

Sollie, O., Jeppesen, P. B., Tangen, D. S., Jernerén, F., Nellesmann, B., Valsdottir, T., Madsen, K. ... Jensen, J. (2018). Protein intake in the early recovery period after exhaustive exercise improves performance the following day. *Journal of applied physiology*, 125, s.1731-1742.

---

Dette er siste tekst-versjon av artikkelen, og den kan inneholde små forskjeller fra forlagets pdf-versjon. Forlagets pdf-versjon finner du her:  
<http://dx.doi.org/10.1152/jappphysiol.01132.2017>

---

This is the final text version of the article, and it may contain minor differences from the journal's pdf version. The original publication is available here:  
<http://dx.doi.org/10.1152/jappphysiol.01132.2017>

---

# Protein intake in the early recovery period after exhaustive exercise improves performance the following day

Ove Sollie<sup>1</sup>, Per B. Jeppesen<sup>2</sup>, Daniel S. Tangen<sup>1</sup>, Fredrik Jerneerén<sup>3</sup>, Birgitte Nellesmann<sup>1</sup>, Ditta Valsdottir<sup>1,4</sup>, Klavs Madsen<sup>5</sup>, Cheryl Turner<sup>3</sup>, Helga Refsum<sup>3,6</sup>, Bjørn S. Skålhegg<sup>6</sup>, John L. Ivy<sup>7</sup>, & Jørgen Jensen<sup>1,\*</sup>

1 Department of Physical Performance, Norwegian School of Sports Sciences, Oslo, Norway

2 Department of Clinical Medicine, Aarhus University, Aarhus, Denmark

3 Department of Pharmacology, University of Oxford, UK

4 Department of Medical Sciences, Atlantis Medical University College, Oslo, Norway

5 Department of Public Health - Sport Science, Aarhus University, 8000 Aarhus C

6 Department of Nutrition, Section for Molecular Nutrition, University of Oslo, Norway

7 Department of Kinesiology and Health Education, University of Texas at Austin, Austin, TX; USA

Running Title: Protein enhances recovery of time trial performance

Address For correspondence:

Jørgen Jensen,

Department of Physical Performance,

Norwegian School of Sport Sciences,

Sognsveien 220,

PO Box 4014 Ullevål Stadion,

0806 Oslo, Norway

e-mail: jorgen.jensen@nih.no

## **Abstract**

The aim of the present study was to investigate the effect of protein and carbohydrate ingestion during early recovery from exhaustive exercise on performance after 18 h recovery. Eight elite cyclists ( $\text{VO}_{2\text{max}}$   $74.0 \pm 1.6 \text{ ml} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ) completed two exercise and diet interventions in a double-blinded, randomized, crossover design. Participants cycled first at 73% of  $\text{VO}_{2\text{max}}$  ( $W_{73\%}$ ) followed by one-min intervals at 90% of  $\text{VO}_{2\text{max}}$  until exhaustion. During the first two hours of recovery, participants ingested either 1.2 g carbohydrate  $\cdot \text{kg}^{-1} \cdot \text{h}^{-1}$  (CHO) or 0.8 g carbohydrate + 0.4 g protein  $\cdot \text{kg}^{-1} \cdot \text{h}^{-1}$  (CHO+PROT). The diet during the remaining recovery period was similar for both interventions and adjusted to body weight. After an 18 h recovery, cycling performance was assessed with a 10 s sprint test, 30 min of cycling at  $W_{73\%}$ , and a cycling time trial (TT). The TT was 8.5% faster ( $41:53 \pm 1:51 \text{ min}$  vs  $45:26 \pm 1:32 \text{ min}$ ;  $p < 0.03$ ) after CHO+PROT compared to CHO. Mean power output during the sprints was 3.7% higher in CHO-PROT compared to CHO ( $1063 \pm 54 \text{ W}$  vs  $1026 \pm 53 \text{ W}$ ;  $p < 0.01$ ). Nitrogen balance in the recovery period was negative in CHO and neutral in CHO+PROT ( $-82.4 \pm 11.5$  vs  $7.0 \pm 15.4 \text{ mg} \cdot \text{kg}^{-1}$ ;  $p < 0.01$ ). In conclusion: TT and sprint performances were improved 18 h after exhaustive cycling by CHO-PROT supplementation during the first two hours of recovery compared with isoenergetic CHO supplementation. Our results indicate that intake of carbohydrate plus protein after exhaustive endurance exercise more rapidly converts the body from a catabolic to an anabolic state than carbohydrate alone, thus speeding recovery and improving subsequent cycling performance.

## **News and Noteworthy**

Prolonged high intensity endurance exercise depends on glycogen utilization and high oxidative capacity. Still, exhaustion develops and effective recovery strategies are required to compete in multi-day stage races. We show that co-ingestion of protein and carbohydrate during the first two hours of recovery is superior to isoenergetic intake of carbohydrate to stimulate recovery, and improves both endurance time-trial and 10 s sprint performance the following day in elite cyclists.

**Keywords:** Diet, sprint, glucose, branched-chain amino acids, nitrogen balance

## **Introduction**

Competitive stage race cycling consists of consecutive days with high physiological stress and limited time for recovery (19). The feeding strategies during recovery are essential to optimize performance and may determine the final outcome of endurance competitions the following day. Carbohydrate and fat are the major fuels during exercise (14; 44; 50; 59). However, amino acids are also oxidized during exhaustive exercise (6; 62), and amino acid oxidation and protein degradation are elevated in muscles with low glycogen content (6; 25; 33). Furthermore, protein breakdown continues in skeletal muscles after exercise if energy intake is insufficient or lacks proteins or amino acids (9).

The importance of carbohydrate intake after exercise for improved recovery of performance is well established (5; 26; 27). However, co-ingestion of carbohydrate and protein after endurance exercise has emerged as a key nutrition practice for many cyclists (21; 52). Intake of both protein and carbohydrate after exercise has been reported to be superior to carbohydrate only to stimulate the rate of glycogen synthesis (3; 28; 64) and increase the rate of protein synthesis (10; 24; 45). Moreover, studies have shown improved recovery of performance after co-ingestion of protein plus carbohydrate compared to carbohydrate only (2; 18; 46; 50; 53; 58; 61), but this has not been a universal finding (3; 4; 20; 43; 48; 49). The reasons for this discrepancy may be due to the type of exercise performed, the severity of metabolic challenge during the exercise prior to the dietary interventions, the recovery period and the test used to evaluate performance after the intervention. Obviously, this topic needs more study.

Typically, recovery time during stage race cycling is 16-20 h, and in this time-frame, improved performance after co-ingestion of protein and carbohydrate has only been reported in two studies using time to exhaustion (TtE) as a performance test (50; 53). Importantly, time trial (TT) performance is considered a more relevant physiological test as it is an effective predictor of cycling performance, is an extremely reliable and repeatable test, and has been used to assess skeletal muscle oxidative phosphorylation capacity (29). However, the beneficial effect of co-ingestion of protein and carbohydrate on time trial performance after 18 h recovery has never been reported.

Sprint performance is an important quality in cycling and requires high power production. Maximal strength and muscle cross-sectional area determine to a large degree maximal power

(38). High cycle sprint performance depends on high maximal strength (56), and myofibrillar quality and neuromuscular activation are determinants of strength (13). Synthesis of myofibrillar proteins contributes to high maximal power and sprint performance. Protein intake after endurance exercise stimulates myofibrillar protein synthesis and proteins in other myocellular compartments (10; 45), which may contribute to improved recovery after exhaustive exercise. Although sprint capacity determines the final outcome of many cycle competitions, the effect of co-ingestion of carbohydrate and protein after exhaustive exercise on sprint performance the following day is unknown.

To further investigate the physiological processes that benefit from co-ingestion of protein and carbohydrate during the early recovery phase after exhaustive exercise, we evaluated the effect of co-ingestion of protein and carbohydrate on sprint and time trial performance 18 h after exhausting exercise. We hypothesized that sprint and TT performances would be higher after co-ingestion of protein and carbohydrate during the first 2 hours of recovery, compared to isoenergetic intake of carbohydrate only.

## **Materials and Methods**

### **Subjects**

Eight male elite endurance cyclists (mean $\pm$ SEM; age: 22.9 $\pm$ 1.2 yr.; height: 182 $\pm$ 2 cm; body mass: 79.5 $\pm$ 3 kg;  $\text{VO}_{2\text{max}}$  5.9 $\pm$ 0.3 L $\cdot$ min $^{-1}$ , 74.0 $\pm$ 1.6 ml $\cdot$ kg $^{-1}$  $\cdot$ min $^{-1}$ ;  $\text{W}_{\text{max}}$  463 $\pm$ 11.2 W, 5.9 $\pm$ 0.1 W $\cdot$ kg $^{-1}$ ) were recruited for the study. The subjects had competitive experience at national and some at international level competitions and trained an average of 16 $\pm$ 1 h weekly, of which 12 $\pm$ 2 h consisted of cycling. The protocol was reviewed by Regional Ethic Committee of Norway (2011/1298) with the decision that the research project was outside the Act on Medical Health Research, confirmed in letter of exemption (2011/1298; IRB ref: IRB00006245). The study was conducted according to the Declaration of Helsinki and participants gave their informed written consent after oral and written information about the study.

### **Study design**

*Overview:* The study was completed in a double-blinded, randomized, balanced, crossover experimental design. Subjects completed one session of physiological measures and two familiarization sessions before the experimental trials. Two experimental interventions,

separated by at least six days, consisted of two consecutive days of testing and dietary control (Fig. 1). The first testing day consisted of an exhaustive cycling exercise bout (EXH) followed by experimental interventions that consisted of participants provided with carbohydrate (CHO) or carbohydrate + protein (CHO+PROT) drinks during the first 2 h of recovery. From 2 to 18 h after the exhaustive exercise, there was dietary control only, which was matched for energy and macronutrient intake for both interventions. During the second testing day, ~18 h after the exhaustive exercise, the athletes completed both sprint and endurance (preloaded time-trial) performance tests.

