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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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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.

1   **Abstract**

2   *Background:* Efficacy of protein absorption and subsequent amino acid utilization may be

3   reduced in elderly. Higher protein intakes have been suggested to counteract for this.

4   *Objective:* To elucidate how habituated level of protein intake affects the fasted state and the

5   stimulatory effect of a protein-rich meal on protein absorption, whole body protein turnover

6   and splanchnic amino acid metabolism.

7   *Design:* 12 males (65-70 years) were included in a double-blinded crossover intervention

8   study, consisting of a 20-day habituation period to a protein intake at recommended dietary

9   allowance (RDA) or high-level (1.1 g/kg lean body mass (LBM)/day or >2.1 g/kg LBM/day),

10   each followed by an experimental trial with a primed, constant infusion of D_8-phenylalanine

11   and D_2-tyrosine. Arterial and hepatic venous blood samples were obtained after an overnight

12   fast and repeatedly four hours after a standardized meal including intrinsically labeled whey

13   protein concentrate and calcium-caseinate proteins. Blood was analyzed for amino acid

14   concentrations and phenylalanine and tyrosine tracer enrichments wherefrom whole-body and

15   splanchnic amino acid and protein kinetics were calculated.

16   *Results:* High compared to the recommended level of protein intake resulted in a higher fasting

17   whole body protein turnover with a resultant 0.03 (±0.01 SEM) μmol/kg LBM/min lower net

18   balance (P<0.05), which was not rescued by the intake of a protein dense meal. The plasma

19   protein fractional synthesis rate was 0.13 (±0.06 SEM) %/h lower (P<0.05) following

20   habituation to high protein. Further a higher fasting and postprandial amino acid removal was

21   observed following habituation to high protein, yielding higher urea excretion and increased

22   phenylalanine oxidation rates (P<0.01).

23 *Conclusion:* Three weeks of habituation to high protein intake (>2.1 g protein/kg LBM/day)

24 led to a significantly higher net protein loss in the fasted state. This was not compensated for in

25 the 4-hour -post-prandial period after intake of a meal high in protein.

26 *Keywords:* Habitual protein intake, recommended protein intake, protein turnover, protein

27 breakdown, whole body protein turnover, intrinsically labelled proteins, stable-isotope tracers,

28 whey protein, caseinate protein

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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,

66 Gorissen and colleagues habituated older individuals for two weeks to high (1.5 g/kg body

67 weight (BW)/d) vs. low (0.7 g/kg BW/d) protein intake and observed neither an effect on

68 fasting whole body protein synthesis and breakdown rates nor a response to plain protein

69 feeding: However, a significantly increased amino acid oxidation was observed following

70 habituation to high protein (20).

71 With reference to the suggestions to elevate the dietary protein for older adults, it remains to

72 be elucidated whether habituation to higher protein intake have an impact different from the

73 currently recommended on the whole body protein turnover in a fasted state and how it

74 affects the post-prandial protein handling .The primary outcome was the difference in plasma

75 protein enrichment from intrinsically labeled whey protein 4 hours after ingestions. This was

76 measured to assess the post-prandial response to a mixed meal following habituation for 20

77 days to a normal (RDA) and high dietary protein intake in older individuals. Secondary

78 outcomes included whole body and plasma specific protein metabolism both in a fasted and

79 post-prandial state. We hypothesized that a high protein diet would increase the amino acid

80 oxidation and the nitrogen removal apparatus but simultaneously improve digestion/absorption

81 driving a more positive postprandial net protein synthesis response.

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88 **Subjects, materials and methods**

89 *Participants*

90 Twelve men (65-70 years) completed this double-blinded randomized crossover trial **(Figure**

91 **1)**. Before inclusion, all participants were screened by a physician and deemed overall healthy

92 based on blood samples, medical history and an interview. The exclusion criteria were

93 diabetes; pain in the skeletal muscle, connective tissue or back; known arthrosclerosis; alcohol

94 intake above 21 units per week; and regular intake of drugs or dietary supplements anticipated

95 to affect body protein turnover. Figure 1 shows a flowchart of the enrollment. 69 males where

96 assessed for eligibility, 12 were included and went through both habituation periods. There

97 were no dropouts.

