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AN ANALYSIS OF THE EFFECT OF β-HYDROXY-β-METHYLBUTYRATE ON THE FLIGHT ABILITY AND LIFESPAN OF *DROSOPHILA MELANOGASTER*

A Thesis Presented

by

Alexandra Beattie

to

The Faculty of the Graduate College

of

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In Partial Fulfillment of the Requirements
for the Degree of Master of Science,
Specializing in Biology

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Accepted by the Faculty of the Graduate College, The University of Vermont, in partial fulfillment of the requirements for the degree of Master of Science, specializing in Biology.

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June 13, 2014

Abstract

As muscle function deteriorates with age, the discovery of new ways to enhance the quality of human life by stunting inevitable aging processes, such as sarcopenia, is a subject of great interest to aging populations, to health care professionals and to nutritional companies. β-hydroxy- β -methylbutyrate (HMB) enhances muscle strength in humans and attenuates disease-induced and disuse-dependent atrophy in rodents. We investigated the feasibility of utilizing *Drosophila* as a model organism to study the biological effects of HMB in aging muscle. Using flight ability as an index of muscle function and monitoring the lifespan of flies, we found that normal food supplemented with 10 mg/mL HMB, supplied from eclosion through adulthood, attenuates the age-dependent decline in flight ability and enhances longevity of flies.

To further discern the dietary optimization of HMB supplementation, we examined the effect of 10 mg/mL HMB supplementation from the larval stages throughout adulthood under various dietary conditions. As dietary restriction without malnutrition and HMB supplementation independently increase longevity in flies, we investigated the relationship between dietary restriction and HMB supplementation. Flies were subject to non-restricted, reduced yeast (protein), or intermittently cycled feeding regimens, with or without HMB.

Both modes of dietary restriction improved flight ability, while only cycling flies on and off food increased lifespan and improved survivorship of the flies. HMB supplementation increased flight ability later in life in all groups, but had differential effects on lifespan; HMB improved the survivorship of females fed a reduced yeast diet, but decreased the survivorship of both non-restricted and intermittently fed flies. This not only suggests that HMB may act via different pathways to influence fly flight and survivorship, but that these mechanisms may differ under various dietary conditions, in different sexes. Because HMB supplemented dietary restriction had different effects on flight ability and survival than dietary restriction alone, HMB likely acts via different mechanisms than dietary restriction. Sex specific effects were found, suggesting that future HMB and dietary restriction studies should make distinctions between effects in males and females.

As the flight ability and longevity of *D. melanogaster* was affected by HMB supplementation under various dietary conditions, future studies may use *D. melanogaster* as a model organism to explore the effects of HMB on age-related muscle deterioration and to help uncover the biological mechanisms of such observations, as well as potential treatments for age-associated muscle dysfunction and disease.

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Chapter 1. An Introduction to HMB Supplementation in Aging Muscle

1.1. Aging and muscle function

Aging is a ubiquitous process, characterized by the inevitable decline of organismal function over time that is thought to be the result of an accumulation of reactive oxygen species (ROS) in tissues via oxidative phosphorylation.[1] These ROS put oxidative stress on cellular systems, decreasing mitochondrial function, and decreasing sensitivity and overall activity of cellular signaling pathways essential for growth and metabolism.[2]

Resistance to oxidative stress is conferred through the activity of superoxide dismutases, catalases and glutathione-peroxidases. However, the antioxidant activity of these enzymes subsides with age, allowing accumulated ROS to disrupt chromosomes, destabilize membranes, denature proteins, promote protein aggregation in various tissues, including the brain and muscle, and deplete ATP stores.[1,3] Such activity increases organismal susceptibility to neurodegenerative and musculoskeletal disease states.[1] While the decline in cognitive ability with age is well documented, the mechanisms lie beyond the scope of this thesis. Relevant to this thesis are the muscular deficits associated with aging.

Muscle has many essential functions. It dictates movement and posture at the organismal level, and serves as the body's biomechanical sensor and metabolic hub at the cellular level, storing amino acids, regulating energy expenditure, thermogenesis, glucose homeostasis, insulin sensitivity, and systemic growth via endocrine and paracrine signaling. As organisms age, muscle function declines in tandem with systemic homeostasis. Increases in DNA and mitochondrial damage, ubiquitin-protein aggregation,

fiber atrophy, and apoptosis, as well as decreases in protein synthesis, protein degradation, and nuclear, sarcoplasmic reticulum, and sarcomere organization and integrity, contribute to the decline in muscle function and strength observed with age. [4]

The muscular deficits associated with aging invariably decrease daily functional ability, increase risk of injury, increase health-care costs, decrease quality of life, and increase mortality. Because the rate of aging is governed by both genetic and environmental factors, research efforts have focused on these factors as ways to attenuate the negative physical and socio-economic outcomes of the aging process.[1,4] While altering one's genome is not yet a realistic means to counter the rate of senescence, altering the effect of one's environment is. Good nutrition and exercise appear to influence gene expression and metabolic signaling pathways associated with aging in a positive manner. Making an effort to improve physical activity and nutritional intake may help diminish some of the negative side effects of aging.

The search for anti-oxidant, anti-cachectic, and anabolic dietary supplements has become a topic of interest for nutritional and biopharmaceutical companies. The development of products to promote healthy aging is becoming increasingly more important as humans today are living longer than ever before. With the ever-increasing size of the elderly population comes an increased demand to improve the quality of life of this population via the development of novel exercise and nutritional interventions.

1.2. Promoting healthy aging by enhancing muscle function through nutrition and exercise: role of the TOR signaling pathway

Sarcopenia, or the decrease in muscle mass and function with age, is one of the most common negative outcomes of the aging process.[1, 5] Muscle has many physical and

metabolic functions that dictate the quality of life for an individual. Thus, if the rate of muscle wasting can be curtailed, so too can associated negative physical and socio-economic side effects.[6]

The stability of muscle function and growth depends on cellular proteostasis. That is, there needs to be a balance between protein synthetic and degradation signaling pathways in the muscle in order for muscle to function properly.[7] This balance is dependent on the availability of amino acids at cellular concentrations.[2] Concentrations of amino acids are maintained either by dietary intake or cellular recycling; when amino acid levels are low, muscle proteins are broken down to maintain consistent cellular amino acid levels.[2] Both synthesis of functional proteins and proteolysis of damaged proteins are essential for optimal muscle function.[8]

It has been shown that amino acid supplementation, in combination with carbohydrate supplementation and resistance exercise, is able to enhance muscle protein synthesis.[9] Maximization of protein synthesis depends on the specific amino acid composition of the supplement, the protein source, the timing of administration, and the age and sex of the target populations. Supplements that contain a high concentration of leucine, among other essential amino acids, mixed with a carbohydrate, administered to young men immediately after resistance exercise, have been shown to be most effective in promoting muscle protein synthesis.[8] Although diminished, a positive response has also been seen in elderly populations,[8] suggesting that supplementation with essential amino acids, in tandem with resistance exercise, may be a means to promote healthy muscles in both young and old individuals.

