

# Metabolomic impacts of branched-chain amino acid supplementation during endurance exercise: a crossover study

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## Research Article

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# Abstract

## Background

Serotonin synthesis in the brain requires a steady supply of tryptophan. Branched chain amino acids (BCAA) and tryptophan are transported across the blood-brain barrier by the amino acid transporter LAT1. BCAA supplementation is predicted to decrease serotonin biosynthesis through LAT1 competition and reduce central fatigue during exercise. Despite a strong theoretical basis for BCAA to attenuate serotonin production and fatigue during exercise, a number of human clinical trials have failed to demonstrate these benefits. To shed light on this discrepancy, we measured the impact of BCAA supplementation on serotonin and associated metabolites during exercise.

## Methods

A cohort of endurance runners (n=10) participated in a randomized, placebo-controlled, crossover trial to determine impact of BCAA supplementation during a 60-minute run at 65% of  $VO_2$  max. Metabolomic analysis targeted for serotonin and untargeted analysis for biomarkers of BCAA supplementation using LCMS were performed on serum samples collected immediately before and after exercise.

## Results

Serum BCAA levels were greater in the supplement group compared to placebo ( $p < 0.05$ ). Serum serotonin was lower immediately after BCAA supplementation and before exercise ( $p < 0.05$ ) but not after exercise. L-ornithine increased during exercise with BCAA treatment compared to placebo. Ratings of perceived exertion were no different in BCAA and placebo groups.

## Conclusions

BCAA supplementation led to a rapid decrease in serum serotonin concentration relative to placebo, which may be indicative of a central nervous system (CNS) mediated process. After exercise with BCAA supplementation, endurance athletes did not show lower serum serotonin concentration, but did present an almost three-fold increase in L-ornithine which has metabolic connections to cortisol and central fatigue.

**Trial Registration:** ClinicalTrials.gov NCT04969536, retrospectively registered 20 July 2021, <https://clinicaltrials.gov/ct2/show/NCT04969536>

## Introduction

Endurance exercise is an intricate nexus of metabolic networks with links to every aspect of physiology, including those involving the CNS. Serotonin, a neurotransmitter specifically associated with lethargy, sleep, and drowsiness in humans (Meeusen, 2006) exhibits increased synthesis during exercise resulting in higher blood concentration (Newsholme 1996, Steinberg 1998). Exercise induced blood serotonin

elevation has been postulated to intensify central fatigue through a loss of cognitive drive and reduction of muscle motor unit recruitment during physical activity (Newsholme 1996, Newsholme 2006). While there are likely many physiological components that impact central fatigue (blood glucose levels, free fatty acids, etc.), serotonin is assumed to be a major contributor after prolonged bouts of endurance exercise (Blomstrand 2006). This has inspired interventions to limit serotonin build up in blood such as supplementation of BCAA (AbuMoh'd 2020).

The rationale for BCAA supplementation to attenuate exercise-induced central fatigue is based on competitive inhibition of tryptophan uptake in the brain. BCAA supplementation during exercise in rats decreased the synthesis of serotonin by providing competitive inhibition to the transport of the serotonin precursor, tryptophan, across the blood-brain barrier (Choi 2013). Tryptophan crosses the blood-brain barrier via the large neutral amino acid transporter 1 (LAT1) along with BCAA including leucine, isoleucine and valine (Verrey 2003). In the brain, tryptophan is converted to serotonin via the enzyme TRP hydroxylase after which it crosses the blood brain barrier through the 5-hydroxytryptophan transporter to circulating blood (Nakatani 2008). Increasing BCAA serum concentrations through oral supplementation saturates the LAT1 transporter reducing tryptophan transport to the brain which limits serotonin synthesis. Therefore, BCAA supplementation is expected to decreased serotonin concentrations in circulating blood.

Although the mechanism by which serotonin synthesis is decreased by BCAA is well documented, the metabolic consequences of fluctuations in serotonin and other neurotransmitters in central fatigue are less clear. A physiological process as complex as central fatigue likely involves neurological mediators such as norepinephrine, epinephrine, and dopamine, in addition to serotonin (Meeusen 2006). The concept of central fatigue is predicated on the idea that the concentration of key neurological metabolites is influenced by exercise and leads to a decrease in motor unit recruitment (Newsholme 1987). To elucidate a more comprehensive impact of BCAA supplementation on neurological metabolites, this study determined the effects of BCAA supplementation on serum serotonin and global metabolic outcomes during endurance exercise using an untargeted metabolomics analysis.

## **Methods**

### **Participants**

Males and females aged 18-45 years who participated in at least five hours of cardiovascular activity (running, biking, swimming, etc.) per week were recruited for this study. Exclusion criteria included the presence of any underlying injury as well as the prescription of selective serotonin reuptake inhibitor (SSRI) medications. SSRIs inhibit the reuptake of serotonin within the synaptic cleft, resulting in increased levels of serotonin in the brain (Sangkuhl 2009). Safety and Ethics approval was granted by the Montana State University Review Board (Project No. DF081319). All participants gave written informed consent prior to initiation of study activities.

### **Research Design**

This study utilized a randomized, placebo-controlled, double-blind crossover design comparing supplementation of BCAA versus placebo beverages consumed immediately before and halfway through a 60-minute run at 65% of maximal aerobic capacity. To isolate the impact of BCAA on serotonin and metabolism during exercise, blood samples were collected immediately before and after exercise. A targeted metabolomic analysis was designed for BCAA and serotonin to verify supplementation induced increases in BCAA serum concentration, as well as for serotonin to assess the impacts of BCAAs. An untargeted metabolomic analysis was performed to identify global metabolic impacts of BCAA supplementation on metabolism during endurance exercise.

## **Maximal Aerobic Capacity**

A graded exercise test was used to measure maximal oxygen consumption ( $VO_2$  max) using a motorized treadmill. Each participant was fitted with an electronic heart rate (HR) monitor, a noseclip and mouthpiece connected to a one-way Rudolph valve and breathing tube to deliver expired air to a metabolic cart (TrueOne 2400, ParvoMedics, Sandy, Utah). Participants self-selected a speed between 5 and 7.5 miles per hour (MPH) and began running at a 0% incline. Each minute, the treadmill incline was increased by 1.5% while speed remained constant until the participant voluntarily terminated the test due to exhaustion. Each participant's  $VO_2$  max was defined as the highest 30 second average value collected during the test. Heart rate at 65% of  $VO_2$  max was then determined and used for the experimental trials.