98 Study design, purpose, risks and discomforts involved were explained (written and orally) to

99 each participant in accordance with the Helsinki declaration, after which the subjects gave

100 written consent of participation. The study took place at Bispebjerg and Frederiksberg

101 Hospital, Copenhagen. The local ethical committee of the Capital Region of Denmark

102 approved the study, protocol number H-15005598 and the study was registered at

103 Clinicaltrials.org under journal number NCT02587156.

104 Pre-screening involved dual-energy X-ray absorptiometry (DXA) scanning (Lunar iDXA; GE

105 Medical Systems, Pewaukee, WI, USA, with enCORE v.16 software) to determine the body

106 composition of the participants, with emphasis on the lean body mass (LBM) used to calculate

107 the desired dietary protein intake and the tracer amount needed. Participant characteristics can

108 be seen in **Table 1.**

109

110 *Experimental design*

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

112   The crossover intervention consisted of 20 days habituation to the recommended level of

113   protein in one period (recommended protein, RP) and habituation to a high level of protein

114   intake in another period (high protein, HP). The order of the habituation periods was randomly

115   assigned by a draw in a double blinded fashion.

116   In the 20-day habituation period the participants received four daily intakes of identically

117   packed supplements containing 20 g of whey hydrolysate and 10 g of sucrose in the HP period

118   and an isocaloric identically packed supplement consisting of 20 g maltodextrin and 10 g

119   sucrose in the RP period. The supplements came in powder form and the participants were

120   instructed to dissolve them a cold beverage of their choosing. The four daily supplements were

121   spread out during the day based on the participants customary protein distribution (e.g. two

122   supplements with breakfast, one with lunch and one with dinner if the participant ingested least

123   protein at breakfast in his daily diet. The pattern of supplement intake was decided by the

124   investigator) aiming at an even protein intake at all 3 main meals in the HP period. In the RP

125   period, participants were instructed in the exact same manner, however as their supplement

126   contained carbohydrate, their protein distribution throughout the day was not even.

127   Each habituation period started with a start-up period of seven days, where the only dietary

128   alterations were the four daily supplements. Subjects were guided towards a basic diet of 1.1 g

129   protein/kg LBM/day based on a 3-day dietary registration during the seven start up days. The

130   remaining 13 days of the 20-day habituation period were controlled by regular online dietary

131   registrations. Protein intake was adjusted to lean body mass instead of total body weight to

132   account for varying body compositions assuming that the lean body mass (LBM) is mainly

133   responsible for amino acid and protein metabolism. The 1.1. g protein/kg LBM/day

134   corresponds to 0.8 g protein/kg BW/day assuming a whole body fat content of 27%.

135

136 From day 19, participants refrained from any sort of strenuous physical activity. At 8 a.m. on

137 day 20, a 24-hour urine collection was started. At the last meal of day on day 20, all

138 participants had a standardized meal. The experimental trial on day 21 was identical for all

139 participants irrespective of the preceding habituation period. Following day 21, the participants

140 had a washout period of minimum 45 days, before crossing over to the alternate protein intake

141 intervention in a double blinded fashion. An overview of the 21-day period is illustrated in

142 figure 2.

143

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

145 Five Danish Holstein Friesian cows were infused with stable isotopes, four with L-[ring-D_5]-

146 phenylalanine and 1 cow with L-[^{15}N]-phenylalanine, the protocol is described in detail

147 elsewhere (21). In brief, milk was collected from 11 milkings, pasteurized and caseinate and

148 whey proteins were purified, yielding L-[ring-D_5]-phenylalanine labeled whey with an

149 enrichment of 15.44 ± 0.24 mole percent excess (MPE) ($\pm$SEM) and L-[^{15}N]-phenylalanine

150 labeled caseinate with an enrichment of 20.81 ± 0.02 MPE ($\pm$SEM).

