Impact of essential amino acid intake, resistance exercise, and aging on the concentration of Achilles peritendinous amino acids and procollagen I␣1 in humans

Nathan W.C. Campbell
Purdue University West Lafayette
Shivam H. Patel
Purdue University West Lafayette
Peter Ferrandi
Purdue University West Lafayette
Samantha Couture
Purdue University West Lafayette
Dominick O. Farino
Purdue University West Lafayette
Julianne Stout
Purdue University West Lafayette
Arman Sabbaghi
Purdue University West Lafayette
Chad C. Carroll (carrol71@purdue.edu)
Purdue University West Lafayette

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Abstract

Several recent studies have shown that consuming amino acid-rich compounds improves tendon collagen content and biomechanical properties. Yet, it is not clear if the consumption of amino acids alters local (peritendinous) amino acid concentrations. If aging or exercise influence local amino acid concentrations in conjunction with an amino acid bolus is also not known. We conducted two studies. In Study 1, young women (n=7, 25+/-2 yrs.) completed two identical resistance training sessions with either essential amino acid (EAA) or placebo consumption. In Study 2, an EAA bolus identical to Study 1 was given to younger (n=7; 27+/-1 yr.) and older adults (n=6; 68+/-2 yrs.). Microdialysis was used to determine Achilles peritendinous amino acid and pro-collagen I α1 (a marker of collagen synthesis) concentrations. In Study 1, amino acid consumption increased peritendinous concentrations of all EAA except histidine ($p<0.05$). In Study 2, the peritendinous concentration of EAAs except for methionine, histidine, and lysine ($p>0.05$) increased with time ($p<0.05$). Further, the concentrations of most measured amino acids were greater in older adults ($p<0.05$). Pro-collagen I α1 concentration ($p>0.05$) was unaffected by exercise, EAA, or aging ($p>0.05$). Our findings demonstrate that: 1) when not combined with exercise, an oral EAA bolus leads to only modest increases in Achilles peritendinous amino acid concentrations, 2) when combined with resistance exercise, EAA consumption resulted in greater peritendinous amino acid concentrations compared to no exercise, 3) the basal concentrations of most amino acids were greater in older adults, and 4) neither the EAA bolus nor exercise altered peritendinous pro-collagen concentrations.

INTRODUCTION

Like skeletal muscle, studies have shown that chronic exercise training can lead to increased tendon cross-sectional area (CSA), stiffness, and collagen cross-linking (Kongsgaard et al. 2007; Carroll et al. 2012). However, the adaptability of tendon tissue to chronic exercise stimulus appears to less robust in women compared to men (Magnusson et al. 2007). Evidence also suggests that exercise protocols which induce skeletal muscle hypertrophy in older adults do not result in concomitant adaptations in tendons (Carroll et al. 2011). Enhancing tendon adaptations to exercise may reduce age-related declines in tendon function and morphological properties (Carroll et al. 2008; Couppe et al. 2009), optimize musculoskeletal performance, and reduce the incidence of tendinopathies. Amino acid supplementation could be a possible approach to enhance exercise tendon adaptations in women, older adults, and other populations.

In humans, men consuming a leucine-rich beverage exhibited a greater increase in tendon CSA after chronic resistance training compared to those given an isocaloric placebo (Farup et al. 2014). Amino acid supplementation also improved outcomes in patients with tendinopathy (Gumina et al. 2012; Notarnicola et al. 2012; Praet et al. 2019). In pre-clinical work with rodents, amino acid supplementation enhanced tendon collagen synthesis and improved biomechanical properties in a model of tendon inflammation (Vieira et al. 2015). A limitation to this promising area of research is the lack of knowledge regarding the extent consumption of amino acids increases the delivery of amino acids to tendons. It is well established that amino acid intake increases serum and skeletal muscle amino acid concentrations

(Gutierrez et al. 1999; McCormack et al. 2017), yet work in human tendons *in vivo* has not been completed. Whether peritendinous amino acid content is influenced by aging or exercise has also not been determined. Knowing this information could guide the development of future amino acid beverages for optimizing amino acid delivery to tendon tissues.

We hypothesized that an oral amino acid bolus would increase Achilles peritendinous concentrations to a similar extent as seen in serum and skeletal muscle in young and older adults. We also hypothesized that exercise and amino acid intake would result in greater peritendinous concentrations of pro-collagen Ia1. We chose to focus our exercise study on young women due to data demonstrating the minimal impact of exercise alone on tendons (Magnusson et al. 2007). We utilized microdialysis to determine the impact of oral amino acid consumption on Achilles peritendinous amino acid concentrations in young and older adults and young women completing an acute bout of resistance exercise. We also assessed peritendinous concentrations of pro-collagen Ia1 as a marker of local collagen synthesis in response to the amino acid provision. To our knowledge, no studies have assessed the effects of amino acid supplementation on peritendinous amino acid concentrations in younger or older adults or after exercise *in vivo*. Future clinical studies investigating the impact of amino acid supplementation on tendon health would benefit from a better understanding of the relationship of amino acid intake to changes in peritendinous amino acid concentrations.

