Effects of Exercise and Dietary EGCG and β-Alanine on Skeletal Muscle in Aged Mice

<table>
<thead>
<tr>
<th>Journal:</th>
<th>Applied Physiology, Nutrition, and Metabolism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manuscript ID</td>
<td>apnm-2015-0372.R1</td>
</tr>
<tr>
<td>Manuscript Type:</td>
<td>Article</td>
</tr>
<tr>
<td>Date Submitted by the Author:</td>
<td>08-Sep-2015</td>
</tr>
<tr>
<td>Complete List of Authors:</td>
<td>Pence, Brandt; University of Illinois at Urbana, Kinesiology and Community Health</td>
</tr>
<tr>
<td></td>
<td>Gibbons, Trisha; University of Illinois at Urbana, Animal Sciences</td>
</tr>
<tr>
<td></td>
<td>Bhattacharya, Tushar; University of Illinois at Urbana, Psychology</td>
</tr>
<tr>
<td></td>
<td>Mach, Houston; University of Illinois at Urbana, Psychology</td>
</tr>
<tr>
<td></td>
<td>Ossyra, Jessica; University of Illinois at Urbana, Psychology</td>
</tr>
<tr>
<td></td>
<td>Petr, Geraldine; University of Illinois at Urbana, Animal Sciences</td>
</tr>
<tr>
<td></td>
<td>Martin, Stephen; University of Illinois at Urbana, Kinesiology and Community Health</td>
</tr>
<tr>
<td></td>
<td>Wang, Lin; University of Illinois at Urbana, Chemistry</td>
</tr>
<tr>
<td></td>
<td>Rubakhin, Stanislav; University of Illinois at Urbana, Chemistry</td>
</tr>
<tr>
<td></td>
<td>Sweedler, Jonathan; University of Illinois at Urbana, Chemistry</td>
</tr>
<tr>
<td></td>
<td>McCusker, Robert; University of Illinois at Urbana, Animal Sciences</td>
</tr>
<tr>
<td></td>
<td>Kelley, Keith; University of Illinois at Urbana, Animal Sciences</td>
</tr>
<tr>
<td></td>
<td>Rhodes, Justin; University of Illinois at Urbana, Psychology</td>
</tr>
<tr>
<td></td>
<td>Johnson, Rodney; University of Illinois at Urbana, Animal Sciences</td>
</tr>
<tr>
<td></td>
<td>Woods, Jeffrey A.; University of Illinois at Urbana-Champaign,</td>
</tr>
<tr>
<td>Keyword:</td>
<td>aging &lt; aging, exercise nutrition &lt; exercise, muscle function &lt; muscle,</td>
</tr>
<tr>
<td></td>
<td>animal model(s) &lt; research models, ergogenic aids &lt; athlete performance</td>
</tr>
</tbody>
</table>
Effects of Exercise and Dietary EGCG and β-Alanine on Skeletal Muscle in Aged Mice

Brandt D. Pence\textsuperscript{1,2}, Trisha E. Gibbons\textsuperscript{2,3}, Tushar K. Bhattacharya\textsuperscript{4,5}, Houston Mach\textsuperscript{4,5}, Jessica M. Ossyra\textsuperscript{4,5}, Geraldine Petr\textsuperscript{2,3}, Stephen A. Martin\textsuperscript{1,2}, Lin Wang\textsuperscript{5,6}, Stanislav S. Rubakhin\textsuperscript{5,6}, Jonathan V. Sweedler\textsuperscript{5,6}, Robert H. McCusker\textsuperscript{2,7,8}, Keith W. Kelley\textsuperscript{2,7,8}, Justin S. Rhodes\textsuperscript{4,5}, Rodney W. Johnson\textsuperscript{2,3,7}, Jeffrey A. Woods\textsuperscript{1,2,3,8}

\textsuperscript{1}Department of Kinesiology & Community Health, \textsuperscript{2}Integrative Immunology & Behavior Program, \textsuperscript{3}Division of Nutritional Sciences, \textsuperscript{4}Department of Psychology, \textsuperscript{5}Beckman Institute, \textsuperscript{6}Department of Chemistry, \textsuperscript{7}Department of Animal Sciences, \textsuperscript{8}Department of Pathology, University of Illinois, Urbana, IL 61801.

Corresponding Author:

Jeffrey A. Woods, Ph.D.
Professor
906 S. Goodwin Ave.
Urbana, IL 61801
Ph: (217) 244-8815
Fax: (217) 244-7322
woods1@illinois.edu

Running Title:
Wheel running and EGCG/Beta-Ala in Aged Mice
Aging leads to sarcopenia and loss of physical function. We examined whether voluntary wheel running when combined with dietary supplementation with (-)-epigallocatechin-3-gallate (EGCG) and β-alanine (β-ALA) could improve muscle function and alter gene expression in the gastrocnemius of aged mice. 17-month-old Balb/cByJ mice were given access to a running wheel or remained sedentary for 41 days while receiving either AIN-93M (standard feed) or AIN-93M containing 1.5 mg·kg⁻¹ EGCG and 3.43 mg·kg⁻¹ β-ALA. Mice underwent tests over 11 days from day 29 to 39 of the study period, including muscle function testing (grip strength, treadmill exhaustive fatigue, rotarod). Following a rest day, mice were euthanized and gastrocnemius were collected for analysis of gene expression by quantitative PCR. Voluntary wheel running (VWR) improved rotarod and treadmill exhaustive fatigue performance and maintained grip strength in aged mice, while dietary intervention had no effect. VWR increased gastrocnemius expression of several genes, including those encoding interleukin (IL)-6 (p=0.001), superoxide dismutase (SOD)1 (p=0.046), peroxisome proliferator-activated receptor gamma-coactivator-1α (Ppargc1a, p=0.013), forkhead box O3 (Foxo3, p=0.005), and brain-derived neurotrophic factor (Bdnf, p=0.008), while reducing gastrocnemius levels of lipid peroxidation marker 4-Hydroxynonenal (4-HNE, p=0.019). Dietary intervention alone increased gastrocnemius expression of genes encoding Ppargc1a (p=0.033), the de-acetylase sirtuin (Sirt)1 (p=0.039), insulin-like growth factor (Igf1, p=0.003), and macrophage marker CD11b (Itgam, p=0.016). Exercise and a diet containing β-ALA and EGCG differentially regulated gene expression in the gastrocnemius of aged mice, while VWR but not dietary intervention improved muscle function. We found no synergistic effects between dietary intervention and VWR.

