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The collagen synthesis response to an acute bout of resistance exercise is greater when ingesting 30 g versus 15 g and 0 g hydrolyzed collagen in resistance-trained young men

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Running title: Resistance exercise and the collagen dose-response

Data availability

Data described in the article, code book, and analytic code will be made available upon request from the corresponding author pending application and approval.

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Abbreviations

AUC, area under the curve

β-CTX, β-isomerized C-terminal telopeptide of type I collagen

CI, confidence interval

CV, coefficient of variation

ELISA, enzyme-linked immunosorbent assay

HFBA, heptafluorobutyric acid

η_p^2, partial eta squared

FSR, fractional synthetic rate

HC, hydrolysed collagen

IGF-I, insulin-like growth factor-1

LC-MS-MS, liquid chromatography with tandem mass spectrometry

mTORC1, mammalian target of rapamycin complex 1

MTU, muscle-tendon unit

PICP, procollagen type I C-terminal propeptide

PINP, procollagen type I N-terminal propeptide

QC, quality controls

RE, resistance exercise

TGF-β, transforming growth factor beta

10-RM, 10 repetition maximum

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Abstract

Background: Resistance exercise (RE) stimulates collagen synthesis in skeletal muscle and tendon but there is limited and equivocal evidence regarding an effect of collagen supplementation and exercise on collagen synthesis. Furthermore, it is not known if a dose-response exists regarding the effect of hydrolyzed collagen (HC) ingestion and RE on collagen synthesis.

Objective: We aimed to determine the HC dose-response effect on collagen synthesis following high-intensity RE in resistance-trained young men.

Methods: Using a double-blind, randomized cross-over design, 10 resistance-trained men (age: 26±3 years; height: 1.77±0.04 m; mass: 79.7±7.0 kg) ingested 0g, 15g or 30g HC with 50mg vitamin C 1h prior to performing four sets' barbell back-squat RE at 10-repetition maximum load, after which they rested for six hours. Blood samples were collected throughout each of the three interventions to analyse procollagen type I N-terminal propeptide (PINP) and β-isomerized C-terminal telopeptide of type I collagen (β-CTX) concentration, and the concentration of 18 collagen amino acids.

Results: The serum PINP concentration×time area-under-the-curve (AUC) was greater for 30g (267±79 $\mu g \cdot L^{-1} \cdot h$) than 15g (235±70 $\mu g \cdot L^{-1} \cdot h$, P=0.039) and 0g HC (219±88 $\mu g \cdot L^{-1} \cdot h$, P=0.005) but there was no difference between 0g and 15g HC (P=0.675). The AUCs of glycine and proline were greater for 30g than for 15g and 0g HC (P<0.05). Plasma β-CTX concentration decreased from -1h to +6h (P<0.05), with no differences between interventions.

Conclusion: The greater PINP AUC suggests 30g HC ingested prior to high-intensity RE augments whole body collagen synthesis more than 15g and 0g HC in resistance-trained young men.

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Introduction

Musculoskeletal tissues, such as skeletal muscle and tendon are crucial for generating and transmitting force to the bone, enabling movement. The structure and function of these tissues are therefore essential for musculoskeletal health and physical performance. Unfortunately, however, injuries to these tissues are common in athletes, with soft-tissue injuries making up the majority of all injuries in male athletes (1). One of the biggest risk factors for soft-tissue injury is muscle weakness (2, 3) and one of the most common methods used by athletes to mitigate this risk factor is to perform chronic resistance exercise (RE) (4). Chronically overloading the muscle-tendon unit in this way causes the muscle to adapt by hypertrophying and getting stronger (5), while the tendon also adapts by hypertrophying and increasing its stiffness and elastic modulus (6, 7).

A stiffer tendon has a higher loading capacity, as there is a linear relationship between Young's modulus and ultimate stress (8). Given that collagen (mainly type I) makes up 60–85% tendon dry weight (9), it is considered a crucial component in the tendon's adaptation to RE, particularly as gains in tendon stiffness are thought to be influenced by both tendon hypertrophy and an increase in collagen fibril density (10, 11). An increase in type I collagen fibril content over time is likely the product of an overload-induced increase in collagen synthesis after each bout of RE.

Collagen synthesis can be assessed either directly from the overloaded tissue, e.g. by measuring skeletal muscle or tendon collagen fractional synthetic rate (FSR), or indirectly from serum concentration of procollagen type I C-terminal propeptide (PICP) or procollagen type I N-terminal propeptide (PINP), which are both cleaved off during the maturation of procollagen to collagen. Indeed, an acute bout of RE in young men has been shown to increase patellar

tendon collagen FSR (12) and serum PINP concentration (13). This response is likely due to RE initiating mechanotransduction (i.e. mechanical stress initiating fibroblast intracellular signalling) (14), and the secretion of growth factors [e.g. transforming growth factor beta (TGF-β) and insulin-like growth factor-1 (IGF-I)], with these growth factors being crucial for procollagen formation in tendon (15, 16). Furthermore, these newly synthesized procollagen molecules undergo post-translational modifications, for which the presence of vitamin C is an essential co-factor during collagen synthesis (17), transport and assembly into tendon (18).

Thus, RE appears crucial for inducing increases in serum PICP/PINP concentration and muscle-tendon collagen FSR, which may lead to changes in connective tissue properties in the longer term. Indeed, concomitant increases in serum PICP concentration and human Achilles tendon collagen content after two months' chronic RE, followed by an increase in Achilles tendon stiffness with a further month's RE training (19), suggest that augmented tendon collagen synthesis and content are necessary to cause an increase tendon stiffness. Not only is tendon stiffness important for mitigating soft-tissue injury risk but it can also influence performance during 'explosive' actions, as a stiffer tendon can transmit muscle force more effectively to the bone, thus increasing the rate of force development (20).

In addition to the role of exercise on collagen synthesis, ingestion of vitamin C-enriched collagen appears to further augment an exercise-induced increase in collagen synthesis in a dose-response manner, i.e. 15 g gelatin increased serum PINP concentration by more than two-fold compared to 5 g and 0 g gelatin (21). This is currently the only study to investigate a dose-response effect of collagen ingestion on changes in collagen synthesis following exercise, albeit in jump-rope exercise not RE. Although no study has examined a collagen dose-response relationship without exercise, chronic collagen supplementation alone has been shown to

induce improvements in bone mineral density (22) and cartilage health (23), suggesting collagen ingestion might stimulate human connective tissue collagen synthesis independently of exercise. Thus, just as ingestion of 40 g whey protein has been shown to augment the muscle protein synthesis response to RE more than 20 g (24), it is possible that collagen ingestion may further augment the RE-induced rise in collagen synthesis (12, 13) in a dose-response manner.

The aim of this study was therefore to investigate the effect of 30 g *vs*. 15 g *vs*. 0 g HC ingested prior to high-intensity back squat RE on whole body collagen synthesis. We hypothesized that 30 g HC would elicit a greater serum PINP response than 15 g HC, which would induce a greater response than 0 g HC. We also hypothesized that 30 g HC would lead to a greater blood availability of the amino acids necessary for collagen synthesis to occur, e.g. glycine and proline.

