The Journal of Nutrition
Nutrient Requirements and Optimal Nutrition

Protein Intake at Breakfast Promotes a Positive Whole-Body Protein Balance in a Dose-Response Manner in Healthy Children: A Randomized Trial

Leonidas G Karagounis,[1,3] Kimberly A Volterman,[2] Denis Breuillé,[1] Elizabeth A Offord,[1] Shahram Emady-Azar,[1] and Daniel R Moore[2]

[1]Institute of Nutritional Science, Nestlé Research Centre, Lausanne, Switzerland; [2]Faculty of Kinesiology and Physical Education, University of Toronto, Toronto, Canada; and [3]Experimental Myology and Integrative Physiology Cluster, Plymouth Marjon University, Plymouth, United Kingdom

Abstract

Background: Protein ingestion promotes whole-body net protein balance (NB) in children, which is a prerequisite for growth. Determining how much protein is required at breakfast to promote a positive NB, which may be negative after the traditional overnight fast in children, has yet to be determined.

Objective: We determined the impact of incremental doses of milk protein at breakfast as well as the impact of daily dietary protein distribution on NB in children.

Methods: A total of 28 children [14 boys, 14 girls; age range: 7–11 y; body mass index (mean ± SD, in kg/m^2): 16.0 ± 1.9] completed 2 intervention trials. During the breakfast meal, participants consumed an isoenergetic beverage with different amounts of protein (0, 7, 14, or 21 g for Groups A–D, respectively) and [^{15}N]-glycine to measure whole body protein metabolism. Whole-body nitrogen turnover, protein synthesis (PS), protein breakdown, and NB were measured over 9 and 24 h.

Results: Following an overnight fast, children were in negative NB (–64.5 mg · kg^{-1} · h^{-1}). Protein ingestion at breakfast induced a stepwise increase in NB over 9 h [Groups A (6.2 mg · kg^{-1} · h^{-1}) < B (27.9 mg · kg^{-1} · h^{-1}) < C (46.9 mg · kg^{-1} · h^{-1}) < D (66.0 mg · kg^{-1} · h^{-1})] with all conditions different from each other (all $P < 0.01$). PS was 42% greater in Group D than in Group A over 9 h ($P < 0.05$).

Conclusions: Consuming ≥7 g of the total daily protein intake at breakfast attenuates the observed overnight protein losses in children during the subsequent 9 h following breakfast consumption. The dose-dependent increase in NB over a daytime fed period, inclusive of breakfast and lunch, highlights the importance of breakfast protein intake on acute anabolism in healthy active children. This trial was registered at clinicaltrials.gov as NCT02465151. *J Nutr* 2018;148:729–737.

Keywords: children, breakfast, protein distribution, protein timing, growth

Introduction

Dietary protein provides the requisite amino acid building blocks to support lean tissue growth throughout life. Lean tissue is continually being remodeled through the reciprocal actions of protein synthesis and protein breakdown. The algebraic difference between these opposing but complementary processes represents the net balance and determines whether protein mass is increasing, decreasing, or being maintained. It is well established that in healthy adults, net balance is negative in the fasting state with protein ingestion stimulating protein synthesis and inducing a positive net protein balance in lean tissues (including muscle) (1–3). Moreover, the overnight fasting protein losses are offset by fed-state gains (4, 5), demonstrated by the diurnal variations observed in nitrogen and protein metabolism in weight-stable adults. However, in contrast to adults, children are in a relative state of constant growth, typically ∼5 cm/y and ∼3 kg/y until ∼10 y of age (6). This growth rate is ultimately underpinned by a chronically greater protein synthesis compared with protein breakdown that serves to support the increased protein mass. Due to these specific physiologic and metabolic demands of growing children, transferring the findings from studies carried out in adults to a given child population may not be appropriate. Previous research suggests that children display similar anabolic responses in acute lean tissue accrual following physical activity to those previously observed in adults

Manuscript received September 22, 2017. Initial review completed October 19, 2017. Revision accepted January 30, 2018.
First published online May 2, 2018; doi: https://doi.org/10.1093/jn/nxy026.

(7–9). Moreover, protein ingestion has been shown to increase net leucine balance during constant oral feeding in healthy children and adolescents (10). Although these previous studies in children support the use of dietary protein to enhance net protein balance under some physiologic settings, less is known about the effects of discrete (i.e., meal) protein intake on protein metabolism at rest in healthy children.

It has long been acknowledged that the pattern and not just the absolute amount of dietary protein can influence nitrogen retention in both adults and children (11, 12), as well as leucine balance in adults (13). Recently emphasis has been placed on breakfast eating, with particular interest in the role of dietary protein intake at breakfast to enhance protein balance, which may be negative following an overnight fast (14, 15). The practical relevance of breakfast protein intake is that the diets of many Western societies feature an unequal distribution of both total protein and energy, with the majority of children consuming more at dinner than at other times of the day (16). Recent research has demonstrated that distributing 90 g protein evenly throughout the day (3 × 30 g) results in greater muscle fraction synthetic rates than consuming the same amount of protein (90 g) in a skewed fashion (10, 15, and 65 g at breakfast, lunch, and dinner, respectively) (17). Collectively, this research suggests that meal protein intakes and distribution may help to optimize protein metabolism and net protein balance in adults (18, 19). Thus, although constant oral feeding (10) and post-exercise bolus feeding (7) results in increased protein metabolism in children, to date there is little research on the impact of discrete meal protein intake and daily distribution on protein metabolism and whole-body net protein balance in healthy active children.

Therefore, the purpose of this study was to identify how different doses of milk protein ingestion during breakfast were able to offset the overnight fasting net protein balance in healthy, physically active children. We hypothesized that, similar to our previous work on post-exercise protein intake in children (7), incremental protein ingestion at breakfast following an overnight sleep would induce a graded dose–response in protein balance. In addition, in line with our previous work on post-exercise dietary protein distribution (20), we hypothesized that daily dietary protein intake distribution would impact the 24-h net protein balance.

Methods

Participants. A total of 32 children (17 boys, 15 girls) volunteered to participate in the study (see **Figure 1** for flow diagram); 1 participant (male) withdrew from the study on the first intervention day due to a refusal to drink the study product, and 3 participants (2 boys, 1 girl) were excluded from the final dataset as the time between intervention visits fell outside the allowable visit window range (>21 d apart) for 2 of the participants, and 1 participant received more protein than planned. Therefore, a total of 28 children (14 boys, 14 girls; see **Table 1** for subject characteristics) provided a complete dataset and were included in the per-protocol analysis. Nevertheless, very similar results were observed for the full analysis dataset. Prior to collecting any data, all the details of the study were verbally explained to each participant and they subsequently provided written informed assent; written informed consent was obtained from each parent and/or guardian prior to the child's enrollment in the study. Participants had to meet the following criteria for inclusion: *1*) to be free of any existing medical conditions, as determined by medical questionnaire; *2*) to be between the ages of 7 and 12 y; *3*) to be healthy and recreationally physically active, defined by obtaining a 3-d average moderate-to-vigorous physical activity (MVPA) of ≥30 min/d (see below); *4*) to have a body mass ≥10th percentile for 7-y-olds and ≤85th percentile for 12-y-olds [based upon the 2000 CDC Growth Charts for the USA (21)]; and *5*) to have a habitual daily protein consumption of between 1.6 and 2.4 $g \cdot kg^{-1} \cdot d^{-1}$ with these values being reflective of their current consumption reality. Participants were excluded based on the following criteria: *1*) current use of medication; *2*) food allergy to milk proteins (e.g., whey or casein); *3*) within 1.5 y of peak height velocity (YPHV) in order to avoid the pubertal growth spurt and increase response homogeneity (22); *4*) currently partaking in any special diet or weight-loss program; *5*) consuming a breakfast containing >21 g protein/d of the 3-d screening period; and *6*) current participation or having participated in another nutritional-clinical trial within 2 mo of the current study. This study was approved by the University of Toronto Health Sciences Research Ethics Board.