### **Physiological testing and familiarization sessions**

*Physiological testing:* On the first visit to the laboratory, subjects completed incremental cycling exercises to determine the relationship between workload (W) and oxygen uptake ( $\text{VO}_2$ ), maximal work capacity ( $W_{\text{max}}$ ) and maximal oxygen uptake ( $\text{VO}_{2\text{max}}$ ). Each subject's custom bike geometry was adjusted on the ergometer (Lode Excalibur Sport, Groningen, The Netherlands) and used throughout the study. Prior to testing, subjects performed a ten min easy self-paced warm up. To determine the relationship between W and  $\text{VO}_2$ , the initial ergometer workload was set at 200-250 W, and increased 25 W every 5 min. Between 2.5-4 min of each step,  $\text{VO}_2$  was measured (Oxycon Pro, Jager Instr; Hoechberg, Germany) and capillary blood samples were taken and analyzed for blood lactate (1500 Sport, YSI Inc., Yellow Springs Instr; Ohio, USA). Heart rate (HR) was measured with a Polar RS 800-CX (Kempele, Finland) at a 5 s average rate throughout the study. Linear regression analysis from the incremental cycling test was used to determine the workload corresponding to 73% of  $\text{VO}_{2\text{max}}$  ( $W_{73\%}$ ) and this value was re-assessed during the first familiarization session. The cycling test was terminated when blood lactate concentration reached 3 mM. The participants selected a cadence between 90 and 105 rpm during this test ( $94 \pm 2$ ); this cadence ( $\pm 5$  rpm) was used subsequently for all familiarization and experimental trials. Subjects rested 5-10 min before being tested for  $W_{\text{max}}/\text{VO}_{2\text{max}}$ . For this test, starting workload was the same as the second last step of the first test and increased 25 W per min until voluntary exhaustion.  $\text{VO}_2$  was measured over 30 s periods and the mean of the two highest consecutive measurements was used as  $\text{VO}_{2\text{max}}$ . A failure for  $\text{VO}_2$  to increase with increasing workload was used as criteria for a successful  $\text{VO}_{2\text{max}}$  measurement.

$W_{\text{max}}$  was determined as:

Workload of the last stage completed + [(25 W/60 s) x s at the final stage].

*Familiarization sessions:* Two to three days after physiological testing, participants completed the first familiarization session, which included all elements of day 2 of experimental trials (see “Day 2- Performance testing”) except for standardized breakfast, and blood and urine sampling. During the first 30 min at fixed intensity, power output was adjusted if necessary to elicit subject’s 73% of  $VO_{2max}$ . The adjusted workload was used as  $W_{73\%}$  for the rest of the study.

Approximately one week before the intervention period, athletes completed a second familiarization session (CON) that replicated all exercise demands and measures of the experimental protocol of day 2 of the experimental period.

### **Experimental trials period**

Subjects recorded their dietary intake and exercise training for 24 h prior to the start of the first experimental trial and were requested to follow the same diet and exercise prior to the second experimental trial. For this 24 h period, subjects were instructed to do very light or no exercise and to repeat this strategy before their second trial.

*Day 1 – Exhaustive exercise session (EXH):* The athletes reported to the laboratory at 11:30 AM for weigh-in and baseline blood sampling before starting their warm-up at 12:00 PM. Another weighing of the athletes was done after EXH to detect weight loss. Water was ingested *ad libitum* during EXH. The exercise session was initiated with a warm-up consisting of consecutive steps of 4 min at a workload eliciting 50, 55 and 60% of  $VO_{2max}$ . After a 5 min recovery, subjects performed a 10 s sprint (Sprint 1, Fig 1) followed by another 5 min recovery period. All sprints during the study were all-out efforts performed using Lode Ergometry Manager 9.4.7.0 software with a torque factor of 1.0 Nm/kg. Throughout the study, recovery periods were complete rest or at a work-rate of 100 W, self-selected by the subjects. The exhaustive exercise bout started with 30 min at  $W_{73\%}$ . After a 5 min recovery, subsequent intervals of 20 min at  $W_{73\%}$  and 5 min recovery between each interval were completed until subjects were unable to maintain their pre-determined selected cadence  $\pm 5$  RPM despite verbal encouragement. After exhaustion, participants recovered for 5 min and then performed 1 min intervals at a power output eliciting 90% of  $VO_{2max}$  followed by 1 min recovery, repeatedly, until voluntary exhaustion (unable to maintain power for 1 min). After the 1 min intervals, subjects recovered for 5 min before completing a second 10 s sprint (Sprint 2, Fig 1).  $VO_2$ , RER, blood lactate, blood glucose (HemoCue Glucose 201<sup>+</sup>, Ängholm, Sweden), HR and rate

of perceived exertion (RPE) were assessed between min 3-4 and the last 2 min of the first 30 min interval. Thereafter, measurements were conducted only at the last 2 min of each 20 min interval and the last 2 min before exhaustion.

*Recovery and intervention supplement ingestion period (0-2 h):* After EXH, a Teflon catheter 18GA (BD Venflon Pro, New Jersey, USA) was inserted into an antecubital vein for serial blood sampling. Supplement drinks were ingested immediately after the first blood sample, and every 30 min for the first 2 h (total 4 drinks). Isovolumetric supplements matched for flavor ( $7.06 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ ) provided  $1.2 \text{ g carbohydrate}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$  (CHO) or  $0.8 \text{ carbohydrate}\cdot\text{kg}^{-1}\cdot\text{h}^{-1} + 0.4 \text{ g whey protein}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$  (CHO+PROT). The carbohydrate portion of the drinks consisted of equal parts of maltodextrin (Maltodextrin White pure, AppliChem, Darmstadt, Germany) and glucose, (Glukose AnalaR, VWR Prolabo, Leuven, Belgium). The whey protein used was Lacprodan, SP-9225 Instant from (Arla Foods, Aarhus, Denmark).

*Prolonged recovery and controlled diet period (2- ~18 h):* Following the first 2 h of recovery, diets for CHO and CHO + PRO were identically matched for energy ( $127 \text{ kJ}\cdot\text{kg}^{-1}$ ) and macronutrient intake ( $4.89 \text{ g carbohydrate}\cdot\text{kg}^{-1}$ ,  $1.08 \text{ g protein}\cdot\text{kg}^{-1}$  and  $0.77 \text{ g fat}\cdot\text{kg}^{-1}$ ). Post-supplement nutrition was custom-made, pre-packaged and provided in the form of dinner, evening carbohydrate drink, supper and breakfast, at 2, ~4, ~6 and ~16 h post EXH, respectively. Dinner was consumed in the laboratory. Evening carbohydrate drink, supper and breakfast were provided for subjects to consume at their homes at the indicated times. Water was ingested *ad libitum* and additional food or drinks were not allowed. Detailed composition of meals for the 2-~18 h period can be found in Table 1.

*Total energy and macronutrient intake:* Total nutrient intake (supplements + meals) of the interventions was isoenergetic ( $169 \text{ kJ}\cdot\text{kg}^{-1}$ ) but differed in composition due to the 0-2 h supplement intervention. The CHO diet contained  $7.29 \text{ g carbohydrate}\cdot\text{kg}^{-1}$ ,  $1.08 \text{ g protein}\cdot\text{kg}^{-1}$  and  $0.77 \text{ g fat}\cdot\text{kg}^{-1}$ , and CHO+PROT diet contained  $6.49 \text{ g carbohydrate}\cdot\text{kg}^{-1}$ ,  $1.88 \text{ g protein}\cdot\text{kg}^{-1}$  and  $0.77 \text{ g fat}\cdot\text{kg}^{-1}$ .

*Day 2- Performance testing:* Subjects ingested breakfast at 7:00 AM in their homes, 2 h prior to the start of the warm-up. Upon arrival to the laboratory at ~08:30 AM, subject's body mass was recorded, and a Teflon catheter was inserted in an antecubital vein for serial, blood sampling. The catheter was flushed with saline solution when samples were not taken to keep



patency. At 9:00 AM, approximately 18 h after the end of EXH, subjects completed the same warm-up as on day 1, recovered for 5 min, and completed a 10 s sprint (Sprint 3, Fig 1) followed by a 5 min recovery. Thereafter, subjects started the pre-loaded TT that consisted of 30 min of cycling at a fixed intensity ( $W_{73\%}$ ), a 5 min recovery, followed by a TT to complete a specific amount of mechanical work. The total work to be completed was equivalent to 30 min (1800 s) at a workload corresponding to 100% of  $VO_{2max}$  (Work output (kJ) = Power at  $VO_{2max}$  (W) \* 1800 s).  $VO_2$ , RER and HR were measured between 3-4, 16-18 and 28-30 min. Blood lactate, blood glucose (capillary blood) and RPE were measured at 4, 18 and 30 min. Blood samples collected from the Teflon catheter were taken at 15 and 30 min.

The TT started at  $W_{73}$ , but the subjects could adjust the workload on a panel mounted on the bike as they preferred. Time was blinded during the TT, but accumulated work was shown on a screen in front of the athletes. The average work output during the TT was  $738 \pm 29$  kJ. Subjects were also informed at every ten percent how much of the TT they had completed.  $VO_2$  and RER were measured between 14-15 min and during the last ten percent of the TT. RPE and HR were taken at 10%, 20%, at 15 min, 60%, 80% and the end of the TT. Blood lactate, blood glucose and blood samples from the Teflon catheter were collected at the end of the TT and 15 min after the TT. After the TT, a 5 min recovery followed before the athletes performed a second 10 s sprint (Sprint 4, Fig 1).

### **Biological Samples**

*Blood:* Venous blood samples were taken from a Teflon catheter previously inserted into an *antecubital* vein except the fasting morning sample, which was taken using a retractable safety winged steel needle (BD Vacutainer, New Jersey, USA). Blood samples were taken in 6.5 ml  $K_2EDTA$  tubes (BD Vacutainer, New Jersey, USA), placed on ice and centrifuged for 10 min at  $4^\circ C$  (2500 g). Plasma was then pipetted into 1.5 ml Eppendorf tubes and stored at  $-80^\circ C$  until subsequent analysis. On day 1, blood samples were taken upon arrival in the laboratory, and immediately after EXH and at 15, 30, 60, 90 and 120 min post EXH. On day 2, blood samples were taken upon arrival in the laboratory, at 15 and 30 min of  $W_{73\%}$ , and at the end of the TT and 15 min after the TT.

*Urine:* All of the urine samples from the athletes during the recovery period were collected in plastic containers in four consecutive batches: 1) from start of day 1 until end of EXH, 2) 0-2 h after EXH, 3) from 2 h after EXH until midnight, and 4) from midnight until start of warm-up

of the pre-loaded TT. Subjects were instructed to void their bladders before the start of EXH. Volume was measured for each period and a 12 ml sample was frozen at -20°C for analysis.

### **Analytical procedures**

All analytic processes were conducted according to the manufacturer's instructions.

*Glucose:* Plasma glucose was determined by an enzymatic procedure (GLUC2) (Roche Diagnostics GmbH, Mannheim, Germany) using a Cobas C-111 autoanalyzer (Roche, Germany).