## **Experimental procedure**

Participants were asked to return to the lab no earlier than 72 hours after the completion of the  $VO_2$  max test to complete the first of two trials in the experimental procedure. In the 48 hours prior to each trial, participants were asked to record what they were eating, drinking and if they took any medications, and they were asked to match pre-testing conditions from trial 1 to trial 2. All participants were asked to refrain from consuming alcohol within the 48 hours prior to the trials. With the exception of the experimental beverage, each of the two trials were identical in procedure and were separated by at least 72 hours. Testing was performed at the same time of day for each participant and all testing was completed between 4:00 and 8:00 p.m.

Participants were provided with a randomly assigned beverage containing either BCAA or a placebo solution. Participants consumed the experimental beverage three minutes prior to their running trial. Each participant was then fitted with a HR monitor and blood was drawn three minutes after ingestion. The blood draw was immediately followed by a warm-up on the treadmill at a self-selected speed for two to five minutes. Each participant then started their 60-minute running trial at 0% incline at 65% of their established  $VO_2$  max. Halfway into the 60-minute trial, each participant ingested another serving of the assigned placebo or BCAA beverage. Heart rate and the rate of perceived exertion (RPE) were collected every 10 minutes during the 60-minute trial. At the conclusion of the 60 minutes, participants cooled down for two minutes before exiting the treadmill and completing the post-exercise blood draw. Blood samples were allowed to clot for 15 minutes followed by centrifugation at 1200 RPM for 15 minutes at

4°C. The serum supernatant was then collected in clean vials and immediately stored at -80°C until liquid chromatography mass spectrometry (LCMS) analysis. Treadmill speed during the first trial were recorded and replicated during the second trial.

### **Experimental beverages**

Each participant was provided a BCAA supplement solution or a placebo solution in a double-blind, randomized crossover design. The BCAA solution was 8 oz of water mixed with approximately 8 grams of a standard BCAA supplement in powder form. Each 8-gram dose contained 2.5 grams of leucine, 1.25 grams of isoleucine and 1.25 grams of valine. The presence of BCAA was confirmed by LCMS. The placebo solution was 8 oz of water mixed with approximately 2.0 mL of a sucralose-based drink mix. Both the BCAA solution and the placebo solution were similar in color and in taste. Participants were allowed to drink additional, plain water during the 60-minute running trial if desired.

### **Metabolite extraction**

Serum samples were thawed and 20µL of serum was removed and placed in a clean vial. Protein precipitation was completed with the addition of 80µL of cold acetone followed by agitation on a vortex machine and two hours in a -80°C freezer. Serum was then centrifuged at 20,000g for 10 minutes at -4°C. The metabolite rich supernatant was collected and concentrated using negative pressure to dryness (ConcentratorPlus, Eppendorf, Hamburg, Germany). Samples were then stored at -80°C for no more than 24 hours until ready for LCMS analysis. Directly before LCMS analysis, metabolite samples were reconstituted with 40µL of methanol:water (50:50) and placed in a clean mass spectrometry vial.

### **LCMS conditions**

LCMS analysis was performed on an Agilent 6538 Q-TOF MS (Agilent Technologies, Santa Clara, CA) coupled to an Agilent 1290 UHPLC (Agilent Technologies, Santa Clara, CA) using a 1.8µm, 2.1mm X 150mm Waters HSST-3 UPLC column (Waters Corp., Milford, MA). Electrospray ionization was in positive mode. LC mobile phases were water (A), and acetonitrile (B), both with 0.1% formic acid. Flow was kept constant at 300µL per minute. The mobile phase gradient began with 95% A and finished with 5% A after seven minutes before returning to 95% at eight minutes and continuing to the end of the ten-minute run time. Column compartment temperature was kept constant at 30°C. MSMS analysis was completed using identical conditions with pooled extracted serum samples.

### **Data analysis**

Serotonin standards (Thermo Fisher Scientific, Waltham, MA) were analyzed under the described LCMS conditions and the retention time and m/z value were determined. Concentrations of 0.001µM, 0.01µM, 0.05µM, 0.1µM, 0.5µM, 1µM and 5µM were analyzed to generate a standard concentration curve and to determine the limit of detection. Peaks for serotonin from participant serum samples were then integrated and the concentration was calculated from the standard curve using MassHunter (Agilent Technologies, Santa Clara, CA). For the untargeted data analysis, the raw data files were converted to .mzML and .mgf

files using MSConvert and then mined using mzMine (Chambers 2012, Katajamaa 2006). Blank samples were also created using the same metabolite procedure without serum and were analyzed under identical LCMS condition concurrently. The resulting sample blank data was also converted and mined with the sample data and were used to remove machine background and mobile phase contributions to the data. Cleaned datasets and MSMS data were then statistically analyzed using MetaboAnalyst and Sirius software, respectively (Chong 2019, Dührkop 2015, Dührkop 2019). The BCAA supplement was also examined using LCMS and the results were analyzed to confirm the presence of BCAA and additives including flavoring and sweetening agents.

## **Secondary study to assess immediate impact of BCAA consumption on serotonin**

The timing of blood samples immediately before and after exercise was intended to isolate the impact of BCAA on serotonin and metabolism during exercise. Supplements were consumed three minutes prior to the pre-exercise blood sample. It was anticipated that three minutes was too short for BCAA absorption to impact tryptophan uptake and serotonin release into the blood. However, lower serotonin concentrations were measured in the pre-exercise blood samples in the BCAA compared to placebo condition. To determine if there was an impact of the experimental drink on blood serotonin concentration three minutes after ingestion, ten healthy males and females aged 23 to 28 years were selected for participation in a validation study to determine the impact of the BCAA supplement drink on serotonin. Potential participants were screened for the absence of underlying pathologies and the presence of an active lifestyle. Several of the participants were endurance athletes fitting the original criteria although some were not. Blood was drawn from each participant after which they ingested an experimental beverage containing either BCAA or the placebo solution. Three minutes after consuming the beverage, a second blood draw was performed. Serum was collected from the blood samples as previously described and stored at -80°C until LCMS analysis. All samples were extracted and analyzed concurrently within 24 hours of metabolite extraction. LCMS and data analysis methods were identical to those from the original cohort.

## **Statistical Analysis**

Serotonin concentrations were normalized and center scaled using the caret R package. After normalization, a nested analysis of variance (ANOVA) was completed with serotonin concentration as the dependent variable and treatment and time as independent variables. The significant results of the nested ANOVA analysis comparing treatment by time led to a post-hoc analysis of the groups and t-tests were performed between treatment and temporal groupings. A second statistical analysis, including normalization, a nested ANOVA and paired t-tests, was performed with the second cohort as with the first cohort.

# **Results**

## **Participants**

Ten endurance trained runners (5 women and 5 men) between age 22-44 years completed the randomized crossover trial. Maximal oxygen consumption ranged from 43.3 to 57.7 ml·kg·min<sup>-1</sup> in women and from 58.1 to 77.4 ml·kg·min<sup>-1</sup> in men.

