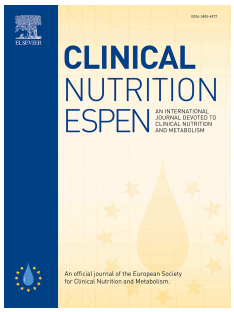
Journal Pre-proof



Estimation of the protein requirements of young men and women using the indicator amino acid oxidation method

Shiori Oda, Kazuko Ishikawa-Takata, Hina Sato, Hidetoshi Kumata, Kohsuke Hayamizu, Haruka Ochiai, Anna Tanaka, Tomonari Umemura, Yuki Tada, Azumi Hida, Rajavel Elango

PII: S2405-4577(26)02198-4

DOI: https://doi.org/10.1016/j.clnesp.2026.105101

Reference: CLNESP 105101

To appear in: *Clinical Nutrition ESPEN*

Received Date: 15 July 2026

Revised Date: 26 August 2026

Accepted Date: 3 September 2026

Please cite this article as: Oda S, Ishikawa-Takata K, Sato H, Kumata H, Hayamizu K, Ochiai H, Tanaka A, Umemura T, Tada Y, Hida A, Elango R, Estimation of the protein requirements of young men and women using the indicator amino acid oxidation method, *Clinical Nutrition ESPEN*, https://doi.org/10.1016/j.clnesp.2026.105101.

This is a PDF of an article that has undergone enhancements after acceptance, such as the addition of a cover page and metadata, and formatting for readability. This version will undergo additional copyediting, typesetting and review before it is published in its final form. As such, this version is no longer the Accepted Manuscript, but it is not yet the definitive Version of Record; we are providing this early version to give early visibility of the article. Please note that Elsevier's sharing policy for the Published Journal Article applies to this version, see: https://www.elsevier.com/about/policies-and-standards/sharing#4-published-journal-article. Please also note that, during the production process, errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

© 2026 European Society for Clinical Nutrition and Metabolism. Published by Elsevier Ltd. All rights are reserved, including those for text and data mining, AI training, and similar technologies.

Journal Pre-proof

Full-length article

Estimation of the protein requirements of young men and women using the indicator amino acid oxidation method

Shiori Oda [a,b], Kazuko Ishikawa-Takata [a,c*], Hina Sato [c], Hidetoshi Kumata [d], Kohsuke Hayamizu [e], Haruka Ochiai [f], Anna Tanaka [f], Tomonari Umemura [d], Yuki Tada [a], Azumi Hida [a] and Rajavel Elango [g]

[a] Faculty of Applied Bioscience, Tokyo University of Agriculture, Tokyo, Japan

[b] Graduate School of Health and Sports Sciences, Toyo University, Tokyo, Japan

[c] Faculty of Health and Sports Science, Toyo University, Tokyo, Japan

[c] Graduate School of Health and Sports Sciences, Toyo University, Tokyo, Japan

[d] School of Life Sciences, Tokyo University of Pharmacy and Life Sciences, Tokyo, Japan

[e] Department of Pharmacy, Yokohama University of Pharmacy, Yokohama, Japan

[f] Graduate School of Tokyo University of Pharmacy and Life Sciences, Tokyo, Japan

[g] Department of Pediatrics, University of British Columbia; BC Children's Hospital Research Institute, Vancouver, BC, Canada

Journal Pre-proof

Journal Pre-proof

19

20 S.O. and K.I-T. contributed equally to the study

21

Journal Pre-proof

Journal Pre-proof

Abstract

The indicator amino acid oxidation (IAAO) method is an alternative method of assessing protein requirements. However, there have been few studies of its use in healthy young adults, and the potential for a sex difference has not been evaluated. The primary purpose of the present study was to evaluate the protein requirements of healthy young men and women using the IAAO method and a basic study diet including a crystalline amino acid mixture, and the secondary purpose was to identify any sex difference in the protein requirement.

Methods: Healthy young men (n=7) and women (n=6) were randomly allocated to consume a study diet containing differing levels of protein (0.24–1.58 g/kg/day) in the form of a crystalline amino acid mixture, along with L-[1-^{13}C] phenylalanine. Their mean protein requirements were determined using a biphase linear regression crossover analysis of the exhaled $^{13}CO_2$, which identified a breakpoint at the minimum rate of appearance of $^{13}CO_2$ for the various protein intakes. This protocol was preregistered with the UMIN Clinical Trials Registry as UMIN000050212.

Results: The breakpoints were estimated to be 1.17 (95% confidence interval (CI), 0.95–1.39) g/kg body mass/day for the male participants and 1.04 (95% CI, 0.84–1.24) g/kg

body mass/d for the female participants. When their protein intake was expressed per unit fat-free mass, the breakpoints were estimated to be 1.50 (95% CI, 1.20–1.80) g/kg fat-free mass/day for the men and 1.35 (95% CI, 1.11–1.60) g/kg fat-free mass/day for the women. There was no significant difference in the breakpoint between the sexes.

Conclusions: The estimated breakpoints obtained during the present study was higher than requirements obtained using the results of nitrogen balance studies. There was no sex difference in the breakpoints using protein intake per unit body mass or fat-free mass for healthy young adults.

Keywords: sex difference; fat-free mass; crystalline amino acid; protein requirement; indicator amino acid oxidation method

Abbreviations

95% CI, 95% confidence interval; APE, atom percent excess; BM, body mass; DLW, doubly-labeled water; DRI, dietary reference intake; EAR, estimated average requirement; FFM, fat-free mass; IAAO, indicator amino acid oxidation; IFR, infrared spectrometer; IRMS, isotope ratio mass spectrometry; PAL, physical activity level;

Journal Pre-proof

RDA, recommended dietary allowance; REE, resting energy expenditure; TEE, total energy expenditure.

Journal Pre-proof

Journal Pre-proof

1. Introduction

The dietary protein requirement is defined as the minimum daily intake necessary to provide indispensable amino acids and nitrogen for the maintenance of protein balance for a healthy adult. In the Dietary Reference Intakes (DRIs) for the US and Japan, protein requirements were calculated using the results of nitrogen balance studies (1, 2). The estimated average requirement (EAR) and recommended dietary allowance (RDA) are 0.66 and 0.8 g/kg body mass (BM)/day, respectively, in the US; and the EAR and RDA are 0.73 g/kg BM/day for a daily mixed diet and 0.91 g/kg BM/day, respectively, in Japan. However, the nitrogen balance method has been shown to have several limitations when used to assess protein requirements (3-5), and alternative approaches have been suggested.

The Indicator Amino Acid Oxidation (IAAO) method has previously been used to identify amino acid and protein requirements in animals and humans (4). The IAAO method is minimally invasive and requires shorter study periods than a nitrogen balance study. Because of this, the IAAO method has often been used for individuals who were considered to be poor candidates for a nitrogen balance study, including children, pregnant women, athletes, and older people (6).

There have been few studies using the IAAO method in healthy adults, but all of these (3, 7-9) have shown that the calculated protein requirements are higher than the current RDA published as part of the DRIs (1, 2). However, direct comparisons of these IAAO studies is not possible because they involved different study diets: a crystalline amino acid mixture (3), casein and whey (9), or the habitual Chinese diet (7, 8). In addition, there has been no comparison of the protein requirements of the sexes.