*Free fatty acids (FFA):* The plasma FFA content was measured by an enzymatic colorimetric assay for the quantitative determination of non-esterified fatty acids (NEFA-HR) (Wako Chemicals GmbH, Neuss, Germany) a Cobas C-111 autoanalyzer (Roche, Germany).

*Glycerol:* The plasma glycerol content was measured by a direct colorimetric procedure (Glycerol, Randox Laboratories Ltd, Crumlin, UK) using a Cobas C-111 autoanalyzer (Roche, Germany).

*Insulin:* Plasma insulin concentrations (unit: pM) were measured with an enzyme-linked immunosorbent assay (ELISA) (Dako, Glostrup, Denmark).

*Uric Acid:* The amount of plasma uric acid was detected with a UA2 kit from Roche (Roche Diagnostics GmbH, Mannheim, Germany) and measured on a Cobas C-111 autoanalyzer.

*Amino acids:* Plasma amino acids including branch chain amino acids (BCAA), were analyzed in a single run using liquid chromatography tandem-mass spectrometry (LC-MS/MS) with a modified version of a previously described method for sulfur amino acid measurements (1). Briefly, deuterium-labelled isotopes were added to plasma as internal standards, followed by the addition of dithioerythritol (as the method is also used for sulfur amino acid measurements) and then protein precipitation using perchloric acid. The acid supernatant was diluted with a solution containing heptane sulfonic acid as an ion pair reagent. LC-MS/MS of all extracts was carried out using a Shimadzu LC-20ADXR Prominence LC system (Kyoto, Japan) coupled to a Sciex QTRAP5500 mass spectrometer with a Turbo V ion source (Framingham, MA, USA). Chromatographic separation was achieved on a Phenomenex Kinetex Core Shell C18 (100 x 4.6 mm, 2.6 µm) LC column (Torrance, CA, USA) with water and methanol gradient mobile phase spiked with formic acid

[0.05%]. Positive mode multiple reaction monitoring was used for detection. Linear calibration curves of the peak area ratios of analytes and internal standards were used for quantification. Coefficients of variation for all amino acid analyses were  $\leq 7\%$ .

*Nitrogen:* Urea and urea nitrogen were measured with a QuantiChrom Urea Assay Kit (DIUR-500), BioAssaySystem. Nitrogen balance was calculated based on protein intake and urinary nitrogen excretion and assuming non-urinary nitrogen losses of 23.2% in CHO and 22.9% in CHO+PROT (46). Nitrogen intake was calculated assuming the nitrogen to amino acid constant of 6.25 (35). Nitrogen concentration in the urine was analyzed with the Kjeldahl method (32).

*Calculation of carbohydrate and fat oxidation:* Carbohydrate and fat oxidation rates were calculated from RER and oxygen uptake together with the *Table of Nonprotein Respiratory Quotient* assuming non-protein metabolism as described by Peronnet and Massicotte (41).

*Statistics:* All values are expressed as mean  $\pm$  SEM. The between treatment difference (CHO vs. CHO+PROT) from the performance test was tested with a two tailed students T-test. Two-way repeated measures analysis of variance (ANOVA) was used to determine treatment differences, with treatment and time used as factors. When differences were detected, Tukey's honestly significant difference post hoc test was performed. Differences were considered significant when  $p < 0.05$ . Pearson's Product Moment Correlation Analysis was used to detect significant relationships between variables. Statistical analyses were performed using SigmaPlot 12.5 software.

## Results

*Exhaustive exercise on day one (EXH):* The workload used during EXH was  $298 \pm 12$  W and corresponded to  $73.0 \pm 3.0\%$  of  $VO_{2max}$ . Voluntary exhaustion at  $W_{73\%}$  occurred after  $112 \pm 9$  and  $108 \pm 6$  min prior to CHO and CHO+PROT, respectively. The athletes thereafter performed 1 min sprints at  $372 \pm 14$  W ( $13 \pm 3$  and  $9 \pm 1$  prior to CHO and CHO+PROT, respectively). There were no significant differences in EXH time or number of sprints before interventions ( $p=0.66$  and  $p=0.37$ , respectively). Total carbohydrate oxidation during EXH was  $426 \pm 34$  and  $405 \pm 32$  g before CHO and CHO+PROT, respectively, and with no significant difference in carbohydrate oxidation between interventions ( $p=0.36$ ) (Table 2). These calculations did not include the 1 min sprints at 90% of  $VO_{2max}$ . Energy utilization during EXH (1 min sprints included) were  $11554 \pm 1009$  kJ before the CHO intervention and

10768±498 kJ before the CHO+PROT intervention ( $p=0.44$ ). Energy utilization during sprints were calculated from the ending workload and assuming an energy efficiency of 23%.

Plasma glucose was steady during the first 30 min interval. Thereafter, it gradually decreased during EXH to 3.8±0.4 (CHO) and 3.4±0.3 mM (CHO+PROT) immediately after EXH.  $VO_2$ , HR and RPE increased during EXH, but there were no differences between CHO compared to CHO+PROT prior to dietary interventions. RER was lower at 70 min compared to 30 min during EXH, but were similar between the treatment groups. Plasma insulin, leucine, isoleucine and valine decreased, while plasma FFA, glycerol and uric acid increased during EXH (Fig. 2).

Before and after EXH, the athletes performed a 10 s sprint. Mean power was lower during the sprint after EXH compared to before EXH, but there was no difference between the treatment groups (CHO: 1062±55 W vs. 998±55 W, CHO+PROT: 1068±50 W vs. 1000±52 W, pre EXH  $p=0.63$  and post EXH  $p=0.88$ ). Water intake during EXH prior to the intervention was similar in both treatment groups (CHO, 2527±275 ml; CHO+PROT, 2791±214 ml,  $p=0.30$ ). Weight loss was 1.3±0.4 kg and 1.1±0.3 kg in the CHO and CHO+PROT, respectively ( $p=0.35$ ).

*Intervention and Recovery Period:* Plasma glucose rose rapidly after the first intervention drink irrespective of the drink provided, and there was no treatment effect or difference in area under the curve ( $p=0.14$ ) (Fig. 2A). Plasma insulin increased during the first 120 min of recovery in both treatment groups but was higher during CHO+PROT compared with CHO (Fig. 2B, Time:  $p<0.001$ ; Treatment:  $p=0.194$ ; Interaction:  $p<0.002$ ). Plasma FFA and glycerol decreased and reached baseline level after 90 min (Fig. 2C, D). Plasma uric acid continued to increase during the 2 h of recovery ( $p<0.05$ ) (Fig. 2E). There was no difference between treatments for FFA, glycerol or uric acid  $p=0.51-0.9$ ). Leucine, isoleucine and valine rose during the first 2 hours of recovery in CHO+PROT, and were significantly difference between treatments ( $p<0.001$ ) (Fig. 2F-H).

*Performance after 18 h recovery:* Before the TT, subjects performed a 10 s modified Wingate sprint. The mean power during this sprint was higher after CHO+PROT compared to CHO (1063±54 W and 1026±53 W, respectively,  $p<0.01$ ; Fig. 3C).

Before the TT, subjects cycled 30 min at a workload corresponding to 73% of  $VO_{2max}$  ( $W_{73\%}$ ). During this 30 min, RPE was higher after CHO compared to CHO+PROT at 18 min ( $p=0.03$ )

and 30 min ( $p=0.001$ ) (Fig. 4A). Heart rate at  $W_{73\%}$  tended to be higher after CHO compared to CHO+PRO (Treatment effect:  $p=0.08$ ). T-test comparisons showed that heart rate was higher after CHO compared to CHO+PROT at 18 min ( $p=0.04$ ) and a tendency for a higher HR after 30 min ( $p=0.09$ ) (Fig. 4B). There was no difference between the interventions for  $VO_2$ , RER, oxidation of carbohydrate or fat, levels of blood lactate or plasma glucose ( $p=0.11-0.98$ ) (Fig. 4C-H). FFA, glycerol, uric acid and insulin were also similar between groups ( $p=0.38-0.8$ ) (Fig. 4L-O). Cycling economy was similar during  $W_{73\%}$  in CHO and CHO+PROT ( $\sim 4.2 \pm 0.2 \text{ kJ} \cdot \text{L O}_2^{-1}$ ).

After 30 min of completing the  $W_{73\%}$  test, the athletes performed the TT. CHO+PROT completed the TT 8.5% faster than CHO (Fig. 3A). The TT time after CHO+PROT was  $41:53 \pm 1:51$  min corresponding to a mean power of  $299 \pm 15$  W. The time to complete the TT after CHO was  $45:26 \pm 1:32$  min and corresponded to a mean power of  $275 \pm 15$  W ( $p < 0.01$  compared to CHO+PROT). The TT time one week before the interventions performed in a rested state (CON) was  $39:39 \pm 1:08$ , corresponding to a mean power of  $313 \pm 11$  W. The TT time for the CHO+PROT group was not significantly different from CON ( $p=0.12$ ), while the CHO group used a significantly longer time to complete the TT compared to CON ( $p=0.01$ ).

The power during the TT was higher after CHO+PROT compared to CHO in the interval of 26% to 93% of completed the TT (Fig. 3B). RPE during the TT was higher at 10 and 20% in the CHO group compared to the CHO+PROT group (Fig. 4A). HR was higher during the TT at 60% and 80% after CHO+PROT compared to CHO (Fig. 4B).  $VO_2$  was higher after CHO+PROT, compared to CHO at 15 min into, and at the end of the TT (corresponding to 50 and 80 min, respectively in Fig. 4C). There was no difference between interventions for carbohydrate ( $p=0.97$ ) or fat oxidation ( $p=0.11$ ) during the TT although the CHO+PROT group worked at a significantly higher power (Fig. 4E, 4F). At the end of the TT, athletes utilized more carbohydrate per min than at 4, 18 and 30 min of  $W_{73\%}$  as well as at 15 min of TT.

The athletes finished the performance session on day 2 with a second 10 s sprint 5 min after the TT. The mean power during the sprint was higher in the CHO+PROT group compared to the CHO group ( $1068 \pm 53$  W and  $1037 \pm 60$  W, respectively; Fig. 3C). Maximal power during the 10 s sprint tended to be higher after CHO+PROT compared to CHO ( $1864 \pm 93$  W and  $1770 \pm 91$  W,  $p=0.06$ ).