MATERIALS and METHODS

Overview: To compare the impact of amino acid consumption in conjunction with exercise (Study 1) young women (n=7, 25±2 yrs. Height: 161±2 cm; Weight: 60±2 kg; BMI: 24±1) completed two identical resistance training sessions that included 8 sets of 15 repetitions of single-leg calf press exercise on a seated leg press machine at 70% of 15RM (Astill et al. 2017). The eight sets were preceded by two warm-up sets at ~40% of maximum effort. Each exercise session was separated by at least one week. Immediately following the exercise bout, subjects consumed an oral bolus of essential amino acids (EAA) or a placebo. Each exercise session was conducted after an overnight fast. At least one week before the exercise studies, the calf muscle strength of the dominant leg was assessed using a 15-repetition maximum (RM), i.e., the maximum weight that can be completed 15 times (Astill et al. 2017). All exercise sessions were supervised by a research team member. For Study 2, a single EAA bolus identical to Study 1 was given to young (n=7; 4 men, 3 women; 27±1 yr.; Height: 169±4 cm; Weight: 69±6 kg; BMI: 24±1) and older adults (n=6; 1 man, 5 women; 68±2 yrs.; Height: 172±5 cm; Weight: 71±4 kg; BMI: 24±2). Participants in Study 2 did not exercise. Both studies were approved by the Institutional Review Board of Purdue University, West Lafayette, IN (IRB#1704019133 and IRB#1904022075) and registered on ClinicalTrials.gov (NCT04067479 and NCT04064528).

Amino Acid Bolus: Subjects in both studies consumed an identical bolus of EAA containing 3.5 grams of leucine (Dickinson et al. 2014; Dickinson et al. 2017; Glynn et al. 2010), 3 g 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. Amino acids (Ajinomoto Health & Nutrition North America, Inc) were mixed in

a noncaloric, non-caffeinated carbonated beverage (Crystal Light). The placebo beverage consisted of Crystal Light only. The leucine dose was chosen based on work demonstrating its effectiveness at stimulating skeletal muscle protein synthesis (Dickinson et al. 2014), and for comparison to previous studies (Dickinson et al. 2017). We decided to include greater glycine because of the recent preclinical evidence implying that glycine can improve tendon properties (Vieira et al. 2015).

Microdialysis Study 1: Immediately after the exercise session, an ethylene oxide sterilized microdialysis fiber was inserted in the peritendinous space anterior to the Achilles tendon after preparation of the skin with an antiseptic (povidone-iodine) and local anesthetic [lidocaine 1%; (Astill et al. 2017; Gump et al. 2013)]. Subjects consumed the EAA bolus 90-minutes after fiber insertion or two-hours post-exercise. The two hours allowed for one hour of fiber equilibration, a thirty-minute baseline collection, and 30 minutes to insert the fiber. Microdialysis samples were collected every 30-minutes for five hours after amino acid consumption. For each experimental day, subjects fasted for 12-hours before arrival at Purdue University. Two subjects also completed a pilot microdialysis trial with no exercise no earlier than one week from the exercise experiments. This pilot experiment was included to determine if fiber insertion resulted in substantial changes in peritendinous amino acid concentrations. The results of these pilot experiments were not included in statistical analysis.

Microdialysis Study 2: A microdialysis fiber was inserted in the peritendinous space of the Achilles tendon after a 12-hour fast. One hour after fiber insertion, subjects consumed a bolus of EAA identical to Study 1. Microdialysis samples were collected every 30-minutes for four hours after amino acid consumption.

Sample Analysis. Amino acid concentrations were determined with high-performance liquid chromatography (Agilent Technologies 1100 HPLC System, Santa Clara, CA). Microdialysis samples deproteinized with 10%TCA (1:1 dilution) and further diluted with 1:1 with 0.1N HCl. Diluted samples were immediately centrifugated for 10 minutes at 4°C (10,000 g). The supernatant was removed and transferred to an HPLC vial. Amino acids were eluted using gradient elution with mobile phase A (10 mM Na_2HPO_4, 10 mM $Na_2B_4O_7$, pH 8.2, and 5 mM NaN_2, pH 8.2) and mobile phase B (45:45:10 of HPLC-grade acetonitrile, methanol, and water (Long 2017). Separation of amino acids was achieved using an Eclipse Plus C18 4.6x100 mm, 3.5mm column (Agilent) with a Restek Ultra C18 Guard Column (Restek Corporation, Bellefonte, PA). Peaks were monitored at 230 nm excitation/450 nm emission (G1321A, Agilent). Individual amino acid concentrations were determined by comparison with a standard curve (AAS18, MilliporeSigma, St. Louis, MO). The concentration of pro-collagen 1a1 concentration in the peritendinous space was determined at select time points after amino acid consumption using a DuoSet^{Ò} ELISA from R&D Systems (DY6220-05, Minneapolis, MN

Statistics. *Study 1:* The concentration pro-collagen Ia1 was analyzed using a linear mixed-effects regression model. The fixed effects in this model were birth control usage, the group indicator, BMI, and time. In addition, the mixed model for pro-collagen Iα1 had random effects for a subject and a spatial power covariance structure to account for the unequally spaced time points in which the measurements were collected. We performed residual diagnostics to evaluate the assumptions of normal error terms,

constant variance for the random error terms, and independent errors for the linear mixed-effects regression models. The residual diagnostics for the analyses of the raw outcome variables indicated that these assumptions were violated. In contrast, the diagnostics for the analyses of the logarithmically transformed outcomes indicated that the assumptions were satisfied. As such, we analyzed the logarithmically transformed outcome variables in our mixed-effects model.

A small number of amino acid time points were randomly lost because participants required a restroom break during the microdialysis experiment. The individual amino acid concentrations were analyzed separately using linear mixed-effects regression models that accounted for the correlation in a subject's repeated measurements. In these models, the fixed effects were birth control usage, the group indicator, BMI, time, and the initial value of the respective amino acids. An autoregressive covariance structure of order 1 was used. As before, residual diagnostics were performed to assess the validity of the model assumptions and based on these diagnostics. The logarithmically transformed amino acid concentrations were ultimately analyzed. Multiple comparison tests were performed based on the Dunnett-Hsu adjustment approach for the fixed effects for strong control of Type 1 errors. The Kenward-Rogers adjustment was used to perform the mixed-effects models' statistical tests. Interactions between group and time were evaluated using tests and model fit statistics such as AIC, BIC, and AICc.

