Impact of an oral amino acid provision on Achilles peritendinous amino acid concentrations in young and older adults

Chad C. Carroll, PhD[1]
Samantha Couture, MS[1]
Dominick O. Farino[1]
Shivam H. Patel, PhD[1]
Nathan W.C. Campbell[1]
Julianne Stout, MD[2]
Arman Sabbaghi, PhD[3]

[1]Department of Health and Kinesiology, Purdue University, West Lafayette, IN
[2]College of Veterinary Medicine, Purdue University, West Lafayette, IN
[3]Department of Statistics, Purdue University, West Lafayette, IN

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Address for correspondence:

Chad C. Carroll, PhD
Assistant Professor
Purdue University
Department of Health and Kinesiology
800 W. Stadium Ave
West Lafayette, IN 47907
Phone: (765) 496-6002
carrol71@purdue.edu

ABSTRACT

Recent studies have indicated that consumption of amino acid-rich compounds can increase tendon collagen content and enhance biomechanical function. Still, it is not clear as to what extent oral consumption of amino acids alters peritendinous amino acid concentrations. Whether aging alters the delivery of amino acids to tendon tissue after oral consumption is also not known. Using microdialysis, we determined the impact of a single oral essential amino acid bolus on Achilles peritendinous amino acid concentrations in younger (n=7; 27±1 yr.) and older adults (n=6; 68±2 yrs.) over four hours. The peritendinous concentration of all amino acids in the beverage except methionine ($p=0.136$) and glycine ($p=0.087$) increased with time ($p<0.05$). Additionally, the concentrations of glycine and arginine were greater in older adults ($p\leq0.05$). We also accessed the impact of amino acid consumption on peritendinous concentrations of pro-collagen Iα1, a marker of collagen synthesis. Pro-collagen Iα1 tended to change with time ($p=0.071$) but was not altered age ($p=0.226$). We demonstrate that an oral amino acid bolus leads to modest increases in Achilles peritendinous amino acid concentrations in young and older adults. The concentration of some amino acids was also greater in older adults. However, the amino acid bolus did not significantly impact peritendinous pro-collagen concentrations.

Keywords: tendon, amino acids, exercise, pro-collagen, microdialysis

INTRODUCTION

Recent investigations have implied that tendons are responsive to amino acids. Oral consumption of amino acids may improve clinical outcomes in patients with tendinopathy (15, 30) and optimize tendon adaptations to exercise training (13). Work in rodents has implied that dietary amino acid supplementation can provide an effective means to promote recovery from tendon injury recovery and enhance collagen synthesis (28, 32). In contrast to skeletal muscle, the impact of amino acid consumption on the health of tendon tissue has not been extensively evaluated, especially in humans.

Tendon collagen content is greater in rats given a leucine-rich diet during recovery from malnourishment, suggesting that a diet rich in leucine could increase collagen synthesis. Additionally, a glycine-enriched diet improved tendon collagen content and biomechanical properties in rats after the induction of inflammation via collagenase injection (32). In support of these preclinical findings, patients treated with an arginine-rich oral supplement had modest improvements in pain (15, 25) and rotator cuff repair integrity (15) over those not consuming the supplement. Further, oral supplementation with glycine-rich collagen-peptides improved clinical outcomes in Achilles tendinopathy patients completing a calf-strengthening program (27).

Healthy tendons also appear to be responsive to dietary amino acid/protein-based interventions. Consumption of a vitamin C-enriched gelatin by healthy young men increased serum glycine, proline, and hydroxyproline concentrations, as well as collagen synthesis (30). The serum collected after subjects consumed the vitamin C-enriched gelatin was also used to treat engineered ligaments *ex vivo*. Treated ligaments had greater collagen content and superior biomechanical properties than ligaments treated with serum take before gelatin consumption (30). Consistent with the anabolic effect of leucine on skeletal muscle (10), tendon cross-sectional area (CSA) increased to a greater extent in young adults consuming a leucine-rich whey isolate during a resistance training program compared to a placebo group (13).

However, Achilles tendon stiffness was lower in mice given branched-chain amino acids during an exercise intervention than mice on a standard diet (3).