There were no differences between interventions during the session on day 2 concerning plasma levels of glucose FFA, glycerol, uric acid or insulin, ( $p=0.38-0.80$ ) (Fig. 4H, 4L-N, 4O). Plasma valine was consistently higher the day after the CHO+PROT compared to the CHO intervention (Fig. 4J; Treatment effect:  $p<0.001$ ; post hoc analyses:  $p<0.05$  at all time points). Plasma leucine was also higher the day after CHO+PROT compared to CHO (Fig. 4I; Treatment effect:  $p=0.017$ ), but the concentration before exercise did not differ. Plasma isoleucine did not differ between diets, but all branched-chain amino acids decreased during the performance tests.

To test the potential effect of plasma BCAA on performance, the differences in BCAA between CHO and CHO+PROT before the performance test on day 2 were correlated with differences in performance. The change in workload was calculated as the relative increase after intake of CHO+PROT compared to CHO (CHO=100%). Interestingly, the difference in performance (mean power during time trial) between CHO and CHO+PROT tended to correlate with differences in plasma valine ( $r=0.68$ ;  $p=0.062$ ; power 0.461) and plasma leucine ( $r=0.61$ ;  $p=0.11$ ), but there was no correlation for isoleucine ( $r=-0.09$ ;  $p=0.84$ ). The difference in performance at the 10 s sprint test did not correlate with differences in plasma BCAA ( $r=-0.03-0.31$ ;  $p=0.45-0.95$ ).

*Nitrogen balance and Urea:* Nitrogen intake during the recovery period was  $13.7\pm 0.5$  and  $23.9\pm 0.8$  g during CHO and CHO+PROT, respectively. Total nitrogen excretion was  $20.2\pm 1.0$  g and  $23.3\pm 1.4$  g during CHO and CHO+PROT, respectively, and significantly different between interventions. Thus, nitrogen balance was negative during CHO ( $-82.4\pm 11.5$  mg  $\cdot$  kg<sup>-1</sup>, -6.6 g) and significantly different from CHO+PROT ( $7.0\pm 15.4$  mg  $\cdot$  kg<sup>-1</sup>, 0.6 g), which was not different from zero (Fig. 5). In the CHO+PROT group, five athletes were in a positive nitrogen balance, while three were in a negative nitrogen balance. In the CHO group, all athletes were in a negative nitrogen balance. Furthermore, individual data showed that all athletes in the CHO group had a more negative nitrogen balance than the CHO+PROT group (Fig. 5A). Urinary creatinine excretion was  $1.9\pm 0.1$  and  $1.7\pm 0.1$  g in the CHO and CHO+PROT groups, respectively ( $p=0.06$ ). Corresponding values for urine nitrogen excretion and urine urea excretion were  $16.4\pm 0.8$  and  $19.0\pm 1.1$  g and  $29.6\pm 1.3$  and  $37.4\pm 2.4$  g ( $p<0.01$ ), respectively ( $p<0.01$ ) (Fig. 5C, D). Urine urea excretion correlated with urine nitrogen excretion ( $r=0.98$ ;  $p<0.01$ ).

## Discussion

We are the first to show that time trial and sprint performance 18 h after exhaustive exercise improves after co-ingestion of protein and carbohydrate during the 2 h window immediately after exercise, compared to carbohydrate only. We report three lines of evidences supporting our hypothesis that CHO+PROT supplementation is superior to isoenergetic CHO supplementation for performance recovery. Compared to carbohydrate alone, co-ingestion of carbohydrate and protein 1) improved time-trial performance, 2) improved sprint performance, and 3) reduced perceived exertion and HR during a standardized submaximal workload ( $W_{73\%}$ ). The lower performance after CHO, furthermore, coincided with a negative nitrogen balance despite intake of 1.1 g/kg protein during the 18 h recovery period, while nitrogen balance was neutral after CHO+PROT when intake of protein was 1.9 g/kg in the same period.

The present study is the first to report improved time trial performance after co-ingestion of protein and carbohydrate after a recovery period of 16-20 h. This finding is noteworthy because time trial performance tests have high reliability and validity relative to actual performance (15). The effect of CHO+PROT on the recovery of performance was convincing with TT performance improved by about 3.5 min (8.5%) compared to CHO. Williams et al. were among the first to show that co-ingestion of carbohydrate plus protein during a 5 h recovery from exhaustive exercise improved cycling time to exhaustion at 85% of  $VO_{2max}$  by 11 min (~55%) (61). Saunders and coworkers also used cycling to exhaustion at 85%  $VO_{2max}$  after a 16 h recovery, and showed that time to exhaustion was 12 min longer (~55%) after carbohydrate plus protein intake compared to carbohydrate only (53). Importantly, the percent increase in performance is not comparable in TT and time to exhaustion performance tests. TT performance tests have also been used to document a positive effect of co-ingestion of carbohydrate and protein on recovery of performance, but only after much shorter recovery periods. Berardi and coworkers found improved performance after intake of carbohydrate and protein compared to carbohydrate alone on a 60 min TT after 6 h of recovery (2), and Ferguson-Stegall et al. found improved performance on a 40 km TT after 4 h of recovery (~7%) when carbohydrate and protein were co-ingested (18). Our recent study showing improved time to exhaustion performance 18 h after exhaustive exercise when protein and carbohydrate were co-ingested compared to carbohydrate only (50), indicated that exhaustion occurred without depletion of carbohydrate stores after intake of carbohydrate only

immediately after exercise, suggesting that co-ingestion of protein and carbohydrate improved muscle function. The current study expands on these findings showing improved TT performance 18 h after exhaustive exercise when carbohydrate and protein were co-ingested compared to carbohydrate only.

To get further insight into the physiological effect of co-ingestion of protein and carbohydrate on performance, we used in the current study a TT performance test, which has been reported to be highly repeatable and to negatively correlated with muscle oxidative capacity in highly trained cyclists (29). Otherwise, the design of the present study was similar to our previous study in relation to exercise to exhaustion, dietary intervention and recovery period (50). Importantly, we demonstrate the superiority of CHO+PROT, compared to CHO, to improve TT performance 18 h after exhaustive exercise. After CHO+PROT and CHO intervention, the athletes were able to cycle at ~74% and ~68% of  $VO_{2max}$ , respectively. This is a major difference in performance in the competitive setting. The mean load during the TT was ~25 W higher after CHO+PROT compared to CHO, however, fat oxidation tended to be higher ( $p=0.08$ ) during the CHO+PROT trial, although elevating exercise intensity normally reduces fat oxidation. The changes in blood concentrations of glucose, lactate, FFA and glycerol during the TT were similar after CHO+PROT and CHO. It is therefore tempting to speculate that mitochondrial function is better after co-ingestion of protein and carbohydrate compared to carbohydrate only, as high mitochondrial oxidative capacity is directly associated with fat oxidative capacity (55). The facts that mitochondrial protein synthesis increases after protein intake (8) and exercise (10; 16; 17; 60) support this idea.

Additional, support of improved recovery after CHO+PROT compared to CHO was that RPE for the 30 min cycling at  $W_{73\%}$  was significantly lower for CHO+PROT compared with CHO. There was also a tendency for higher heart rate after CHO compared to CHO+PROT (treatment effect:  $p=0.086$ ) and T-tests showed significantly higher heart rates after CHO compared to CHO+PROT at 18 min ( $p<0.05$ ) and a tendency for a higher HR after 30 min ( $p=0.09$ ) of the  $W_{73\%}$  segment. The tendency for a higher heart rate at a standardized workload indicates that the physiological challenge was higher after CHO compared to CHO+PROT, and suggests better muscle function after CHO+PROT.

This is the first study to report that CHO+PRO supplementation during the first 2 h of recovery compared with CHO supplementation alone resulted in greater sprint performance



18 h later. High maximal force is a determinant for high maximal power (38) and cycling sprint performance (56). Equally important, sprint performance was higher both before as well as 5 min after the TT performance test after CHO+PROT supplementation compared to CHO supplementation. Since the final outcome in cycling often depends on sprint capacity, intake of protein in the recovery phase may determine the difference between becoming a winner or a loser in multiple stage races on consecutive days. In support of our data, protein intake (milk) has been shown to improve sprint performance 48 h after repeated sprint exercise (31). In contrast, Breen et al. did not show any effect of co-ingestion of protein and carbohydrate on maximal isometric strength the day following endurance exercise (11). However, maximal isometric strength may be a poor measure for recovery of muscle function, or ability to generate muscle power.

It is well known that protein intake immediately after exercise stimulates myofibrillar and mitochondrial protein synthesis of skeletal muscle (10; 24; 45). In the present study, we confirmed previous data (39; 50) that plasma concentrations of BCAA increase after co-ingestion of carbohydrate and protein, and are unchanged after carbohydrate alone. Elevated plasma amino acids post exercise prevent loss of amino acids from skeletal muscle (7) and stimulates protein synthesis (24). Many studies have shown that protein intake after exercise elevates phosphorylation of mTOR, p70S6-kinase and other signaling molecules that stimulate protein synthesis (36; 45; 47; 51; 60). Furthermore, elevated PGC-1 $\alpha$  mRNA has been reported in some (23; 47), but not in all studies (51) after protein supplementation. Unfortunately, the effect of protein intake on performance was not investigated in these studies. In fact, muscle biopsies have only been collected in one study where increased performance was found after co-ingestion of carbohydrate and protein (18). Importantly, Ferguson-Stegall et al. reported higher activation of anabolic signaling after co-ingestion of carbohydrate and protein compared to carbohydrate alone or placebo (18). However, there is a need for more studies that investigate the effect of co-ingestion of protein and carbohydrate supplementation post exercise on signaling mechanisms with protocols that improve recovery of performance.

We found that the nitrogen balance was negative after CHO (protein intake of 1.1 g/kg over 18 h) and neutral after CHO+PROT (1.9 g/kg over 18 h), suggesting that protein synthesis increased after co-ingestion of carbohydrate and protein. In this regard, it has been suggested that nitrogen balance status may determine performance outcome after dietary interventions

(46; 50). The negative nitrogen balance after CHO in our study is likely to indicate a catabolic state in skeletal muscles that reduces functional capacity and performance. We found a similar pattern in our previous study (50), i.e., that improved performance was linked to a positive nitrogen balance after CHO+PROT and a negative nitrogen balance after CHO alone (50). In the current study, nitrogen balance was negative in CHO where protein intake was 1.1 g/kg, and others have found that endurance athletes require 1.6-1.8 g/kg of protein daily to maintain nitrogen balance (30; 57). In our study, protein intake was substantially higher during the first 2 h during CHO+PROT than the dose required to stimulate protein synthesis maximally (37). Consequently, part of the ingested protein was metabolized and urinary excretion of nitrogen and urea were higher after co-ingestion of protein and carbohydrate compared to carbohydrate alone.