Methods

Participants

Thirteen healthy young men volunteered to take part in the study. However, three were excluded prior to participation due to not meeting the inclusion criteria (n = 1) and declining to proceed with participation (n = 2) (**Figure 1**). Therefore, 10 resistance-trained, healthy young men (mean ± SD; age: 26 ± 3 years, height: 1.77 ± 0.04 m, body mass: 79.7 ± 7.0 kg, 4 ± 3 years' RE experience), who performed RE 4 ± 1 times per week, provided written informed consent before completing this study. The study was registered at https://clinicaltrials.gov/ (identifier: NCT05932771), was approved by Liverpool John Moores University Ethics Committee (approval number: 18/SPS/059) and complied with the Declaration of Helsinki. Participants were recruited from a university student population and recruitment began in

January 2019 and data collection was completed in August 2019. To be eligible to participate, volunteers had to be male, have at least 12 months' resistance training experience (including barbell back squat exercise performed at least once a week) and to be free from musculoskeletal injury. Volunteers were excluded if they had a history of patellar tendon pathology, were vegan (due to the bovine source of HC), consumed nutritional supplements or medication purported to have beneficial effects on muscle-tendon properties (e.g. antioxidants, protein, etc.), had sustained a lower limb injury in the previous six months, smoked or were <18 or >30 years old.

*Insert **Figure 1** near hear*

Experimental design

This study was a double-blind, randomized cross-over design. All participants attended the laboratory on four occasions, each separated by a week, and no strenuous physical activity was performed 48 h prior to each visit. Visit 1 was used to assess barbell back squat 10-RM; while visits 2-4 began with participants consuming a drink containing 0 g, 15 g or 30 g HC (each containing 50 mg vitamin C), followed by four sets' 10-RM barbell back squat RE (with 2 min rest in between sets), which typically took 20 min to complete. The three interventions (with a seven-day wash-out period interspersed between each intervention) were performed at the same time of day (08:00 – 15:00), following a 10 h overnight fast. After consuming the supplement and completing the RE, participants rested for 6 h and 10 × 5-mL blood samples were collected at different times points over a 7 h period (**Figure 2**). In addition to the supplement, only water was allowed to be consumed (ad libitum) during each intervention. Participants were instructed to record their dietary intake on the day before their first intervention and to replicate that dietary behaviour on the day preceding each of the subsequent interventions.

*Insert **Figure 2** near hear*

The barbell back squat 10-repeitition maximum (RM) assessment and 10-RM bout during each intervention

The squat depth during the barbell back squat was standardized for all participants to induce the same mechanical loading on the quadriceps femoris muscle-tendon unit during all three experimental interventions. Participants were instructed to place a 20 kg Olympic barbell on their shoulders (the high bar position), place their feet shoulder-width apart (foot location was marked on the floor for subsequent sets) and descend until their knee joint angle reached 90°, measured using a goniometer. While participants held the position at 90° knee flexion, the vertical distance from the floor to the ischial tuberosity was measured. The 10-RM assessment was performed in a squat rack and a resistance band was stretched across both sides of the squat rack to indicate the participant's 90° depth (**Figure 3**). A warm-up comprised two dynamic exercises (low lunge and squat to stand) prior to the actual 10-RM assessment, which comprised the following sets of barbell back squat: 10 repetitions with the 20 kg barbell, 8 repetitions at 50% of the estimated 10-RM, 4 repetitions at 70% and 1 repetition at 90% of the estimated 10-RM). After a 5-min rest period, participants performed 10-RM attempts separated by 5-min rest periods until 10-RM load was obtained. Two researchers observed each test procedure to provide a cue when the participant's proximal hamstrings/gluteus maximus touched the elastic band and to spot the participant. The 10-RM bout during each experimental intervention was preceded by a similar warm-up, i.e. two dynamic exercises followed by 10 repetitions' barbell back squat with the 20 kg barbell, 8 repetitions at 50% of the measured 10-RM, 4 repetitions at 70% 10-RM and 1 repetition at 90% 10-RM. The barbell back squat 10-RM load was 118 ± 21 kg during all three interventions.

*Insert **Figure 3** near here*

Nutritional supplementation

Before commencing each intervention, a laboratory technician (independent to the study) made up the supplement and randomly assigned the order of HC dose (Excel 2016, Microsoft, Washington, USA) for each participant. For each intervention, the technician recorded the date, randomly allocated intervention number (1, 2 or 3) and corresponding HC dose. The study investigators and participants were blinded to HC dose until after all analyses were completed, after which time the technician provided the lead researcher with the participants' intervention numbers and corresponding HC doses. Three doses of HC (0 g, 15 g and 30 g, Myprotein, Cheshire, UK) with 50 mg vitamin C powder (Holland and Barrett Retail Limited, Warwickshire, UK) were dissolved in 300 mL water in an opaque drinks bottle. To match the calories of 30 g HC in the other two interventions, 34.1 g and 15.4 g maltodextrin (Myprotein, Cheshire, UK) was used in the 0 g and 15 g HC interventions, respectively. Although the supplements were described by the manufacturers as "flavourless", 4 g non-caloric sweetener (Truvia®, SilverSpoon, London, UK) was added in all drinks to mask any potential taste difference between interventions. The amino acid profile of the HC supplement is shown in **Table 1**.

*Insert **Table 1** near here.*

Blood sampling

The BD Nexibia™ closed IV catheter system (22 G, Becton, Dickinson and Company, Franklin Lakes, USA) was inserted into a peripheral vein in the right antecubital fossa by a trained phlebotomist. A dressing band (3M™ Tegaderm™ I.V. Advanced Securement Dressing, 3M Health Care, Loughborough, UK) then covered the catheter in order to secure the catheter site and to keep it clean. Eight 5 mL venous blood samples were collected in specialized serum

collection tubes (BD Vacutainer™ Serum Separation Tube (SST™) II Advance, Dickinson and Company, Franklin Lakes, USA) at the following time points: at rest immediately prior to HC ingestion, 0.5 h post HC ingestion, 1 h post HC ingestion, 0.5 h post RE, 1 h post RE, 2 h post RE, 4 h post RE and 6 h post RE for serum preparation (Figure 2). The samples were used to analyse serum PINP and amino acid concentration. Two × 5 mL venous blood samples were collected in EDTA plasma collection tubes (BD Vacutainer™ Hemogard Closure Plastic K2-Ethylenediaminetetraacetic acid (EDTA) Tubes, Dickinson and Company, Franklin Lakes, USA) at rest immediately prior to HC ingestion and 6 h post RE for plasma preparation. These samples were used to analyse plasma β-CTX. The catheter was flushed by 3 mL sterile pre-filled flush syringes containing sodium chloride 0.9% (BD PosiFlush™ Pre Filled Saline Syringe, Dickinson and Company, Franklin Lakes, USA) every 30 min to clean and prevent blood from clotting and blocking the catheter. The SSTs were stored in a tube rack for 30 min for clotting at room temperature and the EDTA tubes were immediately placed on ice before being centrifuged at 1000 g at 4°C for 10 min. The serum and plasma samples were then aliquoted into 5 mL round-bottom polystyrene tubes (Falcon™, Thermo Fisher Scientific, Whitby Canada) and stored at -80 °C until subsequent analysis.