### **Heart rate and perceived exertion after consumption of BCAA**

To determine the effects of BCAA supplementation in endurance runners, we collected physiological and mental metrics during a prolonged workout. Data consisted of MPH treadmill speed, heart rate HR and RPE as well as targeted and untargeted LCMS profiles from serum. Initial analysis centered on HR, MPH, and RPE to determine if performance and/or fatigue were influenced by BCAA supplementation. Starting treadmill speeds were matched for both treatments to make exercise bouts as similar as possible between BCAA and placebo conditions. Data was collected for all three metrics every 10 minutes during the 60-minute running trial (**Figures 1A-C**). ANOVA analyses for HR, MPH treadmill speed and RPE were performed to ascertain differences between our experimental groups. This analysis indicated that there were no significant differences between conditions at comparable times.

### **Serotonin concentration**

To determine if serum serotonin concentration or metabolites differentiated our groups, serum samples were analyzed via LCMS. Total ion chromatograms (TICs) from the LCMS data indicated similar and consistent metabolomic profiles (**Figure 2A**). After validation of our LCMS data, serotonin concentrations were determined using a standard curve and extracting the area under the peak corresponding to serotonin for each sample (**Figure 2B**). Following the determination of the serotonin concentration, statistical comparisons between the BCAA and placebo trials were performed. We began by comparing the pre- and post-workout concentrations for both treatment groups using t-tests and although some slight differences were observed, the analysis did not provide any statistically significant relationships  $p > 0.05$  (**Table 1**). However, a comparison of both treatments pre- and post-exercise using a nested ANOVA revealed significant differences between the groups at  $p = 0.05$ .

Following the results of the nested ANOVA, paired t-tests which reduce the variation between our participants and focus on differences in individual participant variation across the two treatments were explored and significant relationships were determined. We began by comparing the pre- and post-workout concentrations for the placebo treatment group which indicated no statistical difference between the placebo condition pre- and post-exercise (**Table 1**). However, serotonin concentrations exhibited significant differences using the same paired t-test when comparing the pre- and post-exercise BCAA condition (**Table 1**). Additionally, a paired t-test of the change in serotonin concentration between the BCAA and placebo condition was performed and found to be significant ( $p < 0.05$ ) (**Figure 3B**). A closer examination of the pre- and post-workout serotonin concentrations revealed an interesting trend. Seven of the ten participants had a lower starting serotonin concentration after ingestion of the BCAA beverage. This translated into the pre-workout serotonin concentrations being lower in the BCAA condition relative

to placebo ( $p < 0.05$ ). Post-exercise serotonin concentrations in the BCAA condition were not significantly different ( $p > 0.05$ ) from those of the post-exercise placebo condition.

To confirm if the pre-exercise serotonin decrease was caused by consumption of the BCAA supplement, a second cohort was tested for serum serotonin concentration immediately before and three minutes after BCAA supplementation. Paired t-tests indicated no difference between the BCAA and placebo groups initial serotonin concentrations or between the initial and post concentration in the placebo group (**Table 2**). However, there was a significant difference ( $p < 0.05$ ) between the initial and post concentration in the BCAA supplemented group (**Table 2, Figure 3C**). The analysis of the second cohort confirmed the initial finding that BCAA supplementation significantly lowers serum serotonin concentrations within three minutes.

### Untargeted analysis

Our unexpected findings with respect to serotonin concentration challenged us to investigate metabolic changes using an untargeted LCMS approach. Using HILIC chromatography, we were able to track 653 unique features in serum across all participants. Using a partial least-squares discriminating analysis (PLSDA), pre-workout BCAA and placebo groups clustered tightly together while the post-workout groups showed more variation and separation (**Figure 4A**). Post-workout placebo and BCAA conditions showed separation from the pre-workout groups via component two, while post-workout placebo and BCAA indicated partitioning via component 1. This is indicative of some common metabolomic changes between the pre- and post-workout time points, but also shows differences between the post-workout BCAA and placebo conditions.

To investigate the post-exercise changes in greater detail, a differential analysis of individual metabolite concentrations between the BCAA and placebo post-exercise conditions was conducted. This revealed 14 metabolites that had concentration change of at least 2-fold and a significant difference between groups using a paired t-test ( $p < 0.05$ ). Statistically selected metabolites included the BCAA valine and leucine as well as L-ornithine. To better visualize specific metabolite contributions to the differences observed, an in-depth examination was conducted using a heatmap of the top discriminating metabolites. The heatmap showed a cluster of upregulated features in the BCAA post-workout group relative to the placebo post-workout group (**Figure 4C**). The experimental groups clustered almost perfectly based on the 10 most discriminating features although two participants who received the BCAA supplement grouped with the placebo clustering. Upregulated metabolites in the BCAA post-workout group include the BCAA valine and leucine as well as L-ornithine. The flavoring and sweetening agents (dicyclohexyl sulfide and acesulfamo) listed on the label for the BCAA mix were also part of the group of upregulated metabolites in the BCAA post workout samples.

An analysis predicated on a receiver-operating characteristic (ROC) curve was undertaken to see if any of the metabolites differing between the post-workout conditions had potential biomarker value. This examination yielded several promising biomarkers associated with BCAA supplementation. The metabolite with the highest area under the curve (AUC) score was confirmed using an authentic standard

as L-ornithine (**Figure 5A**). L-ornithine had an AUC of 0.94, an excellent score for a biomarker and increased by 2.9-fold in the post-workout BCAA group compared to placebo (paired t-test  $p= 0.0012$ ) (**Figure 5B**). Paired t-tests between the pre-consumption groups indicated that there was no difference between the BCAA and placebo L-ornithine concentration ( $p= 0.34$ ) (**Figure 5B**). The second highest AUC (0.9) was for leucine, which could be predicted as leucine was in the BCAA supplementation. Leucine had a fold change increase of 1.5 in the BCAA group.

## Discussion

BCAA supplementation in endurance runners has metabolic consequences that could lead to decreases in fatigue and increases in athletic performance. To investigate this idea, we analyzed serum of endurance runners using LCMS to determine serotonin concentration and generate metabolic profiles pre- and post-exercise. Our findings indicate that BCAA supplementation almost immediately decreased serotonin concentration in circulating blood, however, this difference was erased after 60 minutes of exercise. A deep, untargeted exploration of the metabolic profiles of the endurance runners led to the identification of a strong biomarker for BCAA supplementation. The biomarker, L-ornithine, increased dramatically with BCAA supplementation. RPE and HR were recorded throughout the experiment, without being significantly different between the BCAA and placebo groups.

