Study Title: A Comparison of the Muscle Protein Synthetic Response to Single Bolus or Split Feeding Following Whole-Body Resistance Exercise

Principal Investigator: Stuart M. Phillips

Co-investigators: Dominique L. Greyvenstein, James P. Newbold, Matthew J. Lees

Institution: Department of Kinesiology, McMaster University, Hamilton, Ontario, L8S 4K1, Canada

1 Study Purpose and Rationale:

Skeletal muscle plays crucial roles in locomotion and metabolism. Muscle protein turnover – protein synthesis and breakdown – governs phenotypic adaptations to loading (i.e. hypertrophy and atrophy). The difference between rates of muscle protein synthesis (MPS) and muscle protein breakdown (MPB) determines net protein balance, such that skeletal muscle is in a net anabolic (MPS>MPB), catabolic (MPS<MPB) or stable (MPS=MPB) state [1]. Resistance exercise training (RET) is a potent anabolic stimulus that increases MPS, the effect of which is enhanced when RET is combined with protein consumption [2].

In young, healthy individuals, it has been widely accepted that a protein dose of ~20-25 g (~0.25-0.30 g protein/kg body mass) maximally stimulates MPS following RET, and higher doses result in irreversible amino acid oxidation [3]. Several studies have obtained similar results, showing increased amino acid oxidation and urea production when larger doses of protein that cannot be fully utilized for MPS are consumed [4, 5]. Following full-body RET as opposed to only lower-limb RET, a slightly larger dose of protein (40 g) may be more effective at stimulating MPS (compared to 20 g) [6]. Despite the literature suggesting an upper limit of ~20-25 g of protein/meal following RET, a recent study concluded that the ingestion of 100 g of protein at a single meal (so-called one-meal a day – "OMAD" – style feeding) provides a larger and more sustained MPS response compared to 25 g of protein [7]. These findings challenge the notion that protein meal distribution is important in the post-exercise MPS response [8, 9]. However, we propose that a per meal paradigm is correct and that even 100g OMAD is not as effective as 3 evenly distributed meals. Therefore, the purpose of this study is to compare the MPS response between a single high-dose protein bolus and a split feeding (3 meals per day) protocol following RET in both male and female participants.

2 Description of the Population

Inclusion Criteria:

- English-speaking
- Male or Female, aged 18-30 years
- Healthy, non-smoking/vaping
- BMI between 20 and 30 kg/m^2
- Not taking any medication or with any medical condition that, in the opinion of the investigators, would compromise the study outcome or the safety of the research participant. For example, taking any corticosteroids or antibiotics and individuals with any metabolic disorders like diabetes.
- No contraindications to proteins provided – whey, pea, soy, egg – in meals
- Ability to provide informed consent

Exclusion Criteria:

- Subject has any concurrent medical, orthopedic, or psychiatric requirements that, in the opinion of the investigators, would compromise their ability to comply with the study requirements
- Allergy or sensitivity to study ingredients
- Individuals who are incompetent and/or who are unable to give informed consent
- Any other condition that, in the opinion of the investigators, may adversely affect the subject's ability to complete the study or its measures or may pose a significant risk to the subject
- Any cancer, or related condition, or any genetic muscle diseases or disorders
- Current gastrointestinal disorder that could interfere with the study (e.g., IBS/IBD, diarrhea, acid reflux disease, dysphagia, etc.)
- Excessive alcohol consumption (>21 units/week) and/or a smoker (cigarettes or vaping)
- Use of corticosteroids, antibiotics, any anabolic steroid, creatine, whey protein supplements, casein, branched-chain amino acids (BCAAs) or any other natural health product (NHP), medication or supplement used for muscle strengthening/building within 45 days prior to screening
- Personal or family history of a clotting disorder or deep vein thrombosis

Recruitment Strategy

Participants will be recruited via self-referral through public advertisements (posters) at McMaster University campus, and additional advertisements will be displayed on social media and at local grocery stores/restaurants.