151

152 *Day 21 – experimental trial day*

153 Participants fasted for 12 h overnight and arrived at 8 a.m. by taxi to the hospital ward. Upon

154 arrival they finished their first 24-hour urine sample period and a second 24-hour urine sample

155 period was started. Hereafter, the participants changed into hospital attire and were placed in a

156 bed in the supine position and a catheter was inserted in an antecubital vein and a background

157 blood sample was taken (-100 min, end of meal intake is set to 0 min). Hereafter, primed

158 continuous infusions were started of D_8-phenylalanine (prime: 4 μmol/kg LBM, continuous: 4

159 μmol/kg LBM/h in a fasted state, 4.8 μmol/kg LBM//h in postprandial state), D_2-tyrosine

160 (prime: 2.2 μmol /kg BW, continuous: 2.3 μmol/kg BW/h in a fasted state, 2.75 μmol/kg

161 LBM/h in a postprandial state), $^{15}N_2$-urea (prime 84 µmol/kg BW, continuous 9 µmol/kg

162 BW/h) and indocyanine green (ICG, prime: 1 mg, continuous infusion: 7-14 mg/h). At -40

163 minutes a catheter was placed in a radial artery under local anesthesia. Fluoroscopy was used

164 to place a catheter in a hepatic vein (through vena femoralis). The radial artery catheter as well

165 as the liver catheter were used for blood collection which were taken simultaneously. The

166 catheters were kept clear by frequent flushing with saline.

167 No sooner than 90 minutes after the start of the stable isotope tracer infusion, two fasting blood

168 samples were taken from each of the sampling sites (hepatic vein and artery). The consecutive

169 blood samples were taken at -12 and -10 minutes, analysis of these results were pooled and

170 collectively referred to as -10. Following blood sampling, a protein dense mixed breakfast

171 meal was served. The meal had to be ingested within 10 minutes and contained 0.61 g

172 protein/kg LBM, of which 0.23 g/kg LBM came from ^{15}N-phenylalanine intrinsically labeled

173 caseinate protein, and 0.31 g/kg LBM came from D_5-phenylalanine intrinsically labeled whey

174 protein. The D_5-labeled whey protein was dissolved in water and served as a protein drink. The

175 ^{15}N-labeled caseinate was mixed with raspberry jam, which was spread with butter on a bun

176 and served with the whey drink. The protein content in the butter and the bun constituted the

177 remaining 0.07 g/kg LBM of the total 0.61 g protein /kg LBM. **Table 2** shows the

178 macronutrient content in the breakfast.

179

180 After meal ingestions, blood samples were drawn simultaneous from the hepatic vein and

181 radial arteria at time 30, 60, 90, 120, 150, 180, and 240 min. After the last sample was

182 obtained, all catheters were removed, participants received a standardized lunch containing

183 0.80 g protein/kg LBM and a urine sample was collected, and the participants got a taxi home.

184 At home they ingested a standardized dinner ensuring an equal protein content of 0.61 g /kg

185 LBM. Thus, all participants ingested 2.02 g protein/kg LBM across day 21 independent of

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

187 hour urine collection ended.

188

189 *Blood analysis*

190 Arterial plasma samples were converted to serum by use of 1unit thrombin pr. 200 µl EDTA

191 plasma. The serum samples were analyzed for insulin, using ALPCO Insulin ELISA assay

192 (Catalog number: 80-INSHU-E01.1, E10.1, ALPCO).

193 Free amino acids were extracted from arterial plasma for measures of amino acid

194 concentrations and amino acid tracer abundances. An internal standard in a 500 µL 50% acetic

195 acid solution was added to 100 µl plasma, following which plasma was poured over cation

196 exchange columns with resin (AG 50W-X8 resin, Bio-Rad laboratories, Hercules, Ca, USA),

197 which had been prepped by adding 3 x 2ml 1M HCl creating an acidic environment. The

198 columns were washed 5 times with 3 mL of deionized water before the amino acids were

199 eluted by adding 2 x 2 mL 4M NH_4OH collected in vials. Solvent was evaporated under a

200 stream of N_2 flow at 70°C and derivatized using PITC derivatization agent, converting the

201 samples into their phenylthiocabamyl (PITC) derivative. 10 µl of the 100 µl derivatized

202 samples were loaded and analyzed on an ultra performance liquid chromatography system

203 coupled to a triple stage quadrupole mass spectrometer (LC-MS/MS) (Thermo Fischer

204 Scientific, San Jose, CA, USA) as described by Bornø et al. (22). Plasma protein bound tracer

205 enrichments, were analyzed from plasma protein precipitated with 500 µl ice cold acetone pr

206 50 µl plasma, hydrolyzed by 1 ml 1 M HCl and 1 ml resin slurry, and left overnight at 110°C.