General overview. Participants reported to the Iovate/Muscletech Metabolism & Sports Science Laboratory at the University of Toronto on 3 separate occasions: a preliminary visit and 2 intervention visits (**Figure 2**). Participants underwent an initial screening visit in order to establish their eligibility to participate in the study based on the aforementioned inclusion and exclusion criteria. Subsequently, participants underwent a consecutive 3-d screening period during which they were provided with a 3-d diet log to record their habitual dietary intake and an accelerometer to monitor their habitual activity levels. On the evening of day 3, participants ingested an oral [^{15}N]-glycine tracer (to measure whole-body protein kinetics) and collected all urine over the subsequent 10-h overnight period in order to enable the measurement of overnight protein metabolism. Eligible participants then completed 2 separate intervention visits (separated by a 4- to 21-d washout period). The intervention visits were conducted in a randomized, double-blind manner. At ~0730 and following their arrival to the laboratory all participants consumed 100 mL of water containing 2 mg/kg [^{15}N]-glycine to noninvasively measure whole-body protein kinetics. All urine was collected over the subsequent 9 h (for primary outcome) and 24 h (secondary outcome) in order to determine whole-body net protein balance. Following the consumption of the tracer beverage, each participant then orally ingested an isoenergetic (140 kcal) milk-based protein beverage containing a variable amount of protein and carbohydrate (described below and in **Table 2**). On the day of the intervention, diet and physical activity were controlled and mirrored the dietary intake and physical activity levels previously recorded for each participant.

Preliminary visit. Participants completed a preliminary session during which standing and sitting height (centimeters), body mass (kilograms), BMI, chronological age, and maturity offset (calculated as YPHV) (22) were determined (Table 1).

3-d screening period. To characterize habitual physical activity levels, participants were asked to wear an accelerometer (ActiGraph GT3X; AtiGraph, Pensacola, FL) for 3 d following the preliminary visit. Total and moderate-to-vigorous physical activity (MVPA) levels were quantified with the use of the cut points developed by Evenson et al. (23). To estimate habitual dietary intakes, participants were also asked to complete a 3-d dietary record (over the same 3-d period as wearing the accelerometer) that was analyzed with Food Processor SQL software (ESHA, Salem, OR) for energy and macronutrient intakes.

Prior to going to bed on the evening of day 3 of the screening period, participants collected a spot urine sample and voided their bladder completely (~2100). Following the spot urine sample, participants consumed 100 mL of water that contained 2 mg/kg body weight of

LGK and KAV contributed equally to this work.
This work was supported by a research grant from Nestec Ltd.
Author disclosures: LGK, DB, EO are employees of Nestec Ltd.; DRM was an employee of Nestec Ltd. The authors have no financial conflicts of interest to declare.
Address correspondence to LGK (e-mail: leonidas.karagounis@rdls.nestle.com).
List of abbreviations: MVPA, moderate-to-vigorous physical activity; NB, whole-body net protein balance; PB, whole-body protein breakdown; PS, whole-body protein synthesis; Q, whole-body nitrogen turnover; YPHV, years from peak height velocity.

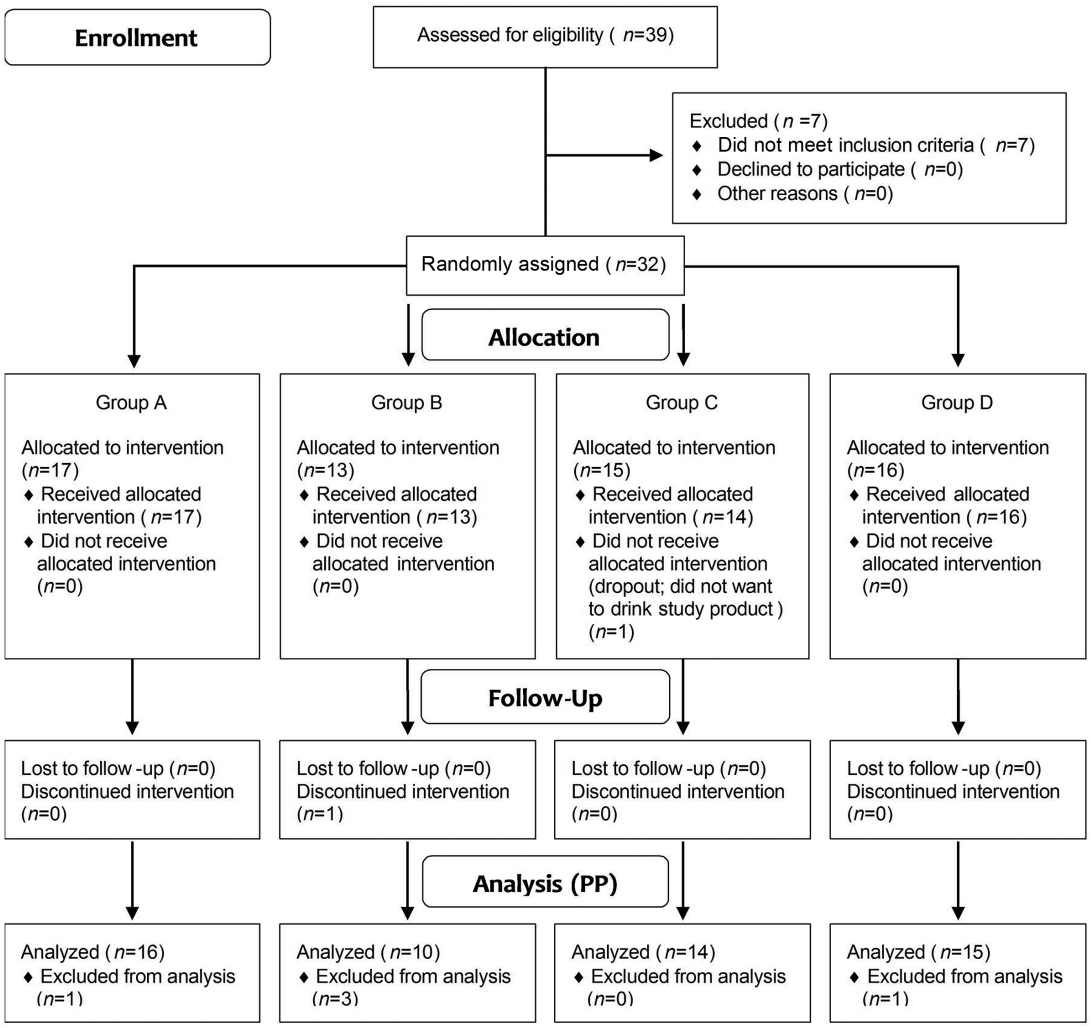

FIGURE 1 CONSORT flow diagram. Flow of participants and study analysis. CONSORT, CONsolidated Standards Of Reporting Trials; PP, per protocol.

