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Human Kinetics
ORIGINAL RESEARCH

Presleep Protein Supplementation Does Not Improve Recovery During Consecutive Days of Intense Endurance Training: A Randomized Controlled Trial

Mads S. Larsen
Aarhus University and Arla Foods Ingredients P/S

Dagmar Clausen and Astrid Ank Jørgensen
Aarhus University

Ulla R. Mikkelsen
Arla Foods Ingredients P/S

Mette Hansen
Aarhus University

Recent studies demonstrate that protein ingestion immediately before sleep improves muscle recovery during the night following resistance exercise. Whether this feeding strategy benefits recovery from endurance training has yet to be established. The aim of this study was to investigate the effects of whey protein isolate ingested every night before sleep on subsequent performance and circulatory markers of muscular recovery during a week of intensified endurance training mimicking a training camp. In a parallel design, 32 trained runners underwent a 1-week intervention with a rigorously controlled diet (carbohydrate = 7.2 $g \cdot kg^{-1} \cdot day^{-1}$, protein = 1.8 $g \cdot kg^{-1} \cdot day^{-1}$, and fat = 1.0 $g \cdot kg^{-1} \cdot day^{-1}$) and exercise program (11 sessions) while receiving either a protein (0.5 $g \cdot kg^{-1} \cdot day^{-1}$) or carbohydrate (0.5 $g \cdot kg^{-1} \cdot day^{-1}$) beverage every night before sleep. Blood samples were obtained on the morning of Days 1, 4, 7, and 8 and analyzed for markers of muscle damage (creatine kinase, lactate dehydrogenase, and myoglobin). The postintervention 5-km time-trial performance was significantly impaired in both groups (11 ± 24 s, $p < .01$). Plasma creatine kinase (227% ± 221%, $p < .01$), lactate dehydrogenase (18% ± 22%, $p < .01$), and myoglobin (72% ± 62%, $p < .01$) increased gradually throughout the week with no difference between the groups ($p > .05$). In conclusion, the presleep protein ingestion did not reduce the decline in performance or ameliorate the rise of circulatory markers of muscle damage during a week of intensified training when compared with the isocaloric carbohydrate ingestion.

Keywords: dietary protein, endurance performance, muscle damage

Periodically, most elite endurance athletes undertake high-intensity exercise, for example, during training camps or meets. Uncustomary intense and frequent exercise places the musculoskeletal system under great strain. To maintain training intensity and/or performance and reduce the risk of overuse injuries, efficient muscle recovery is of special importance during such periods. Although the cellular mechanisms driving the acute regenerative processes are not well elucidated, a growing number of studies have unveiled the benefits of protein feeding strategies in regard to optimizing recovery from endurance exercise (Moore et al., 2014), including attenuated circulatory markers of sarcolemma disruption (Hansen et al., 2015; Saunders et al., 2004, 2007; Valentine et al., 2008), improvements in protein balance (Howarth et al., 2009; Koopman et al., 2004), glycogen resynthesis (Levenhagen et al., 2001; van Loon et al., 2000), and markers of immune function (Witard et al., 2014). Furthermore, studies show an accelerated regain of strength and function following a single bout of muscle-damaging resistance exercise when supplementing with whey protein (Buckley et al., 2010; Cooke et al., 2010). Traditionally, protein-feeding strategies have focused on the time immediately surrounding or during the exercise session. However, emerging evidence indicates that ingestion of dietary protein prior to overnight sleep may potentiate recovery. During the last decade, a series of studies have shown that protein provision immediately prior to or during overnight sleep is digested and released into the circulation, therefore, stimulating muscle protein synthesis (Groen et al., 2012; Kouw et al., 2017; Trommelen et al., 2018). This approach adds to the conventional way of administering protein in the hours surrounding exercise sessions. Following resistance exercise, presleep protein feeding promotes overnight recovery by enhancing muscle protein synthesis (Res et al., 2012). Furthermore, a 12-week resistance training study demonstrated additional gains in muscle mass and strength when protein, rather than a noncaloric placebo, was ingested before sleep (Snijders et al., 2015). Thus, the

Larsen, Clausen, Jørgensen, and Hansen are with the Section for Sports Science, Department of Public Health, Aarhus University, Aarhus, Denmark. Larsen and Mikkelsen are with Arla Foods Ingredients P/S, Viby J, Denmark. Hansen (mhan@ph.au.dk) is corresponding author.

beneficial effects of presleep protein supplementation on muscle protein synthesis rates seem well substantiated. However, the potential impact of presleep protein feeding may reach beyond improved gains in muscle mass and strength. It is well established that protein ingestion increases both myofibrillar and mixed muscle protein synthesis after endurance exercise (Moore et al., 2014). This response is clearly an expression of increased muscle protein turnover inducing tissue repair, tissue remodeling, and adaptation to endurance training, more so than promoting muscle protein accretion. Previous findings from our laboratory suggest that the ingestion of 0.3 g protein per kg body weight immediately before and after endurance training sessions improves performance and reduces markers of muscle damage in elite runners during a strenuous 1-week training camp (Hansen et al., 2015). The benefits of the protein-feeding strategy in the study of Hansen et al. (2015) were observed in spite of the control group meeting the current recommendations of daily protein intake for endurance athletes (~1.7 $g \cdot kg^{-1} \cdot day^{-1}$; Jager et al., 2017).

The present trial aimed to study if protein feeding before sleep would aid athletes in maintaining performance capacity during a period of intensified training. We hypothesized that the ingestion of protein supplements every night prior to sleep would reduce muscle damage and the decline in performance after 1 week of intensified training compared with controls receiving an isocaloric carbohydrate supplement. To our knowledge, this is the first study to assess the effect of presleep protein supplements on recovery from endurance training.

Methods

Experimental Protocol

The study was designed as a controlled, double-blinded randomized paired design (Figure 1). Following preliminary testing, the subjects were divided into two groups, ingesting either a whey protein isolate drink (PRO, $n = 17$) or an isocaloric carbohydrate drink (CHO, $n = 17$) immediately before sleep every night of the 1-week training intervention. A 5-km time trial (TT) was performed on Day 1 and Day 8.

