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**Impact of habituated dietary protein intake on fasting and postprandial whole body protein turnover and splanchnic amino acid metabolism in elderly men: a randomized controlled, crossover trial.**

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the funding entities have neither been taking part in the study design, trial conduction, sample analyses nor data interpretation in the present study.

Running head: Habituated protein intake & whole body turnover.

List of abbreviations: Body weight (BW), fractional synthesis rate (FSR), high protein (HP), lean body mass (LBM), net balance (NB), plasma flow (PF), partial volume of distribution (pV), rate of appearance ($R_a$), rate of disappearance ($R_d$), recommended dietary allowance (RDA), recommended protein (RP).

Clinical Trial Registry: Journal number NCT02587156, Clinicaltrials.org.

Data described in the manuscript, code book, and analytic code will be made available upon request pending application and approval.

**Abstract**

*Background:* Efficacy of protein absorption and subsequent amino acid utilization may be reduced in elderly. Higher protein intakes have been suggested to counteract for this.

*Objective:* To elucidate how habituated level of protein intake affects the fasted state and the stimulatory effect of a protein-rich meal on protein absorption, whole body protein turnover and splanchnic amino acid metabolism.

*Design:* 12 males (65-70 years) were included in a double-blinded crossover intervention study, consisting of a 20-day habituation period to a protein intake at recommended dietary allowance (RDA) or high-level (1.1 g/kg lean body mass (LBM)/day or >2.1 g/kg LBM/day), each followed by an experimental trial with a primed, constant infusion of $D_8$-phenylalanine and $D_2$-tyrosine. Arterial and hepatic venous blood samples were obtained after an overnight fast and repeatedly four hours after a standardized meal including intrinsically labeled whey protein concentrate and calcium-caseinate proteins. Blood was analyzed for amino acid concentrations and phenylalanine and tyrosine tracer enrichments wherefrom whole-body and splanchnic amino acid and protein kinetics were calculated.

*Results:* High compared to the recommended level of protein intake resulted in a higher fasting whole body protein turnover with a resultant 0.03 (±0.01 SEM) μmol/kg LBM/min lower net balance ($P<0.05$), which was not rescued by the intake of a protein dense meal. The plasma protein fractional synthesis rate was 0.13 (±0.06 SEM) %/h lower ($P<0.05$) following habituation to high protein. Further a higher fasting and postprandial amino acid removal was observed following habituation to high protein, yielding higher urea excretion and increased phenylalanine oxidation rates ($P<0.01$).

*Conclusion:* Three weeks of habituation to high protein intake (>2.1 g protein/kg LBM/day) led to a significantly higher net protein loss in the fasted state. This was not compensated for in the 4-hour -post-prandial period after intake of a meal high in protein.

*Keywords:* Habitual protein intake, recommended protein intake, protein turnover, protein breakdown, whole body protein turnover, intrinsically labelled proteins, stable-isotope tracers, whey protein, caseinate protein

## Introduction

The recommended daily requirement of 0.83 g dietary protein/kg body weight/day (1–3) for supporting lean mass maintenance in all adults have repeatedly been challenged and suggested to be elevated for individuals above 65 years of age (4). The basis for this awareness arises from follow-up and observational cross-sectional studies (5–7). A limitation of these findings is that differences most often are found between moderately high and lower than recommended dietary allowance (RDA) protein intakes (5,6,8). Suggestions made to elevate the protein intake above the current recommendations for the elderly assume that an additive effect of protein intake will also be seen between recommended and higher protein intakes. This assumption has not been tested.

One cause for an enhanced protein requirement for elderly individuals is anabolic resistance (9–11). The mechanism for which is unknown but may be found in the route from oral intake of protein to peripheral efficacy to enhance protein synthesis and or reduce protein breakdown. The first pass splanchnic tissues extraction has been shown to increase with age (12,13), thus following a protein containing meal, less amino acids is presented to the circulation and made available to promote protein synthesis. The amount of amino acids in the circulation is affected by the absorption and digestion rates of proteins as well as the ability of the peripheral tissue to take up amino acids from the circulation and incorporate them into body proteins and vice versa to release amino acids into the circulation.

Metabolic homeostasis can occur within rather wide ranges of protein exposures. This is reflected by e.g. increased capacity to degrade and oxidize amino acids and hence scavenge nitrogen by increasing urea production and excretion when protein intake is increased (14–16). Fasting whole body protein kinetics have also been shown to be enhanced after habituation to divergent dietary protein levels in both younger (17,18) and older (19) individuals. In contrary,

Gorissen and colleagues habituated older individuals for two weeks to high (1.5 g/kg body weight (BW)/d) vs. low (0.7 g/kg BW/d) protein intake and observed neither an effect on fasting whole body protein synthesis and breakdown rates nor a response to plain protein feeding: However, a significantly increased amino acid oxidation was observed following habituation to high protein (20).

With reference to the suggestions to elevate the dietary protein for older adults, it remains to be elucidated whether habituation to higher protein intake have an impact different from the currently recommended on the whole body protein turnover in a fasted state and how it affects the post-prandial protein handling .The primary outcome was the difference in plasma protein enrichment from intrinsically labeled whey protein 4 hours after ingestions. This was measured to assess the post-prandial response to a mixed meal following habituation for 20 days to a normal (RDA) and high dietary protein intake in older individuals. Secondary outcomes included whole body and plasma specific protein metabolism both in a fasted and post-prandial state. We hypothesized that a high protein diet would increase the amino acid oxidation and the nitrogen removal apparatus but simultaneously improve digestion/absorption driving a more positive postprandial net protein synthesis response.

## Subjects, materials and methods

### *Participants*

Twelve men (65-70 years) completed this double-blinded randomized crossover trial **(Figure 1)**. Before inclusion, all participants were screened by a physician and deemed overall healthy based on blood samples, medical history and an interview. The exclusion criteria were diabetes; pain in the skeletal muscle, connective tissue or back; known arthrosclerosis; alcohol intake above 21 units per week; and regular intake of drugs or dietary supplements anticipated to affect body protein turnover. Figure 1 shows a flowchart of the enrollment. 69 males where assessed for eligibility, 12 were included and went through both habituation periods. There were no dropouts.

Study design, purpose, risks and discomforts involved were explained (written and orally) to each participant in accordance with the Helsinki declaration, after which the subjects gave written consent of participation. The study took place at Bispebjerg and Frederiksberg Hospital, Copenhagen. The local ethical committee of the Capital Region of Denmark approved the study, protocol number H-15005598 and the study was registered at Clinicaltrials.org under journal number NCT02587156.

Pre-screening involved dual-energy X-ray absorptiometry (DXA) scanning (Lunar iDXA; GE Medical Systems, Pewaukee, WI, USA, with enCORE v.16 software) to determine the body composition of the participants, with emphasis on the lean body mass (LBM) used to calculate the desired dietary protein intake and the tracer amount needed. Participant characteristics can be seen in **Table 1.**

### *Experimental design*

The experimental design is shown in **Figure 2.**

The crossover intervention consisted of 20 days habituation to the recommended level of protein in one period (recommended protein, RP) and habituation to a high level of protein intake in another period (high protein, HP). The order of the habituation periods was randomly assigned by a draw in a double blinded fashion.

