TITLE

Post-exercise dietary leucine retention for whole-body anabolism is greater with whey protein isolate and fish-derived protein hydrolysate than non-essential amino acids in trained young men

RUNNING TITLE

Whole-body anabolism of a fish protein hydrolysate

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AUTHOR CONTRIBUTIONS

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Methodology: BPC, DRM, BE
Formal analysis: ME, MJL, JAA, DWD, GWPF, MAB
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The authors declare that the results of the study are presented clearly, honestly, and without fabrication, falsification, or inappropriate data manipulation.

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INSTITUTIONAL REVIEW BOARD STATEMENT

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Review Board (or Ethics Committee) of Dublin City University (DCUREC2021/152, 13/08/2021).

CLINICAL TRIAL REGISTRATION

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ABSTRACT

Marine-derived protein powder, such as blue whiting-derived protein hydrolysates (BWPH), represent high-quality sources of dietary protein, but an ability to support post-exercise anabolism is not established. The impact of BWPH on whole-body anabolism was compared with an isonitrogenous whey protein isolate (WPI) and non-essential amino acid (NEAA) control in ten trained young males (31±4 y) who, on three separate visits, performed a session of whole-body resistance exercise and then consumed, in randomized crossover fashion, BWPH, WPI, or NEAA (0.33 g kg^{-1}; 19, 33, and 0 mg kg^{-1} leucine, respectively) with L-[1-^{13}C]leucine. Breath, blood, and urine samples were collected for 6-h postprandial to assess dietary leucine oxidation (OX), AA concentrations, and 3-methylhistidine:creatinine ratio. Peak and AUC concentrations for leucine, BCAAs and EAAs were greater in WPI compared with BWPH (all $P<0.05$) but with no differences in time to peak concentration. Total OX reflected leucine intake (WPI>BWPH>NEAA; $P<0.01$) whereas relative OX was greater ($P<0.01$) in WPI (28.6±3.6%) compared to NEAA (21.3±4.2%), but not BWPH (28.6±8.8%). Leucine retention, a proxy for whole-body protein synthesis, was greater in WPI (185.6±9.5 μmol kg^{-1}) compared with BWPH (109.3±14.1 μmol kg^{-1}) and NEAA (5.74±0.30 μmol kg^{-1}) (both $P<0.01$), with BWPH being greater than NEAA ($P<0.01$). Urinary 3-methylhistidine:creatinine ratio did not differ between conditions. Both WPI and BWPH produced essential aminoacidemia and supported whole-body anabolism after resistance exercise, but a higher intake of BWPH to better approximate the leucine and EAA content of WPI may be needed to produce an equivalent anabolic response.

KEYWORDS

amino acids; breath test; recovery; resistance exercise; skeletal muscle

INTRODUCTION

Post-exercise protein ingestion is essential to stimulate muscle and whole body protein synthesis (Koopman *et al.*, 2005; Moore *et al.*, 2009). Essential amino acids (EAA; especially leucine) are the primary drivers of protein synthesis (Tipton *et al.*, 1999; Børsheim *et al.*, 2002), which generally results in dietary proteins enriched in these AAs, such as whey protein, being especially anabolic when consumed after resistance exercise (Tipton *et al.*, 2004; Tang *et al.*, 2009; Witard *et al.*, 2014). Additionally, proteins that are rapidly digested and absorbed have been reported to support a greater post-exercise increase in muscle protein synthesis (Tipton *et al.*, 2004; West *et al.*, 2011), although the rate of increase in circulating AA concentration does not always modulate anabolism after resistance exercise (Burd *et al.*, 2015; Chan *et al.*, 2019). Therefore, there is an interest in evaluating the anabolic potential of dietary protein beyond the traditional dairy-based sources to provide insight into nutritional strategies to augment post-exercise recovery and adaptation (Burd *et al.*, 2019; Morgan and Breen, 2021).

Fish-derived protein hydrolysates may offer potential as functional foods to benefit skeletal muscle metabolism, overall metabolic health, and healthy ageing in humans, whilst enhancing economic and environmental sustainability (Lees and Carson, 2020). In healthy young individuals a Nile tilapia-derived protein hydrolysate induced a similar pattern of post-exercise aminoacidaemia when compared to an established whey protein hydrolysate formulation (Cordeiro *et al.*, 2020). More recently, work from our laboratory has shown that ingestion of a blue whiting-derived protein hydrolysate (BWPH) produces postprandial aminoacidaemia in older adults at rest, concurrent with markers of stimulation of skeletal muscle anabolism in a cell-based model using *ex vivo* serum (Lees *et al.*, 2021). However, the postprandial increases in the serum concentrations of AAs that are important for skeletal muscle anabolism *in vivo*, namely EAA and leucine, were greater with whey protein compared to BWPH. Taken together, these *in vitro* findings suggest that BWPH could represent a viable

high-quality source of dietary protein to support postprandial anabolism. However, additional *in vivo* human research is warranted to determine whether BWPH may support post-exercise anabolism.

The primary fates of dietary AAs are for supporting whole-body protein synthesis or utilization as an immediate energy source (i.e. oxidation), as they represent poor gluconeogenic precursors (Fromentin *et al.*, 2013). These observations have contributed to the development of stable isotope methodologies that determine the partitioning of an EAA-tracer between oxidation and non-oxidative disposal (Boirie *et al.*, 1996; Elango, Ball and Pencharz, 2008), the latter of which is used as a proxy for protein synthesis. Given that up to ~70% of dietary leucine is utilized for protein synthesis and that it is preferentially metabolized within peripheral (e.g. muscle) tissues (Boirie *et al.*, 1996; Tessari *et al.*, 1996), we have demonstrated that a [^{13}C]leucine 'breath test' effectively discriminates leucine retention (intake minus oxidation) between rested and post-resistance exercise (Mazzulla *et al.*, 2022). In response to COVID-19 related research restrictions, we also utilized this non-invasive test remotely to determine the anabolic potential of blends of EAA (Waskiw-Ford *et al.*, 2022). Therefore, using that [^{13}C]leucine breath test methodology, the present study investigated the anabolic response to BWPH ingestion in young resistance-trained males compared with whey protein isolate (WPI) and an isonitrogenous non-essential amino acid (NEAA) control. We hypothesized that, due to their greater leucine and EAA contents, WPI and BWPH would support a greater whole-body leucine retention than NEAA when consumed immediately after whole-body resistance exercise, and that this surrogate measure of muscle anabolism would be sensitive to differences in leucine and EAA content between WPI and BWPH.