At the cellular level, it is thought that leucine rich/essential amino acid supplements act through the TOR signaling pathway.[10] The target of rapamycin complex 1 (TORC1) is the central signaling hub of the TOR pathway; various signaling pathways converge on this complex. TORC1 is sensitive to physiological stresses and changes in energy, nutrient, or growth factor levels, responding to the convergent signaling of insulin, growth factor, Wnt, glucose, cytokine, and amino acid receptors.[11]

Input from these upstream signals allows TORC1 to regulate its two prominent downstream targets, p70S6-Kinase (p70S6K) and 4E-Binding Protein (4E-BP), which enhance and repress translation, respectively.[10] Other downstream targets are known to regulate cell growth, proliferation, survival, apoptosis, energy metabolism, lipid synthesis, lysosome biogenesis, and cytoskeletal structure (Figure 1.1).[11] Thus, the TOR pathway plays a critical regulatory role in both muscle function and aging, linking nutrient sensing to protein synthesis, cell growth, autophagy and apoptosis. Understanding the mechanisms through which TOR modulates aging and muscle protein synthesis is important if advancements in identifying potential therapeutic targets for age-related disease are to be implemented.

As previously mentioned, the branched chain amino acid, leucine, is thought to play a significant role in promoting muscle growth by up-regulating mTORC1, thereby increasing protein synthesis, and inhibiting autophagy and proteolysis of muscle protein.[9,10] Exactly how cells sense leucine and other amino acid signals, and how signals interact with mTORC1, remains unclear and requires further research.

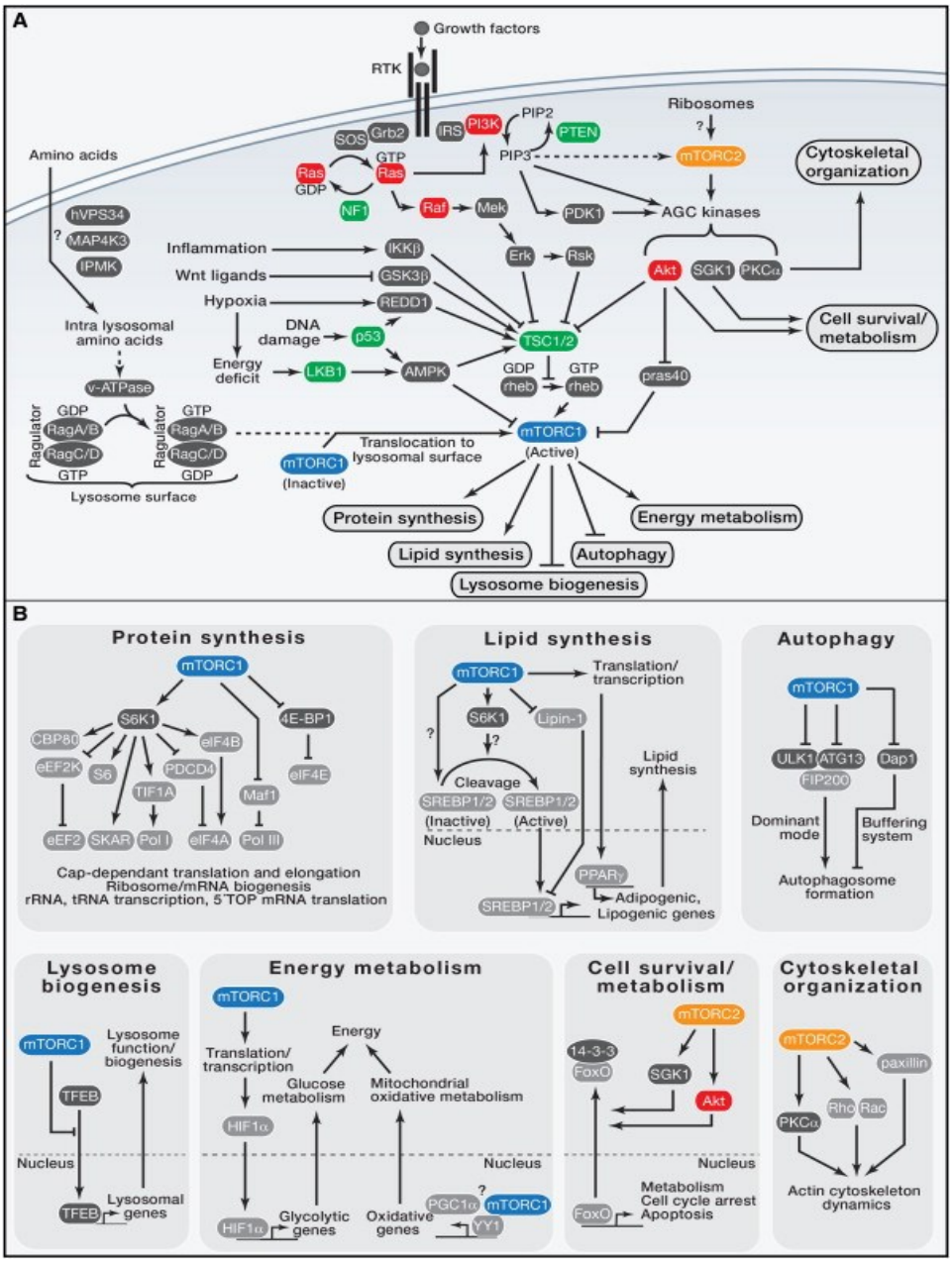

Figure 1.1: The mTOR pathway. (A) mTOR is the primary serine/threonine kinase that distinguishes mTORC1 and mTORC2. The mammalian target or rapamycin complex 1 (mTORC1) (shown in blue), is a nutrient and energy sensitive protein complex that is regulated by various upstream signaling pathways. Growth factors, amino acids, cytokines, oxygen availability, and energy levels dictate the activation of these signaling pathways, thus regulating mTORC1 and subsequent downstream effectors to either promote or inhibit protein synthesis, lipid synthesis, autophagy, lysosome biogenesis, and energy metabolism.[9] Unlike mTORC1, mTORC2 (shown in yellow) is insensitive to energy and nutrients and plays a role in regulating cell metabolism and cytoskeletal organization. (B) Activated mTOR complexes (mTORC1 and mTORC2) signal various downstream effectors to promote or inhibit protein synthesis, lipid synthesis, autophagy, lysosome biogenesis, and energy metabolism. Figure reprinted from reference 11.

When metabolized, leucine breaks down into α-ketoisocaproate (KIC); the KIC intermediate is further metabolized into isovaleryl-CoA or β-hydroxy-β-methylbutyrate (HMB).[12] HMB is of particular interest as it, like leucine, has been shown to produce positive effects in muscle mass and strength in several rat and human trials.[13,14,15-22, 28-35, 40-47] These studies present HMB as an anabolic and anti-catabolic molecule that it is able to recover muscle under cachectic and atrophic conditions, as well as prevent muscle wasting.

Because HMB appears to produce similar outcomes as leucine, the question of whether HMB acts as the metabolite responsible for the phenotypes of leucine supplementation arises.[12] Furthermore, because only 5% of leucine is metabolized into HMB [13] (Figure 1.2), if HMB truly is the driving force of anabolic/anti-catabolic effects of leucine supplementation, then HMB would be a more potent and efficient nutritional supplement than leucine.

