% protein and 29% fat). To reflect competition conditions and maximize performance, each subject consumed small and identical body mass corrected meals on test days (5.7 kcal·kg bm^{-1}, 65% CHO, 14% protein and 21% fat), containing 17.3 ± 0.2 g protein, 74.2 ± 0.8 g CHO and 11.5 ± 0.1 g fat every second hour from 7 AM until 60 mins prior to the initiation of the test. Thus, subjects consumed 2 to 4 small meals before tests.

Diet manipulation

On the days involving CHO manipulation, each subject received a time schedule and meals and drinks taking body mass, allergies and dislikes into consideration. Each subject consumed a small CHO-enriched breakfast, similar to the meal on test days (see above), 60 mins before the high intensity morning sessions (5.7 kcal·kg bm^{-1}). During the subsequent 7 hrs of recovery, the High and Low CHO groups were provided with isocaloric diets (2217-2495 kcal) containing 6g and 1g CHO·kg bm^{-1}, corresponding to 414 ± 2.4 g and 79.5 ± 1.0 g CHO, respectively (13%

CHO, 17% protein and 70% fat vs. 72% CHO, 16% protein and 12% fat, respectively). Protein intake was similar in both groups during the 7 hrs of recovery to prevent bias from this route for gluconeogenesis and to avoid manipulation with three different macronutrients. During the subsequent 2 hrs of moderate training, the Low CHO group consumed only water ad libitum, while the High CHO group consumed a beverage containing 1g CHO·kg bm^{-1}·h^{-1}. To match overall energy balance between groups, the diet was based on body mass.

VO$_2$ measurements

The participants refrained from exercise during the 36 hrs preceding all tests. On the first and last day of the study (test day 1 and 5, Fig. 1), body composition was determined by dual-energy x-ray absorptiometry (DEXA) (Lunar Prodigy Advance, GE Healthcare, Little Chalfont, United Kingdom) and a submaximal ramp test and a maximal cycling test were performed. Cycle tests were performed on an electronically braked ergometer (Schoberer Rad Messtechnik (SRM), 117 GmbH Julich, Germany), calibrated before each test.

The submaximal ramp test, undertaken following 10 mins of easy warm-up (100-150 W), involved 4-min intervals at an initial workload of 135 W. The workload was elevated by 35 W every fourth minute until the RER value remained above 1.00 for one minute. A maximal test was conducted 15 mins after termination of the submaximal test, with the initial 2-min workload corresponding to the workload during the penultimate step of the submaximal test and thereafter increased 25 W every minute until exhaustion.

VO$_2$ and VCO$_2$ were estimated using an Oxygraf CPET OEM system for AMIS 2001 (Innovision, Glamsbjerg, Denmark) with sampling every 10 sec. Prior to each test, the gas

analyzer was calibrated with two standard mixtures of gases containing 21 and 15% of O_2 and 0 and 5% of CO_2. Ventilation sensors were calibrated manually with a 3L syringe. The VO_2 and RER values of each workload were defined as the mean VO_2 and RER during the last two minutes of that workload. The highest mean 30-sec value for VO_2 during the maximal test was defined as VO_{2max}. During the final 30 secs of each workload, capillary blood was drawn from a fingertip for subsequent determination of lactate.

Fat oxidation rate

The fat oxidation rates at submaximal power outputs (170, 205 and 240W) were calculated using the stoichiometric equation developed by Frayn: fat oxidation = (1.67 x VO_2) – (1.67 x VCO_2), assuming that urinary nitrogen excretion was negligible (14). Moreover, the association between the relative utilization of VO_{2max} during the graded test and fat oxidation rate was determined by polynomial curve fitting. The relative utilizations of VO_2 were divided into 7.5% intervals from 30% to 75% of VO_{2max}. Maximal fat oxidation was calculated as the apex of the best-fit polynomial curves.

Endurance performance test

Endurance performance was determined three days after the first VO_2 measurements, as well as six and three days before the final measurements of these same parameters (Fig. 1). These tests were conducted on the same ergometer as described above (SRM) and consisted of a 30-min maximal TT performed after 90 mins of intermittent exercise. During this initial intermittent exercise, the workload alternated continuously between 4 mins 30 secs at 45% of pre W_{max} (145-209W) and 30 secs at 90% of this same pre W_{max} (290-413W). For direct comparison of

endurance performance pre to post, the workload during the 90-min pre-load phase was identical during the pre and post tests. The athletes were used to employ power meters and heart rate monitors and well aware of their bike capabilities. For the purpose of familiarization and to optimize the pacing strategy, the subjects conducted a 20-km outdoor TT during the last two weeks prior to the study using a heart rate monitor. During the test itself, subjects monitored time as well as instantaneous heart rate and power output on a screen throughout the test and they were instructed to attain the highest possible mean power output. During the initial 90 mins of intermittent exercise before the TT at baseline (Pre) and before the TT in one of the last two endurance performance tests (Post CHO) the athletes ingested $0.7g\ CHO \cdot kg^{-1} \cdot h^{-1}$. In contrast, the athletes consumed a placebo beverage (i.e. artificially sweetened beverage) during the initial 90 min of intermittent exercise before the other TT following the intervention (Post placebo). The order of the two post-intervention endurance performance tests was randomized and double-blinded. VO_2, power output and heart rate were measured continuously during the 30-min TT. Endurance performance is presented as both absolute (W) and relative power output (W·kg body mass^{-1}). Because of an injury, one subjects missed the endurance performance test with placebo after the training period.