Japanese people are known to have lower resting energy expenditures (REEs) (10) and poorer β-cell function than people of other ethnicities (11), implying that they may have different metabolic characteristics. The only one previous study of young Japanese men to use the IAAO method to determine protein requirements used a whey-based test diet and did not compare the sexes (9).

The primary purpose of the present study was to evaluate the protein requirements of healthy young Japanese men and women using the IAAO method and a study diet based on a crystalline amino acid mixture, and the secondary purpose was to identify any sex difference.

2. Material & Methods

Journal Pre-proof

2.1 Participants

A total of 13 healthy participants, who were university students (seven men and six women) were recruited to participate in the study, and they completed 3–7 trials each. The study was conducted between December 2022 and December 2024 at the Tokyo University of Agriculture. The participants had no history of a recent change in BM, undertook no habitual exercise, and had no chronic disease or acute illness that could have affected their protein or energy metabolism. In the present study, a sample size calculation was not conducted because this study was exploratory. Then the sample size was decided according to the previous studies (6) which most studies used 6 to 9 participants for each subgroup. All the procedures were performed in accordance with the standards stated in the most recent version of the Declaration of Helsinki, and the study protocol was approved by the Ethics Committee of Tokyo University of Agriculture (Nov 4, 2022; no. 2217, Jan 21, 2023; no. 2228). The protocol was registered with the UMIN Clinical Trials Registry as UMIN000050212 (https://center6.umin.ac.jp/cgi-open-bin/ctr/ctr_view.cgi?recptno=R000057152). The participants were fully informed about the protocol and the possible associated risks, then they provided their written informed consent.

Journal Pre-proof

Journal Pre-proof

2.2 Study protocol

Before the experiment, the participants arrived at the laboratory, underwent REE and anthropometric measurements, and submitted a 3-day dietary record. In addition, the male participants were asked to estimate their physical activity level (PAL) before the trial using the doubly-labeled water (DLW) method. Protein requirements were assessed using the minimally invasive IAAO protocol. The participants underwent 3–7 trials using different test protein intake levels, each of which involved 2 adaptation days, followed by an IAAO study day. Each trial was separated by more than 2 weeks. The women participated in one trial per menstrual cycle, during their luteal phase. On the study day, the participants were asked to visit the laboratory without having consumed anything except water, and then they consumed one twelfth of their daily energy and protein intake in each of eight meals, which were consumed at hourly intervals as test meals.

2.3 Measurement of REE and PAL and anthropometry

The participants fasted for at least 7 hours before coming to the laboratory. Their height was measured to the nearest 1 mm using an AD-6227 (A & D Company, Tokyo, Japan). Their BM and fat-free mass (FFM) were measured using the bioimpedance method (Innerscan RD-800; Tanita Corporation, Tokyo, Japan). After resting in a supine position

for 30 minutes in a quiet, dark room, REE was measured using an indirect calorimeter (Quark RMR; Cosmed, Lazio, Italy) for at least 15 minutes. The values at which breathing was stable and the deviation was <5% were used to estimate REE. Height, BM, and FFM were measured during the morning of every IAAO study day using the same equipment.

The DLW method was used to measure PAL. The male participants consumed approximately 0.06 g/kg BM $^{2}H_2O$ at 99.8 atom % (Cambridge Isotope Laboratories, MA, USA) and 1.4 g/kg BM $H_2{}^{18}O$ at 10.0 atom % (Nippon Sanso, Tokyo, Japan). A urine sample was collected before these doses were ingested and at the same time on days 1, 2, 14, and 15 days afterwards. The isotopic ratio was analyzed using an isotope ratio mass spectrometry (ANCA-GSL; Sercon, Crewe, UK). rCO_2 was calculated using equations 2 and 3 of Speakman *et al.* (12), and the total energy expenditure (TEE) was calculated using Weir's equation (13). A food quotient of 0.869 was calculated from the 3-day food record using the equation of Black *et al* (14). The PAL was calculated as the TEE divided by the REE. (see supplementary file) For the female participants, the PAL was assumed to be 1.75, according to the DRI for Japan (1), because all the participants were university students without high-intensity exercise habits.

Habitual protein intake was assessed using a 3-day food record. The dietary records were checked by research dietitians, and intakes were calculated using nutrition

150 calculation software (Eiyo Plus ver.2; Kenpakusya, Tokyo, Japan) and the Standard
151 Tables of Food Composition for Japan (15).

152

153 *2.4 Basal body temperature and salivary progesterone concentration*

154 The female participants were asked to measure their basal body temperature sublingually
155 every morning using an electronic thermometer (ET-W525DZ; Terumo Corporation,
156 Tokyo, Japan) to keep track of their menstrual cycle and to keep a record. Saliva samples
157 were collected after waking for the measurement of salivary progesterone concentrations
158 2–3 days after the end of menstruation and on the IAAO study days. On the day of
159 measurement, within 30 minutes of waking and at least 10 minutes after rinsing the mouth
160 with water, saliva samples of at least 1 mL each were collected into two tubes (Saliva
161 Collection Aid; Salimetrics, Carlsbad, CA, USA). After collection, the samples were
162 immediately placed into a freezer. The participants were requested to bring their samples
163 to the laboratory, where they were stored at −80°C until analyzed. They were analyzed
164 using an enzymatic immunoassay method (Salivary Progesterone Enzyme Immunoassay
165 Kit; Salimetrics, CA, USA). The salivary and circulating progesterone concentrations
166 have been shown to be closely correlated ($r=0.87$, $p<0.001$) (16). The phase of the
167 menstrual cycle was assessed using the method of Schmalenberger *et al* (17).

Journal Pre-proof

Journal Pre-proof

168

169 2.5 *Adaptation and study diets*

170 To ensure that the participants consumed sufficient energy and a consistent amount of
171 protein, they were provided with an adaptation diet for 2 days prior to the IAAO study
172 day, as in a previous study (18). The adaptation diet contained 0.9–1.0 g protein/kg
173 BM/day, and an amount of energy calculated as the REE multiplied by the PAL for each
174 participant. Commercially available, familiar foods were provided by the researchers as
175 the adaptation diet.

176 The participants arrived at the research site by 08:00 and consumed the first test diet
177 between 08:30 and 09:00. The study day test diet consisted of amino acid drinks, protein-
178 free cookies, and caramel blocks. The amino acid drink contained the crystalline amino
179 acid mixture and powdered juice (Matsuyama Seika, Ehime, Japan), maltodextrin
180 (Harbor Laboratories, Tokyo, Japan) and/or sugar. The protein-free cookies were made
181 with starch pancake mix (Oto Corporation, Kanagawa, Japan), shortening, sugar, and
182 water; and the caramel block was made of coconut oil (Life Science Research Corporation,
183 Fukuoka, Japan) and powdered juice (Matsuyama Seika, Ehime, Japan). In addition,
184 multivitamin and mineral tablets (Dear-Natura Multivitamins & minerals; Asahi Group
185 Foods, Ltd., Tokyo, Japan) were taken to avoid vitamin and mineral deficiencies.