METHODS

Participants

Ten young healthy males (age, 31±4 y; height, 1.79±0.08 m; body mass, 86.0±10.6 kg, body mass index, 26.8±3.1 $kg.m^{-2}$; body fat, 19.8±6.6%) with at least two years of resistance exercise training experience undertaking two or more sessions per week, gave written informed consent to participate after written and verbal explanation of the study procedures. Ethical approval (permit number: ANONYMISED) was obtained from the ANONYMISED Research Ethics Committee in accordance with the Declaration of Helsinki (ISRCTN registry trial number: ISRCTN15460158).

Experimental design

Data collection took place in the ANONYMISED between mid-August and mid-November 2021, and sample analysis and data processing took place up to December 2023. In a crossover design, participants visited the laboratory on three separate occasions for experimental trials, with each trial separated by either 7 or 14 d. The three experimental trials each comprised of a whole-body resistance exercise training session followed by a 6-h recovery period (Figure 1). Participants consumed isocaloric drinks at +0 h of recovery, containing isonitrogenous quantities of BWPH, WPI or NEAA. The individual AA concentrations of the protein sources have been previously described in a separate study from our group on older adults (Lees *et al.*, 2021). Trials were conducted in a double-blind manner, and the trial order was randomised. Venous blood, breath and urine samples were collected throughout the 6-h recovery period (Figure 1).

Pre-trial preparation

All experimental trials were performed between 06:30 am and 4:30 pm, but on an individual basis, participants performed their second and third trials at the same time +/-1 h as their first trial. Pre-trial preparation was the same for each experimental trial. Participants were asked to abstain from alcohol for 48 h and caffeine for 18 h, and refrain from strenuous exercise training on the day before each trial. Participants were provided with a standardised diet

(Gourmet Fuel, Ireland) for the day before each experimental trial, which provided 35 kcal kg^{-1} at a macronutrient ratio of 60% carbohydrate, 20% protein, and 20% fat.

Experimental trials

The experimental trials were identical except for the drinks consumed. Each drink contained 0.33 g protein kg^{-1} body mass of BWPH, WPI, or NEAA, dissolved in 300 mL water, This dose was chosen in order to provide an ecologically-valid and efficacious dose of protein to support post-exercise muscle protein synthesis (Moore, 2019). The BWPH, WPI, and NEAA drinks contained 19, 33, and 0 mg kg^{-1} of leucine, respectively. The drinks also contained L-[1-^{13}C]leucine enriched to the value of 5% of total leucine content in BWPH and WPI (in line with our previous work (Mazzulla *et al.*, 2022) and 99.9% in NEAA, which provided the same total tracer intake as in the BWPH trial. The drinks were prepared by an investigator not involved with the experimental trials to maintain blinding. During each trial, the assigned drink was ingested at the start of the 6-h recovery period and within 5 min of cessation of the exercise training session. All drinks were administered in opaque drinks bottles, and participants were instructed to complete ingestion of the drink within the 5 min period.

On the day of experimental trials, participants arrived at the laboratory following an overnight fast (>10 h). Upon arrival, participants provided baseline urine, breath, and blood samples. Body composition was determined by bioelectrical impedance analysis (SOZO Digital Health Platform; ImpediMed Inc, Carlsbad, CA USA) during the first experimental trial. Participants then completed the resistance exercise training session, which was designed as a whole-body stimulus with the aim of maximising the quantity of muscle mass recruited during the session. Each session was split into two blocks of exercises performed in "circuit" style. The first block consisted of 1 warm up set and 3 working sets of 4 exercises (chest press; leg press; lat pull-down; leg curl). The second block consisted of 3 working sets of 3 exercises (shoulder press; leg extension; seated row). Participants were instructed to complete 10

repetitions of each exercise for each working set and aim for 2 repetitions in reserve after each working set, while working at a tempo of 2 second concentric and 2 second eccentric contractions. The loads lifted during the first experimental trial were replicated during the second and third experimental trials. After completion of the exercise session, an indwelling catheter (20G Insyte Autoguard; Becton Dickinson, Franklin Lakes, NJ USA) was inserted into an antecubital vein for serial blood sampling.

Venous blood samples were collected in clot-activating and lithium heparin containers (VACUETTE, Greiner Bio-One, Kremsmünster, Austria) every 20 min for the initial 4 h of recovery and every 30 min thereafter until cessation of each trial. Breath samples were collected in plastic tubes (VACUETTE, Greiner Bio-One) using a breath collection kit (Easysampler Breath Test Kit; Quintron Instrument Company, Milwaukee, WI USA) every 20 min for the initial 4 h of recovery and every 30 min thereafter until cessation of each trial. Urine samples were collected in 3 L containers and produced 2 x 2.5 mL pooled samples for later analysis.