Analysis of BCAA supplementation in our cohort of endurance runners provided a wealth of information, and while some of the findings were expected, others were not. The most unanticipated result was that BCAA supplementation decreased circulating blood serotonin concentrations within three minutes of ingestion. This outcome was retested in a second cohort and the rapid decrease in serotonin was validated. While the rapid decrease in serum serotonin after consumption of the BCAA drink is unlikely to be the result of reduced production in the brain (Burke 2012), other mechanisms for rapid modulation of this neurotransmitter are plausible. Activation of type III taste receptors in response to sour stimuli can trigger release of serotonin (Larson 2015). Differences between the two drinks may have been sufficient to induce this response, although the BCAA drink and placebo were carefully balanced to avoid this and neither group had an increase in serotonin. Alternatively, the relatively short time between ingestion and decrease in serotonin concentration may indicate CNS involvement.

A CNS-mediated response to oral exposure of macromolecules, including carbohydrates, has been demonstrated (Jeukendrup 2013). For example, a carbohydrate oral “swish” without ingestion increases blood glucose and enhances athletic performance (Carter 2004, Rollo 2008). This effect has been linked to CNS activation of metabolic pathways that occurs pre-ingestion (Chambers 2009). The consistent response to BCAA consumption in our two cohorts, one comprised of endurance runners and the second made up of healthy adults, would be expected if the effect was CNS mediated. To our knowledge, this is the first report that oral administration of BCAA can alter serum serotonin concentration. It remains to be determined if this is a direct or indirect effect from the activation of oral receptors.

Although our results demonstrated significant differences in serotonin concentration three minutes after supplementation, those differences were not present after 60 minutes of running at 65%  $\text{VO}_2$  max. We also failed to detect significant change in RPE or HR. This general pattern has been observed in at least one previous study in which lower serotonin levels were measured with BCAA supplementation pre-exercise but not post-exercise (Kim 2013). Further, there were no differences between conditions in perceived exertion during the run. A recent meta-analysis found that BCAA supplementation had mixed effects on central fatigue and that methods used to measure this varied from study to study (Hormoznejad 2019). Not only did the measures of central fatigue vary, but BCAA supplementation protocols varied widely from study to study, including timing of ingestion, dosing, whether or not there was a loading period, and type of exercise. A recent research study focused on the effect of BCAA on serotonin levels described variations based on a variety of factors including time of day, timing of ingestion, and differing dosages (AbuMod'd 2020). Therefore, our findings indicate that BCAA supplementation did not alter fatigue or performance under our specific exercise and supplementation conditions.

In addition to exploring the impact of BCAA on serotonin, we sought to elucidate the impact of BCAA supplementation on metabolism. Statistical analysis of the untargeted LCMS metabolomic data grouped by treatment post-workout, indicated L-ornithine as a potential biomarker for BCAA supplementation. L-ornithine had the highest AUC using a ROC analysis of the LCMS data and has a metabolic relationship with serotonin. L-ornithine is synthesized from arginine and glutamine and is a precursor for the production of polyamines like putrescine and spermine (Urdiales 2001). Biosynthesis of polyamines from L-ornithine begins with the enzyme ornithine decarboxylase (ODC), an inducible enzyme which decarboxylates L-ornithine. Serotonin activates ODC, increasing L-ornithine catabolism leading to increased production of polyamines (Kodama 1987, Mates 1989). The decreased serum levels of serotonin immediately after ingestion of the BCAA supplement could lead to a decrease in ODC activity and therefore higher levels of L-ornithine after exercise.

An alternative explanation for the increase in L-ornithine is the role it has in the urea cycle (Rodwell 2003). L-ornithine is a major component of the urea cycle and supplementation has been shown to reduce serum ammonia levels (Bai 2013). As BCAA availability increased with supplementation in our trial, oxidation of BCAA for energy production would be expected increase during running. Plasma levels of BCAA and L-ornithine have been shown to decrease during exercise in the absence of exogenous nutrient supplementation (Brodan 1976). Along with the increase in short term energy from oxidation of BCAA, an increase in nitrogen concentration in the blood and a corresponding need for disposal would be expected (Rennie 2006). Brodan *et. al* showed that a correlation between serum amino acid and L-ornithine concentrations reflected a positive relationship between amino acid concentrations and ureagenesis. BCAA and L-ornithine supplementation and nitrogen accumulation has also been specifically investigated (Mikulski 2015, Sugino 2008). In these cases, BCAA supplementation along with increases in L-ornithine led to decreased levels of ammonia. Ammonia levels have been implicated in central fatigue as ammonia

can cross the blood-brain barrier and disrupt neurotransmitter metabolism, leading to an increase in central fatigue (Nybo 2005).

With a rise in L-ornithine levels, associated catecholamine regulated pathways could be modulated (Figure 6). This modulation is largely due to the effect of L-ornithine on the hypothalamic-pituitary-adrenal (HPA) axis. L-ornithine supplementation increases HPA activity leading to an increase in systemic cortisol levels (Konishi 2015). Cortisol increases have been correlated with a decrease in stress and fatigue as well as improved mood (Papadopulous 2012).

Our analysis indicates that BCAA supplementation leading to variations in serotonin concentration may influence L-ornithine abundance such that HPA induced cortisol increases and ammonia decreases could reduce fatigue. However, ratings of perceived exertion did not differ between BCAA and placebo trials, suggesting that this potential impact was not detectable during 60 minutes of running at a moderate (65% of  $VO_2$  max) exercise intensity. L-ornithine supplementation and central fatigue intensity has previously been tested with mixed results. With a cohort of healthy people, Sugino et al. found that L-ornithine supplementation decreased fatigue while Mikulski et al. determined that L-ornithine had no effect on endurance athletes. Our cohort was also composed of endurance athletes and BCAA supplementation leading to L-ornithine increases in circulating blood also showed no effect on RPE. These differing results between endurance athletes and non-endurance athletes could be the result of a training adaption. Further research is needed to clarify the impact of elevated L-ornithine levels on central fatigue.

Although we had robust datasets that allowed for deep coverage of metabolic profiles and a secondary validation group, our study did have several limitations. Collection of a blood sample prior to consumption of the experimental beverages may have avoided uncertainty as to whether the lower serotonin pre-exercise in the BCAA condition was induced by the BCAA beverage. Another limitation was the composition of the validation group which was not homogenous when compared with the initial cohort. The validation group was comprised of active individuals who may or may not possess some of the metabolic adaptations found in the initial group of trained endurance runners. Regardless, the validation group did provide proof of concept that BCAA consumption was potentially responsible for the difference in serotonin measured prior to the exercise bout in the endurance runners. Another limitation of our study is that the endurance runners did not show significant differences in RPE during the running trial based on treatment with BCAA or placebo supplementation. One hour at 65% $VO_2$  max may not have provided enough exertion to generate fatigue such that our groups could achieve separation. Further research is recommended to determine whether downstream metabolites from BCAA supplementation impact central fatigue during endurance activities of sufficient intensity and or duration to increase RPE.