[^{15}N]-glycine to determine the baseline state of protein balance and whole-body protein kinetics during a 10-h overnight fasting period in children (see below for details). Participants were instructed to collect all urine produced during the remainder of the evening, throughout the night, and the first urination the following morning (inclusive). Participants were also instructed not to consume any food or drink (except water) until after the first urine collection the following morning (at ~0700).

TABLE 1 Participant characteristics at baseline[1]

	Mean ± SD	Range
Age, y	8.7 ± 1.2	7–11
Body mass, kg	30.3 ± 6.9	19.9–46.4
Height, cm	136.9 ± 10.0	118.8–157.9
BMI, kg/m^2	16.0 ± 1.9	12.6–21.3
Weight percentile,[2] %	50.2 ± 25.4	0.5–88.7
Height percentile,[2] %	61.7 ± 27.3	4.8–99.3
Years from PHV, y	2.5 ± 0.6	−3.5 to −1.5
Body fat, %	14.0 ± 6.1	1.2–32.1
Fat-free mass, kg	25.7 ± 5.1	17.6–35.5

[1] Sample size and gender distribution, n = 28 (14 girls, 14 boys). PHV, peak height velocity.
[2] Percentile rankings relative to 2000 CDC growth charts for the United States (21).

Intervention visit. Following ≥4 d of washout after the 3-d screening period, participants reported to the laboratory (~0730) after an overnight fast and provided a spot urine sample prior to voiding their bladders. Following the spot urine sample, participants then underwent a BOD POD test to assess body mass, lean body mass, and percentage of body fat (BOD POD; Cosmed USA Inc., Chicago, IL). Participants were once again asked to void their bladders prior to being provided with their experimental beverage (see below for details and Figure 2) and were instructed to consume the beverage in its entirety within 5 min of commencing ingestion. In addition, 100 mL of water were consumed that contained 2 mg/kg body weight of [^{15}N]-glycine to determine whole-body protein kinetics (see below for details). Participants were also provided with a small low-protein breakfast (e.g., half an apple or half an orange), which provided 1.0% of total daily protein intake.

Each participant remained at the laboratory until 1630. All urine up until this point was collected and used to quantify the protein balance up to the 9-h time point (see below for details). During this 9-h period, each participant was provided with a controlled lunch providing 30% and 35% of daily protein and energy, respectively (deduced from the dietary records for protein), and a protein-free afternoon snack (providing 0% and 15% of daily protein and energy, respectively) to be

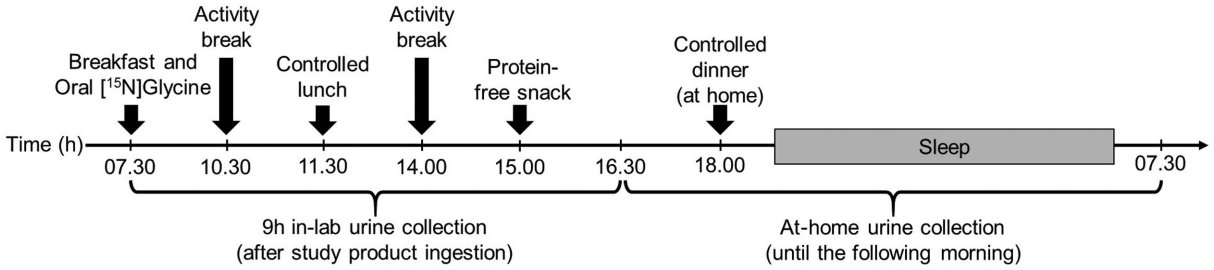

FIGURE 2 Schematic representation of the study outline.

consumed at regular intervals (see Figure 2). While in the laboratory, participants were also provided with 2 × 30-min periods in which they performed supervised physical activity during the morning (~1030–1100) and afternoon (~1400–1430) to simulate a normal schoolday routine.

After the last urine collection at 1630, each participant was allowed to return home and was provided with a preset dinner to be consumed at home. The dinner provided 40% of daily energy; however, the distribution of protein at the dinner meal was determined by the amount of protein consumed at breakfast so that the total daily protein intake throughout the study day remained constant (Table 2). All urine excreted at home up until ~0730 the following morning (or before the first meal of the day) was collected and kept refrigerated until collection by an allocated member of the investigational team. The exact time of the final urination before the first meal of the day was recorded, which allowed for the correction of whole-body protein metabolism and protein balance to 24 h.

Beverage preparation and composition. All beverages were prepared by dissolving the preprepared powders (Nestec Ltd, Vevey, Switzerland) containing different ratios of carbohydrate and protein in deionized water to a fixed volume of 200 mL. The beverage was then heated at 50°C for 60 min to ensure complete reconstitution of the experimental beverage. Beverages were prepared the evening prior to the trials and kept chilled at 4°C until ingestion. The experimental beverages contained either 0 g (Group A), 7 g (Group B), 14 g (Group C), or 21 g (Group D) bovine skim milk protein (containing both whey and casein protein fractions in a ratio of ~1:4, respectively). Beverages were isoenergetic (~140 kcal) and provided a variable amount of carbohydrate (sucrose): 35, 28, 21, and 14 g in Groups A–D, respectively.

Controlled diet. Protein intake throughout the experimental day was maintained constant for every participant as predetermined from his or her habitual 3-d dietary intake record. Daily energy intake was calculated with the use of the FAO/WHO/UNU equations to estimate metabolic rate in adolescent populations (24) corrected with an activity factor of 1.7. To ensure dietary compliance, all food was provided to the participants in prepackaged containers. While in the laboratory, a member of the investigational team visually confirmed compliance and any food item that was not eaten was recorded and its macronutrient content was subtracted from the daily macronutrient intake. For food

TABLE 2 Macronutrient and energy intake at individual daily eating occasions and across the entire 24 h of the intervention day[1]

