*J Nutr Health Aging*
*Volume 21, Number 5, 2017*

# EFFECTS OF RUNNING WHEEL ACTIVITY AND DIETARY HMB AND B–ALANINE CO-SUPPLEMENTATION ON MUSCLE QUALITY IN AGED MALE RATS

D.W. RUSS[1,2], C. ACKSEL[1,3], K.W. MCCORKLE[1], N.K. EDENS[4,5], S.M. GARVEY[4]

1. Laboratory for Integrative Muscle Biology, Division of Physical Therapy, Ohio University, Athens, OH USA; 2. Ohio Musculoskeletal and Neurological Institute (OMNI), Heritage College of Osteopathic Medicine, Athens, OH USA; 3. Division of Nutrition, School of Applied Health Sciences and Wellness, Ohio University, Athens, OH USA; 4. Abbott Nutrition R&D, 3300 Stelzer Road, Columbus, OH USA; 5. Current Affiliation: Common Threads, 3811 Bee Cave Rd, Ste. 108, Austin, TX USA. Corresponding author: David W. Russ, PT, Ph.D. Associate Professor, Division of Physical Therapy , School of Rehab & Communication Sciences, Ohio University, W279 Grover Center, Athens, OH 45701, (ph.)740-566-0022, (fax)740-593-0293, russd@ohio.edu

**Abstract:** *Objective:* Loss of skeletal muscle function is linked to increased risk for loss of health and independence in older adults. Dietary interventions that can enhance aging muscle function, alone or in combination with exercise, may offer an effective way to reduce these risks. The goal of this study was to evaluate the muscular effects of beta-hydroxy-beta-methylbutyrate (HMB) and beta-alanine (β-Ala) co-supplementation in aged Sprague-Dawley rats with voluntary access to running wheels (RW). *Methods:* Aged (20 months) rats were housed with ad libitum access to RW while on a purified diet for 4 weeks, then balanced for RW activity and assigned to either a control or an experimental diet (control + HMB and β-Ala) for the next 4 weeks (n = 10/group). At the end of the study, we assessed muscle size, in situ force and fatigability in the medial gastrocnemius muscles, as well as an array of protein markers related to various age- and activity-responsive signaling pathways. *Results:* Dietary HMB+β-Ala did not improve muscle force or fatigue resistance, but a trend for increased muscle cross-sectional area (CSA) was observed (P = 0.077). As a result, rats on the experimental diet exhibited reduced muscle quality (force/CSA; P = 0.032). Dietary HMB+β-Ala reduced both the abundance of PGC1-α (P = 0.050) and the ratio of the lipidated to non-lipidated forms of microtubule-associated protein 1 light chain 3 beta (P = 0.004), markers of mitochondrial biogenesis and autophagy, respectively. Some alterations in myostatin signaling also occurred in the dietary HMB+β-Ala group. There was an unexpected difference (P = 0.046) in RW activity, which increased throughout the study in the animals on the control diet, but not in animals on the experimental diet. *Conclusions:* These data suggest that the short-term addition of dietary HMB+β-Ala to modest physical activity provided little enhancement of muscle function in this model of uncomplicated aging.

**Key words:** Fatigue, sarcopenia, exercise, myostatin, autophagy.

## Introduction

Increasing, or maintaining physical activity (PA) is considered an effective strategy for preventing or slowing age-associated functional declines in multiple organs and physiological systems. The neuromuscular system in particular both contributes to, and benefits from, PA as age increases. Responses to PA are thought to include: improved muscle insulin sensitivity (1), reduced risk of cardiovascular morbidity and mortality (2, 3), decreased incidence of type 2 diabetes in high risk individuals (4), and even increased brain volume and improved cognitive function (5, 6). However, while these systemic effects of muscular activity are of great importance, it is equally critical to maintain the contractile (i.e., force-producing) function of skeletal muscle itself (7-9).

Resistance exercise is typically considered to be the intervention of choice to address age related impairments specific to muscle force-production by increasing muscle size (i.e., hypertrophy) (10, 11). However, accumulating data suggest aging muscles can lose their capacity for force production, even when muscle mass is increased or maintained (12, 13). Moreover, increasing evidence suggests that the hypertrophic response is blunted in older relative to younger individuals (14-16). There has thus been much interest in the potential use of nutritional supplements to maintain or increase aging muscle mass, in particular protein and amino acid supplements (17). Predictably, the results of these studies vary, with experimental differences in dose and timing of the supplement, mode and intensity of exercise, study population and method of evaluation of muscle function all likely to contribute to inconsistencies in results. Nevertheless, there is sufficient evidence to suggest that protein and amino acid supplementation can augment resistance exercise in terms of increasing muscle size and strength (18).