Study 2: Individual amino acid concentrations were evaluated with a mixed-effects regression model with random intercepts for subjects and continuous-time first-order autoregressive correlation structure [AR(1)] (Simpson et al. 2010). A Kenward-Roger adjustment was used for hypothesis testing of fixed effects (Time and Age). For amino acids and pro-collagen, we noted that the residuals were not normally distributed, and that the constant variance assumption was not valid. Thus, the raw outcome data were log-transformed before the analysis. Multiple comparisons were performed using a Dunnett's test. Individual time points were compared to the 30-minute sample collection for any significant time effects. All statistical analyses (Study 1 and 2) were completed in SAS (SAS Institute, Inc), and figures were generated using GraphPad Prism 9.0.1 (GraphPad Software, San Diego, CA).

RESULTS

Study 1: Achilles peritendinous pro-collagen Ia1 concentration did not change across time and was not influenced by amino acid consumption (Figure 1, $p>0.05$). Aspartic acid was not consistently detectable in all samples due to the low fluorescence of this amino acid and the generally low concentrations in peritendinous samples. Measures of amino acids not included in the oral bolus, glutamic acid, asparagine, glutamine, arginine, alanine, and tyrosine were not influenced by amino acid consumption. Still, they did gradually decline with time ($p<0.05$) to varying degrees (Figure 2).

Peritendinous histidine concentrations were not influenced by amino acid consumption ($p>0.05$, Figure 3). Isoleucine, valine, methionine, phenylalanine, and lysine concentrations were greater at 90 and 150 minutes during the amino acid trail when compared to placebo ($p<0.05$, Figure 3), but returned to placebo levels by 210 minutes. Threonine and glycine concentrations were greater during the amino acid trial at

90, 150, and 210 minutes, returning to placebo levels by 270 minutes ($p<0.05$, Figure 3). Leucine was also greater in the Achilles peritendinous space when amino acids were given, and levels continued to be elevated through the end of the experiment ($p<0.05$, Figure 3).

Study 2: Peritendinous pro-collagen Ia1 concentration tended to change with time ($p=0.071$) but were not altered by age (mixed-effect analysis, Figure 4). Generally, peritendinous concentrations of most amino acids not provided in the essential amino acid bolus were higher in older adults than young (Figure 5). Glutamic acid concentrations were greater in older adults at 60, 90, and 150 minutes ($p<0.05$). Serine, glutamine, tyrosine, and alanine concentrations were greater in older adults but only at 90 and 150 minutes ($p<0.05$). Arginine concentrations were also greater in older adults than young adults, only at 30, 60, 90, and 120 minutes. Asparagine, tryptophan concentrations were not different between young and older adults ($p>0.05$). No differences across time were noted for the amino acids not included in the amino acid bolus (Figure 5, $p>0.05$).

Of the amino acids provided in the cocktail, glycine concentrations were greater in older adults than young at 60, 90, 120, and 150 minutes ($p<0.05$, Figure 6). Glycine concentrations were also greater at 120 minutes compared to the 30-minute time point. Threonine and valine concentrations were not different between young and old ($p>0.05$) but were greater at 120-minutes compared to 30-minutes ($p<0.05$, Figure 6). Leucine concentrations increased with time and were greater at 60, 90, 120, and 150-minutes when compared to 30-minutes ($p<0.05$, Figure 6). Leucine concentrations were also greater in older adults, but only the 90-minute time point reached statistical significance ($p<0.05$). Isoleucine concentrations were greater at 60, 90, and 120-minutes compared to 30-minutes ($p<0.05$) and were greater in older adults at 90-minutes ($p<0.05$, Figure 6). Methionine concentrations did not increase with time ($p<0.05$) but were greater in older adults at 90 and 150-minutes post-bolus consumption ($p<0.05$, Figure 6). Further, phenylalanine concentrations increased with time and were greater than the 30-minute time points at 60, 90, and 120-minutes post-bolus consumption ($p<0.05$, Figure 6). Histidine did not change with time ($p>0.05$) but was greater in older adults compared to young at 90 and 150-minutes ($p<0.05$, Figure 6). Lysine concentrations were not influenced by amino acid consumption or age ($p>0.05$, Figure 6).

DISCUSSION

It is well-established that consumption of protein-rich foods or essential amino acids results in elevations of serum and skeletal muscle amino acid concentrations (Carroll et al. 2005; Dickinson et al. 2014; Tang et al. 2009; Bohe et al. 2003). A limitation of the current literature is that the extent to which amino acid ingestion alters local (peritendinous) concentrations of amino acids in humans has not been described, especially in the context of exercise or aging. Further, few studies have evaluated the impact of essential amino acid consumption on markers of tendon collagen synthesis in humans. We sought to determine 1) if an oral bolus of essential amino acids rich in leucine and glycine consumed after an acute bout of resistance exercise would increase Achilles peritendinous amino acid levels and increase pro-collagen Ia1 in young women and 2) if aging would influence the impact of an EAA bolus on peritendinous amino acid and pro-collagen concentrations.