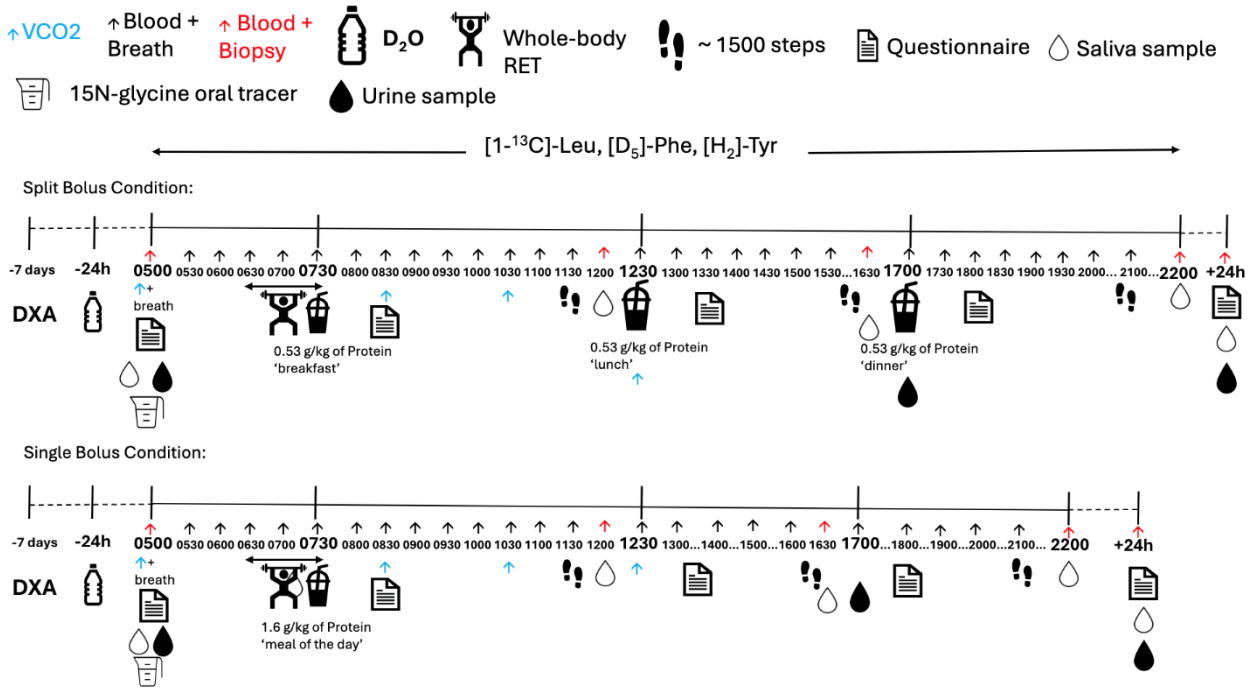
Sample Size

A power analysis was performed using G*Power (version 3.1.9.6). Based on previous dose-response trials (including from our facility) measuring muscle protein synthesis, we assumed a moderate effect size (F = 0.25, equivalent to Cohen's d = 0.50) [3-7]. For the present investigation, we determined 11 participants per group (22 total participants) would be sufficient to attain 80% power and an alpha at 0.05. To account for potential subject dropout, sex balancing, and missed samples, 24 participants will be recruited (12 per group).

3 Design and Detailed Description of Methodology

Trial Overview: Twenty-four healthy young men and women (age: 18-30 years; BMI: 20-30kg/m^2) will be randomized using computer-generated random numbers to either a single bolus (one meal with 1.6 g/kg protein) or a split bolus group (three meals spread across the day, each with 0.53 g/kg protein). Participants will undergo D_2O heavy water ingestion, stable isotope infusion, venous blood draws, breath samples, and skeletal muscle biopsies of the *vastus lateralis*. Primary endpoints, muscle protein synthesis

(MPS), and plasma amino acid concentration will be measured throughout the day. The entire trial duration will take place over 72 hours. A schematic of the trial is provided below.