On the morning of Days 1, 4, 7, and 8, the subjects reported to the lab for blood sampling, body weight measurements, and questionnaires. The blood samples were analyzed for markers of muscle damage (creatine kinase [CK], lactate dehydrogenase [LDH], and myoglobin [Mb]) and cortisol.

All meals during the study were provided by the research team and controlled for energy and macronutrient content.

Prior to giving their written consent to participate, all subjects were informed of the possible risks of all procedures. The study was registered at www.clinicaltrials.gov (identifier ID: NCT03147833), approved by the ethics committee of the Central Region of Denmark (1-10-72-292-16), and adhered to the Declaration of Helsinki.

Subjects and Pairing

A total of 34 trained male runners were included in the study. The subjects were paired based on their fitness level (VO_2peak), 5-km TT performance, physical characteristics (age and weight), and training history.

To get an accurate estimate of the participants' training status, they were asked to upload their last 2 months of training history from their sport watches to an Internet-based training management software (Sportlyzer, Tartu, Estonia). In addition, all participants filled out a questionnaire regarding their training habits.

Within each pair, the subjects were randomly assigned to either PRO or CHO and completed the exact same training program. The subjects were unaware of the pairing throughout the study period. Subject characteristics are presented in Table 1.

Exercise Testing

Prior to inclusion, the volunteers underwent a screening protocol consisting of a 5-km TT and assessment of peak oxygen consumption.

5-km TT. A 5-km TT was performed on three occasions: (a) prior to the inclusion of the subjects, (b) on Day 1 of the intervention (baseline test, PRE), and (c) at the end of the intervention (Day 8, POST). The participants were asked to perform the run as fast as possible on all three occasions. The first TT was used to determine if the volunteer was eligible for inclusion in the study and as a familiarization trial. Only those subjects able to complete the run in less than 21 min underwent further assessments. To avoid anyone pacing their run, the subjects were unaware of these inclusion criteria. To minimize the influence of changing weather conditions on the ground surface, the 5-km TT was performed on a 2.5-km paved path (out and back) instead of synthetic track surfacing. All runners wore a heart rate monitor. To avoid pacing, the heart rate monitors were covered with black tape, and the start times were staggered by 1 min.

VO_2peak. The VO_2peak was determined using a progressive running test on a treadmill, during which gas exchange measurements were carried out every 10 s using a computerized mixing bag system (O2CPX with Innocore software; Innovision ApS, Glamsbjerg, Denmark). The VO_2peak was defined as the highest oxygen uptake averaged during any 30 s of test. Before commencing the test, a 10-min self-chosen warm-up was allowed. The test was

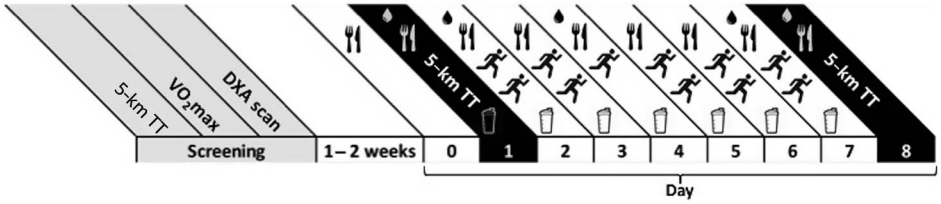

Figure 1 — Study design. Overview of study design including 8 days of intervention. Diet control. Training session. Two symbols indicate training in the morning and in the afternoon. Blood samples and body weight measurements in the morning. Protein ($n = 14$) or carbohydrate ($n = 16$) supplement. DXA = dual-energy X-ray absorptiometry; TT = time trial.

Table 1 Subject Characteristics, Diet, and Training

	Characteristics							Diet				Training		
	Age (years)	Body mass (kg)	Body mass DXA (kg)	Lean body mass (kg)	BMI (kg/m^2)	VO_2peak (LO_2/min)	Relative VO_2peak ($LO_2 \cdot min^{-1} \cdot kg^{-1}$)	Energy (MJ/day)	Protein (g/day)	Carbohydrate (g/day)	Fat (g/day)	Sessions (per week)	Duration (min/week)	Distance (km/week)
CHO	26.3 ± 6.8	74.0 ± 7.0	74.9 ± 7.0	65.7 ± 6.5	21.8 ± 1.8	4.7 ± 0.5	64 ± 7	14.3 ± 0.9	134 ± 12	524 ± 30	71 ± 6	10.9 ± 0.5	575 ± 94	102 ± 25
PRO	27.3 ± 7.4	73.6 ± 8.6	74.4 ± 8.5	64.1 ± 7.1	22.0 ± 1.8	4.5 ± 0.5	62 ± 6	14.3 ± 0.8	134 ± 15	527 ± 31	70 ± 6	10.6 ± 1.0	542 ± 100	99 ± 22
p	.70	.88	.84	.52	.75	.39	.38	.70	.84	.44	.16	.39	.36	.75

Note. Data are presented as mean ± *SD*. DXA = dual-energy X-ray absorptiometry; BMI = body mass index; CHO = carbohydrate; PRO = protein.

initiated at a speed of 15 km/hr. After 2 min, the speed was increased by 1 km/hr, which was repeated every minute until volitional exhaustion.

Assessment of Body Weight and Composition

Body composition was determined using dual-energy X-ray absorptiometry (GE Lunar DXA scan; GE Healthcare, Chicago, IL). Body weight was monitored at all laboratory visits using a bioelectrical impedance analyzer (TBF-310GS Body Composition Analyzer; Tanita Corporation of America, Inc., Chicago, IL).

Diet Control

During the study, all meals, snacks, and drinks, excluding water, were provided by the research team. Diet compositions were analyzed using a software program (Vitakost ApS, Kolding, Denmark). The total daily energy expenditure was estimated as basal metabolic rate (Cunningham, 1980) × daily physical activity–level factor besides training (set to 1.5) + estimated energy expenditure during training (Keytel et al., 2005). All suppers were consumed at the university cafeteria. An evening snack was to be consumed before 8 p.m. Only the water and the experimental drink were allowed after 8 p.m.