Surprisingly, studies examining the impact of amino acid consumption on tendon properties are limited to young and middle-aged adults. Age-related declines in tendon function (9) and morphological properties (5, 8) and a higher incidence of tendinopathies with age (2, 20) make older adults excellent candidates for amino acid interventions that are proposed to improve tendon properties, yet critical knowledge gaps remain. The extent to which oral consumption of amino acids increases the local delivery of amino acids to tendons is unknown. Knowing this information could guide the development of future oral amino acid beverages to optimize amino acid delivery to tendon tissues. Additionally, whether peritendinous amino acid content is influenced by aging has not been determined. It is well established that oral amino acid provisions increase serum and skeletal muscle amino acid concentrations (17, 22), but such work in human tendon *in vivo* has not been completed.

In this investigation, we utilized microdialysis to determine the impact of oral amino acid consumption on Achilles peritendinous amino acid concentrations in young and older adults (1, 16). We also assessed peritendinous concentrations of pro-collagen Iα1 as a marker of local collagen synthesis in response to the amino acid provision (29). Based on the work described above and work in skeletal muscle (10, 12), we utilized an amino acid bolus rich in leucine and glycine. To our knowledge, no studies have assessed the effects of oral amino acid supplementation on peritendinous amino acid concentrations in young or older adults *in vivo*. To set the framework for future clinical investigations and the optimization of amino acid beverages for therapeutic purposes, establishing the extent to which peritendinous amino acid concentrations change after oral consumption is critical.

METHODS

Subjects. Seven young (4 men, 3 women; 27±1 yr.; BMI: 24±1) and six elderly (1 man, 5 women; 68±2 yrs.; BMI: 24±2) adults participated in this research study. Exclusion criteria included body mass index (BMI) greater than 35 kg/m^2, chronic use of medications known to affect protein or collagen metabolism, a previous history of tendinopathies or diabetes, and sensitivity to study beverage ingredients. All subjects were sedentary (one day or less per week of aerobic or resistance exercise for at least one year) or recreationally active (not training for competitive events). All participants provided voluntary informed written consent. The Institutional Review Board of Purdue University, West Lafayette, IN (IRB#1904022075), approved this research study. This study was registered at ClinicalTrials.gov (NCT04064528).

Microdialysis. To assess amino acid concentrations in the peritendinous space of the Achilles tendon, we utilized microdialysis (1, 16, 18). For each microdialysis experiment, subjects fasted for 12-hours before arrival at Purdue University. After preparation of the skin with an antiseptic (povidone-iodine) and local anesthetic (lidocaine 1%), an ethylene oxide sterilized microdialysis fiber was inserted in the peritendinous space anterior to the Achilles tendon (1, 16). One hour after fiber insertion, subjects consumed a mixed amino acid beverage prepared from individual amino acid stocks (Ajinomoto Health & Nutrition North America, Inc, Raleigh, NC). Microdialysis samples were collected every 30-minutes for four hours after amino acid consumption.

Amino Acid Supplement. Subjects received an oral bolus of essential amino acids containing 3.5 grams of leucine (11, 12, 14), 3 grams of proline, 2 g glycine, 1.1 g histidine, 1.0 g isoleucine, 1.55 g lysine, 0.30 g methionine, 1.55 g phenylalanine, 1.45 g threonine, and 1.2 g valine (11). Amino acids (Ajinomoto Health & Nutrition North America, Inc) were mixed in a noncaloric, non-caffeinated carbonated beverage (Crystal Light, Kraft Foods, Inc). The leucine dose was chosen based on work demonstrating its effectiveness at stimulating skeletal muscle protein synthesis (10) and for comparison to previous studies (10, 12). We decided to include