In addition to the search for ways to enhance the response of aging muscle to resistance exercise, there is an increasing appreciation that aerobic exercise, and possibly more general PA, may improve size and strength of older muscles in a way they do not for young muscles (19-21). Thus, just as with resistance exercise, it is worth examining the potential of dietary supplements to enhance the effects of PA on aging muscle contractile function. One dietary intervention, β-hydroxy-β-methylbutyrate (HMB), a leucine metabolite, is believed to increase muscle size by blunting muscle protein breakdown, although some data also support a stimulation of muscle protein synthesis (for recent review, c.f., (22)). Studies

*Received February 2, 2016*
*Accepted for publication April 5, 2016*

indicate that HMB shows promise for increasing muscle mass in older adults, though the effects on strength are inconsistent (23-25). In rodent models of aging, HMB has been shown to increase muscle size (26) and force production during recovery from a disuse-reloading injury (27). Thus, combining HMB to enhance muscle size with aerobic activity to increase muscle quality (i.e., force per unit muscle size), as reported by Harber and colleagues (20), could be an optimal strategy to improve contractile function in aging muscle.

In addition to muscle weakness, increased fatigue is another common complaint of older adults (28). The extent to which muscle fatigue per se contributes to this symptomatic fatigue is equivocal (29), but improving muscular endurance would be expected to contribute to improved quality of life and overall physical function in older individuals. Aerobic exercise or increased PA are standard interventions for reducing fatigue (30, 31). In addition, dietary beta-alanine (β-Ala) may improve fatigue resistance by supporting the synthesis of carnosine (32). Improvements in submaximal exercise performance with β-Ala have been reported for both healthy, relatively sedentary older individuals (33) and master's athletes (34). In rodent models, β-Ala improves some measures of fatigue (35), but the results in older animals are inconsistent (36).

We recently investigated the potential for dietary co-supplementation with HMB and β-Ala (HMB+β-Ala) to address both age-related weakness and fatigue in rats (40). Contrary to our hypotheses, HMB+β-Ala did not significantly improve in situ muscle force or fatigability, despite using a dosage comparable to those shown to produce significant effects elsewhere (25, 37). However, the animals in our study were cage-housed and thus quite sedentary. It is possible that the additional stimulus of increased physical activity might enhance potential positive effects of HMB+β-Ala on aging muscle contractile function. Accordingly, the primary goal of the present study was to evaluate the effect of the addition of physical activity (via access to voluntary running wheels (RW)) to HMB+β-Ala co-supplementation on muscle size, contractile force and fatigability in aged rats. We chose RW activity as our intervention, as we have previously shown that even modest amounts of RW activity can induce several potentially beneficial metabolic and molecular adaptations in aging rat skeletal muscle (38, 39). We also evaluated several protein markers associated with a wide range of age-, diet- and training-responsive muscular adaptations, as our recent study (40) did find that HMB+β-Ala influenced one such marker (MURF1), despite the lack of effect on muscle function or morphology.

## Materials and methods

### *Experimental Animals*

Aged, male Sprague-Dawley (SD) rats were purchased from Harlan (20 months-of-age upon arrival) and housed in an environmentally controlled facility (12–12 h light–dark cycle, 22°C) at Ohio University (Athens, OH). All animals acclimated to the animal facility for two weeks, with ad libitum access to water and standard natural chow (Harlan #T8640 Teklab 22/5). Access to food and running wheels was not withheld at any time during the study. Animal use and all procedures were approved by the Ohio University Institutional Animal Care and Use Committee, and the "Principles of Laboratory Animal Care" (NIH publication No. 86-23, revised 1985) were followed throughout the study.

### *Running Wheels*

Observation of RW activity began after the 2 weeks of acclimation, and was monitored on instrumented RW's as we have described elsewhere (38). After 4 weeks of RW activity on the purified diets, animals were ranked according to average daily distance run and all were assigned to either the Ctl or Exp diet group, so that RW distance was balanced across groups. This assignment also resulted in the two groups having comparable average body mass, so no further balancing in this regard was needed.

### *Dietary Intervention*

Following 2-week acclimation, animals were all placed on a purified diet (AIN-93M purified diet) for 4 weeks, at which time they were assigned to either a control (Ctl) diet (continued use of the purified diet) or an experimental (Exp) diet (AIN-93M formulated with the combination of calcium HMB monohydrate (10.5g/kg chow, hereafter referred to as HMB) and β–Ala (8.39g/kg chow)). This formulation was chosen to approximate a daily intake of 343 mg HMB/kg body weight and 274 mg β–Ala/kg body weight, or the metabolic equivalent of 3 g HMB and 2.4 g β–Ala per day in a 70 kg human. Diets were color-coded to allow blinding regarding dietary assignment. In contrast to the 8 week dietary intervention previously tested in sedentary rats (40), we chose to reduce the duration of dietary intervention in this study to 4 weeks for several reasons. We wanted to examine the effect of the addition of dietary supplementation to an already-ongoing exercise routine, such as an active older adult might do; but we also wanted to test the animals in the same age range at which we tested the sedentary animals in our earlier study (40).