The energy content of the basic diet balanced the estimated individual energy expenditure. Energy provisions were evaluated and adjusted in case of changes in body weight exceeding ±0.5 kg from the baseline and based on the individual subject's perception of satiety. The macronutrient composition of the basic diet is presented in Table 1. The food intake 24 hr prior to the two performance tests was exactly matched. Water was allowed ad libitum at all times. No subject reported any waste of food.

Presleep Drinks

Each subject consumed a presleep drink immediately before bedtime every day during the study period. The subjects were blinded to the content of the presleep beverages. To mask the content, each drink (protein and carbohydrate) came in two flavors (protein: apple and grapefruit; carbohydrate: berry and pomegranate). Each subject received the same flavored drink every night by random allocation. The drinks contained either 0.5 g per kg body weight of whey protein isolate (Lacprodan ALPHA-20; Arla Foods Ingredients Group P/S, Viby J, DK) or maltodextrin. The drinks were matched for caloric content (kJ per kg body weight). The subjects added water to their liking, but were advised to use ~250 ml.

Training

All runners were set to complete 11 training sessions, excluding the two TTs. No additional training was allowed. The training was planned for each individual pair of runners to challenge their training status without increasing the risk of overreaching and injuries. Two daily running sessions (morning and afternoon) were performed except on Day 4, when only one session was completed.

The morning run was planned by the research team and coaches but undertaken by the subjects individually. The afternoon sessions were planned and supervised by experienced coaches and performed collectively (the specified training program is provided as Supplementary Tables [available online]). All training sessions were monitored using sport watches. The training data were uploaded every evening to Sportlyzer. To reduce the risk of overuse injuries, three subjects complaining of musculoskeletal pain in the lower extremities performed some training sessions as cycling at the intensities and durations similar to those of the superseded running sessions.

Blood Samples

On the morning of Days 1, 4, 7, and 8, blood samples were drawn from a cubital vein into Li-Heparin blood collection tubes. After centrifugation (10 min, 5 °C, 1,200 rpm), plasma was stored at −80 °C until analysis. Levels of cortisol, CK, LDH, and Mb were analyzed at Aarhus University Hospital, Denmark. The coefficient of variation for the analyses was as follows: CK = 5.6–7.8%, LDH = 6.0–8.2%, Mb = 11.0–12.7%, and cortisol = 15.8–18.0%.

Questionnaires

At each laboratory visit, the subjects were asked to rate their *sense of performance* and their level of *motivation for training* on a scale from 1 (lowest score) to 100, designed by the authors.

Statistics

The data were analyzed using Stata version 14.2 Stata (version 14.2; StataCorp, College Station, TX). Normality of all data was checked by Q–Q plots and the plots of residuals versus the fitted values. The data were log transformed where appropriate (CK, LDH, Mb, cortisol, and training history outcomes). The data collected repeatedly through the study period were analyzed for Time × Treatment interaction by a repeated-measures mixed-effects model. Unequal *SD*s and correlations within and between subjects were included in the analyses by letting the *SD*s and correlations vary between and within subjects.

Subject characteristics were analyzed using an unpaired *t* test. The data are presented as mean ± *SD*s if nothing else is stated. A p value <.05 was considered to be statistically significant.

Results

A total of 30 subjects (PRO: 14; CHO: 16) completed the study. Prior to the intervention, two subjects dropped out due to time constraints. During the intervention, two subjects were excluded (illness unrelated to the study; poor compliance), both from the PRO group.

Performance and Training

An overall decline in TT performance (11 ± 24 s, $p = .01$) was observed between Day 1 (18 min 31 s ± 1 min 19 s) and Day 8 (18 min 42 s ± 1 min 21 s), with no significant difference in decline between groups ($p > .05$; Figure 2). The difference in weather conditions between the two test days was minor (Day 1: 13 °C and dry; Day 8: 8 °C and damp from the previous night's rain).

At the baseline, the VO_2max (Table 1) and training history (Supplementary Tables [available online]) did not differ between the groups ($p > .05$). On average, the subjects increased their weekly running distance threefold during the intervention compared with the reported training history, with no differences between the groups ($p > .05$). This increase refers to running, exclusively. On average, the participants increased their weekly training volume (all activities

included; expressed as weekly training duration) from 7.6 ± 4.5 hr to 9.2 ± 1.8 hr, with no difference between groups ($p = .9$). Further data on the training history are provided in the Supplementary Tables [available online].

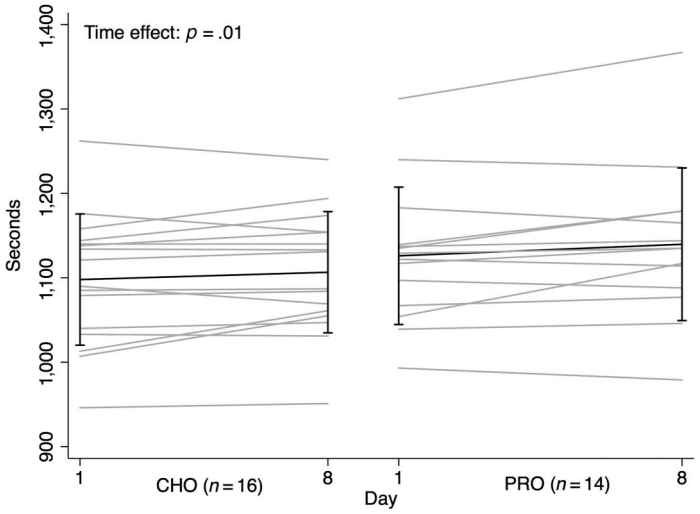

Figure 2 — Performance tests. Five-kilometer time-trial performance (s) at Day 1 (PRE) and Day 8 (POST) by group. Gray lines are individual tests, and black lines are mean ± *SD* values. Overall time effect ($p = .01$). CHO = carbohydrate; PRO = protein.