	Group A ($n = 16$)	Group B ($n = 10$)	Group C ($n = 14$)	Group D ($n = 15$)
Protein, g				
Breakfast	0.6 ± 0.1	7.6 ± 0.1	14.6 ± 0.1	21.6 ± 0.1
Lunch	18.4 ± 3.9	16.8 ± 4.5	19.5 ± 3.2	17.2 ± 3.5
Snack	0.0 ± 0.1	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.1
Dinner	37.1 ± 11.8	29.9 ± 10.0	28.5 ± 11.1	18.8 ± 8.5
During 24 h	56.2 ± 14.3	54.3 ± 14.2	62.5 ± 14.0	58.2 ± 12.0
Carbohydrate, g				
Breakfast	43.6 ± 0.7	36.8 ± 0.1	29.8 ± 0.0	22.8 ± 1.0
Lunch	120 ± 19.0	116 ± 22.5	112 ± 20.1	116 ± 19.0
Snack	57.2 ± 8.1	50.6 ± 10.0	58.3 ± 6.2	56.5 ± 8.4
Dinner	86.4 ± 32.6	97.7 ± 25.9	107 ± 33.5	126 ± 28.9
During 24 h	272 ± 46.9	273 ± 40.4	287 ± 52.8	307 ± 40.4
Fat, g				
Breakfast	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
Lunch	19.9 ± 6.0	14.0 ± 7.8	21.1 ± 5.4	21.4 ± 4.2
Snack	9.4 ± 2.2	8.9 ± 2.7	8.6 ± 0.9	9.0 ± 1.9
Dinner	27.5 ± 11.1	23.2 ± 4.9	25.6 ± 10.3	20.0 ± 10.5
During 24 h	55.9 ± 15.1	46.1 ± 11.3	55.4 ± 13.6	50.5 ± 13.7
Energy, kcal				
Breakfast	173 ± 3.0	174 ± 0.0	174 ± 1.0	174 ± 3.0
Lunch	705 ± 77.0	635 ± 87.0	693 ± 94.0	693 ± 95.0
Snack	301 ± 33.0	270 ± 45.0	298 ± 32.0	294 ± 36.0
Dinner	744 ± 206.0	711 ± 147.0	769 ± 243.0	746 ± 176.0
During 24 h	1923 ± 277.0	1791 ± 228.0	1936 ± 347.0	1907 ± 271.0

[1] Data are presented as means ± SDs, $n = 28$ (14 girls, 14 boys).

items to be eaten at home, the participants or guardians confirmed the ingestion of each item by selecting it from a provided checklist upon eating. Participants or guardians were instructed that no food or drink (except water) other than what was provided was to be ingested during the evening of the study day. All food containers were kept and returned to the laboratory to ensure consumption, and any food item that was uneaten (and subsequently returned) was recorded and its macronutrient content was subtracted from the daily macronutrient intake.

Urine sample collection and analysis. On the evening of day 3 of the screening period, a spot urine sample was collected and 3 × 3-mL aliquots were collected and frozen at –80°C prior to analysis for background [^{15}N]-ammonia enrichment. Participants were provided with a 2-L container in order to collect all urine produced overnight until the first urination the following morning (inclusive). They were then instructed to store this urine at 4°C; this urine represented the 10-h overnight urine collection. The volume of the 10-h urine sample was measured with a graduated measuring cylinder and the volume was recorded to the nearest milliliter. Three × 3-mL (for isotopic enrichment analysis) and 3 × 1.5-mL (for urea and creatinine concentrations) aliquots of the 10-h pooled urine were collected and frozen at –80°C prior to analysis.

On the morning of the intervention day, a fasting spot urine sample was collected and 3 × 3-mL aliquots were collected and frozen at –80°C prior to analysis for background [^{15}N]-urea and [^{15}N]-ammonia enrichment. All urine produced by the participants while in the laboratory was collected into a labeled container, and then transferred (and pooled) into a 3-L jug; the total laboratory urine sample represented the 9-h urine collection. The volume of the 9-h urine sample was measured with a graduated measuring cylinder and the volume was recorded to the nearest milliliter. Three × 3-mL (for isotopic enrichment analysis) and 3 × 1.5-mL (for urea and creatinine concentrations) aliquots of the 9-h urine were collected and frozen at –80°C prior to analysis. The remaining 9-h urine was then stored at 4°C until the following day. Upon leaving the laboratory, each participant was provided with a 3-L container to collect all urine produced during the remainder of the day until the first urination the following morning (inclusive), and instructed to store it at 4°C; this urine was then combined with the urine collected during the 9-h in-laboratory period to produce the full 24-h sample. The volume of the 24-h urine sample was measured to the nearest milliliter with a graduated cylinder and recorded. Three × 3-mL (for isotopic enrichment analysis) and 3 × 1.5-mL (for urea and creatinine concentrations) aliquots of the 24-h pooled urine were collected and frozen at –80°C prior to analysis.

The concentrations of the major nitrogen-containing metabolites (urea and creatinine) were determined colorimetrically with the use of commercially available kits (Quantichrom, Bioassay Systems, USA), as an estimate of urinary nitrogen excretion. In addition, the [^{15}N]-enrichments (i.e., ratio of tracer:tracee, t:Tr) of urinary ammonia (at baseline and 10-h overnight on the screening day, as well as baseline, 9 h and 24 h on the intervention days) and urea (at baseline and 24 h on the intervention days) were determined in duplicate by isotope ratio MS by Metabolic Solutions Incorporated (Nashua, NH, USA) in order to determine whole-body measures of nitrogen turnover (Q), protein synthesis (PS), protein breakdown (PB), and net balance (NB) by the end-product method (25), similar to our previous work (8). Briefly, Q based on the [^{15}N]-ammonia end-product for the 9-h analysis was calculated as described previously (8). Q based on the [^{15}N]-harmonic mean approach for the 24-h analysis was similar to our previous work (8), though here corrected for time in order to express units as mg · kg^{-1} · h^{-1}. Protein synthesis was calculated as PS (mg protein · kg^{-1} · h^{-1}) = (Q – E)/(t × body mass) × 6.25, where E is measured and estimated nitrogen excretion. Measured nitrogen excretion was the sum of urinary urea nitrogen and creatinine nitrogen excretion over the 9- and 24-h periods, as required. Miscellaneous nitrogen excretion (e.g., fecal) was estimated according to previously published values in children consuming a 1.2 g protein · kg^{-1} · d^{-1} diet (26). PB and NB were calculated as previously described (8).

Sample size. To determine the required sample size, a simulation was performed based on previously published data (8).

The simulation revealed that with $n = 30$ participants systematically consuming 2 out of the 4 doses (accounting for a 20% drop-out rate), and with protein balance increasing with protein intake, then the minimal statistically significant dose could be estimated to be 7.10 g protein (95% CI: 2.9 g, 10.4 g) and the optimal dose 11.5 g protein (95% CI: 8.2 g, 16.6 g).

Randomization and blinding. Participant recruitment and testing occurred between July 2015 and May 2016. Upon enrollment to the study, the investigator in charge of beverage preparation (whose only other responsibility within the trial was to prepare the controlled diets) systematically randomly assigned each participant to 1 of the 12 different sequences (in order to consume 2 of the possible 4 doses) via Medidata Balance. Participants were randomly allocated to 2 of the 4 different intervention groups, named Groups A, B, C, or D. Medidata Balance provided a different nonspeaking code to represent each of the 4 groups (A, B, C, or D). Hence, the person in charge of beverage preparation had knowledge of the subject randomization but remained blinded to the protein content of the specific beverages, the trial statistician configuring Medidata Balance was semiblind, and the remainder of the study staff and participants were double-blind. The randomization codes were only broken after all data were collected and cleaned and the statistical analysis plan was confirmed. The trial was completed when all participants were recruited and tested.