137 greater glycine because of the recent preclinical evidence implying that glycine can improve
138 tendon properties (32). The proline and glycine were meant to provide additional material for
139 collagen synthesis without providing unhealthy levels of these amino acids.
140 **Sample Analysis.** Amino acid concentrations were determined with high-performance liquid
141 chromatography (Agilent Technologies 1100 HPLC System, Santa Clara, CA). Microdialysis
142 samples (15 μl) were deproteinized with 15 μl of 10%TCA and further diluted with 30 μl 0.1N
143 HCl. Diluted samples were immediately centrifugated for 10 minutes at 4°C (10,000 g). The
144 supernatant was removed and transferred to an HPLC vial. Amino acids were eluted using
145 gradient elution with mobile phase A (10 mM Na_2HPO_4, 10 mM $Na_2B_4O_7$, pH 8.2, and 5 mM
146 NaN_2, pH 8.2) and mobile phase B (45:45:10 of HPLC-grade acetonitrile, methanol, and water
147 (21). Separation of amino acids was achieved using an Eclipse Plus C18 4.6x100 mm, 3.5μm
148 column (Agilent) with a Restek Ultra C18 Guard Column (Restek Corporation, Bellefonte, PA).
149 Peaks were monitored at 230 nm excitation/450 nm emission (G1321A, Agilent). The
150 concentration of individual amino acid concentrations was determined by comparison with a
151 standard curve (AAS18, MilliporeSigma, St. Louis, MO).
152 The concentration of pro-collagen 1α1 concentration in the peritendinous space was
153 determined at select time points after amino acid consumption using a DuoSet® ELISA from
154 R&D Systems (DY6220-05, Minneapolis, MN). Due to the large dialysate volume needed for the
155 pro-collagen assay, we could not include every sampling point. Microdialysis samples were
156 diluted 1:9.5 with Reagent Dilutant and assayed in duplicate per the manufacture instructions.
157 **Statistics**
158 Several participants required a restroom break during the microdialysis experiment resulting
159 in a small number of missed collection points after amino acid consumption. Thus, individual
160 amino acid concentrations were evaluated with a mixed-effects model. For amino acids and pro-
161 collagen, noted residuals were not normally distributed and assumptions of excepted non-

constant variance were not correct, thus raw data was log-transformed before analysis. Multiple comparisons were performed using a Fisher's LSD test. The area under the curve values were compared using an unpaired, two-tailed t-test. All statistical analysis was completed in GraphPad Prism 9.0.1.

167 **RESULTS**
168
169 Mean values for all measured amino acids are provided in Table 1. When comparing
170 individual amino acids, peritendinous glycine concentrations ($p=0.015$, Figure 1) were greater in
171 older adults than young adults after beverage consumption through 150 minutes post-beverage
172 consumption. The age-related difference in glycine concentrations was no longer significant
173 beginning at 180 minutes post-beverage consumption (Figure 1). In contract, glycine did not
174 significantly change with time ($p=0.087$, Figure 1). Threonine, valine, isoleucine, lysine, leucine,
175 histidine, and phenylalanine concentrations increased with time ($p\leq0.05$, Figure 1) with all of
176 these amino acids returning to baseline by 180 minutes post-beverage consumption. For
177 threonine, valine, isoleucine, lysine, leucine, histidine, and phenylalanine, no differences
178 between young and older adults were detected ($p>0.05$, Figure 1). The dose of methionine in
179 the study bolus was not sufficient to increase peritendinous concentrations ($p=0.136$, Figure 1)
180 and no significant difference was noted between young and older adults ($p=0.077$, Figure 1). No
181 differences across time were noted for the amino acids that were not included in the essential
182 amino acid bolus ($p>0.05$, Table 1). Additionally, except for arginine ($p=0.05$), no differences
183 between young and older adults were noted in the peritendinous concentrations of amino acids
184 not included in the essential amino acid bolus.
185 In contrast to the mixed model analysis, AUC values for glycine, histidine, methionine, and
186 phenylalanine were greater in older adults compared to young (Table 2). AUC for lysine and
187 threonine were also higher in older adults but did not reach statistical significance ($p=0.06$ and
188 $p=0.07$, respectively). Peritendinous pro-collagen Iα1 concentrations tended to change with time
189 ($p=0.071$) but were not altered by age (mixed-effect analysis, $p=0.226$ and AUC, $p=0.109$,
190 Figure 2).

DISCUSSION

It is well-established that an elevation of serum amino acids can have a significant anabolic effect on skeletal muscle (6, 11, 31). A limited number of investigations in humans and rodents have indicated that tendon tissue is also sensitive to elevations in amino acids. Amino acid or protein beverages have increased tendon CSA in adults completing resistance training (13) and improved outcomes during tendinopathy rehabilitation (15, 30). In rodents, amino acids solutions, especially those rich in leucine or glycine, improve tendon properties in various disease models (28, 32). Consumption of amino acid or protein beverages increases serum and skeletal muscle amino acids. However, to our knowledge, the extent to which oral amino acid ingestion alters local (peritendinous) concentrations of amino acids in humans has not been described. The potential impact of amino acids on tendon properties in older adults is also relevant, given the changes in tendon properties with aging (5). Thus, we also considered the impact of aging on the ability of oral amino acids to increase peritendinous amino acid concentrations. Additionally, we determined if a provision of amino acids would increase the peritendinous concentration of a common collagen synthesis marker.