**KEY WORDS**

Voluntary wheel running, gene expression, muscle function, animal model, supplementation, aging
INTRODUCTION

Physiological aging includes a number of morphological and functional changes to many organs, including skeletal muscle. Total muscle mass decreases in humans starting at approximately age 30 (Short et al. 2003), and this change is associated with deficits in muscle strength. Mechanistically, a number of studies have noted an age-related decrease in the rate of protein synthesis in skeletal muscle with resistance exercise (Yarasheski et al. 2002), although the rate of protein synthesis in young versus old is thought to be unchanged at rest (Fry and Rasmussen 2011, Volpi et al. 2001). Additionally, skeletal muscle mitochondria become increasingly dysfunctional with advancing age (Rooyackers et al. 1996). Age-related loss of muscle mass and function (sarcopenia) is associated with impairments in mobility and locomotion (Morley et al. 2011). Whether these impairments are the consequence or the cause of sarcopenia is a matter of debate. Age-associated mitochondrial dysfunction in skeletal muscle (Rooyackers et al. 1996) is associated with an age-related increase in oxidative stress (Capel et al. 2005), and these findings are thought to be causally linked. Indeed, overexpression of the antioxidant enzyme catalase has been shown to prevent age-associated metabolic impairments in mice (Lee et al. 2010), and mitochondrial DNA deletions have been shown to co-localize with oxidative damage in sarcopenic muscle fibers isolated from aged rats (Wanagat et al. 2001). However, the exact link between oxidative stress and mitochondrial dysfunction in aging remains under investigation.

β-alanine (β-ALA) is the non-proteinogenic β form of the amino acid alanine and is a precursor for carnosine, a dipeptide of β-ALA and L-histidine. In muscle, carnosine acts as a pH buffer and functions as an antioxidant (Boldyrev et al. 1993), which suggests a potential role for carnosine in reversing or limiting the effects of oxidative stress and cellular senescence. Carnosine additionally increases Ca\(^{2+}\) sensitivity and aids Ca\(^{2+}\) release from the sarcoplasmic reticulum in muscle (Dutka et al. 2012), thus directly enhancing muscle contractility and performance. Thus, supplementation with β-ALA has been widely examined for its effects on muscle carnosine content and physical function.
Supplementation with β-ALA or carnosine is a strategy commonly used by athletes, as β-ALA-induced increases in muscle carnosine have been shown to decrease fatigue and improve muscular function (Derave et al. 2010). Recently, β-ALA has been investigated for its effects on skeletal muscle function in the elderly. β-ALA supplementation for 12 weeks increased muscle carnosine content and improved time-to-exhaustion in several tests of physical capacity in 60-80 year olds (del Favero et al. 2012). Furthermore, there was a positive correlation between improved physical capacity and increases in skeletal muscle carnosine content. As muscle carnosine and methylated analogue anserine contents naturally decrease with age (Stuerenburg and Kunze 1999), including in rodents (Derave et al. 2008, Johnson and Hammer 1992), β-ALA supplementation is an attractive strategy to minimize or reverse age-associated deficits in muscle functional capacity. However, recent data indicate that β-ALA supplementation has also been demonstrated to have no effect on skeletal muscle function after a 6 week interval training program (Cochran et al. 2015), no effect on Wingate performance in women (Kresta et al. 2014), and no effect on measures of muscle function in young mice after a 4-week voluntary wheel running intervention (Bhattacharya et al. 2015). Thus, the value of β-ALA supplementation for improving muscle function remains somewhat controversial.

In addition to β-ALA, we used the abundant biologically active catechin, (-)-epigallocatechin-3-gallate (EGCG) due to its well-established anti-oxidant action. EGCG is the major polyphenolic catechin constituent of green tea and has been widely investigated for its antioxidant properties, as this is the major health benefit attributed to green tea (Yang 1999). EGCG acts as a scavenger of a number of free radicals and has been shown to exert anti-inflammatory effects (Aktas et al. 2004). Thus, there is considerable interest in the potential effects of EGCG on ameliorating a variety of oxidative and pro-inflammatory conditions, including normal aging. In contrast with β-ALA, there is limited information available on the role of EGCG in muscle, especially from an aging standpoint. In aged rats, EGCG increased skeletal muscle activity of several antioxidant enzymes (including superoxide dismutase,
catalase, glutathione peroxidase, and glutathione reductase) and increased skeletal muscle levels of non-enzymatic antioxidants such as ascorbate and α-tocopherol (Senthil Kumaran et al. 2008). Several studies have reported that green tea extract or EGCG supplementation reduces muscle loss and/or enhances muscle recovery during or after hind-limb suspension-induced unloading in aged rats (Alway et al. 2014, Alway et al. 2015). However, the effect of EGCG on skeletal muscle dysfunction resulting from normal physiological aging has yet to be adequately examined.

Exercise training has long been utilized as an intervention to improve muscle function in aged subjects. Although resistance exercise training is commonly used in this population, aerobic exercise training also has benefits in aged skeletal muscle. In the aged, aerobic exercise training increases skeletal muscle mitochondrial number and activity, enhances muscle protein synthesis, improves insulin sensitivity, and increases oxidative capacity (Short et al. 2003). As these exercise-induced improvements are related to decreased oxidative stress and increased exercise capacity in aging (Leeuwenburgh and Heinecke 2001), aerobic exercise is a powerful modality by which to improve physical function in the elderly. An exercise-induced increase in mitochondrial number (mitochondrial biogenesis) is thought to be of importance in many of the above effects of exercise. Mitochondrial biogenesis increases due to enhanced activity of peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC-1α, encoded by Ppargc1a), which coordinates the transcriptional activity of several factors related to mitochondrial biogenesis, is increased by exercise, and decreases in skeletal muscle with aging (Puigserver and Spiegelman 2003). Thus, it is thought that aerobic exercise mediates its positive effects on skeletal muscle through increasing activity of PGC-1α. However, training adaptations are still reduced in aged individuals compared to young (Lanza et al. 2008), thus adjunct strategies (e.g. nutritional supplementation) which increase the training response in aged individuals are of interest.