Due to the previously noted anabolic effect of leucine and glycine on tendon collagen (Vieira et al. 2015) and mass (Farup et al. 2014), we evaluated peritendinous pro-collagen Ia1 as a marker of collagen synthesis. Exercise did not impact pro-collagen Ia1 in the placebo group and the addition of the amino acid bolus did not result in greater peritendinous pro-collagen concentrations during the exercise condition. We also did not observe a significant increase in peritendinous levels of pro-collagen Ia1 in the young and older participants in Study 2 nor a difference with age. The minimal effect of exercise on pro-collagen Ia1 is consistent with previous work in women where exercise did not increase tendon collagen synthesis when assessed using stable isotope methods (Miller et al. 2005). A lack of an age effect is surprising, given reports in rodents demonstrating a gradual decline in collagen synthesis with aging in the heart, lung, skeletal muscle, and skin (Mays et al. 1991). A significant portion of newly synthesized collagen is rapidly degraded, which could limit the interpretation of peritendinous pro-collagen measures (Laurent et al. 1985; Mays et al. 1991). With the peritendinous microdialysis approach, we assume that any pro-collagen detected reflects intratendinous events. Extensive work by Langberg and colleagues (Langberg et al. 2002) has validated the peritendinous microdialysis technique providing evidence that peritendinous measures correlate with intratendinous measures. Using stable isotopes (Miller et al. 2005) to assess collagen synthesis directly would provide more detailed results but require invasive tissue sampling.

In addition to the study of pro-collagen Ia1, we demonstrate that a bolus of EAAs can indeed increase peritendinous amino acid concentrations. In Study 1, values peaked at 90 minutes post-consumption and gradually returned to baseline levels over 1-2 hours. We observed the strongest response in peritendinous leucine and glycine concentrations, likely due to the much larger dose of these amino acids relative to other amino acids in the beverage. Histidine was the only amino acid included in the EAA bolus that was not greater than placebo conditions. Study 2 noted that the same oral amino acid bolus increased peritendinous amino acid concentrations in young and older individuals. However, the increase in amino acid concentrations appeared to be more robust and sustained when amino acid consumption was combined with resistance exercise. Possibly increased peritendinous blood flow after exercise (Boushel et al. 2000a; Boushel et al. 2000b) aided in increasing amino acid delivery to the tendon tissue. These data imply that the combination of amino acids with exercise could be more advantageous to enhancing tendon properties. Future work could optimize the timing of amino acid consumption relative to an exercise session.

In Study 2, peritendinous concentrations of glutamic acid, serine, glutamine, histidine, glycine, arginine, alanine, tyrosine, methionine, phenylalanine, isoleucine, and leucine were greater in older adults at select time points. A greater baseline peritendinous amino acid concentration is surprising as serum amino acid concentrations are typically lower in older adults than young (Pitkanen et al. 2003). Only leucine, isoleucine, and phenylalanine concentrations increased consistently after EAA bolus consumption. In addition, glycine, threonine, and valine concentrations were only statistically different from 30-minutes at 120-minutes post-beverage consumption. The change in Achilles peritendinous amino acid concentrations across time followed a similar pattern to those previously reported in serum and skeletal

muscle (Gutierrez et al. 1999; McCormack et al. 2017) with levels peaking at 1-2 hours post-ingestion then returning to baseline levels at approximately three hours.

The observed peritendinous amino acid concentrations in both studies were substantially lower than previously reported in serum and skeletal muscle (Gutierrez et al. 1999; McCormack et al. 2017). While we did not assess probe recovery, we previously reported a 50-65% probe recovery range for the amino acid sarcosine (Gump et al. 2013). Even accounting for recovery estimation, the values obtained in the Achilles peritendinous space are lower than those obtained in serum or skeletal muscle. Our findings suggest that delivery of amino acids to the Achilles tendon is limited compared to skeletal muscle and serum. Future work should focus on establishing the amino acid concentrations needed to provide a biological effect on tendon tissue *in vivo* to optimize amino acid beverages for tendon health. A lower concentration of peritendinous amino acids after oral consumption is consistent with our previous work with acetaminophen (Gump et al. 2013). Achilles peritendinous levels of acetaminophen achieved maximum values approximately 50% lower than those seen in serum or skeletal muscle after oral consumption of the drug (Muller et al. 1995). It is not yet clear why the peritendinous concentration of compounds is lower than serum or skeletal muscle compared to the Achilles peritendinous space.

There are some limitations to the current studies. First, we did not include a cohort of men in Study 1 for comparison. A leucine-rich isolate has been shown to induce tendon hypertrophy in men completing a resistance training program (Farup et al. 2014), implying that amino acids can indeed induce an anabolic response. The lack of effect of amino acids on pro-collagen could be a sex-specific resistance to amino acids. Second, the EAA bolus provided in this study, while offering an anabolic effect in skeletal muscle (Dickinson et al. 2014), may not raise peritendinous amino acid to the degree that results in an anabolic effect on tendon tissue. Our data indicate that larger gram quantities of some amino acids may be needed to result in significant peritendinous increases.

In future work, it would be interesting to determine if variations in the source of protein (e.g., whey or soy) or beverage amino acid content would alter peritendinous amino acid concentrations, as reported for serum (Tang et al. 2009). Direct comparisons between men and women are also warranted. It would be exciting to determine if a larger bolus or repeated smaller amounts of amino acids would increase peritendinous amino acid concentrations to a greater extent than seen in the current investigation.

In summary, our findings demonstrate that: 1) when not combined with exercise, an oral EAA bolus leads to only modest increases in Achilles peritendinous amino acid concentrations in young and older adults, 2) when combined with resistance exercise, EAA consumption resulted in greater peritendinous amino acid concentrations compared to no exercise, 3) the basal concentration of most amino acids was greater in older adults, 4) neither the EAA bolus nor exercise altered peritendinous pro-collagen concentrations. The observed peritendinous amino acid concentrations were substantially lower than previously reported in serum and skeletal muscle (Gutierrez et al. 1999; McCormack et al. 2017).