186 On the study day, the test diet provided the REE (kcal/day) multiplied by the PAL
187 (1.5, for PAL level 1, as shown in the Japanese DRIs, because the participants remained
188 sedentary in the laboratory during the study period), with a fat/energy ratio of 30% (3,
189 19). The amount of carbohydrate consumed in the amino acid drink was adjusted using
190 powdered maltodextrin and sugar according to the protein intake level. The protein drinks
191 included 0.3–1.6 g/kg BM/day protein for the men and 0.2–1.6 g/kg BM/day protein for
192 the women as a crystalline amino acid mixture (Table 1). The actual protein test intake
193 was calculated using the measured amount of crystalline amino acid in the amino acid
194 drink divided by the BM of the participant on the study day. The crystalline amino acid
195 composition was based on the amino acid composition of an egg, except for the
196 phenylalanine and tyrosine contents, as previously described (20).
197
198 *2.6 Tracer protocol*
199 $NaH^{13}CO_3$ (0.176 mg/kg) and [1-^{13}C] phenylalanine (1.86 mg/kg) were administered as
200 part of the fifth meal on the IAAO study day, then a dose of [1-^{13}C] phenylalanine (1.2
201 mg/kg) was administered with the sixth-to-eighth meals. As in the previous studies, fixed
202 amounts of phenylalanine (30.5 mg/kg BM/day) and tyrosine (40.0 mg/kg BM/day) were
203 provided, irrespective of the test protein intake level (21, 22).

Journal Pre-proof

Journal Pre-proof

204

205 *2.7 Sample collection and analysis*

206 Approximately 2 hours after the start of the study, resting exhaled gas analysis was
207 performed with the participants in a seated position using an indirect calorimeter (Quark
208 RMR; Cosmed, Lazio, Italy) to measure the carbon dioxide output (VCO_2) on the IAAO
209 study day (Figure 1). To establish a baseline, a breath sample was obtained once before
210 the fifth meal. From 30 minutes after the seventh meal (6.5 hours after the start of the
211 IAAO protocol), breath samples were obtained eight times at 15-minute intervals. Breath
212 samples were collected into a breath collection bag (Breath Collection Bag 20; Otsuka
213 Pharmaceutical, Tokyo, Japan).

214 The stable isotope ratios in the breath samples were analyzed in duplicate using an
215 infrared spectrometer (IFR) (POCone; Otsuka Electronics, Osaka, Japan) (23). Twenty-
216 four breath samples (three test intakes for eight participants) were obtained on each study
217 day and analyzed using both an isotope ratio mass spectrometer (IRMS) (Hydra 20-20
218 Stable Isotope Mass Spectrometer; SerCon Ltd, Crewe, UK) and IFR. The mean isotope
219 ratios were 4.72±1.92‰ and 5.00±1.93‰ for IRMS and IFR, respectively. IFR yielded
220 significantly higher ratios (p=0.004), but the correlation coefficient for the relationship
221 between the IRMS and IFR data was high (r=0.986, p<0.001). The breakpoints calculated

222 from the data obtained using IRMS and IFR were 1.02 (95% CI: 0.61–1.43) g/kg/day and
223 1.13 (95% CI, 0.71–1.55) g/kg/day, respectively, and did not significantly differ.

224 Urine samples were collected once before the administration of the fifth test meal, to
225 act as a baseline, and then three times, every 30 minutes, from 6.5 hours after the first
226 meal (24). The urinary ^{13}C-Phe enrichment was analyzed using a previously described
227 method (25). In brief, urinary samples were reacted with pentafluorobenzyl bromide in a
228 monophase aqueous-acetone system to yield di-substituted pentafluorobenzyl derivatives
229 of Phe, which were then analyzed by GC/EI-MS (SIM) using an Agilent 5975C-inert
230 system equipped with a HP-5MS (30 m × 0.25 mm internal diameter × 0.25 μm; Agilent)
231 capillary column. Phe and ^{13}C-Phe were detected and quantified using ions with *m/z* of
232 434 and 435, respectively.

233

234 *2.8 Estimation of isotope kinetics*

235 The atom percent excess (APE) was calculated using the mean value and coefficients of
236 variation of the stable isotope concentration in the exhaled breath of at least four samples
237 if the coefficient of variation was <5% for eight samples. The phenylalanine flux, quantity
238 of exhaled $^{13}CO_2$, and phenylalanine oxidation were calculated using the model of
239 Matthews *et al* (26). Briefly, the phenylalanine flux (μmol/kg BM/h) was calculated using

Journal Pre-proof

240 the ingested L-[1-^{13}C] phenylalanine and urinary L-[1-^{13}C] phenylalanine concentrations.
241 The rate of appearance of $^{13}CO_2$ generated by the oxidation of ingested L-[1-^{13}C]
242 phenylalanine ($F^{13}CO_2$, μmol/kg BM/h) was calculated using the model of Matthews *et*
243 *al* (26), with a factor of 0.82 to account for the retention of $^{13}CO_2$ in the body bicarbonate
244 pool in the fed state (27). The oxidation rate of phenylalanine (μmol/kg BM/h) was
245 calculated from the concentration of $F^{13}CO_2$ and the urinary ^{13}C-phenylalanine
246 enrichment (24, 26).
247
248 *2.9 Statistical analysis*
249 The results are expressed as mean ± SD, and differences in datasets between the men and
250 women participants were identified using the Mann–Whitney U test. The phenylalanine
251 flux at the various levels of protein intake was compared using one-way analysis of
252 variance. The breakpoint for $F^{13}CO_2$ was estimated using a biphasic linear mixed-effects
253 model (28). The model accounted for internal correlations among the measurements for
254 the same participant by treating the participant as a random effect. The breakpoint was
255 estimated for a specific protein intake level, for which the Akaike information criterion
256 was minimized in the mixed-effects change-point regression model. The 95% CI of the
257 breakpoint was obtained using the standard asymptotic theory of the maximum profile

Journal Pre-proof

likelihood method. To evaluate the model's goodness-of-fit, the marginal and conditional R squares (R^2_m and R^2_c) were calculated using a previously established model (29). R^2_m explained the variance of the fixed effects and R^2_c explained the variance of both the fixed and random effects. Statistical analyses were performed using R (version 4.4.0; R Foundation for Statistical Computing, Vienna, Austria) and the nlme package (https://cran.r-project.org/).