Statistics. The 9-h protein balance (primary outcome) was analyzed by ANOVA in a mixed-effects linear model setting with the product as fixed effect and the subject as random effect with the aim of examining the relation between the incremental dosing regime and the 9-h protein balance. The analyses for the 9-h protein balance were conducted on both the full analysis set and the per protocol populations. Supportive analysis for the primary outcome was also performed with ANCOVA in a mixed model effect by adjusting the model for MVPA and total physical activity. Statistical analysis of 24-h protein balance distribution (secondary outcome) was done similarly to the primary outcome. To determine differences between group means, all protein kinetic measures (i.e., Q, PS, PB, and NB) for 9 and 24 h were subjected to pairwise *t* tests based on a mixed model taking into account the intersubject variability.

All statistical analyses were performed with SAS version 9.4 by SOCAR (a contract research organization) under the supervision of the Nestlé Research Centre. Data are presented as means ± SDs, unless otherwise indicated. Statistical significance level was set at $P \leq 0.05$ and all *P* values presented throughout the manuscript are unadjusted for multiplicity.

Results

Energy and protein intake. The average 3-d protein intake for the participants was 2.0 ± 0.3 g · kg^{-1} · d^{-1}, whereas habitual breakfast dietary protein intake was 0.4 ± 0.2 g · kg^{-1} · d^{-1}. Specifically, participants consumed 1.8 ± 0.3 g · kg^{-1} · d^{-1} of protein on day 3 of the 3-d screening, prior to the overnight protein metabolism measures and 12.2 ± 0.7 g at breakfast. Total energy and macronutrient intakes on the intervention day are presented in Table 2. One participant refused to consume the investigational beverage in its entirety due to the taste and complained of a stomachache on the trial day, resulting in termination of participation in the study. All remaining participants tolerated the investigational beverage with no reported adverse events.

Physical activity. Total habitual physical activity and habitual MVPA from the 3-d accelerometer were 243.5 ± 43.5 and 72.0 ± 21.9 min/d, respectively. Total habitual physical activity on the intervention day was 204.6 ± 43.6, 193.9 ± 34.1,

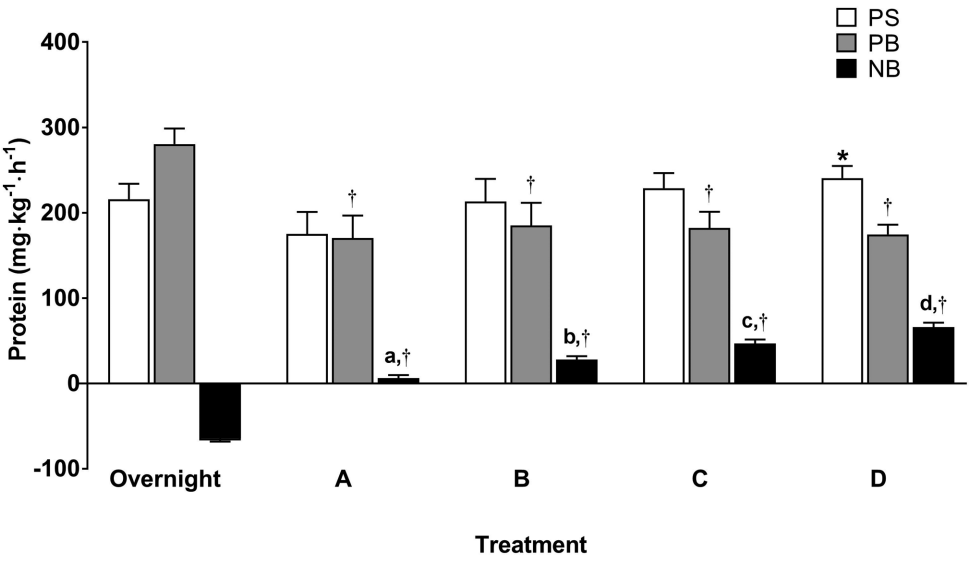

FIGURE 3 Relative whole-body protein turnover during the 9-h period after test beverage (breakfast) ingestion calculated using the [^{15}N]-ammonia end-product method expressed relative to body mass in children who consumed 0 g protein at breakfast (Group A, $n = 16$), 7 g protein at breakfast (Group B, $n = 10$), 14 g protein at breakfast (Group C, $n = 14$), or 21 g protein at breakfast (Group D, $n = 15$). Values are presented as means ± SEMs. Labeled means without a common letter differ, $P < 0.05$. *Different from Group A, $P < 0.001$; †different from corresponding overnight data, $P < 0.01$. NB, net balance; PB, protein breakdown; PS, protein synthesis.

191.2 ± 45.3, and 196.2 ± 55.7 min/d for Groups A–D, respectively. Total physical activity did not differ between groups ($P > 0.05$). Total MVPA on the intervention day was 62.1 ± 23.6, 59.4 ± 20.8, 64.2 ± 28.7, and 61.5 ± 23.1 min/d for Groups A–D, respectively. Total MVPA did not differ between groups ($P > 0.05$).

Overnight whole-body protein metabolism by [^{15}N]-ammonia end-product enrichment. During the 9-h overnight period of the baseline visit PS and PB were 216 ± 94.8 and 280.5 ± 96.4 mg · kg^{-1} · h^{-1}, respectively, with overnight net protein balance being negative at –64.5 ± 19.8 mg · kg^{-1} · h^{-1}.

Whole-body 9-h protein metabolism by [^{15}N]-ammonia end-product enrichment during the day. Protein synthesis was greater in Group D than in Group A over 9 h ($P < 0.05$). There were no significant differences observed between any of the study protein dose groups for PB. Protein ingestion at breakfast induced a stepwise increase in NB over 9 h, with all conditions different from Group A and from each other (all $P < 0.001$) (**Figure 3**).

The adjusted linear regression showed that none of the covariate estimates were statistically different for MVPA ($P > 0.05$) or for total physical activity ($P > 0.05$), suggesting the unadjusted model is preferred.

Whole-body 24-h protein metabolism by harmonic mean of [^{15}N]-ammonia and [^{15}N]-urea end-product enrichment. Over 24 h, there were no differences between groups for either PS or PB. The 24-h NB was positive for all study groups; however, the mean NB was lower ($P < 0.05$) for children who ingested 21 g protein at breakfast (Group D) than for the children who received 7 g protein at breakfast (Group B) (**Figure 4**).