## Conclusion

In our cohort of subjects, a connection emerged between BCAA supplementation and a rapid decrease in serum serotonin. A possible explanation is a CNS mediated response to BCAA supplementation involving

sensory pathways in the mouth. The fluctuations in serotonin concentration seen in the BCAA group also strongly correlated with variations in the concentrations of L-ornithine. Further study of the relationship between BCAA supplementation and L-ornithine levels should be completed along with investigation into the metabolic associations between L-ornithine and central fatigue. Analysis of possible CNS specific pathways leading to BCAA induced serotonin decrease should be explored as well.

## **Abbreviations**

ANOVA: Analysis of variance

AUC: Area under the curve

BCAA: Branched-chain amino acid

CNS: Central nervous system

HPA: Hypothalamic-pituitary-adrenal

HR: Heart rate

LAT1: Large neutral amino acid transporter 1

LCMS: Liquid chromatography-mass spectrometry

MPH: Miles per hour

ODC: Ornithine decarboxylase

PLSDA: Partial least-squares discriminating analysis

ROC: Receiver-operating characteristic

RPE: Rate of perceived exertion

SSRI: Selective serotonin reuptake inhibitor

TIC: Total ion chromatogram

## **Declarations**

### **Ethics approval and consent to participate**

Safety and Ethics approval was granted by the Montana State University Review Board (Project No. DF081319). All participants gave written informed consent prior to initiation of study activities.

### **Consent for publication**

Not applicable

## **Availability of data and materials**

The datasets generated and/or analyzed during the current study are available in the Mendeley Data repository DOI: 10.17632/pftrs8wbc5.1 or are included in this published article and its supplementary information files.

## **Competing interests**

The authors declare no competing interests.

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## **Authors' contributions**

JP extracted metabolites from serum, analyzed samples using LCMS, interpreted LCMS results, completed the statistical analysis and wrote the manuscript. DF collected blood samples and RPE and heart rate data from the initial cohort. LF extracted metabolites from serum and assisted in the statistical analysis. HF extracted metabolites from serum, analyzed samples using LCMS analysis and collected blood samples from the second cohort. IR collected blood samples from the second cohort and assisted in manuscript preparation. BB conceived the study, interpreted LCMS data and assisted in manuscript preparation. MM conceived the study, collected blood samples for both cohorts, interpreted the results and assisted in manuscript preparation.

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## Tables

**Table 1. Serotonin concentration comparisons**

Treatment	Pre-exercise( $\mu\text{M}$ )	Post-exercise( $\mu\text{M}$ )	t-test p-value	Paired t-test p-value
Placebo	0.77 $\pm$ 0.16	0.71 $\pm$ 0.16	0.39	0.21
BCAA	0.65 $\pm$ 0.13	0.81 $\pm$ 0.12	0.069	0.0081

Error values are based on the standard deviation

**Table 2. Validation study paired t-tests**

Treatment	Pre-consumption( $\mu\text{M}$ )	Post-consumption( $\mu\text{M}$ )	p-value
Placebo	0.84 $\pm$ 0.17	0.87 $\pm$ 0.17	0.14
BCAA	0.87 $\pm$ 0.14	0.66 $\pm$ 0.11	0.00038

Error values are based on the standard deviation

## Figures

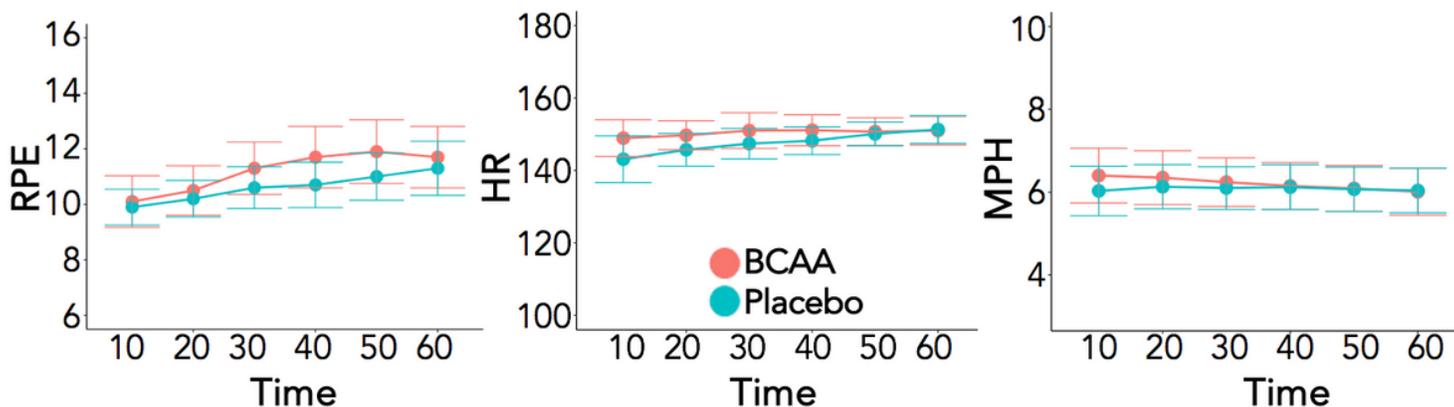


Figure 1

Metrics obtained from 60-minutes of treadmill activity at 65% VO<sub>2</sub> max. Data was collected and is shown in ten-minute increments. Red points and lines indicate participants who ingested the BCAA supplement while blue points and lines indicate participants who ingested the placebo drink. (A) RPE for participants. (B) HR for participants. (C) MPH pace selected by participants. Error bars are included for each group at each time point and represent the standard deviation.