Given the potential benefits of EGCG and β-ALA to muscle function, the purpose of our study was to examine the effects of a dietary intervention supplemented with β-ALA and EGCG in
combination of a voluntary exercise intervention on skeletal muscle function and body composition in aged mice. We hypothesized that, in addition to beneficial independent effects, dietary supplementation with β-ALA and EGCG would either additively or synergistically improve muscle function, body composition, markers of mitochondrial biogenesis and gene expression of antioxidant enzymes in response to voluntary exercise in aged mice.

**METHODS**

**Animals**

Male Balb/cByJ retired breeder mice (8-10 months old) were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained until 17 months of age. Mice were then randomized to treatments as described below and began the feeding portion of the study. Mice were maintained in an AAALAC-accredited animal care facility and allowed *ad libitum* access to water and food. The mice were individually housed on a 12 hour reversed light-dark cycle (dark period 10:00 AM to 10:00 PM) at a constant temperature of 24 °C. All procedures used were approved by the Institutional Animal Care and Use Committee at the University of Illinois Urbana-Champaign.

**Study Design**

The study design is diagrammed in **Supplementary Figure S1**. Mice were switched to either a control (Ctrl, N=28) diet or an experimental (EGCG+β-ALA, N=30) diet containing β-ALA and EGCG for 41 days. Body weight and food and water intake (by disappearance) were recorded twice weekly. Mice were randomized to have access to a running wheel (volunteer running wheel: VWR, Ctrl N=15, EGCG+β-ALA N=15) or maintained in standard housing (sedentary: SED, Ctrl N=13, EGCG+β-ALA N=15) during this period. Thus, mice were randomly assigned to one of 4 treatment groups (Ctrl-SED, Ctrl-VWR, EGCG+β-ALA-SED, EGCG+β-ALA-VWR).
Beginning on day 29, mice underwent a battery of behavioral and functional tests. These tests included 7 days of Morris Water Maze (days 29-35) and 2 days of Contextual Fear Conditioning (days 38-39) testing, the results of which are reported elsewhere (Gibbons et al. 2014). On experimental day 36, mice underwent functional muscle testing, including grip strength, rotarod, and treadmill test to exhaustion. Grip strength and rotarod only were performed on day 37. Following the cessation of behavioral testing on day 39, mice were given a rest day on day 40 and were euthanized for tissue collection on day 41 prior to the onset of the dark cycle. Mice were euthanized by CO$_2$ asphyxiation. Muscle samples were excised from both hind limbs and the gastrocnemius complex was separated from the soleus, frozen on dry ice and stored at -80 °C for analysis. The gastrocnemius was chosen for its importance in running movements and for its mixed fiber type composition, which we suggest gives a more representative picture of skeletal muscle adaptations to exercise than would the use of a predominantly oxidative (soleus) or glycolytic (quadriceps) muscle group (Jackaman et al. 2007).

**Diets**

Ctrl and EGCG+β-ALA diets were purchased from Research Diets, Inc. (New Brunswick, NJ) and were based on the purified AIN-93M mature rodent diet. Mice receiving Ctrl were given ad libitum access to the AIN-93M diet throughout the study. EGCG+β-ALA was manufactured by Research Diets by mixing 1.7 mg Teavigo (90% EGCG, DSM Nutritional Products, Basel, Switzerland) and 3.43 mg β-ALA (NutraBio, Middlesex, NJ) per g of AIN-93M diet, which was then pelleted to match the consistency and appearance of Ctrl. Dietary constituents have been previously published (Gibbons et al. 2014). If the EGCG+β-ALA had yielded positive effects, the individual dietary components would have been examined to determine their contribution. Since this was not the case, we did not test the individual diets as they were unlikely to have positive effects where the combined diet did not, as was shown in our previous study in young mice (Bhattacharya et al. 2015).
The diets were independently assayed by Covance, Inc. (Princeton, NJ), and the experimental diet was found to contain 1.49 mg of EGCG per gram of diet (99.3% of expected) and 3.34 mg of β-ALA per gram of diet (97.4% of expected). The control diet AIN-93M diet was found to be free of both EGCG and β-ALA. Mice consumed an average of 182 mg·kg$^{-1}$·day$^{-1}$ and 417 mg·kg$^{-1}$·day$^{-1}$ of EGCG and β-ALA respectively, based on average daily diet disappearance and mean body weight. Target EGCG dosage was determined based on previous studies demonstrating beneficial cognitive effects of EGCG in mice (Li et al. 2009). As studies examining β-ALA supplementation and its effects on cognition or muscle function in mice are lacking, we calculated β-ALA dosage using the 2.4 g·day$^{-1}$ dose that has been shown to improve physical work capacity in humans (Stout et al. 2008). For a 70 kg human, this equates to 34 mg·kg$^{-1}$·day$^{-1}$ and was adjusted for mice using the FDA-recommended conversion of 12.3 (Us 2005). Thus, the target dose for β-ALA in this study was 418 mg·kg$^{-1}$·day$^{-1}$.

**Wheel Running**

VWR mice were given constant access to a running wheel (Respironics, Bend, OR), while SED mice were housed in standard shoebox cages. Both VWR and SED remained in their respective conditions throughout the entirety of the study period. Wheel turns were monitored by computerized software (VitalView, Respironics, Bend, OR) in 1 hour increments, and resulting data was analyzed as wheel distance (km) covered in a 24 hour period. VWR and SED mice were subjected to similar handling throughout the experiment.