In this study, we demonstrate that amino acids can be detected in the Achilles peritendinous space of humans. The concentrations of the amino acids not included with the beverage were stable across time, suggesting a minimal impact of fiber insertion (Table 1). Of the amino acids in the drink, all increased with time ($p<0.05$), except methionine and glycine. The change in Achilles peritendinous amino acid concentrations across time followed a similar pattern to those previously reported in serum and skeletal muscle (1, 10) with levels peaking at 1-2 hours post-ingestion then returning to baseline levels at approximately three hours (Figure 2).

While direct statistical comparisons are not possible, the observed basal peritendinous amino acid concentrations (Table 1) were substantially lower than previously reported in serum and skeletal muscle (7, 10). Additionally, the peak concentrations obtained in the peritendinous space after consuming the amino acid bolus were generally lower than skeletal muscle or serum

218 (10). For example, utilizing a similar amino acid bolus, Dickinson et al. (10) reported peak serum
219 concentrations of approximately 1000 μM and 250 μM for leucine and phenylalanine,
220 respectively. In comparison, the highest concentration obtained in the Achilles peritendinous
221 space for leucine was 115 μM for young and 143 μM for older adults. Phenylalanine Achilles
222 peritendinous concentrations peaked at 20 μM for young and 38 μM for older adults. We did not
223 account for probe recovery due to the large number of analytes examined. However, we have
224 previously reported a probe recovery range of 50-65% for the amino acid sarcosine (16). Even
225 accounting for estimation of recovery, the values obtained in the Achilles peritendinous space
226 are lower than those obtained in serum or skeletal muscle. Further, the increase in
227 peritendinous amino acid concentrations after the oral bolus was modest when compared to
228 serum or skeletal muscle. Even with the larger dose of glycine, we did not observe a significant
229 increase in peritendinous glycine concentrations with time. This suggests that delivery of amino
230 acids to the Achilles tendon is limited when compared to skeletal muscle and serum. Larger oral
231 doses of amino acids may be needed to achieve greater benefits for tendons.
232 A lower concentration of amino acids after oral consumption is consistent with our previous
233 work with acetaminophen (16). Achilles peritendinous levels of acetaminophen achieved
234 maximum values that were approximately 50% lower than those seen in serum or skeletal
235 muscle after oral consumption of the drug (24). It is not yet clear why the peritendinous
236 concentration of compounds is lower than serum or skeletal muscle compared to the Achilles
237 peritendinous space. While the tendon is poorly vascularized, blood flow in the peritendinous
238 space during non-exercise conditions is similar to the surrounding calf muscle (4) implying that
239 delivery of amino acids to the tendon would not be impaired.
240 Interestingly, when accounting for the area under the curve, the concentration of several of
241 the amino acid included in the beverage achieved higher peritendinous concentrations in older
242 adults than in young (Figure 1). Arginine, which was not included in the amino acid bolus, was

243 also higher in the older participants compared to young. A greater baseline peritendinous amino
244 acid concentration is surprising as serum amino acid concentrations are typically lower in older
245 adults than young (26).

246 With the apparent anabolic effect of leucine and glycine on tendon collagen (32) and mass
247 (13), we evaluated peritendinous pro-collagen Iα1 as a marker of collagen synthesis. Even with
248 the large proportion of leucine and glycine in the amino acid beverage, we did not observe a
249 significant increase in peritendinous levels of pro-collagen Iα1 ($p=0.071$). One limitation of the
250 peritendinous microdialysis approach is that we assume that any pro-collagen detected is a
251 reflection of intratendinous events. Extensive work by Langberg and colleagues (19) has
252 validated the peritendinous microdialysis technique providing evidence that peritendinous
253 measures correlate with intratendinous measures. However, using stable isotope methods to
254 directly assess collagen synthesis (23) would provide more detailed results but would require
255 invasive tissue sampling.