1.3. Physical effects and potential molecular mechanisms of β-hydroxy-β-methylbutyrate supplementation

1.3.1. Various outcomes of HMB supplementation

HMB is found naturally in some foods, such as catfish, citrus fruit, avocado, alfalfa, cauliflower and meat, and has been marketed as an anabolic/anti-catabolic nutritional supplement by companies such as Abbot Nutrition. For the last 2 decades, HMB has been used primarily by young athletes and body builders to improve various parameters of muscle health.

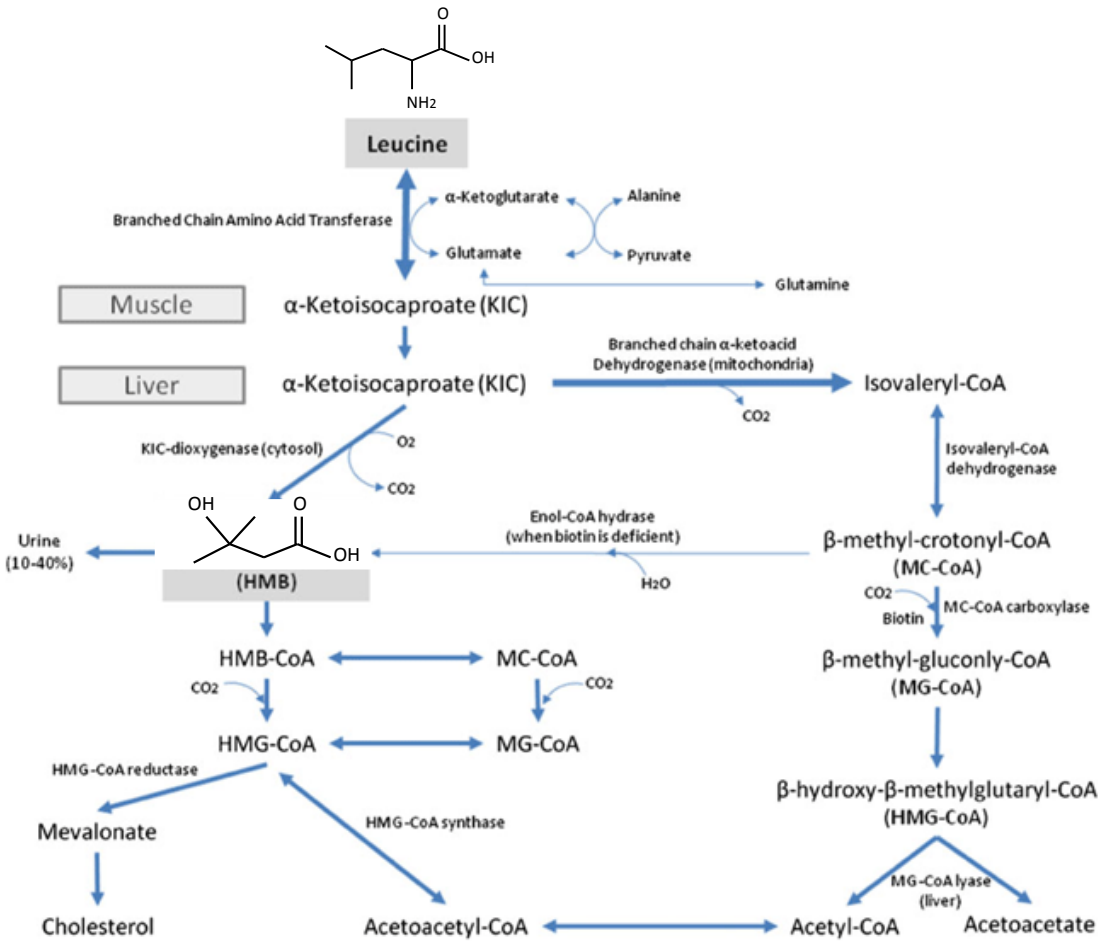

Figure 1.2: Metabolism of Leucine and HMB. HMB is a precursor in the synthesis of cholesterol and Acetyl-CoA. Figure modified from reference 19.

Many studies have shown that HMB supplementation, at a dose of 3g/day, is sufficient to improve muscle strength and function, increase lean body mass, and decrease fat mass in young untrained and trained, male and female, adults subject to various aerobic or resistance exercise regimens (Table 1.1).[15-22] Other studies, however, do not report any difference in muscle performance with HMB supplementation. The studies that showed no effect were performed in trained male populations, suggesting that HMB may affect untrained populations to a greater extent than trained populations, and that this effect may occur in a sex specific manner (Table 1.2).[23-27] It remains unclear whether HMB has a differential effect in males versus females, however, as most studies only examined the effect of HMB in male populations, and of the studies that examined the effect of HMB in both male and female populations, none make any comparison of its effect in different sexes.

More recently, the question of whether HMB could be used to promote muscle health in elderly individuals, susceptible to sarcopenia and cachexia, has been posed. Table 1.3 summarizes several studies that have reported significant improvements in muscle strength, functional mobility, lean body mass and fat mass in elderly populations supplemented with HMB, with or without exercise.[28-35] It appears that HMB has similar effects in elderly as it does in young, untrained populations. Studies by Clark *et al* and May *et al* have shown HMB, in combination with arginine and glutamine, to increase lean body mass (LBM) in other populations susceptible to sarcopenia and cachexia, such as HIV and cancer patients.[36-38]

As mentioned, not only has HMB been used in combination with exercise regimens, but it has been used in combination with other supplements, including arginine,

lysine, glutamine, or creatine, in both young, aged, and diseased populations (Table 1.4).[30, 31, 33, 36-39] Most studies compare the effects of the "supplement-cocktail" to a control, without examining the effects of the cocktail to an HMB treated group, making it difficult to draw conclusions about how effective HMB was in producing the observed effects in these studies. Interestingly, many studies that provide individuals with a supplement cocktail produce similar effects, including increases in LBM, strength and functional mobility, as well as decreases in creatine kinase and muscle damage, as studies that provide individuals with an HMB supplement alone.

There are a broad range of methodologies used in the field of HMB research, exemplified by the studies summarized in Tables 1.1-1.5. Factors such as age, sex, dose, timing of dose, type of exercise, timing of dose relative to exercise, levels of exercise intensity, level of participant training, levels of supervision during training interventions, use of secondary supplements, and type of effects monitored differ substantially from study to study.[14] Although methodologies vary, the majority of study outcomes are similar, suggesting that HMB prevents further decline in wasting muscle. How exactly HMB exerts such physical effects, that is, what molecular mechanisms underlie its effects, remains unclear.