### *Muscle Contractile Measurements*

Prior to contractile testing, animals were anesthetized (Ketamine + Xylazine; 40 + 10 mg kg -1 body mass), then mounted in a rigid frame that securely immobilizes the leg and pelvis. Force-frequency relationships (FFRs) were determined using in situ neuromuscular electrical stimulation of the medial gastrocnemius (MG) muscle, as previously described (40). Maximum rates of tetanic force development and relaxation were determined from the force responses to 100-Hz stimulation. Following FFR testing, muscle fatigue was induced using a modified Burke fatigue protocol (41), consisting of a 3-minute bout of 330-ms, 40-Hz trains delivered at a rate of 1/s. Testing trains of 1- and 100-Hz

were administered before and after the fatigue protocol. The use of testing trains allowed us to control for any frequency-specific effects of the fatiguing protocols. Fatigue was reported using the fatigue index (final force/initial force) for all three frequencies (1, 40 and 100 Hz). In addition, we examined the rate of fatigue using a curve-fitting routine that we have previously applied to a similar electrically-elicited fatigue protocol (42). Muscle quality was calculated post-hoc by expressing muscle force relative to the cross-sectional area (CSA) of the muscle (40).

Both MG muscles were dissected after completion of the fatigue testing, blotted dry, weighed and snap frozen in liquid nitrogen. Frozen samples were stored at -80° C for subsequent immunoblotting and myosin heavy chain (MHC) determination. We also quantified total intramuscular lipid and water content, as we have previously described (40).

***Myosin Heavy Chain Analysis***

Myofibrillar proteins were extracted from a portion of the stimulated muscle samples and used for electrophoretic determination of the relative expression of myosin heavy chain (MHC) isoforms as described previously (39, 43). Gels were stained with Coomassie Brilliant Blue, scanned and quantified densitometrically on a LiCor Odyssey system.

***Immunoblotting***

For the bulk of the immunoblotting, the unstimulated MG was used. We also processed the stimulated MG to allow assessment of certain acute responses to contraction (e.g., AMPK and p70s6K phosphorylation). Frozen MG samples were processed for immunoblotting, then subjected to SDS-PAGE and transferred to transferred to polyvinylidene fluoride (PVDF) membranes (39). Antibodies against ribosomal protein S6 kinase, 70kDa, polypeptide 1 (P70S6K), phospho-P70S6K (Thr 421/Ser 424), and myostatin (MSTN) were purchased from Santa Cruz Biotechnology (Dallas, TX). Antibodies against protein kinase, AMP-activated, alpha 1 catalytic subunit (AMPK,), phospho-AMPK (Thr172,), eukaryotic initiation factor-4e (EIF-4e), EIF-4e binding protein 1 (4eBP1), Parkin, Serine Palmitoyltransferase (SPT), Glucose-regulated protein 78 (Grp78) and muscle RING-finger protein-1 (MURF1), were purchased from Abcam (Cambridge, MA). The antibody against microtubule-associated protein 1 light chain 3 beta (LC3B) was purchased from OriGene (Rockville, MD). The antibody against peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (PGC1α) was purchased from EMD Millipore/Calbiochem (Billerica, MA). The antibodies against Mitochondrial Transcription Factor A (TFAM) was purchased from Millipore-EMD (Billerica, MA). Antibodies against ubiquitin-binding protein p62/sequestosome1 (p62) and apoptosis regulator Bcl2 (Bcl2) were purchased from Sigma-Aldrich (St. Louis, MO). All primary antibodies were diluted 1:2,000 in blocking buffer plus Tween 20. After primary incubation, membranes were washed 5 x 5 minutes in tris-buffered saline plus Tween 20 (TBS-T) and incubated for 1 hour at room temperature with appropriate secondary antibodies (LI-COR, Lincoln, NE) which were diluted in blocking buffer (1:10,000-1:20,000). Following secondary incubation, membranes were once again washed 5 x 5 minutes in TBS-T, then rinsed for 5 minutes with TBS. Membranes were dried in the dark overnight prior to scanning and densitometric band analysis with a LI-COR Odyssey system. After scanning, the membranes were stained with Coomassie Brilliant Blue R250 and within blot band intensities were normalized to total protein per lane determined from the stained, scanned membrane. The blots were all performed in duplicate. Known amounts of rabbit and mouse IgG were run on each gel as a standard for normalization of bands from different blots.