Muscle biopsies

Three muscle biopsies (i.e. Pre, acute after the 7th carbohydrate manipulation day, and Post, Fig. 1) were obtained randomly from *m. quadriceps femoris* of the right and left thighs using 5 mm Bergström needles as previously described (6, 15). Pre and Post biopsies were obtained two hours before the VO_2 measurements and 1 hour after a standard meal (see above). The acute biopsy was obtained 1 hr after the moderate 2 hr training session in a subgroup of the athletes (*n*

= 17; Low n = 9, High n = 8). The procedure for extraction of muscle tissue was identical at all time points. Muscle tissue was placed on filter paper upon an ice-cooled Petri dish, blotted and dissected free from fat and connective tissue. Two parts of the biopsy were immediately frozen in liquid nitrogen and stored at -80ºC for subsequent analysis of enzyme activity and glycogen content, as well as immunoblotting. Another part was manually homogenized with a potter-elvehjem glass-glass homogenizer (Kontes Glass Industry, Vineland, NJ, USA) for determination of MHC distribution. Parts of the biopsies and also blood samples were stored for later analysis in companion studies.

Muscle Glycogen and enzyme activity

Muscle glycogen content was determined spectrophotometrically (Beckman DU 650) as previously described (15). Freeze-dried muscle tissue (1.5mg) was boiled in 0.5ml 1M HCL for 150 mins before it was quickly cooled, whirl-mixed and centrifuged at 3500g for 10 mins at 4ºC, before analysed for glucose content. Muscle glycogen content is expressed as mmol·kg dw^{-1}.

Enzyme activities were measured at 30°C in freeze-dried muscle dissected to be free from non-muscle constituents (28). Citrate synthase (CS) activity was determined by the addition of oxaloacetate to a buffer solution containing muscle homogenate, DTNB buffer, acetyl-CoA and H_2O. β-Hydroxyacyl-coenzyme A Dehydrogenase (HAD) activity was measured after the addition of acetoacetyl CoA to a buffer solution containing Imidazol, NADH and EDTA. Absorbance of CS and HAD was recorded for 600 secs, converted into enzyme activity rates and expressed as µmol·g dw^{-1}·min^{-1}. Because of limited amounts of biopsy material, CS and HAD activity was determined in 11 and 10 subjects from the Low and High group, respectively.

Immunoblotting

20mg of frozen muscle tissue was freeze-dried and subsequently homogenized, separated and electroblotted as previosuly described (32). The following primary antibody was purchased from Cell Signalling Technology (Danvers, MA, USA) and utilized as follows; phospho-specific ACC[Ser79] (cat # 3661, conc. 1:1000 in 5% BSA). The following primary antibody was purchased from ABCAM (Cambridge, UK) and utilized as follows; CPT1B (cat # AB134135, conc. 1:1000 in 5% BSA). With regards to secondary antibodies for both targets, membranes were then incubated for 1 hr with Horseradish peroxidase-conjugated goat anti-rabbit (Cat # 2054, Santa Cruz,TX, USA) and utilized as follows: phosphospecific ACC[Ser79] (conc. 1:5000 in 1% BSA) and CPT1B (conc. 1:4000 in 1% BSA). Proteins were visualized by chemiluminiscence (Thermo Scientific, MA, USA) and quantified with a UVP imaging system (UVP, CA, USA). Precision Plus Protein All Blue standards were used as markers of molecular weight (Bio-Rad, CA, USA). Values derived for quantification of immunoblotting for each protein target were normalized to the total amount of protein loaded for each sample, using the Stain Free Technology approach previously described (16,17).

Fiber type distribution.

Myosin heavy chain (MHC) composition was determined from homogenate using gel electrophoresis as previously described (11) and modified for humans (26). Briefly, muscle homogenate and sample-buffer (10% glycerol, 5% 2-mercaptoethanol and 2.3% SDS, 62.5mM Tris and 0.2% bromophenolblue at pH 6.8), was boiled for 3 mins and loaded with three different amounts of protein on a SDS-PAGE gel and run at 80V for at least 42 hrs at 4°C. The gels were stained with Coomassie and MHC bandswere quantified densitometrically (Phoretix 1D,

nonlinear, Newcastle, UK) by scanning (Lino-scan 1400 scanner, Heidelberg, Germany). The MHC band density was estimated as an average of the three loaded protein amounts. MHC II was identified with Western Blot using monoclonal antibody (Sigma M 4276) with the protocol Xcell IITM (Invitrogen, Carlsbad, CA, USA).