Table 1.1. Studies in young subjects that report improvements in body composition and performance with HMB supplementation

Study	Subject/Age	Dose HMB/ Duration	Exercise	Outcome of HMB Supplementation
Nissen, 1996[15]	Trained and Untrained Males 18-24 y/o	1.5 - 3 g/day 3 & 7 weeks	Resistance	• Decrease in exercise induced muscle proteolysis (decrease CK, LDH and 3MH) • Increase LBM and strength
Knitter, 2000 [16]	Males and Females 20-50 y/o	3g/day 6 weeks	Aerobic	• Decrease creatine phosphokinase and LDH response • Helps prevent exercise-induced muscle damage
Gallagher, 2000 [17]	Untrained Males 18-24 y/o	3 or 6 g/day 8 weeks	Resistance	• Increase in LBM • Slight increase in strength • Slight decrease in FM • No difference between 3 and 6 g dose
Panton, 2000 [18]	Untrained Males and Females 20-40 y/o	3g/day 4 weeks	Resistance	• Reduce CK levels • Increase LBM and upper body strength • Decrease FM
Lamboley , 2007 [19]	Active Males and Females 18-24 y/o	3g/day 5 weeks	Aerobic	• Enhanced aerobic capacity
Thomson, 2009 [20]	Trained Males 18-24 y/o	3g/day 9 weeks	Resistance	• Increase lower body strength
Portal, 2010 [21]	Trained female volleyball players 13-18 y/o	3g/day 7 weeks	Resistance and Aerobic	• Increase LBM • Increase power and strength • Decrease FM
Wilson, 2014 [22]	Trained Males 20 y/o	1 g x3 / day 12 weeks	Resistance	• Increase LBM • Increase strength • Increase vertical jump power • Prevents increases in CK and cortisol during high intensity resistance training

CK: Creatine Kinase. LDH: Lactate Dehydrogenase. 3MH:3-methyl-histidine. LBM: lean body mass. FM: fat mass.

Table 1.2. Studies in young subjects that report no effect on body composition or performance with HMB supplementation

Study	Subject/Age	Dose HMB/ Duration	Exercise	Outcome of HMB Supplementation
Kreider, 1999 [23]	Trained Males 18-24 y/o	0/3/6 g/day 4 weeks	Resistance	• No change in strength, LBM or FM
Slater, 2001 [24]	Trained Males 18-24 y/o	3g/day 9 weeks	Resistance	• No change in strength, body composition or molecular markers
Ransone, 2003 [25]	Male Football Players 18-24 y/o	3g/day 4 weeks	Resistance	• No change in strength (bench press, squats, and power cleans) or body composition (body weight and body fat)
Hoffman, 2004 [26]	Male Football Players 18-24 y/o	3g/day 10 days	Resistance and Aerobic	• No change in strength or testosterone, cortisol, or myoglobin levels
O'Connor, 2007 [27]	Male Rugby Players 25 y/o	3g/day 6 weeks	Resistance	• No change in strength or endurance

LBM: lean body mass. FM: fat mass.

Table 1.3. Studies in elderly populations that report improvements in body composition and performance with HMB supplementation

Study	Subject/Age	Dose HMB/ Duration	Protocol	Outcome of HMB Supplementation
Vukovich, 2001 [28]	Males and Females 70 y/o	3g/day 8 weeks	Resistance	• Increase LBM and upper and lower body strength • Reduce FM
Coelho and Carvalho, 2001 [29]	Hypercholesterolemic Males 50-72 y/o	3g/day 4 weeks	Resistance	• Increase LBM • Increase Strength • Decrease LDL cholesterol
Flakoll, 2004 [30]	Females 65+ y/o	2 g/day 12 weeks	Additional supplements (arginine and lysine)	• Increase protein synthesis • Increase LBM • Increase functional mobility (GUG test) • Increase lower body strength
Baier, 2009 [31]	Sedentary Males and Females 75-77 y/o	2-3 g/day 1 year	Additional supplements (arginine & lysine)	• Increase LBM and protein turnover • No change in strength or function
Hsieh, 2010 [32]	Bedridden, Tube-fed Males and Females 65+ y/o	2g/day 4 weeks	No exercise or additional supplement	• Decrease blood and urine urea nitrogen excretion
Fuller, 2011 [33]	Males and Females 75-77 y/o	2-3 g/day 1 year	Additional supplements (arginine, lysine, vitamin D)	• Increase LBM with HMB cocktail • Increase strength with Vitamin D supplementation
Deutz, 2013 [34]	Males and Females 60-76 y/o	1.5g x2/day 10 weeks	Resistance	• Prevent decline in LBM during 10 days of bedrest • Variable baseline strength/function
Stout, 2013 [35]	Males and Females 65+ y/o	3g x 2 /day 24 weeks	Resistance	• Increase strength and "muscle quality" with and without resistance exercise

LBM: lean body mass. FM: fat mass. LDL: low density lipoprotein.

Table 1.4. Effects of HMB supplementation in combination with other supplements on muscle health parameters in various populations

Study	Subjects	Dose HMB/ Duration	Additional Supplement	Exercise	Outcome of HMB Supplementation
Clark, 2000 [36]	Male and Female HIV patients (>5% weight loss in last 3 months)	1.5g x2/day 8 weeks	+14g/day arginine +14g/day glutamine	Not monitored	• Increase LBM • Increase body weight • Increase immune function (# C3+ and C8+ cells)
Jowko, 2001 [37]	Active Males 18-24y/o	3g/day 3 weeks	+ 20g creatine/day 10 days +10g creatine/day 14 days	Resistance	• Decrease CK and blood and urine urea nitrogen • Increase LBM and strength
May, 2002 [38]	Cancer patients	3 g/day 24 weeks	+14g/day arginine +14g/day glutamine	Not monitored	• Increase LBM
Flakoll, 2004 [30]	Elderly Females 65+ y/o	2 g/day 12 weeks	+ 5g/day arginine + 1.5g/day lysine	Not monitored	• Increase protein synthesis • Increase LBM • Increase functional mobility (GUG test) • Increase lower body strength
Van Someren, 2005 [39]	Untrained Males 18-25y/o	3g/day 2 weeks	+0.3g/day KIC	Resistance	• Prevent decreases in 1RM • Prevent increases in CK (Prevent muscle damage)
Baier, 2009 [31]	Sedentary Males and Females 75-77y/o	2-3 g/day 1 year	+L-arginine +L-lysine	Not monitored	• Increase LBM and protein turnover
Fuller, 2011 [33]	Males and Females 75-77y/o	2-3 g/day 1 year	+ 5-7.5g/day arginine +1.5-2.25g/day lysine)	Not monitored	• Increase LBM with HMB cocktail • Increase strength with Vitamin D supplementation

LBM: lean body mass. CK: creatine kinase. GUG: Get up and Go. RM: repetition maximum.

1.3.2. Potential mechanisms of HMB

Various experimental models have been used to investigate the biochemical mechanism of HMB (Table 1.5).[40-47, 50] The three predominant hypotheses regarding the mechanism of HMB are that HMB increases muscle protein synthesis, prevents muscle protein degradation, or supports muscle cell proliferation and differentiation to produce net gains in muscle mass.