207 Following hydrolysis, the samples were purified over cation exchange resin, as described

208 above. For measures of deuterium enrichments, samples were PITC derivatized and run on LC-

209 MS/MS system as described above. For determining the ^{15}N abundances, amino acids were

210 converted to the N-acetyl-propyl (NAP) derivatives and analyzed on a gas chromatography-

211 combustion-isotope ratio mass spectrometer (GC-C-IRMS) system as previously described by

212 Bornø et al. (23).

213 Plasma and urine samples for analysis of urea concentration as well as urea tracer enrichments

214 were prepared with internal standards and run over resin columns just like the samples for

215 phenylalanine and tyrosine enrichments. Following cation exchange on the resin columns and

216 overnight drying under N_2 stream the samples were derivatized by adding 40 µl acetonitrile

217 and 40 µl N-methyl-N-(tert-butyldimethylsilyl) trifluroroacetamide (MtBSTFA) + 1% tert-

218 butyl-dimethylchlorosilane (tBDMCS), followed by vortex mixing and kept at 70ºC for 30

219 minutes. The derivatized samples were separated on a GC column and isotope ratios were

220 analyzed on a triple-stage quadrupole mass spectrometer.

221

222 *Splanchnic flow*

223 The splanchnic blood flow was measured via Fick's principle, using a primed continuous

224 infusion of indocyanine green (ICG) as indicator as previously described (24). The plasma ICG

225 concentration was determined by spectrophotometry at wavelengths 805 and 900 nm. The light

226 absorption at 900 nm is a measure of turbidity and is thus regarded as the background "noise".

227 The calculation of blood flow is based on the difference in ICG concentration found in the

228 artery and hepatic venous blood. Thus, the splanchnic plasma flow is calculated using Fick's

229 principle (25).

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

230 Where C_{ICG-a}=ICG concentrations in the artery, and C_{ICG-hv}=ICG concentrations in the hepatic

231 vein.

232

233 *Tracers*

234   Concentrations of the labeled phenylalanine and tyrosine are calculated as their tracer to tracee

235   ratio (TTR) multiplied by the concentrations of unlabeled compounds. Enrichments are given

236   as mole percent excess (MPE=TTR/(1+TTR)). For D_8-phenylalanine enrichments the

237   transamination product (D_7-phenylalanine), and oxidation products (D_7- and D_6-tyrosine) were

238   also measured and the sum D_8- and D_7-phenylalanine as well as D_7- and D_6-tyrosine were used

239   for all calculations.

240   In the postprandial period, the tracers are in non-steady state and all amino acid kinetics in this

241   period are based on both ingestion of D_5-phenylalanine labeled whey, ^{15}N-phenylalanine

242   labeled caseinate, continuously infused D_8-phenylalanine and continuously infused D_2-

243   tyrosine. The calculations are done using Steele equation (26) with modification introduced by

244   Proietto et al. (27). An approach which is generally accepted for calculating whole body amino

245   acid and protein kinetics (20,28–30). The Steele equation incorporates the change in pool size

246   defined as: $Pool\ size = pV * \frac{dC}{dt}$, where pV is the partial volume of distribution set at 0.125,

247   dC is the difference in concentrations [μmol/l] across the time interval, dt. This means that for

248   all calculations which include non-steady state conditions, the values are calculated as delta

249   values between timepoints, thus the time resolution for these will be -5, 15, 45, 75, 105, 135,

250   165 and 210 minutes.

251

252   <u>Plasma protein FSR:</u> The direct incorporation of amino acids into plasma protein was given by:

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

253   Where ΔE protein is the change in enrichment in plasma protein, and the precursor is the

254   enrichment in the plasma taken from the hepatic vein.