Blood Samples

The plasma CK ($p < .001$), LDH ($p < .001$), and Mb ($p < .001$) increased during the study period, but no significant differences were observed between the groups at any time point (Figure 3). Although the plasma cortisol levels did not change during the intervention in either group, a significant difference was observed at the baseline, with lower levels in PRO versus CHO (531.1 ± 117.2 vs. 616.8 ± 88.8 nmol/L; $p < .05$).

Diet Control and Body Weight

Body weight (Figure 4) did not differ significantly at the baseline between the two groups. Surprisingly, an overall time ($p < .001$) and Time × Treatment effect ($p < .05$) was observed, but the post hoc pairwise comparison did not reveal any differences between treatments at specific time points. Yet a small but significant decrease in body weight was observed on Day 8 for both treatments (CHO: −0.7 ± 0.6 kg; PRO: −0.3 ± 0.5 kg; $p \leq .05$).

The macronutrient content in the standardized diet (i.e., excluding the intervention beverages) was not different between the groups (all parameters $p > .05$; Table 1).

Motivation and Sense of Performance Capacity

Both motivation ($p < .05$) and the sense of performance capacity ($p < .001$) dropped throughout the study week, with no significant

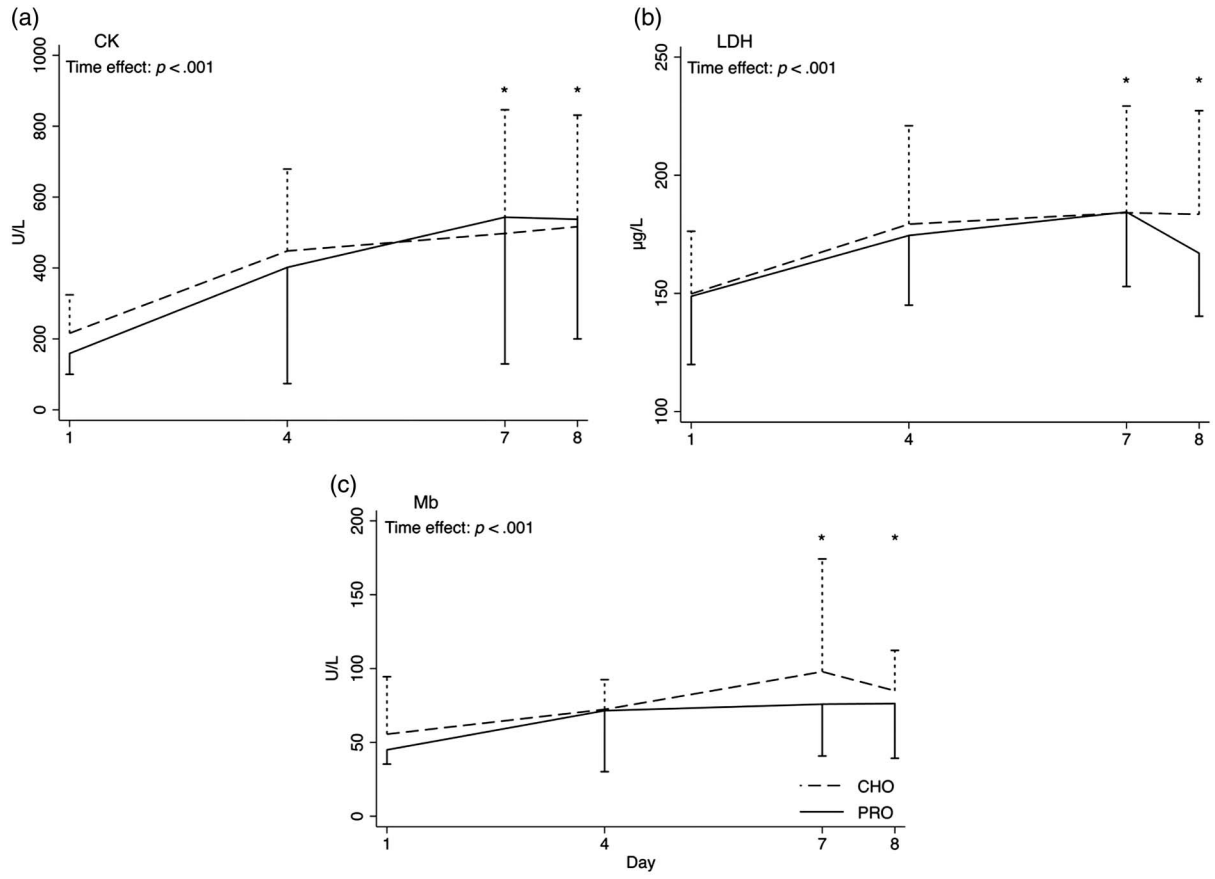

Figure 3 — Markers of sarcolemma disruption. Development in markers of sarcolemma disruption (CK, LHD, and Mb). Values are presented as mean ± *SD*. Overall time effects were observed for CK, LDH, and Mb ($p < .01$). *Significant difference from Day 1 ($p < .01$). CK = creatine kinase; LDH = lactate dehydrogenase; Mb = myoglobin; CHO = carbohydrate; PRO = protein.

differences between the groups (Figure 5). However, the motivation tended to be higher in CHO than in PRO at the baseline ($p = .07$).

Discussion

This study provides novel data regarding the effect of presleep protein supplementation during a prolonged endurance training intervention. In addition to the presleep beverages, the participants were subjected to a rigorously controlled diet and training program. Contrary to our hypothesis, similar impairments (~1%) in the 5-km TT performance after the intervention were observed in the two groups. This finding coincided with similar rises in circulatory markers of muscle damage and decline in motivation and the sense of performance capacity.