256 In future work, it would be interesting to determine if a different source of protein (e.g., whey
257 or soy) or variations in beverage amino acid content would alter peritendinous amino acid
258 concentrations, as reported for serum (31). The limited rodent and human work suggest that
259 tendon tissue is indeed sensitive to serum amino acids changes. However, given the lower
260 concentrations obtained in the peritendinous space, we should carefully explore the dose-
261 response impact of amino acids on tendon tissue using *in vitro* or *ex vivo* models. Such
262 information is critical for optimizing oral doses of amino acids for human consumption to
263 maximize the stimulation of collagen synthesis. Also, understanding the anabolic potential of
264 each amino acid would aid in optimizing beverage content. It would be exciting to determine if a
265 larger bolus or repeated smaller doses of amino acids would increase peritendinous amino acid
266 concentrations to a greater extent than seen in the current investigation. Understanding the

impact of these beverages on peritendinous amino acid concentrations will optimize beverage content to maximize the clinical benefit of such compounds.

While much work has attempted to define an optimal protein dose to stimulate skeletal muscle protein synthesis, such information is not yet available for the tendon. Developing a nutritional cocktail to optimize tendon and skeletal muscle health is appropriate given the importance of both tissues to overall musculoskeletal function. The small number of human studies suggest the exciting possibility that amino acid beverages could be optimized to increase peritendinous amino acids level for optimization of tendon health and recovery while providing the established benefits for skeletal muscle.

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288 **Figure Legend**

289 **Figure 1:** Line graphs of Achilles peritendinous amino acid concentrations over time. Data
290 expressed as mean±standard error. No interactions were noted. Main effect p values for time
291 and age are presented above each figure insert. Multiple comparisons were performed on
292 significant main effects. *$p<0.05$ versus Young vs. Old; $^{\#}p<0.05$ versus 30 minutes. $^{\$}p=0.06$
293 versus 30 minutes.

294 **Figure 2:** Line graph of Achilles peritendinous pro-collagen concentrations over time. Data
295 expressed as mean±standard error.