In the 20-day habituation period the participants received four daily intakes of identically packed supplements containing 20 g of whey hydrolysate and 10 g of sucrose in the HP period and an isocaloric identically packed supplement consisting of 20 g maltodextrin and 10 g sucrose in the RP period. The supplements came in powder form and the participants were instructed to dissolve them a cold beverage of their choosing. The four daily supplements were spread out during the day based on the participants customary protein distribution (e.g. two supplements with breakfast, one with lunch and one with dinner if the participant ingested least protein at breakfast in his daily diet. The pattern of supplement intake was decided by the investigator) aiming at an even protein intake at all 3 main meals in the HP period. In the RP period, participants were instructed in the exact same manner, however as their supplement contained carbohydrate, their protein distribution throughout the day was not even.

Each habituation period started with a start-up period of seven days, where the only dietary alterations were the four daily supplements. Subjects were guided towards a basic diet of 1.1 g protein/kg LBM/day based on a 3-day dietary registration during the seven start up days. The remaining 13 days of the 20-day habituation period were controlled by regular online dietary registrations. Protein intake was adjusted to lean body mass instead of total body weight to account for varying body compositions assuming that the lean body mass (LBM) is mainly responsible for amino acid and protein metabolism. The 1.1. g protein/kg LBM/day corresponds to 0.8 g protein/kg BW/day assuming a whole body fat content of 27%.

From day 19, participants refrained from any sort of strenuous physical activity. At 8 a.m. on day 20, a 24-hour urine collection was started. At the last meal of day on day 20, all participants had a standardized meal. The experimental trial on day 21 was identical for all participants irrespective of the preceding habituation period. Following day 21, the participants had a washout period of minimum 45 days, before crossing over to the alternate protein intake intervention in a double blinded fashion. An overview of the 21-day period is illustrated in figure 2.

*Production of intrinsically labeled proteins (given on day 21)*

Five Danish Holstein Friesian cows were infused with stable isotopes, four with L-[ring-$D_5$]-phenylalanine and 1 cow with L-[$^{15}$N]-phenylalanine, the protocol is described in detail elsewhere (21). In brief, milk was collected from 11 milkings, pasteurized and caseinate and whey proteins were purified, yielding L-[ring-$D_5$]-phenylalanine labeled whey with an enrichment of 15.44 ± 0.24 mole percent excess (MPE) (±SEM) and L-[$^{15}$N]-phenylalanine labeled caseinate with an enrichment of 20.81 ± 0.02 MPE (±SEM).

*Day 21 – experimental trial day*

Participants fasted for 12 h overnight and arrived at 8 a.m. by taxi to the hospital ward. Upon arrival they finished their first 24-hour urine sample period and a second 24-hour urine sample period was started. Hereafter, the participants changed into hospital attire and were placed in a bed in the supine position and a catheter was inserted in an antecubital vein and a background blood sample was taken (-100 min, end of meal intake is set to 0 min). Hereafter, primed continuous infusions were started of $D_8$-phenylalanine (prime: 4 µmol/kg LBM, continuous: 4 µmol/kg LBM/h in a fasted state, 4.8 µmol/kg LBM//h in postprandial state), $D_2$-tyrosine (prime: 2.2 µmol /kg BW, continuous: 2.3 µmol/kg BW/h in a fasted state, 2.75 µmol/kg

LBM/h in a postprandial state), $^{15}N_2$-urea (prime 84 µmol/kg BW, continuous 9 µmol/kg BW/h) and indocyanine green (ICG, prime: 1 mg, continuous infusion: 7-14 mg/h). At -40 minutes a catheter was placed in a radial artery under local anesthesia. Fluoroscopy was used to place a catheter in a hepatic vein (through vena femoralis). The radial artery catheter as well as the liver catheter were used for blood collection which were taken simultaneously. The catheters were kept clear by frequent flushing with saline.

No sooner than 90 minutes after the start of the stable isotope tracer infusion, two fasting blood samples were taken from each of the sampling sites (hepatic vein and artery). The consecutive blood samples were taken at -12 and -10 minutes, analysis of these results were pooled and collectively referred to as -10. Following blood sampling, a protein dense mixed breakfast meal was served. The meal had to be ingested within 10 minutes and contained 0.61 g protein/kg LBM, of which 0.23 g/kg LBM came from $^{15}$N-phenylalanine intrinsically labeled caseinate protein, and 0.31 g/kg LBM came from $D_5$-phenylalanine intrinsically labeled whey protein. The $D_5$-labeled whey protein was dissolved in water and served as a protein drink. The $^{15}$N-labeled caseinate was mixed with raspberry jam, which was spread with butter on a bun and served with the whey drink. The protein content in the butter and the bun constituted the remaining 0.07 g/kg LBM of the total 0.61 g protein /kg LBM. **Table 2** shows the macronutrient content in the breakfast.

After meal ingestions, blood samples were drawn simultaneous from the hepatic vein and radial arteria at time 30, 60, 90, 120, 150, 180, and 240 min. After the last sample was obtained, all catheters were removed, participants received a standardized lunch containing 0.80 g protein/kg LBM and a urine sample was collected, and the participants got a taxi home. At home they ingested a standardized dinner ensuring an equal protein content of 0.61 g /kg LBM. Thus, all participants ingested 2.02 g protein/kg LBM across day 21 independent of

their habituated protein intake. Next morning at 8 a.m. and prior to food intake, the second 24-hour urine collection ended.

*Blood analysis*

Arterial plasma samples were converted to serum by use of 1unit thrombin pr. 200 µl EDTA plasma. The serum samples were analyzed for insulin, using ALPCO Insulin ELISA assay (Catalog number: 80-INSHU-E01.1, E10.1, ALPCO).

Free amino acids were extracted from arterial plasma for measures of amino acid concentrations and amino acid tracer abundances. An internal standard in a 500 µL 50% acetic acid solution was added to 100 µl plasma, following which plasma was poured over cation exchange columns with resin (AG 50W-X8 resin, Bio-Rad laboratories, Hercules, Ca, USA), which had been prepped by adding 3 x 2ml 1M HCl creating an acidic environment. The columns were washed 5 times with 3 mL of deionized water before the amino acids were eluted by adding 2 x 2 mL 4M $NH_4OH$ collected in vials. Solvent was evaporated under a stream of $N_2$ flow at 70°C and derivatized using PITC derivatization agent, converting the samples into their phenylthiocabamyl (PITC) derivative. 10 µl of the 100 µl derivatized samples were loaded and analyzed on an ultra performance liquid chromatography system coupled to a triple stage quadrupole mass spectrometer (LC-MS/MS) (Thermo Fischer Scientific, San Jose, CA, USA) as described by Bornø et al. (22). Plasma protein bound tracer enrichments, were analyzed from plasma protein precipitated with 500 µl ice cold acetone pr 50 µl plasma, hydrolyzed by 1 ml 1 M HCl and 1 ml resin slurry, and left overnight at 110°C. Following hydrolysis, the samples were purified over cation exchange resin, as described above. For measures of deuterium enrichments, samples were PITC derivatized and run on LC-MS/MS system as described above. For determining the $^{15}N$ abundances, amino acids were converted to the N-acetyl-propyl (NAP) derivatives and analyzed on a gas chromatography-

combustion-isotope ratio mass spectrometer (GC-C-IRMS) system as previously described by Bornø et al. (23).