3. Results

3.1 Characteristics of the participants

The participants were 20–24 years old, and the mean ages of the men and women did not differ (Table 2). The FFM of the men was significantly larger than that of the women (p=0.002), and the percentage body fat of the men was significantly lower (p=0.008). The BM and FFM did not significantly change during the study (Supplementary table 1). The habitual protein intakes of both the men and women were 1.3 g/kg/day. All the women had regular menstrual cycles of 27–34 days in length (30). Nine trials were conducted during the mid-luteal phase, and the other 21 trials were conducted during the entire luteal phase, according to the backward-count method (17). Seven trials were conducted 17–21

Journal Pre-proof

276 days before menstruation, when the menstrual cycles were longer than usual. All the trials
277 were confirmed to have been conducted between the day of ovulation and the onset of the
278 following period of menstruation. The progesterone concentration during the luteal phase
279 (mean 280 pg/mL) was significantly higher than that during the follicular phase (116
280 pg/mL) (p=0.020). The mean PAL of the men during habitual daily life was 1.75±0.07,
281 and using a PAL of 1.5 for the study test diet, the mean energy intakes associated with
282 the study test diet were 8.88 MJ (2,121 kcal) and 7.32 MJ (1,750 kcal) for the men and
283 women, respectively. Because the protein intake associated with the study test diet varied
284 between 0.2 and 1.6 g/kg BM/day, the energy obtained from protein varied from 2.9% to
285 18.4%.
286
287 *3.2 Phenylalanine flux and oxidation*
288 There was no significant difference in the phenylalanine flux, regardless of the protein
289 intake level (Table 3), suggesting that the size of the precursor pool for indicator amino
290 acid oxidation was not affected by the protein intake level, as required in the IAAO study
291 design, and shown earlier (3, 19-21).
292
293 *3.3 L[1-^{13}C] Phenylalanine oxidation*

Journal Pre-proof

294 The amount of $^{13}CO_2$ excreted in the breath was plotted against the protein intake level,
295 expressed per unit BM and per unit FFM (Figure 2, 3). As the protein intake level
296 increased, the concentration of $^{13}CO_2$ exhaled in the breath decreased and then stabilized,
297 and the value of the breakpoint was obtained. The breakpoints were estimated to be 1.17
298 (95% CI: 0.95–1.39, R^2_m=0.484, R^2_c=0.664) g/kg BM/day for the men (Figure 2A) and
299 1.04 (95% CI: 0.84–1.24, R^2_m=0.490, R^2_c=0.699) g/kg BM/day for the women (Figure
300 2B), and there was no significant difference between the two. When the data for the men
301 and women were combined, the breakpoint was estimated to be 1.13 (95% CI: 0.98–1.29,
302 R^2_m=0.504, R^2_c=0.684) g/kg BM/day. Breakpoints were also estimated for the protein
303 intake per unit FFM, and were 1.50 (95% CI, 1.20–1.80, R^2_m=0.517, R^2_c=0.650) g/kg
304 FFM/day for the men and 1.35 (95% CI: 1.11–1.60, R^2_m=0.490, R^2_c=0.698) g/kg
305 FFM/day for the women (Figure 3), and these also did not differ. When the data for the
306 men and women were combined, the breakpoint was estimated to be 1.39 (1.21–1.58,
307 R^2_m=0.500, R^2_c=0.686) g/kg FFM/day.
308
309 **4. Discussion**
310

Journal Pre-proof

311 In the present study, we examined the protein requirement for healthy young Japanese
312 men and women using the IAAO method. We found no significant difference between
313 the men and women using protein intake expressed per unit BM or FFM, and the
314 breakpoints in the relationships between protein intake and $^{13}CO_2$ were estimated to be
315 1.17 (95% CI: 0.95–1.39) and 1.04 (95% CI: 0.84–1.24) g/kg BM/day, respectively.

316 In the present study, we did not find a significant difference in the values of the
317 breakpoints between the men and women, although there were large inter-individual
318 differences in the relationship between $F^{13}CO_2$ and protein intake, which might explain
319 this. However, the earlier study conducted in young Chinese adults yielded similar results
320 (no difference in the breakpoints of the men and women, which were 0.88 and 0.85 g/kg
321 BM/day, respectively) (7). A subanalysis conducted as part of a meta-analysis of nitrogen
322 balance studies suggested that the protein requirement per unit BM of men is higher than
323 that of women (31). In the present study, even though protein intake was calculated on
324 both a per-unit BM and FFM basis, small differences in the body size of the men and
325 women might have affected the results. Indeed, a previous study showed that adjustment
326 for total energy expenditure using BM, FFM, or REE does not fully adjust for individual
327 differences (32). The women in the present studies had similar body sizes to those
328 reported following the National Health and Nutrition Survey in Japan (33), but the men

Journal Pre-proof

were shorter and weighed less in the present study. In addition, all of the trials were conducted for the women only during the luteal phase of their menstrual cycles, because the phase has been shown to affect the protein or amino acid requirement. The loss of nitrogen in the urine has been reported to be higher during the follicular phase, and at its lowest at ovulation (34). Furthermore, the lysine requirement was higher in the luteal phase than in the follicular phase (35). Thus, if the trials had been conducted during another phase of the menstrual cycle, a sex difference might have been identified.

The breakpoint estimated in the present study was slightly higher than those estimated during previous studies (3, 7-9). The previous study in which protein requirements for healthy men were estimated using the IAAO method had a similar design to the present study (using crystalline amino acid mixture similar to that of an egg) yielded a breakpoint of 0.93 g/kg BM/day and an upper limit of the 95% CI of 1.24 g/kg BM/day (3). However, it was difficult to compare the present findings with the previous findings for the following reasons. First, even after adjustment for BM, the participants in the present study had shorter stature and lower body mass. In particular, the men were shorter and lighter than those who participated in the National Health and Nutrition Survey (169.0 cm and 60.8 kg, respectively, for 22-year-old men) (33). Second, Japanese people in general have higher PALs than those in other countries (36). The men in the present study

Journal Pre-proof

had a PAL of 1.75, which is higher than the level recommended for the prevention of obesity (37). Previous studies that used the IAAO method for athletes (38-42) yielded higher breakpoint values, implying that relatively active individuals might have higher protein requirements. Third, it was possible that differences in habitual protein intake affected the results. In the present study, the participants confirmed an adaptation diet, associated with a protein intake of 0.9–1.0 g/kg BM/day, and based on diets used in previous studies (3, 19, 20, 40) and the DRIs for Japan (1), for 2 days. In contrast, in studies conducted in China, a 5–6-day adaptation period was used, and the diet contained the same protein content as the test diet (7, 8). For most of the studies that used the IAAO method, the habitual protein intake was not clear (6). Both the men and women in the present study consumed 1.4 g/kg BM/day protein. The habitual protein intake of the participants in the similar previous study was not clear, but in a study by the same research group, similar participants consumed 1.6 g/kg BM/day protein (43). Metabolic demands are thought to be affected by high habitual protein intake (44), and therefore this would affect the results of studies using the IAAO method. Finally, the composition of the test diet differed from that of the diets used in previous studies. Thirty percent of energy requirements were provided by fat in the test meal used in the present study, which was lower than in the study conducted in Canada (33%) (3), and comparable to the studies

conducted in China (26.5%–33.5%) (7, 8). The effect of the macronutrient composition of diets on protein requirements is considered to be very limited, although the available evidence is conflicting, and is considered to be very limited. In addition, previous studies of the effect of macronutrient composition used relatively extreme conditions(45, 46). When a BM maintenance diet was consumed, a diet with a carbohydrate-to-fat ratio of 1:1 was associated with less nitrogen excretion than a diet with a carbohydrate-to-fat ratio of 2:1 (45). However, consumption of an extremely low carbohydrate and high fat diet (2% of energy from carbohydrate and 83% of energy from fat) for 11 days was associated with greater urinary nitrogen excretion than intermediate- (44% of energy) or high- (85% of energy) carbohydrate diets, even when the amount of energy obtained from protein was the same in each (46).