As a derivative of leucine, the question of whether HMB acts through insulin/TOR signaling pathways to enhance muscle anabolism arises. Eley *et al* carried out a study in murine myotubes and found that HMB was able to rescue protein synthesis inhibited by proteolysis inducing factor (PIF).[40] This study also found that rapamycin, a potent inhibitor of the TOR pathway, inhibited HMB induced protein synthesis, suggesting that HMB acts through TOR signaling. Furthermore, mTOR, p70S6K, and 4E-BP1 phosphorylation was increased in the presence of HMB, resulting in increased protein synthesis.[40] Another study by Fanelli *et al* showed that HMB supplementation increased phosphorylated p70S6K and phosphorylated mTOR in the gastrocnemius of cachexic mice, again suggesting that HMB plays a role in the enhancement of mTOR signaling.[41] Interestingly, Smith *et al* showed that HMB supplementation increased protein synthesis and decreased proteasome activity, both of which may occur through an up regulation of TOR, in the gastrocnemius and soleus muscles of a cancer-induced mouse model.[42]

Other than causing an increase in protein synthesis and/or decrease in protein degradation via TOR, HMB may also increase proliferation of myoblasts to generate net gains in muscle. Kormasio *et al* examined HMB's involvement in the PI3K/Akt and

MAPK/ERK signaling pathways; both pathways signal TOR to promote muscle cell proliferation and survival. In their study, the addition of HMB to serum-starved human myoblasts leads to increased MAPK/ERK phosphorylation, IGF-I transcription, PI3K interactions with tyrosine phosphorylated proteins, Akt phosphorylation, MyoD mRNA expression, MRF expression, inhibition of apoptosis and increased cell proliferation[43], suggesting that HMB may work upstream of TOR to prevent apoptosis and promote muscle cell proliferation and differentiation.

Two separate studies by Hao *et al* [44] and Supinski and Callahan[45] showed decreases in levels of cleaved caspase-3 with HMB supplementation in atrophied mouse soleus and plantaris after hind limb suspension,[44] and in the diaphragm of septic mice.[45] Hao *et al* found decreases in additional apoptotic proteins, including Bax and caspase-9 with HMB supplementation. Both studies suggest that HMB may protect against apoptotic signaling to increase muscle cell proliferation.[44,45] It is not known, however, whether these effects occurred via TOR signaling.

It is possible that HMB does not act via the TOR signaling pathway, and instead, acts via IGF-1/FOXO signaling to prevent protein degradation. Increased IGF-1 results in phosphorylated Akt, which negatively regulates FOXO, thus decreasing Atrogin-1 transcription and ubiquitin ligase activity.[12] In support of this hypothesis are studies by Wilson *et al* and Park *et al;* both have shown that HMB supplementation decreases Atrogin-1 mRNA expression in old and calorically restricted mice, respectively.[46,47] Smith *et al* found that HMB supplementation caused the weight of soleus muscle to increase and E2 ubiquitin ligase activity to decrease in the gastrocnemius of normal and cachexic mice.[42] Another study conducted by Smith *et al,* [48] similar to the

aforementioned study conducted by Eley *et al,* [40] found that HMB attenuates increases in proteolysis induced by PIF in mouse myotubes, lending support to the hypothesis that HMB may interfere with ubiquitin-proteasome activity.

Two lesser supported hypotheses consider the role of HMB metabolites in producing the previously described enhanced muscle phenotypes. One hypothesis suggests that HMB metabolites may improve mitochondrial respiration, while the other proposes that they improve sarcolemmal integrity; both may improve muscle function.[13] HMB is a precursor in the pathway of cholesterol synthesis (Figure 1.2). Before HMB is metabolized into cholesterol, it is first converted into HMB-CoA, which is converted into HMG-CoA, which is converted into either mevalonate or acetoacetyl-CoA. Mevalonate can be converted into cholesterol, or can eventually metabolized into coenzyme Q.[12] Coenzyme Q plays a role in both myocyte proliferation and the electron transport chain, thus, presumably, and increase in HMB would cause an increase in CoQ, which may support myocyte proliferation and respiration. Additionally, because acetoacetyl-CoA is converted into acetyl-CoA,[12] which feeds into the citric acid cycle, producing electron carriers for the electron transport chain, HMB supplementation may indirectly enhance aerobic respiration. Vukovich and Dreifort found that trained cyclists supplemented with HMB increased their time to VO2 max and VO2 at 2mM blood lactate, suggesting that HMB may positively influence respiration and performance.[49]

Being a precursor of cholesterol, HMB may be able to enhance the sarcolemmal membrane integrity by supporting cholesterol synthesis.[13] While possible, this hypothesis is unlikely in light of a study performed on hypercholesteremic patients, where HMB supplementation surprisingly lowered total and LDL levels of cholesterol.[29]

In summary, there are several potential mechanisms through which HMB may exert its effects (Figure 1.3). Because the molecular mechanism of HMB remains unknown, further research in an effective model organism is needed to more accurately pinpoint the mechanism of HMB supplementation, before HMB can be confidently marketed by nutritional companies.

Table 1.5. Effects of HMB supplementation in experimental animal models

Study	Test Organism	Dose HMB	Outcome of HMB Supplementation
Smith, 2005 [42]	Mouse cancer cachexia model	2, 5, 10 g/day	• Increase soleus weight of normal and CC mice • Decrease proteasome activity/decrease protein degradation in GSN • Decrease E2 Ubiquitin enzyme • Increase protein synthesis (increase Phe recycling/g muscle/ 2 hours)
Eley, 2007 [40]	Mouse myotubes	0, 25, 50μM	• Attenuates PIF inhibited protein synthesis (by preventing autophosphorylation of PKR and phosphorylation of eIF2α and eEF2) • Rapamycin inhibits HMB stimulated protein synthesis • Increases mTOR, S6K, and 4EBP1 phosphorylation • Increases association of eIF4G with eIF4E • Reduces eEF2 phosphorylation
Eley, 2008 [50]	Mouse myotubes	0, 25, 50 μM	• HMB attenuates LPS, TNFα, and ANGII inhibited protein synthesis
Kornasio, 2009 [43]	Human, Chick and Mouse myoblasts	0, 10, 20, 50, 100 μM	• Induces myoblast proliferation via MAPK/ERK pathway • Induces PI3K/Akt phosphorylation necessary to promote differentiation of myoblasts • Reduces muscle cell apoptosis induced by serum starvation or staurosporine-treatment • Promotes synthesis of IGF-I mRNA
Hao, 2011 [44]	Rat muscle fiber atrophy model	13-15g/day	• Increase CSA of plantaris and soleus fibers • Prevent decrease in maximal isometric force during recovery • Decrease Bax protein, cleaved caspase-3 and cleaved caspase-9 during reloading
Fanelli, 2011 [41]	Mouse cancer cachexia model	4% of food	• Increased GSN and heart weight in normal and CC mice • Decrease p-eIF2α levels in normal mice • Increase phosphorylated p70S6K and phosphorylated mTOR in normal and CC mice
Wilson, 2012 [46]	Rat aging model	15-20g/day	• Prevent increase in FM in middle aged rats • Decrease FM in old aged rats • Maintain grip strength in young and middle aged rats • Increase grip strength in old and very old rats • Maintain myofiber size as rats age • Decrease Atrogin-1 mRNA expression in very old rats • Increase myogenin in very old rats
Park, 2013 [47]	Trained or Trained/ Calorically Restricted Mice	20 g/day	• Increased LBM and sensorimotor function in trained mice • Maintained grip strength in trained CR mice • Increase GSN mass and myofiber CSA in trained CR mice • Decrease Atrogin 1 mRNA expression, but not protein expression, in GSN of CR mice
Supinski, 2014 [45]	Male mouse sepsis (LPS) model	150μmol/kg	• Prevent sepsis-induced activation of caspase 3, 20S proteasome and PKR • Did not prevent sepsis-induced calpain activation • Prevent sepsis-induced diaphragm weakness/preserve diaphragm strength

CC: cancer cachexia. GSN: gastrocnemius. PIF: proteolysis inducing factor. PKR: protein kinase R. eIF: eukaryotic initiation factor. eEF: eukaryotic elongation factor. mTOR: mechanistic target of rapamycin. S6K: S6 Kinase. 4EBP: 4E binding protein. LPS: liposaccharide. TNFα: tumor necrosis factor α. ANGII: angiotensin II. MAPK: mitogen activated protein kinase. ERK: extracellular-signal regulated kinase. PI3K: phosphoinosotide-3-kinase. Akt: protein kinase B. IGF: insulin growth factor. CSA: cross sectional area. FM: fat mass. LBM: lean body mass. CR: calorically restricted.

