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

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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 peritendionus 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 ($p\leq0.05$). The amino acid concentrations in the Achilles peritendinous space were lower than those previously reported in serum or skeletal muscle after a bolus with a similar amino acid content. 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 increase 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 several amino acids was also greater in older adults. However, the amino acid bolus did not 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 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 the incidence of tendinopathies increase with aging (2, 20). Thus, older adults are excellent candidates for amino acid interventions, stimulating collagen synthesis, and improving tendon properties. 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 tissue 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 a 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

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

variance were not correct, thus raw data was log-transformed before analysis. No significant interactions were detected. 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.

168 **RESULTS**

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170 Mean values for all measured amino acids are provided in Table 1. When comparing

171 individual amino acids, peritendinous glycine concentration ($p=0.015$, main effect, Figure 1) was

172 greater in older adults than young adults after beverage consumption but failed to significantly

173 increase with time ($p=0.087$, main effect, Figure 1). Threonine, valine, isoleucine, lysine,

174 leucine, histidine, and phenylalanine concentrations increased with time ($p \leq 0.05$, main effect,

175 Figure 1), but no differences between young and older adults were detected. The dose of

176 methionine in the study bolus was not sufficient to increase peritendinous concentrations

177 ($p=0.136$, Figure 1) and no significant difference was noted between young and older adults

178 ($p=0.077$, Figure 1). No differences across time were noted for the amino acids that were not

179 included in the essential amino acid bolus ($p>0.05$, Table 1). Additionally, except for arginine

180 ($p=0.05$), no differences between young and older adults were noted in the peritendinous

181 concentrations of amino acids not included in the essential amino acid bolus.

182 In contrast to the mixed model analysis, AUC values for glycine, histidine, methionine, and

183 phenylalanine were greater in older adults compared to young (Table 2). AUC for lysine and

184 threonine were also higher in older adults but did not reach statistical significance ($p=0.06$ and

185 $p=0.07$, respectively). Peritendinous pro-collagen Iα1 concentration tended to increase with time

186 ($p=0.071$) but was not altered age (mixed-effect analysis, $p=0.226$ and AUC, $p=0.109$, 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

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

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

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

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

263 impact of these beverages on peritendinous amino acid concentrations will optimize beverage
264 content to maximize the clinical benefit of such compounds.
265 While much work has attempted to define an optimal protein dose to stimulate skeletal
266 muscle protein synthesis, such information is not yet available for the tendon. Developing a
267 nutritional cocktail to optimize tendon and skeletal muscle health is appropriate given the
268 importance of both tissues to overall musculoskeletal function. The small number of human
269 studies suggest the exciting possibility that amino acid beverages could be optimized to
270 increase peritendinous amino acids level for optimization of tendon health and recovery while
271 providing the established benefits of skeletal muscle.
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273
274

	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. *$p \leq 0.05$ main effect for age, #$p \leq 0.05$ 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. *$p<0.05$, [#]$p=0.0642$, [&]$p=0.0673$