Plasma concentrations of all the BCAAs decreased during the exhaustive exercise prior to the dietary intervention, and plasma valine remained lower in the CHO group compared to CHO+PROT group the following day. This finding agrees with our previous study (50). Leucine oxidation increases during exercise (42; 63) and branched chain amino acids are mainly metabolized in skeletal muscle by mitochondrial branched chain amino transferase (mBCAT) (12). Furthermore, deletion of mBCAT dramatically reduces endurance capacity in mice (54). In the present study, both valine and leucine were lower during the performance tests in CHO compared to CHO+PROT, and it is tempting to speculate that metabolism of BCAA is required for optimal mitochondria function. Moreover, the difference in performance between CHO+PROT and CHO groups showed a strong correlation with differences in plasma valine ( $r=0.68$ ,  $p=0.062$ ) and plasma leucine ( $r=0.61$ ,  $p=0.11$ ). The lack of formal statistical significances probably reflects low power. Thus, our results add support to the premise that BCAA metabolism is important for exercise capacity (40), and that valine flux should be further studied during exercise and related to fatigue and to recovery.

Uric acid is the end product of purine degradation. In agreement with our previous study, uric acid increased after exhaustive exercise without a difference between the dietary interventions (50). These results indicate that intake of protein immediately after exercise does not influence degradation of purines and formation of uric acid.

The exercise prior to dietary intervention was demanding and other studies have shown that glycogen content decreases to a low level (22) under such physiological demands. Exercise

with low glycogen content stimulates protein degradation (6; 25; 33), and severe endurance exercise requires substantial protein to maintain nitrogen balance (57). In the present study, protein and carbohydrate were co-ingested in the early phase of recovery, which stimulates protein synthesis more intensely than when provided later (34). Strengths of the present study include the fact that the participants were experienced cyclists, familiarized with the tests, and adhered to standardized diet and training prior the two interventions. Moreover, the study had a randomized crossover design, and the performance tests were optimized by avoiding muscle biopsies. However, the lack of biopsies can also be considered a weakness since we lack data on glycogen, mitochondrial function and rate of protein synthesis in muscle. Another limitation is the relatively low sample number and low statistical power on some variables. In future studies, it will be important to study amino acid flux in muscle and at the whole body level as well as protein degradation.

In conclusion, supplementation with protein combined with carbohydrates within the first few hours after exhaustive cycling improves cycling time trial and sprint performance the following day. These improvements were correlated with a positive nitrogen balance during recovery, and suggest an increased catabolism of endogenous proteins in the absence of dietary protein intake after exercise. The mechanism for the benefits of carbohydrate plus protein intake supplementation following exercise remains unclear, but may be related to mitochondrial or myofibrillar protein synthesis, reduced protein breakdown or a combination of these events. The importance of the findings in this study cannot be overstated as they relate protein intake to endurance performance during competitions occurring over consecutive days.

## **Acknowledgements**

We thank Astrid Bolling, Eva Mølgaard Jensen and Lene Trudsø (Department of Clinical Medicine, Aarhus University) for technical assistance. Arla Food, Aarhus Denmark, provided the whey protein, Lacprodan, SP-9225 Instant. The study was in part supported by Arla Foods Ingredients Group P/S and Team Denmark, which is an organization funded by the Danish government with the purpose of promoting elite sports in Denmark. Present address for BN: Department of Internal Medicine, Bærum Hospital, Vestre Viken, Norway. Present address for FJ: Department of Pharmaceutical Biosciences, Uppsala University, Uppsala, Sweden.

## Reference List

1. **Antoniades C, Shirodaria C, Leeson P, Baarholm OA, Van-Assche T, Cunnington C, Pillai R, Ratnatunga C, Tousoulis D, Stefanadis C, Refsum H and Channon KM.** MTHFR 677 C>T Polymorphism reveals functional importance for 5-methyltetrahydrofolate, not homocysteine, in regulation of vascular redox state and endothelial function in human atherosclerosis. *Circulation* 119: 2507-2515, 2009.
2. **Berardi JM, Noreen EE and Lemon PW.** Recovery from a cycling time trial is enhanced with carbohydrate-protein supplementation vs. isoenergetic carbohydrate supplementation. *J Int Soc Sports Nutr* 5: 24, 2008.
3. **Berardi JM, Price TB, Noreen EE and Lemon PW.** Postexercise muscle glycogen recovery enhanced with a carbohydrate-protein supplement. *Med Sci Sports Exerc* 38: 1106-1113, 2006.
4. **Betts J, Williams C, Duffy K and Gunner F.** The influence of carbohydrate and protein ingestion during recovery from prolonged exercise on subsequent endurance performance. *J Sports Sci* 25: 1449-1460, 2007.
5. **Betts JA and Williams C.** Short-term recovery from prolonged exercise: exploring the potential for protein ingestion to accentuate the benefits of carbohydrate supplements. *Sports Med* 40: 941-959, 2010.

6. **Blomstrand E and Saltin B.** Effect of muscle glycogen on glucose, lactate and amino acid metabolism during exercise and recovery in human subjects. *J Physiol* 514 ( Pt 1): 293-302, 1999.
7. **Blomstrand E and Saltin B.** BCAA intake affects protein metabolism in muscle after but not during exercise in humans. *Am J Physiol Endocrinol Metab* 281: E365-E374, 2001.
8. **Bohe J, Low A, Wolfe RR and Rennie MJ.** Human muscle protein synthesis is modulated by extracellular, not intramuscular amino acid availability: a dose-response study. *J Physiol* 552: 315-324, 2003.
9. **Borsheim E, Cree MG, Tipton KD, Elliott TA, Aarsland A and Wolfe RR.** Effect of carbohydrate intake on net muscle protein synthesis during recovery from resistance exercise. *J Appl Physiol (1985 )* 96: 674-678, 2004.
10. **Breen L, Philp A, Witard OC, Jackman SR, Selby A, Smith K, Baar K and Tipton KD.** The influence of carbohydrate-protein co-ingestion following endurance exercise on myofibrillar and mitochondrial protein synthesis. *J Physiol* 589: 4011-4025, 2011.
11. **Breen L, Tipton KD and Jeukendrup AE.** No effect of carbohydrate-protein on cycling performance and indices of recovery. *Med Sci Sports Exerc* 42: 1140-1148, 2010.
12. **Brosnan JT and Brosnan ME.** Branched-chain amino acids: enzyme and substrate regulation. *J Nutr* 136: 207S-211S, 2006.
13. **Cormie P, McGuigan MR and Newton RU.** Developing maximal neuromuscular power: Part 1--biological basis of maximal power production. *Sports Med* 41: 17-38, 2011.

14. **Coyle EF, Coggan AR, Hemmert MK and Ivy JL.** Muscle glycogen utilization during prolonged strenuous exercise when fed carbohydrate. *J Appl Physiol* 61: 165-172, 1986.
15. **Currell K and Jeukendrup AE.** Validity, reliability and sensitivity of measures of sporting performance. *Sports Med* 38: 297-316, 2008.
16. **Di Donato DM, West DW, Churchward-Venne TA, Breen L, Baker SK and Phillips SM.** Influence of aerobic exercise intensity on myofibrillar and mitochondrial protein synthesis in young men during early and late postexercise recovery. *Am J Physiol Endocrinol Metab* 306: E1025-E1032, 2014.
17. **Donges CE, Burd NA, Duffield R, Smith GC, West DW, Short MJ, Mackenzie R, Plank LD, Shepherd PR, Phillips SM and Edge JA.** Concurrent resistance and aerobic exercise stimulates both myofibrillar and mitochondrial protein synthesis in sedentary middle-aged men. *J Appl Physiol (1985 )* 112: 1992-2001, 2012.
18. **Ferguson-Stegall L, McCleave EL, Ding Z, Doerner PG, III, Wang B, Liao YH, Kammer L, Liu Y, Hwang J, Dessard BM and Ivy JL.** Postexercise carbohydrate-protein supplementation improves subsequent exercise performance and intracellular signaling for protein synthesis. *J Strength Cond Res* 25: 1210-1224, 2011.
19. **Fernandez-Garcia B, Perez-Landaluce J, Rodriguez-Alonso M and Terrados N.** Intensity of exercise during road race pro-cycling competition. *Med Sci Sports Exerc* 32: 1002-1006, 2000.
20. **Goh Q, Boop CA, Luden ND, Smith AG, Womack CJ and Saunders MJ.** Recovery from cycling exercise: effects of carbohydrate and protein beverages. *Nutrients* 4: 568-584, 2012.

21. **Hansen M, Bangsbo J, Jensen J, Krause-Jensen M, Bibby BM, Sollie O, Hall UA and Madsen K.** Protein intake during training sessions has no effect on performance and recovery during a strenuous training camp for elite cyclists. *J Int Soc Sports Nutr* 13: 9, 2016.
22. **Hermansen L, Hultman E and Saltin B.** Muscle glycogen during prolonged severe exercise. *Acta Physiol Scand* 71: 129-139, 1967.
23. **Hill KM, Stathis CG, Grinfeld E, Hayes A and McAinch AJ.** Co-ingestion of carbohydrate and whey protein isolates enhance PGC-1alpha mRNA expression: a randomised, single blind, cross over study. *J Int Soc Sports Nutr* 10: 8, 2013.
24. **Howarth KR, Moreau NA, Phillips SM and Gibala MJ.** Coingestion of protein with carbohydrate during recovery from endurance exercise stimulates skeletal muscle protein synthesis in humans. *J Appl Physiol* 106: 1394-1402, 2009.
25. **Howarth KR, Phillips SM, MacDonald MJ, Richards D, Moreau NA and Gibala MJ.** Effect of glycogen availability on human skeletal muscle protein turnover during exercise and recovery. *J Appl Physiol* 109: 431-438, 2010.
26. **Ivy JL.** Muscle glycogen synthesis before and after exercise. *Sports Med* 11: 6-19, 1991.
27. **Ivy JL.** The regulation and synthesis of muscle glycogen by means of nutrient intervention. In: *The Encyclopaedia of Sports Medicine: An IOC Medical Commission Publication, Volume 19*, edited by Maughan RJ. Oxford, UK: Wiley-Blackwell Science Ltd, 2013, p. 113-125.