Blood analyses

Markers of collagen synthesis and breakdown were analysed by measuring the circulating concentration of PINP and β-CTX, respectively. Further, circulating collagen amino acid concentrations were measured throughout the entirety of each intervention. PINP analyses were performed at Liverpool John Moores University, while β-CTX and amino acid profile analyses were performed at the Bioanalytical Facility, University of East Anglia.

PINP

Six serum samples (at rest prior to HC ingestion, 0.5 h-post RE, 1-h post RE, 2 h-post RE, 4 h-post RE and 6 h-post RE) were used to measure serum PINP concentrations using an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (USCN Life Sciences, Wuhan, China). The intra-assay coefficient of variation (CV) was <10% and the inter-assay CV was <12%, with a detection range of 2.47-200 $\mu g \cdot L^{-1}$, and sensitivity of <0.91 $\mu g \cdot L^{-1}$. The ELISA absorbance readings were performed at 450 nm, using a Clariostar microplate reader (BMG Labtech, Ortenberg, Germany). The concentration × time total area under the curve (AUC) for PINP and amino acids (see below) were calculated using Prism software (version 9.4.1, GraphPad Inc., San Diego, San Diego, California USA).

β-CTX

EDTA plasma concentrations of β-CTX were measured using electrochemiluminescence immunoassay on a Cobas e601 analyser (Roche Diagnostics, Germany). The inter-assay CV for β-CTX was ≤3% between 0.2 and 1.5 $\mu g \cdot L^{-1}$ with the sensitivity of 0.01 $\mu g \cdot L^{-1}$.

Amino acid profile

Eight serum samples (at rest immediately prior to HC ingestion, 0.5 h post HC ingestion, 1 h post HC ingestion, 0.5 h post RE, 1 h post RE, 2 h post RE, 4 h post RE and 6 h post RE) were used to assess the concentration of 18 amino acids associated with collagen composition (glycine, proline, hydroxyproline, glutamic acid, alanine, arginine, aspartic acid, lysine, serine, leucine, valine, phenylalanine, threonine, isoleucine, histidine, tyrosine, methionine, and glutamine, but not hydroxylysine). All 18 amino acid concentrations were measured simultaneously using anionic ion-pair reverse phase liquid chromatography tandem mass spectrometry (LC-MS/MS) system following derivatisation of the amino acid with *n*-butanol hydrogen chloride. The LC-MS/MS system used a Micromass® Quattro Ultima™ Pt

(Manchester, UK) coupled to an Agilent 1100 series (Cheadle, UK) high performance liquid chromatography binary pump. Electrospray ionisation source operating in positive ion mode, mass detection for each amino acid butyl ester was achieved in multiple reaction monitoring mode. Certified amino acid standards were purchased from Wacko Chemicals GmbH (Neuss, Germany) and Sigma-Aldrich (Dorset, UK). Internal standards used were glucosaminic acid and S-(2-Aminoethyl)-L-cysteine hydrochloride (Sigma-Aldrich, Dorset, UK) and L-Citrulline-2,3,3,4,4,5,5-d7 (Isoscience, King of Prussia, PA, USA). Three internal quality controls (QC) at low, medium and high concentrations were made from pooled human serum. For each batch of analysis, 10 µL of standards, QC and test samples were added to a microcentrifuge tubes, to which 440 µL of internal standards made up in 0.1M hydrochloride in methanol was added. The mixture was vortexed twice, each time allowed to stand for 10 min, then centrifuged at 10,800 × g for 5 mins. The supernatant was then transferred into a borosilicate tube and dried to completeness under nitrogen gas at a temperature of 60ºC. 100 µL of 3N *n*-butanol hydrogen chloride was added to the dried residue, vortex mixed, capped and incubated at 60ºC for 7 min. Following butylation, the mixture was dried completely under nitrogen gas, and then reconstituted with 250 µL of 12% acetonitrile:water containing 0.025% heptafluorobutyric acid (HFBA). After a final vortex mix, the samples were transferred to a polypropylene autosampler vial for injection into the LC-MS/MS.

Chromatographic separation was achieved using a Modus AAC 100 x 2.1mm 3µm column (Chromatography Direct Ltd, Runcorn, UK) maintained at 40ºC. Anionic ion-pair reagent HFBA was added to the mobile phases to improve analyte interaction with the stationary phase. A gradient elution profile at a flow rate of 350 $\mu L \cdot min^{-1}$ was used throughout. Initial conditions were 88% mobile phase A (0.025% HFBA in water) and 12% mobile phase B (0.025% HFBA in acetonitrile). This was held for 30 s. Mobile phase B was increased linearly to 20% at 10

min, with a further linear increase in mobile phased B to 60% at 15 min. This was held constant until 16.9 min and returned to the initial conditions at 17 min. Injection volume was 10 µL with an injection cycle time of 20 min. The assay range was 0 – 2000 µmol·L^{-1} for all 18 amino acids studied. Inter-assay precision coefficient of variation (CV) for all amino acids were between 3.3% to 10.3%.

Statistical analyses

Data were analysed using the statistical software package SPSS (Version 26, IBM Inc., Armonk, NY, USA). Sample size was estimated prior to conducting the study with G*Power software (version 3.1.9.6, Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany). The estimation was performed using a large effect size (η_p^2 = 0.22), based on the results from Shaw et al. (21), which demonstrated a two-fold increase in the serum PINP concentration × time area under the curve (AUC) following exercise with 15 g vs. 5 g gelatin ingestion. The results from our a priori power calculation deemed a minimum of eight participants was necessary to detect an effect of HC dose (one-way repeated measures analysis of variance (ANOVA); α: 0.05; power: 0.80). We recruited 10 participants to account for an expected 10-20% drop out. Using the Shapiro-Wilk test, all data were deemed to be normally distributed except for the amino acid data. The latter data were therefore log transformed prior to undergoing subsequent statistical analyses. One-way within-subject ANOVA models were performed to compare baseline (-1 h) concentrations of PINP and β-CTX in all three trails. Two-way within-subject ANOVAs (dose × time) were performed to detect changes in serum PINP and amino acid concentrations over time. To detect changes in plasma β-CTX concentration, a two-way within-subject ANOVA (dose × time) was performed. One-way repeated measures ANOVA models were performed to detect dose-dependent differences in concentration × time AUCs for PINP

and each of the 18 amino acids analysed. Where Mauchly's test of sphericity had been violated, Greenhouse-Geisser ($\varepsilon < 0.75$) or Huynh-Feldt ($\varepsilon > 0.75$) corrections were applied. Where a main effect of HC dose existed, Fisher's LSD post-hoc pairwise comparisons were performed to reveal which doses differed. Partial eta squared effect sizes (η_p^2) were reported for each statistical model, and the thresholds for η_p^2 are defined as small ($\eta_p^2 = 0.01$), medium ($\eta_p^2 = 0.06$) and large ($\eta_p^2 = 0.14$) (25). All data analyses matched the research design, as there were no missing data for any of the dependent variables. The level of statistical significance was set at $P < 0.05$ and all data are presented as mean ± standard deviations with 95% confidence intervals (CI, where applicable), unless stated otherwise.