Analysis of blood samples

Blood samples for serum were allowed to clot at room temperature for 30 min, and then stored on ice prior to centrifugation (1800 x *g* for 15 min at 4°C). Aliquots were prepared and stored at -80°C for subsequent analysis. Serum AAs were analysed using the Agilent 1200 reversed-phase ultra-performance liquid chromatography (RP-UPLC) system (Agilent Technologies Inc., Santa Clara, CA USA) equipped with an Agilent 1260 binary pump and a G1367C automated liquid handling system, with AA separation (C_{18} ZORBAX rapid resolution column (4.6mm×50mm,1.8μm; Agilent Technologies Inc.), data acquisition (Chemstation software; Agilent Technologies Inc.), and quantitative analysis all carried out as previously described (Power-Grant *et al.*, 2016). In addition to time-series data, peak concentration (Cmax), time to peak concentration (Tmax) and incremental area under the curve

(iAUC; determined using the trapezoidal method) were calculated for total AAs, total NEAAs, total EAAs, total branched-chain amino acids (BCAAs), and each of the individual AAs.

Analysis of breath samples and whole-body protein metabolism

$^{13}CO_2$ breath enrichments were measured using isotope-ratio mass spectrometry (IR-MS, ID-Microbreath, Compact Science Systems, Newcastle-under-Lyme, UK) as previously described (Mazzulla *et al.*, 2022). Briefly, breath samples were analysed in sequence with two technical replicates ordered in series. The atom % excess (APE) of $^{13}CO_2$ was calculated from delta Pee Dee Belemnite (PDB) values as follows, whereby Δ = delta PDB and Rref = 0.0112372 for PDB:

$$\text{APE} = 100/\left\{\frac{1}{\left[\left(\frac{\Delta}{1000}\right)+1\right]\text{x Rref}} + 1\right\}$$

Exogenous leucine oxidation (ExoOx) was calculated using modified Steele's equations as previously described:

$$\text{Exo Ox} = \frac{\text{Ei CO2}}{\text{diet Leu Ei}} \text{ x } \dot{V}CO_2 \text{ x } \frac{1}{k}$$

whereby Ei CO_2 is ^{13}C enrichment obtained from expired CO_2, diet Leu Ei is the enrichment of ingested leucine, $\dot{V}CO_2$ is the rate of CO_2 production, and *k* is the correction factor for the incomplete recovery of $^{13}CO_2$ in breath (Boirie *et al.*, 1996) as previously described (Mazzulla *et al.*, 2022).

An estimate of resting VCO_2 (Reckman *et al.*, 2019), in μmol kg^{-1} min^{-1}, was obtained by multiplying body surface area (BSA) by 300 mmol CO_2 h^{-1} (Shreeve, Cerasi and Luft, 1970). This approach yields similar data to resting VCO_2 measured via indirect calorimetry (Shreeve, Cerasi and Luft, 1970). BSA was estimated according to Haycock's equation (Haycock, Schwartz and Wisotsky, 1978):

$$\text{BSA} = 0.024265 \times \text{height}^{0.3964} \times \text{body mass}^{0.5378}$$

where height is expressed in cm and body mass is expressed in kg.

Total exogenous leucine oxidation was calculated from the ingested leucine enrichment using the trapezoidal AUC method. As a marker of whole-body anabolism, postprandial leucine retention was calculated as the difference between leucine intake and total exogenous leucine oxidation.

Analysis of urine samples

Aliquots were prepared from the pooled urine samples and stored at -80°C for subsequent analysis. The 3-methylhistidine (3MH) concentration was measured in duplicate using a commercially-available enzyme-linked immunosorbent assay (ELISA) kit (#MBS7606614, MyBioSource, San Diego, CA, USA). Urinary 3MH concentrations were normalised to urinary creatinine (3MH:Cr; nmol $mmol^{-1}$) to increase precision and account for sample dilution as previously described (Waskiw-Ford *et al.*, 2022). Urinary creatinine concentration was measured in duplicate using a QuantiChrom Creatinine Assay Kit (#DICT-500, BioAssay Systems, Hayward, CA USA). The percentage coefficient of variation (%CV) for both assay kits was <10%.

Statistical analyses

Using leucine retention as the primary outcome, the *a priori* sample size calculation was based on data from our previous study using this $^{13}CO_2$ breath test method that demonstrated a sensitivity to detect differences between fasted, fed (0.25 g kg^{-1} crystalline AAs) and fed+exercised conditions (Mazzulla *et al.*, 2022). Using these effect sizes, an α of 0.05 and β of 0.8, analysis using one-way repeated measures ANOVA would have required n=6 participants (GPower v3.1). To further increase the statistical power, the target sample size was set at n=10.

Statistical analyses were performed using SPSS Statistics (Version 26, IBM, Armonk, NY). Differences in time course variables were assessed using a mixed-design two-factor (Time*Condition) ANOVA with repeated measures on Time. Where sphericity was violated,

a Greenhouse-Geisser correction was applied to all main effects and interactions, and if data were not normally-distributed, logarithmic transformations were conducted. Where significant interactions were identified in the ANOVA, Tukey's post-hoc test was performed to determine differences between means for all significant main effects and interactions. A one-way repeated measures ANOVA was used to test differences in AUC variables and leucine retention. Where significance was identified in the ANOVA, a Bonferroni post-hoc test with multiple comparisons was used to identify differences between conditions. For all analyses, the level of significance was $P<0.05$, and all results are presented as mean±standard deviation.

RESULTS

Serum AA concentrations

There were significant main effects of Time, Condition, and a Time*Condition interaction (all $P<0.01$) for serum leucine concentrations. Post-hoc testing revealed that leucine was similar at baseline between conditions (all $P>0.91$). After 20 min, leucine was significantly elevated in both WPI and BWPH compared with NEAA, and these differences persisted until 160 min for BWPH and until 240 min for WPI (all $P<0.05$). After 20 min, leucine was also significantly elevated in WPI compared with BWPH, which persisted until 180 min (all $P<0.05$).