***Statistical analysis***

Data are presented as means ± SE, unless otherwise noted. Animal mass, food intake and running distance were analyzed using 2-way (time X diet) ANOVAs, with time as a repeated factor. The FFR and muscle quality-frequency responses were analyzed using 2-way (group X frequency) ANOVAs, with frequency as a repeated factor. Acute phosphorylation responses (i.e., AMPK and p70s6K phosphorylation) were compared using 2-way (stimulation x diet) ANOVAs with stimulation as a repeated factor. For Post hoc comparisons were tested with unpaired t-tests. Muscle morphology (i.e., mass, lipid content, etc.) and immunoblot data were compared using unpaired t-tests. Threshold for statistical significance was set at $P \leq 0.05$ for all analyses.

## Results

***Mortality***

A total of 20 experimental animals were received and 3 animals expired prior to contractile testing (2 in the Exp diet group and 1 in the Ctl diet group). This mortality rate of 15% was not unexpected, and was the same as that observed in our earlier study of HMB+β-Ala (37).

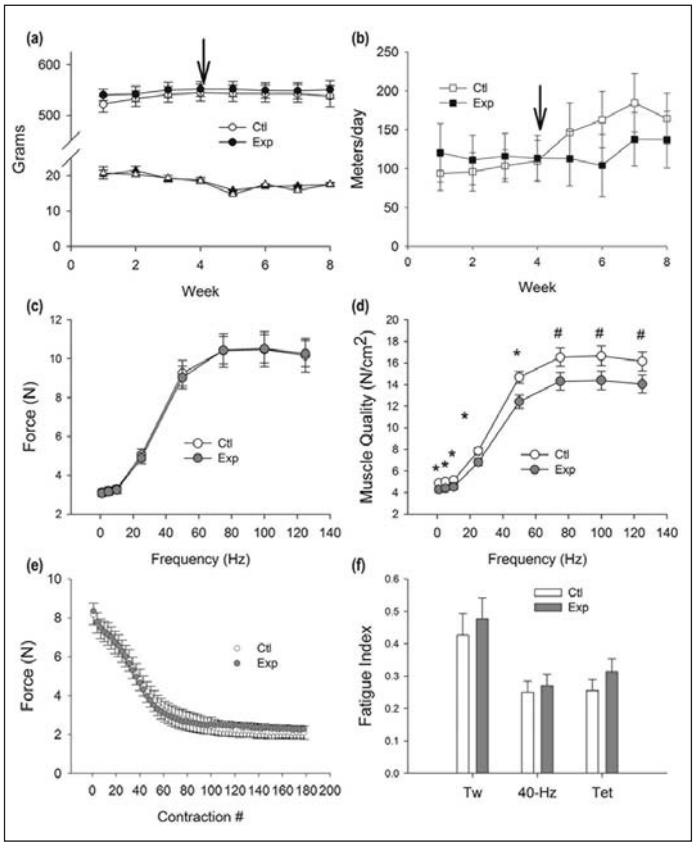
***Body mass, food intake and RW activity***

Food intake and body mass over the course of the experiments are presented in Figure 1a. The average food intake during the intervention phase was ~18.2 g/d, corresponding to an HMB dose of 190 mg/d (roughly 350 mg/kg body mass/day), and a β-Ala dose of 153 mg/day (roughly 282 mg/kg body mass/day). There were significant effects of time for body mass ($P = 0.022$) and food intake ($P < 0.001$), but there were no significant effects of diet or diet X time interactions. The main effect of time and the diet X time interaction were significant ($P < 0.001$ & $P = 0.019$, respectively) (Figure 1b). A comparison of the change in average RW activity on the 4-week purified diet phase vs. that on the dietary intervention phase within each group (by paired t-test) indicated that animals on the Ctl diet increased running

distance (P = 0.012), while those on the Exp diet did not (P = 0.434).

**Figure 1**

(a) Mean (±SE) body mass (circles) and daily food intake (triangles); arrow indicates switch to dietary intervention phase. (b) Mean (±SE) daily running distance per week; arrow indicates switch to dietary intervention phase. (c) Mean (±SE) absolute forces for stimulation frequencies tested. (d) Mean (±SE) forces normalized to CSA (Muscle Quality) for stimulation frequencies tested; * = significant for difference between dietary groups, # = trend (0.100 ≥ P ≥ 0.085) for difference between dietary groups. (e) Mean (±SE) peak forces during fatigue testing. (f) Mean (±SE) fatigue indices for 1-, 40- and 100-Hz testing trains. Filled symbols = Ctl diet; Open symbols = Exp Diet