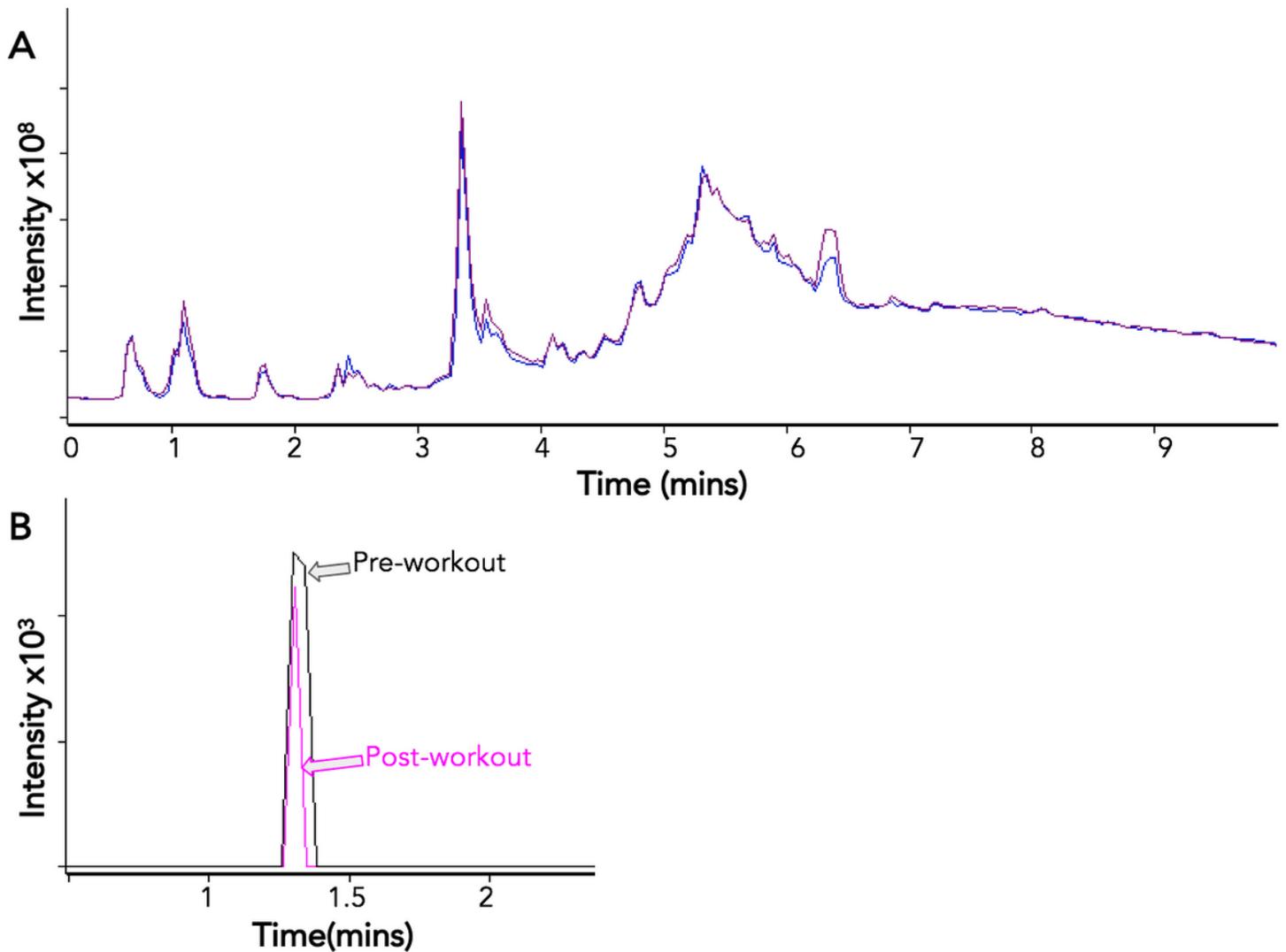
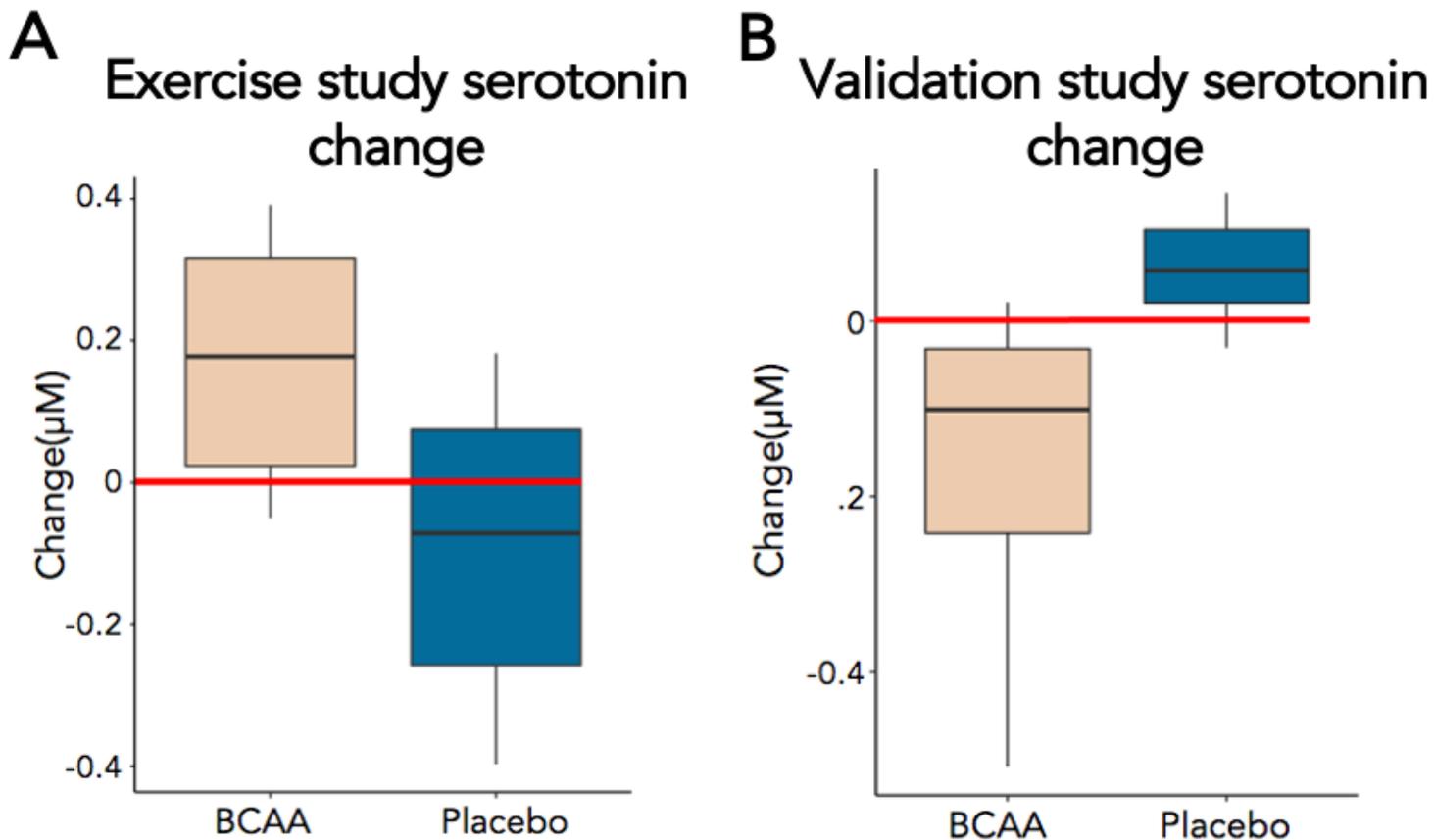