Statistical analysis

Statistical analyses of treatment effects were carried out using a two-way ANOVA with repeated measures (group vs. time) and the Sidak post hoc test correcting for multiple comparison (Graph Pad Prism 6.07). Values are expressed as mean $\pm$ SEM and $P < 0.05$ were considered significant. Data from immunoblotting were log-transformed due to lack of normal distribution and then analyzed by a two-way ANOVA. Correlations between variables were tested by linear regression analysis.

Results

Training compliance and intensities

All 26 subjects completed the 11 carbohydrate manipulation days. The average intensities during the initial 6 x 5-mins in the morning and the moderate 2-hr training in the afternoon were 86.3 $\pm$ 0.5% of HR_{max} (Low CHO: 86.5%; High CHO: 86.0%) and 66.7 $\pm$ 0.3% of HR_{max} (Low CHO: 66.9%; High CHO: 66.7%), respectively. There were no differences in training intensities between the groups. Using the W–HR relationship, power output during the morning sessions was estimated to increase from 293 $\pm$ 9 W to 346 $\pm$ 14 W (76 $\pm$ 2% to 89 $\pm$ 3% of W_{max} pre) during the intervention period in the Low CHO group and from 293 $\pm$ 9 W to 327 $\pm$ 11 W (76 $\pm$ 3% to 85 $\pm$ 2% of W_{max} pre) in the High CHO group. Likewise, power output during the

moderate 2-hr training sessions was estimated to increase during the training period (Low CHO: 165 ± 5 W to 205 ± 8 W; High CHO: 154 ± 7 W to 191 ± 6 W). Estimated power output during both training sessions increased to a similar extent in both groups. Besides the nine hours of training associated with the CHO manipulation, average training volume of the easy-to-moderate training sessions was 6 ± 1 hrs per week and identical between the groups because of the matched design (Fig. 1), thus entailing a total training volume of 16 ± 1 hrs per week.

Body composition and MHC distribution

Body mass was maintained in both groups after the intervention period (Low CHO: 74.9 ± 1.9 to 75.1 ± 2.0kg; High CHO: 75.3 ± 1.7 to 75.4 ± 1.7kg). However, fat mass was reduced (Low CHO: 9.7 ± 0.8 to 8.9 ± 0.9kg; High CHO: $10.4 \pm 0.$ to 10.0 ± 0.6kg; $P = 0.001$), while fat free mass increased to a similar extent in both groups (Low CHO: 68.7 ± 1.8 to 69.3 ± 1.8kg; High CHO: 68.5 ± 1.5 to 69.2 ± 1.5kg; $P < 0.0001$).

Prior to the training period, the relative distribution of MHC I and II isoforms was not different between the Low CHO group (MHC I: $49 \pm 2\%$, MHC II $51 \pm 2\%$) and the High CHO group (MHC I $51 \pm 3\%$, MHC II $49 \pm 3\%$).

VO_{2max} and Endurance performance

After the training period, VO_{2max} was increased by $5 \pm 2\%$ and $6 \pm 1\%$ in the Low and High CHO groups, respectively, with no difference between the groups (overall time effect: $P = 0.0003$) (Fig. 2A). From the W-VO_2 relationship, W_{max} was estimated to increase from 395 ± 10 W to 422 ± 12 W in the Low CHO group and from 395 ± 11 W to 428 ± 11 W in the High CHO group during the intervention period (overall time effect: $P < 0.0001$).

Mean power output during the 30-min TT including CHO provision increased by $6 \pm 1\%$ and $5 \pm 2\%$ in the Low and High CHO group, respectively, with no difference between the groups (overall time effect: $P < 0.001$) (Fig. 2B). Also, with CHO provision, the relative power output increased to a similar extent in the Low CHO group ($+5 \pm 2\%$; 4.08 ± 0.1 to 4.28 ± 0.1 W·kg^{-1}) as in the High CHO group ($+4 \pm 2\%$; 4.05 ± 0.1 to 4.20 ± 0.1 W·kg^{-1}) (overall time effect: $P < 0.001$) (Fig. 2C).

Mean power output during the two 30-min post-intervention TTs was similar after consuming CHO and placebo during the 90-min preload phase in both the Low CHO (320 ± 9 W vs 318 ± 10 W) and the High CHO group (317 ± 10 W vs 316 ± 12 W) (Fig. 2B). Furthermore, relative power output during the two post-intervention TTs was similar with CHO and placebo provision (Low CHO: 4.28 ± 0.1 W·kg^{-1} vs. 4.25 ± 0.1 W·kg^{-1}; High CHO: 4.21 ± 0.1 W·kg^{-1} vs. 4.22 ± 0.1 W·kg^{-1}) (Fig. 2C).