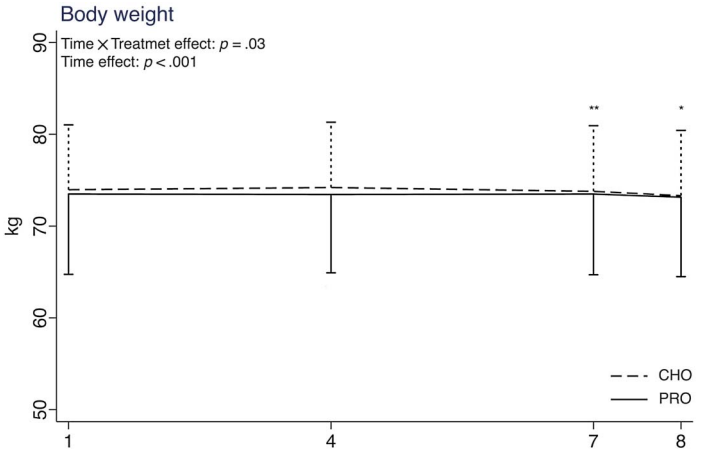

Figure 4 — Body weight. Data are presented as mean ± *SD*. Overall time and Time × Treatment effect. CHO = carbohydrate; PRO = protein. *Significantly different from all other days within group ($p < .05$). **Significantly different from Day 4 in CHO exclusively ($p = .003$).

Performance

Unlike our previous study, where protein supplements were given in temporal relation to the exercise, we did not observe a beneficial effect of presleep protein feeding in the present trial, even though the sample size was comparable (Hansen et al., 2015). Like us, others have reported performance benefits of increased protein intake ingested close to training sessions during consecutive days of intensified endurance training (Rowlands et al., 2008; Skillen et al., 2008; Thomson et al., 2011; Witard et al., 2011). However, no consensus has been established on this topic (Hill et al., 2013; Luden et al., 2007; Nelson et al., 2012). Likewise, consistency is lacking among the more acute studies measuring performance capacity within the 24 hr following a single exercise bout combined with protein supplementation, both when compared with isocaloric (Betts et al., 2007; Ferguson-Stegall et al., 2011; Hall et al., 2013; Millard-Stafford et al., 2005; Rowlands et al., 2007) or nonisocaloric placebo treatments (Betts et al., 2007; Breen et al., 2010; Goh et al., 2012; Millard-Stafford et al., 2005; Saunders et al., 2004).

Muscle Damage

As expected, a continuous rise in the circulatory markers of muscle cell disruption was observed throughout the training period, as observed previously (Hansen et al., 2015). This implies that the subjects had been exposed to considerable strain throughout the week. Nevertheless, this study did not reveal any difference between the groups in any of the blood markers. This finding is contrary to a number of previous longitudinal studies showing an ameliorating effect of protein/amino acid ingestion on markers of sarcolemma disruption when ingested immediately before and/or after training (Hansen et al., 2015; Luden et al., 2007; Thomson et al., 2011). The remarkable difference between this study and previous training studies showing the positive effects of protein supplementation on muscle recovery and performance is the timing of the supplementary protein intake. Although one of the studies

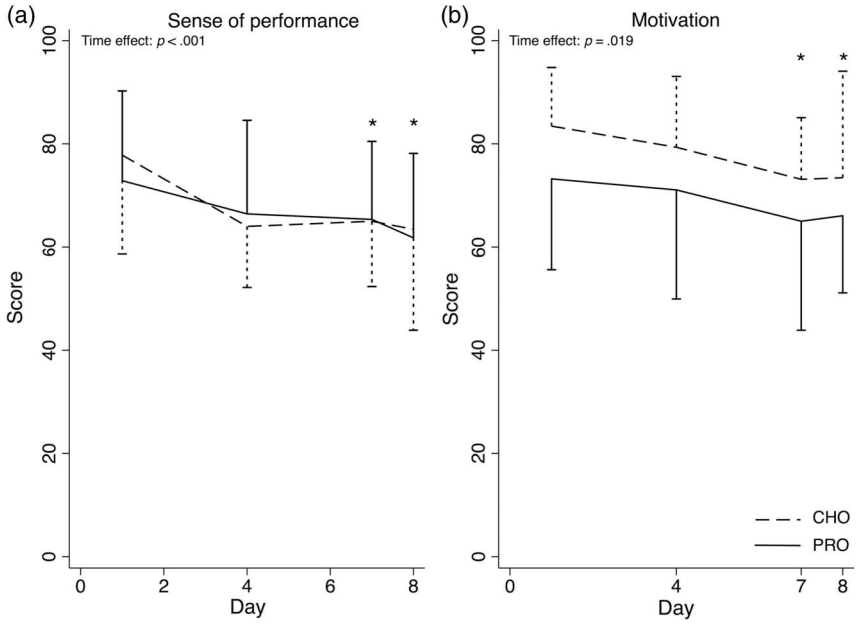

Figure 5 — Daily sense of performance capacity and motivation. Data are presented as mean ± *SD*. CHO = carbohydrate; PRO = protein. *Significantly different from Day 1 ($p < .05$).

showing the beneficial effects of protein on recovery from endurance exercise provided the supplementation at night, this was still immediately after the exercise (Thomson et al., 2011). Thus, although this study was not designed to investigate the impact of protein timing per se, we permit ourselves to speculate that the supplementation of protein-derived amino acids beyond the hours immediately surrounding the exercise is of little importance when aiming to improve recovery from endurance training performed during the day. Supporting this notion, Levenhagen et al. (2001) showed that immediate protein feeding following a 60-min bike ride at 60% of VO_2max induced a greater uptake of amino acids across the leg and greater leg and whole-body protein synthesis than when the protein supplement was given 3 hr later. Furthermore, substantial evidence suggests that the protein net balance during (Rennie et al., 1981) and/or in the early hours after endurance training in the fasted (Levenhagen et al., 2002; Sheffield-Moore et al., 2004) or carbohydrate-fed state (Howarth et al., 2009; Koopman et al., 2004; Levenhagen et al., 2002) is negative. However, acute protein ingestion postexercise shifts the whole-body net protein balance to positive and increases muscle protein synthesis (Breen et al., 2011; Howarth et al., 2009; Koopman et al., 2004; Levenhagen et al., 2001, 2002). These latter findings seem to support that protein-derived amino acids ingested immediately following endurance exercise, compared with several hours later, has a greater potential in attenuating muscle damage and/or facilitating faster repair by accelerated muscle protein turnover. In line with our speculations, it is noteworthy that, in the study by Res et al. (2012) showing improvements in overnight recovery (muscle protein synthesis) following resistance exercise, the training sessions were completed at 09:00 p.m., and both the posttraining drink (60 g carbohydrate and 20 g whey protein) and the presleep drink (40 g casein vs. water) were consumed within 2.5 hr of completing the exercise. Thus, in this study, we may have seen a positive effect of the protein supplementation, had the timing of the exercise sessions been in close connection to the protein feeding (i.e., at night). However, undertaking intense training late at night does not mimic the typical training pattern of an elite athlete. Thus, such a design would not serve our purpose.