255

256 <u>Ingested amino acid appearance rate:</u> 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 <u>Net amino acid balance across the splanchnic tissues:</u> 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

272 <u>Whole body protein turnover:</u> All measures in this section are based on concentrations and

273 enrichments in the arterial blood. The endogenous R_a represents phenylalanine appearing from

274 within the body, used as a measure of protein breakdown. It is given by:

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

275 The formula is the same for the fasted, steady state and the postprandial, non-steady state.

276 However, the appearance rates used are the ones calculated in the steady and non-steady state,

277 respectively.

278 The rate limiting step in phenylalanine oxidation is the irreversible hydroxylation of

279 phenylalanine to tyrosine (32). This conversion is given by:

280 In the fasted, steady state

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

281 In the postprandial, non-steady state

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

282 Where $\hat{E}_{tyr}$ and $\hat{E}_{phe}$ are the mean enrichments of either D_7-tyrosine or D_8-phenylalanine

283 between two surrounding timepoints.

284 Subtracting the 'phenylalanine-to-tyrosine conversion rate' from the total Rate of

285 disappearance (R_d), gives a measure of the phenylalanine removed by other processes than

286 oxidations, hence a measure of synthesis. The R_d is given by:

287 In the fasted, steady state

$$Total\ R_d = Total\ R_a$$

288 In the postprandial, non-steady state

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

289 As for the endogenous R_a, the formula for the endogenous R_d is the same in the fasted steady

290 state and the postprandial non-steady state. However, the disappearance rate and the

291 conversion of phenylalanine to tyrosine used are the ones calculated in the steady and non-

292 steady state respectively. Thus, the endogenous R_d is given by:

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

293

294 *Deviations*

295 All 12 subjects completed all trial days. However, on the experimental day 21, three subjects

296 did not receive infusion of D_2-tyrosine and are not included in calculations of phenylalanine

297 conversion to tyrosine and whole-body protein synthesis measurements. One participant was

298 excluded from all postprandial measurements due to mixing error with the labeled proteins.

299 D_7-phenylalanine and D_6-tyrosine (deriving from transamination of D_8-phenylalanine and

300 conversion from D_7-phenylalanine to D_6-tyrosine) was only measured for 5 participants. Based

301 on the known D_8-phenylalaine/D_7-phenylalanine and D_7-tyrosine/D_6-tyrosine ratios for these

302 five participants, the D_7-phenylalanine and D_6-tyrosine have been calculated for all

303 participants.

304 There were no dropouts.

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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.

325

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332

333   **Results**

334   The participants were on average 66.6 year of age and were overall healthy based on normal

335   blood pressure, lipid blood profile and hemoglobin A1c. Their average protein intake (1.5 g/kg

336   LBM/day) was higher than the current recommendation. All participant characteristics are

337   shown in Table 1.

338   With an intake of 1.22 ± 0.04 g/kg LBM/day (0.82 ± 0.03 g/kg BW/day), the full 20-day

339   dietary composition for the RP period was slightly higher than the aim (1.1 g/kg LBM/day).

340   However, the last 13 days of the habituation period, were right on point. For the HP period the

341   aim was >2.1 g/kg LBM/day. The actual intake covering the full 20-day period was 24%

342   higher, with an intake of 2.61 ± 0.04 g/kg LBM/day (1.76 ± 0.04 g/kg BW/day). The protein

343   intakes in the two habituation periods are seen in **Table 3.**

344

345   *Nitrogen and amino acid losses*

346   The urine urea quantity during the last 24 hours of the habituation was significantly higher

347   following habituation to HP (**Figure 3A**, P<0.01), emphasizing a high compliance to the

348   dietary protocol among the participants. During the 24 hours which included the trial day all

349   participants had the exact same relative protein intake irrespective of the habituation period.

350   Urine collected during the 8 h of the trial had a similar urea quantity independent of

351   habituation period (**Figure 3B**). In contrast, the total 24 hour urine of the entire trial day 21

352   had a higher urine urea after habituation to HP (**Figure 3C**, P<0.05) in spite of the same

353   protein intake through the entire day.