Amino acid supplementation is emerging as a potential therapeutic option for enhancing tendon adaptations to exercise and improving tendon health in patients recovering from tendinopathy. 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 levels to improve tendon health and recovery while providing the established benefits for skeletal muscle.

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Figures

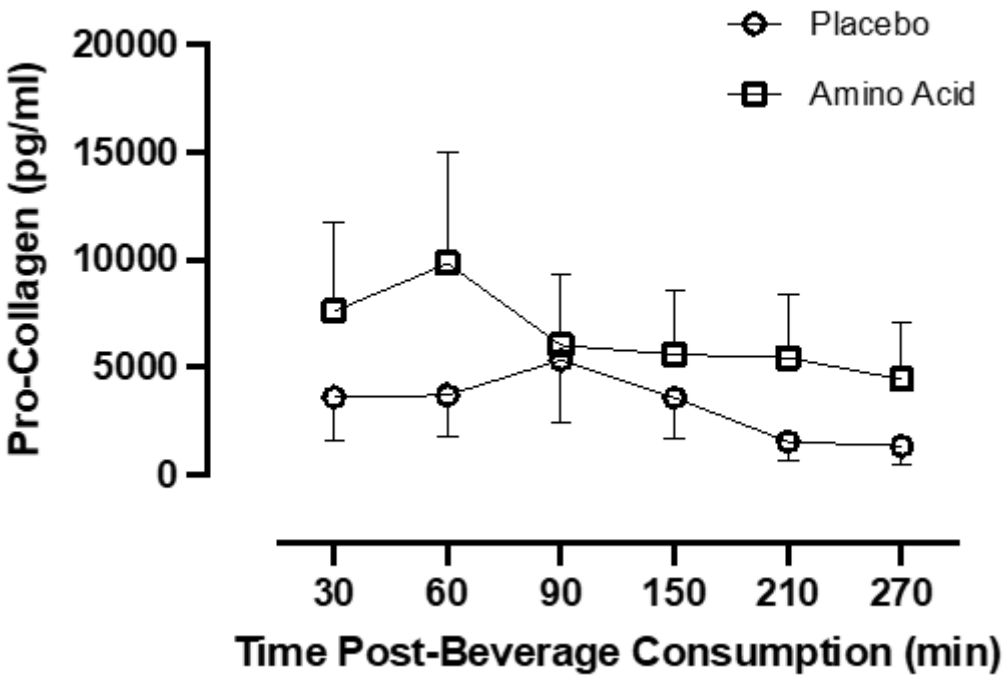

Figure 1

Line graph of Achilles peritendinous pro-collagen Ia1 concentration over time for Study 1. Data expressed as mean±standard error. Up error bars (Placebo) and Down error bars (Amino Acid) were removed for clarity.