Results

Serum PINP concentration and AUC

Baseline serum PINP concentrations for the 0 g, 15 g and 30 g HC interventions did not differ ($P = 0.990$, $\eta_p^2 = 0.001$; 0 g HC 32.7 ± 28.0 (95% CI: 12.7 – 52.7) $\mu g \cdot L^{-1}$; 15 g HC 32.7 ± 24.2 (95% CI: 15.4 – 49.9) $\mu g \cdot L^{-1}$; 30 g HC 32.3 ± 21.6 (95% CI: 16.9 – 47.7) $\mu g \cdot L^{-1}$. Regarding serum PINP concentration, there was a main effect of HC dose ($P = 0.004$, $\eta_p^2 = 0.462$) and time ($P = 0.013$, $\eta_p^2 = 0.458$) but no dose × time interaction effect ($P = 0.732$, $\eta_p^2 = 0.071$, **Figure 4A**). These results suggest the dose effect was not time specific. Post-hoc pairwise comparisons revealed that 30 g HC had a greater PINP response than 0 g HC ($P = 0.002$) and 15 g HC ($P = 0.020$), while 15 g HC did not different from 0 g HC ($P = 0.245$). Regarding the serum PINP concentration × time AUC, there was a main effect of HC dose ($P = 0.001$, $\eta_p^2 = 0.517$), and post-hoc pairwise comparisons revealed that 30 g HC had a greater AUC (267 ± 79 [95% CI: 211 – 323] $\mu g \cdot L^{-1} \cdot h$ than 15 g HC (235 ± 70 [95% CI: 184 – 284] $\mu g \cdot L^{-1} \cdot h$, P =

277 0.013) and 0 g HC (219 ± 88 [95% CI: 155 – 281] μg·L^{-1}·h, P = 0.002), but 15 g HC AUC did
278 not differ from 0 g HC AUC (P = 0.225, **Figure 4B**).

279 *Insert **Figure 4** near here.*

280 ***Plasma β-CTX***

281 Baseline plasma β-CTX concentrations for the 0 g, 15 g and 30 g HC interventions did not
282 differ (P = 0.311, η_p^2 = 0.122; 0 g HC 0.8 ± 0.3 (95% CI: 0.6 – 1.0) μg·L^{-1}; 15 g HC 0.8 ± 0.3
283 (95% CI: 0.6 – 0.9) μg·L^{-1}; 30 g HC 0.8 ± 0.2 (95% CI: 0.6 – 0.9) μg·L^{-1}. There was a main
284 effect of time (P = 0.007, η_p^2 = 0.577) but no main effect of HC dose (P = 0.286, η_p^2 = 0.127)
285 and no dose × time interaction (P = 0.748, η_p^2 = 0.031), i.e. plasma β-CTX concentration
286 decreased from -1 h (prior to HC ingestion and RE) to 6 h post RE for all three interventions,
287 with no difference between intervention (**Figure 5**).

288 *Insert **Figure 5** near hear.*

289 ***Serum amino acid concentrations***

290 Serum concentrations over the 7-h period of each intervention of the 18 amino acids that
291 constitute type I collagen are shown in **Figure 6**. The main effects of time, dose, and dose ×
292 time interaction effects for each amino acid are denoted in **Figure 6**. There were main effects
293 of dose for 14 amino acids (glycine, proline, hydroxyproline, glutamic acid, alanine, arginine,
294 aspartic acid, lysine, serine, leucine, valine, isoleucine, methionine, and glutamine), with 30 g
295 HC demonstrating higher serum concentrations than 0 g. Of those amino acids, glycine, proline,
296 hydroxyproline, arginine, lysine, serine, leucine, valine, isoleucine, and methionine in 30 g HC
297 showed higher serum concentrations than 15 g HC. All amino acids except for histidine (P >
298 0.05) showed a dose × time interaction effect, and there was no main effect of dose for histidine

299 (P = 0.451).

300 *Insert **Figure 6** near here.*

301 **Discussion**

302 This study is the first to investigate the effect of *high-intensity* resistance exercise (RE)
303 and 0, 15 and 30 g hydrolyzed collagen (HC) supplementation on whole body collagen turnover
304 in an homogenous group of resistance-trained, healthy, young men. We found that the serum
305 PINP concentration × time area-under-the-curve (AUC) for the 30 g HC intervention was
306 greater than for the 15 g and 0 g HC interventions. Further, these results were consistent with
307 greater increases in the appearance of key amino acid constituents of collagen (e.g. glycine and
308 proline) within the blood following ingestion of 30 g HC versus 15 g and 0 g. Therefore, at
309 least 30 g HC is required to provide greater exogenous collagen amino acid availability, which
310 appears to be a key factor for optimising collagen synthesis following high-intensity RE in
311 resistance-trained, young men.

312 To address the aims of our study, we measured serum PINP following RE with different
313 doses of HC. We chose high-intensity back squat RE to target the quadriceps muscle-tendon
314 unit (MTU), because the human patellar tendon appears to hypertrophy only following
315 prolonged periods of high-intensity (6, 7) and not moderate-intensity (26) resistance training.
316 In the current study, the 7-h experimental design was based on a significant increase in muscle
317 and tendon collagen fractional synthetic rate (FSR) at 6 h post-exercise following 1-h RE in
318 healthy young men (12). Although the significantly elevated muscle collagen FSR was similar
319 at 6 h post- and 24 h post-RE (12), tendon collagen FSR appeared to be further augmented at
320 24 h post- compared to 6 h post-RE, although it is not stipulated in the article whether tendon
321 collagen FSR measured at these two time points differed significantly. In a separate study,

serum PICP concentration was significantly higher 48 h post-exercise following 50 maximal concentric knee extensions in healthy young men (27). Thus, it is possible that, had each of our three interventions lasted 24 – 48 h post-RE, we may have observed further increases in serum PINP concentration, and possibly a larger effect of HC ingestion on these increases.

The 30 min high-intensity RE model we employed in our study was associated with peak serum PINP concentrations of ~60 $\mu g \cdot L^{-1}$ (regardless of dose), which occurred 30-60 min after the onset of RE (and 90-120 min after supplement ingestion). We chose to measure serum PINP concentration because it is a reliable biomarker of collagen synthesis, being a procollagen peptide that is cleaved off during maturation from procollagen to collagen (28). The similar PINP concentration at +0.5 and +1 h post-RE for all three doses (including 0 g HC) suggests the increase in PINP concentration observed within the first hour after starting the RE occurred as a consequence of the RE, rather than HC ingestion. However, it is possible that our data at +0.5 h post-RE may have been influenced by an increase in blood flow. A distinction should be made between flux, i.e. the total amount of PINP passing through the blood registered at any given time, and concentration, i.e. the ratio of PINP to the volume of serum. Blood flow rises 20-fold and 7-fold in the calf muscle and peritendinous area of the Achilles tendon, respectively, during repeated plantar flexion contractions in healthy individuals (29). However, an increase in blood flow to the peritendinous tendon has been shown to return to resting levels within a few minutes of finishing the same type of exercise (30). Therefore, the fact that serum PINP concentration was still at its peak at +1 h in the current study, i.e. 30 min after RE ended, when cardiac output would be expected to have returned to resting rates following lower-limb RE (31), it is likely that this increase in PINP concentration was due to an increase in RE-induced collagen synthesis, rather than blood flow. Serum PINP concentration decreased to baseline values in the 0 g and 15 g interventions for the remainder of those interventions, while

346 it decreased but remained more elevated in the 30 g intervention, which resulted in the higher
347 AUC in the 30 g intervention compared to the 0 g and 15 g interventions.