Serum BCAA concentrations demonstrated main effects for Time, Condition, and a Time*Condition interaction (all $P<0.01$). Post-hoc testing showed no differences between groups at baseline (all $P>0.88$) but after 20 min, BCAA were greater in both WPI and BWPH compared with NEAA, and remained so until 160 min for BWPH and until 220 min for WPI (all $P<0.05$). After 20 min, BCAA were also greater in WPI compared with BWPH until 140 min (all $P<0.05$).

For serum EAA, significant main effects were found for Time, Condition, and a Time*Condition interaction (all $P<0.01$). There were no differences at baseline (all $P>0.98$). After 20 min, EAA were significantly higher in both WPI and BWPH compared with NEAA, and these differences remained until 120 min for BWPH and until 220 min for WPI (all $P<0.05$). In WPI, EAA were elevated compared with BWPH from 20 min to 140 min, and again at 360 min (all $P<0.05$).

Peak concentrations (Cmax) for leucine, BCAA, and EAA were all significantly greater in WPI compared with BWPH, and both WPI and BWPH were significantly greater than NEAA (Table 1; all $P<0.05$). Time to peak concentration (Tmax) for leucine, BCAA, and EAA did not differ between BWPH and WPI. The AUC for leucine, BCAA, and EAA concentrations were greatest in WPI, followed by BWPH and NEAA, with all conditions significantly different from each other (all $P<0.01$).

Exogenous leucine metabolism and marker of protein breakdown

There were significant Time, Condition, and Time*Condition interaction effects for exogenous leucine oxidation (expressed in % h^{-1}; Figure 3, all $P<0.01$). Exogenous leucine oxidation was greater in WPI ($P<0.01$) and BWPH ($P<0.05$) compared with NEAA for t=20-140 min but there were no differences for t=160-360 min. Exogenous leucine oxidation was greater in BWPH compared with WPI at t= 40 min ($P<0.05$) but there were no differences between BWPH and WPI for all other timepoints. There was a significant main effect of Condition for total exogenous leucine oxidation (expressed in μmol kg^{-1}; $P<0.001$) with WPI being greater than both BWPH and NEAA, and BWPH being greater than NEAA (Figure 4A, all $P<0.001$). When expressed as a percentage of the ingested leucine, total exogenous leucine oxidation was higher for WPI (28.6±3.6%) compared with NEAA (21.3±4.2%; $P<0.01$) with no differences between WPI and BWPH (28.6±8.8%), or between BWPH and NEAA (Figure 4B). Total exogenous leucine retention as a proxy for whole body anabolism (AUC expressed

as μmol kg^{-1}; Figure 4C) was greater in WPI (185.6±9.5 μmol kg^{-1}) compared with BWPH (109.3±14.1 μmol kg^{-1}) and NEAA (5.74±0.30 μmol kg^{-1}), and was greater in BWPH compared with NEAA (all $P<0.01$). There were no differences in urinary 3MH:Cr between conditions ($P=0.532$) (Figure 5).

DISCUSSION

The present study is the first investigation to explore the anabolic potential of BWPH in human participants following resistance exercise. Consistent with our hypothesis, we observed that total exogenous leucine oxidation and retention (i.e., estimates of whole-body anabolism) over the 6 h postprandial period were both greatest in WPI compared with BWPH, with each of these conditions also greater than the NEAA control. These differences in leucine retention were accompanied by differences in peak concentration and AUC for serum leucine, BCAAs, and EAAs between conditions (WPI>BWPH>NEAA). Urinary analysis of a surrogate marker of myofibrillar protein catabolism (3MH:Cr) revealed no significant differences between conditions suggesting that this outcome was not impacted by different protein sources being ingested in the post-exercise period.

We utilised a non-invasive $^{13}CO_2$ 'breath test' methodology, which we have previously developed (Mazzulla *et al.*, 2022; Waskiw-Ford *et al.*, 2022), to estimate the retention of dietary leucine for whole-body protein synthesis. The principle of this method is that the retained portion of [^{13}C]leucine reflects the amount of unlabelled dietary leucine directed towards non-oxidative leucine disposal, which is a proxy for protein synthesis (Boirie *et al.*, 1996). While the incorporation of a tracer into a dietary protein would be the ideal method to determine the metabolic fate of dietary leucine (Pennings *et al.*, 2011; van Vliet *et al.*, 2016), these proteins are logistically-challenging and costly to generate. However, the rate and total exogenous oxidation of dietary leucine from a protein that is rapidly-digested and absorbed

(i.e. whey) is similar when free tracer ([^{13}C]leucine) is added to the unlabelled protein as compared to when the protein is intrinsically-labelled (Boirie *et al.*, 1996). In the present study, ingestion of WPI and BWPH resulted in a rapid aminoacidaemia and leucinaemia, the latter of which was somewhat delayed compared to our previous study that employed crystalline AA (i.e. Tmax at 20 vs. 60 min, respectively) (Mazzulla *et al.*, 2022), but peaked at a similar time as $^{13}CO_2$ enrichment. While breath enrichment remained elevated throughout the entire postprandial period, this effect is likely a function of the retention of the ^{13}C label within the bicarbonate pool, rather than a delayed metabolism of the free tracer relative to the protein-derived unlabelled leucine. Finally, total ^{13}C recovery was similar in NEAA to our recently published tracer-only trials (~20%) (Mazzulla *et al.*, 2022), and similar in WPI and BWPH as intrinsically-labelled whey protein (~31%) (Boirie *et al.*, 1996). Thus, the tracer was able to sufficiently model the fate of the protein-derived leucine in these rapidly-digested protein sources and provide an estimate of the relative retention of dietary leucine for whole-body protein synthesis.