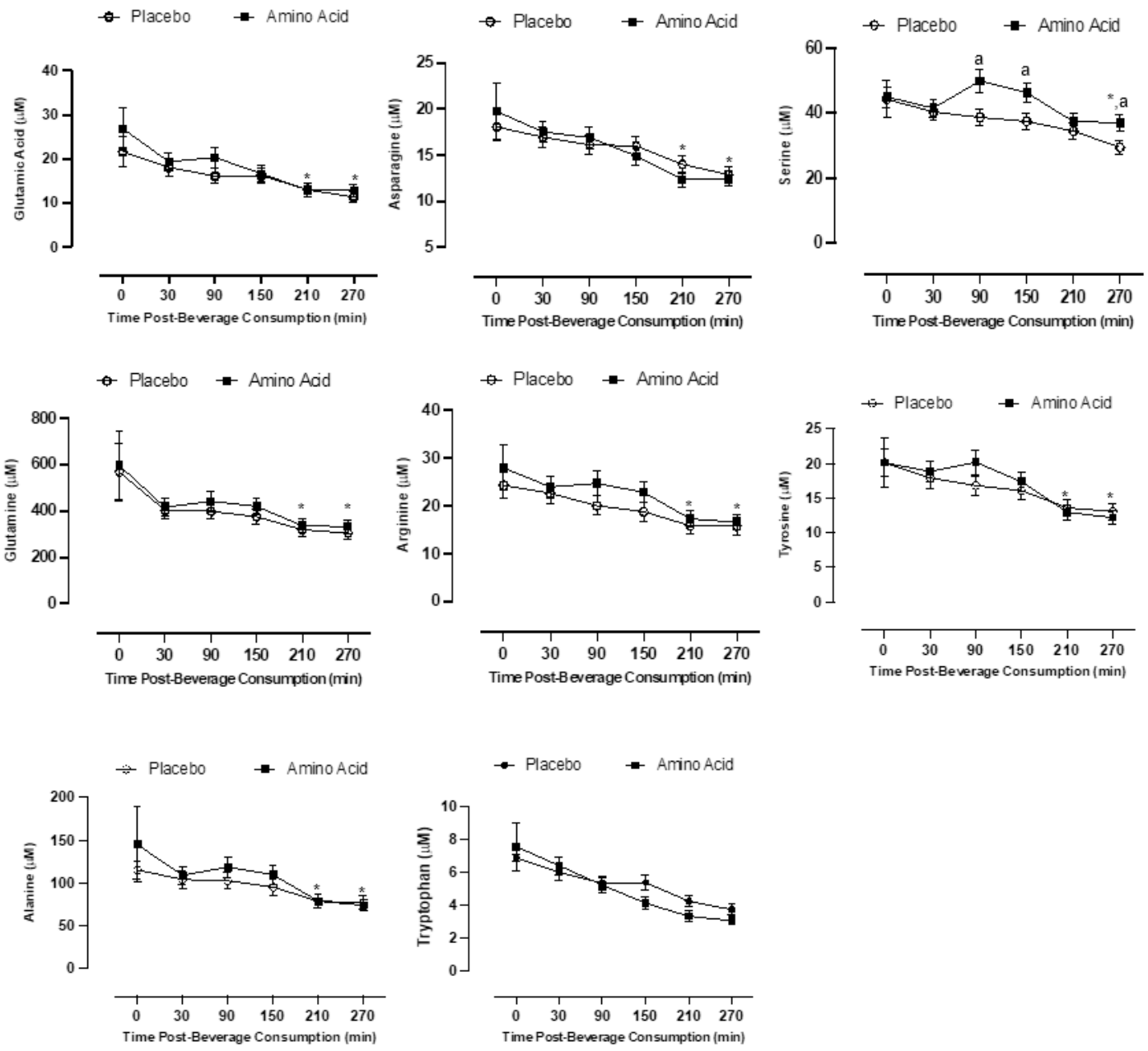

Figure 2

Line graphs for the amino acids not included in the study beverage for Study 1. Data presented as Achilles peritendinous amino acid concentrations across time during the placebo and amino acid conditions. Data expressed as mean±standard error. No interactions were noted. Multiple comparisons were performed on significant main effects. *p<0.05 versus 0-minutes independent of amino acid consumption. [a]p£0.05, Placebo vs. Amino Acid at noted time point.

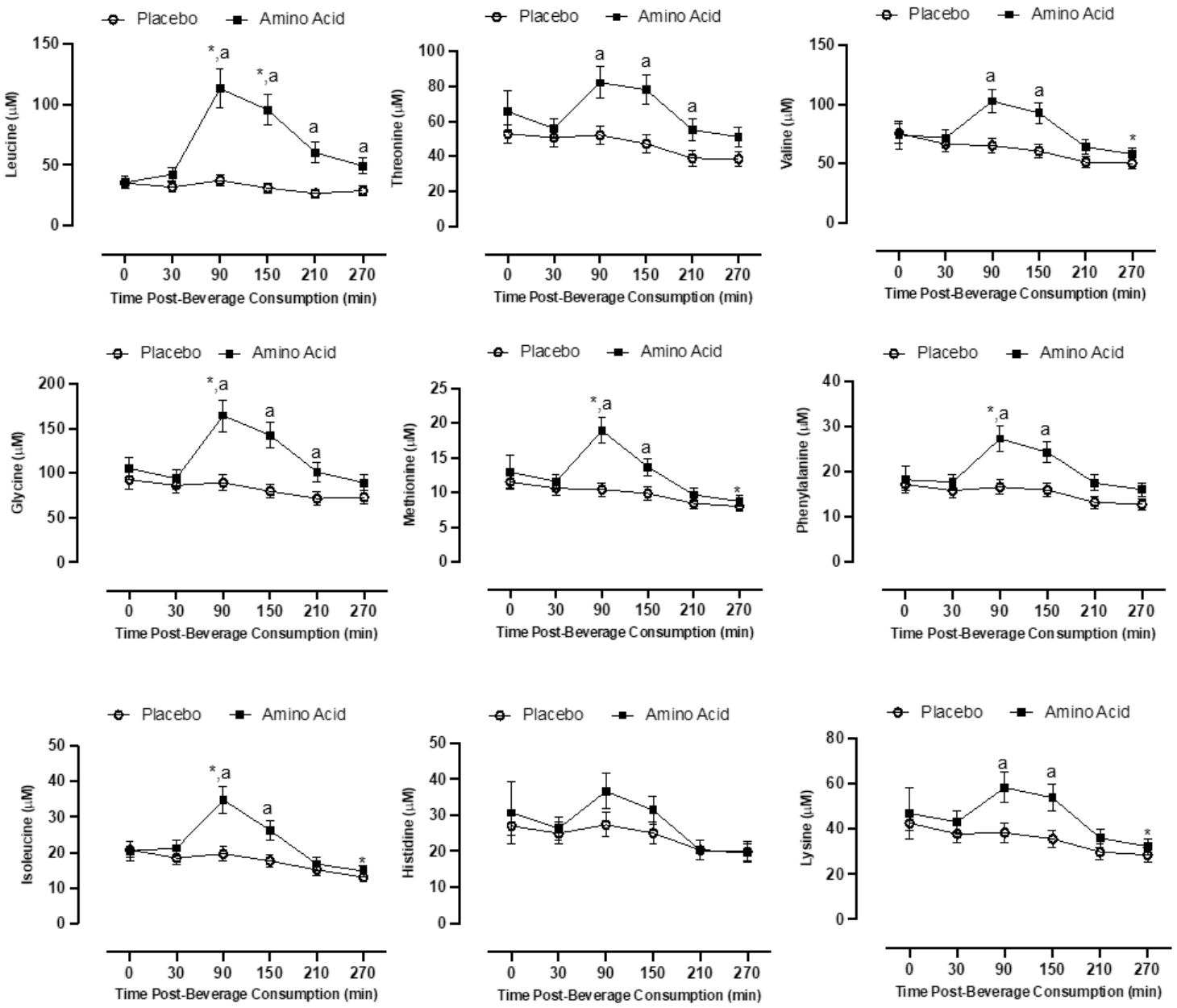

Figure 3

Line graphs of the amino acids included in the essential amino acid bolus for Study 1. Data presented as Achilles peritendinous amino acid concentrations across time during the placebo and amino acid conditions. Data expressed as mean±standard error. [a]p£0.05, Placebo vs. Amino Acid at noted time point. *p<0.05 versus 0-minutes independent of amino acid consumption.