348 In contrast to our results, Aussieker et al. (32) recently found that 30 g HC ingestion
349 with six sets of 8–15 repetitions at 60% estimated 1-RM barbell back squat did not augment
350 *vastus lateralis* muscle connective tissue protein FSR or circulating PINP concentration more
351 than RE with 30 g whey protein ingestion or RE alone in different groups of young men and
352 women. A number of differences in study design may help explain this discrepancy between
353 studies. Firstly, the between-group design used by Aussieker et al. (32)may have introduced
354 more within and between intervention variability (thus potentially confounding an effect of HC)
355 compared to a within-group cross-over design, as used in the current study. Secondly, estrogen
356 is known to affect skeletal muscle and tendon collagen synthesis in women (33, 34), and the
357 use of a mixed-sex cohort by Aussieker et al. (32), rather than a 100% male cohort as used in
358 the current study, may have increased within- and between-group variability in connective
359 tissue protein FSR. Thirdly, vitamin C was not consumed during the interventions by Aussieker
360 et al. (32), which began after an overnight fast. As vitamin C is required for the biosynthesis
361 of collagen (17) and humans are unable to store it in the body or synthesize it endogenously
362 (35), this may have limited muscle connective tissue protein FSR. Finally, it should be noted
363 that Aussieker et al. (32) measured connective tissue protein FSR in skeletal muscle and not
364 tendon or ligament. The latter tissues have a 70-85% type I collagen content (9) compared to
365 just ~5% in skeletal muscle (36).

366 Contrary to Aussieker et al. (32) and in accordance with our findings, Shaw et al. (21)
367 found that serum PINP concentration was greater following jump-rope exercise with gelatin
368 supplementation in a dose-response manner. Due to different time points used to measure
369 serum PINP concentration, a direct comparison of peak concentration between studies is not

possible. Nevertheless, the serum PINP AUC was greater in our 30 g HC intervention compared to our 15 g and 0 g interventions (with no difference between our 0 g and 15 g interventions, **Figure 4**), while Shaw et al. (21) found a greater effect of 15 g versus 5 g and 0 g gelatin. This suggests that the different exercise models used may require different doses of exogenous collagen to optimise the collagen synthetic response for that particular exercise.

Regarding the amino acids that constitute collagen (e.g. glycine, proline, hydroxyproline, etc.), these peaked in circulation around 1 – 1.5 h after ingestion of 30 g HC in our study (**Figure 6**). This was in line with previous studies, which involved the ingestion of 15 g gelatin or 20 g or 30 g collagen peptides in healthy young populations (21, 32, 37). This similarity between studies indicates the maximal rate of amino acid absorption occurs approximately an hour after ingestion of collagen in healthy young men, regardless of dose and type of collagen supplementation. We also observed that the average concentrations of glycine, proline, hydroxyproline, arginine, lysine, serine, leucine, valine, isoleucine, and methionine were greater after ingestion of 30 g HC compared to 15 g and 0 g HC. The high availability of collagen amino acids in the 30 g HC intervention might have promoted a greater collagen synthetic response in two ways. Firstly, the greater abundance of key amino acids may have simply provided more of the essential components to increase collagenous tissue content following an overload-induced stimulation of collagen synthesis in the lower-limb MTUs. Secondly, they may have stimulated mammalian target of rapamycin complex 1 (mTORC1) phosphorylation independently of muscle contraction/stretch-activated mechanisms, in a similar manner to amino acid stimulation of skeletal muscle myofibrillar protein synthesis via mTORC1 activation (38). For example, after treating chondrocytes from bovine cartilage with 1.5 mM glycine, proline, or lysine for 15 days, type II collagen synthesis was 1.6 times greater following glycine treatment compared to proline treatment and 2 times greater compared to

lysine treatment (39). Further, human skin fibroblasts treated with 5 mM proline for 48 h demonstrated an increase in collagen type I α1 expression (40), while proline and hydroxyproline also increased TGF-β expression in human fibroblasts (41), which would be expected to cause phosphorylation of protein kinase B (Akt) and mTORC1 (thus explaining the increase in collagen synthesis and gene expression). Considering the above mechanisms and that collagen synthesis is stimulated via phosphorylation of Akt and mTORC1 in response to mechanical loading in cultured human tendon-derived stromal cells (42), and that mechanical loading increases in TGF-β expression and type I collagen expression in rat Achilles tendon (43), independent RE- and amino acid-associated signalling pathways likely explain our findings that high-intensity RE with 30 g HC supplementation stimulates collagen synthesis more than RE with 15 g HC ingestion or RE alone.

Concerning collagen breakdown, β-CTX is released into circulation from mature type I collagen during degradation, and therefore serves as a reliable biomarker of collagen breakdown (44). In the current study, regardless of HC dose, plasma β-CTX concentration decreased by ~30% from -1 to 6 h post RE (**Figure 5**). This may have been caused by the high-intensity RE stimulating collagen synthesis (**Figure 4**), subsequently inhibiting collagen breakdown. This hypothesis is supported by currently unpublished data from our laboratory in middle-aged men, suggesting the decrease in circulating β-CTX concentration occurs immediately after RE and remains lower for the subsequent six hours of rest, regardless of HC dose. Alternatively, the reduction may have been associated with circadian rhythm, as Qvist et al. (45) reported that resting serum β-CTX concentrations in men and pre- and postmenopausal women (aged 24-73 years) peaked at 08:00, then sharply decreased between 11:00 and 14:00. Nevertheless, as tissue turnover is determined by the rates of both synthesis and breakdown, the role of collagen degradation on the regulation of collagen turnover is crucial. For example,

418 avian skeletal muscle exposed to mechanical loading led to an increase in collagen synthesis,
419 which was accompanied by a decrease in the degradation of newly synthesized collagen, as
420 well as an increase in the degradation of mature collagen (46). Thus, further research is
421 necessary to elucidate the effects of RE and HC supplementation on degradation of newly
422 synthesized and mature collagen for the regulation of collagen turnover.

423 A limitation of this study is that our assessment of collagen synthesis was indirect, i.e.
424 we measured serum PINP concentration rather than harvesting tendon biopsies, with which we
425 could have assessed tendon PINP concentration or collagen FSR directly. However, human
426 tendon (47) and serum PINP concentration (13) increases after an acute bout of exercise, and
427 serum PINP can remain elevated for up to four days following resistance-type exercise in
428 healthy young men (27). Nevertheless, future studies should investigate the effect of HC
429 ingestion with RE on connective tissue collagen synthesis by measuring circulating and tendon
430 PINP concentration, and tendon collagen FSR simultaneously.