Muscle protein synthesis and whole-body net protein balance demonstrate a dose-response to ingested protein (Moore *et al.*, 2009; Witard *et al.*, 2014; Holwerda *et al.*, 2019; Park *et al.*, 2020), which may be influenced in part by the leucine content of the protein and/or subsequent circulating leucinaemia (Churchward-Venne *et al.*, 2014). Consistent with the anabolic potential of leucine, a leucine-dose response was observed for dietary leucine oxidation that, due to a similar relative percentage ingested oxidation between conditions, translated into a reciprocal dose-response in leucine retention that was greatest in WPI and lowest in NEAA. These data suggest that, on an isonitrogenous basis, BWPH was more anabolic than NEAA, but less anabolic than WPI, due in part to a lower *relative* leucine content. While the relative contribution of skeletal muscle to whole body outcome cannot be precisely determined, we previously demonstrated that this $^{13}CO_2$ breath test can detect the expected

exercise-induced increase in leucine retention that would be consistent with the stimulation of muscle protein synthesis (Mazzulla *et al.*, 2022). Furthermore, protein synthesis, and ultimately net protein balance, is generally saturated at a lower protein dose than whole-body protein balance (Holwerda *et al.*, 2019; Churchward-Venne *et al.*, 2020), which suggests that AA deposition into skeletal muscle is prioritized during recovery from exercise. Finally, using an *ex vivo* model we have previously demonstrated that WPI and BWPH ingestion stimulate protein synthesis and myotube growth in C2C12 myocytes with no effect of NEAA ingestion (Patel *et al.*, 2019; Lees *et al.*, 2021). Therefore, our results are consistent with the ability of complete proteins (WPI and BWPH) to support whole-body, and presumably skeletal muscle, anabolism after a single session of resistance exercise. Because our goal was to provide an ecologically-valid and efficacious dose of protein (0.33 g kg^{-1}) to support post-exercise muscle protein synthesis (Moore, 2019), the differences in EAA and leucine content between the protein sources resulted in ~71% greater total leucine intake with WPI (9.87 g 100 g^{-1}) compared to BWPH (5.78 g 100 g^{-1}). WPI was greater than BWPH in total leucine retention by a similar magnitude (~73%), consistent with other research suggesting that leucine intake has a prominent role in stimulating post-exercise skeletal muscle anabolism. For example, supplementing a suboptimal dose of whey protein with additional leucine can induce an anabolic response similar to a saturating dose of whey protein, despite providing only 25% of the whey protein content and 62% of the EAA content (Churchward-Venne *et al.*, 2014). Thus, we speculate that the difference in leucine retention between WPI and BWPH may be overcome by a slightly greater (and thereby, leucine-matched) quantity of BWPH ingestion given that the percentage of leucine oxidized was similar (Figure 4B).

In the absence of exogenous AAs, resistance exercise increases skeletal muscle protein breakdown to provide substrates to support increased rates of muscle protein synthesis (Phillips *et al.*, 1997). The post-translational methylation of histidine within actin and myosin proteins

prevents these AAs from being recycled for protein synthesis, which has led to the suggestion that 3MH is a biomarker of myofibrillar protein catabolism (Young and Munro, 1978). We (Waskiw-Ford *et al.*, 2022) and others (Bird, Tarpenning and Marino, 2006) have demonstrated that consuming a complete complement of EAA in crystalline form can attenuate post-exercise increases in urinary 3MH, suggesting that EAA consumption attenuates the exercise-induced stimulation of myofibrillar protein breakdown. Contrary to these findings, no differences in urinary 3MH:Cr between conditions were observed in the present study. The exercise stimulus may not have sufficiently stimulated muscle protein catabolism given that we studied a trained population (Phillips *et al.*, 1999), and/or alternative mass spectrometry-based assessments of 3MH may be more appropriate to detect subtle differences in this outcome (Cegielski *et al.*, 2021). Regardless, changes in myofibrillar protein synthesis are generally more predictive of muscle hypertrophy particularly when assessed under free-living conditions (Damas *et al.*, 2016; Abou Sawan *et al.*, 2022), and therefore targeting nutrition strategies to attenuate myofibrillar protein catabolism may be of lower practical relevance for athletes aiming to enhance gains in lean body mass with resistance exercise training.

A limitation of the present study is the lack of females in our sample. As we are currently investigating the validity of this $^{13}CO_2$ breath test in females, recruiting males only avoided potentially introducing additional variability to the study. Given the absence of a sex-based differences in the muscle protein synthesis response to RE in the fed state (West *et al.*, 2012), the $^{13}CO_2$ breath test will likely become a viable tool for assessing the whole-body anabolic response in females, and facilitate increasing female representation in future non-invasive exercise research trials. Another limitation to restate is that while skeletal muscle may contribute ~34% to whole-body leucine metabolism (Tessari *et al.*, 1996), whether differences in whole-body leucine retention reflect the deposition into skeletal muscle *per se* is not specifically established in this study.

In summary, this is the first study in humans to explore the anabolic potential of BWPH compared to WPI and a NEAA control, and employed a novel '$^{13}CO_2$ breath test method to estimate post-exercise anabolism. Essential aminoacidaemia and whole-body anabolism were robustly induced after resistance exercise by both WPI and BWPH ingested at a dose of 0.33 g kg^{-1}. A more pronounced effect in WPI compared with BWPH was likely due to there being a divergent AA composition between the protein sources despite the conditions being isonitrogenous. A greater absolute quantity of BWPH is speculated to be required to elicit a similar anabolic response to WPI by better approximating the leucine and EAA content of the 0.33 g kg^{-1} dose of WPI. Based on current and prior evidence, BWPH represents a viable alternative source of bioavailable dietary protein to support anabolism in young healthy males following resistance exercise.