**Body Composition**

Body composition was analyzed prior to and on the final day of the pre-testing portion of the study (day 0 and day 28, respectively) by small animal magnetic resonance imaging (EchoMRI, Houston, TX). Total fat mass and lean mass were assessed for each mouse at each time point by this method. Data were expressed as % fat mass or % lean mass by dividing fat mass or lean mass by total body mass at each time point.
Muscle Function Testing

Forelimb grip strength was assessed using a commercially available force gauge (Columbus Instruments, Columbus, OH) and was assessed in 5 separate trials per day over 2 consecutive days both at the beginning of the study (day 1 and day 2) and during the muscle function testing period (day 36 and 37) by the same investigator. Grip strength was quantified as the average of the highest recorded grip force on each testing day at both time points and as the percent change in maximal grip strength from the beginning to the end of the study in each group. Grip strength is expressed as peak force in Newtons (N).

An exhaustive treadmill test was performed to assess fatigability (day 36). Mice ran on an inclined (5%), motorized treadmill (Jog-a-Dog, Ottawa Lake, MI) using an incremental running velocity protocol as previously described (Martin et al. 2013). Fatigue was defined by an inability to continue running despite gentle prodding for at least 10 seconds. The test ended at 120 minutes if mice had not reached fatigue. No electric shock was used. Data were expressed as time-to-exhaustion (min).

An automated rotarod unit (Accuscan, Columbus, OH) with a 30 mm diameter rotating dowel and a 63 cm fall height was utilized. Mice were placed on the dowel, and rotation started at 0 rpm with constant acceleration to a maximum of 60 rpm. Timing was controlled by photobeam, and timing for each mouse was stopped automatically by the system when the falling mouse broke the plane of the photobeam. Mice underwent 4 consecutive trials per day at the initiation of the dietary/exercise intervention (days 1 and 2) and after intervention (days 37 & 38). Data were expressed as the average performance across all 8 trials at pre-intervention (pre) and the average performance across all 8 trials at post-intervention (post).

Gastrocnemius Gene Expression

Total RNA was isolated from frozen gastrocnemius samples by Trizol (Invitrogen, Carlsbad, CA) according to manufacturer’s instructions. RNA was reverse-transcribed to cDNA using a commercially
available high-capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, CA). The cDNA was stored at -20 °C until gene expression analysis.

Gene expression analysis was performed by Taqman Low Density Array (TLDA, Applied Biosystems, Carlsbad, CA) according to manufacturer’s instructions. A total of 1000 ng of cDNA was loaded per sample. Commercially-available, pre-validated primers (Applied Biosystems, Carlsbad, CA) were used for TLDA analysis (Supplementary Table S1). TLDA cards were run on a high-throughput real-time polymerase chain reaction system (7900HT, Applied Biosystems, Carlsbad, CA), and Ct values were determined using SDS 2.4 and RQ Manager 1.2.1 software packages (Applied Biosystems, Carlsbad, CA).

All samples were run in duplicate, and gene expression was expressed relative to the housekeeping gene (Gapdh) and expressed as fold change from the referent Ctrl-Sed group by the $2^{\Delta\Delta Ct}$ method.

4-Hydroxynonenal Assay

The level of 4-hydroxynonenal (4-HNE) was analyzed in gastrocnemius extracts by enzyme-linked immunosorbant assay (ELISA) according to manufacturer’s instructions (OxiSelect HNE Adduct ELISA Kit, Cell Biolabs, San Diego, CA). Gastrocnemius samples were homogenized in sterile phosphate-buffered saline (PBS) supplemented with 5 μl protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) and stored at -80°C until analysis. Total protein content in homogenized samples was quantified by DC Protein Assay (Bio-Rad, Hercules, CA), and samples were diluted in PBS to a concentration of 10 μg protein·ml$^{-1}$ for use in the ELISA kit. All samples assayed for 4-HNE were tested in duplicate. Color development was read at 450 nm on a spectrophotometric plate reader (BioTek, Winooski, VT).

Sample Preparation for Muscle Carnosine/β-Alanine Detection

All reagents used, except specifically mentioned, were obtained from Sigma-Aldrich (St. Louis, MO). 200 proof ethanol was from Decon Laboratories (King of Prussia, PA).

Muscle samples were pre-chilled with liquid nitrogen and pulverized using a tissue pulverizer (BioSpec Products, Bartlesville, OK). Analytes were extracted using a multistage extraction procedure.
During the first stage, 200 μl absolute ethanol containing 25 mg/L antioxidant butylated hydroxytoluene (BHT) was added into the tube containing the pulverized, frozen, and weighted tissue. Tissue samples were manually stirred/homogenized with 10-15 strokes of pestle, incubated for 15 min on ice, and centrifuged at 21000×g using an Eppendorf 5810R refrigerated benchtop centrifuge (Eppendorf, Hauppauge, NY) for 10 min at 4°C. Supernatant was collected and 100 μl water containing 25 mg/L BHT were added to the pellet. 15 min incubation was followed by 10 min centrifugation at 21000×g. Supernatant was collected and combined with the supernatant from the first extraction. The last step of analyte extraction from remaining pellet resembles the first step except that only 50 μl ethanol containing 25 mg/L BHT was added. All three supernatants were combined and analyzed by LC-MS within 12 hours. Unless otherwise indicated, each procedure was performed either on ice or at 4°C.

Quantitative measurements of analytes using Multiple Reaction Monitoring (MRM) assay

MRM assay was performed using atmospheric pressure ionization (API) ultrahigh performance liquid chromatography-triple quadrupole-mass spectrometry (UHPLC-TQ-MS) system from Bruker (Bruker Daltonics, Billerica, MA ) consisting of Advance™ UHPLC module and EVOQ Elite™ triple quadrupole-mass spectrometer. A Kinetex 2.6 μm HILIC 50 x 2.1 mm internal diameter column (Phenomenex, Torrance, CA) was used for LC analyte separation. Mobile phase A was composed of 50 mM ammonium acetate (pH 4) and acetonitrile (5/95 v/v). Mobile phase B was acetonitrile, water and 50 mM ammonium acetate (pH 4) at ratio of 50/40/10 v/v. Flow rate was set to 250 μL/min. The details of LC conditions were as follows (time in minutes/mobile phase A %/mobile phase B %): 0.00/100/0, 0.05/100/0, 1.10/5/95, 5.30/5/95, 5.40/100/0.