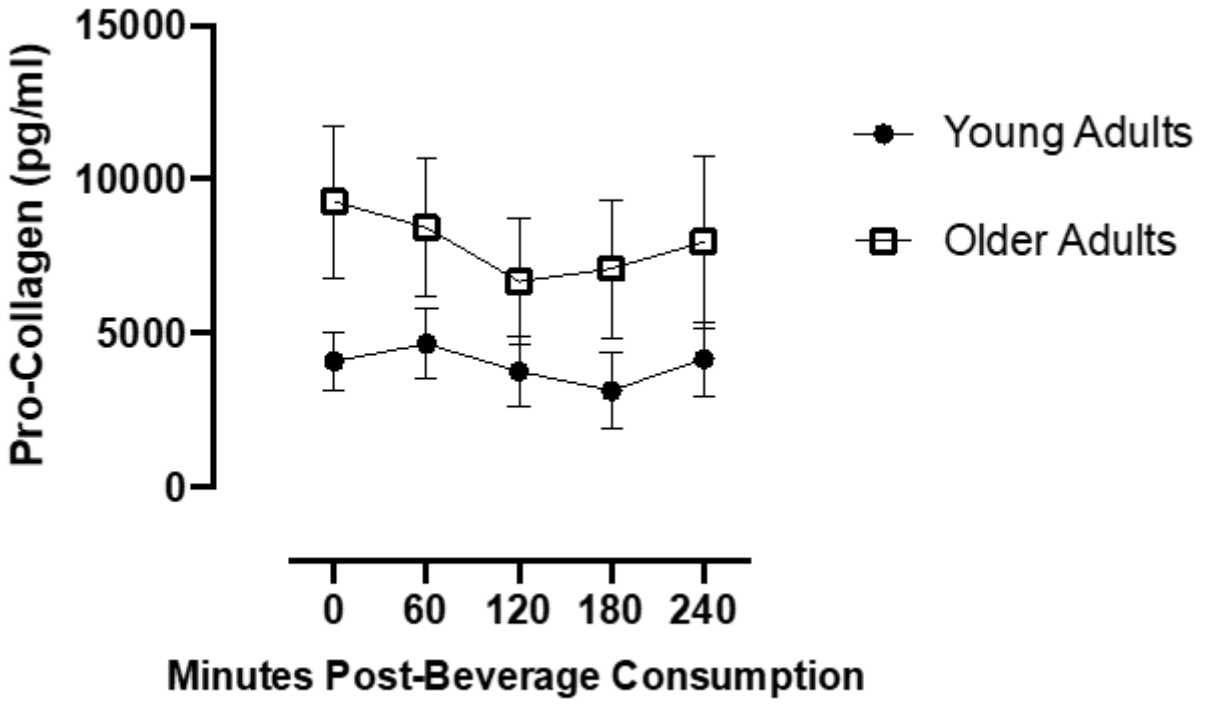

Figure 4

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

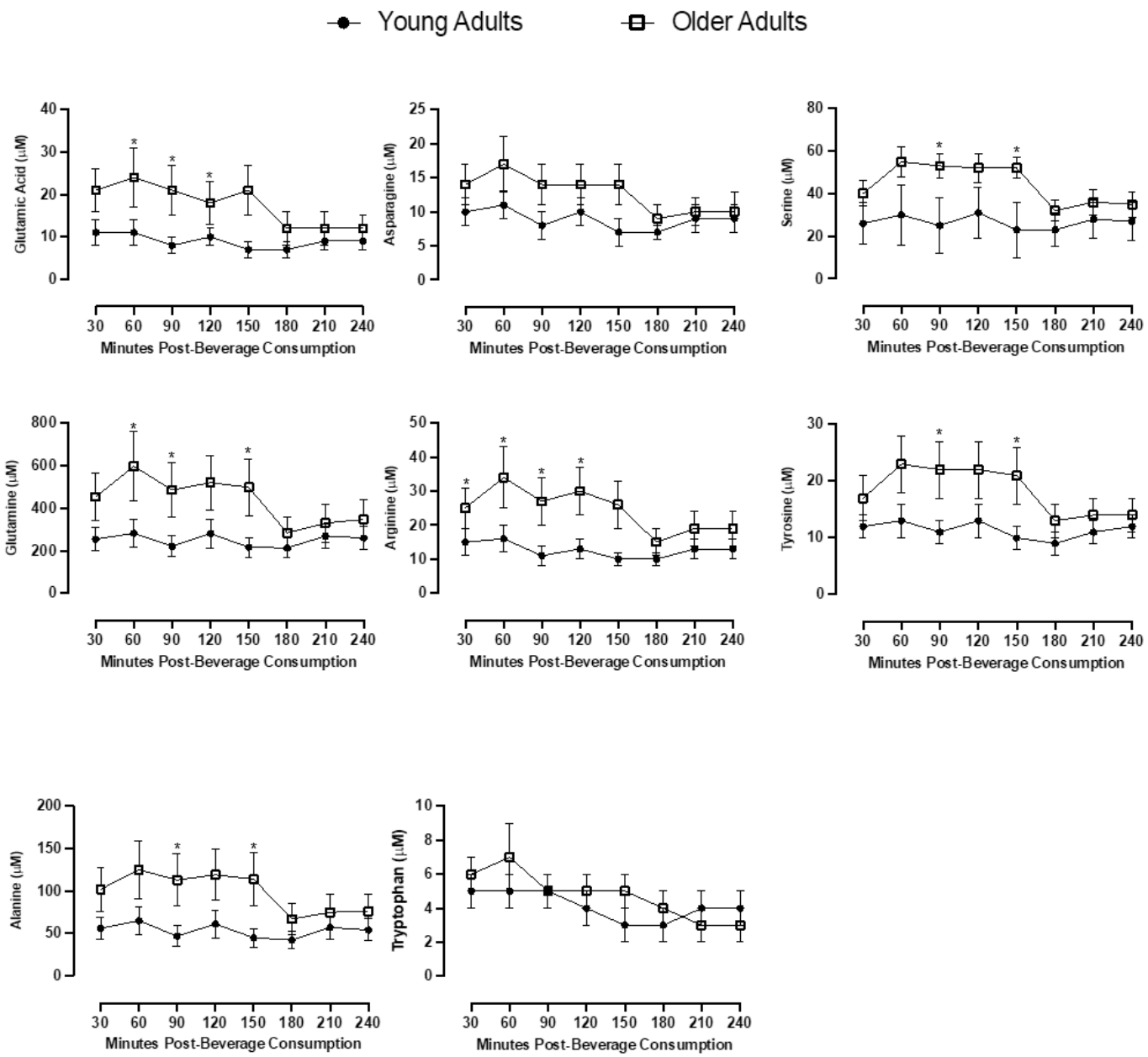

Figure 5

Line graphs for the amino acids not included in the study beverage for Study 2. Data presented as Achilles peritendinous amino acid concentrations across time during the placebo and amino acid conditions. Data expressed as mean±standard error. No interactions were noted. Multiple comparisons were performed on significant main effects. *p<0.05 versus Young vs. Old at specified time point