A schematic outlining the experimental design for the present study. DXA: Dual-energy X-ray absorptiometry, D_2O: Deuterated water.

Procedure Overview

This study involves the following procedures: (i) venous blood sampling via venipuncture to assess tracer, amino acid, insulin, and glucose concentrations, (ii) skeletal muscle biopsy to assess tracer incorporation (myofibrillar and sarcoplasmic fractional synthesis rates), (iii) breath samples to assess amino acid oxidation, (iv) dual-energy X-ray absorptiometry (DXA) scan to assess body composition, (v) deuterated water (D_2O) and saliva sampling to assess the incorporation of the tracer in the body water pool, (vi) gastrointestinal distress questionnaires, (vii) one repetition maximum (1-RM) on leg press, leg extension, chest press, and lat pulldown to familiarize the participant with the trial exercise, (viii) whole-body resistance exercise bout, performing 3 sets of 8-12 repetitions at 70-80% of their pre-determined one-repetition maximum, of the leg press, leg extension, chest press, and lat pulldown to increase protein sensitization.

Feeding

On the day of the infusion, participants will consume a high-protein beverage and cookies in either a single bolus or three evenly split doses (meals). Participants will consume a total of 1.6g/kg of protein, 2.5g/kg of carbohydrate, and 0.5g/kg of fat. The protein beverage will be composed of milk protein (50%), pea protein (15%), soy protein (15%)

egg protein (20%), and maltodextrin to resemble a complete meal. The cookies will provide the remaining carbohydrates and fat to fulfill the participants' daily recommended intake.

Pre-Study Assessment

The purpose and experimental design of the study will be delivered via phone or email conversations, and all questions participants may have will be addressed.

Visit 1 (1 week prior to visit 2) Consent, Screening, DXA, 1 Repetition Maximum Testing: Participants will come to McMaster University to meet with one of the researchers who will explain the study protocol. Upon the provision of written informed consent, the researcher will confirm the participant's eligibility through a review of the inclusion/exclusion criteria and medical history and participants will have their height, weight, heart rate, and blood pressure measured. Participants will then undergo a DXA scan to assess body composition. Participants will then provide a venous blood sample for laboratory testing and undergo 1-repetition maximum (1RM) testing on leg press, leg extension, chest press, and lat pulldown exercises. Participants will make a list of all the types and quantities of food they eat for 3 days during this week (3-day food diary), and before leaving the laboratory, participants will be outfitted with an accelerometer to monitor their physical activity for three days before their next visit. This visit will take ~2.5 hours.

Visit 2 (24 hours prior to visit 3) - D_2O Loading: Participants will report to the laboratory following an overnight fast at ~0700 and will provide a saliva sample and undergo the D_2O loading protocol. The D_2O loading protocol involves drinking 350 mL of D_2O (diluted with water, split up into 8 equal servings, taken every 90 minutes. The participants will be sent home with the last 5 doses (i.e. # 4, 5, 6,7,8) of D_2O and will be instructed to consume them on the 90-minute schedule until complete. Participants will be asked to bring their empty D_2O bottles to their next visit. This visit will take ~5 hours.

Visit 3 (0430 – 2200) - Tracer Infusion, Blood Draws, Muscle Biopsies, Exercise, Feeding: Participants will report to the laboratory following an overnight fast at 0430 and undergo a baseline breath sample, muscle biopsy, blood sample, and urine sample. In this visit, participants will undertake a whole-body resistance exercise session, muscle biopsies, stable isotope-labelled amino acid infusion, serial breath sampling, and serial blood sampling for aminoacidemia profiling in response to feeding. Following the baseline measures, a catheter will be inserted into an antecubital vein on each arm for blood sampling and infusions, and participants will then complete a full-body resistance training session consisting of leg press, leg extension, chest press, and lat pulldown. Immediately following resistance training, participants will consume a high-protein beverage with cookies, as described earlier. Subsequent meals, if in the 3-meal group, will be consumed at 1230h and 1700h. Following the first biopsy, blood and breath samples will be taken

every 30 minutes until the first postprandial biopsy (1200). Following this biopsy, blood and breath samples will be taken every 30 minutes for the first 3 hours after each subsequent meal in the 3-meal group. In the single bolus group, blood and breath samples will be taken every hour after the first postprandial biopsy. The third and fourth biopsies will be taken at 1630 and 2200, respectively. Saliva sampling will be time-matched to the muscle biopsies. At 0500 and 1700, participants will be asked to provide a spot urine sample. Following the fourth biopsy, the catheter will be removed, concluding the visit. This visit will take ~17 hours.