Atmospheric-pressure chemical ionization (APCI) source was used in MS/MS analysis. The analysis was performed using the positive ionization polarity. Monitored MRM transitions for beta-alanine were 90->72.2, 90->45.2, and 90->43.2. Transitions for carnosine were 227->110.1, 227->156.1, and 227->122. The spray current was 15 μA. Temperatures of cone and heated probe were set in
correspondence to 150 °C and 450 °C. Gas flows were 10 units on cone, 15 units on probe, and 60 units on nebulizer.

The stock solutions containing standards of interest – ß-ALA and carnosine – were prepared in ethanol. The stock solutions were diluted with described above mobile phase A creating a set of samples with concentrations of the analyte standards ranging from ppb to ppm.

Absolute concentrations of the analytes were determined using the standard addition approach to reduce the effects of sample matrix on the measured values without the use of stable isotope labeled standards. Briefly, the same volumes of various concentrations of standards were spiked into a set of aliquots of two muscle samples and measured along with one aliquot for each sample, which was spiked with only solvent A. 2 ppm, 5 ppm, 10 ppm, 20 ppm, 50 ppm of carnosine standard and 100 ppb, 200 ppb, 500 ppb, 1 ppm, 2 ppm ß-ALA standard were spiked into the aliquots. For each aliquot, two technical replicates were acquired.

**Data Analysis**

Body weight, body composition, and grip strength were analyzed by repeated measures (RM-) analysis of variance (ANOVA) in a $2 \times 2 \times 2$ design (time $\times$ diet $\times$ activity). Food and water disappearance as well as running distance were analyzed by factorial ANOVA using a $2 \times 2$ (diet $\times$ activity) design. Grip strength ratios and rotarod performance scores were analyzed by factorial ANOVA using a $2 \times 2$ (diet $\times$ activity) design. Treadmill time to fatigue was analyzed by $2 \times 2$ (diet $\times$ activity) ANOVA on rank-transformed data due to non-normality of the data and the failure of standard transformations to transform the data to normal.

MRM data were processed using Bruker MS software (Bruker MS Workstation 8.1 for EVOQ™) calculating and processing peak areas and plotting them on calibration curves with final statistics calculated in Excel. The concentrations of ß-ALA and carnosine in each sample were normalized to the sample’s wet weight.
Data for gene expression and 4-HNE in the gastrocnemius were analyzed by factorial ANOVA using a 2 × 2 (diet × activity) design. Mice displaying splenomegaly indicative of immune activation at tissue collection were excluded from these analyses (N=2, one each from Ctrl-Sed and EGCG+β-ALA-Sed). For all ANOVA analyses in this study, post-hoc mean separation was performed by Tukey’s HSD in the event of a significant main effect or interaction. As there were no significant interactions detected in our data, we have reported only post-hoc tests for main effects in the results section. All data analysis was performed using SPSS software v. 22 (IBM, Armonk, NY) with significance set at p≤0.05. All results are expressed as mean ± SEM.

RESULTS

Descriptive Data and Muscle Carnosine/β-ALA

Lean and fat mass and other descriptive data have been published previously (Gibbons et al. 2014) and are summarized here. There were no differences in body weights between groups prior to intervention. VWR reduced body weights from pre- to post-intervention ($F_{1,52}=4.691$, $p=0.035$, not shown). Post-hoc analysis revealed that pre-to-post body weights were decreased in both Ctrl-VWR and EGCG+β-ALA-VWR groups ($p<0.05$, not shown). Table 1 shows lean and fat mass pre- and post-intervention. For percentage body fat mass, there was a significant time × VWR interaction ($F_{1,52}=6.062$, $p=0.017$). Post-hoc analysis indicated a near-significant reduction in fat mass percentage from pre- to post-intervention in the Ctrl-VWR group ($p=0.057$). Percentage lean mass did not differ between groups at either time point. The dietary treatment increased muscle concentration of carnosine at sacrifice with no significant effect of exercise (Ctrl-Sed 2.45 ± 0.15 μg/mg, EGCG+β-ALA-Sed 2.85 ± 0.21 μg/mg, Ctrl-VWR 1.76 ± 0.32 μg/mg, EGCG+β-ALA-VWR 2.91 ± 0.15 μg/mg, diet main effect $F_{1,16}=12.498$, $p=0.003$, N=5/group). Gastroc β-ALA did not differ between groups.

Muscle Function Tests
There was a time × VWR ($F_{1,52}=7.360$, $p=0.009$) and a near-significant EGCG+ß-ALA × VWR ($F_{1,52}=3.753$, $p=0.058$) interaction for grip strength from pre- to post-intervention (Figure 1A). Post-hoc analysis revealed no significant differences when grip strength was expressed as absolute force. Expression of grip strength normalized to lean mass for each individual mouse at each time point did not alter interpretation of these data (not shown). When forelimb grip strength was analyzed on a relative basis by calculating the ratio of post-intervention to pre-intervention values (Figure 1B), there was an effect of VWR ($F_{1,52}=6.761$, $p=0.012$). VWR maintained maximal grip strength from pre- to post-intervention compared to their SED counterparts. EGCG+ß-ALA alone or in combination with VWR had no effect on grip strength from pre- to post-intervention. Pre-intervention absolute grip strength was not significantly different between groups (one-way ANOVA $F_{3,63}=0.612$, $p=0.610$, not shown).

In the treadmill test to exhaustion, there was a main effect of VWR ($F_{1,54}=12.932$, $p=0.001$) such that VWR mice ran longer than their SED counterparts (Figure 2A). EGCG+ß-ALA had no effect on performance on the treadmill test in either VWR or SED groups nor was there a synergy between EGCG+ß-ALA and VWR.

In the rotarod test, there was a main effect of VWR ($F_{1,52}=6.115$, $p=0.017$) and a trend toward an effect for EGCG+ß-ALA ($F_{1,52}=2.814$, $p=0.099$) such that VWR increased and EGCG+ß-ALA decreased average rotarod performance compared to their respective SED or Cntl counterpart groups (Figure 2B).