All subjects in the present trial ingested a standardized diet and thus achieved the recommended amount of daily protein (1.7 g/kg; Jager et al., 2017; Kerksick et al., 2017). It could be argued that the ample amount of protein supplied in the basic diet may have obfuscated the potential benefit of the presleep supplementation. Had the basic dietary provision of protein been suboptimal, a beneficial effect of the presleep supplementation may have been present by counteracting a negative nitrogen balance. However, as this was designed as an applied study, meant to reflect the typical behavior of a competitive athlete focusing on a high level of performance during training and competition, feeding the control group a suboptimal diet would not serve our purpose.

Despite our best efforts, the mean body mass of the runners dropped slightly (CHO: –0.9%; PRO: –0.5%) at the end of the study. However, as the decline in body mass occurred between Day 7 and Day 8 and not as a development throughout the study, it could be related to insufficient hydration. One may argue that this might have influenced the POST-test performance negatively (Armstrong et al., 1985). However, the effect of slight hypohydration on performance seems trivial at temperatures of ~8 °C (Day 8; Murray, 2007). Regardless, the loss of body mass was not different between groups, and the energy availability was standardized across groups.

Surprisingly, cortisol was lower in PRO versus CHO at the baseline. Notably, the biological (>40%) and diurnal variation in plasma cortisol are relatively large in the morning. In this regard, two CHO subjects showed relative high values (787 and 809 nmol/L), whereas one PRO subject had a particularly low value (232 nmol/L). Thus, the difference may be related to a statistical Type I error, due to a relatively small sample size.

Limitations

Given the design of this study and recovery in general, we would have liked to monitor sleep. Unfortunately, we were unable to acquire equipment to do so accurately. Furthermore, this study only included men. This, of course, complicates the extrapolation of our findings to the population of female runners. The decision regarding the inclusion of men only was based on our own (2017, unpublished) observations and the observations of others, suggesting that the presence of estrogen may attenuate the levels of CK in the circulation following muscle-damaging exercise (Enns & Tiidus, 2010). However, the lack of female participants may be viewed as a limitation to the study.

In summary, we provided moderately trained runners a whey protein isolate or an isocaloric carbohydrate beverage every night before sleep, along with a standardized diet during a strenuous training week. Presleep whey protein ingestions did not reduce impairments in performance or ameliorate the increase in markers of muscle cell disruption. Compared with the existing literature, we speculate that, when supplemented by a well-balanced diet, the timing of protein intake is of importance to elicit an ergogenic effect.

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References

Armstrong, L.E., Costill, D.L., & Fink, W.J. (1985). Influence of diuretic-induced dehydration on competitive running performance. *Medicine & Science in Sports & Exercise, 17*(4), 456–461. PubMed ID: 4033401 doi:10.1249/00005768-198508000-00009

Betts, J., Williams, C., Duffy, K., & Gunner, F. (2007). The influence of carbohydrate and protein ingestion during recovery from prolonged exercise on subsequent endurance performance. *Journal of Sports Sciences, 25*(13), 1449–1460. PubMed ID: 17852694 doi:10.1080/02640410701213459

Breen, L., Philp, A., Witard, O.C., Jackman, S.R., Selby, A., Smith, K., ... Tipton, K.D. (2011). The influence of carbohydrate-protein

co-ingestion following endurance exercise on myofibrillar and mitochondrial protein synthesis. *Journal of Physiology, 589*(Pt 16), 4011–4025. PubMed ID: 21746787 doi:10.1113/jphysiol.2011.211888

Breen, L., Tipton, K.D., & Jeukendrup, A.E. (2010). No effect of carbohydrate-protein on cycling performance and indices of recovery. *Medicine & Science in Sports & Exercise, 42*(6), 1140–1148. PubMed ID: 19997018 doi:10.1249/MSS.0b013e3181c91f1a

Buckley, J.D., Thomson, R.L., Coates, A.M., Howe, P.R., DeNichilo, M.O., & Rowney, M.K. (2010). Supplementation with a whey protein hydrolysate enhances recovery of muscle force-generating capacity following eccentric exercise. *Journal of Science and Medicine in Sport, 13*(1), 178–181. PubMed ID: 18768358 doi:10.1016/j.jsams.2008.06.007

Cooke, M.B., Rybalka, E., Stathis, C.G., Cribb, P.J., & Hayes, A. (2010). Whey protein isolate attenuates strength decline after eccentrically-induced muscle damage in healthy individuals. *Journal of the International Society of Sports Nutrition, 7*, 30. PubMed ID: 20860817 doi:10.1186/1550-2783-7-30

Cunningham, J.J. (1980). A reanalysis of the factors influencing basal metabolic rate in normal adults. *American Journal of Clinical Nutrition, 33*(11), 2372–2374. PubMed ID: 7435418 doi:10.1093/ajcn/33.11.2372

Enns, D.L., & Tiidus, P.M. (2010). The influence of estrogen on skeletal muscle: Sex matters. *Sports Medicine, 40*(1), 41–58. PubMed ID: 20020786 doi:10.2165/11319760-000000000-00000

Ferguson-Stegall, L., McCleave, E.L., Ding, Z., Doerner, P.G., 3rd, Wang, B., Liao, Y.H., ... Ivy, J.L. (2011). Postexercise carbohydrate-protein supplementation improves subsequent exercise performance and intracellular signaling for protein synthesis. *Journal of Strength and Conditioning Research, 25*(5), 1210–1224. PubMed ID: 21522069 doi:10.1519/JSC.0b013e318212db21