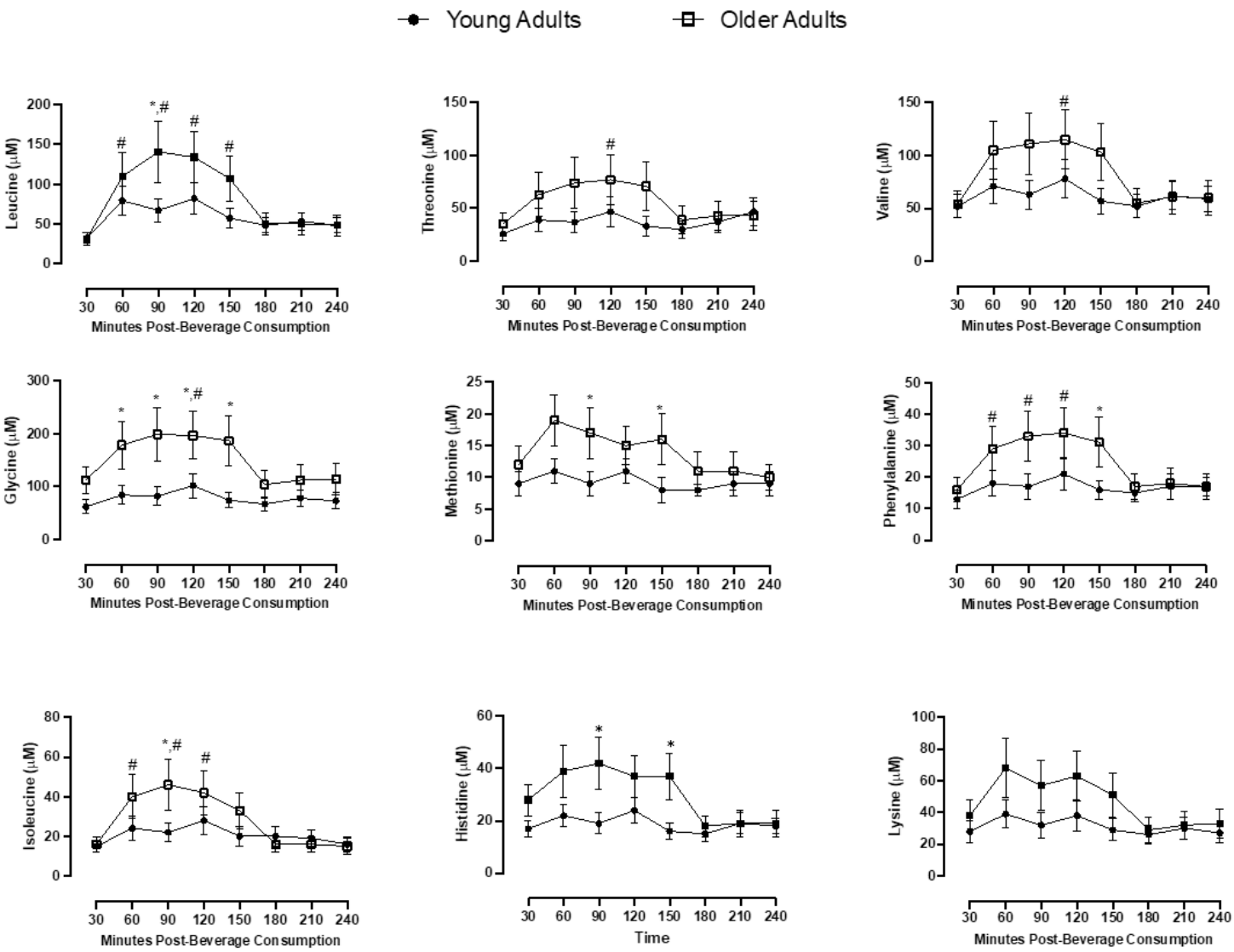

Figure 6

Line graphs of Achilles peritendinous amino acid concentrations over time for Study 2. Data expressed as mean±standard error. No interactions were noted. Main effect p values for time and age are presented above each figure insert. Multiple comparisons were performed on significant main effects. *p<0.05 versus Young vs. Old at specified time point; #p<0.05 versus 30 minutes.