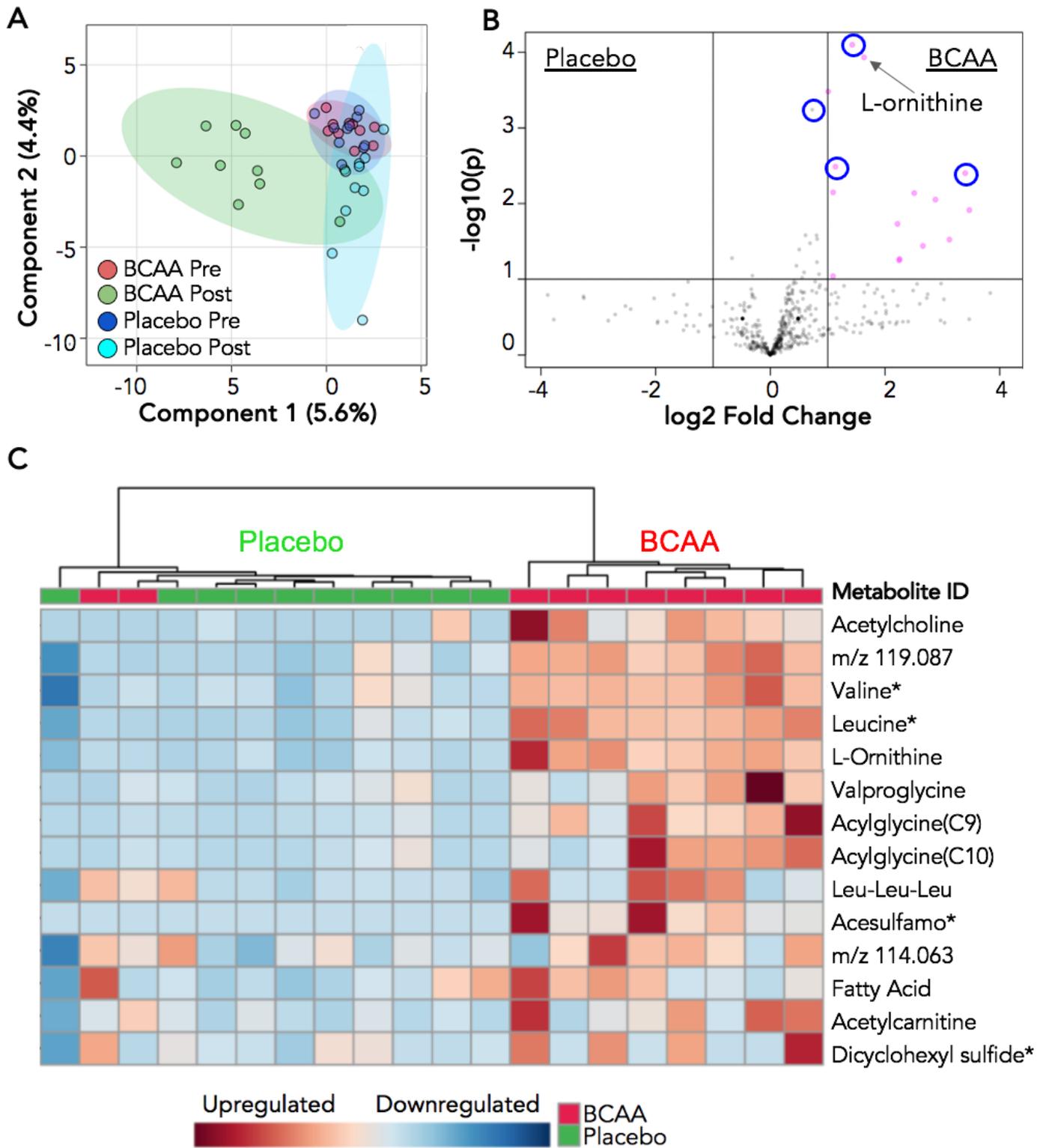
Figure 2

Serum chromatography. (A) Total ion chromatograms of serum samples for an individual before and after the 60-minute workout. This chromatogram is from a subject who received a BCAA supplementation prior to the workout. (B) Extracted ion chromatogram for the m/z value of 177.102 corresponding to serotonin in the same individual as in panel A. Note the decrease in serotonin post workout relative to the starting concentration.



**Figure 3**

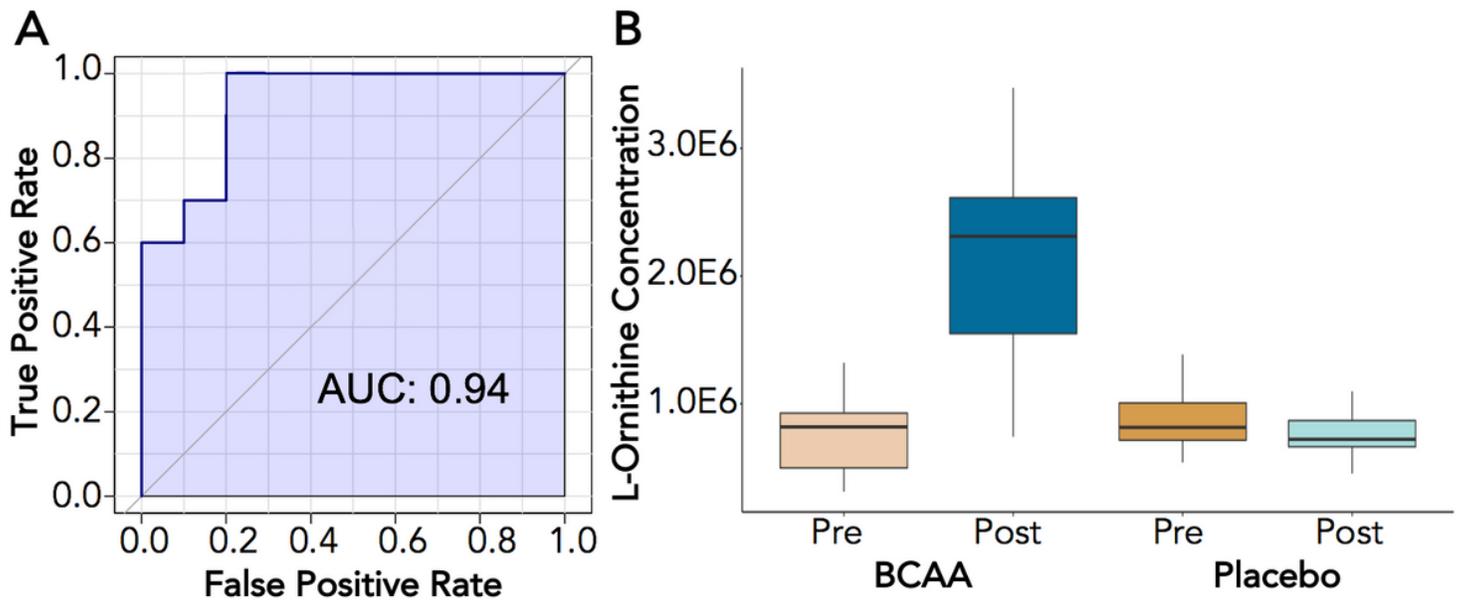
Serum chromatography. (A) Total ion chromatograms of serum samples for an individual before and after the 60-minute workout. This chromatogram is from a subject who received a BCAA supplementation prior to the workout. (B) Extracted ion chromatogram for the  $m/z$  value of 177.102 corresponding to serotonin in the same individual as in panel A. Note the decrease in serotonin post workout relative to the starting concentration.