Goh, Q., Boop, C.A., Luden, N.D., Smith, A.G., Womack, C.J., & Saunders, M.J. (2012). Recovery from cycling exercise: Effects of carbohydrate and protein beverages. *Nutrients, 4*(7), 568–584. PubMed ID: 22852050 doi:10.3390/nu4070568

Groen, B.B., Res, P.T., Pennings, B., Hertle, E., Senden, J.M., Saris, W.H., & van Loon, L.J. (2012). Intragastric protein administration stimulates overnight muscle protein synthesis in elderly men. *American Journal of Physiology-Endocrinology and Metabolism, 302*(1), E52–E60. PubMed ID: 21917635 doi:10.1152/ajpendo.00321.2011

Hall, A.H., Leveritt, M.D., Ahuja, K.D., & Shing, C.M. (2013). Coingestion of carbohydrate and protein during training reduces training stress and enhances subsequent exercise performance. *Applied Physiology, Nutrition, and Metabolism, 38*(6), 597–604. PubMed ID: 23724875 doi:10.1139/apnm-2012-0281

Hansen, M., Bangsbo, J., Jensen, J., Bibby, B.M., & Madsen, K. (2015). Effect of whey protein hydrolysate on performance and recovery of top-class orienteering runners. *International Journal of Sport Nutrition and Exercise Metabolism, 25*(2), 97–109. PubMed ID: 25029703 doi:10.1123/ijsnem.2014-0083

Hill, K.M., Stathis, C.G., Grinfeld, E., Hayes, A., & McAinch, A.J. (2013). Co-ingestion of carbohydrate and whey protein isolates enhance PGC-1alpha mRNA expression: A randomised, single blind, cross over study. *Journal of the International Society of Sports Nutrition, 10*(1), 8. PubMed ID: 23402493 doi:10.1186/1550-2783-10-8

Howarth, K.R., Moreau, N.A., Phillips, S.M., & Gibala, M.J. (2009). Coingestion of protein with carbohydrate during recovery from endurance exercise stimulates skeletal muscle protein synthesis in humans. *Journal of Applied Physiology (1985), 106*(4), 1394–1402. doi:10.1152/japplphysiol.90333.2008

Jager, R., Kerksick, C.M., Campbell, B.I., Cribb, P.J., Wells, S.D., Skwiat, T.M., ... Antonio, J. (2017). International society of sports nutrition position stand: Protein and exercise. *Journal of the International Society of Sports Nutrition, 14*, 20. PubMed ID: 28642676 doi:10.1186/s12970-017-0177-8

Kerksick, C.M., Arent, S., Schoenfeld, B.J., Stout, J.R., Campbell, B., Wilborn, C.D., ... Antonio, J. (2017). International society of sports nutrition position stand: Nutrient timing. *Journal of the International Society of Sports Nutrition, 14*, 33. PubMed ID: 28919842 doi:10.1186/s12970-017-0189-4

Keytel, L.R., Goedecke, J.H., Noakes, T.D., Hiiloskorpi, H., Laukkanen, R., van der Merwe, L., & Lambert, E.V. (2005). Prediction of energy expenditure from heart rate monitoring during submaximal exercise. *Journal of Sports Sciences, 23*(3), 289–297. PubMed ID: 15966347 doi:10.1080/02640410470001730089

Koopman, R., Pannemans, D.L., Jeukendrup, A.E., Gijsen, A.P., Senden, J.M., Halliday, D., ... Wagenmakers, A.J. (2004). Combined ingestion of protein and carbohydrate improves protein balance during ultra-endurance exercise. *American Journal of Physiology-Endocrinology and Metabolism, 287*(4), E712–E720. PubMed ID: 15165999 doi:10.1152/ajpendo.00543.2003

Kouw, I.W., Holwerda, A.M., Trommelen, J., Kramer, I.F., Bastiaanse, J., Halson, S.L., ... van Loon, L.J. (2017). Protein ingestion before sleep increases overnight muscle protein synthesis rates in healthy older men: A randomized controlled trial. *Journal of Nutrition, 147*(12), 2252–2261. PubMed ID: 28855419 doi:10.3945/jn.117.254532

Levenhagen, D.K., Carr, C., Carlson, M.G., Maron, D.J., Borel, M.J., & Flakoll, P.J. (2002). Postexercise protein intake enhances whole-body and leg protein accretion in humans. *Medicine & Science in Sports & Exercise, 34*(5), 828–837. PubMed ID: 11984302 doi:10.1097/00005768-200205000-00016

Levenhagen, D.K., Gresham, J.D., Carlson, M.G., Maron, D.J., Borel, M.J., & Flakoll, P.J. (2001). Postexercise nutrient intake timing in humans is critical to recovery of leg glucose and protein homeostasis. *American Journal of Physiology-Endocrinology and Metabolism, 280*(6), E982–E993. PubMed ID: 11350780 doi:10.1152/ajpendo.2001.280.6.E982

Luden, N.D., Saunders, M.J., & Todd, M.K. (2007). Postexercise carbohydrate-protein- antioxidant ingestion decreases plasma creatine kinase and muscle soreness. *International Journal of Sport Nutrition and Exercise Metabolism, 17*(1), 109–123. PubMed ID: 17460336 doi:10.1123/ijsnem.17.1.109

Millard-Stafford, M., Warren, G.L., Thomas, L.M., Doyle, J.A., Snow, T., & Hitchcock, K. (2005). Recovery from run training: Efficacy of a carbohydrate-protein beverage? *International Journal of Sport Nutrition and Exercise Metabolism, 15*(6), 610–624. PubMed ID: 16521846 doi:10.1123/ijsnem.15.6.610

Moore, D.R., Camera, D.M., Areta, J.L., & Hawley, J.A. (2014). Beyond muscle hypertrophy: Why dietary protein is important for endurance athletes. *Applied Physiology, Nutrition, and Metabolism, 39*(9), 987–997. PubMed ID: 24806440 doi:10.1139/apnm-2013-0591