Plasma and urine samples for analysis of urea concentration as well as urea tracer enrichments were prepared with internal standards and run over resin columns just like the samples for phenylalanine and tyrosine enrichments. Following cation exchange on the resin columns and overnight drying under $N_2$ stream the samples were derivatized by adding 40 µl acetonitrile and 40 µl N-methyl-N-(tert-butyldimethylsilyl) trifluroroacetamide (MtBSTFA) + 1% tert-butyl-dimethylchlorosilane (tBDMCS), followed by vortex mixing and kept at 70ºC for 30 minutes. The derivatized samples were separated on a GC column and isotope ratios were analyzed on a triple-stage quadrupole mass spectrometer.

*Splanchnic flow*

The splanchnic blood flow was measured via Fick's principle, using a primed continuous infusion of indocyanine green (ICG) as indicator as previously described (24). The plasma ICG concentration was determined by spectrophotometry at wavelengths 805 and 900 nm. The light absorption at 900 nm is a measure of turbidity and is thus regarded as the background "noise". The calculation of blood flow is based on the difference in ICG concentration found in the artery and hepatic venous blood. Thus, the splanchnic plasma flow is calculated using Fick's principle (25).

$$Splanchnic\ plasma\ flow = \frac{ICG\ influx}{C_{ICG-a} - C_{ICG-hv}}$$

Where $C_{ICG\text{-}a}$=ICG concentrations in the artery, and $C_{ICG\text{-}hv}$=ICG concentrations in the hepatic vein.

*Tracers*

Concentrations of the labeled phenylalanine and tyrosine are calculated as their tracer to tracee ratio (TTR) multiplied by the concentrations of unlabeled compounds. Enrichments are given as mole percent excess (MPE=TTR/(1+TTR)). For $D_8$-phenylalanine enrichments the transamination product ($D_7$-phenylalanine), and oxidation products ($D_7$- and $D_6$-tyrosine) were also measured and the sum $D_8$- and $D_7$-phenylalanine as well as $D_7$- and $D_6$-tyrosine were used for all calculations.

In the postprandial period, the tracers are in non-steady state and all amino acid kinetics in this period are based on both ingestion of $D_5$-phenylalanine labeled whey, $^{15}N$-phenylalanine labeled caseinate, continuously infused $D_8$-phenylalanine and continuously infused $D_2$-tyrosine. The calculations are done using Steele equation (26) with modification introduced by Proietto et al. (27). An approach which is generally accepted for calculating whole body amino acid and protein kinetics (20,28–30). The Steele equation incorporates the change in pool size defined as: $Pool\ size = pV * \frac{dC}{dt}$ , where pV is the partial volume of distribution set at 0.125, dC is the difference in concentrations [µmol/l] across the time interval, dt. This means that for all calculations which include non-steady state conditions, the values are calculated as delta values between timepoints, thus the time resolution for these will be -5, 15, 45, 75, 105, 135, 165 and 210 minutes.

Plasma protein FSR: The direct incorporation of amino acids into plasma protein was given by:

$$FSR = \left(\frac{\Delta E\ protein\ [MPE]}{E\ Precursor\ pool\ [MPE] * \Delta time\ [h]}\right) * 100\%$$

Where ΔE protein is the change in enrichment in plasma protein, and the precursor is the enrichment in the plasma taken from the hepatic vein.

256 Ingested amino acid appearance rate: The exogenous rate of appearance ($R_a$), is the appearance
257 rate into the systemic circulation of phenylalanine derived from the ingested proteins, thus
258 phenylalanine from whey and caseinate, into the hepatic vein. In order to calculate this, the
259 total $R_a$ is needed. In the fasting, steady state, the total and exogenous $R_a$ is given by:

$$Total\ R_a = \frac{IR}{E_{(t)}}$$

$$Exogenous\ R_a = \frac{Total\ R_a \cdot dE_{OT(t)}}{E_{protein}}$$

260 In the postprandial, non-steady state

$$Total\ R_a = \frac{IR}{\hat{E}_{(t)}} - \frac{pV \cdot \left[\frac{\hat{C}_{(t)}}{1+\hat{E}_{(t)}}\right] \cdot \left[\frac{dE_{(t)}}{dt}\right]}{\hat{E}_{(t)}}$$

$$Exogenous\ R_a = \frac{Total\ R_a \cdot\ dE_{OT(t)} + pV\ \cdot dC_{(t)} \cdot \left[\frac{dE_{(t)}}{dt}\right]}{E_{protein}}$$

261 Where IR= tracer infusion rate [µmol/kg LBM/min], $E_{(t)}$= enrichment at time t, $dE_{OT(t)}$= delta
262 enrichment from the oral tracer ($D_5$-phenylalanine or $^{15}N$-phenylalanine) at time t, $\hat{C}_{(t)}$=
263 average concentration between values surrounding time t, $\hat{E}_{(t)}$=average enrichment between
264 values surrounding time t, $dE_{(t)}$=delta enrichment, dt=delta time and $E_{protein}$=the enrichment in
265 the ingested protein. All concentrations and enrichments are measured in the hepatic vein.
266
267 Net amino acid balance across the splanchnic tissues: The net balance (NB) across the
268 splanchnic tissues is given by (31):

$$Splanchnic\ NB = (C_a - C_{hv}) \cdot PF$$

269 Where $C_a$ and $C_{hv}$ are phenylalanine concentrations measured in the artery and hepatic vein,
270 respectively. PF is the plasma flow in the hepatic vein.
271

Whole body protein turnover: All measures in this section are based on concentrations and enrichments in the arterial blood. The endogenous $R_a$ represents phenylalanine appearing from within the body, used as a measure of protein breakdown. It is given by:

$$Endogenous\ R_a = Total\ R_a - Exo\ R_a - IR$$

The formula is the same for the fasted, steady state and the postprandial, non-steady state. However, the appearance rates used are the ones calculated in the steady and non-steady state, respectively.

The rate limiting step in phenylalanine oxidation is the irreversible hydroxylation of phenylalanine to tyrosine (32). This conversion is given by:

In the fasted, steady state

$$Phe \rightarrow tyr = Total\ R_a \cdot \frac{E_{tyr}}{E_{phe}}$$

In the postprandial, non-steady state

$$Phe \rightarrow tyr = Total\ R_a \cdot \frac{\hat{E}_{tyr}}{\hat{E}_{phe}}$$

Where $\hat{E}_{tyr}$ and $\hat{E}_{phe}$ are the mean enrichments of either $D_7$-tyrosine or $D_8$-phenylalanine between two surrounding timepoints.