**Gastrocnemius Gene Expression**

VWR increased gene expression of *Il6*, the gene encoding interleukin (IL)-6 ($F_{1,49}=13.410$, $p=0.001$, Figure 3A) within the gastrocnemius muscle. There were no significant main effects or interactions for other cytokines (Figure 3A) including *Il1b* (IL-1β) or *Tnf* (tumor necrosis factor, TNFα).

Expression of *Il10* was negligible in muscles from all treatment groups (mean Ct > 35, not shown). Interestingly, EGCG+ß-ALA increased gene expression of *Itgam*, the gene encoding macrophage marker CD11b ($F_{1,49}=6.268$, $p=0.016$, Figure 3A), with no significant main effects or interactions in gene
expression of other macrophage markers (not shown), including \textit{Itgax} (CD11c), \textit{Mrc1} (CD206), or \textit{Retnla} (FIZZ1). Neither EGCG+ß-ALA nor VWR affected gene expression of chemokines for macrophages (\textit{Ccl2}, monocyte chemoattractant protein-1) or neutrophils (\textit{Cxcl1}, keratinocyte chemoattractant) in the gastrocnemius (data not shown).

For genes associated with oxidative stress (Figure 3B), there was a main effect of VWR for \textit{Sod1}, the gene encoding superoxide dismutase (SOD)-1 ($F_{1,49}=4.193$, $p=0.046$), as well as a near-significant main effect of VWR for \textit{Gpx1}, which encodes glutathione peroxidase (GPX)-1 ($F_{1,49}=3.750$, $p=0.059$), in which VWR increased expression of both of these genes. There was a trend for EGCG+ß-ALA to increase expression of \textit{Sod2} (SOD2), although this effect did not reach significance ($F_{1,49}=3.423$, $p=0.070$). Neither EGCG-ß-ALA nor VWR impacted gene expression of \textit{Nos2}, the gene encoding nitric oxide synthase-2.

Both VWR and EGCG-ß-ALA affected several genes associated with important intracellular signaling pathways (Figure 3C). There were significant main effects for both EGCG+ß-ALA ($F_{1,49}=4.802$, $p=0.033$) and VWR ($F_{1,49}=6.672$, $p=0.013$) for \textit{Ppargc1a}, the gene encoding PGC-1α, such that both EGCG-ß-ALA and VWR increased expression of this gene compared to normal chow-fed or sedentary mice, respectively. Likewise, EGCG-ß-ALA increased expression of \textit{Sirt1} (SIRT-1, $F_{1,49}=4.481$, $p=0.039$), while there was a near-significant effect of VWR ($F_{1,49}=3.805$, $p=0.057$) in increasing expression of this gene. VWR also increased expression of \textit{Foxo3} (FOXO3, $F_{1,49}=8.446$, $p=0.005$). There was no significant main effect or interaction with respect to expression of \textit{Prkag2}, a gene encoding the gamma-2 subunit of 5’ AMP-activated protein kinase (AMPK), although EGCG-ß-ALA tended to increase expression of this gene ($F_{1,49}=2.849$, $p=0.098$).

Finally, VWR increased gene expression of \textit{Bdnf}, the gene encoding brain-derived neurotrophic factor (BDNF, $F_{1,49}=7.568$, $p=0.008$, Figure 3D). There was also a significant main effect of EGCG-ß-ALA for \textit{Igf1} expression (insulin-like growth factor (IGF)-1), such that EGCG-ß-ALA increased expression of this
gene in the gastrocnemius ($F_{1.49}=9.451, p=0.003$, **Figure 3D**). Neither EGCG+β-ALA nor VWR impacted expression of *Vegfa*, the gene encoding vascular endothelial growth factor-A (data not shown).

**Oxidative Stress**

Analysis of 4-HNE as a measure of oxidative stress in gastrocnemius samples revealed a significant EGCG+β-ALA × VWR interaction ($F_{1.52}=5.583, p=0.022$) and a significant main effect of VWR ($F_{1.52}=5.815, p=0.019$) such that VWR reduced 4-HNE in the gastrocnemius muscle compared to SED controls (**Figure 4**). Post-hoc mean separation by Tukey HSD revealed that Ctrl-VWR had reduced 4-HNE compared to Ctrl-SED ($p=0.009$).

**DISCUSSION**

We have demonstrated an effect for voluntary wheel running in improving muscle function and increasing expression of antioxidant genes. Although the results are perhaps unsurprising given the extant literature on exercise and aging, they are relatively novel in the context of aging and VWR. A recent study examining the impact of 4 weeks of VWR in extremely old (28-30 months) C57Bl/6J mice showed similar VWR-mediated improvements in grip strength, endurance, and rotarod performance (Graber et al. 2014). However, the effects of exercise on muscle function tests can be highly strain-dependent (Merritt and Rhodes 2015), and we believe that the current study is the first to demonstrate VWR-mediated improvements in rotarod and grip strength in aged Balb/cByJ mice.

In addition to muscle function tests, we examined gene expression for markers of a number of pathways which might affect muscle (dys)function in aged animals. For ease of presentation, we have grouped these into 4 categories: inflammation, oxidative stress, cellular signaling, and growth factors. Both inflammation (Kalinkovich and Livshits 2015) and oxidative stress (Wohlgemuth et al. 2010) are known to be associated with impaired muscle function in aging, and treatments which reduce inflammation and oxidative stress have been shown to improve muscle growth and function (Marzani et al. 2008, Mourkioti et al. 2006). Likewise, growth factors such as IGF-1 are important for promotion of
muscle growth and are shown to be reduced with age (Kalinkovich and Livshits 2015, Sharples et al. 2015). Finally, both mitochondrial function (Marzetti et al. 2013) and autophagy (Wohlgemuth et al. 2010) are shown to be dysregulated in skeletal muscle during aging, and we examined expression of markers related to these pathways as well.