Visit 4 (24 hours post visit 3) - Muscle Biopsy: 24 hours following the first biopsy in visit 3, participants will report to the laboratory following an overnight fast. During this visit, participants will undergo a final biopsy and provide a blood,breath, and urine sample. In addition, a final gastrointestinal distress questionnaire and appetite questionnaire will be completed. This visit will take around ~1 hour.

4 Definition of End-Point Measures

Primary:

- Muscle protein synthesis rates

Secondary:

- Plasma amino acid concentrations
- Plasma amino acid enrichment
- Histological analysis of proteins of interest
- Amino acid oxidation

5 Hypotheses

Primary

- There will be no difference in MPS between the two feeding regimes

Secondary

- There will be no significant sex-based differences.

6 Measurements and Analytical Instruments

Anthropometrics, Body Composition: Body height (m) and mass (kg) will be measured with a stadiometer and scale, respectively, to calculate BMI (kg/m2). Body composition (i.e. fat (%), appendicular lean mass (kg), and lean body mass (kg)) will be determined by dual-energy x-ray absorptiometry (DXA).

Skeletal Muscle Biopsies and Analysis: During the study, five skeletal muscle biopsies of the vastus lateralis will be collected using the suction-modified Bergström needle technique. The procedure involves the removal of a small piece of skeletal muscle tissue using a sterile, hollow needle under local anesthetic (lidocaine). A small piece of the muscle, approximately 100 mg, will be collected at each time point. After each biopsy,

incisions will be closed with an instant medical adhesive and steri-strips, and covered with a sterile bandage. Integrated rates of MPS will be determined using the D_2O method. Amino acid enrichment will be determined using isotope ratio mass spectrometry, and myofibrillar fractional synthetic rate will be determined.

Plasma amino acids, glucose and insulin: Plasma amino acid concentrations will be analyzed via gas-chromatography-mass spectrometry. Serum glucose and insulin will be assessed using fluorimetric methods as per the manufacturer's instructions.

Deuterated water: Deuterium is an isotope of hydrogen, and small amounts of deuterium are present in normal water consumed daily (~0.02%). Based on the quantities ingested during this study, the consumption of deuterium poses no health risk to the participants. 20-30 days after the participant stops drinking this water, the body water will return to its normal concentration. During the study, participants will be required to drink 350 ml of deuterated water (D_2O), split into eight equal servings.

Saliva Sampling: Saliva samples will be collected during visit 2, immediately before D_2O ingestion, in order to determine deuterium enrichments in the body water and protein-bound (albumin) pool, respectively. Participants will provide saliva samples prior to each D_2O dose (8 saliva samples on visit 2). This can be done at the lab and home. Saliva will be collected using a salivette (i.e., chewing on a piece of cotton). Saliva samples will also be collected on visit 3 and visit 4, time-matched to the muscle biopsies.

Stable isotope-labelled amino acid infusion: During visit 3, catheters will be placed on both arms or hands to collect blood samples and inject amino acid solutions throughout the day. After collecting the first blood sample, an infusion of the amino acids will be started. The amino acids will be dissolved in saline. The infusion will last for 17h. The components of the infusion will be [1-^{13}C]-Leu, [D_5]-Phe, and [H_2]-Tyr. Details of the amino acids ingested are described in Figure 3.