Subtracting the 'phenylalanine-to-tyrosine conversion rate' from the total Rate of disappearance ($R_d$), gives a measure of the phenylalanine removed by other processes than oxidations, hence a measure of synthesis. The $R_d$ is given by:

In the fasted, steady state

$$Total\ R_d = Total\ R_a$$

In the postprandial, non-steady state

$$Total\ R_d = pV \cdot \frac{dC}{dt} + Total\ R_a$$

As for the endogenous $R_a$, the formula for the endogenous $R_d$ is the same in the fasted steady state and the postprandial non-steady state. However, the disappearance rate and the conversion of phenylalanine to tyrosine used are the ones calculated in the steady and non-steady state respectively. Thus, the endogenous $R_d$ is given by:

$$Endogenous\ R_d = Total\ R_d - (Phe \rightarrow tyr)$$

*Deviations*

All 12 subjects completed all trial days. However, on the experimental day 21, three subjects did not receive infusion of $D_2$-tyrosine and are not included in calculations of phenylalanine conversion to tyrosine and whole-body protein synthesis measurements. One participant was excluded from all postprandial measurements due to mixing error with the labeled proteins. $D_7$-phenylalanine and $D_6$-tyrosine (deriving from transamination of $D_8$-phenylalanine and conversion from $D_7$-phenylalanine to $D_6$-tyrosine) was only measured for 5 participants. Based on the known $D_8$-phenylalaine/$D_7$-phenylalanine and $D_7$-tyrosine/$D_6$-tyrosine ratios for these five participants, the $D_7$-phenylalanine and $D_6$-tyrosine have been calculated for all participants.

There were no dropouts.

311 **Statistics**

312 Intervention groups' responses to feeding over time were compared using two-way ANOVA
313 with repeated measures. Interaction effects were tested using SIDAK post hoc test and time
314 effects were tested using Dunnet's post hoc test. Difference between the two habituation
315 periods in the fasted state was compared using a paired t-test. The insulin data is presented as
316 the numerical values, however as data was not normally distributed the statistical analysis were
317 performed on log transformed data. The primary outcome was the difference in plasma protein
318 enrichment of $D_5$-phenylalanine from the intrinsically labeled whey protein 4 hours after
319 ingestions. This was studied in 12 individuals, allowing us to detect a difference of 0.01 MPE
320 with the expected standard deviation of 0.01, a significance level of 5% and a power of 80%.
321 GraphPad Prism 7.0 was used for all statistical tests. Data is presented as means ±SEM unless
322 otherwise stated. Significant level was set to $p<0.05$. The intervention effect was assessed by
323 two-tail Student's *t*-test on within-subject dissimilarity as a response to high- and low-protein
324 intake.
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## Results

The participants were on average 66.6 year of age and were overall healthy based on normal blood pressure, lipid blood profile and hemoglobin A1c. Their average protein intake (1.5 g/kg LBM/day) was higher than the current recommendation. All participant characteristics are shown in Table 1.

With an intake of 1.22 ± 0.04 g/kg LBM/day (0.82 ± 0.03 g/kg BW/day), the full 20-day dietary composition for the RP period was slightly higher than the aim (1.1 g/kg LBM/day). However, the last 13 days of the habituation period, were right on point. For the HP period the aim was >2.1 g/kg LBM/day. The actual intake covering the full 20-day period was 24% higher, with an intake of 2.61 ± 0.04 g/kg LBM/day (1.76 ± 0.04 g/kg BW/day). The protein intakes in the two habituation periods are seen in **Table 3.**

### *Nitrogen and amino acid losses*

The urine urea quantity during the last 24 hours of the habituation was significantly higher following habituation to HP (**Figure 3A**, $P<0.01$), emphasizing a high compliance to the dietary protocol among the participants. During the 24 hours which included the trial day all participants had the exact same relative protein intake irrespective of the habituation period. Urine collected during the 8 h of the trial had a similar urea quantity independent of habituation period (**Figure 3B**). In contrast, the total 24 hour urine of the entire trial day 21 had a higher urine urea after habituation to HP (**Figure 3C**, $P<0.05$) in spite of the same protein intake through the entire day.

In line with these findings, the urea $R_a$ is significantly higher following habituation to HP in the fasted state (**Figure 4A**, $P<0.01$). The urea $R_a$ remained elevated following habituation to

HP as compared to RP throughout the 4-hour postprandial period (**Figure 4B**). However, there was no difference in the absolute change from fasted to postprandial urea $R_a$ between the two habituation periods (baseline corrected; data not shown). In accordance with this, the phenylalanine hydroxylation rate to tyrosine (**Figure 4C and D**) was significantly higher following habituation to HP both in the fasted (Figure C, $P<0.01$) and in the postprandial four hour period (Figure 4D, time: $P<0.0001$, intervention $P<0.001$).

*Plasma protein fractional synthesis rate and nutrient protein-derived tracer abundances*

The fractional synthesis rates (FSR) of plasma proteins are shown in **Figure 5A**. There was a significant higher plasma FSR in the 4-hour postprandial period following the RP period compared to the HP period ($P=0.046$).

The enrichment from the intrinsically labeled whey ($D_5$-phenylalanine labeled) and caseinate ($^{15}$N-phenylalanine labeled) proteins were assessed in the plasma protein 1 and 4 hours postprandial. Values are presented as mole percent excess (MPE) for $D_5$-phenylalanine and atom percent excess (APE) for $^{15}$N-phenylalanine enrichments. The incorporation of phenylalanine deriving from both whey and caseinate was greater at 4 hours compared to 1 hour postprandial (**Figure 5B and C**, $P<0.0001$). For the incorporation of phenylalanine deriving from the whey protein into plasma proteins there was a significantly higher incorporation following RP compared to HP intake 4 hours postprandial (Figure 5C, $P=0.0007$).

*Insulin concentrations*

Serum insulin increased with the meal intake and remained elevated from baseline in the first 3 hours postprandial. It was unaffected by the preceding protein habituation period both in the fasted and the postprandial state (**Figure 6**).

380

381 *Phenylalanine and protein kinetics*

382 The $R_a$ of phenylalanine from the whey and caseinate was measured in blood samples both
383 from the hepatic vein and the radial artery. While there is a higher appearance rate measured in
384 the hepatic vein, the $R_a$ pattern is the same from the two sampling sites. There is a significant
385 time effect of the $R_a$ of phenylalanine from the whey and the caseinate. However, no
386 differences existed between habituation periods (**Figure 7**).

387 The whole-body protein breakdown rate was significantly higher following habituation to HP
388 compared with RP ($P<0.05$) in the fasted state (**Figure 8A**). In the postprandial period, the
389 whole-body protein breakdown was significantly decreased (**Figure 8B**, time: $P<0.0001$) with
390 no difference between habituation periods. The whole-body protein synthesis was higher in the
391 fasted state following habituation to HP as compared to RP (Figure 8A, $P<0.01$). Post-
392 prandially, the whole-body protein synthesis increased (**Figure 8C**, time: $P<0.0001$) but more
393 so following habituation to HP as compared to RP ($P<0.05$). As an overall result, the whole-
394 body protein turnover rate was higher when habituated to HP compared to RP. However, and
395 most important for lean body mass maintenance, the net protein turnover balance in the fasted
396 state was less negative following habituation to RP (**Figure 8A**, $P<0.05$). The net protein
397 balance was increased in the postprandial period ($P<0.0001$), albeit no differences between
398 habituation periods (Figure 8D).

399 There were no differences between interventions in the change from fasting to the postprandial
400 state for all protein kinetic parameters (the endogenous rate of appearance, phenylalanine to
401 tyrosine conversion, endogenous rate of disappearance, and the resultant net balance, data not
402 shown).