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588

589 **TABLES**

590 **Table 1.** Peak concentration (Cmax) and time to peak concentration (Tmax) of individual
591 amino acids in serum samples.
592

	NEAA (n=10)		WPI (n=10)		BWPH (n=10)	
Variable	**Cmax (µM)**	**Tmax (min)**	**Cmax (µM)**	**Tmax (min)**	**Cmax (µM)**	**Tmax (min)**
Total EAA	962±94	18±18	1742±204*	52±14*	1515±175*,#	42±11*
Total NEAA†	2630±254	48±14	1961±294*	36±16	2050±233*	44±8
Total BCAA	460±65	2±6	897±94*	50±14*	735±116*,#	42±11*
Leucine	146±20	4±8	336±40*	52±14*	264±42*,#	42±11*
Isoleucine	65±11	0±0	186±24*	50±14*	127±26*,#	42±11*
Valine	253±35	6±13	376±31*	54±10*	346±50*	44±8*
Arginine	103±19	32±27	146±39*	40±19	175±36*	42±11
Aspartic Acid	14±6	58±11	4±3*	38±24	4±3*	30±24*
Glutamic Acid	114±32	56±13	65±23*	40±16	65±35*	42±15
Asparagine	78±15	40±16	94±20	36±13	85±13	42±11
Serine	247±42	46±10	153±33*	30±11*	151±24*	42±11#
Glutamine	718±34	46±10	707±89	50±11	700±111	44±16
Glycine	640±107	48±10	214±50*	36±16	289±64*	44±8
Alanine	695±107	46±13	527±110*	32±14	515±129*	38±11
Tyrosine	88±16	138±82	89±15	52±14*	93±16	46±10*
Histidine	79±25	32±34	93±30	46±13	84±40	42±15
Methionine	28±5	30±46	50±5*	38±15	59±9*,#	42±15
Threonine	157±24	44±8	190±44	48±14	158±25	44±8
Tryptophan	68±8	26±43	123±20*	54±13	84±16#	36±8
Phenylalanine	58±9	2±6	78±6*	28±10*	82±8*	36±8*
Lysine	168±27	32±23	319±54*	46±16	317±39*	44±8

593 Data are presented as mean±standard deviation. BCAA, branched-chain amino acids; BWPH,
594 blue whiting protein hydrolysate; EAA, essential amino acids; NEAA, non-essential amino
595 acids; WPI, whey protein isolate. * P<0.05 vs. NEAA; # P<0.05 vs. WPI. † Available non-
596 essential amino acids only.

597 **FIGURE LEGENDS**

598 **Figure 1.** Overview of the experimental trials.

599

600 **Figure 2.** Serum amino acid concentrations and area under the curve (AUC) for leucine (A-B),
601 total branched-chain amino acids (C-D), and total essential amino acids (E-F) in response to
602 BWPH, WPI, and NEAA. Data are presented as mean±standard deviation. *Significant
603 differences between conditions at each timepoint (see text for further details). † WPI different
604 from BWPH (P<0.01). For AUC, conditions that do not share a letter are significantly different
605 (all P<0.01). BWPH, blue whiting protein hydrolysate; NEAA, non-essential amino acids;
606 WPI, whey protein isolate.

607

608 **Figure 3.** Time course of exogenous leucine oxidation. Data are presented as mean±standard
609 deviation. # NEAA different from WPI (P<0.01), * NEAA different from BWPH (P<0.05), †
610 WPI different from BWPH (P<0.05). BWPH, blue whiting protein hydrolysate; NEAA, non-
611 essential amino acids; WPI, whey protein isolate.

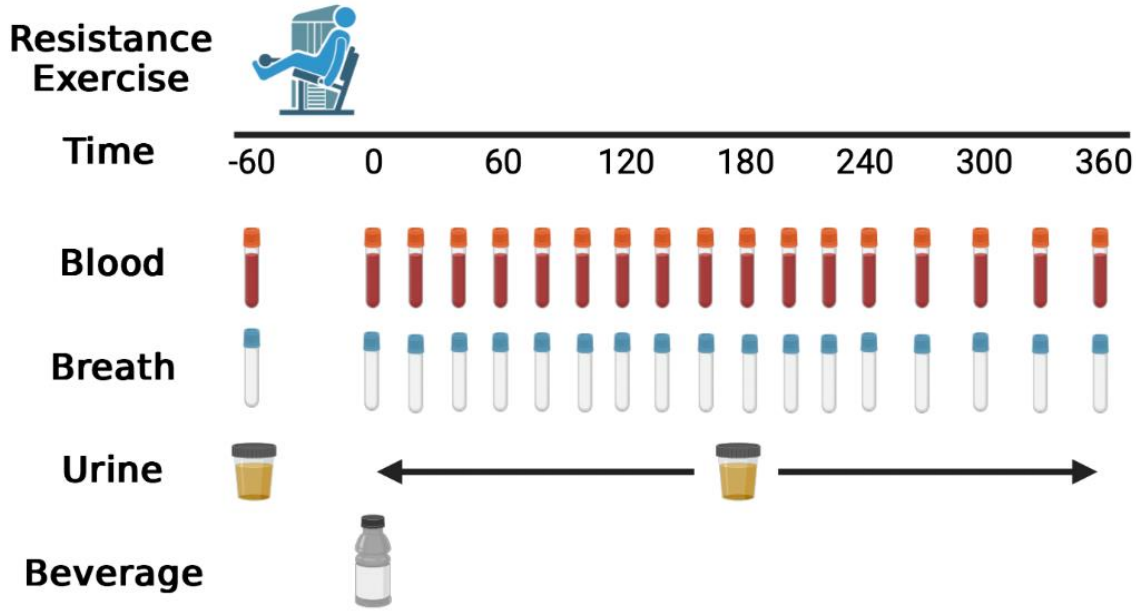
612

613 **Figure 4.** A) Total exogenous leucine oxidation expressed as an absolute amount. B) Relative
614 exogenous leucine oxidation expressed as a percentage of leucine intake. C) Total exogenous
615 leucine oxidation. Data are presented as mean±standard deviation. Conditions that do not share
616 a letter are significantly different (all P<0.01). BWPH, blue whiting protein hydrolysate;
617 NEAA, non-essential amino acids; WPI, whey protein isolate.