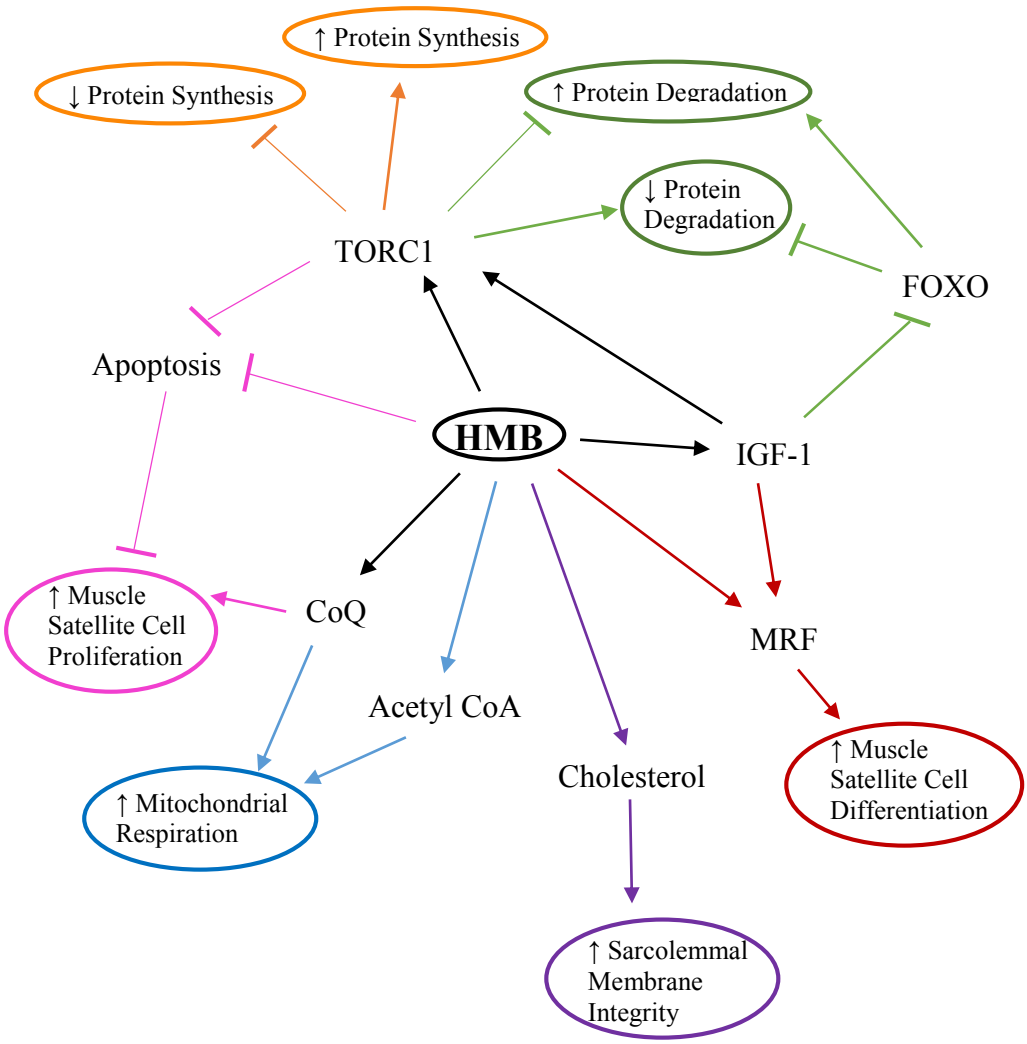

Figure 1.3. Summary of the potential mechanisms of HMB. HMB may enhance muscle health by promoting protein synthesis, preventing protein degradation, promoting muscle cell proliferation and differentiation, increasing mitochondrial respiration, and/or increasing muscle cell membrane integrity.

1.4. *Drosophila melanogaster* as a model organism

Before significant gains can be made in understanding the mechanistic action of HMB in muscle, we must first find a reliable and cost-effective live animal model that is amenable to genetic manipulation.

D. melanogaster has proven to be an extremely useful model organism for many studies.[51] Fruit flies are much less expensive and easier to maintain than mice or rats, and have a short lifespan with a quick generation time.[51] Stocks are easy to sustain and their large brood numbers provide experiments with power.[52] The genetics of *D. melanogaster* are efficient and well-established, and findings in *D. melanogaster* are applicable to various other organisms, including humans, due to the conservation of the majority of physiological pathways among animal genomes.[51,53,54] Similarities between mammalian skeletal muscle and *Drosophila* flight muscle allow for the examination of conserved mammalian mechanisms through fruit fly investigations.[52]

Several studies used *D. melanogaster* as a model organism to examine the effects of anti-oxidizing, or anti-oxidizing enhancing, dietary supplements, such as Carnosine[55], Creatine[56], Curcumin[57], Epicatechin[58], and even blueberry extract[59], on processes of aging. All studies found that supplementation was able to mediate negative effects of oxidative stress, to some extent, in *D. melanogaster*, which encourages the use of *D. melanogaster* in future nutritional supplementation and age-related studies.

For these reasons, the use of *Drosophila* as a test organism in this post-genomic era is becoming more common. Their conserved metabolic pathways offer the opportunity to test hypothesized pharmacological and nutritional treatments prior to their

testing in more costly and time-consuming mammalian models, to ultimately identify similar treatment in humans.[51,54] If this project can identify *D. melanogaster* as an appropriate model organism to explore the effects of HMB on age-related muscle deterioration, then future studies may focus on uncovering the biological mechanisms of such observations, as well as potential treatments for age-induced muscle dysfunctions.

1.5. Objectives

Decreased flight ability and increased mortality in *D. melanogaster* are associated with increased age and subsequent age-dependent muscle deterioration.[52] β-Hydroxy-β-Methylbutyrate is thought to stunt the loss of muscle function in aging organisms. While a number of studies have suggested that nutritional supplementation with HMB in rats, mice, and humans can negate various negative side effects of aging, such as sarcopenia, the extent to which HMB influences muscle atrophy in *Drosophila melanogaster* has not yet been explored.