403

*Amino acid concentrations and net splanchnic tissues amino acid balance*

The arterial amino acid concentrations and splanchnic net balances are presented in **Table 4**. The amino acid concentrations in the fasted state were similar for RP and HP except for glycine. After meal and protein intake the concentrations of glycine (Gly), asparagine (Asn), alanine (Ala), tyrosine (Tyr) and isoleucine (Ile) were significantly higher following habitation to RP at several timepoints compared with subjects following HP diet ($P<0.05$). In addition, tendencies for higher concentrations were also observed for lysine (Lys), tryptophan (Trp), methionine (Met) and threonine (Thr) ($P<0.07$) in RP versus HP habituated participants. The measured net balances across the splanchnic bed revealed no intervention (RP versus HP) effect. It should be acknowledged that the splanchnic balance techniques bear relatively higher variability as for example the single arterial amino acid concentration measurement. In the fed state, the net balances for all amino acids but glutamine (Gln) became more negative indicating a net release of amino acids from the splanchnic tissues to the circulation. However, no changes were observed for glutamine (Gln) in the fasted compared to fed state.

## Discussion

The main findings of the present study are: 1. Twenty days of habituation to HP intake enhances the whole body protein turnover rate; 2. HP habituation leads to a more negative overnight fasted net protein balance; 3. The higher overnight fasted net protein loss with HP was not compensated for in the 4 h postprandial period after a protein-dense meal containing the same amount of protein for HP and RP; 4. Postprandial plasma protein FSR and consequently incorporation of meal derived phenylalanine into plasma proteins were significantly higher following habituation to RP.

Similar results as our finding, that whole body protein turnover is increased in response to habituation to HP, were reported by Pannemans and colleagues in young (17) and older (19) participants, who were habituated to either 12 or 21 E% protein. Whereas after habituation to 0.70 g protein/kg BW/day or 1.53 g protein/kg BW/day for 14 days, Gorissen and colleagues (20) show no difference in whole body protein synthesis nor breakdown, in response to a protein intake, thus no change in protein turnover. The major difference being that Gorrissen et al. (20) studied the postprandial response to only ~0.42 g whey protein/kg LBM without concomitant energy intake. Such amount of protein may have been insufficient to stimulate protein turnover kinetics optimally in older men. Moore and colleagues showed that for plateau stimulation of muscle protein synthesis rates requires an intake of 0.61 g protein/kg LBM in older individuals (33), which we provided in the mixed meal.

An enhanced protein turnover rate may be advantageous as an improved capacity to repair and remodel tissues and maintain protein function or adaption to a physiological challenge. However, in the overnight fasted state HP habituation led to a more negative whole-body net protein balance, which has also been shown by Hursel et al. comparing long term habituation

449 to rather high (2.4 g protein/kg/d) with rather low protein (0.4 g protein/kg/d)(18). In the
450 present study, the more negative whole-body net balance was not counterbalanced by a more
451 positive net balance in the postprandial state. In a conservative scenario, assuming a fasting
452 state condition of 8 hours a day, the negative net balance means a net protein loss of ~3 g of
453 whole body proteins/day. This is equivalent to ~1 kg body protein/year assuming that the body
454 proteins consists of 4.5% phenylalanine (34). It should be noted that this estimated loss is
455 solely based on the fasting condition and does not represent a real-life situation. However, the
456 fact that the adaptions to higher protein intakes may affect the utilization of amino acids in a
457 negative direction should be considered. Combined, these results imply that care should be
458 taken before providing large amounts of proteins. However, it should also be kept in mind that
459 too low protein intake should at all times be prevented, as it has shown to lead to lean body
460 mass erosion (5,35).

461

462 The rate limiting step in phenylalanine degradation hydroxylation of phenylalanine to tyrosine
463 (32) was elevated following habituation to HP both in the fasted and the 4-hour postprandial
464 state. Furthermore, the higher amount of urea excreted in the urine and the higher urea rate of
465 appearance in the blood under fasting conditions at day 21 emphasize that high protein intake
466 results in higher nitrogen excretion. Of importance, even on day 22, where the participants
467 ingested the exact same protein amount at all meals, the urine urea content remained 23%
468 elevated in the HP condition (Figure 3C), emphasizing a reduced ability to retain amino acids
469 and nitrogen when habituated to high protein intake. Such effect can be described as an
470 impaired utilization of amino acids when habituated to HP diet.

471 All circulating amino acids concentrations with the exception of glycine were similar for RP
472 and HP in the fasted state whereas postprandially, the glycine, asparagine, alanine, tyrosine and

isoleucine concentrations were higher in RP. A higher postprandial amino acid concentration can originate from a difference in digestion/absorption rate, splanchnic first by-pass extraction and/or reduced peripheral clearance and increased production rates. The exogenous whey and caseinate phenylalanine appearance rates (Figure 7) reveal similar protein digestion and absorption rates after RP and HP habituation and the postprandial whole body protein synthesis, degradation and net balance were also similar indicating similar peripheral clearance and release rates. Therefore, the higher arterial concentrations of some amino acids after RP habituation most likely originate from a lower net splanchnic first by-pass extraction of these amino acids. To quantify arterial-hepatic venous differences, blood samples were taken from the radial artery and the hepatic vein, reflecting the net balance across the splanchnic tissues (36) despite the mixing of the arterial blood to the liver with portal venous content. In the postprandial period, there was a net release for all amino acids, except glutamine, into the circulation from the splanchnic tissue reflecting the expected net uptake. For glutamine the demand in the splanchnic tissue extract most content in the meal, which is in agreement with previous findings from Stoll et al. estimating that in piglets the gut tissue utilizes more than 95% of dietary glutamine (37). Together with alanine, glutamine is a key nitrogen carrier and intermediate in amino acid transamination processes. Moreover, glutamine is the preferred energy source of rapidly dividing cells such as those present in the intestine (36).

For all of the measured amino acids the net balance across the splanchnic tissues was similar following the two habituation periods. Despite the even net balance across the splanchnic tissues phenylalanine oxidation occurring primarily in the liver was significantly increased following habituation to HP intake (Figure 4). Thus, another process must utilize more amino acids in the RP condition, which appeared to be for plasma protein synthesis, which primarily occurs in the hepatocytes (38).In agreement, the whey-derived amino acid tracer ($D_5$-phenyalanine) was more abundant in the plasma proteins four hours after meal intake after RP

habituation. Overall, these results indicate that during habituation to high protein diets, the amino acid metabolism is higher, leaving less amino acids for the translational apparatus even in the postprandial period.

A limitation of the present study is that we cannot extrapolate our findings to the entire 24-hour protein net balance. Irrespectively, it was clear that the four-hour net protein balance response to the same meal did not compensate for the net protein loss identified during the end of an overnight fasting period, despite the fact that it was a protein rich meal. The postprandial net protein balance might have differed between the two habituations had the breakfast meal not been identical but contained a protein level comparable to the habituated period as seen by Kim et al. (40). In this study, following habituation to a high protein intake we measured the response to a protein intake similar to the habituated level, and following habituation to the recommended protein level we measured the response to an intake of protein which was twice as high as the habituated level. This means that following the RP period, the meal stimuli is out of the ordinary. Hence their response could potentially be exacerbated.