***Contractile Testing & Muscle Morphology***

For the FFR, there was no significant main effect of diet, nor was there a frequency X diet interaction (Figure 1c). When the muscle quality-frequency data were evaluated however, there was a significant effect of diet (P = 0.032) with greater muscle quality in the animals on the Ctl diet. These differences tended to exhibit more robust statistical differences at lower vs. higher frequencies (see Figure 1d). There was a trend (P = 0.077, Table 1) for CSA to be greater in the animals on the Exp diet, and peak force at each frequency tested was significantly correlated with CSA (0.012 ≥ P ≥ 0.004, 0.481 ≥ r2 ≥ 0.388). Thus variance in CSA explained 39-48% of the variance in peak force, depending on the stimulation frequency. No effect of diet was found for rates of contractile force development or relaxation (Table 1), nor was any effect seen on muscle fatigue, either when comparing the fatigue indices of the testing trains or during the test (Figure 1f and 1e, respectively). Comparable total force-time integrals were produced for each dietary condition (data not shown). No significant differences between the groups were observed for muscle size, lipid content or MHC composition, though there was a trend for CSA to be greater in the animals on the Exp diet (Table 1).

**Table 1**

| | Exp. Diet | Ctl. Diet | P-value |
|---|---|---|---|
| Muscle Mass (g) | 1.19 ± 0.05 | 1.08 ± 0.06 | 0.158 |
| l0 (cm) | 3.53 ± 0.09 | 3.66 ± 0.05 | 0.239 |
| CSA (cm2) | 0.72 ± 0.03 | 0.62 ± 0.03 | 0.077 |
| Lipid Content (% ww) | 5.43 ± 0.75 | 3.89 ± 0.53 | 0.110 |
| Water Content (% ww) | 76.47 ± 1.06 | 74.89 ± 0.63 | 0.289 |
| Type IIa MHC (%) | 4.45 ± 1.27 | 3.05 ± 0.71 | 0.366 |
| Type IIx MHC (%) | 22.39 ± 3.00 | 26.20 ± 2.78 | 0.340 |
| Type IIb MHC (%) | 62.61 ± 5.72 | 61.65 ± 4.34 | 0.895 |
| Type I MHC (%) | 10.58 ± 2.75 | 9.09 ± 2.18 | 0.675 |
| MRFD (mN ms-1) | 332.8 ± 65.4 | 312.9 ± 53.0 | 0.506 |
| MRFR(mN ms-1) | 266.5 ± 95.2 | 259.7 ± 107.5 | 0.890 |
| Norm MRFD (% ms-1) | 3.18 ± 0.45 | 3.04 ± 0.36 | 0.509 |
| Norm MRFR (% ms-1) | 2.51 ± 0.67 | 2.42 ± 0.40 | 0.739 |

**Figure 2**

Mean (±SE) phosphorylated/pan protein ratios for p70s6K and AMPK before and after fatiguing stimulation. Horizontal bar indicates significant effect of acute stimulation. Insets show representative blots

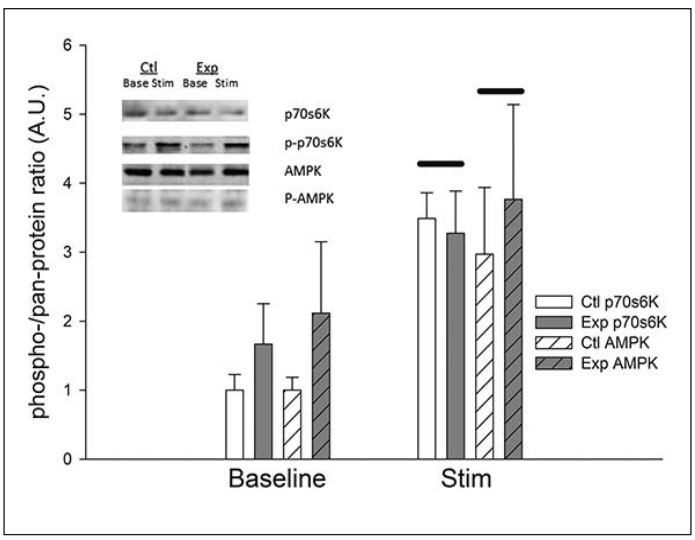
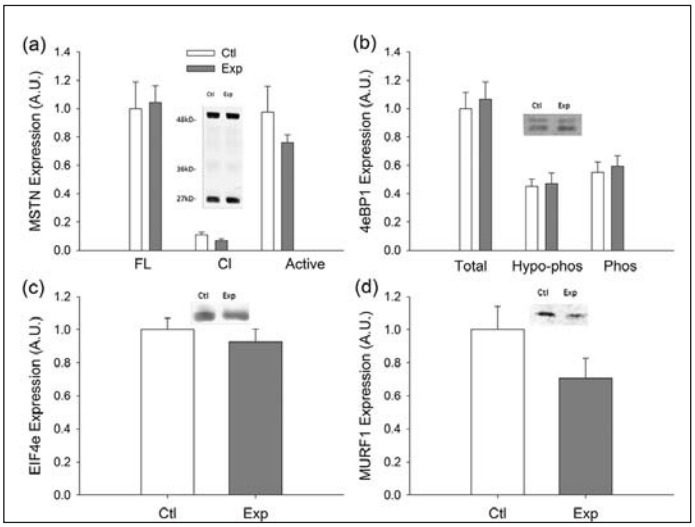