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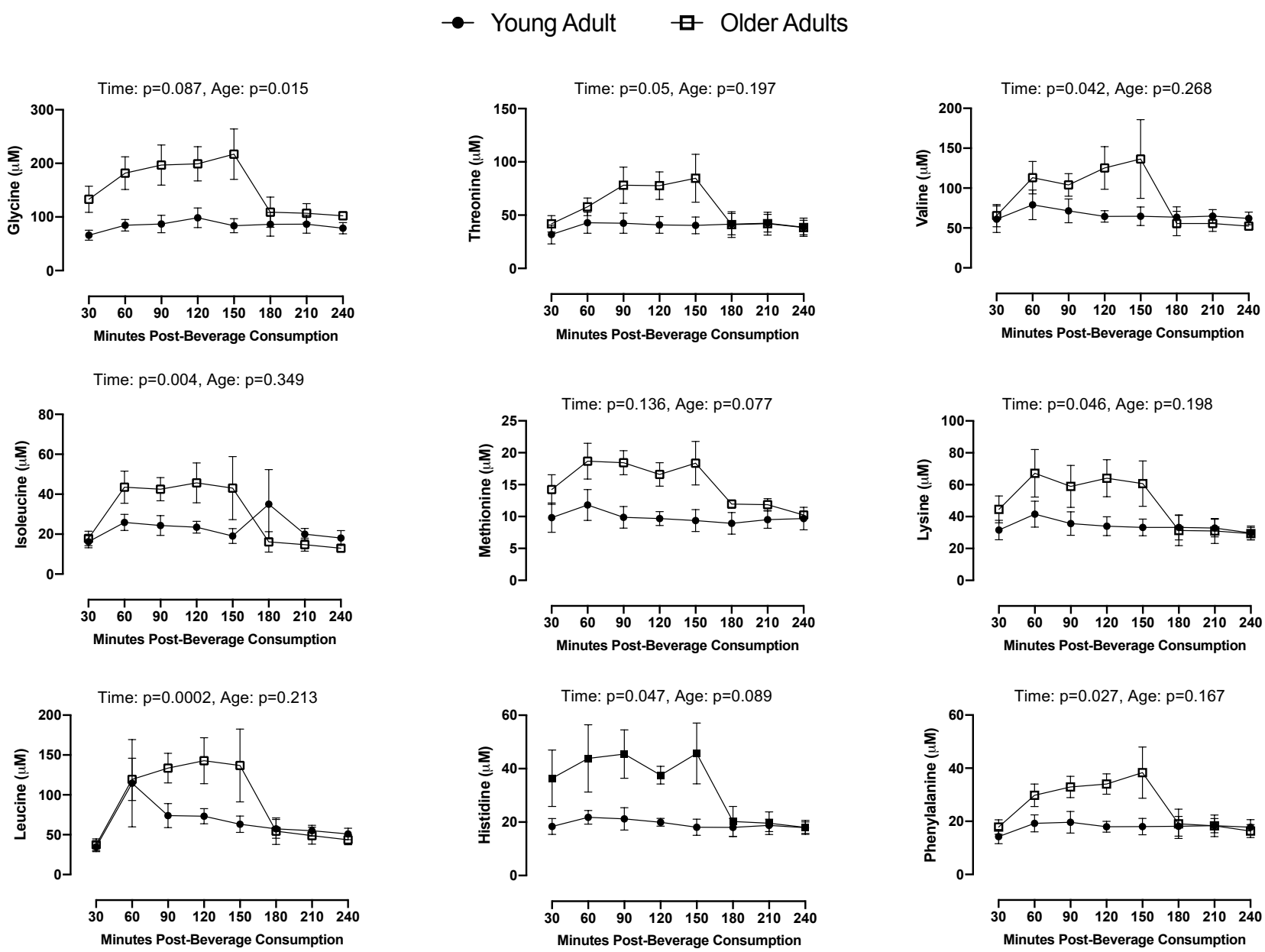
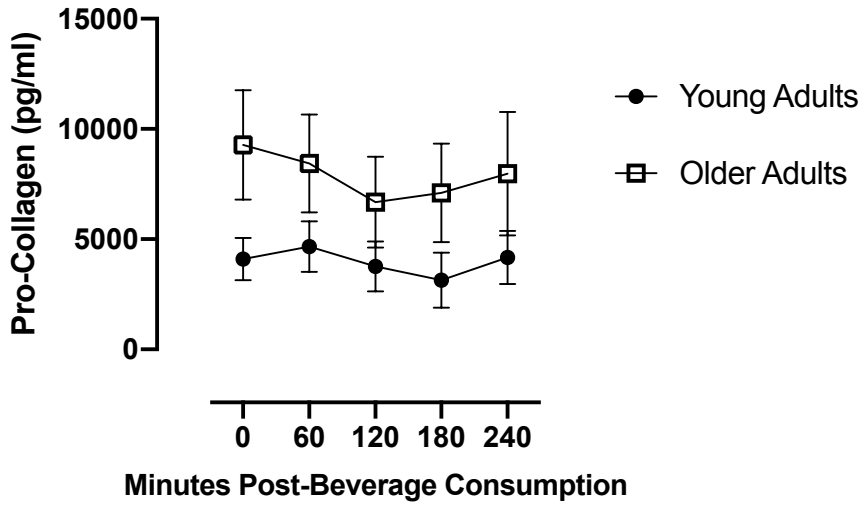
Figure Legend

Figure 1: Line graphs of Achilles peritendinous amino acid concentrations over time. Data expressed as mean±standard error. Main effect p values for time and age are present above each figure insert when appropriate.

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


Young Adult
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)
30 60 90 120 150 180 210 240
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

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