**Figure 4**

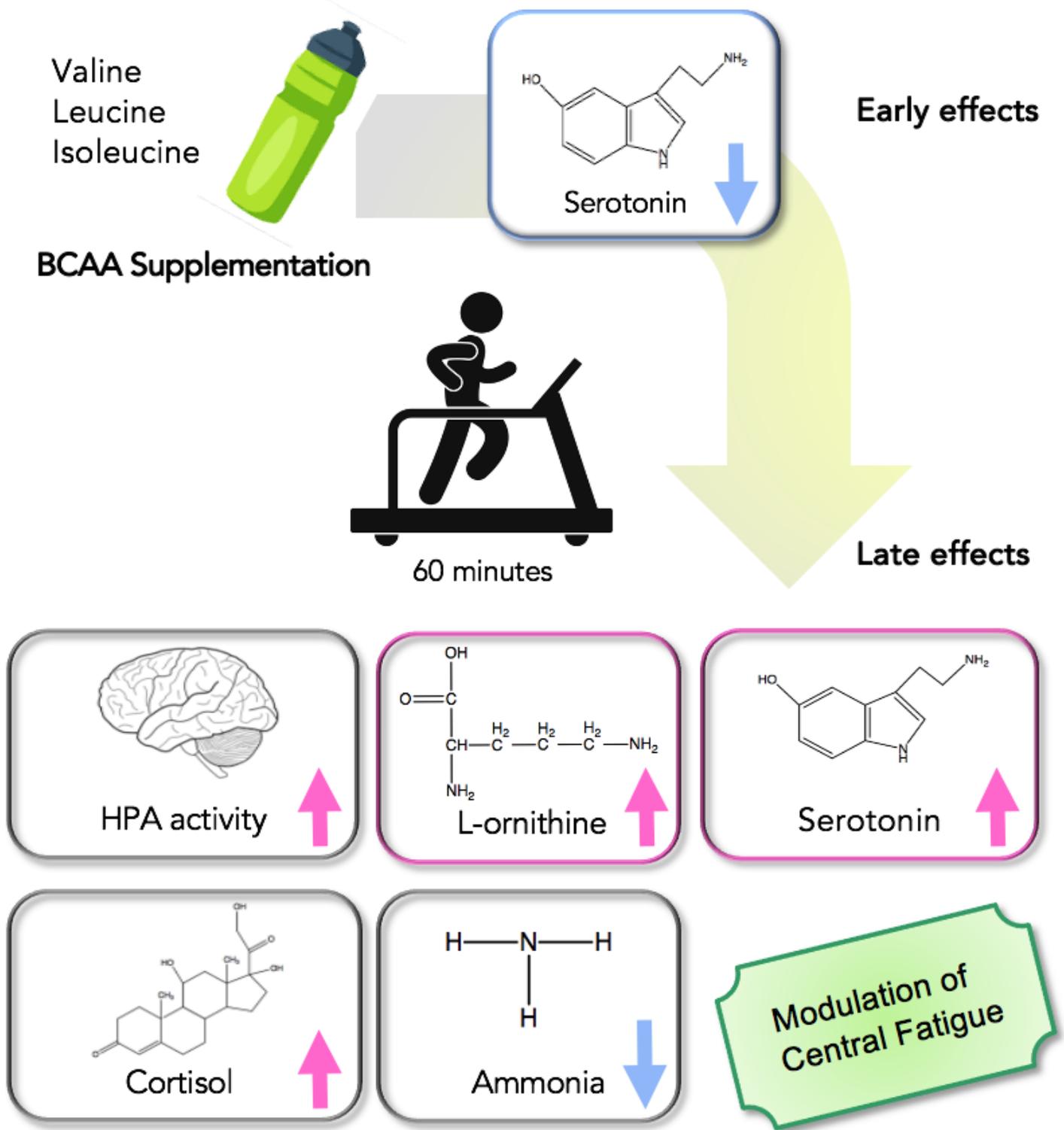
Untargeted metabolomic profile analysis. (A) PLS-DA of BCAA and placebo groups pre- and post-workout. All four groups are shown with 95% confidence intervals. (B) T-test and fold change analysis of the BCAA and placebo post-workout groups. 14 metabolites are significant to a p-value of  $<0.05$  with a fold change of  $>2$  including components of the supplement marked with blue circles. Circled dots correspond to leucine/isoleucine, valine, acesulfamo and dicyclohexyl sulfide. The metabolite L-ornithine is also

statistically significant and is labeled. (C) Heatmap of the top 10 discriminating features selected by t-tests for the BCAA and placebo group post-workout. Asterisks indicate components of the BCAA supplement.



**Figure 5**

Biomarker analysis of untargeted data. AUC and box and whisker plot for the metabolite with the highest AUC score, L-ornithine. (A) AUC determined using a ROC analysis testing the potential of L-ornithine as a biomarker. (B) Box and whisker plot for relative L-ornithine concentration in the BCAA and placebo groups pre-(initial) and post-workout (final). Relative concentration is represented as area under the peak corresponding to L-ornithine.



**Figure 6**

Metabolic impacts of BCAA supplementation. BCAA ingestion leads to a rapid decrease in serum serotonin (early effects) in endurance athletes and untrained participants. decrease pre-exercise and an increase in serotonin during exercise. Following a 60 min. treadmill run there were distinct metabolic changes with BCAA supplementation not observed with placebo. L-ornithine can be used as a biomarker for BCAA supplementation and along with serotonin increased with BCAA supplementation relative to the

placebo group. L-ornithine has established associations with serotonin, HPA activity, cortisol production, as well as decreased ammonia in the blood. Grey boxes show predictions based on our data. The combined effects are likely to impact perception of central fatigue under certain conditions and provides a foundation for future studies.