In conclusion, a protein intake higher than recommended level resulted in a significantly more negative fasting whole-body net protein balance. The ingestion of a mixed protein dense meal did not compensate for the lowered fasting net protein balance in the first four postprandial hours. Further, the findings of a lower plasma protein FSR and less abundance of dietary whey derived phenylalanine in plasma proteins point towards a higher first pass splanchnic extraction of amino acids from the meal when habituated to high protein content in the diet. This is most likely caused by a higher amino acid catabolism in the liver yielding the observed higher urea production and excretion.

Taking the limitations into consideration, these results indicate potential drawbacks of ingesting a 'higher than currently recommended protein level to improve the body's protein utilization. Rather it increases the body's catabolism of amino acids, measured as increased amino acids oxidation and nitrogen excretion.

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**Table 1: Subject characteristics at inclusion**

| Participant characteristics N=12 | |
|---|---|
| Age [years] | 66.6 ± 1.6 |
| Height [m] | 1.79 ± 0.04 |
| Body weight [kg] | 84.3 ± 10.9 |
| BMI [kg/m2] | 26.3 ± 3.3 |
| LBM [kg] | 56.4 ± 4.4 |
| Fat % | 29.9 ± 5.7 |
| Systolic blood pressure [mmHg] | 137 ± 25 |
| Diastolic blood pressure [mmHg] | 83 ± 11 |
| Normal dietary protein intake [g/kg LBM/d] | 1.5 ± 0.3 |
| Daily energy intake [kcal/LBM] | 36.5 ± 7.7 |
| E% protein | 18 ± 2.6 |
| Hemoglobin A1c (IFCC) [mmol/mol] | 35.2 ± 2.6 |
| Thyrotropin (TSH) [$x10^{-3}$IU/l] | 1.5 ± 0.8 |
| Total Cholesterol [mmol/l] | 5.5 ± 0.7 |
| HDL cholesterol [mmol/l] | 1.7 ± 0.4 |
| LDL Cholesterol [mmol/l] | 3.2 ± 0.6 |
| Triglyceride [mmol/l] | 1.3 ± 0.8 |

Values are means ± SD; BMI, body mass index; LBM, lean body mass; E%, energy percent.

**Table 2: Macronutrient composition of the breakfast meal.**

| Trial day breakfast | |
|---|---|
| Calories [kcal] | 441 |
| Protein [g/kg LBM] | 0.61 |
| - $D_5$-phe labeled whey [g/kg LBM] | 0.31 |
| - $^{15}N$-phe labeled casein [g/kg LBM] | 0.23 |
| Carbohydrates [g/kg LBM] | 1.06 |
| Fat [g/kg LBM] | 0.21 |

LBM, lean body mass

**Table 3: Total daily calorie and protein intake for the recommended and high protein intake periods including the supplement.**

| | **20-day habituation period N=12** | |
|---|---|---|
| **Recommended protein period** | Basic diet | Basic diet + supplements |
| Calorie intake week 2-3 [kcal/day] | 2117 ± 84 | 1624 ± 84 |
| Protein intake day 1-7 [g/kg LBM/day] | 1.42 ± 0.09 | 1.42 ± 0.09 |
| Protein intake day 1-7 [g/kg BW/day] | 0.96 ± 0.07 | 0.96 ± 0.07 |
| Protein intake day 8-20 [g/kg LBM/day] | 1.11 ± 0.03 | 1.11 ± 0.03 |
| Protein intake day 8-20 [g/kg BW/day] | 0.75 ± 0.03 | 0.75 ± 0.03 |
| **High protein period** | Basic diet | Basic diet +supplements |
| Calorie intake week 2-3 [kcal/day] | 2207 ± 58 | 1666 ± 58 |
| Protein intake day 1-7 [g/kg LBM/day] | 2.78 ± 0.09 | 1.35 ± 0.07 * |
| Protein intake day 1-7 [g/kg BW/day] | 1.87 ± 0.08 | 0.91 ± 0.06 * |
| Protein intake day 8-20 [g/kg LBM/day] | 2.53 ± 0.05 | 1.10 ± 0.03 * |
| Protein intake day 8-20 [g/kg BW/day] | 1.71 ± 0.05 | 0.74 ± 0.02 * |

Data excluding the supplements are noted in the parentheses. LBM, lean body mass; BW, body weight. Values are mean ± SEM. *denotes $p<0.001$ between interventions.