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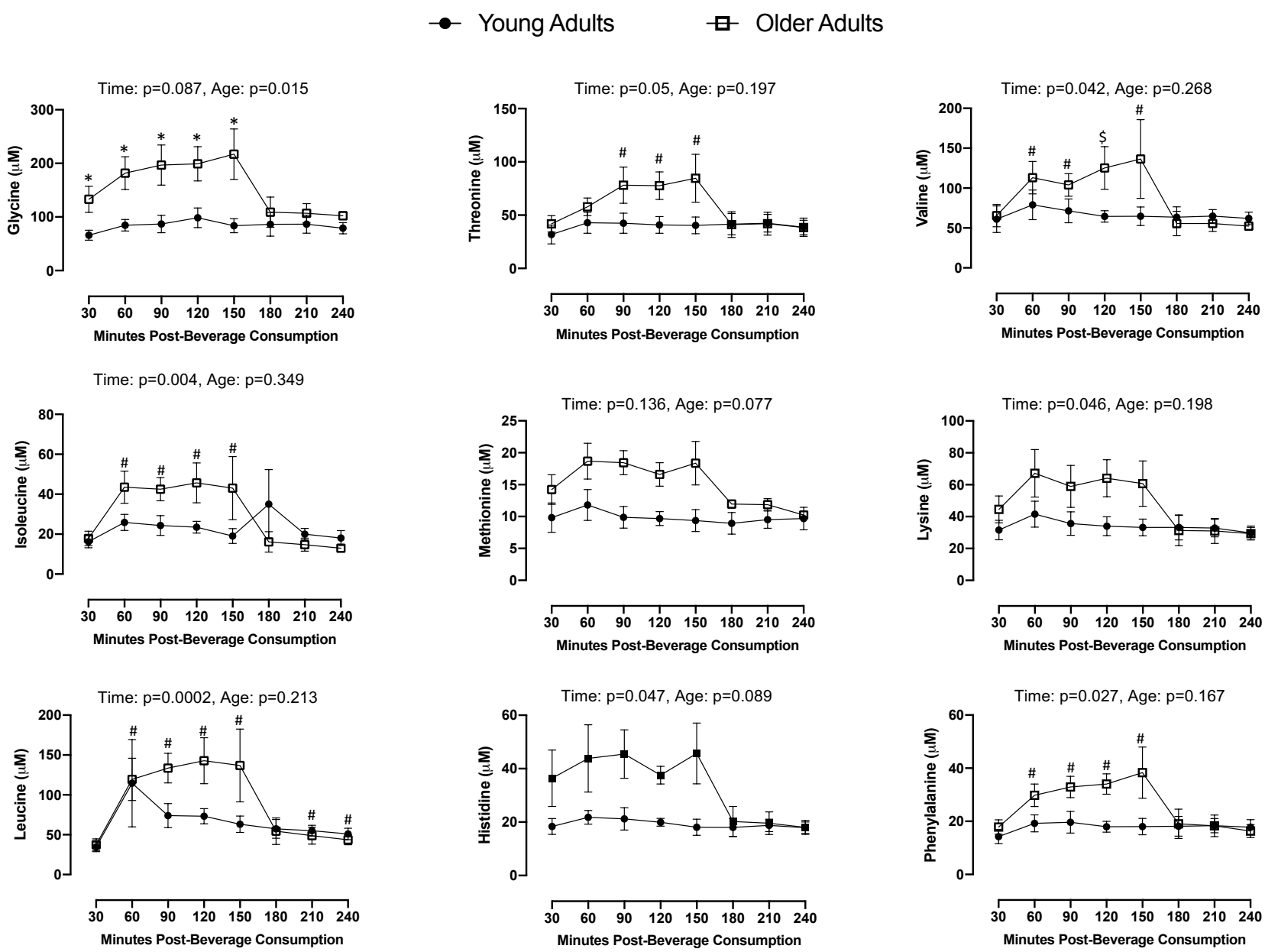
	30		60		90		120		150		180		210		240	
	Y	O	Y	O	Y	O	Y	O	Y	O	Y	O	Y	O	Y	O
Glu	12±2	31±15	11±2	31±11	9±2	22±5	11±1	21±3	8±2	27±9	8±2	17±6	10±2	16±5	12±4	15±4
Asn	11±2	16±2	11±2	17±2	9±2	14±2	9±1	15±1.5	8.5±1	16±4	9±2	9±3	10±2	10±2	10±1	10±1
Ser	28±4	47±9	30±5	56±10	27±6	52±10	28±5	54±10	27±4	67±22	29±7	33±8	31±6	34±7	30±5	30±3
Gln	273±47	555±119	292±50	612±110	241±50	468±59	253±44	557±94	245±40	614±215	272±68	302±75	298±62	322±65	279±42	320±36
His$^{\$}$	18±3	36±11	22±3	44±13	21±4	45±9	20±1	38±3	18±3	46±11	18±3	20±6	20±2	20±4	18±2	18±3
Gly	66±9	133±24*	85±11	182±30*	87±16	197±38*	98±18	199±32*	84±13	217±47*	86±22	109±28	87±17	107±18	79±11	102±7
Thr	32±9	42±8	43±10	58±8	42±9.5$^{\#}$	78±17$^{\#}$	41±8$^{\#}$	78±13$^{\#}$	40±8$^{\#}$	85±22$^{\#}$	42±10	41±12	42±8	42±10	89±51	38±7
*Arg**	17±3	30±6	16±3	33±7	11±2	27±5	14±3	30±5	11±2	31±9	13±4	15±4	15.5±4	18±4	15±3	17±3
Ala	62±13	117±26	67±14	125±13	51±14	116±7	63±13	125±10	53±11	128±22	57±17	84±23	65±17	84±18	58±10	78±10
Tyr	13±2	19±2	14±2	23±2	12±3	21±2	11±2	22±2	11±2	23±4	11±2	15±4	12±2	15±3	12±2	14±1
Cys	70±10	127±31	92±13	166±42	88±20	159±23	105±20	177±32	82±15	202±63	85±20	116±36	94±19	118±32	86±11	105±24
Val	61±17	65±14	79±19$^{\#}$	113±20$^{\#}$	71±15$^{\#}$	104±14$^{\#}$	65±7	125±27	65±12$^{\#}$	136±49$^{\#}$	64±13	56±15	65±8	56±10	62±8	52±5
Met	10±2	14±2	12±2	19±3	10±2	18±2	10±1	17±2	9±2	18±3	9±2	12±0.6	9±1	12±0.9	10±2	10±0.4
Trp	5±1	7±1	5±1	7±1	6±1	5±2	4±1	5±1	4±1	6±2	4±0.9	3±0.9	6±2	3±0.5	4±0.9	2±0.3
Phe	14±3	18±3	19±3$^{\#}$	30±4$^{\#}$	20±4$^{\#}$	33±4$^{\#}$	18±2$^{\#}$	34±4$^{\#}$	18±3$^{\#}$	38±10$^{\#}$	18±4	19±5	18±3	18±4	18±3	16±2
Iso	16±3	18±4	26±4$^{\#}$	43±8$^{\#}$	24±5$^{\#}$	42±6$^{\#}$	23±3$^{\#}$	46±10$^{\#}$	19±4$^{\#}$	43±16$^{\#}$	35±17	16±5	20±3	15±3	18±4	13±2
Leu	35±6	37±7	115±55$^{\#}$	119±26$^{\#}$	74±15$^{\#}$	134±18$^{\#}$	73±10$^{\#}$	143±29$^{\#}$	63±10$^{\#}$	137±46$^{\#}$	57±12	54±17	55±7$^{\#}$	49±11$^{\#}$	51±7$^{\#}$	44±6$^{\#}$
Lys$^{\$}$	32±6	45±8	41±8	67±15	36±7	59±13	34±6	64±12	33±5	61±14	33±8	31±9	33±6	31±8	30±4	29±4