431 In conclusion, we have demonstrated for the first time that a single bout of high-
432 intensity, lower-limb RE with 30 g HC ingestion increased whole body collagen synthesis more
433 than RE with 15 g or 0 g HC in resistance-trained young men. This higher response was likely
434 related to the greater availability of key amino acids following the ingestion of 30 g HC
435 compared to 15 g and 0 g. This may have implications for augmenting tendon adaptation to
436 high-intensity resistance training when 30 g HC is ingested in combination with resistance
437 exercise over a prolonged period of time. Future studies should also investigate if a dose-
438 response exists regarding high-intensity RE with HC supplementation in resistance-trained
439 young women.

440

441 **Conflict of interest**

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442 The authors declare that the research was conducted in the absence of any commercial or
443 financial relationships that could be construed as a potential conflict of interest.

444

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446 Authors contributed to this manuscript as follows: study conception and design (RME); data
447 collection (JL); data curation (JL); data analysis (JL, RME, JCYT, RD, JD); supervision (RME,
448 DRC, CES); writing – original draft (JL); writing – review, editing and approval of final draft
449 (RME, JCYT, RD, JD, WDF, KE, DRC, CES).

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Figure legends and Table

Figure 1. CONSORT flow diagram. HC, hydrolyzed collagen; BS, blood samples collected for serum procollagen type I N-terminal propeptide (PINP) concentration, plasma β-isomerized C-terminal telopeptide of type I collagen (β-CTX) concentration, and serum amino acid concentration.

Figure 2. Schematic diagram of the experimental protocol. HC, hydrolyzed collagen; RE, resistance exercise; -1, rest prior to HC intake; -0.5, 0.5 h-HC ingestion; 0, 1 h-HC ingestion; +0.5, 0.5 h-post RE; 1, 1 h-post RE; 2, 2 h-post RE; 4, 4 h-post RE; 6, 6 h-post RE.

Figure 3. 90° barbell back squat. A resistance band was attached to the squat rack to indicate when the participant had reached 90° knee flexion during each repetition of the back squat 10-RM.

Figure 4. Collagen synthesis following hydrolyzed collagen (HC) ingestion (-1 h) and performing resistance exercise. (A) serum PINP concentrations (B) serum PINP concentration × time area under the curve following 0 g HC (green circles), 15 g HC (blue squares), and 30 g HC (red triangles) ingestion. *Greater than 0 g HC ($P = 0.005$); †Greater than 15 g HC ($P = 0.039$). Values represent mean ± SEM.

Figure 5. Collagen breakdown following hydrolyzed collagen (HC) intake and performing resistance exercise. Plasma CTX-1 concentrations following 0 g HC (green circles), 15 g HC (blue squares), and 30 g HC (red triangles) ingestion. *Lower than -1 h ($P < 0.05$).

Figure 6. Concentrations of eighteen serum amino acid at before ingesting hydrolyzed collagen (-1 h), 1 h after ingesting 0 g (green circles), 15 g (blue squares), or 30 g (red triangles) (HC) (+1 h) and then performing barbell back squat exercise at time point 0 h. Values represent mean

± SEM.

Table 1. Amino acid composition of the hydrolyzed collagen supplement.

Amino acids	Weight (%)
Glycine	21.0
Proline	12.8
Hydroxyproline	12.2
Glutamic acid	10.3
Alanine	8.9
Arginine	7.3
Aspartic acid	6.0
Lysine	3.5
Serine	3.1
Leucine	2.7
Valine	2.4
Phenylalanine	2.1
Threonine	1.9
Hydroxylysine	1.5
Isoleucine	1.5
Histidine	1.1
Tyrosine	1.0
Methionine	0.9

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Enrollment

Assessed for eligibility
n = 13

Excluded
n= 3

- Not meeting inclusion criteria (*n* = 1)
- Declined to participate (*n* = 2)
- Other reasons (*n* = 0)

Allocation

Randomly assigned
n = 10

Follow-Up

0 g HC	15 g HC	30 g HC
n = 10	*n* = 10	*n* = 10
Completed *n* = 10	**Completed** *n* = 10	**Completed** *n* = 10

Analysis

Analysed BS	Analysed BS	Analysed BS
n = 10	*n* = 10	*n* = 10

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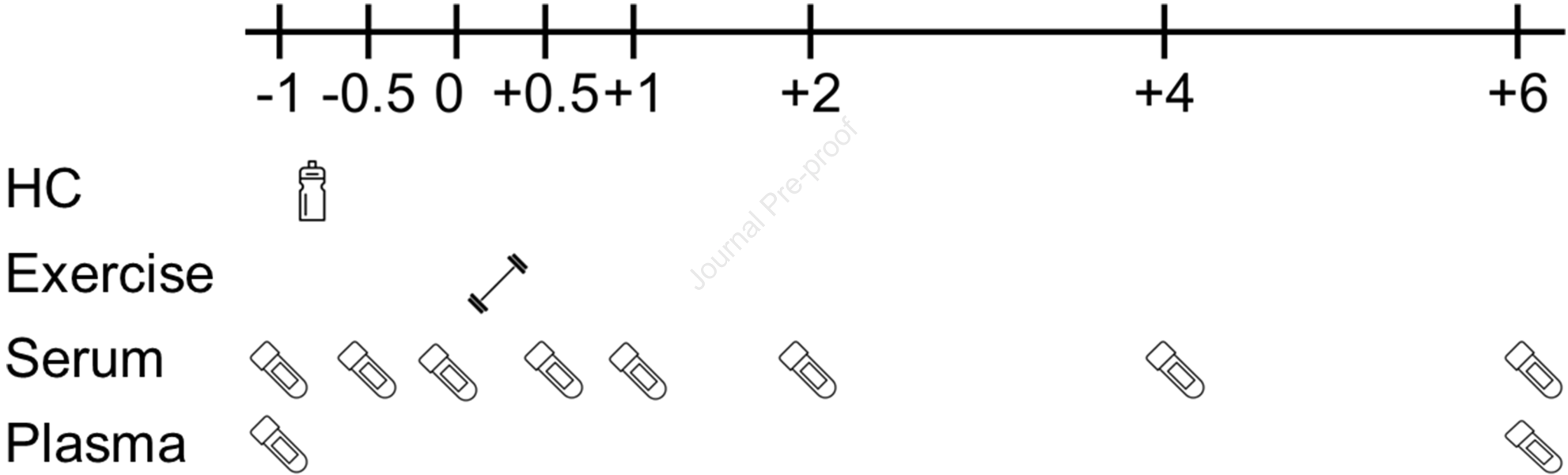