RER values were identical within and between the groups in all 30-min TTs (Low CHO: Pre 0.99 ± 0.01; Post CHO 0.99 ± 0.01; Post placebo 0.99 ± 0.01) (High CHO: Pre 0.99 ± 0.01; Post CHO 0.99 ± 0.01; Post placebo 0.99 ± 0.01)

Muscle Glycogen

After the training period, resting muscle glycogen concentration was similarly increased by 18% in the Low CHO group (543 ± 22 mmol·kg dw^{-1} to 643 ± 31 mmol·kg dw^{-1}) and 15% in the High CHO group (557 ± 23 mmol·kg dw^{-1} to 643 ± 28 mmol·kg dw^{-1}) (overall time effect $P = 0.001$) (Fig. 2D).

To investigate the acute effect of the carbohydrate manipulation on muscle glycogen content, acute biopsies were obtained from 17 athletes after the moderate 2-hr training session on the 7th

carbohydrate manipulation day (i.e. after ≈ 2 weeks of training). Compared to the resting post biopsy, glycogen was reduced to a similar extent in both the Low CHO (-31 ± 7%; 648 to 431 mmol·kg dw^{-1}) and the High CHO group (-39 ± 4%; 652 to 396 mmol·kg dw^{-1}) after the moderate 2-hr session (overall time effect $P < 0.0001$) (Fig. 2D). Glycogen utilization correlated with VO$_{2max}$ ($P = 0.03$, r$^2 = 0.30$), while no associations were observed between glycogen utilization and endurance performance (r$^2 = 0.16$, ns) or enzyme activity: CS: r$^2 = 0.02$, ns ; HAD: r$^2 = 0.14$, ns.

Submaximal measurements

No differences were observed between the Low and High CHO groups in any parameters during the graded submaximal tests before and after the training period. Heart rate was significantly reduced by 10-14 bpm at 135, 170 and 205W in both groups after the training and diet intervention (Table 2). Likewise, blood lactate was reduced to a similar extent in both groups at 205W and 240W. By contrast, VO$_2$, RER and the fat oxidation rate remained unchanged at all submaximal intensities in both groups after the intervention period (Table 2).

The relationship between the fat oxidation rate and relative utilization of VO$_{2max}$ remained unchanged in both groups after the training period, with no differences between the groups (Fig. 3). However, in the High CHO group the fat oxidation rate tended to be lower after the training period ($P = 0.07$). From the polynomial curve fitting, the average maximal fat oxidation was calculated to be 0.25 and 0.27 g · min^{-1} in the Low CHO group and 0.30 and 0.24 g · min^{-1} in the High CHO group before and after the training period, respectively.

Mitochondrial adaptations

Maximal citrate synthase activity increased to the same extent in the Low CHO group (+11%; 134 ± 5 to 147 ± 5 mmol·kg dw^{-1}·min^{-1}, $n = 11$) as in the High CHO group (+12%; 130 ± 7 to 142 ± 7 mmol·kg dw^{-1}·min^{-1}, $n = 10$) during the training period (overall time-effect: $P = 0.05$) (Fig. 4A). HAD activity remained unchanged after the training period in both the Low CHO group (+7%; 185 ± 6 to 196 ± 7 mmol·kg dw^{-1}·min^{-1} $n = 11$, ns) and the High CHO group (+4%; 185 ± 8 to 191 ± 7 mmol·kg dw^{-1}·min^{-1} $n = 10$, ns) (Fig. 4B) (overall time-effect: $P = 0.21$, ns). The pACC protein level exhibited an overall time-effect ($P < 0.01$). Although no difference was observed between the groups, this seemed to be primarily driven by a ~ 65% increase in the Low CHO group, whereas only an 11% increase was observed in the High CHO group (Fig. 4C). The total CPT1b level was unchanged in both the Low and High group after the training period (Fig. 4D).

Discussion

The present investigation demonstrated that superimposing periodic CHO restriction to four weeks of routine endurance training in highly trained endurance athletes does not lead to superior training effects compared to the same training with a CHO enriched diet. Thus, changes in VO_{2max}, endurance performance, resting glycogen levels and mitochondrial adaptations were similar, regardless of CHO availability.

"Train-low" in elite endurance athletes

CHO restriction during exercise and recovery has been shown to enhance signaling for mitochondrial biogenesis, and consequently it has been proposed as a strategy to improve endurance performance in athletes (30,31). However, most training studies have not been able to demonstrate a translation of these cellular adaptations into superior effects on endurance performance (8,12,22,25,36,38). The main part of studies investigating the effects of CHO restriction in combination with training, have been conducted in untrained or moderately endurance trained individuals (10,18,24,12,25,36,38) while only a few recent studies have employed highly trained endurance athletes (8,22). The present study used an applied approach to evaluate the effects of superimposing periodic CHO restriction to routine training in elite triathletes and road cyclists. To meet the complex physiological demands of both triathlon and road cycling, the weekly training programs encompassed important training sessions (i.e. recovery and prolonged bike sessions in cyclists and swim and run sessions in triathletes) in addition to the three days with carbohydrate manipulation. By using this applied design, and in accordance with the recent studies in elite endurance athletes, endurance performance increased similarly by periodic CHO restriction and a normal CHO enriched diet.