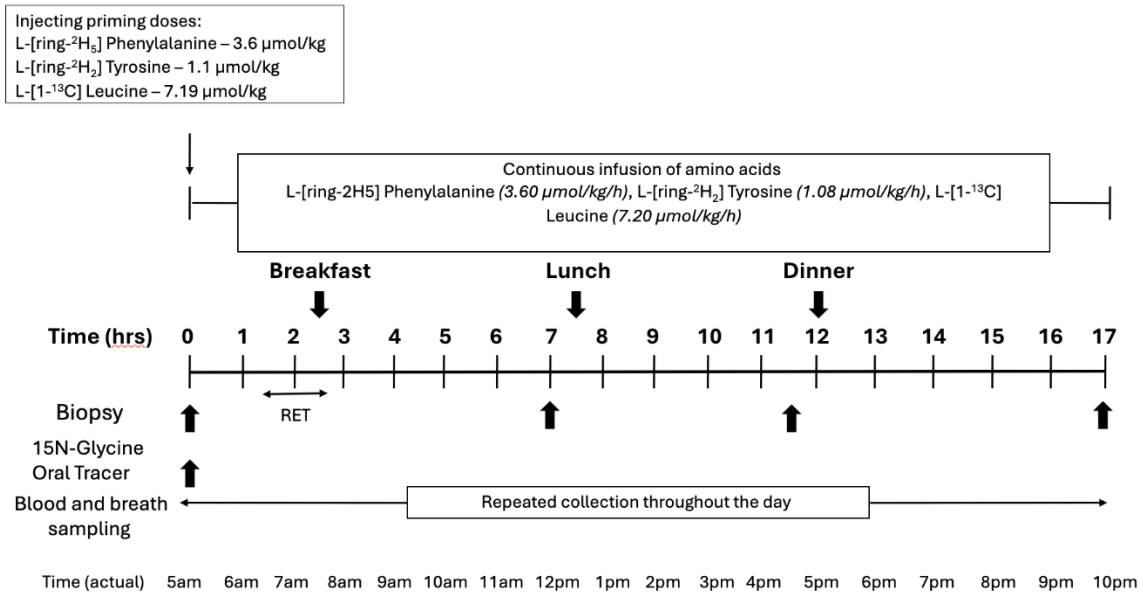


Figure 3. Schematic Representation of Stable Isotope-labelled Amino Acid Infusion. RET: Resistance exercise training.

Blood collection: Blood samples will be collected at 33 (split bolus) and 28 (single bolus) time points during the trials. Split bolus: starting at 0500, and a blood draw every 30 minutes until 1530, then every 30 minutes from 1630 to 2000. Two blood draws will follow at 2200 and +24 hrs. Single bolus: Blood draw every 30 minutes from 0500 to 1300, then one draw every hour from 1400 to 2200. Finally, one blood draw at +24 hrs. Blood draws will be performed using a central venous catheter, performed by a trained and experienced study investigator. The catheter will be placed in an antecubital vein. Approximately 6 mL will be collected for each sample. The split-bolus group will have a total of 198 mL of blood collected, and the single-bolus group will have a total of 168 mL of blood collected. Samples will be used to measure plasma amino acids, glucose and insulin.

Breath Sampling: Breath samples will be collected at the same time points of the blood draws. Samples will be collected into sterile 10mL vacutainers and stored at room temperature before the analysis of $^{13}CO_2$ enrichment by isotope ratio mass spectrometry. CO_2 volume will be collected at baseline and each hour after the first meal for the first three hours. Volume samples will be collected by breathing into a metabolic chamber for 20 minutes.

1-Repetition Maximum Testing and Resistance Training Protocol:

1-repetition maximum testing will be completed on visit 1. Subjects will perform a 5-minute cycling warm-up, before completing a 1-repetition maximum on the leg press, leg extension, chest press, and lat pulldown. The multiple repetitions testing procedure will be utilized [10]. Subjects will perform an unloaded warm-up set of 10 repetitions, allowing for researchers to correct lifting technique if required. The following sets will be performed at progressively increasing loads until the participant reaches failure on that exercise. Repetitions will be considered valid if the participant can complete the entire repetition in a controlled, unassisted manner. Between sets, participants will be allotted a 2-minute resting period. The acute bout of resistance training will occur on visit 3. Subjects will perform a 5-minute cycling warm-up, followed by 4 sets of 10 repetitions of leg press, leg extension, chest press, and lat pulldown. The first set of each exercise will be performed at 65% of the participant's 1-repetition maximum. The following sets of each exercise will be performed at 80% of the participant's 1-repetition maximum until volitional fatigue occurs. For each set, strong verbal encouragement will be used. Participants will be allotted 2-minute rest periods between each set.