28. **Ivy JL, Goforth HW, Jr., Damon BM, McCauley TR, Parsons EC and Price TB.** Early postexercise muscle glycogen recovery is enhanced with a carbohydrate-protein supplement. *J Appl Physiol* 93: 1337-1344, 2002.
29. **Jacobs RA, Rasmussen P, Siebenmann C, Diaz V, Gassmann M, Pesta D, Gnaiger E, Nordsborg NB, Robach P and Lundby C.** Determinants of time trial performance and maximal incremental exercise in highly trained endurance athletes. *J Appl Physiol (1985)* 111: 1422-1430, 2011.
30. **Kato H, Suzuki K, Bannai M and Moore DR.** Protein Requirements Are Elevated in Endurance Athletes after Exercise as Determined by the Indicator Amino Acid Oxidation Method. *PLoS One* 11: e0157406, 2016.
31. **Kirk B, Mitchell J, Jackson M, Amirabdollahian F, Alizadehkhayat O and Clifford T.** A2 Milk Enhances Dynamic Muscle Function Following Repeated Sprint Exercise, a Possible Ergogenic Aid for A1-Protein Intolerant Athletes? *Nutrients* 9: 2017.
32. **Kjeldahl J.** Neue Methode zur Bestimmung des Stickstoffs in organischen Körper. *Analytische Chemie* 22: 366-382, 1883.
33. **Lemon PWR and Mullin JP.** Effect of initial muscle glycogen levels on protein catabolism during exercise. *Exerc Physiol* 48: 624-629, 1980.
34. **Levenhagen DK, Carr C, Carlson MG, Maron DJ, Borel MJ and Flakoll PJ.** Postexercise protein intake enhances whole-body and leg protein accretion in humans. *Med Sci Sports Exerc* 34: 828-837, 2002.

35. **Mariotti F, Tome D and Mirand PP.** Converting nitrogen into protein--beyond 6.25 and Jones' factors. *Crit Rev Food Sci Nutr* 48: 177-184, 2008.
36. **Moberg M, Apro W, Ekblom B, van HG, Holmberg HC and Blomstrand E.** Activation of mTORC1 by leucine is potentiated by branched-chain amino acids and even more so by essential amino acids following resistance exercise. *Am J Physiol Cell Physiol* 310: C874-C884, 2016.
37. **Moore DR, Robinson MJ, Fry JL, Tang JE, Glover EI, Wilkinson SB, Prior T, Tarnopolsky MA and Phillips SM.** Ingested protein dose response of muscle and albumin protein synthesis after resistance exercise in young men. *Am J Clin Nutr* 89: 161-168, 2009.
38. **Moss BM, Refsnes PE, Abildgaard A, Nicolaysen K and Jensen J.** Effects of maximal effort strength training with different loads on dynamic strength, cross-sectional area, load-power and load-velocity relationships. *Eur J Appl Physiol Occup Physiol* 75: 193-199, 1997.
39. **Nelson AR, Phillips SM, Stellingwerff T, Rezzi S, Bruce SJ, Breton I, Thorimbert A, Guy PA, Clarke J, Broadbent S and Rowlands DS.** A protein-leucine supplement increases branched-chain amino acid and nitrogen turnover but not performance. *Med Sci Sports Exerc* 44: 57-68, 2012.
40. **Overmyer KA, Evans CR, Qi NR, Minogue CE, Carson JJ, Chermiside-Scabbo CJ, Koch LG, Britton SL, Pagliarini DJ, Coon JJ and Burant CF.** Maximal oxidative capacity during exercise is associated with skeletal muscle fuel selection and dynamic changes in mitochondrial protein acetylation. *Cell Metab* 21: 468-478, 2015.

41. **Peronnet F and Massicotte D.** Table of nonprotein respiratory quotient: an update. *Can J Sport Sci* 16: 23-29, 1991.
42. **Phillips SM, Atkinson SA, Tarnopolsky MA and MacDougall JD.** Gender differences in leucine kinetics and nitrogen balance in endurance athletes. *J Appl Physiol (1985 )* 75: 2134-2141, 1993.
43. **Romano-Ely BC, Todd MK, Saunders MJ and Laurent TS.** Effect of an isocaloric carbohydrate-protein-antioxidant drink on cycling performance. *Med Sci Sports Exerc* 38: 1608-1616, 2006.
44. **Romijn JA, Coyle EF, Sidossis LS, Gastaldelli A, Horowitz JF, Endert E and Wolfe RR.** Regulation of endogenous fat and carbohydrate metabolism in relation to exercise intensity and duration. *Am J Physiol* 265: E380-E391, 1993.
45. **Rowlands DS, Nelson AR, Phillips SM, Faulkner JA, Clarke J, Burd NA, Moore D and Stellingwerff T.** Protein-leucine fed dose effects on muscle protein synthesis after endurance exercise. *Med Sci Sports Exerc* 47: 547-555, 2015.
46. **Rowlands DS, Rossler K, Thorp RM, Graham DF, Timmons BW, Stannard SR and Tarnopolsky MA.** Effect of dietary protein content during recovery from high-intensity cycling on subsequent performance and markers of stress, inflammation, and muscle damage in well-trained men. *Appl Physiol Nutr Metab* 33: 39-51, 2008.
47. **Rowlands DS, Thomson JS, Timmons BW, Raymond F, Fuerholz A, Mansourian R, Zwahlen MC, Metairon S, Glover E, Stellingwerff T, Kussmann M and Tarnopolsky MA.** Transcriptome and translational signaling following endurance exercise in trained skeletal muscle: impact of dietary protein. *Physiol Genomics* 43: 1004-1020, 2011.

48. **Rowlands DS, Thorp RM, Rossler K, Graham DF and Rockell MJ.** Effect of protein-rich feeding on recovery after intense exercise. *Int J Sport Nutr Exerc Metab* 17: 521-543, 2007.
49. **Rowlands DS and Wadsworth DP.** Effect of high-protein feeding on performance and nitrogen balance in female cyclists. *Med Sci Sports Exerc* 43: 44-53, 2011.
50. **Rustad PI, Sailer M, Cumming KT, Jeppesen PB, Kolnes KJ, Sollie O, Franch J, Ivy JL, Daniel H and Jensen J.** Intake of Protein Plus Carbohydrate during the First Two Hours after Exhaustive Cycling Improves Performance the following Day. *PLoS One* 11: e0153229, 2016.
51. **Samuelsson H, Moberg M, Apro W, Ekblom B and Blomstrand E.** Intake of branched-chain or essential amino acids attenuates the elevation in muscle levels of PGC-1 $\alpha$  mRNA caused by resistance exercise. *Am J Physiol Endocrinol Metab* 311: E246-E251, 2016.
52. **Sanchez-Munoz C, Zabala M and Muros JJ.** Nutritional intake and anthropometric changes of professional road cyclists during a 4-day competition. *Scand J Med Sci Sports* 26: 802-808, 2016.
53. **Saunders MJ, Kane MD and Todd MK.** Effects of a carbohydrate-protein beverage on cycling endurance and muscle damage. *Med Sci Sports Exerc* 36: 1233-1238, 2004.
54. **She P, Zhou Y, Zhang Z, Griffin K, Gowda K and Lynch CJ.** Disruption of BCAA metabolism in mice impairs exercise metabolism and endurance. *J Appl Physiol (1985 )* 108: 941-949, 2010.
55. **Stisen AB, Stougaard O, Langfort J, Helge JW, Sahlin K and Madsen K.** Maximal fat oxidation rates in endurance trained and untrained women. *Eur J Appl Physiol* 98: 497-506, 2006.

56. **Stone MH, Sands WA, Carlock J, Callan S, Dickie D, Daigle K, Cotton J, Smith SL and Hartman M.** The importance of isometric maximum strength and peak rate-of-force development in sprint cycling. *J Strength Cond Res* 18: 878-884, 2004.
57. **Tarnopolsky MA, MacDougall JD and Atkinson SA.** Influence of protein intake and training status on nitrogen balance and lean body mass. *J Appl Physiol (1985 )* 64: 187-193, 1988.
58. **Thomson JS, Ali A and Rowlands DS.** Leucine-protein supplemented recovery feeding enhances subsequent cycling performance in well-trained men. *Appl Physiol Nutr Metab* 36: 242-253, 2011.
59. **van Loon LJ, Greenhaff PL, Constantin-Teodosiu D, Saris WH and Wagenmakers AJ.** The effects of increasing exercise intensity on muscle fuel utilisation in humans. *J Physiol* 536: 295-304, 2001.
60. **Wilkinson SB, Phillips SM, Atherton PJ, Patel R, Yarasheski KE, Tarnopolsky MA and Rennie MJ.** Differential effects of resistance and endurance exercise in the fed state on signalling molecule phosphorylation and protein synthesis in human muscle. *J Physiol* 586: 3701-3717, 2008.
61. **Williams M, Raven PB, Fogt DL and Ivy JL.** Effects of recovery beverages on glycogen restoration and endurance exercise performance. *J Strength Cond Res* 17: 12-19, 2003.
62. **Wolfe RR.** Does exercise stimulate protein breakdown in humans?: isotopic approaches to the problem. *Med Sci Sports Exerc* 19: 172-178, 1987.

63. **Wolfe RR, Goodenough RD, Wolfe MH, Royle GT and Nadel ER.** Isotopic analysis of leucine and urea metabolism in exercising humans. *J Appl Physiol Respir Environ Exerc Physiol* 52: 458-466, 1982.
  
64. **Zawadzki KM, Yaspelkis BB, III and Ivy JL.** Carbohydrate-protein complex increases the rate of muscle glycogen storage after exercise. *J Appl Physiol* 72: 1854-1859, 1992.

## Legends

### Tables

**Table 1:** Energy intake and content of macronutrients for the different standardized meals and supplements during the 16 h of the recovery period, when the diets were similar between groups, and adjusted for bodyweight.

**Table 2:** Blood glucose, blood lactate,  $VO_2$ , RER, carbohydrate and fat oxidation, HR and RPE during EXH. During the first interval (30 min), measurements were taken at 3-4 min and the last two min of the interval. On the rest of the intervals (20 min), measurements were taken during the two last min of each interval. Measurements were also taken the last two min before voluntary exhaustion ( $W_{73\%End}$ ). Furthermore, blood lactate and blood glucose were measured before and after the one min bouts at 90% of  $VO_{2max}$ .