354   In line with these findings, the urea R_a is significantly higher following habituation to HP in

355   the fasted state (**Figure 4A**, P<0.01). The urea R_a remained elevated following habituation to

356 HP as compared to RP throughout the 4-hour postprandial period (**Figure 4B**). However, there

357 was no difference in the absolute change from fasted to postprandial urea R_a between the two

358 habituation periods (baseline corrected; data not shown). In accordance with this, the

359 phenylalanine hydroxylation rate to tyrosine (**Figure 4C and D**) was significantly higher

360 following habituation to HP both in the fasted (Figure C, P<0.01) and in the postprandial four

361 hour period (Figure 4D, time: P<0.0001, intervention P<0.001).

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

363 The fractional synthesis rates (FSR) of plasma proteins are shown in **Figure 5A**. There was a

364 significant higher plasma FSR in the 4-hour postprandial period following the RP period

365 compared to the HP period (P=0.046).

366 The enrichment from the intrinsically labeled whey (D_5-phenylalanine labeled) and caseinate

367 (^{15}N-phenylalanine labeled) proteins were assessed in the plasma protein 1 and 4 hours

368 postprandial. Values are presented as mole percent excess (MPE) for D_5-phenylalanine and

369 atom percent excess (APE) for ^{15}N-phenylalanine enrichments. The incorporation of

370 phenylalanine deriving from both whey and caseinate was greater at 4 hours compared to 1

371 hour postprandial (**Figure 5B and C**, P<0.0001). For the incorporation of phenylalanine

372 deriving from the whey protein into plasma proteins there was a significantly higher

373 incorporation following RP compared to HP intake 4 hours postprandial (Figure 5C,

374 P=0.0007).

375

376 *Insulin concentrations*

377 Serum insulin increased with the meal intake and remained elevated from baseline in the first 3

378 hours postprandial. It was unaffected by the preceding protein habituation period both in the

379 fasted and the postprandial state (**Figure 6**).

380

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

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

405   The arterial amino acid concentrations and splanchnic net balances are presented in **Table 4**.

406   The amino acid concentrations in the fasted state were similar for RP and HP except for

407   glycine. After meal and protein intake the concentrations of glycine (Gly), asparagine (Asn),

408   alanine (Ala), tyrosine (Tyr) and isoleucine (Ile) were significantly higher following habitation

409   to RP at several timepoints compared with subjects following HP diet (P<0.05). In addition,

410   tendencies for higher concentrations were also observed for lysine (Lys), tryptophan (Trp),

411   methionine (Met) and threonine (Thr) (P<0.07) in RP versus HP habituated participants. The

412   measured net balances across the splanchnic bed revealed no intervention (RP versus HP)

413   effect. It should be acknowledged that the splanchnic balance techniques bear relatively higher

414   variability as for example the single arterial amino acid concentration measurement. In the fed

415   state, the net balances for all amino acids but glutamine (Gln) became more negative indicating

416   a net release of amino acids from the splanchnic tissues to the circulation. However, no

417   changes were observed for glutamine (Gln) in the fasted compared to fed state.

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425 **Discussion**

426 The main findings of the present study are: 1. Twenty days of habituation to HP intake

427 enhances the whole body protein turnover rate; 2. HP habituation leads to a more negative

428 overnight fasted net protein balance; 3. The higher overnight fasted net protein loss with HP

429 was not compensated for in the 4 h postprandial period after a protein-dense meal containing

430 the same amount of protein for HP and RP; 4. Postprandial plasma protein FSR and

431 consequently incorporation of meal derived phenylalanine into plasma proteins were

432 significantly higher following habituation to RP.

433

434 Similar results as our finding, that whole body protein turnover is increased in response to

435 habituation to HP, were reported by Pannemans and colleagues in young (17) and older (19)

436 participants, who were habituated to either 12 or 21 E% protein. Whereas after habituation to

437 0.70 g protein/kg BW/day or 1.53 g protein/kg BW/day for 14 days, Gorissen and colleagues

438 (20) show no difference in whole body protein synthesis nor breakdown, in response to a

439 protein intake, thus no change in protein turnover. The major difference being that Gorrissen

440 et al. (20) studied the postprandial response to only ~0.42 g whey protein/kg LBM without

441 concomitant energy intake. Such amount of protein may have been insufficient to stimulate

442 protein turnover kinetics optimally in older men. Moore and colleagues showed that for plateau

443 stimulation of muscle protein synthesis rates requires an intake of 0.61 g protein/kg LBM in

444 older individuals (33), which we provided in the mixed meal.