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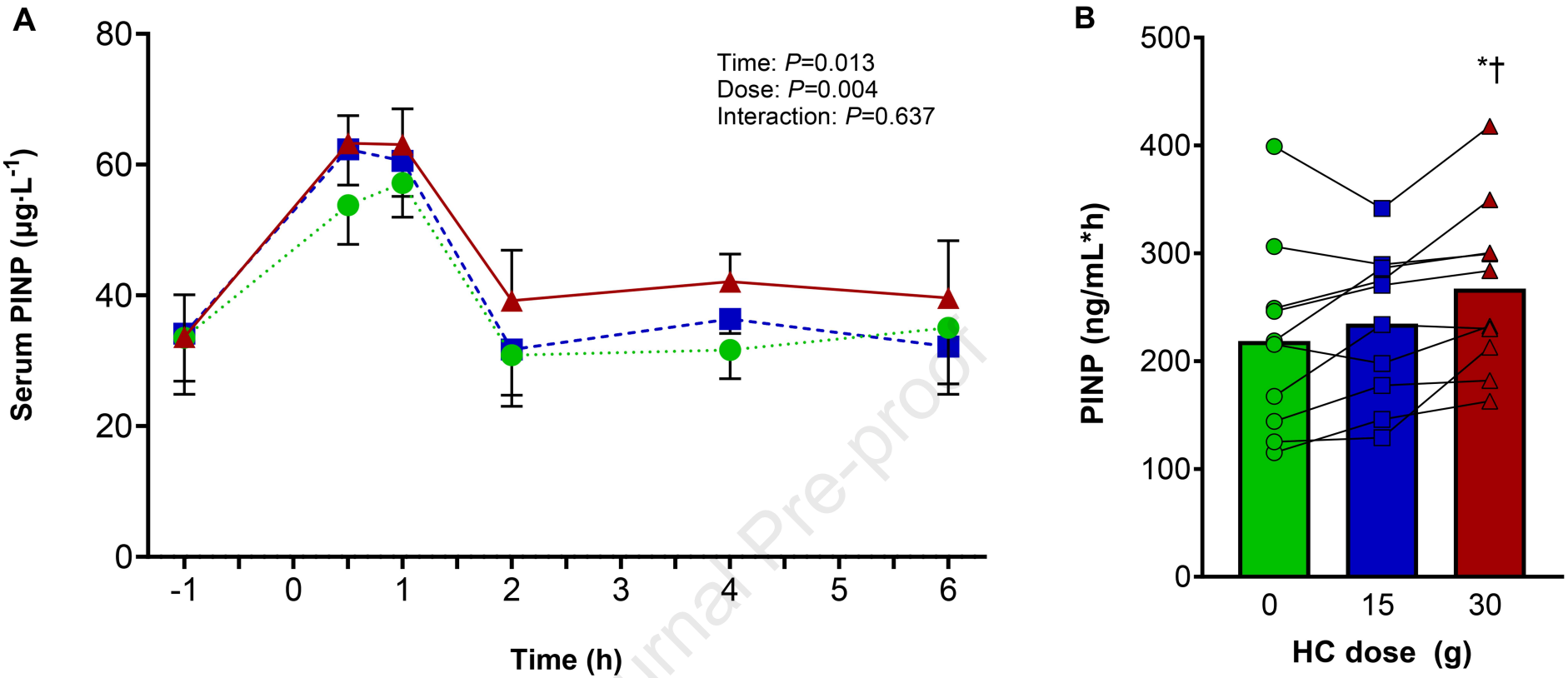

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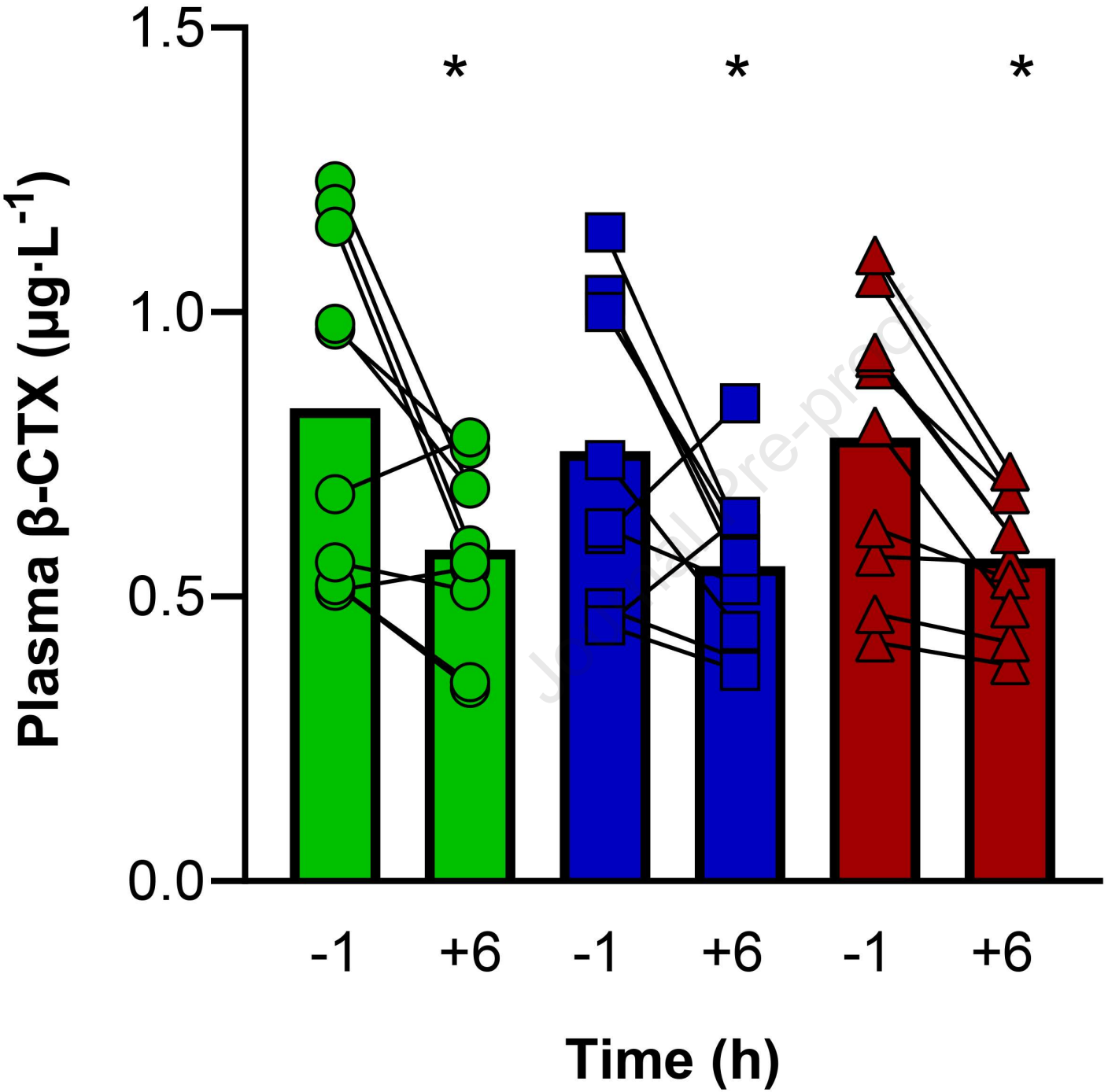