618

619 **Figure 5.** Urinary 3-methylhistidine:creatinine ratio. Data are presented as mean±standard
620 deviation. BWPH, fish protein hydrolysate; NEAA, non-essential amino acids; WPI, whey
621 protein isolate.

622 ***Figure 1.***



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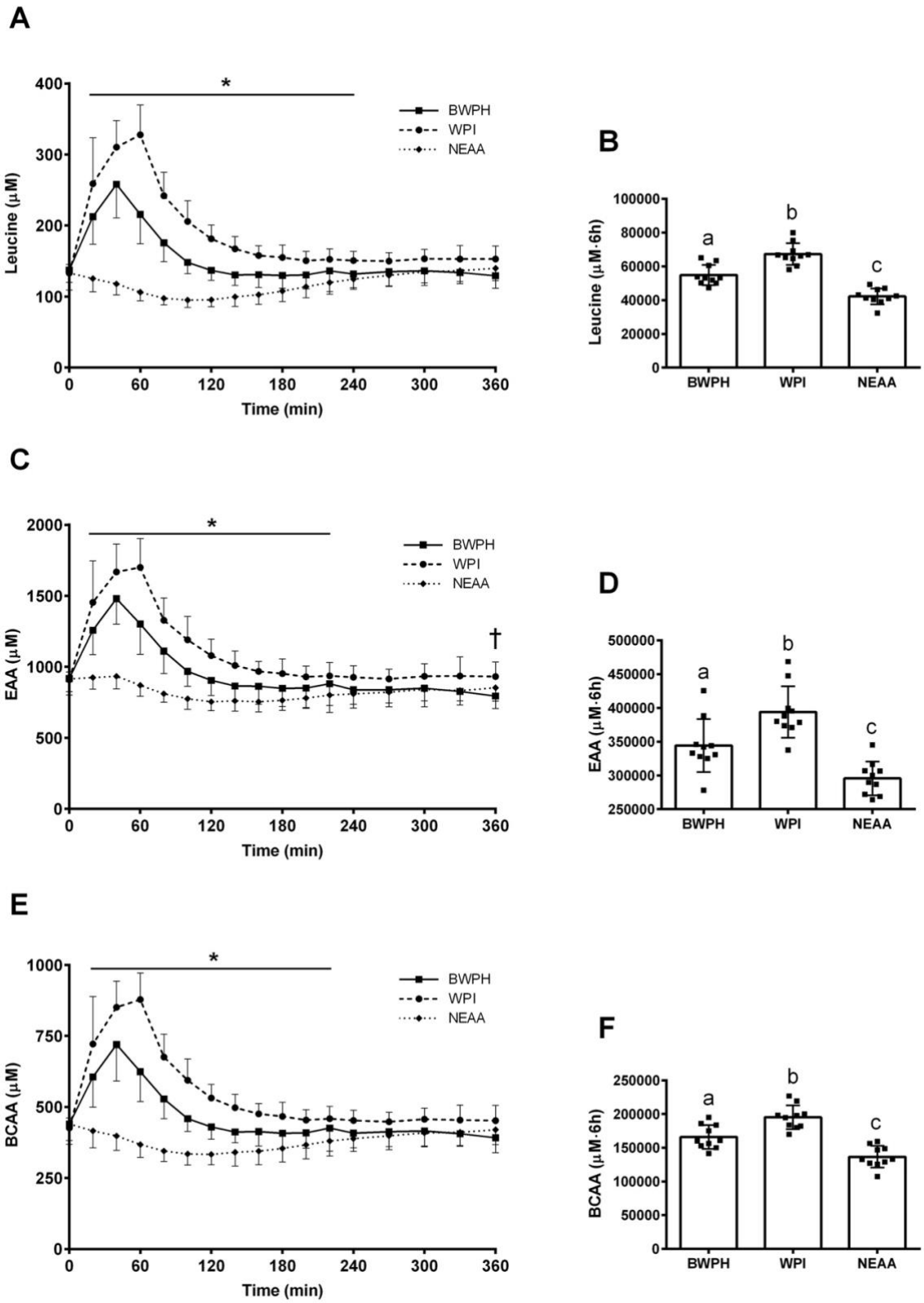
625 ***Figure 2.***