Discussion

We demonstrate that, similar to adults (4, 5), healthy active children are in a state of negative NB during overnight sleep (i.e., a ~10-h overnight fast). However, breakfast dietary protein intake, as part of the daily protein intake, induces a dose-response increase in whole-body net protein balance over the following ~9-h period, which helps to offset overnight protein loss. Consuming as little as 7 g milk-based protein at breakfast (~12% of daily protein intake) in combination with 18 g protein at lunch are sufficient to support a positive NB during the subsequent 9-h period when habitual diet and activity are controlled. Finally, we showed that under habitual free-living conditions, daily dietary protein distribution may impact 24-h whole-body protein balance in healthy children.

Protein metabolism displays a diurnal variation, as a result of which fasting state losses are counterbalanced by fed state gains (4, 5, 27). This is evident during the overnight fasting period, in the course of which the whole-body and muscle protein NB becomes negative (4, 28), a process that takes place in part to provide amino acid substrates to support vital physiological functions (gluconeogenesis, organ tissue synthesis, etc.). It has been suggested that nitrogen flux based on urinary ammonia enrichment more closely models protein metabolism in peripheral tissues such as muscle, which represents the major site of the ammonia precursor glutamine (29). This possibility could result in slightly greater rates of nitrogen flux by ammonia enrichment during the overnight period after tracer ingestion given that muscle glutamine production is generally greatest during fasting. However, compared with urea and creatinine, urinary ammonia excretion represents a relatively minor route of total body nitrogen loss under normal conditions (30), which would have thus had minimal impact on our overnight calculated rates of whole-body net protein balance. Nevertheless, the present results in children mirror those of previous research in adults: the overnight fast induced a shift towards a negative NB that was primarily driven by an increased rate of PB (Figure 3), presumably in both muscle and nonmuscle tissues. This increase in overnight whole-body PB was attenuated by ~50% during the 9-h fed period in all conditions, suggesting the effects were not mediated by the dietary protein intake but likely due to an insulin-mediated suppression of whole-body

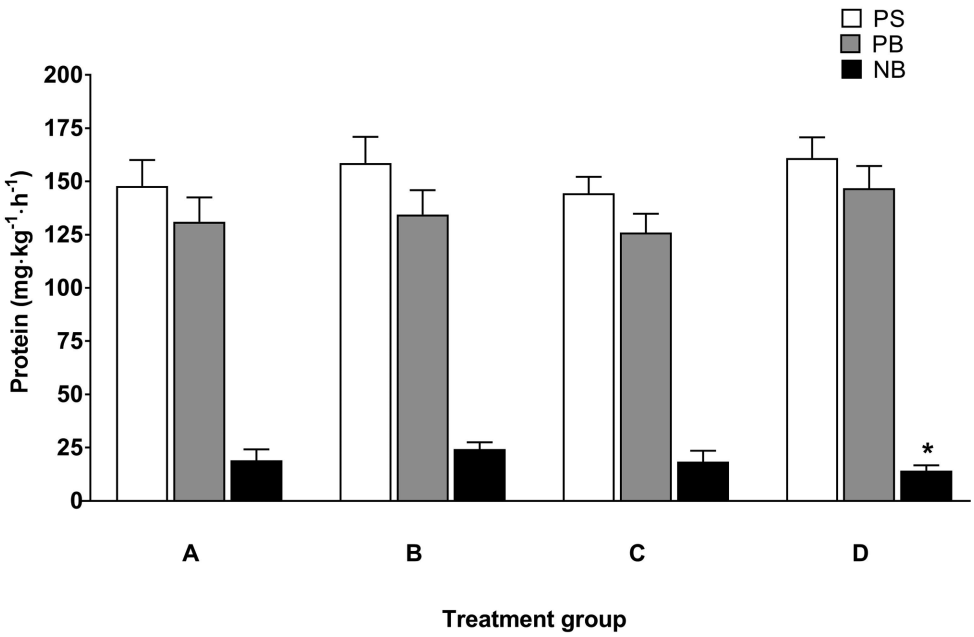

FIGURE 4 Relative whole-body protein turnover during the 24-h period following 4 different daily protein intake distributions. Values are calculated using the harmonic mean of [^{15}N]-ammonia and [^{15}N]-urea expressed relative to body mass in children who consumed 0 g protein at breakfast (Group A, $n = 16$), 7 g protein at breakfast (Group B, $n = 10$), 14 g protein at breakfast (Group C, $n = 14$), or 21 g protein at breakfast (Group D, $n = 15$). Values are presented as means ± SEMs. *Significantly different from Group B ($P < 0.05$). NB, net balance; PB, protein breakdown; PS, protein synthesis.

catabolism (31, 32). These results would be consistent with whole-body PB being the primary variable modified during transition from a fasting to fed state at relatively higher protein intakes (i.e., ~1.6–2.1 g · kg^{-1} · d^{-1}) approximating those in the present study (33). However, the observed attenuation in the rates of whole-body PB was not sufficient to induce an overall shift from negative to positive NB. This suboptimal anabolic environment may have been mediated by a lack of dietary amino acid availability that could have been exacerbated by an insulin-mediated suppression in PB (34, 35). Indeed, the provision of ≥7 g dietary protein at breakfast (Group B) resulted in a greater overall positive NB. Moreover, 21 g protein at breakfast (Group D) was required to elicit a detectable stimulation of whole-body protein synthesis over 9 h. The requirement for a greater protein intake to stimulate whole-body protein synthesis is consistent with observations from fasting to fed transitions in adults consuming higher protein daily protein intakes (i.e., >1.6 g · kg^{-1} · d^{-1}) (33). Thus, replenishment of overnight fasting protein loss is initiated by food consumption during the subsequent day and is enhanced in a dose-dependent manner with dietary protein ingestion.

Although our tracer methodology precludes our ability to establish a true anabolic value of breakfast per se, all participants consumed an identical energy intake over the 9-h period with a similar protein intake at lunch (consumed ~4 h after breakfast). Thus, any differences observed in whole-body protein metabolism and NB between groups would ultimately be driven by the difference in protein consumed at breakfast. In contrast to the statistically significant dose-response increase in NB over the 9-h daytime period, there was little effect of varying the morning beverage protein intake on the rates of PS and PB between groups, with the exception of greater observed rates of PS between Group D and Group A. However, although the change in PS between the other groups was statistically nonsignificant, it may be physiologically important. This lack of robust statistically significant differences in PS and PB is consistent with our previous studies in healthy children after exercise that were based on the use of both oral (8, 9, 20) and intravenous (7) tracer methodologies. Thus, the relatively high rates of protein synthesis and breakdown that are characteristic of growing children (25) may only need to be minimally altered before translating into potentially meaningful differences in net protein balance. Alternatively, given the large body nitrogen pool, which turns over more slowly than carbon-labeled substrates such as [^{13}C]-leucine, it is possible that a longer postprandial period after the lunchtime meal could have allowed for a more complete metabolic response to the previous 9 h of nutrient ingestion and resulted in more robust increases in protein synthesis (36). Nevertheless, the dose-dependent increase in NB in the present study is consistent with our previous studies after exercise (7) and the anabolic effects of dietary protein in adults (3, 5, 37–39).