445 An enhanced protein turnover rate may be advantageous as an improved capacity to repair and

446 remodel tissues and maintain protein function or adaption to a physiological challenge.

447 However, in the overnight fasted state HP habituation led to a more negative whole-body net

448 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

473   isoleucine concentrations were higher in RP. A higher postprandial amino acid concentration

474   can originate from a difference in digestion/absorption rate, splanchnic first by-pass extraction

475   and/or reduced peripheral clearance and increased production rates. The exogenous whey and

476   caseinate phenylalanine appearance rates (Figure 7) reveal similar protein digestion and

477   absorption rates after RP and HP habituation and the postprandial whole body protein

478   synthesis, degradation and net balance were also similar indicating similar peripheral clearance

479   and release rates. Therefore, the higher arterial concentrations of some amino acids after RP

480   habituation most likely originate from a lower net splanchnic first by-pass extraction of these

481   amino acids. To quantify arterial-hepatic venous differences, blood samples were taken from

482   the radial artery and the hepatic vein, reflecting the net balance across the splanchnic tissues

483   (36) despite the mixing of the arterial blood to the liver with portal venous content. In the

484   postprandial period, there was a net release for all amino acids, except glutamine, into the

485   circulation from the splanchnic tissue reflecting the expected net uptake. For glutamine the

486   demand in the splanchnic tissue extract most content in the meal, which is in agreement with

487   previous findings from Stoll et al. estimating that in piglets the gut tissue utilizes more than

488   95% of dietary glutamine (37). Together with alanine, glutamine is a key nitrogen carrier and

489   intermediate in amino acid transamination processes. Moreover, glutamine is the preferred

490   energy source of rapidly dividing cells such as those present in the intestine (36).

491   For all of the measured amino acids the net balance across the splanchnic tissues was similar

492   following the two habituation periods. Despite the even net balance across the splanchnic

493   tissues phenylalanine oxidation occurring primarily in the liver was significantly increased

494   following habituation to HP intake (Figure 4). Thus, another process must utilize more amino

495   acids in the RP condition, which appeared to be for plasma protein synthesis, which primarily

496   occurs in the hepatocytes (38).In agreement, the whey-derived amino acid tracer (D_5-

497   phenyalanine) was more abundant in the plasma proteins four hours after meal intake after RP

498   habituation. Overall, these results indicate that during habituation to high protein diets, the

499   amino acid metabolism is higher, leaving less amino acids for the translational apparatus even

500   in the postprandial period.

501

502   A limitation of the present study is that we cannot extrapolate our findings to the entire 24-

503   hour protein net balance. Irrespectively, it was clear that the four-hour net protein balance

504   response to the same meal did not compensate for the net protein loss identified during the end

505   of an overnight fasting period, despite the fact that it was a protein rich meal. The postprandial

506   net protein balance might have differed between the two habituations had the breakfast meal

507   not been identical but contained a protein level comparable to the habituated period as seen by

508   Kim et al. (40). In this study, following habituation to a high protein intake we measured the

509   response to a protein intake similar to the habituated level, and following habituation to the

510   recommended protein level we measured the response to an intake of protein which was twice

511   as high as the habituated level. This means that following the RP period, the meal stimuli is out

512   of the ordinary. Hence their response could potentially be exacerbated.

513

514   In conclusion, a protein intake higher than recommended level resulted in a significantly more

515   negative fasting whole-body net protein balance. The ingestion of a mixed protein dense meal

516   did not compensate for the lowered fasting net protein balance in the first four postprandial

517   hours. Further, the findings of a lower plasma protein FSR and less abundance of dietary whey

518   derived phenylalanine in plasma proteins point towards a higher first pass splanchnic

519   extraction of amino acids from the meal when habituated to high protein content in the diet.

520   This is most likely caused by a higher amino acid catabolism in the liver yielding the observed

521   higher urea production and excretion.

522   Taking the limitations into consideration, these results indicate potential drawbacks of

523   ingesting a 'higher than currently recommended protein level to improve the body's protein

524   utilization. Rather it increases the body's catabolism of amino acids, measured as increased

525   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) [$\times 10^{-3}$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).