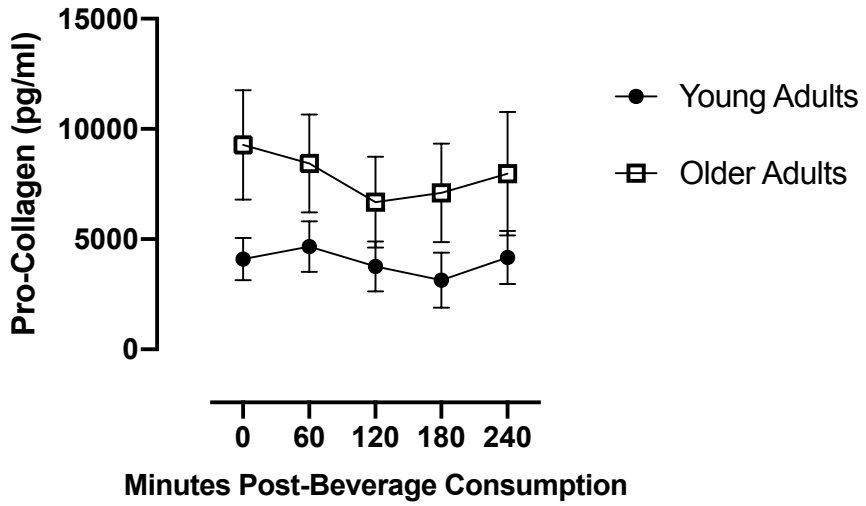
Table 1: Bolded amino acids were included in the study beverage. Values are expressed in μM with mean±SE. Y = young adults; O = older adults. Numbers in first row represent minutes post-amino acid consumption. *$p \leq 0.05$ young versus old, $^{\#}p \leq 0.05$ different from 30 minutes. $^{\$}p<0.05$, main effect for time.

Table 2

Amino Acid	Young Adults	Older Adults
Glycine	17974±2249	33843±3655*
Histidine	4079±414	7188±970*
Isoleucine	4948±1086	6629±970
Leucine	14412±3158	20294±2889
Lysine	7234±938	10504±1327[#]
Methionine	2069±253	3244±239*
Phenylalanine	3821±450	5687±603*
Threonine	8574±1267	12645±1587[&]
Valine	14092±1831	19471±2826

Date presented as mean±standard error. *$p<0.05$, [#]$p=0.0642$, [&]$p=0.0673$


Young Adults
Older Adults
Time: p=0.087, Age: p=0.015
Glycine (μM)
Time: p=0.05, Age: p=0.197
Threonine (μM)
Time: p=0.042, Age: p=0.268
Valine (μM)
Time: p=0.004, Age: p=0.349
Isoleucine (μM)
Time: p=0.136, Age: p=0.077
Methionine (μM)
Time: p=0.046, Age: p=0.198
Lysine (μM)
Time: p=0.0002, Age: p=0.213
Leucine (μM)
Time: p=0.047, Age: p=0.089
Histidine (μM)
Time: p=0.027, Age: p=0.167
Phenylalanine (μM)
Minutes Post-Beverage Consumption


15000
10000
5000
0
Pro-Collagen (pg/ml)
0
60
120
180
240
Minutes Post-Beverage Consumption
Young Adults
Older Adults