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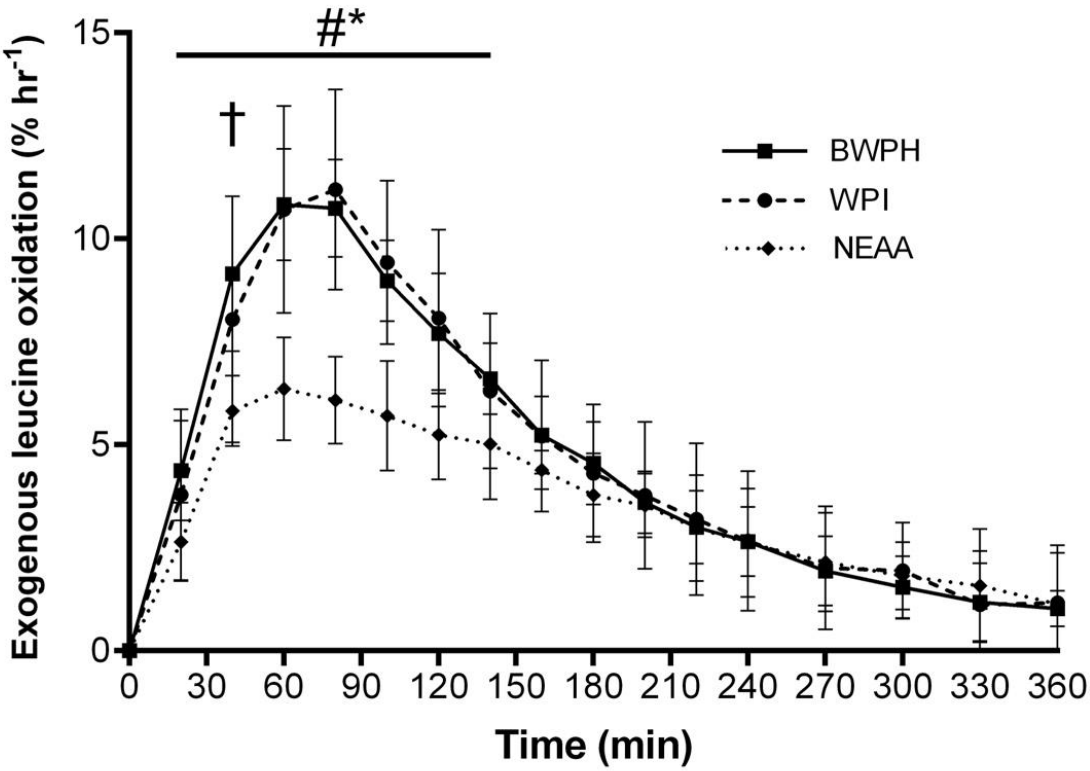
628 ***Figure 3.***



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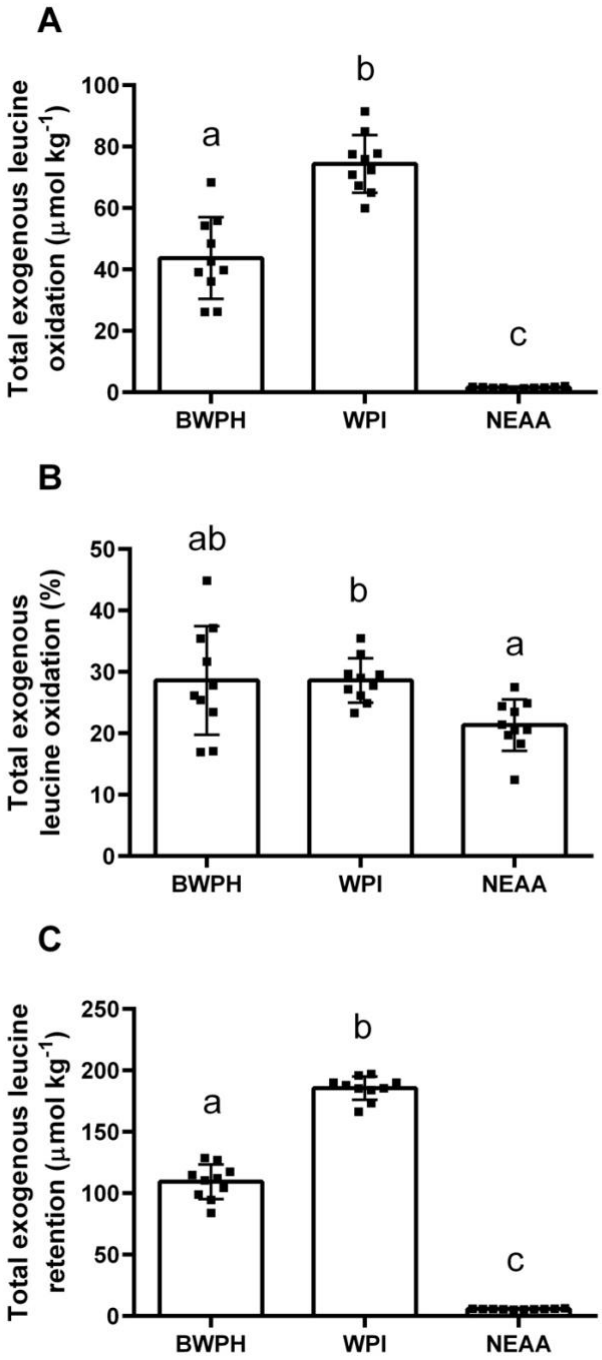
631 ***Figure 4.***



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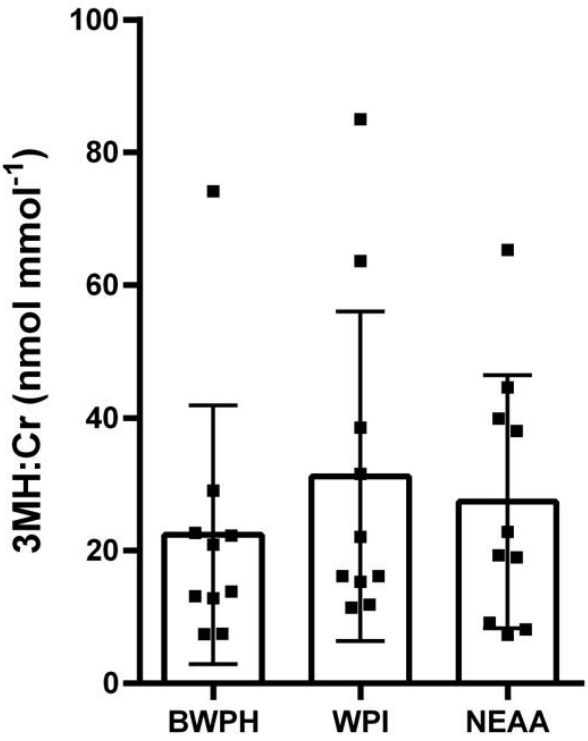
634 ***Figure 5.***



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