Impact of diet manipulation on endurance performance

Superior effects of training and recovery with CHO restriction on performance have previously been shown in some (10,18,24), but not all studies (8,12,22,25,36,38). There can be various explanations for this discrepancy, including differences in study designs, the duration of the performance tests, the magnitude of the training-induced glycogen depletion and the athlete training status. Most of these studies were not matched for timing of energy intake between

groups (i.e. highly fluctuating energy intake in low-groups) (8,12,18,22,24,25,36,38). This implies an exposure to various energy deficiencies rather than just CHO restriction, thus complicating the interpretation of the effects of CHO restriction *per se*.

Potential effects of periodic CHO restriction or fasting on endurance performance may have been underestimated by the use of relatively short test protocols ($\leq$ 60 min.) in several studies (8,10,18,25,36). The superior acute regulatory effects of "train-low" on muscle cell adaptations have generally involved targets related to lipid oxidation and mitochondrial biogenesis, and to investigate the potential of the strategy for endurance athletes, prolonged performance tests therefore seem preferable. Here we used a 2-hr test protocol consisting of a 90min pre-load phase with intermittent exercise followed by a 30-min maximal TT. With CHO provision during the pre-load phase, we registered a 5% increase in mean power output in both groups which can be explained by the increases pre-exercise glycogen, lean-body mass, enzyme activity and VO_{2max} in both groups. Since the superior effects of endurance training with CHO restriction have previously been associated with increased fat oxidation capacity (e.g. HAD activity, fat oxidation rate, mitochondrial biogenesis), enhancements in endurance performance could be expected during prolonged exercise with restricted CHO availability (22,38). We therefore also conducted an endurance performance test with CHO omission during the 90-min preload phase after the training period. Surprisingly, there was no difference in mean power output during the tests with and without CHO provision, neither within nor between the groups. RER values of 0.99 during the final 30-min TT after both CHO and placebo provision clearly demonstrated that energy came almost solely from CHO oxidation and that glycogen and CHO availability were not critically reduced during the performance test. Thus, in these highly trained athletes the performance during 2hrs of high intensity endurance exercise was not limited by CHO

availability. This observation is in disagreement with previous studies, which have shown positive effects of CHO provision during high-intensity exercise lasting up to 120 mins in highly trained athletes (35). This discrepancy may be explained by differences in exercise intensities and the use of overnight fasting before performance tests in many previous studies, which is however not relevant to the "real life" training and competition situation. Future studies in elite athletes, should investigate even longer performance tests, to clarify for how long time these athletes can exercise before CHO availability limits performance and if "train-low" is beneficial during competitive events where glycogen availability becomes markedly reduced.

The duration of the performance tests used by Hansen et al. (18) and Cochran et al. (10) was less than 20 mins, surprisingly still demonstrating superior effects of "train-low" on performance. Hansen and colleagues compared two different training regimes (i.e. twice every second day vs. once every day), and demonstrated a marked advantage of training twice every second day. Muscle glycogen was markedly reduced to ≈ 100 mmol·kg dw^{-1} by the "twice every second day" training protocol, which is much lower than seen generally and in the present study, thus possibly amplifying the training stimuli. Furthermore, the use of isolated knee extensor exercise in training and testing differs from the exercise modes used in other studies and could likely explain why no studies have been able to demonstrate similar performance enhancing effects using whole body exercise. The use of different weekly training distributions between groups in the study by Hansen and colleagues (18) however complicates the evaluation of "train-low" *per se*.

Despite dietary control, losses of body mass have been a consequence of the "sleep-low" strategy in some studies (8,24) which likely explains part of the superior effects of "train-low" on running performance observed by Marquet and colleagues (24). Also, using various "train-low" strategies

in elite race walkers, Burke and colleagues (8) observed a larger reduction in body mass in the "train-low" group compared to the high CHO group, but without superior effects on race walking performance. Together with the present results, the latter could indicate that highly trained endurance athletes are generally less responsive to "train-low", which should be further examined in future studies.

"Train-low" applicability in elite endurance athletes

Anecdotally, elite athletes in a variety of endurance sports employ "train-low" strategies to enhance performance (33). Based on the present results and the results of Burke and colleagues (8) and Hulston and Colleagues (22), the applicability and effects of "train-low" in elite endurance athletes seem limited, however. Conducting 2-4 daily training sessions as part of their normal training programs, athletes of this caliber inevitably experience occasional unintentional glycogen depletion and they may therefore be less responsive to "train-low" stimuli. From the post-exercise muscle glycogen concentrations, measured after seven days with CHO restriction, it moreover seems difficult to achieve sufficiently low glycogen levels for a prolonged period in elite endurance athletes, at least with the present protocol (i.e. high resting glycogen levels and isocaloric diets). Using a slightly more intense training protocol with 11 hrs of fasted recovery between training sessions, Lane and colleagues reported a net glycogen utilization of 334 mmol · kg dw^{-1} in elite endurance athletes, which is 100 mmol · kg dw^{-1} more than observed here (23). This either suggest that energy restriction instead of CHO restriction may be a preferable strategy to reduce muscle glycogen or that the net muscle glycogen utilization was reduced as a consequence of repeated exposure to the same training protocol in the present study.