The breakpoint value estimated in the present study was higher than the EAR and RDA protein recommendations in the DRIs of the US of 0.66 and 0.8 g/kg BM/day (2) and of Japan of 0.73 and 0.91 g/kg BM/day, respectively (1). Both DRIs set protein requirements on the basis of data from previous nitrogen balance studies, and these values are thought to reflect the minimum protein requirements, because such studies evaluated the difference between nitrogen intake and the nitrogen loss *via* the urine, feces, and miscellaneous other routes. In contrast, using the minimally invasive IAAO method, the

Journal Pre-proof

oxidation of the indicator amino acid is measured (L-[1-^{13}C] phenylalanine in the present study) relative to the intakes of all the other amino acids. A decrease in phenylalanine oxidation suggests no further incorporation into protein. Therefore, the breakpoint identified using the IAAO method has been suggested to reflect the protein or amino acid intake level that can be used as a threshold value for the optimization of whole-body protein net balance (47). A reanalysis of the results of nitrogen balance studies using biphase linear regression analysis yielded a higher protein requirement (of 0.91 g/kg BM/day) than that in the current recommendations (3). In the DRIs for Japan, the EAR was calculated using the data from nitrogen balance studies (0.66 g/kg BM/day) (31), and they estimate the protein utilization efficiency for a mixed protein diet to be 90% (48). The RDA for protein was calculated as the EAR multiplied by 1.25, to account for interindividual variability. However, the method used to estimate the RDA using data obtained using the IAAO method should be discussed carefully.

In the present study, we used the IAAO method, which has been widely used to estimate amino acid and protein requirements. However, there were a few limitations. First, the number of participants of each sex might have been too small to identify sex differences in protein requirements, and there was large interindividual variation in the relationship between the $F^{13}CO_2$ and protein intake. Therefore, the present study is best

Journal Pre-proof

regarded as exploratory, and the sample size calculation was not conducted in advance. If we conduct the rough calculation according to the present result which assumed sex difference of 0.013, standard deviation of 0.14 for the calculation of protein requirement from nitrogen balance studies (1), and statistical power of 0.8, the number of participants needed for each sex become 19. Second, the period required to complete the measurements of the protein levels was nearly 1 year, because we conducted the measurements for women only during the luteal phase of their menstrual cycles, and this could have increased intraindividual variability. However, because we used this long period, the present results may more accurately reflect the habitual protein requirement of the participants. In addition, habitual protein intake among the present participants were higher than the measured protein requirement. Third, similar to other studies that have used the IAAO method, the use of crystalline amino acids instead of regular foods, and the administration of test meals every hour, which differs from standard meal consumption patterns, makes the results difficult to extrapolate to people with normal daily protein intake. Fourth, it is unclear whether the protein requirements obtained using the IAAO method represent the amount that is appropriate for the maintenance and improvement of long-term health. Finally, habitual energy expenditure was only measured for the men, because of the cost and intensiveness of the study protocol, and

419 the need for women to undergo studies only during the follicular period of their menstrual
420 cycles. The DRIs for Japan list the requirements for nutrients according to sex, age group,
421 and PAL (three levels are considered). Further studies will be needed to determine
422 whether the protein requirements calculated in the present study differ with the various
423 PALs defined in the DRIs for Japan.
424

425 **5. Conclusion**

426

427 This is the first study, to the best of our knowledge, to use the minimally invasive IAAO
428 method alongside a crystalline amino acid mixture-based study diet in healthy young
429 Japanese men and women. We have calculated a breakpoint using the IAAO method that
430 is higher than the present RDA, which was calculated using the data from nitrogen
431 balance studies. In addition, we found no significant difference in the protein
432 requirements of each sex. The present results should provoke further discussions
433 regarding the dietary protein requirements of humans.
434

435 **Acknowledgments**

Journal Pre-proof

We thank all the participants in the study. We also thank Mark Cleasby, PhD from Edanz (https://jp.edanz.com/ac) for editing a draft of this manuscript.

Funding statement

This work was supported by AMED [grant number JP24ek0210166h0003].

Conflict of interest

None of the authors declare a conflict of interest.

Author contributions

Oda, Ishikawa-Takata: conceptualization, methodology, data curation, formal analysis, writing – original draft, writing – review & editing. **Sato:** data curation, writing – review & editing. **Hayamizu:** formal analysis, writing – review & editing. **Kumata, Ochiai, Tanaka, Umemura:** analysis of the urinary isotope concentrations, writing – review & editing. **Tada:** scheduled women with their menstrual phase, writing – review & editing. **Hida:** supervision, writing – review & editing. **Elango:** conceptualization, writing – review & editing.

References

Journal Pre-proof

Journal Pre-proof

1. Ministry of Health, Labour and Welfare,, Japan. Dietary Reference Intake for Japanese 2025 Tokyo, Japan: Ministry of Health, Labour and Welfare, , Japan; 2024 [Available from: https://www.mhlw.go.jp/content/10904750/001316585.pdf.
2. Institute of Medicine. Dietary Reference Intakes for Energy, Carbohydraate, Fiber, Fat, Fatty Acids, Cholesterol, Protein, and Amino Acids. Washington, DC, USA: National Academies Press; 2005.
3. Humayun MA, Elango R, Ball RO, Pencharz PB. Reevaluation of the protein requirement in young men with the indicator amino acid oxidation technique. Am J Clin Nutr. 2007;86(4):995-1002.
4. Elango R, Humayun MA, Ball RO, Pencharz PB. Evidence that protein requirements have been significantly underestimated. Curr Opin Clin Nutr Metab Care. 2010;13(1):52-7.
5. Weiler M, Hertzler SR, Dvoretskiy S. Is It Time to Reconsider the U.S. Recommendations for Dietary Protein and Amino Acid Intake? Nutrients. 2023;15(4).
6. Matsumoto M, Narumi-Hyakutake A, Kakutani Y, Tsuji M, Hatamoto Y, Higaki Y, et al. Evaluation of protein requirements using the indicator amino acid oxidation method: a scoping review. J Nutr. 2023;153(12):3472-89.
7. Li M, Wang ZL, Gou LY, Li WD, Tian Y, Hu YC, et al. Evaluation of the protein requirement in Chinese young adults using the indicator amino acid oxidation technique. Biomed Environ Sci. 2013;26(8):655-62.
8. Tian Y, Liu J, Zhang Y, Piao J, Gou L, Tian Y, et al. Examination of Chinese habitual dietary protein requirements of Chinese young female adults by indicator amino acid method. Asia Pac J Clin Nutr. 2011;20(3):390-6.
9. Yamamoto Yumen Y, Morita S, Yoshikawa M, Sonoki H, Iwamoto H, Takeda Y, et al. An Evaluation of the Protein Requirements of Japanese Young Men by Consuming Two Types of Dairy Proteins Using the Indicator Amino Acid Oxidation Method. J Nutr Sci Vitaminol (Tokyo). 2022;68(5):383-9.
10. Miyake R, Tanaka S, Ohkawara K, Ishikawa-Takata K, Hikihara Y, Taguri E, et al. Validity of predictive equations for basal metabolic rate in Japanese adults. J Nutr Sci Vitaminol (Tokyo). 2011;57(3):224-32.
11. Moller JB, Dalla Man C, Overgaard RV, Ingwersen SH, Tornoe CW, Pedersen M, et al. Ethnic differences in insulin sensitivity, beta-cell function, and hepatic extraction between Japanese and Caucasians: a minimal model analysis. J Clin Endocrinol Metab. 2014;99(11):4273-80.
12. Speakman JR, Yamada Y, Sagayama H, Berman ESF, Ainslie PN, Andersen LF, et al. A standard calculation methodology for human doubly labeled water studies. Cell Rep Med. 2021;2(2):100203.