7 Potential Harms, Risks, or Discomforts

Body composition testing (DXA Scan): The radiation dose from one DXA scan is ~2 micro-Sieverts, which is about the amount of radiation an average person receives every 6 hours from natural radiation in our environment (i.e., from the sun, television and computer screens etc.). This procedure is painless and non-invasive.

Skeletal muscle biopsy procedure: The biopsy technique is routinely used in our research (>9500 biopsies in the last 20yr), and provided that proper precautions are taken, complications are rare. The muscle biopsy procedure will only be carried out by trained and experienced personnel who have been delegated to conduct the procedure. Participants can stop the procedure at any time if they wish. During the time that the sample is being taken (~5 sec), sensations of deep pressure in the thigh may be felt and, on some occasions, are considered moderately painful. However, the discomfort passes very quickly and participants are quite capable of performing exercise and daily activities afterwards. Once the anesthetic wears off, the leg may feel tight, and often there is the sensation of a deep bruise or "Charlie Horse." Analgesics such as Acetaminophen (Tylenol) or Ibuprofen (Advil) can be taken if the pain associated with the biopsy is experienced. It is also beneficial to periodically apply an ice pack to the biopsy site the following day, as this will help to reduce any swelling and any residual soreness. The following day, the leg may feel uncomfortable when going downstairs. The tightness or discomfort in the muscle usually disappears within 2 days, and subjects routinely begin exercising at normal capacity within a day.

There is a risk of internal bleeding at the site of the biopsy, which can result in bruising and temporary discoloration of the skin. There is also a risk of feeling lightheaded or faint as the procedure is occurring. On occasion, a small lump may form under the site of the

incision, but this normally disappears within 2-3 weeks. As with any incision, there is also a risk of infection; however, this risk is virtually eliminated through proper cleansing of the area and daily changing of wound coverings. To allow the incisions to heal and minimize any risk of infection, participants are instructed to clean the incision daily with 70% isopropyl alcohol wipes, as well as avoid submersing the incision in water. Showers are fine, but avoid baths, swimming, saunas, and jacuzis for at least 3-4 days following the biopsy procedure.

Heavy water (D2O): Heavy water contains deuterium, which is a hydrogen isotope, yet non-radioactive and is, in fact, already present in small amounts in the water we drink daily (i.e. 0.02%). In very large amounts, deuterium-labelled water can have adverse effects. A very high dose (more than 40 cups consumed in a very short time for a person weighing about 150 pounds) may cause a toxic response. In this study, participants will receive 30 times less than this amount. In previous research studies using heavy water, very few people who drank deuterium-labelled water reported temporary dizziness or vertigo. There is a 3.3% chance of encountering these side effects, and only upon the first- or second-time heavy water is ingested. If it does occur, the side effects may last up to two hours and will go away on their own. If this occurs, we ask that participants rest quietly and refrain from driving or operating heavy machinery. About half the water in the body is replaced every week. Once you stop drinking heavy water, deuterium will return to its normal concentration in 20-30 days.

Venous Blood Sampling: The insertion of a needle for blood sampling is a common medical practice and involves minimal risk, provided proper precautions are taken. The needle is inserted under sterile (alcohol will be used to minimize the chance of infection) conditions; however, there is a theoretical risk of infection. There is also a chance of bleeding if enough pressure is not put on the insertion site when the needle is removed. This may cause some minor discomfort and could result in bruising/skin discoloration that could last up to a few weeks. There is also a small risk that damage to the vessel wall could result in the formation of a small blood clot, which could travel through the bloodstream and become lodged in a smaller vessel. However, we have never experienced these types of problems in our laboratory after several thousand venous blood-sampling procedures.