Based on NHANES data, some of the present authors recently reported that, similar to adults (40), children in the United States aged 4–18 y consume the majority of their daily protein intake in the evening on any given day (16). The impact of the distribution, and not just the total daily amount, of a nutrient (e.g., protein) for health promotion has received attention in recent years, as recently reviewed by Al Moosawi et al. (41). However, the ability of protein intake pattern to influence nitrogen and amino acid NB in adults is not a new concept (12, 13) but has received renewed attention. With respect to protein intake, a balanced daily distribution may improve nitrogen retention in children (42), although data from adults are less conclusive and may depend on gender and age (17, 43–45).

Although we cannot discount the possibility of a type I error, the physiologic basis for this apparent difference may be related in part to the ~34–50% lower evening protein intake of Group D compared with the other groups, which would result in a relatively prolonged period under conditions of lower

protein exposure. The 24-h controlled diet in the present study, although greater than the current safe intake (46), was designed to approximate the participants' habitual protein intake in order to minimize any day-to-day variability in protein intake and protein metabolism (47). Thus, it is possible that the repeated increased consumption of protein above the current recommended intake across the day but at the habitual intakes of most North American youth (16) may mask any impact that a balanced protein distribution may have on acute surrogate markers of "growth" (i.e., NB). This would be consistent with a reduced efficiency of postprandial nitrogen retention with a high ($2\ g \cdot kg^{-1} \cdot d^{-1}$) compared with a normal ($1\ g \cdot kg^{-1} \cdot d^{-1}$) protein intake (48). However, our results are at odds with previous research in children consuming $>2\ g \cdot kg^{-1} \cdot d^{-1}$ of protein, which might be related in part to the duration of the controlled diet period (i.e., 2 wk compared with 24 h) (42).

In conclusion, healthy active children are in a net catabolic state in the morning following an overnight fast. The consumption of ≥7 g of protein as part of a breakfast containing carbohydrate is an important nutritional approach enhancing net protein balance over the subsequent 9-h postprandial period. Finally, total protein intake may be more important than the distribution across an entire day for enhancing 24-h net protein balance. Future research including long-term studies is ultimately required to elucidate the most appropriate way for children to consume their daily protein intake as a means to optimize net protein balance.

Acknowledgments

We thank Eric Williamson for his help with the controlled diets, Dinesh Kumbhare for his medical oversight, Amelie Goyer for her clinical trial management, and Dominik Grathwohl for his input into the statistical analysis plan. The authors' responsibilities were as follows—LGK, KAV, and DRM: designed the research and wrote the article; KAV: conducted the research; EO: involved in the initial project conception and research plan and discussion of results and conclusions for the final manuscript; SEA: had responsibility for the statistical analysis and reporting; LGK, DRM, KAV, and DB: had responsibility for the data interpretation; LGK and DMR: had primary responsibility for the final content; and all authors: read and approved the final manuscript.

References

1. Volpi E, Mittendorfer B, Wolf SE, Wolfe RR. Oral amino acids stimulate muscle protein anabolism in the elderly despite higher first-pass splanchnic extraction. Am J Physiol 1999;277:E513–20.
2. Volpi E, Ferrando AA, Yeckel CW, Tipton KD, Wolfe RR. Exogenous amino acids stimulate net muscle protein synthesis in the elderly. J Clin Invest 1998;101:2000–7.
3. Rennie MJ, Edwards RHT, Halliday D, Matthews DE, Wolman SL, Millward DJ. Muscle protein synthesis measured by stable isotope techniques in man: the effects of feeding and fasting. Clin Sci 1982;63:519–23.
4. Pacy PJ, Price GM, Halliday D, Quevedo MR, Millward DJ. Nitrogen homoeostasis in man: the diurnal responses of protein synthesis and degradation and amino acid oxidation to diets with increasing protein intakes. Clin Sci 1994;86:103–18.
5. Price GM, Halliday D, Pacy PJ, Quevedo MR, Millward DJ. Nitrogen homoeostasis in man: influence of protein intake on the amplitude of diurnal cycling of body nitrogen. Clin Sci 1994;86:91–102.
6. Tanner JM, Whitehouse RH. Clinical longitudinal standards for height, weight, height velocity, weight velocity, and stages of puberty. Arch Dis Child 1976;51:170–9.
7. Volterman KA, Moore DR, Breithaupt P, Godin J-P, Karagounis LG, Offord EA, Timmons BW. Postexercise dietary protein ingestion increases whole-body leucine balance in a dose-dependent manner in healthy children. J Nutr 2017;147:807–15.
8. Moore DR, Volterman KA, Obeid J, Offord EA, Timmons BW. Postexercise protein ingestion increases whole body net protein balance in healthy children. J Appl Physiol (1985) 2014;117:1493–501.
9. Volterman KA, Obeid J, Wilk B, Timmons BW. Effects of postexercise milk consumption on whole body protein balance in youth. J Appl Physiol 2014;117):1165–9.
10. Beckett PR, Jahoor F, Copeland KC. The efficiency of dietary protein utilization is increased during puberty1. J Clin Endocrinol Metab 1997;82:2445–9.
11. Gattas V, Barrera GA, Riumallo JS, Uauy R. Protein-energy requirements of prepubertal school-age boys determined by using the nitrogen-balance response to a mixed-protein diet. Am J Clin Nutr 1990;52:1037–42.
12. Leverton RM, Gram MR. Nitrogen excretion of women related to the distribution of animal protein in daily meals. J Nutr 1949;39:57–65.
13. el-Khoury AE, Sánchez M, Fukagawa NK, Gleason RE, Tsay RH, Young VR. The 24-h kinetics of leucine oxidation in healthy adults receiving a generous leucine intake via three discrete meals. Am J Clin Nutr 1995;62:579–90.
14. Leidy HJ, Gwin JA, Roenfeldt CA, Zino AZ, Shafer RS. Evaluating the intervention-based evidence surrounding the causal role of breakfast on markers of weight management, with specific focus on breakfast composition and size. Adv Nutr 2016;7:S563–75.
15. Leidy HJ, Hoertel HA, Douglas SM, Higgins KA, Shafer RS. A high-protein breakfast prevents body fat gain, through reductions in daily intake and hunger, in "breakfast skipping" adolescents. Obesity (Silver Spring) 2015;23:1761–4.
16. Mathias KC, Almoosawi S, Karagounis LG. Protein and energy intakes are skewed toward the evening among children and adolescents in the United States: NHANES 2013–2014. J Nutr 2017;147:1160–6.
17. Mamerow MM, Mettler JA, English KL, Casperson SL, Arentson-Lantz E, Sheffield-Moore M, Layman DK, Paddon-Jones D. Dietary protein distribution positively influences 24-h muscle protein synthesis in healthy adults. J Nutr 2014;144:876–80.
18. Phillips SM, Chevalier S, Leidy HJ. Protein "requirements" beyond the RDA: implications for optimizing health. Appl Physiol Nutr Metab 2016;41:565–72.
19. Layman DK, Anthony TG, Rasmussen BB, Adams SH, Lynch CJ, Brinkworth GD, Davis TA. Defining meal requirements for protein to optimize metabolic roles of amino acids. Am J Clin Nutr 2015;101:S1330–8.
20. Volterman KA, Moore DR, Breithaupt P, Grathwohl D, Offord EA, Karagounis LG, Timmons BW. Timing and pattern of postexercise protein ingestion affects whole-body protein balance in healthy children: a randomized trial. Appl Physiol Nutr Metab 2017;42:1142–8.
21. Kuczmarski RJ, Ogden CL, Guo SS, Grummer-Strawn LM, Flegal KM, Mei Z, Wei R, Curtin LR, Roche AF, Johnson CL. 2000 CDC Growth Charts for the United States: methods and development. Vital and health statistics series 11, Data from the National Health Survey 2002(246):1–190.
22. Mirwald RL, Baxter-Jones AD, Bailey DA, Beunen GP. An assessment of maturity from anthropometric measurements. Med Sci Sports Exerc 2002;34:689–94.
23. Evenson KR, Catellier DJ, Gill K, Ondrak KS, McMurray RG. Calibration of two objective measures of physical activity for children. J Sports Sci 2008;26:1557–65.
24. Dietz WH, Bandini LG, Schoeller DA. Estimates of metabolic rate in obese and nonobese adolescents. J Pediatr 1991;118:146–9.
25. Grove G, Jackson AA. Measurement of protein turnover in normal man using the end-product method with oral [15N]glycine: comparison of single-dose and intermittent-dose regimens. Br J Nutr 1995;74:491–507.
26. Bunout D, de la Maza MP, Barrera G, Leiva L, Gattas V, Hirsch S. Assessment of sarcopenia: longitudinal versus cross sectional body composition data. Aging Clin Exp Res 2007;19:295–9.
27. Garlick PJ, Clugston GA, Swick RW, Waterlow JC. Diurnal pattern of protein and energy metabolism in man. Am J Clin Nutr 1980;33:1983–6.