Journal Pre-proof

13. Weir JB. New methods for calculating metabolic rate with special reference to protein metabolism. J Physiol. 1949;109(1-2):1-9.
14. Black AE, Prentice AM, Coward WA. Use of food quotients to predict respiratory quotients for the doubly-labelled water method of measuring energy expenditure. Hum Nutr Clin Nutr. 1986;40(5):381-91.
15. The Council for Science and Technology, Ministry of Educatin,, Culture,, Sports,, Science and Technology,, Japan. Standard Tables of Food Composition in Japan 2020: Ministry of Educatin,, Culture,, Sports,, Science and Technology,, Japan; 2020 [Available from: https://www.mext.go.jp/a_menu/syokuhinseibun/mext_01110.html.
16. Salimetrics, LLC. Salivary Progesterone Enzyme Immunoassay Kit CA, USA: Salimetrics, LLC; 2025 [Available from: chrome-extension://efaidnbmnnnibpcajpcglclefindmkaj/https://salimetrics.com/wp-content/uploads/2018/03/progesterone-saliva-elisa-kit.pdf.
17. Schmalenberger KM, Tauseef HA, Barone JC, Owens SA, Lieberman L, Jarczok MN, et al. How to study the menstrual cycle: Practical tools and recommendations. Psychoneuroendocrinology. 2021;123:104895.
18. Thorpe JM, Roberts SA, Ball RO, Pencharz PB. Prior protein intake may affect phenylalanine kinetics measured in healthy adult volunteers consuming 1 g protein. kg-1. d-1. J Nutr. 1999;129(2):343-8.
19. Rafii M, Chapman K, Elango R, Campbell WW, Ball RO, Pencharz PB, et al. Dietary Protein Requirement of Men >65 Years Old Determined by the Indicator Amino Acid Oxidation Technique Is Higher than the Current Estimated Average Requirement. J Nutr. 2015;146(4):681-7.
20. Rafii M, Chapman K, Owens J, Elango R, Campbell WW, Ball RO, et al. Dietary protein requirement of female adults >65 years determined by the indicator amino acid oxidation technique is higher than current recommendations. J Nutr. 2015;145(1):18-24.
21. Zello GA, Pencharz PB, Ball RO. Phenylalanine flux, oxidation, and conversion to tyrosine in humans studied with L-[1-13C]phenylalanine. Am J Physiol. 1990;259(6 Pt 1):E835-43.
22. Brunton JA, Ball RO, Pencharz PB. Determination of amino acid requirements by indicator amino acid oxidation: applications in health and disease. Curr Opin Clin Nutr Metab Care. 1998;1(5):449-53.
23. Kato M, Saito M, Fukuda S, Kato C, Ohara S, Hamada S, et al. 13C-Urea breath test, using a new compact nondispersive isotope-selective infrared spectrophotometer: comparison with mass spectrometry. J Gastroenterol. 2004;39(7):629-34.

Journal Pre-proof

24. Zello GA, Pencharz PB, Ball RO. Dietary lysine requirement of young adult males determined by oxidation of L-[1-13C]phenylalanine. Am J Physiol. 1993;264(4 Pt 1):E677-85.
25. Kumata H, Ochiai H, Tanaka A, Oda S, Ishikawa-Takata K, Umemura T. Simple analytical method to determine urinary isotopic enrichment of phenylalanine by GC/EI-MS coupled with pentafluorobenzyl derivatization. Talanta. 2025;287:127697.
26. Matthews DE, Motil KJ, Rohrbaugh DK, Burke JF, Young VR, Bier DM. Measurement of leucine metabolism in man from a primed, continuous infusion of L-[1-3C]leucine. Am J Physiol. 1980;238(5):E473-9.
27. Hoerr RA, Yu YM, Wagner DA, Burke JF, Young VR. Recovery of 13C in breath from NaH13CO3 infused by gut and vein: effect of feeding. Am J Physiol. 1989;257(3 Pt 1):E426-38.
28. Hayamizu K, Kato M, Hattori S. Determining amino acid requirements from repeated observations on indicator amino acid oxidation method by mixed-effect change-point regression models. J Clin Biochem Nutr. 2011;49(2):115-20.
29. Nakagawa S, Schielzeth H. A general and simple method for obtaining R2 from generalized linear mixed-effects models. Methods Ecol Evol. 2013;4(2):115-20.
30. Elliott-Sale KJ, Altini M, Doyle-Baker P, Ferrer E, Flood TR, Harris R, et al. Why We Must Stop Assuming and Estimating Menstrual Cycle Phases in Laboratory and Field-Based Sport Related Research. Sports Med. 2025;55(6):1339-51.
31. Li M, Sun F, Piao JH, Yang XG. Protein requirements in healthy adults: a meta-analysis of nitrogen balance studies. Biomed Environ Sci. 2014;27(8):606-13.
32. Taguri E, Tanaka S, Ohkawara K, Ishikawa-Takata K, Hikihara Y, Miyake R, et al. Validity of physical activity indices for adjusting energy expenditure for body size: do the indices depend on body size? J Physiol Anthropol. 2010;29(3):109-17.
33. Ministry of Health, Labour and Welfare, Japan. The National Health and Nutrition Survey [Available from: https://www.mhlw.go.jp/bunya/kenkou/kenkou_eiyou_chousa.html.
34. Calloway DH, Kurzer MS. Menstrual cycle and protein requirements of women. J Nutr. 1982;112(2):356-66.
35. Kriengsinyos W, Wykes LJ, Goonewardene LA, Ball RO, Pencharz PB. Phase of menstrual cycle affects lysine requirement in healthy women. Am J Physiol Endocrinol Metab. 2004;287(3):E489-96.
36. Ishikawa-Takata K, Tabata I, Sasaki S, Rafamantanantsoa HH, Okazaki H, Okubo H, et al. Physical activity level in healthy free-living Japanese estimated by doubly labelled water method and International Physical Activity Questionnaire. Eur J Clin Nutr. 2008;62(7):885-91.
37. Saris WH, Blair SN, van Baak MA, Eaton SB, Davies PS, Di Pietro L, et al. How much physical activity is enough to prevent unhealthy weight gain? Outcome of the IASO 1st Stock Conference and consensus statement. Obes Rev. 2003;4(2):101-14.