Power output was gradually increased during training throughout the intervention period in the present study, and a higher training intensity or volume was not possible in our highly trained athletes. Thus, the present "train-low" strategy, in combination with the other weekly training sessions (Fig. 1), imposed a significant physiological stress, and increasing the intensity or the number of intervals would probably compromise other important training adaptations, thus deviating from a "real-world" intervention. In order to investigate whether our athletes were able to tolerate a higher intensity during the moderate 2-hr training session in the afternoon, the intended intensity was increased to 70% of HR_{max} on the 4th day with carbohydrate manipulation. Despite the relatively low intensity, average intensity in both groups was only 68% of HR_{max}, with several athletes not being able to exceed 65% of HR_{max}. This indicates that the athletes were sufficiently challenged by the original intensity of 65% of HR_{max}, which may be explained by the high intensity morning session, but also the training load from days without CHO restrictions, especially in those athletes with the highest training volume conducting up to three training sessions a day.

On a weekly basis, the present "train-low" strategy included 6hrs of training (30-50% of total training volume) and 21 hrs of "recovery-low" (Fig. 1). In comparison, the 3-week study by Marquet and colleagues (24) included 3 weekly 1h "train-low" sessions (≈ 30% of total training volume) and three nights of "recover low" (24) while the 3-week study by Burke and colleagues (8) used 6 weekly "train-low" sessions (≈ 50% of total training volume (km)). To ensure maintenance or development of other important training adaptations, it may not be appropriate to perform more than ≈ 50 % of the total training volume as "train-low" for a prolonged period in elite athletes. As shown by the present results, there may furthermore be some difficulties of achieving reduced glycogen availability in elite endurance athletes within an applied "real-

world" training regimen, which challenges the development of "train-low" strategies for daily or weekly use by elite athletes.

Net glycogen breakdown

Superior acute regulatory effects of CHO restriction have previously been associated with relatively low post exercise muscle glycogen availability ([100:300] mmol·kg dw^{-1}) (18,23,30,37), suggesting that the relatively high glycogen content after the CHO restriction in the present study constituted an insufficient stimuli. Based on the literature, we employed a glycogen-depleting cycling protocol, which we assumed would reduce muscle glycogen by $\approx$ 50% in well-trained athletes (34). Nevertheless, muscle glycogen was only reduced by 31% (-217 mmol·kg dw^{-1}) in the Low group, which may be partly explained by the minor intake of 1g CHO·kg bm^{-1} during recovery and that muscle glycogen was resynthesized during recovery, despite the CHO restriction. Moreover, the utilization of resynthesized muscle glycogen during the afternoon session were likely larger in the High CHO group, explaining, at least partly, the similar net glycogen utilization in this group (-39 %; -256 mmol·kg dw^{-1}).

As the present study was conducted in elite athletes, we were not able to obtain multiple muscle biopsies and consequently we were not able to quantify the extent of gluconeogenesis and glycogen re-synthesis during the 7 hr recovery period. Skeletal muscles have the capacity to resynthesize muscle glycogen at relatively high rates during recovery from high intensity exercise, even with the absence of exogenous carbohydrate in untrained and moderately trained individuals (1,2,3,21). Since gluconeogenesis is furthermore positively associated with training status, the rate of gluconeogenesis was presumably high in the present group of elite athletes (5). Lactate has been proposed as the primary source of gluconeogenesis during the initial recovery

from high-intensity exercise and since blood lactate concentrations were markedly increased after the initial 6 x 5 mins ($\approx$ 4 mmol·L^{-1}) and the subsequent 2 x 5 min. ($\approx$ 12 mmol·L^{-1}), it is likely that lactate conversion contributed to muscle glycogen resynthesis (13). Protei). In addition to the minor intake of CHO in the low group, protein may also have served as a source for glycogenesis, resynthesizing glycogen, although the intake of exogenous protein was relatively small (16% of energy intake) in both groups.