Aerobic exercise training has been previously shown to reduce oxidative stress (Leeuwenburgh and Heinecke 2001), and in this study we have demonstrated significant increase in antioxidant gene expression for Sod1, as well as a significant decrease in gastrocnemius levels of 4-HNE, a product of lipid peroxidation used here as a tissue marker of overall oxidative stress. Sod2 was unaffected by these treatments, although there was a trend for exercise to upregulate the expression of the gene. Both Sod1 and Sod2 were examined as Sod1 is abundantly present in the cytosol, while Sod2 is primarily present in the mitochondria (Fukui and Zhu 2010).

Aerobic exercise is also well-known to increase mitochondrial biogenesis (Pilegaard et al. 2003), and in this study we demonstrated significant increase in expression of Ppargc1a in the gastrocnemius of VWR mice. We additionally noted increased expression of Bdnf, the gene encoding BDNF, which acts to increase neurogenesis and angiogenesis (Kermani and Hempstead 2007). BDNF and IL-6, gene expression of which was also increased in our study, additionally increase myogenesis and fat oxidation in muscle (Pedersen 2013), and both have been routinely shown to be upregulated with exercise (Pedersen 2013). However, Il6 is generally upregulated only after acute exercise (Pedersen 2013), thus the finding of an upregulation of Il6 expression 12 hours after the last exercise bout is surprising. The less than 2-fold upregulation of Il6 in this study, while still significant, is far less striking than that induced by acute exercise, and this may potentially be explained by increased cage activity in VWR mice (although this is speculative as cage activity was not monitored).

Interestingly, VWR also increased expression of Foxo3, the gene encoding FOXO3. FOXO3 is a transcription factor that has been implicated in a number of physiological processes. For example,
FOXO3 has been demonstrated to promote DNA repair and antioxidant enzyme function (Kops et al. 2002) and to upregulate gluconeogenesis (Barthel et al. 2005). Additionally, FOXO3 enhances autophagy in atrophying muscle cells (Zhao et al. 2007), and stress-induced autophagy has been shown to be essential to muscle protein and glucose homeostasis during exercise (He et al. 2012).

A limitation of this study was the measurement of gene expression at a single time point. This provides only a snapshot of overall physiological activity and may not completely reflect downstream protein synthesis or functional activity. Additionally, mRNA levels likely vary considerably within subjects across the duration of the treatment. However, measurement of mRNA in mice requires sufficient tissue mass so as to necessitate euthanasia, and the need to maintain statistical power for behavioral testing precluded euthanizing mice for gene expression analysis at multiple time points in this study.

In addition to the described alterations in gastrocnemius gene expression, VWR also improved physical function as defined by increased performance by VWR mice in forelimb grip strength, the rotarod test, and a treadmill test to fatigue. Although these findings are not novel, the combination of VWR-induced improvements in muscle function and increases in gene expression do serve to underscore the utility of VWR as an appropriate model for aerobic exercise training. There has long been debate, both formal and informal, about the propriety of VWR as an exercise modality, given the nature of the activity in mice. Consistent with our study, mice routinely run multiple kilometers in a 24 hour period, while forced treadmill-trained mice generally run less than 400 meters per day.

It is often argued that forced treadmill training offers a better model of human exercise, given the greater volume and reduced intensity in VWR. Despite this, VWR still yields many of the same positive change seen in forced treadmill exercise, which has been shown to include adaptations in cardiac and skeletal muscle (Allen et al. 2001) consistent with other endurance exercise modalities. Our findings reinforce the need for careful selection of exercise modality in rodent exercise studies, as
recent reports have demonstrated differential effects of VWR and forced treadmill running (Cook et al. 2013), despite these strategies sharing many of the same adaptive benefits.

In contrast to VWR, dietary intervention with supplementation of EGCG and ß-ALA had little effect on the parameters measured in this study. Dietary intervention increased gene expression of signaling molecules Ppargc1a and Sirt1, indicating the possibility that our dietary intervention may increase mitochondrial biogenesis in the aged muscle irrespective of exercise training. EGCG+ß-ALA had no effect on expression of antioxidant genes or on gastrocnemius oxidative stress as measured by 4-HNE.

Perhaps the most interesting effect of our dietary intervention is the increase in gene expression of Itgam, the gene encoding CD11b, a common marker for macrophages as well as other leukocytes (Solovjov et al. 2005). Although gene expression by itself is insufficient to demonstrate an increase in gastrocnemius macrophage content, it does suggest that our dietary intervention may act to increase either macrophage influx or proliferation in skeletal muscle. As this hypothesis is tangential to our main aims in this study, we did not pursue the finding further, but it offers an interesting potential avenue for future exploration in this area. The observed increased expression of Itgam may also partially explain the dietary intervention effect of increasing expression of Igf1, as macrophages have previously been demonstrated to produce IGF-1 and to be involved in muscle injury repair (Lu et al. 2011).

Although we noted that the combined intervention (EGCG+ß-ALA-VWR) had the greatest expression of several genes in absolute terms (most notably Ppargc1a, Sirt1, and Foxo3), the lack of statistically significant activity x diet interactions for these genes as well as the relatively low number of significant main effects of diet give us no evidence to support our hypothesis that the combined VWR and EGCG+ß-ALA diet treatments would synergistically act to improve outcomes in this study. Thus we conclude that given the parameters of our study design, a combination diet including supplemental ß-ALA and EGCG is ineffective at improving muscle function in aged mice.
It is possible that alternate strategies may show different results. In rodent studies examining the impact of EGCG on skeletal muscle, doses range from 1 mg·kg\(^{-1}\)·day\(^{-1}\) (Chen et al. 2009) to 1500 mg·kg\(^{-1}\)·day\(^{-1}\) (Friedrich et al. 2012). Additionally, delivery methods vary widely, with strategies such as oral gavage (Senthil Kumaran et al. 2008) and supplementation in the drinking water (Chen et al. 2009) being most common. The number of studies which have used incorporation of EGCG into the diet is relatively limited, especially in relation to the effects of EGCG on skeletal muscle. However, successful studies using diet-incorporated EGCG which target skeletal muscle have reported EGCG concentrations (w/w) in the diet of 0.32% (Sae-Tan et al. 2011) and 0.5% and 1% (Friedrich et al. 2012), higher than the 0.135% concentration used in this study, although these studies used high-fat diet feeding, which may also explain the efficacy of EGCG.