Journal Pre-proof

563 38. Bandegan A, Courtney-Martin G, Rafii M, Pencharz PB, Lemon PW. Indicator Amino
564 Acid-Derived Estimate of Dietary Protein Requirement for Male Bodybuilders on a Nontraining
565 Day Is Several-Fold Greater than the Current Recommended Dietary Allowance. J Nutr.
566 2017;147(5):850-7.
567 39. Bandegan A, Courtney-Martin G, Rafii M, Pencharz PB, Lemon PWR. Indicator amino
568 acid oxidation protein requirement estimate in endurance-trained men 24 h postexercise
569 exceeds both the EAR and current athlete guidelines. Am J Physiol Endocrinol Metab.
570 2019;316(5):E741-E8.
571 40. Kato H, Suzuki K, Bannai M, Moore DR. Protein Requirements Are Elevated in
572 Endurance Athletes after Exercise as Determined by the Indicator Amino Acid Oxidation
573 Method. PLoS One. 2016;11(6):e0157406.
574 41. Malowany JM, West DWD, Williamson E, Volterman KA, Abou Sawan S, Mazzulla M,
575 et al. Protein to Maximize Whole-Body Anabolism in Resistance-trained Females after Exercise.
576 Med Sci Sports Exerc. 2019;51(4):798-804.
577 42. Wooding DJ, Packer JE, Kato H, West DWD, Courtney-Martin G, Pencharz PB, et al.
578 Increased Protein Requirements in Female Athletes after Variable-Intensity Exercise. Med Sci
579 Sports Exerc. 2017;49(11):2297-304.
580 43. Szwiega S, Pencharz PB, Ball RO, Xu L, Kong D, Elango R, et al. Length of Adaptation
581 Has No Effect on the Threonine Requirement Determined in Healthy Young Adult Males Using
582 the Indicator Amino Acid Oxidation Method. J Nutr. 2023;153(7):2016-26.
583 44. Millward DJ. Post-prandial tracer studies of protein and amino acid utilisation: what
584 can they tell us about human amino acid and protein requirements? Br J Nutr.
585 2024;131(12):2005-30.
586 45. McCargar LJ, Clandinin MT, Belcastro AN, Walker K. Dietary carbohydrate-to-fat
587 ratio: influence on whole-body nitrogen retention, substrate utilization, and hormone response
588 in healthy male subjects. Am J Clin Nutr. 1989;49(6):1169-78.
589 46. Bisschop PH, De Sain-Van Der Velden MG, Stellaard F, Kuipers F, Meijer AJ,
590 Sauerwein HP, et al. Dietary carbohydrate deprivation increases 24-hour nitrogen excretion
591 without affecting postabsorptive hepatic or whole body protein metabolism in healthy men. J
592 Clin Endocrinol Metab. 2003;88(8):3801-5.
593 47. Malowany JM, van Lieshout GAA, Verdijk LB, Moore DR, van Loon LJC, Trommelen
594 J. The indicator amino acid oxidation (IAAO) technique: a novel approach to assess protein
595 intakes that maximize whole-body protein anabolism. Crit Rev Food Sci Nutr. 2025:1-10.
596 48. Kaneko K, Ishikawa K, Setoguchi K, Koike G. Utilization and requirement of dietary
597 protein taking into account the dermal and miscellaneous nitrogen losses in Japanese women. J
598 Nutr Sci Vitaminol (Tokyo). 1988;34(5):459-67.

Journal Pre-proof

599

Journal Pre-proof

Journal Pre-proof

Figure legends

Figure 1. Study protocol

*1 Each test meal contributed one twelfth of the daily energy and protein intake, and was administered in eight doses at hourly intervals.

*2 $NaH^{13}CO_3$ (0.176 mg/kg) and [1-13C] phenylalanine (1.86 mg/kg) were administered in the fifth meal (4 hours after the start of the IAAO protocol).

*3 Breath and urine samples were obtained once, before the fifth meal.

From 30 minutes after the seventh meal (6.5 hours after the start of the IAAO protocol), breath samples were obtained eight times at 15-minute intervals, and urine samples were obtained three times, at 30-minute intervals.

Figure 2. Relationship between the protein intake, expressed per unit body mass, and the rate at which ingested L-[1-^{13}C] phenylalanine appeared in the exhaled breath as $^{13}CO_2$

The breakpoint was determined using biphase linear regression crossover analysis.

Figure 3. Relationship between the protein intake, expressed per unit fat-free mass, and the rate at which ingested L-[1-^{13}C] phenylalanine appeared in the exhaled breath as $^{13}CO_2$

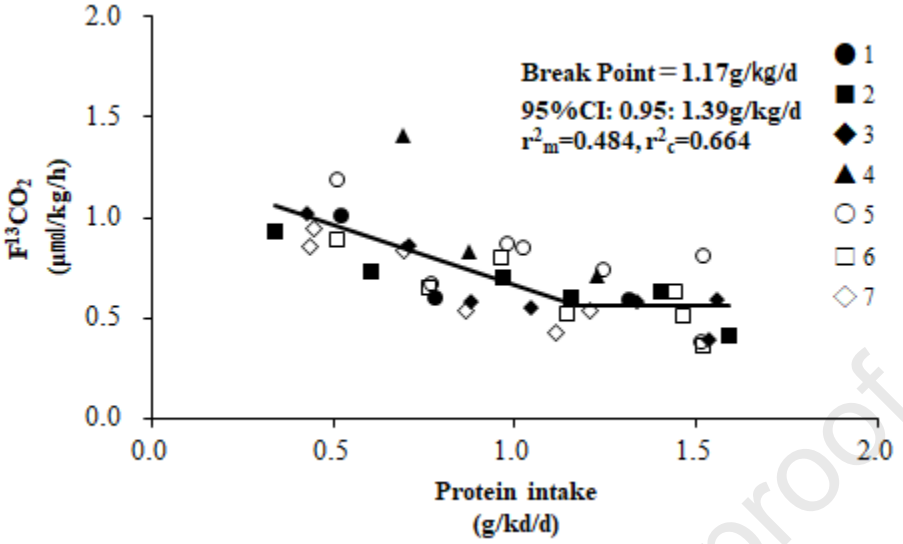
The breakpoint was determined using biphase linear regression crossover analysis.

Hour	0	1	2	3	4	5	6	7							
Test Meal *[1]	↑	↑	↑	↑	↑	↑	↑	↑							
$NaH^{13}CO_3$*[2]					↑										
L-[1-^{13}C] phenylalanine*[2]					↑	↑	↑	↑							
Breath*[3]					↑			↑	↑	↑	↑	↑	↑	↑	↑
Urine*[3]					↑			↑		↑		↑			
VCO_2			↑												

Figure 1

Journal Pre-proof

(men)



(women)