**Table 4: Arterial amino acid concentrations and splanchnic tissues net balances**

| | | | Arterial conc.[µmol/L]±SEM; NB, net balance [µmol/min]±SEM | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | -10min | 30min | 60min | 90min | 120min | 150min | 180min | 240min |
| Asp | Arterial | RP | 5±1 | 10±1 | 12±3 | 6±1 | 9±4 | 6±1 | 5±1 | 8±3 |
| | $ | HP | 5±0 | 10±1 | 7±1 | 5±0 | 5±1 | 5±0 | 5±0 | 5±1 |
| | NB | RP | -4±1 | -23±5 | -5±3 | -5±1 | 1±4 | -6±3 | -3±1 | -6±2 |
| | $ | HP | -3±0 | -14±3 | -5±2 | -3±1 | -2±1 | -2±1 | -2±0 | -2±0 |
| Glu | Arterial | RP | 111±27 | 127±14 | 126±19 | 125±25 | 110±20 | 125±31 | 106±23 | 107±23 |
| | $ | HP | 93±10 | 114±7 | 104±8 | 96±6 | 99±15 | 91±9 | 89±8 | 92±10 |
| | NB | RP | -101±17 | -174±30 | -115±15 | -125±20 | -100±21 | -119±35 | -104±15 | -129±22 |
| | $ | HP | -81±11 | -131±22 | -88±24 | -103±19 | -67±21 | -81±15 | -85±11 | -83±14 |
| Ser | Arterial | RP | 113±15 | 167±15 | 183±23 | 147±14 | 144±27 | 138±19 | 120±15 | 130±20 |
| | $ | HP | 102±5 | 147±7 | 131±8 | 114±7 | 104±4 | 106±6 | 104±7 | 105±6 |
| | NB | RP | 17±3 | -62±17 | 1±15 | 11±11 | 27±19 | -10±18 | -6±6 | -17±16 |
| | $ | HP | 17±2 | -32±22 | -2±17 | 4±3 | 11±5 | 8±16 | 6±6 | 9±9 |
| Gly | Arterial | RP | *223±27 | *244±27 | *263±32 | *234±22 | *219±25 | *234±27 | 209±21 | *229±25 |
| | $ | HP | 184±11 | 187±12 | 176±13 | 169±12 | 166±9 | 179±13 | 180±16 | 191±15 |
| | NB | RP | 26±7 | -14±16 | 20±8 | 17±14 | 18±7 | -8±27 | -11±9 | -22±19 |
| | $ | HP | 15±5 | -19±22 | 10±19 | -2±16 | -2±9 | -7±24 | -4±12 | -1±18 |
| Asn | Arterial | RP | 45±7 | 86±11 | *91±11 | *79±7 | 64±7 | *73±12 | 64±9 | 60±8 |
| | $ | HP | 44±3 | 77±5 | 71±5 | 62±6 | 56±4 | 57±4 | 56±5 | 55±5 |
| | NB | RP | 9±2 | -42±9 | -16±4 | 0±6 | -3±4 | -8±5 | -10±3 | -8±4 |
| | $ | HP | 7±2 | -27±13 | -9±9 | -7±7 | -4±3 | -4±8 | -3±4 | -1±5 |
| Gln | Arterial | RP | 707±78 | 857±100 | 893±105 | 840±76 | 721±68 | 798±83 | 722±74 | 741±76 |
| | $ | HP | 648±29 | 747±41 | 756±51 | 718±50 | 646±45 | 692±47 | 687±49 | 735±52 |
| | NB | RP | 122±27 | 39±53 | 65±27 | 152±51 | 78±32 | 75±62 | 41±28 | 13±51 |
| | | HP | 97±14 | -8±77 | 58±77 | 37±56 | 47±38 | 74±65 | 71±25 | 61±58 |
| His | Arterial | RP | 77±9 | 101±11 | 110±11 | 100±9 | 88±8 | 97±11 | 89±11 | 91±10 |
| | $ | HP | 73±3 | 93±5 | 94±5 | 85±5 | 79±3 | 83±6 | 84±5 | 88±6 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | NB | RP | 8±2 | -18±7 | -10±3 | 4±6 | 0±3 | -9±8 | -9±4 | -14±6 |
| | $ | HP | 3±2 | -21±11 | -7±10 | -7±6 | -3±4 | -5±8 | -6±3 | -4±7 |
| Thr | Arterial | RP | 118±17 | 217±23 | 234±25 | 216±18 | 182±17 | 199±25 | 175±21 | 166±18 |
| | $ | HP | 111±6 | 197±10 | 183±9 | 165±11 | 156±13 | 145±8 | 137±9 | 134±8 |
| | NB | RP | 19±4 | -110±25 | -45±5 | -13±14 | -12±9 | -24±19 | -19±6 | -16±11 |
| | $ | HP | 16±3 | -74±34 | -25±24 | -19±20 | 9±16 | 0±18 | 2±7 | 6±10 |
| Ala | Arterial | RP | 266±29 | *407±50 | *493±54 | *487±43 | 402±40 | *431±39 | 396±38 | 401±42 |
| | $ | HP | 225±17 | 323±29 | 361±28 | 362±34 | 336±24 | 349±29 | 356±31 | 363±30 |
| | NB | RP | 152±21 | 19±37 | -3±19 | 93±41 | 83±20 | 77±27 | 70±19 | 94±29 |
| | $ | HP | 115±10 | 21±45 | 31±39 | 48±36 | 80±18 | 93±46 | 98±19 | 109±30 |
| Pro | Arterial | RP | 167±18 | 290±25 | 322±31 | 332±27 | 293±25 | 335±28 | 306±25 | 300±24 |
| | $ | HP | 155±11 | 277±18 | 281±20 | 278±22 | 263±15 | 271±20 | 272±26 | 269±19 |
| | NB | RP | 9±5 | -142±31 | -85±7 | -68±26 | -68±29 | -98±45 | -87±17 | -82±26 |
| | $ | HP | 0±6 | -127±47 | -72±38 | -89±38 | -56±18 | -55±35 | -47±14 | -32±18 |
| Arg | Arterial | RP | 84±11 | 138±14 | 139±16 | 128±12 | 105±10 | 119±15 | 102±11 | 95±10 |
| | $ | HP | 70±5 | 116±7 | 105±7 | 96±6 | 86±4 | 88±7 | 84±7 | 81±5 |
| | NB | RP | 13±3 | -44±13 | -14±2 | -1±8 | -10±9 | -15±14 | -13±5 | -13±8 |
| | $ | HP | 8±2 | -30±21 | -10±13 | -13±15 | -1±5 | -3±13 | -1±4 | -1±7 |
| Tyr | Arterial | RP | 62±7 | 125±12 | *132±13 | *125±10 | 105±10 | *116±12 | *102±10 | 95±10 |
| | $ | HP | 64±3 | 114±7 | 110±7 | 98±5 | 88±4 | 87±4 | 84±6 | 81±4 |
| | NB | RP | 8±3 | -45±19 | -10±3 | 2±8 | -4±7 | -7±12 | -3±4 | -5±8 |
| | $ | HP | 8±2 | -30±24 | -11±15 | -9±13 | -1±4 | -3±12 | 2±3 | 1±6 |
| Val | Arterial | RP | 235±28 | 409±38 | 429±40 | 417±30 | 349±29 | 388±42 | 341±37 | 323±33 |
| | $ | HP | 260±11 | 427±22 | 419±21 | 386±20 | 344±18 | 345±22 | 326±21 | 315±18 |
| | NB | RP | 7±6 | -154±38 | -81±5 | -35±24 | -49±23 | -64±34 | -58±11 | -62±21 |
| | $ | HP | -4±7 | -144±63 | -71±49 | -85±38 | -49±20 | -42±37 | -34±12 | -28±23 |
| Met | Arterial | RP | 21±2 | 51±5 | 51±5 | 45±2 | 35±3 | 39±5 | 34±3 | 28±3 |
| | $ | HP | 20±1 | 47±3 | 40±3 | 34±3 | 30±1 | 29±2 | 27±2 | 25±2 |
| | NB | RP | 5±1 | -24±5 | -6±2 | 2±3 | -1±2 | -3±3 | -2±1 | -1±2 |
| | $ | HP | 4±0 | -16±10 | -3±5 | -3±5 | 0±2 | 0±4 | 1±1 | 2±2 |
| Ile | Arterial | RP | 52±8 | 177±22 | 175±21 | *152±13 | 112±12 | *129±21 | 111±17 | 91±11 |
| | $ | HP | 55±4 | 176±14 | 154±14 | 121±8 | 108±10 | 91±5 | 82±5 | 74±4 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | NB | RP | 0±1 | -121±28 | -52±5 | -24±14 | -34±13 | -30±15 | -28±11 | -26±7 |
| | $ | HP | -3±3 | -102±44 | -52±26 | -44±18 | -10±14 | -22±13 | -19±5 | -14±7 |
| Leu | Arterial | RP | 124±14 | 376±36 | 394±41 | 336±28 | 258±23 | 283±38 | 242±33 | 201±24 |
| | $ | HP | 135±9 | 400±33 | 358±30 | 287±17 | 242±16 | 220±17 | 203±20 | 180±15 |
| | NB | RP | 5±4 | -247±58 | -98±10 | -38±27 | -50±19 | -64±22 | -51±7 | -37±10 |
| | $ | HP | -7±4 | -224±82 | -106±50 | -82±34 | -28±20 | -37±24 | -26±8 | -10±10 |
| Tryp | Arterial | RP | 54±7 | 97±10 | 106±12 | 98±9 | 79±7 | 86±9 | 72±8 | 65±7 |
| | $ | HP | 51±2 | 87±5 | 85±4 | 76±3 | 67±3 | 66±4 | 61±4 | 58±3 |
| | NB | RP | 3±1 | -31±10 | -10±1 | 1±6 | -4±5 | -7±10 | -5±3 | -7±5 |
| | $ | HP | 1±1 | -28±14 | -6±9 | -9±7 | -3±3 | -2±8 | -1±2 | -1±5 |
| Phe | Arterial | RP | 52±6 | 91±7 | 92±8 | 85±5 | 73±5 | 83±8 | 73±7 | 71±6 |
| | $ | HP | 58±2 | 90±4 | 83±4 | 79±4 | 74±4 | 75±4 | 73±5 | 72±3 |
| | NB | RP | 7±2 | -25±7 | -5±3 | 1±5 | -4±4 | -7±7 | -6±2 | -7±5 |
| | $ | HP | 5±1 | -21±13 | -4±8 | -9±8 | -3±5 | -4±9 | -1±3 | 0±5 |
| Lys | Arterial | RP | 177±19 | 368±33 | 374±34 | 325±21 | 260±20 | 295±34 | 257±26 | 231±22 |
| | $ | HP | 187±9 | 363±19 | 318±17 | 273±15 | 241±9 | 242±13 | 230±15 | 219±12 |
| | NB | RP | 18±5 | -186±47 | -56±4 | -10±22 | -22±19 | -30±23 | -32±9 | -28±14 |
| | $ | HP | 13±5 | -146±70 | -32±39 | -33±33 | -10±13 | -14±32 | -8±10 | -5±19 |
| Ess. | Arterial | RP | 411±49 | 962±93 | 997±96 | 904±65 | 719±61 | 800±98 | 694±85 | 614±66 |
| | $ | HP | 450±21 | 1003±61 | 931±59 | 794±40 | 693±33 | 655±40 | 611±42 | 569±33 |
| BCAA | NB | RP | 13±10 | -521±123 | -232±14 | -97±62 | -132±52 | -158±69 | -137±26 | -125±37 |
| | $ | HP | -16±14 | -470±188 | -229±121 | -212±90 | -86±50 | -101±74 | -78±23 | -51±32 |
| Ess.excl. | Arterial | RP | 591±67 | 1078±99 | 1118±107 | 1009±69 | 835±66 | 934±105 | 820±83 | 764±72 |
| | $ | HP | 577±24 | 1010±46 | 922±45 | 818±43 | 744±23 | 739±38 | 709±43 | 689±33 |
| BCAA | NB | RP | 81±17 | -462±117 | -151±16 | -18±66 | -53±52 | -96±85 | -87±30 | -84±53 |
| | $ | HP | 56±14 | -351±180 | -95±112 | -96±100 | -7±43 | -28±95 | -10±30 | 1±56 |
| Total | Arterial | RP | 2703±313 | 4337±423 | 4618±470 | 4273±326 | 3605±308 | 3971±432 | 3523±365 | 3430±336 |
| | $ | HP | 2541±93 | 3992±193 | 3836±203 | 3501±197 | 3186±127 | 3220±181 | 3137±206 | 3139±177 |
| | NB | RP | 328±76 | -1413±400 | -532±61 | -40±272 | -154±182 | -360±375 | -340±133 | -374±245 |
| | $ | HP | 206±57 | -1188±621 | -410±443 | -432±375 | -89±174 | -105±377 | -56±127 | 9±232 |