Likewise, despite the vast literature examining β-ALA and muscle, surprisingly little information is available on β-ALA supplementation in rodents. A recent study supplemented mice with 0.6% or 1.2% β-ALA w/v in drinking water for 8 weeks and demonstrated increased muscle carnosine and enhanced fatigue resistance at the higher dose (Everaert et al. 2013). This represents a dosage approximately 3.5-fold greater than that used in our study (~1420 mg·kg\(^{-1}\)·day\(^{-1}\) vs. 414 mg·kg\(^{-1}\)·day\(^{-1}\)), and the dietary treatment was applied for twice as long (8 weeks vs. 4 weeks). While we noted no deficit in treadmill or rotarod performance due to dietary intervention in either SED or VWR mice, it is possible that paraesthesia symptoms (“pins and needles”) may have obscured a potential β-ALA benefit to performance on these tests. It is unclear to what extent mice are affected by this phenomenon, but β-ALA supplementation in drinking water at a dose of 40 mg·kg\(^{-1}\) was sufficient to induce itching behavior in mice in a recent study (Liu et al. 2012).

**Conclusions**

In this study, we demonstrated significant positive effects of voluntary wheel running in aged mice on several muscle function measures. Additionally, wheel running enhanced gastrocnemius
expression of a variety of genes known to be related to antioxidant and biogenic mechanisms in skeletal muscle. In contrast, a diet containing epigallocatechin gallate and β-alanine had no effect on muscle function and caused only a slight increase in expression of several genes related to mitochondrial biogenesis. Further, we found no synergistic effects when dietary and exercise interventions were combined.
ACKNOWLEDGEMENTS

Funded by the Center for Nutrition, Learning and Memory, a partnership between the University of Illinois at Urbana-Champaign and Abbott.

The authors would like to thank and acknowledge Drs. Neile Edens and Sean Garvey (Abbott) for their assistance with determining dietary dosages of EGCG and β-alanine and in editing the manuscript.

CONFLICTS OF INTEREST

JAW, RWJ, and JSR are funded by the Abbott-University of Illinois partnership, the Center for Nutrition, Learning, and Memory. For the remaining authors none are declared.
REFERENCES


### Table 1: Descriptive data.

<table>
<thead>
<tr>
<th></th>
<th>Ctrl-SED</th>
<th>EGCG+β-ALA-SED</th>
<th>Ctrl-VWR</th>
<th>EGCG+β-ALA-VWR</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Day 1 body weight (g)</strong></td>
<td>31.1 ± 0.5</td>
<td>30.3 ± 0.6</td>
<td>30.5 ± 0.6</td>
<td>30.7 ± 0.5</td>
</tr>
<tr>
<td><strong>Day 41 body weight (g)</strong></td>
<td>30.0 ± 0.6</td>
<td>29.1 ± 0.6</td>
<td>27.0 ± 0.5*</td>
<td>27.5 ± 0.5*</td>
</tr>
<tr>
<td><strong>Food disappearance (g/day)</strong></td>
<td>3.4 ± 0.1</td>
<td>3.5 ± 0.1</td>
<td>3.6 ± 0.1</td>
<td>3.6 ± 0.1</td>
</tr>
<tr>
<td><strong>Water disappearance (g/day)</strong></td>
<td>2.7 ± 0.2</td>
<td>3.4 ± 0.2†</td>
<td>3.1 ± 0.2</td>
<td>3.3 ± 0.2</td>
</tr>
<tr>
<td><strong>Running distance (km/day)</strong></td>
<td>—</td>
<td>4.8 ± 0.8</td>
<td>4.5 ± 0.4†</td>
<td></td>
</tr>
<tr>
<td><strong>Lean Mass Pre (% body weight)</strong></td>
<td>83.7 ± 0.8</td>
<td>83.1 ± 0.8</td>
<td>83.6 ± 0.8</td>
<td>82.8 ± 0.6</td>
</tr>
<tr>
<td><strong>Lean Mass Post (% body weight)</strong></td>
<td>83.9 ± 1.0</td>
<td>85.3 ± 0.9</td>
<td>84.0 ± 0.6</td>
<td>84.0 ± 0.7</td>
</tr>
<tr>
<td><strong>Fat Mass Pre (% body weight)</strong></td>
<td>10.9 ± 0.8</td>
<td>10.4 ± 0.8</td>
<td>11.2 ± 0.8</td>
<td>11.4 ± 0.7</td>
</tr>
<tr>
<td><strong>Fat Mass Post (% body weight)</strong></td>
<td>9.3 ± 1.0</td>
<td>9.0 ± 0.7</td>
<td>7.8 ± 0.8</td>
<td>8.3 ± 0.7</td>
</tr>
</tbody>
</table>

N=10-15/group for all variables. Data are mean ± SEM. * Main effect of VWR (p < 0.05). † Main effect of diet (p < 0.05). Body weight, food/water disappearance, and running distance data were previously published in Gibbons et al. (2014), and are re-published with permission.
FIGURE CAPTIONS

Figure 1: Voluntary wheel running increased grip strength. (A) Average maximal forelimb grip strength before (Pre) and after (Post) dietary/exercise intervention. (B) Ratio of maximal grip strength measurements from pre- to post-intervention. * Significant main effect of VWR (p=0.009). N=12-15/treatment combination. Data are mean ± SEM.

Figure 2: Voluntary wheel running enhanced performance on the treadmill and rotarod tests. (A) Performance on the treadmill test to fatigue. (B) Average performance on 4 rotarod tests per day over a period of 2 days. * Significant main effect of VWR (p<0.05). N=12-15/treatment combination. Data are mean ± SEM.

Figure 3: Gastrocnemius gene expression of (A) inflammatory markers, (B) oxidative stress markers, (C) intracellular signaling molecules, and (D) growth factors. Significant main effect of †VWR and ‡EGCG+ß-ALA (p<0.05). N=12-14/treatment combination. Data are mean ± SEM.

Figure 4: Gastrocnemius 4-HNE. † Significant main effect of VWR (p=0.019). N=12-15/group. Data are mean ± SEM.