28. Greenhaff PL, Karagounis LG, Peirce N, Simpson EJ, Hazell M, Layfield R, Wackerhage H, Smith K, Atherton P, Selby A, et al. Disassociation between the effects of amino acids and insulin on signaling, ubiquitin ligases, and protein turnover in human muscle. Am J Physiol Endocrinol Metab 2008;295:E595–604.
29. Fern EB, Garlick PJ, Waterlow JC. The concept of the single body pool of metabolic nitrogen in determining the rate of whole-body nitrogen turnover. Hum Nutr Clin Nutr 1985;39: 85–99.
30. Weiner ID, Mitch WE, Sands JM. Urea and ammonia metabolism and the control of renal nitrogen excretion. Clin J Am Soc Nephrol 2015;10:1444–58.
31. Arslanian SA, Kalhan SC. Protein turnover during puberty in normal children. Am J Physiol 1996;270:E79–84.
32. Kien CL, Zipf WB, Horswill CA, Denne SC, McCoy KS, O'Dorisio TM. Effects of feeding on protein turnover in healthy children and in children with cystic fibrosis. Am J Clin Nutr 1996;64: 608–14.
33. Jackson AA, Soares MJ, Grove G, Waterlow JC. Enrichment in urinary ammonia and urea with hourly oral doses of [^{15}N]glycine: evidence for a step function and a circadian rhythm in protein turnover. Clin Sci 1997;93:265–71.
34. Tessari P, Inchiostro S, Biolo G, Trevisan R, Fantin G, Marescotti MC, Iori E, Tiengo A, Crepaldi G. Differential effects of hyperinsulinemia and hyperaminoacidemia on leucine-carbon metabolism in vivo. Evidence for distinct mechanisms in regulation of net amino acid deposition. J Clin Invest 1987;79:1062–9.
35. Trommelen J, Groen BBL, Hamer HM, de Groot LCPGM, van Loon LJC. Mechanisms in endocrinology: exogenous insulin does not increase muscle protein synthesis rate when administrated systemically: a systematic review. Eur J Endocrinol 2015;173:R25–R34.
36. Duggleby SL, Waterlow JC. The end-product method of measuring whole-body protein turnover: a review of published results and a comparison with those obtained by leucine infusion. Br J Nutr 2007;94:141–53.
37. Moore DR, Robinson MJ, Fry JL, Tang JE, Glover EI, Wilkinson SB, Prior T, Tarnopolsky MA, Phillips SM. Ingested protein dose response of muscle and albumin protein synthesis after resistance exercise in young men. Am J Clin Nutr 2009;89:161–8.
38. Deutz NE, Wolfe RR. Is there a maximal anabolic response to protein intake with a meal? Clin Nutr 2013;32:309–13.
39. Macnaughton LS, Wardle SL, Witard OC, McGlory C, Hamilton DL, Jeromson S, Lawrence CE, Wallis GA, Tipton KD. The response of muscle protein synthesis following whole-body resistance exercise is greater following 40 g than 20 g of ingested whey protein. Physiol Rep 2016;4:e12893.
40. Almoosawi S, Winter J, Prynne CJ, Hardy R, Stephen AM. Daily profiles of energy and nutrient intakes: are eating profiles changing over time? Eur J Clin Nutr 2012;66:678–86.
41. Almoosawi S, Vingeliene S, Karagounis LG, Pot GK. Chrono-nutrition: a review of current evidence from observational studies on global trends in time-of-day of energy intake and its association with obesity. Proc Nutr Soc 2016;FirstView:1–14.
42. Barja I, Araya H, Muñoz P, Vega L, Arteaga A, Tagle MA. Effect of spacing protein intake on nitrogen balance in normal children. Am J Clin Nutr 1972;25:506–11.
43. Arnal MA, Mosoni L, Boirie Y, Houlier ML, Morin L, Verdier E, Ritz P, Antoine JM, Prugnaud J, Beaufrere B, et al. Protein feeding pattern does not affect protein retention in young women. J Nutr 2000;130: 1700–4.
44. Arnal M-A, Mosoni L, Boirie Y, Houlier M-L, Morin L, Verdier E, Ritz P, Antoine J-M, Prugnaud J, Beaufrère B, et al. Protein pulse feeding improves protein retention in elderly women. Am J Clin Nutr 1999;69:1202–8.
45. Bollwein J, Diekmann R, Kaiser MJ, Bauer JM, Uter W, Sieber CC, Volkert D. Distribution but not amount of protein intake is associated with frailty: a cross-sectional investigation in the region of Nurnberg. Nutr J 2013;12:109.
46. Elango R, Humayun MA, Ball RO, Pencharz PB. Protein requirement of healthy school-age children determined by the indicator amino acid oxidation method. Am J Clin Nutr 2011;94:1545–52.
47. Millward DJ. Metabolic demands for amino acids and the human dietary requirement: Millward and Rivers (1988) revisited. J Nutr 1998;128:S2563–76.
48. Morens C, Bos C, Pueyo ME, Benamouzig R, Gausserès N, Luengo C, Tomé D, Gaudichon C. Increasing habitual protein intake accentuates differences in postprandial dietary nitrogen utilization between protein sources in humans. J Nutr 2003;133:2733–40.