The overarching goal of this research is to determine whether *D. melanogaster* may be used as an appropriate test organism for studies that question the effects of HMB on muscle sustainability, so that in the future, cost-effective studies may be conducted to elucidate the genetic basis of HMB action. Because of the high degree of homology between fly and human disease genes, as well as the well-established genetics of *D. melanogaster*, uncovering conserved molecular mechanisms in *D. melanogaster* that nutritional supplements, such as HMB, act through may allow for the development of nutritional treatments for cachectic, age-related muscle dysfunctions and disorders in humans.

As flight ability can be used as proxy for muscle function, the specific aims of this project are to determine whether supplementing fly food with various concentrations of HMB will 1) delay the onset of age-related flight loss in *D. melanogaster* when administered from eclosion to death, 2) retard the loss of muscle-dependent flight ability when administered from median-age to death, 3) delay the onset of age-related flight loss when administered from the larval stages throughout adulthood, and 4) delay the onset of age-related flight loss when administered from the larval stages throughout a calorically restricted adult life.

Chapter 2. Journal Article: β-Hydroxy-β-Methylbutyrate extends lifespan and attenuates age-dependent loss of flight ability in *Drosophila*

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2.1. Abstract

Muscle function deteriorates with age. β-hydroxy- β -methylbutyrate (HMB) enhances muscle strength in humans and attenuates disease-induced and disuse atrophy in rodents. We investigated the feasibility of utilizing *Drosophila* as a model organism to study the biological effects of HMB in aging muscle. Using flight ability as an index of muscle function, we found that HMB attenuates the age-dependent decline in flight ability. Both male and female flies fed a diet supplemented with 10 mg/ml HMB (MB10) had higher flight scores than control flies fed a normal diet (MB00) after median age ($p<0.01$). Significant differences in flight ability were found in week 6 when comparing male flies fed 5mg/ml HMB (MB05) v 1 mg/ml HMB (MB01) ($p<0.01$), but not when comparing female flies. Unexpectedly, MB10 increased maximum lifespan by ~15%. The life extension effect was seen in females (12%) and males (17%). MB10 increased the median age of males ($p<0.05$) but not of females. A similar lifespan extension effect was seen when comparing MB05 to MB01 ($p<0.01$); however, there was no difference in the median age between the two groups. We propose that HMB attenuates the age-dependent decline in flight ability and prolongs lifespan by influencing muscle health.

2.2. Introduction

The aging process is characterized by overall decline in bodily functions. A progressive decline in muscle function with age has been observed in organisms ranging from fruit flies to humans. The decline in muscle function is largely attributed to sarcopenia, or loss of muscle mass[4], but a decrease in muscle strength independent of muscle mass (dynapenia) is also considered a major contributor.[60] The muscular deficits associated with aging invariably decrease daily functional ability, increase risk of injury, decrease quality of life, increase health-care costs, and increase mortality. Thus, if the rate of decline in muscle function can be curtailed, so too can associated physical and socio-economic consequences. Because the rate of aging is governed by both genetic and external factors, research efforts have focused on these factors as ways to attenuate the negative physical and socio-economic outcomes of the aging process. Two significant interventions, exercise and protein (amino acid) supplementation, have been shown to influence muscle health in young and old adults, separately and in combination, by enhancing muscle protein synthesis.[8,61]

Maximization of protein synthesis depends on the specific amino acid composition of the supplement, the protein source, the timing and dose of administration, and the age and sex of the target populations. Supplements that contain a high concentration of leucine, among other essential amino acids, mixed with a carbohydrate, administered to young men immediately after resistance exercise, have been shown to be most effective in promoting protein synthesis.[8] Although diminished, a positive response was also seen in elderly populations, suggesting that supplementation with essential

amino acids, in tandem with resistance exercise, may be a means to promote healthy aging in both young and old individuals.[8]

The development of products to promote healthy aging is becoming increasingly more important as the general increase in awareness of the benefits of a healthy lifestyle, as well as advances in medical technology over the past 50 years, have enabled humans to live longer than ever before. Muscle function and growth is dependent on the availability of amino acids at cellular concentrations to promote protein synthesis. Concentrations of amino acids are maintained either by dietary intake or cellular recycling; when amino acid levels are low, proteins in muscle cells are broken down into their constituent amino acids to maintain consistent cellular amino acid levels [62]. Both synthesis of functional proteins and proteolysis of defective proteins are essential for optimal muscle function.[8]

The leucine metabolite β-hydroxy-β-methylbutyrate (HMB) has been shown to have a positive effect on muscle function in several rat[44,46] and human trials.[15-22, 28-35] The molecular mechanism of HMB remains controversial. HMB has been proposed to have an anabolic effect and an anti-catabolic effect that is able to recover muscle under cachectic and atrophic conditions, as well as prevent muscle wasting. Several studies indicate HMB acts through the TOR pathway to induce protein synthesis[40] and more recent studies have indicated an effect suppressing protein degradation.[48]

The effects of HMB on exercise performance in humans are also controversial. While many studies have shown a positive effect, other studies have not.[24] These differences can be partly explained by the age and sex of test subjects, as well as the levels of exercise, type of exercise, dose and timing of HMB administration, that test

subjects are exposed to in each study.[14] Additional studies are needed to pinpoint the molecular mechanism and physiological effects of HMB administration, particularly with respect to the elderly.

Here, we use *Drosophila melanogaster* to examine the effect of HMB supplementation on muscle function with age. *D. melanogaster* has proven to be an extremely useful model organism for studies of human diseases and aging due to the high conservation of genetic, regulatory, physiological, and metabolic pathways.[51,53,63,64] Specifically, similarities between mammalian skeletal muscle and *Drosophila* indirect flight muscle allow for the examination of conserved mechanisms that could reveal targets for therapeutic and nutritional interventions. We show that a dose of 10mg/mL HMB in fly food, supplied from eclosion to death, is sufficient to provoke a physiological response in *D. melanogaster* that attenuates the age-associated decline in flight ability, and extends lifespan, in a sex-dependent manner. In light of these results, we propose that *D. melanogaster* serves as a reliable, rapid, and cost-effective model organism to further investigate the biological mechanism of HMB action.

2.3. Materials and Methods

2.3.1. Fly Stocks

The Oregon R (OR) wild-type strain of *Drosophila melanogaster* was used to test the effects of HMB on fly flight and lifespan. Three broods of stock flies were maintained in separate 200 ml plastic bottles containing standard cornmeal fly food (5.4 g/L agar, 12.8 g/L yeast, 60.9 g/L cornmeal, 8% (v/v) molasses, and 1.3% (v/v) propionic

acid), and were kept in a room set at 20ºC, 70% humidity, and a 12/12 h light/dark cycle throughout the course of the study.

2.3.2. Study design

Adult flies were collected from bottles upon eclosion (day 1) and transferred to fifteen 35-ml plastic vials containing standard cornmeal fly food with no HMB (MB00; control), or standard cornmeal fly food containing 1 mg HMB/mL (MB01), 5mg HMB/mL (MB05), or 10mg HMB/mL (MB10). CaHMB (Lonza) was obtained from Abbott Nutrition. The 1mg/mL dose corresponds to approximately a molar "double dose" of leucine found in the yeast extract of fly food. We conducted four separate trials, two comparing MB00 to MB10 and another two comparing MB01 to MB05.