Enzyme activity and long-chain fatty acid uptake

To our knowledge, this 4-week "train-low" study is the first to collect muscle biopsies to investigate myocellular responses in elite athletes. Contrary to our hypothesis, enzymatic changes were independent of carbohydrate availability during training and recovery. CS activity and glycogen content increased equally in both groups by $\approx$15% and $\approx$14%, respectively. By contrast, HAD activity remained unchanged in both groups. Moreover, even though total CPT1b protein remained unchanged in both groups, the observed chronic increase in pACC (a proxy of ACC activity), by 65% in the Low group and 11% in the High group, indicates that the capacity for long-chain fatty acid mitochondrial entry was enhanced. Although the increase in pACC did not differ statistically, it was obviously driven predominantly by CHO restriction. The comparable myocellular adaptations between the groups are in accordance with the identical improvements in endurance performance between the groups. A similar lack of chronic difference in metabolic proteins after training in a CHO-restricted versus a CHO-fed state has previously been observed in two studies that included physically active, but not elite trained, individuals (10,12). On the contrary, training studies in both untrained or endurance trained individuals by Morton et al. (25), Yeo et al. (38) and Hulston et al. (22) reported that oxidative

enzyme activity (i.e. SDH, CS and HAD activity) was enhanced more by CHO restriction compared to a CHO enriched diet. Manipulating energy intake by fasting and not just by CHO intake may partly explain the enhanced response in enzyme activity observed in some previous studies (22,38). Collectively, the present and previous results indicate that differences may exist in the responsiveness of specific metabolic enzymes due to differences in training status and "train-low" strategies. Our data suggest that highly trained individuals are less responsive to changes in enzyme activity induced by "train-low", at least when the energy intake, and the timing hereof, is standardized and equal to control.

Conclusion

In conclusion, four weeks of regular endurance training in elite athletes, including either periodic CHO restriction or a CHO-enriched diet, induced similar effects on performance and the myocellular response. With the present matched and isocaloric design, endurance performance, VO_{2max}, glycogen availability and markers of mitochondrial adaptations all increased to the same extent with high and periodically low CHO availability. The lack of group differences could be explained by the modest glycogen depletion in the Low group after the CHO manipulation, suggesting that more aggressive manipulation protocols may be needed to achieve superior effects in elite endurance athletes.

Conflict of interest

The authors report no conflict of interest and the results of the present study do not constitute endorsement by ACSM. We declare that the results of the study are presented clearly, honestly and without fabrication, falsification, or inappropriate data manipulation.

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Figure legends

Figure 1. Schematic illustration of the 36-day study period, including the timing of the five laboratory visits and the training period including periodic CHO manipulation and moreover an example of a weekly training schedule, including routine training and three days with CHO manipulation. The schedule is representative for those triathletes completing the highest training volume. In addition to the three CHO manipulation days, the triathletes conducted swim and run sessions, while road cyclists carried out supplemental bike sessions. Training schedules were similar within pairs but different between pairs throughout the 4-week training period. More information about training plans is described in "Methods". ST = strength training.

Figure 2. VO_{2max} (A), power output during the 30-min TT undertaken after 90 mins of intermittent exercise with either CHO or placebo provision (B & C) and resting glycogen content before and after 4 weeks of endurance training including a periodic low CHO intake ((Low, $n = 13$) or a high CHO intake (High, $n = 13$) as well as muscle glycogen 1 hr after the moderate 2-hr afternoon session on the seventh day with CHO manipulation ($n = 17$; Low $n = 9$, High $n = 8$) (D). # Overall time effect from pre to post with no differences between the groups, $P \leq 0.001$. * Different from pre and post.

Figure 3. Association between relative exercise intensity and fat oxidation rates during the graded submaximal test. The association between the relative utilization of VO_{2max} and the fat oxidation rate was determined by polynomial curve fitting. The relative utilizations of VO_{2max} were divided into 7.5% intervals from 30% to 75% of VO_{2max} and associated with corresponding fat oxidation rates. The fat oxidation curves did not differ between the Low and High CHO groups before and after the training period. However, the fat oxidation rate tended to be lower in the High CHO group after compared to before the training period ($P = 0.07$).

Figure 4. Effects of 4 weeks of endurance training with either a periodic low CHO diet (Low) or a high CHO diet (High) in elite endurance on (A) citrate synthase activity (CS, Low: $n = 11$; High: $n = 10$); (B) 3-hydroxyacyl-CoA dehydrogenase activity (HAD, Low: $n = 11$; High: $n = 10$); (C) pACC^{Ser79} (Low: $n = 13$; High: $n = 12$); and (D) CPT1b (Low: $n = 13$; High: $n = 12$). # Overall time effects from pre to post with no differences between the groups: citrate synthase: $P = 0.05$; pACC^{Ser79}: $P = 0.004$.