### Figures

**Figure 1:** Design of the interventions. The protocol was completed twice in a double-blinded, randomized, balanced, crossover experimental design. The athletes reported to the laboratory 11:30 AM on day 1 and started an exhaustive training protocol (EXH) at 12:00 PM. EXH consisted of exhaustive cycling at 73% and 90% of  $VO_{2max}$ , and two 10 s sprints (at the start and at the end of EXH). After EXH, an 18 h standardized isoenergetic recovery period started, where the subjects were fed either carbohydrate alone (CHO group) or carbohydrate and protein (CHO+PROT group) the first 2 h. The diet during the rest of the recovery period was similar for both interventions. On day 2, the athletes ate a standardized breakfast at 07:00 AM and reported to the laboratory 08:30 AM. The performance tests started 09:00 AM, and consisted of a 10 s sprint, 30 min at  $W_{73\%}$ , a time-trial performance test, and a second 10 s sprint 5 min after the time-trial.

**Figure 2:** Plasma metabolites before and after exhaustive exercise, and during the dietary interventions. Carbohydrate or carbohydrate and protein were provided during the immediate recovery (0-120 min) as described. Plasma levels of glucose (A), insulin (B), FFA (C), glycerol (D), uric acid (E), leucine (F), isoleucine (G), valine (H) were measured before exercise (-110 min) and during the 2 h of recovery from exhaustive exercise when participants received energy drinks. Values are mean  $\pm$  SEM. E:  $p < 0.05$  compared to basal value before

exercise. a:  $p < 0.05$  compared to immediately after exercise ( $t=0$ ). b:  $p < 0.05$  compared to CHO.

**Figure 3.** Performance from the time-trial and 10 s sprint tests the day after the diet interventions. Average time spent on the time trial (A), power curve during TT (B), mean power during the sprints (C). Values are mean  $\pm$  SEM. \* In Fig. 3A and 3C  $p < 0.05$  compared to CHO. In Fig. 3B, \*  $p < 0.05$  comparing CHO and CHO+PROT for each 10% interval.

**Figure 4:** Measurements from the performance test on day 2. Time 0 is at arrival before warm up. Time 15 and 30 is after 15 and 30 min cycling at of  $W_{73\%}$ . Time 80 is at the end of TT, and 95 is 15 min after completion of TT. RPE (A) and heart rate (B) were taken at 4, 18, 30 min of  $W_{73\%}$ , and at 10%, 20%, 60%, 80% and at 15 min and at the end of the time trial.  $VO_2$  (C), RER (D), carbohydrate oxidation (E), fat oxidation (F), blood lactate (G), plasma glucose (H), were taken at 4, 18 and 30 min of  $W_{73\%}$ , and at 15 min and at the end of TT. Glucose was also taken at time 0. Leucine (I), valine (J), isoleucine (K), FFA (L), glycerol (M), uric acid (N) and insulin (O) were taken before, at 15 min, and at the end of  $W_{73\%}$ , and at 15 min and at the end of TT and 15 min after TT. Values are mean  $\pm$  SEM. T= Time effect; P=treatment effect between groups. \* Time points with differences between CHO and CHO+PROT ( $p < 0.05$ ).

**Figure 5:** Nitrogen balance and urine excretion of nitrogen, urea and BCAA during the 18 h recovery period. Nitrogen balance was calculated from protein intake and urine nitrogen. Nitrogen balances for CHO and CHO+PROT during the recovery period with individual responses (A) and responses for the different time periods for urine collection (B), total nitrogen excretion (C), total urea excretion (D), \* Significant differences between groups. Values are mean  $\pm$  SEM.  $p < 0.05$ .



## Tables

**Table 1**

	Time	Time after EXH	Carbohydrate (g·kg <sup>-1</sup> )	Protein (g·kg <sup>-1</sup> )	Fat (g·kg <sup>-1</sup> )	Energy content (kJ·kg <sup>-1</sup> )
Dinner	~ 5:00 pm	~ 2 h	1.4	0.47	0.29	39.01
Evening carbohydrate drink	~ 7:00 pm	~ 4 h	1.2	0	0	20.40
Supper	~ 9:00 pm	~ 6 h	0.97	0.29	0.24	30.54
Breakfast	~ 7:00 am	~ 16 h	1.32	0.32	0.24	37.00

**Table 2**

	0 min	3-4 min	28-30 min	48-50 min	68-70 min	W <sub>73%</sub> End	Exhaustion
<b>Blood glucose (mM)</b>							
CHO	4.9±0.3	4.9±0.3	5.5±0.3	4.6±0.3	3.8±0.3 <sup>c,α</sup>	4.0±0.2 <sup>a,b,c</sup>	3.8±0.3 <sup>a,b,c,d</sup>
CHO+PRO	5.0±0.2	5.0±0.2	5.3±0.3	4.5±0.3	4.2±0.2 <sup>c</sup>	3.6±0.3 <sup>a,b,c</sup>	3.4±0.3 <sup>a,b,c,d</sup>
<b>Blood lactate (mM)</b>							
CHO	1.19±0.10	2.88±0.44 <sup>a</sup>	2.42±0.36 <sup>a</sup>	2.24±0.42 <sup>a</sup>	1.97±0.39 <sup>α</sup>	2.60±0.33 <sup>a</sup>	2.89±0.36 <sup>a,*</sup>
CHO+PRO	0.91±0.14	2.75±0.48 <sup>a</sup>	2.26±0.44 <sup>a</sup>	1.88±0.38 <sup>a</sup>	1.82±0.39	2.24±0.37 <sup>a</sup>	2.45±0.32 <sup>a,*</sup>
<b>VO<sub>2</sub> (ml·min<sup>-1</sup>)</b>							
CHO		4163±162	4354±172 <sup>b</sup>	4352±173 <sup>b</sup>	4336±203 <sup>b,α</sup>	4509±213 <sup>b,c,d,e</sup>	
CHO+PRO		4149±177	4280±164 <sup>b</sup>	4360±177 <sup>b</sup>	4422±192 <sup>b</sup>	4516±248 <sup>b,c,d,e</sup>	
<b>RER (VO<sub>2</sub>·CO<sub>2</sub><sup>-1</sup>)</b>							
CHO		0.89±0.01	0.90±0.01	0.89±0.01	0.88±0.01 <sup>c,α</sup>	0.89±0.01	
CHO+PRO		0.88±0.01	0.90±0.02	0.89±0.02	0.88±0.02 <sup>c</sup>	0.88±0.02	
<b>Carbohydrate oxidation (g·min<sup>-1</sup>)</b>							
CHO		3.7±0.3	4.0±0.2	3.9±0.3	3.6±0.3 <sup>α</sup>	3.9±0.2	
CHO+PRO		3.4±0.2	4.0±0.3	3.5±0.2	3.7±0.3	3.8±0.5	
<b>Fat oxidation (g·min<sup>-1</sup>)</b>							
CHO		0.8±0.1	0.8±0.1	0.8±0.1	0.9±0.1 <sup>α</sup>	0.9±0.1	
CHO+PRO		0.9±0.1	0.7±0.1	1.0±0.1	0.9±0.1	0.9±0.2	
<b>HR (beats·min<sup>-1</sup>)</b>							
CHO		156±4	169±3 <sup>b</sup>	170±4 <sup>b</sup>	167±2 <sup>b,α</sup>	178±3 <sup>b,c,d,e</sup>	
CHO+PRO		155±4	166±3 <sup>b</sup>	166±4 <sup>b</sup>	168±4 <sup>b</sup>	175±3 <sup>b,c,d,e</sup>	
<b>RPE (Borg Scale)</b>							
CHO		12.8±0.5	13.9±0.6	14.6±0.7 <sup>b</sup>	14.6±0.4 <sup>b,c,α</sup>	18.8±0.3 <sup>b,c,d,e</sup>	
CHO+PRO		12.6±0.5	13.6±0.5	14.1±0.7 <sup>b</sup>	15.0±0.7 <sup>b,c</sup>	18.4±0.4 <sup>b,c,d,e</sup>	

Data is mean ± SEM. N=8 (<sup>α</sup> N=7 for CHO 68-70 min; one athlete did not manage to cycle for 70 min before CHO intervention) <sup>a</sup> Significant different from 0 min <sup>b</sup> Significant different from 4 min <sup>c</sup> Significant different

from 30 min. <sup>d</sup> Significant different from 50 min. <sup>e</sup> Significant different from 70 min. \*Treatment effect between groups.  $p < 0.05$ .