**Figure 3**

(a) Mean (±SE) Myostatin expression for full-length (50 kD), cleaved (38 kD) and active (27 kD) forms. Though no differences between dietary groups were present for any specific marker, the ratios of the active and cleaved to the full-length form did exhibit an effect of diet (see text for details). (b) Mean (±SE) 4eBP1 expression. (c) Mean (±SE) EIF-4e expression. (d) Mean (±SE) MURF1 expression. Insets show representative immunoblots




***Immunoblotting***

Immunoblot data comparing the stimulated to the unstimulated muscles are presented in Figure 2. The rest of the immunoblot results involve comparisons of the dietary groups in the unstimulated muscles only, and are presented in Figures 3-5. Data are presented as arbitrary units, and were normalized to the baseline, Ctl diet values.

***Acute Signaling Responses to Muscle Contraction***

No differences between the dietary groups for the pan- or phosphorylated forms of p70s6K or AMPK were present at baseline. Accordingly, we present the phospho-/pan- ratios as indices of signaling responses to contractile activity. Fatiguing stimulation induced significant increases in the phospho-/pan-protein ratios for both p70s6K ($P < 0.001$) and AMPK ($P = 0.007$) (Figure 2). No significant effect of diet or diet X time interaction was observed for either p70s6K or AMPK.

***Protein Synthesis & Breakdown***

No significant effect of diet was found for abundance of total, phosphorylated or slower-migrating, hypo-phosophorylated (44) 4eBP1 protein; nor the ratio of the two bands. Similarly, expression of EIF-4e and MURF1 were unaffected by diet. Although no significant differences were observed for any of the individual forms (i.e., full-length, cleaved pro-peptide or active) of myostatin, the active:full-length and cleaved:full-length ratios were reduced in the animals on the Exp diet ($P = 0.043$ and 0.095, respectively).

**Figure 4**

(a) Mean (±SE) LC3b-I, LC3b-II and II/I ratio; * = significant for difference between dietary groups. (b) Mean (±SE) p62 expression. † = Trend for difference from Ctl group ($P = 0.097$). (c) Mean (±SE) Bcl2 expression. (d) Mean (±SE) Parkin expression. Insets show representative blots

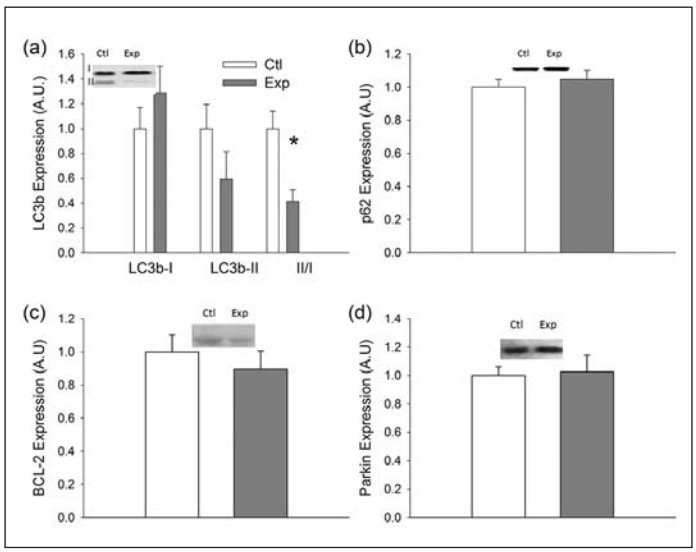
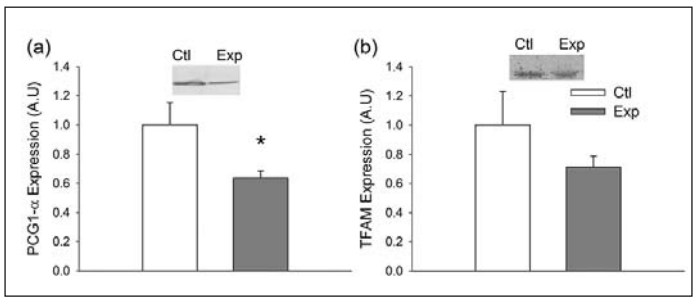



**Figure 5**

Mean (±SE) PGC-1α expression; * = significant for difference between dietary groups. (b) Mean (±SE) TFAM expression. Insets show representative blots




***Autophagy***

There was a significant effect of diet on one common marker of autophagic flux (LC3b-II/I ratio, $P = 0.004$), but not p62 (Figure 4a&b, respectively). Interestingly, there were no significant differences between the dietary group in LC3b-I and –II individually, despite the lower ratio in the Exp diet group. Expression of Parkin, a protein whose expression is believed to target organelles for autophagy (45), was not affect by diet; nor was expression of Bcl2, which has been shown to inhibit autophagy, though it is better known for its anti-apoptotic properties (46).