Figure 1

Figure 2

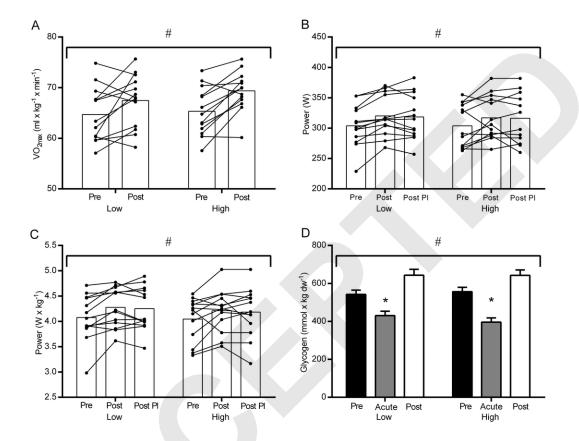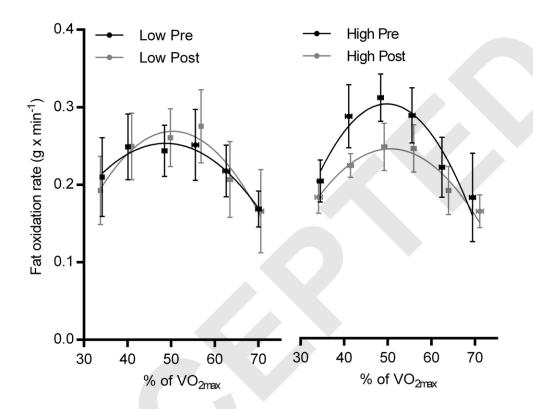**Figure 3**

Figure 4
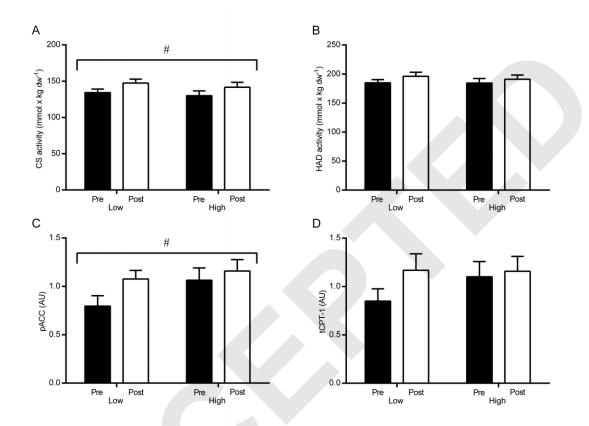
Table 1. Descriptive variables for Low and High CHO groups

	Low CHO			High CHO		
Subjects (*n*)		13			13	
Age, years	24	±	1	25	±	1
Body mass, kg	74.9	±	1.9	75.3	±	1.7
Height, cm	184	±	2	183	±	2
Body fat, %	9.7	±	0.8	10.4	±	0.7
LBM, kg	68.7	±	1.8	68.5	±	1.5
VO_{2max} (mL·min^{-1})	4835	±	142	4920	±	153
VO_{2max} (mL·kg^{-1}·min^{-1})	64.7	±	1.4	65.3	±	1.3
Training volumen (h·week^{-1})	17	±	1	17	±	1

Values are from baseline and means ± SEM. No differences were observed between groups.

Table 2. Submaximal measurements during cycling exercise.

		170 W Low	170 W High		205 W Low	205 W High		240 W Low	240 W High	
Heart rate (bpm)	Pre	130 ± 3	134 ± 3		143 ± 2	144 ± 4		154 ± 3	155 ± 4	
	Post	119 ± 3	122 ± 3	*	129 ± 3	132 ± 3	*	140 ± 3	146 ± 3	*
VO_2 (ml · min^{-1})	Pre	2348 ± 35	2439 ± 45		2728 ± 37	2800 ± 33		3121 ± 41	3174 ± 38	
	Post	2303 ± 51	2374 ± 44		2662 ± 51	2773 ± 44		3044 ± 70	3174 ± 45	
RER (VCO_2. VO_2^{-1})	Pre	0.93 ± 0.01	0.93 ± 0.01		0.95 ± 0.01	0.95 ± 0.01		0.97 ± 0.01	0.97 ± 0.01	
	Post	0.93 ± 0.01	0.94 ± 0.00		0.94 ± 0.01	0.94 ± 0.01		0.96 ± 0.01	0.96 ± 0.01	
Fat oxidation (g·min^{-1})	Pre	0.27 ± 0.04	0.29 ± 0.03		0.22 ± 0.04	0.23 ± 0.04		0.15 ± 0.04	0.18 ± 0.05	
	Post	0.25 ± 0.04	0.24 ± 0.02		0.26 ± 0.04	0.24 ± 0.03		0.21 ± 0.04	0.19 ± 0.05	
Blood lactate (mmol · L^{-1})	Pre	1.22 ± 0.07	1.40 ± 0.14		1.41 ± 0.12	1.51 ± 0.16		2.06 ± 0.16	2.43 ± 0.30	
	Post	1.08 ± 0.07	1.21 ± 0.16		1.15 ± 0.07	1.28 ± 0.13	*	1.55 ± 0.13	1.60 ± 0.22	*

Oxygen consumption (VO_2), Repiratory Exchange Ratio (RER), fat oxidation rate and blood lactate at 170, 205, 240 W before and after the training period in the Low and High CHO group. Subjects consumed identical meals every second hour from 7am until 60 min. prior to the submaximal test. The meals (5.7 kJ · kg bw^{-1}) contained 0.9 g CHO · kg bw^{-1}, with a relative contribution of 65% CHO, 14% protein and 21% fat. Values are means ± SEM. * Overall time effect pre to post, $P < 0.05$.