## Figures

Figure 1

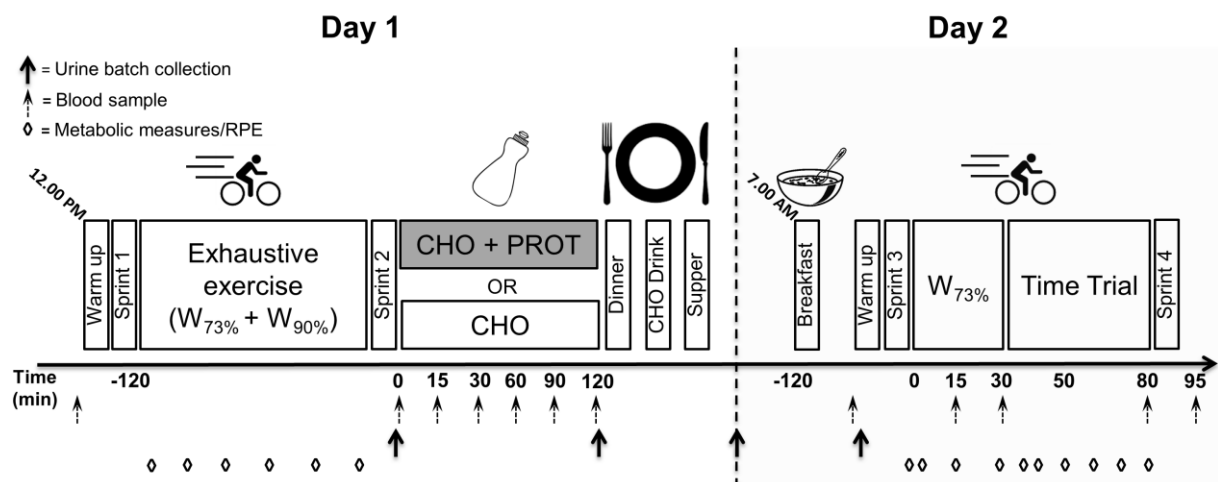


Figure 2

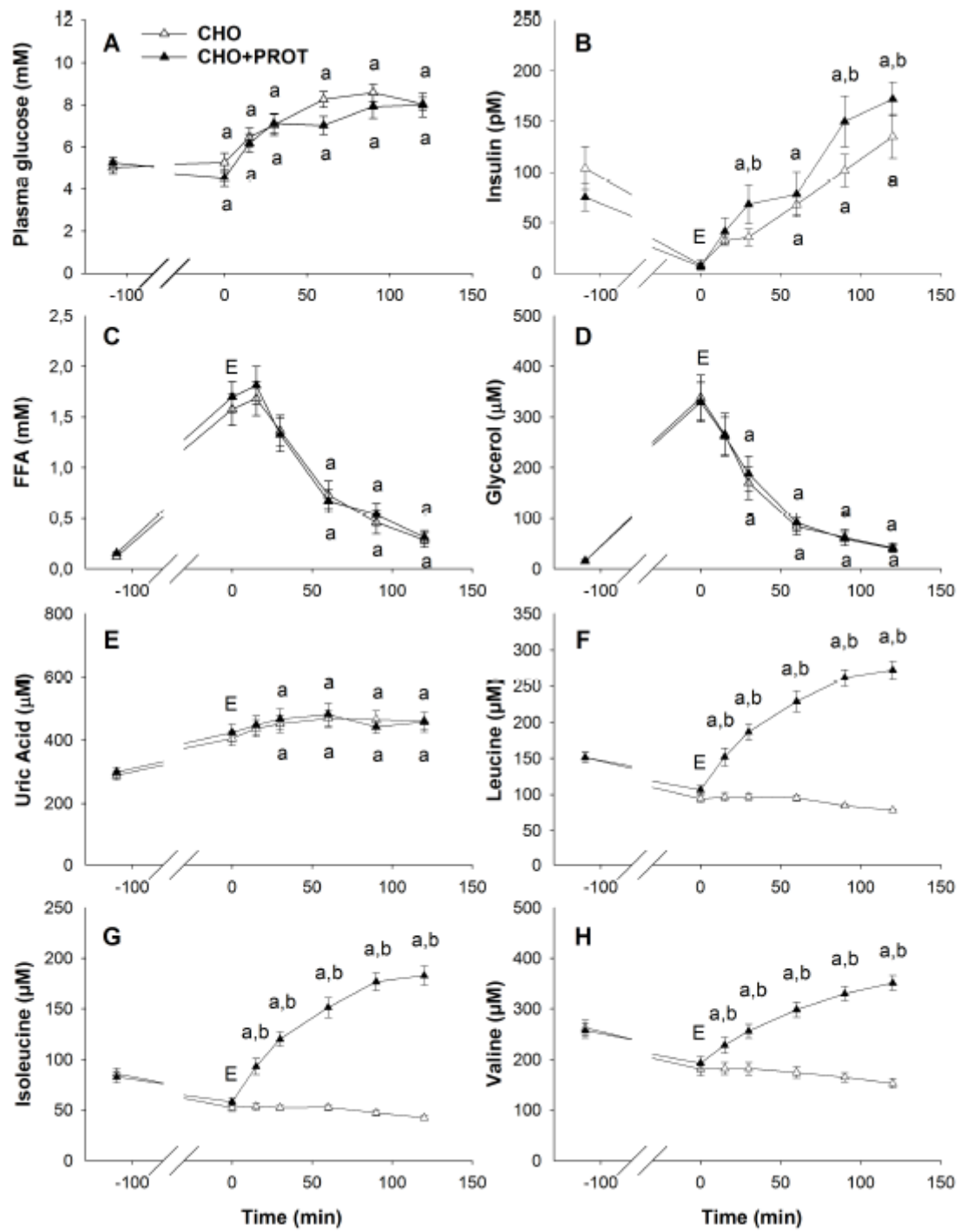
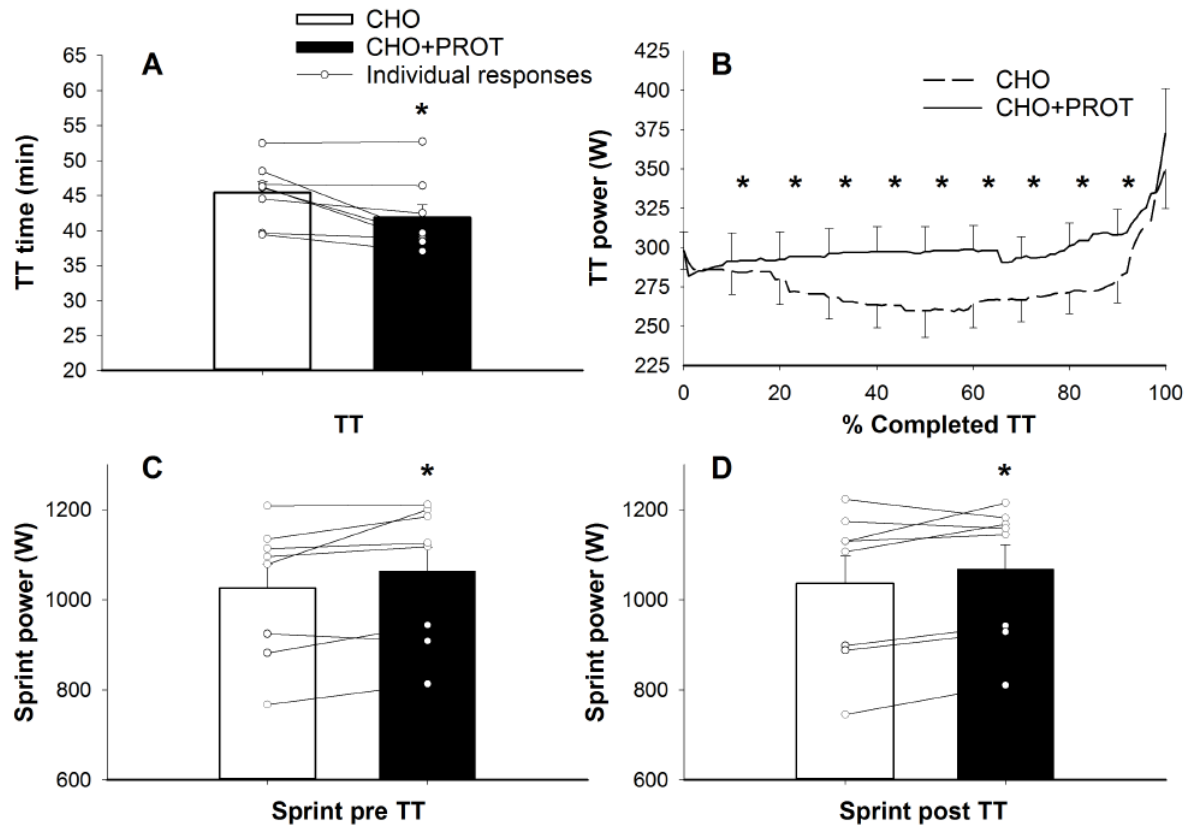


Figure 3



**Figure 4**

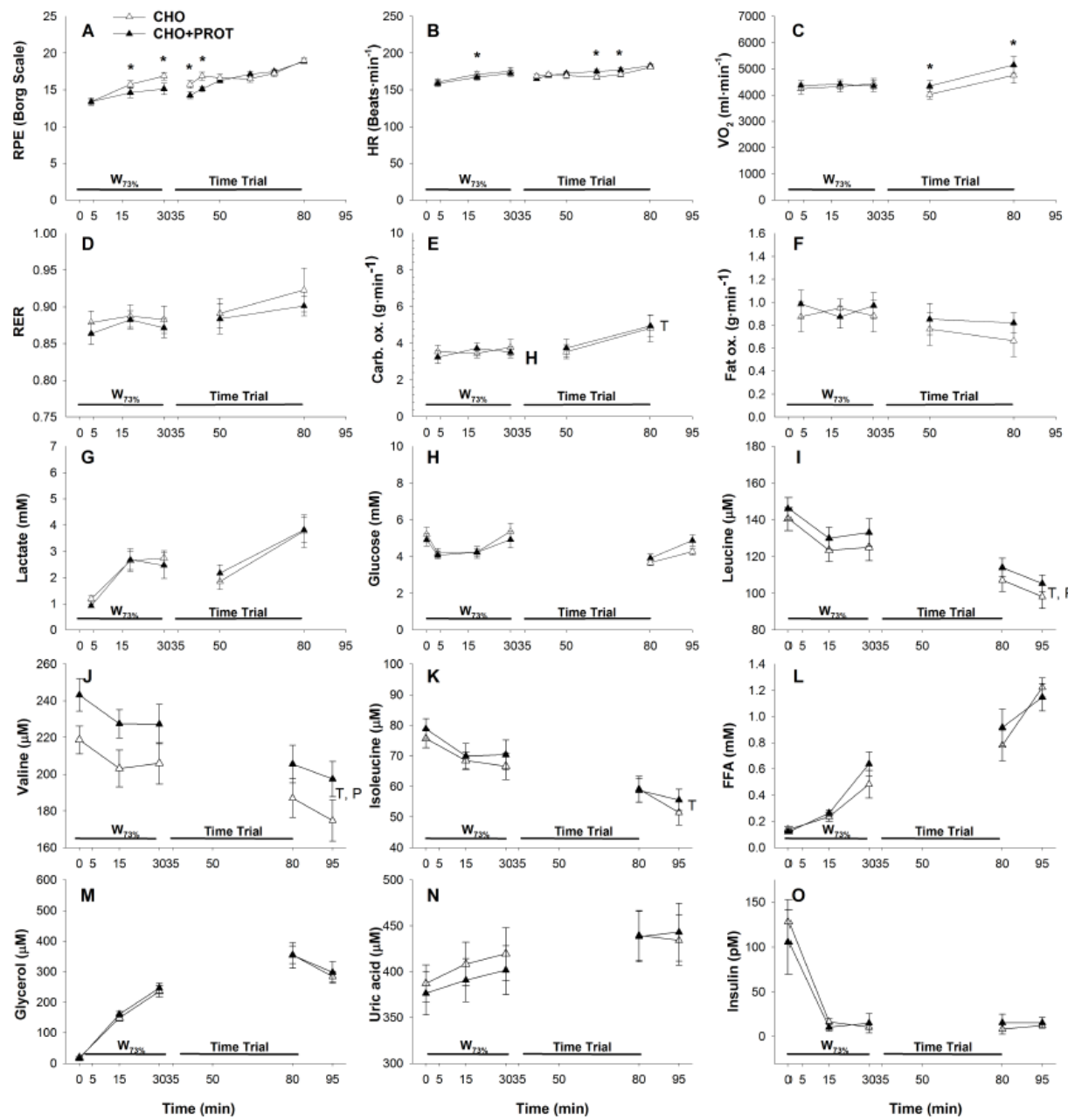


Figure 5