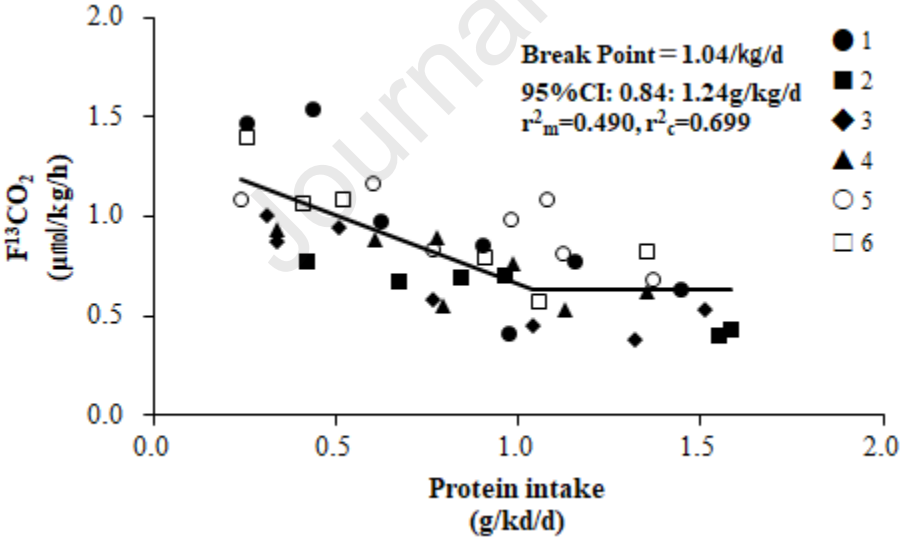
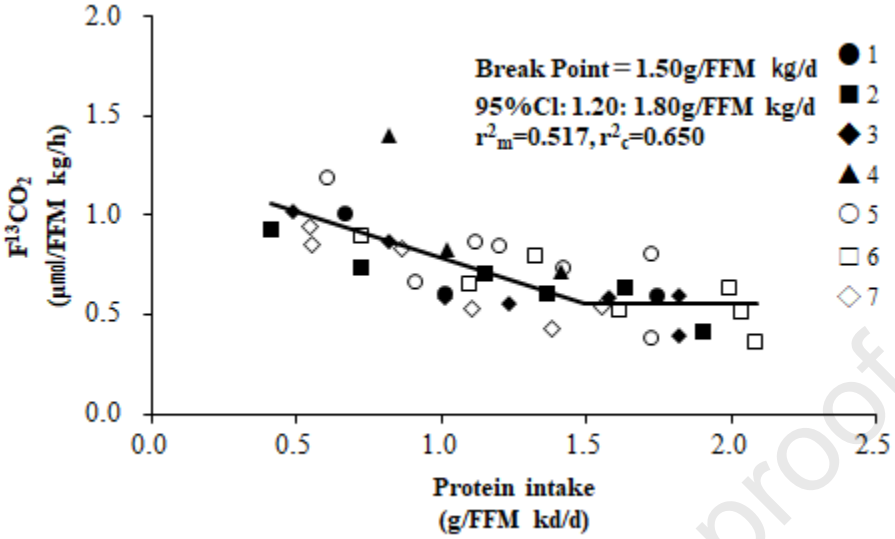


Figure 2

Journal Pre-proof

(men)



(women)

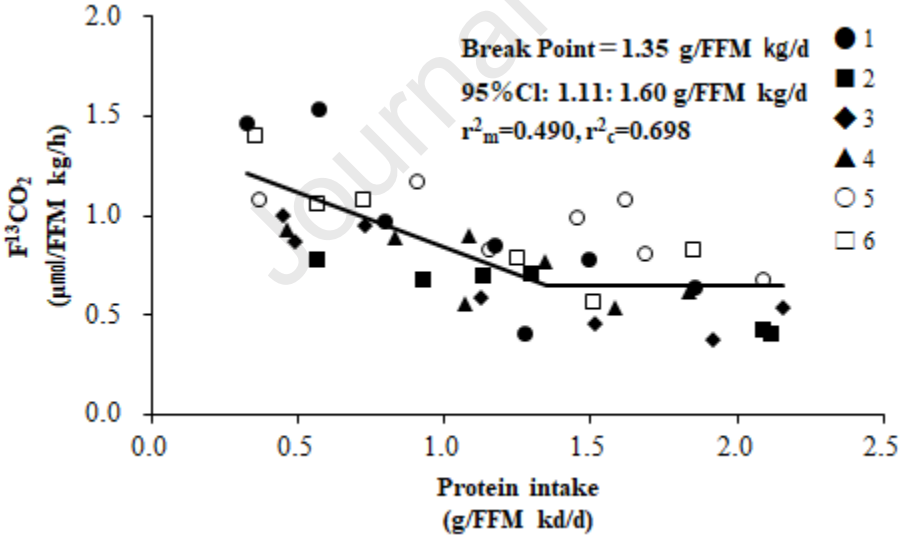


Figure 3

Journal Pre-proof

Journal Pre-proof

Table 2. Characteristics of the subjects

	Males (n=7)	Females (n=6)	p
Age (y)	21.9±1.1	21.7±0.9	0.731
Height (cm)	164.7±4.6	159.9±5.0	0.181
Body mass (kg)	57.6±7.8	52.4±3.8	0.445
Body mass index (kg/m²)	21.2±2.4	20.6±1.9	0.945
Fat free mass (kg)	47.0±4.2	37.6±2.2	0.002
Proportion of body fat (%)	18.4±5.1	28.1±4.1	0.008
Resting metabolic rate (MJ)	5.86±0.85	5.07±0.46	0.101
/Body mass (kJ/kg)	101.8±7.9	96.9±5.9	0.181
/Fat free mass (kJ/kg)	125.3±14.7	134.6±6.9	0.101

All values are shown as mean ± standard deviation
p value is calucurated by Man-Whitney u-test

Journal Pre-proof

Journal Pre-proof

Table 3. Protein intakes and Phenylalanine flux

	Protein intakes (g/kg BM/day)	Phenylalanine flux[1] (μmmol/kg BM/day)
Males	＜0.45 (n=4)	57.7± 8.8
	0.45≦, ＜0.75 (n=7)	43.9±12.7
	0.75≦, ＜1.05 (n=11)	64.3±17.2
	1.05≦, ＜1.35 (n=8)	52.8±11.9
	1.35≦ (n=9)	66.4±21.0
Females	＜0.45 (n=9)	49.1± 7.1
	0.45≦, ＜0.75 (n=6)	59.6±18.8
	0.75≦, ＜1.05 (n=12)	54.3±21.8
	1.05≦, ＜1.35 (n=8)	67.9±32.4
	1.35≦ (n=5)	46.7±17.1

Phenylalanine flux are expressed as mean ± standard error

[1] Protein intake levels had no statistically significant effect on phenylalanine flux.

BM: body weight

Journal Pre-proof

Table 1. Amount of protein intake used in individual participants

Subjects ID	Protein intakes (g/kg BM/day)
Males	
1	0.5, 0.8, 1.3
2	0.3, 0.6, 1.0, 1.2, 1.4, 1.6
3	0.4, 0.7, 0.9, 1.1, 1.3, 1.5, 1.6
4	0.7, 0.9, 1.3
5	0.5, 0.8, 0.9, 1.0, 1.3, 1.5, 1.6
6	0.5, 0.8, 1.0, 1.2, 1.4, 1.5, 1.6
7	0.4, 0.5, 0.7, 0.9, 1.1, 1.3
Females	
1	0.2, 0.4, 0.6, 0.9, 1.0, 1.2, 1.5
2	0.4, 0.7, 0.9, 1.0, 1.3, 1.5
3	0.3, 0.4, 0.5, 0.8, 1.1, 1.4, 1.5
4	0.3, 0.6, 0.7, 0.8, 1.0, 1.1, 1.4
5	0.2, 0.6, 0.8, 1.0, 1.1, 1.2, 1.4
6	0.2, 0.4, 0.5, 0.9, 1.1, 1.4

BM: body mass

Journal Pre-proof