NB=Splanchnic tissues net balance, a negative value means a higher amino acid concentration in the hepatic vein, i.e. a net amino acid release from the splanchnic tissue into the main circulation. Ess. BCAA, essential branched-chain amino acids; ess.excl. BCAA, essential amino acids excluding branched-chain amino acids. $ denotes an overall time effect, with a significant change from fasting, * denotes an interaction effect with a Sidak post hoc test revealing significant difference between RP and HP ($P<0.05$). N=11, data is mean ± SEM. Ess. =essential .

**Figure legends:**

**Figure 1: Flow chart of the enrollment process.**

**Figure 2: Overview of the 20-day trial period, with emphasis on the day 21 trial day**.

**Figure 3: Urea amount in the urine A) 24 hour urine day 20-21, post '20 day habituation', B) 8 hour urine during the trial, including lunch C) 24 hour urine day 21-22, including the trial period.** RP, recommended protein period; HP, high protein period. * denotes significant differences between interventions assessed by a paired t-test. Fig. A+B N=10, fig. C N=12, values are mean ± SEM.

**Figure 4: Nitrogen and amino acid loss as well as phenylalanine conversion in the fasted and postprandial state**. Figure A and B show the urea rate of appearance in the hepatic vein in the post habituation, fasted state (A), pre and four-hour postprandial (B). (N=10, values are mean ± SEM). Figure C and D shows the phenylalanine conversion to tyrosine. RP, recommended protein period; HP, high protein period; $R_a$, rate of appearance. (N=8, values are mean ± SEM). $ denotes significant change from fasting, * denotes significant difference between habituated protein levels. In the fasted state comparison between groups are done by a paired t-test while changes over time and between groups are done by a two-way ANOVA with repeated measures.

**Figure 5: 0-4 hours fractional synthesis rate (FSR) for plasma proteins (A) and incorporation of amino acids from ingested caseinate ($^{15}$N-phenylalnine) (B) and whey ($D_5$-phenylalanine) (C) into plasma proteins.** The plasma protein FSR in the 4 hours postprandial period (A) is significantly higher following habituation to RP compared HP intake ($P<0.05$). Phenylalanine from the breakfast meal into plasma protein is significantly increased with time (B and C). RP, recommended protein period; HP, high protein period; FSR, fractional synthesis rate;

APE, atom percent excess; MPE, mole percent excess. $ denotes significant change from 1 hour ($P<0.05$). The enrichment deriving from the $D_5$-phenylalanine from the whey protein is significantly higher in the plasma proteins 4 hours post meal intake when participants are habituated to RP intake. * denotes significant difference between habituations ($P<0.001$). Plasma FSR comparison between groups are done by a paired t-test while changes in enrichment over time and between groups are done by a two-way ANOVA with repeated measures. Values mean ± SEM, N=11.

**Figure 6: The insulin concentrations in the post habituation, fasted state (A), and immediately pre and four hours postprandial (B).** RP, recommended protein period; HP, high protein period. $ denotes significant change from fasting. In the fasted state comparison between groups are done by a paired t-test while changes over time and between groups are done by a two-way ANOVA with repeated measures. (N=12, values are mean ± SEM).

**Figure 7: Exogenous rate of appearance of the ingested protein in the hepatic vein (A) representing the rate at which the intrinsically labeled phenylalanine enters the circulation And in the radial artery (B), representing the rate of apperance into the radial artery**. For the apperance rate in both the hepatic vein and the radial artery, there is a significant time effect ($p<0.0001$) and a difference between $R_a$ of Whey and $R_a$ of casein on time, intervention and interaction ($p<0.0001$). RP, recommended protein period; HP, high protein period; Exo, exogenous: $R_a$, rate of appearance. Changes over time and between groups are done by a two-way ANOVA with repeated measures. (N=11, values are mean ± SEM)

**Figure 8: Whole body protein turnover in the fasted (A) and immediately pre and postprandial state (B, C, D).** The endogenous release of phenylalanine represents the whole body protein breakdown in fasted (A) and fed state (B). The endogenous rate of disappearance

represents the protein synthesis in the fasted (A) and fed state (C). The net balance is shown in the fasted (A) and fed state (D). RP, recommended protein period; HP, high protein period; endo, endogenous; $R_a$, rate of appearance; $R_d$, rate of disappearance. $ denotes significant change from fasting, * denotes significant difference between habituated protein levels. In the fasted state comparison between groups are done by a paired t-test while changes over time and between groups are done by a two-way ANOVA with repeated measures. (N=8, values are mean ± SEM).