Murray, B. (2007). Hydration and physical performance. *Journal of the American College of Nutrition, 26*(Suppl. 5), 542S–548S. PubMed ID: 17921463 doi:10.1080/07315724.2007.10719656

Nelson, A.R., Phillips, S.M., Stellingwerff, T., Rezzi, S., Bruce, S.J., Breton, I., ... Rowlands, D.S. (2012). A protein-leucine supplement increases branched-chain amino acid and nitrogen turnover but not performance. *Medicine & Science in Sports & Exercise, 44*(1), 57–68. PubMed ID: 21685813 doi:10.1249/MSS.0b013e3182290371

Rennie, M.J., Edwards, R.H., Krywawych, S., Davies, C.T., Halliday, D., Waterlow, J.C., & Millward, D.J. (1981). Effect of exercise on protein turnover in man. *Clinical Science (London), 61*(5), 627–639. doi:10.1042/cs0610627

Res, P.T., Groen, B., Pennings, B., Beelen, M., Wallis, G.A., Gijsen, A.P., ... van Loon, L.J. (2012). Protein ingestion before sleep improves postexercise overnight recovery. *Medicine & Science in Sports & Exercise, 44*(8), 1560–1569. PubMed ID: 22330017 doi:10.1249/MSS.0b013e31824cc363

Rowlands, D.S., Rossler, K., Thorp, R.M., Graham, D.F., Timmons, B.W., Stannard, S.R., & Tarnopolsky, M.A. (2008). 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. *Applied Physiology, Nutrition, and Metabolism, 33*(1), 39–51. PubMed ID: 18347652 doi:10.1139/H07-136

Rowlands, D.S., Thorp, R.M., Rossler, K., Graham, D.F., & Rockell, M.J. (2007). Effect of protein-rich feeding on recovery after intense exercise. *International Journal of Sport Nutrition and Exercise Metabolism, 17*(6), 521–543. PubMed ID: 18156659 doi:10.1123/ijsnem.17.6.521

Saunders, M.J., Kane, M.D., & Todd, M.K. (2004). Effects of a carbohydrate-protein beverage on cycling endurance and muscle damage. *Medicine & Science in Sports & Exercise, 36*(7), 1233–1238. PubMed ID: 15235331 doi:10.1249/01.MSS.0000132377.66177.9F

Saunders, M.J., Luden, N.D., & Herrick, J.E. (2007). Consumption of an oral carbohydrate-protein gel improves cycling endurance and prevents post-exercise muscle damage. *Journal of Strength and Conditioning Research, 21*(3), 678–684. PubMed ID: 17685703 doi:10.1519/R-20506.1

Sheffield-Moore, M., Yeckel, C.W., Volpi, E., Wolf, S.E., Morio, B., Chinkes, D.L., ... Wolfe, R.R. (2004). Postexercise protein metabolism in older and younger men following moderate-intensity aerobic exercise. *American Journal of Physiology-Endocrinology and Metabolism, 287*(3), E513–E522. PubMed ID: 15149953 doi:10.1152/ajpendo.00334.2003

Skillen, R.A., Testa, M., Applegate, E.A., Heiden, E.A., Fascetti, A.J., & Casazza, G.A. (2008). Effects of an amino acid carbohydrate drink on exercise performance after consecutive-day exercise bouts. *International Journal of Sport Nutrition and Exercise Metabolism, 18*(5), 473–492. PubMed ID: 19033610 doi:10.1123/ijsnem.18.5.473

Snijders, T., Res, P.T., Smeets, J.S., van Vliet, S., van Kranenburg, J., Maase, K., ... van Loon, L.J. (2015). Protein ingestion before sleep increases muscle mass and strength gains during prolonged resistance-type exercise training in healthy young men. *Journal of Nutrition, 145*(6), 1178–1184. PubMed ID: 25926415 doi:10.3945/jn.114.208371

Thomson, J.S., Ali, A., & Rowlands, D.S. (2011). Leucine-protein supplemented recovery feeding enhances subsequent cycling performance in well-trained men. *Applied Physiology, Nutrition, and Metabolism, 36*(2), 242–253. PubMed ID: 21609286 doi:10.1139/h10-104

Trommelen, J., Kouw, I.W.K., Holwerda, A.M., Snijders, T., Halson, S.L., Rollo, I., ... van Loon, L.J.C. (2018). Pre-sleep dietary protein-derived amino acids are incorporated in myofibrillar protein during post-exercise overnight recovery. *American Journal of Physiology-Endocrinology and Metabolism, 314*(5), E457–E467. doi:10.1152/ajpendo.00273.2016

Valentine, R.J., Saunders, M.J., Todd, M.K., & St Laurent, T.G. (2008). Influence of carbohydrate-protein beverage on cycling endurance and indices of muscle disruption. *International Journal of Sport Nutrition and Exercise Metabolism, 18*(4), 363–378. PubMed ID: 18708686 doi:10.1123/ijsnem.18.4.363

van Loon, L.J., Saris, W.H., Kruijshoop, M., & Wagenmakers, A.J. (2000). Maximizing postexercise muscle glycogen synthesis: Carbohydrate supplementation and the application of amino acid or protein hydrolysate mixtures. *American Journal of Clinical Nutrition, 72*(1), 106–111. PubMed ID: 10871568 doi:10.1093/ajcn/72.1.106

Witard, O.C., Jackman, S.R., Kies, A.K., Jeukendrup, A.E., & Tipton, K.D. (2011). Effect of increased dietary protein on tolerance to intensified training. *Medicine & Science in Sports & Exercise, 43*(4), 598–607. PubMed ID: 20798660 doi:10.1249/MSS.0b013e3181f684c9

Witard, O.C., Turner, J.E., Jackman, S.R., Kies, A.K., Jeukendrup, A.E., Bosch, J.A., & Tipton, K.D. (2014). High dietary protein restores overreaching induced impairments in leukocyte trafficking and reduces the incidence of upper respiratory tract infection in elite cyclists. *Brain, Behavior, and Immunity, 39*, 211–219. PubMed ID: 24120932 doi:10.1016/j.bbi.2013.10.002