Acute exercise: The potential risks and discomforts associated with the acute exercise bout are similar to those associated with any form of strenuous physical activity. These include fatigue, muscle strain, fainting, abnormal blood pressure, irregular heart rhythm, and in very rare instances, heart attack, stroke or death. We never had a serious incident while strength test in either younger or older adults in our laboratory.

7 Data Analysis Plan

Data will be tested for normality using the Shapiro-Wilk test. A three-way mixed-design repeated measures analysis of variance (ANOVA), incorporating time (h) and condition (bolus vs split) as within-subject factors and sex as a between-subjects factor, will be employed to assess differences across the variables. If there are no sex-by-other level interactions, data will be collapsed across sexes and males and females will be analyzed together. Post hoc tests will be used when significant interactions are found. Statistical significance will be determined as $P \leq 0.05$. All data analysis will be completed using R.

8 References

1. McKendry, J., et al., Resistance Exercise, Aging, Disuse, and Muscle Protein Metabolism. Compr Physiol, 2021. 11(3): p. 2249-2278.
2. Phillips, S.M., A brief review of critical processes in exercise-induced muscular hypertrophy. Sports Med, 2014. 44 Suppl 1(Suppl 1): p. S71-7.\
3. Moore, D. R., Robinson, M. J., Fry, J. L., Tang, J. E., Glover, E. I., Wilkinson, S. B., Prior, T., Tarnopolsky, M. A., & Phillips, S. M. (2009). Ingested protein dose response of muscle and albumin protein synthesis after resistance exercise in young men. *Am J Clin Nutr, 89*(1), 161-168. https://doi.org/10.3945/ajcn.2008.26401
4. Witard, O. C., Jackman, S. R., Breen, L., Smith, K., Selby, A., & Tipton, K. D. (2014). Myofibrillar muscle protein synthesis rates subsequent to a meal in response to increasing doses of whey protein at rest and after resistance exercise. Am J Clin Nutr, 99(1), 86-95. https://doi.org/10.3945/ajcn.112.055517
5. Mallinson, J.E., et al., *Protein dose requirements to maximize skeletal muscle protein synthesis after repeated bouts of resistance exercise in young trained women.* Scand J Med Sci Sports, 2023. **33**(12): p. 2470-2481.
6. Macnaughton, L. S., Wardle, S. L., Witard, O. C., McGlory, C., Hamilton, D. L., Jeromson, S., Lawrence, C. E., Wallis, G. A., & Tipton, K. D. (2016). 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, 4*(15). https://doi.org/10.14814/phy2.12893
7. Trommelen, J., van Lieshout, G. A. A., Nyakayiru, J., Holwerda, A. M., Smeets, J. S. J., Hendriks, F. K., van Kranenburg, J. M. X., Zorenc, A. H., Senden, J. M., Goessens, J. P. B., Gijsen, A. P., & van Loon, L. J. C. (2023). The anabolic response to protein ingestion during recovery from exercise has no upper limit in magnitude and duration in vivo in humans. *Cell Rep Med, 4*(12), 101324. https://doi.org/10.1016/j.xcrm.2023.101324
8. Moore, D.R., et al., *Daytime pattern of post-exercise protein intake affects whole-body protein turnover in resistance-trained males.* Nutr Metab (Lond), 2012. **9**(1): p. 91.

9. Areta, J.L., et al., *Timing and distribution of protein ingestion during prolonged recovery from resistance exercise alters myofibrillar protein synthesis.* J Physiol, 2013. **591**(9): p. 2319-31
10. Mayhew, J.L., Prinster, J.L., Ware, J.S., Zimmer, D.L., Arabas, J.R., and Bemben, M.G. (1995). Muscular endurance repetitions to predict bench press strength in men of different training levels. J. Sports Med. Phys. Fitness 35, 108–113.