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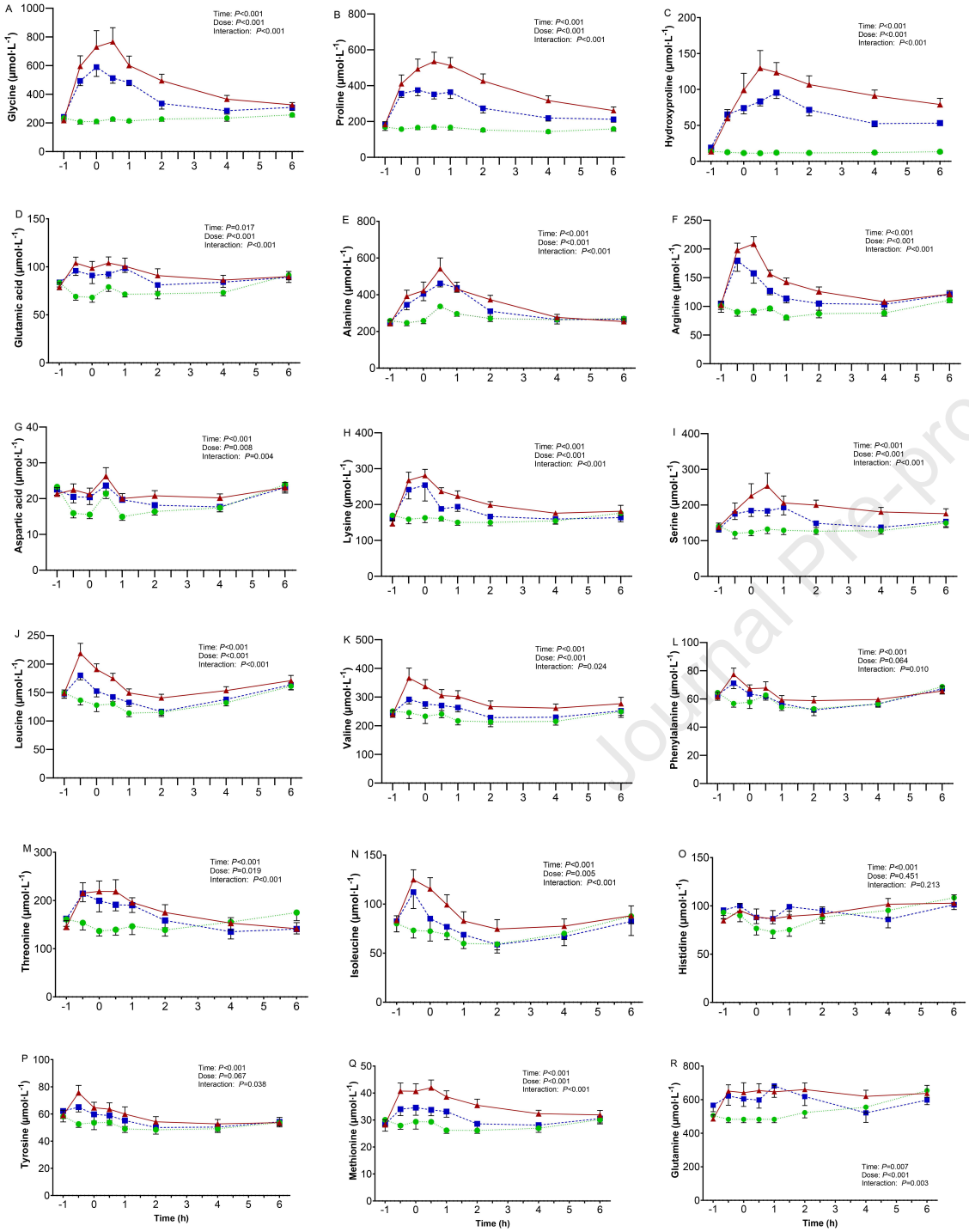

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Table 1. Checklist of information to include when reporting research in sport nutrition and exercise metabolism. This table was adopted from Betts et al. (2020)

Section	Item	Checklist	Page (pp.)/line number (LN)
Title			
	1a	State the independent (groups/conditions) and dependent (outcome) variables	pp.1
	1b	Identify the study population or case	pp.1
Abstract			
	2a	Specify the research design, methods, and characteristics of study population	LN 8 – 14
	2b	Report a balanced account of the results and cite actual data	LN 15 – 19
	2c	Restrict conclusions to measured variables, without speculation or unsupported recommendations	LN 20 – 21
Introduction			
	3a	Present a scientific rationale based on an objective review of available evidence	LN 25 – 84
	3b	State the aims, objectives, research questions, and/or hypotheses	LN 84 – 89
Methods			
Ethics	4	Provide details of ethical approval (citing conduct of human research in accordance with the Declaration of Helsinki)	LN 98 – 100
Design	5	Summarize the research design (e.g., parallel trial/cross-over, randomized, counterbalanced, blinding, observational)	LN 110 – 112
Sampling	6a	List the eligibility (inclusion/exclusion) criteria and sampling method	LN 102 – 108
	6b	Characterize the study sample (e.g., demographics, anthropometry, lifestyle)	LN 95 – 87
	6c	Report the setting/location and periods of recruitment and data collection	LN 101 – 102

		Justify the sample size (presenting the selected target effect size and error variances to replicate sample size estimates)	LN 245 – 252
	6d	Detail all aspects of the groups/conditions (considering the need to verify the composition of ingested substances)	LN 126 – 145 LN 148 – 161
Interventions	7	Define the pre-specified primary, secondary and/or mechanistic outcome variables	LN 185 – 188
Measurement	8a 8b	Rationalize the selection of test protocols, considering validity and reliability (e.g., coefficient of variation, familiarization)	LN 195 – 196 LN 204 – 205 LN 241 – 242
	8c	Justify the smallest worthwhile effect or minimal clinically important difference	LN 264 – 268
Randomization	9	Detail the exact mechanisms of generating and concealing the random allocation sequence	LN148 – 151
Blinding	10	Document whether participants and/or researchers were aware of allocation (e.g., exit questionnaire)	LN 151 – 154
Standardization	11	Describe within- and between-participant controls (e.g., replication/reporting of diet, physical activity, sleep, menstrual cycle)	LN 120 – 122
Order effects	12	Detail control of systematic influences of serial measurements (e.g., sequence effect in analysis model, wash-out interval)	LN 110 – 112

Statistics	13a	Specify the contrast for primary inferences (i.e., relative to the appropriate control, not changes from baseline in each group/condition)	LN 275 – 276 LN 296 – 297
	13b	Clearly distinguish and fully justify any unplanned, interim or exploratory subgroup analyses	n/a
	13c	Describe any adjustments for violated statistical assumptions and for relevant covariates (e.g., baseline measures)	n/a
Results			
Participant flow	14a	Report the sample size at each phase from recruitment to analysis (with reasons for losses and exclusions)	Figure 1
	14b	Ensure data analysis matches research design, avoiding data pooling across groups/conditions (i.e., pseudoreplication	Figure 1
Outcomes	15a	Report SI units and report measures of central tendency, variability, and effect size/precision (confidence intervals)	LN 275 – 312
	15b	Report individual data/responses (e.g., draw figures showing the raw data in each group/condition)	Figures 4 and 5
	15c	Document all relevant harms and unintended consequences observed	n/a
Discussion			
	16a	Present an objective and balanced interpretation of the observed data within the context of existing evidence	LN 315 – 434
	16b	Consider the applicability and/or practical relevance of the research findings (e.g., external validity)	n/a
	16c	Acknowledge strengths and limitations of the research relevant to accurate interpretation (e.g.,	LN 435 – 448

		internal validity)	
Other			
Disclosures	17	State any relevant relationships (e.g., financial, technical, material support)	LN 459 – 460
Protocol	18	Identify any publicly registered or published protocol (explaining any deviations)	n/a

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