***Oxidative Metabolism/Mitochondrial Function***

Expression of PGC1-α was significantly greater in the animals on the Ctl diet ($P = 0.050$), but expression of TFAM was not (Figure 5).

## Discussion

Both HMB and β-ala supplementation are purported to enhance aspects of muscle function that have been found to exhibit age-related declines (i.e., contractile force and endurance), and so combining them to improve aging muscle function makes theoretical sense. However, we have previously reported that 8 weeks of dietary HMB+β-Ala in aged rats produced no significant enhancement of in situ muscle function, though it did alter the expression of some protein markers consistent with reduced protein degradation. However, those animals were sedentary, with no access to physical activity beyond simple in-cage mobility. The effects of many dietary supplements, including protein, are enhanced by exercise/physical activity (47-49). The present study reports the response of aged rats to 4 weeks of dietary HMB+β-Ala co-supplementation in combination with ongoing, RW activity. As in our earlier study, we evaluated muscle contractility and morphology, as well as broad spectrum of protein markers.

The effects of HMB+β-Ala plus volitional RW activity on in situ muscle function and morphology were different from those in our previous study of aged, sedentary rats. In the earlier study, dietary HMB+β-Ala was associated with non-significant increases in muscle size and force production. Here, HMB+β-Ala had no effect on in situ peak force production, which was extremely similar across groups (Figure 1c), but produced a trend toward increased muscle CSA. As a result, the rats on the Exp diet exhibited reduced muscle quality (Figure 1d). The observation of greater MG CSA without an increase in force in the Exp diet group may appear paradoxical at first blush, but these findings are not inconsistent with other data from aging muscles. As noted earlier, maintaining muscle mass does not guarantee maintenance of strength in aging muscles. It is interesting to note that at least one study of myostatin inhibition (13) has reported a similar decline in muscle quality, and we observed here that dietary HMB+β-ala lowered the ratio of active to full-length myostatin. In addition, one group has reported that dietary supplementation with whey protein during resistance exercise did not improve muscle strength or size in modestly active older women (50). Interestingly, though this group did not specifically test for muscle quality, they found, similar to what we observed here, that the control group exhibited somewhat greater increases in strength, despite smaller increases in muscle size.

To assess the potential effect of whole muscle composition on CSA (and by extension muscle quality), we performed separate analyses of covariance (ANCOVAs) for the effect of diet group on CSA using percent water and percent lipid wet weight (Table 1) as covariates. Interestingly, when either percent water or lipid were included as covariates, the effect of diet on CSA was significant ($P = 0.039$ and 0.043, for water and lipid as covariates, respectively). These data are consistent with the differences in muscle quality and suggest the possibility that the muscles of animals on the Exp diet contain greater non-contractile volume (water and lipid) than those on the control diet. Differences in other non-contractile tissue (i.e., connective tissue) may have contributed to the difference in muscle quality as well, but we did not conduct any assays to measure connective tissue.

As with our previous study, we observed no effect of dietary HMB+β-ala on muscle fatigue. It may well be that any benefits of HMB+β-Ala with regard to muscle fatigue do not become apparent during short-term, high demand contractile tasks such as those induced with in situ muscle stimulation. In vivo tests of sustained, volitional activity (e.g., forced swimming or distance treadmill running) may be more appropriate for modeling potential effects of HMB+β-ala or β-ala alone on fatigue in humans.

In our previous study of sedentary rats of the same age and strain, the principal changes in the protein markers following 8 weeks HMB+β-Ala co-supplementation were consistent with a decrease in muscle protein breakdown (i.e., reduced MURF1). In the present study, the shorter 4-week dietary intervention did not significantly alter MURF1 ($P = 0.141$), a response that may require a longer HMB+β-Ala loading phase in aged rats. Instead, the major effects of the Exp vs. the Ctl diet included decreases in the active/full-length myostatin ratio, the LC3b-II/I ratio and PGC1α. Interestingly, we have previously found that RW activity increased the LC3b-II/I ratio and PGC1α, as well as active myostatin in the MG muscle (38). Thus, it appears that HMB+β-Ala tended to mitigate changes in protein markers associated with increased physical activity, though it should be noted that this previous study of RW activity was conducted in late middle-aged rats. It should be further noted that differences in RW activity occurred over the course of the present study, despite balancing the animals for this behavior prior to starting the dietary intervention phase. Rats on the Ctl diet increased their RW activity more than those on the Exp diet (Figure 1). It is unclear why this difference in RW activity emerged in the Ctl group. It might be that paresthesiae induced by β-Ala (a known side effect in humans (51)) made running unpleasant for some of the animals, further increasing the variability. As the absolute RW activity for all the animals was low, the potential effect would be magnified. However, it could also be that RW activity is simply highly variable and difficult to predict, short of measures such as selectively breeding for it (52).

Regardless of the cause, this difference in RW activity may have contributed to some of the experimental differences observed in the present studies. Accordingly, we included change in RW activity (average weekly distance after the dietary switch – average weekly distance prior to the dietary switch) as a covariate in our analyses of those dependent variables for which an effect of diet was present: muscle quality, the ratio of active:full-length and myostatin expression, the LC3b-II/I ratio and PCG1α expression. In two cases, muscle quality and the active:full-length myostatin ratio, inclusion of these covariates eliminated the significant effect of diet, and in another (LC3b-II/I ratio) it increased the p-value,

though difference remained statistically significant. These data are not surprising, in that we have previously shown that RW activity increases active (27 kD) myostatin in late middle-aged rats (38), and increases the LC3b-II/I ratio in aged rats (39). In the case of PGC1α, inclusion of RW activity as a covariate actually decreased the p-value from 0.050 to 0.011. The mechanism for this result is less clear, as our previous work in late-middle aged rats found that RW activity increased PGC1α abundance (38). Thus, it appears that differences in RW activity may have contributed to many of the observed differences between the dietary groups.

In summary, 4 week dietary HMB+β-ala co-supplementation in aged, volitionally running rats exhibited markedly different effects than those we have previously reported following 8 weeks of the same intervention in sedentary rats of the same strain. At the level of muscle function and morphology, rats receiving dietary HMB+β-ala during ongoing RW activity showed a tendency to increase muscle size without increasing force production. At the molecular level, they tended to exhibit reductions in abundance of a number of proteins that we have previously found to increase with RW activity. Though it is never simple to extrapolate data in animals to humans, these results suggest that dietary supplementation with HMB+β-ala may not represent an effective strategy to improve muscle function during uncomplicated aging in older, well-nourished adults. However the present data do not rule out the possibility that older adults that are either malnourished or subjected to injury or illness that requires bedrest, immobilization or institutionalization may benefit from such dietary interventions. Animal studies indicate that HMB may improve recovery from disuse (though disuse atrophy is unaffected) (27). Moreover, dietary HMB and branched-chain amino acid supplementation have been reported to increase indices of muscle mass in healthy older adults undergoing 10 days of bedrest and elderly residents in a convalescent setting with a range of co-morbidities (53, 54). Indeed, as it has been recently suggested that periodic episodes of reduced physical activity are an important contributor to age-related muscle function (55), dietary supplements, such as HMB+β-ala might be useful supplements for older adults to take in a prophylactic manner. One can rarely, if ever, predict when illness or injury might curtail physical activity and these supplements might improve recovery, even if the present data suggest they may not enhance baseline muscle function.

Given the fact that muscle tissue composition played a role in effect of diet on muscle quality, and the observation that dietary HMB+β-ala reduced the abundance of several protein markers that we have previously found to be elevated by physical activity (LC3b-II/I ratio, PGC1α and the active form of myostatin), it is tempting to speculate that the dietary intervention interfered with the physical activity-associated signaling that influences the balance of contractile to non-contractile tissue in aging muscle. However, these findings must be interpreted cautiously in light of two major factors. First, the data presented are largely related to cross-sectional measures (excepting RW activity, body mass and food consumption), that could not be assessed at baseline. We thus cannot account for the potential effect that baseline levels might have had on the results. Second, the unexpected increase of RW activity in the animals on the Ctl diet and the effect of RW activity when included as a covariate make it difficult to ascribe any differences to diet alone. Further studies involving a more tightly controlled dose of physical activity (e.g., treadmill running) or perhaps a pharmaceutical exercise mimetic such as AICAR might help provide further insights regarding the findings of the present study and the potential underlying mechanisms.

*Acknowledgements:* We thank Paul Johns (Abbott Nutrition) and Dr Robert G. Brannan (Ohio University, Division of Nutrition) for technical assistance.

*Disclosures/Conflicts of Interest:* S.M.G. is an employee of Abbott Nutrition, a division of Abbott Laboratories, Inc. At the time of study, N.K.E. was also an employee of Abbott Nutrition. Funding for this project was provided by Abbott Nutrition. K.W.M was the recipient of an Ohio University College of Health Science & Professions Graduate Assistantship.

*Ethical approval:* All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures were in accordance with the ethical standards of the Ohio University Institutional Animal Care and Use Committee, and the "Principles of Laboratory Animal Care" (NIH publication No. 86-23, revised 1985) were followed throughout the study.

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