For Study I, the HMB supplemented diet was started at day 1. For the Study II, the HMB supplemented diet was started at approximately the median age determined from the first study (Figure 2.1).

2.3.3. Flight Performance

Flies were tested for flight ability beginning at day 3 and once every week thereafter. For each treatment group, a total of 100 flies were maintained in groups of 20 (10 males and 10 females) in 35 ml vials. To perform the flight test, all lights in the designated room were turned off, except for a single column of light that shone down into the Plexiglas flight chamber; this single light source provided the flies with a stimulus to encourage upward flight. Flies were tested individually and their flight path was scored as up (6), horizontal (4), down (2), or not at all (0).[65] Flies were retrieved and retested a

total of six times. At least ten flies (~5 males and 5 females) were tested for each time point and the average group flight score was calculated.

2.3.4. Lifespan

Each treatment group consisted of approximately two hundred flies (100 males and 100 females) divided proportionally into ten food vials. To track longevity, flies were transferred every other day to new food vials and the number of flies that died since the last transfer was recorded. The process was repeated until all flies died.

2.3.5. Statistical Analyses

The median and maximum lifespans of each treatment group were determined from generated lifespan curves. Median age of each treatment group was reported with the data set's associated interquartile range (IQR). Because flies were transferred every other day, both the median and maximum lifespans were reported as ±1day. Median tests and log-rank tests were used to compare the median ages and overall survival of MB00 and MB10 treatment groups, as well as MB01 and MB05 treatment groups. Flight scores were recorded as mean ± SE. A one-way analysis of variance (ANOVA) was used for flight data comparing more than two age groups, Student t-tests were used to determine age differences between two groups (MB00 v MB10 and MB01 v MB05), and a Student-Neuman-Keuls post hoc test was used for data comparing more than two groups. All statistical tests were performed using JMP Pro10 Software. Results were considered significant at $p < 0.05$.

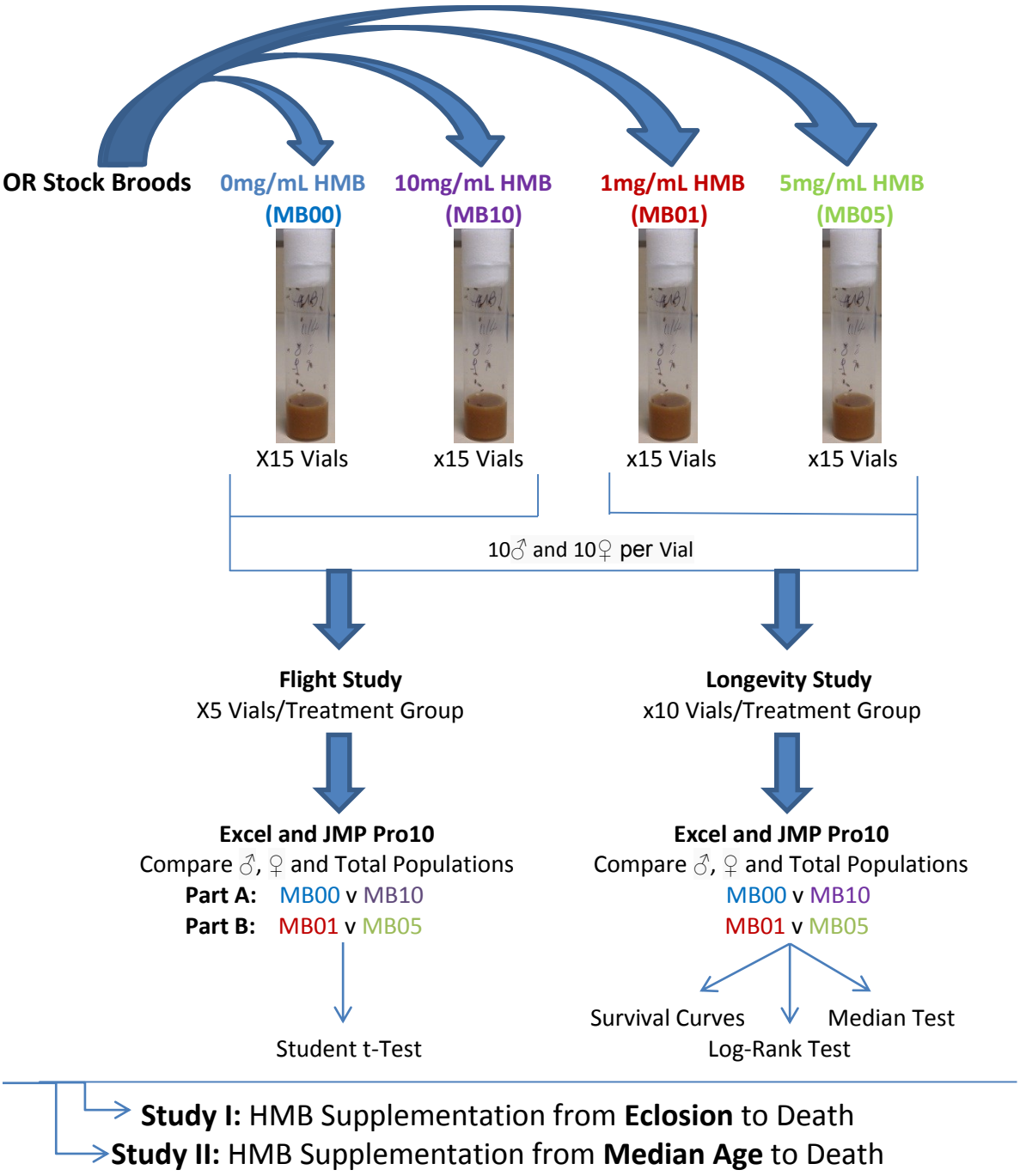

Figure 2.1. Experimental outline of Study I and II. For Study I, CaHMB feeding began at the onset of adult life. For Study II, CaHMB feeding began at approximately adult median age. In Study I, MB00 and MB10 treatment groups were tested together and statistically compared, while MB01 and MB05 treatment groups were tested together and statistically compared. Study II only compared MB00 and MB10 treatment groups.

2.4. Results

Figure 2.1 presents an overview of the study design. The effect of HMB on flight ability and lifespan was examined through adult lifetime feeding (Study I) or feeding starting at adult median age (Study II). Study I was divided in two parts. In Part A, flies maintained in normal food without HMB (MB00) were compared to flies maintained in food with 10 mg/ml HMB (MB10). In Part B, flies maintained in normal food with 1mg/mL HMB (MB01) were compared to flies maintained in food with 5 mg/ml HMB (MB05).

2.4.1. Effect of HMB supplementation on flight performance

Flies lose their flight ability with age due to increased muscle damage.[52] To determine if HMB has any effect on delaying the decline in flight performance, we measured flight ability in flies maintained in food with and without HMB. In this assay, flight scores range from 0 (flightless) to 6 (upward flight).

Figure 2.2 shows the average flight scores of MB00 (no dose) and MB10 (high dose) flies from Study IA, trials 1 and 2 combined. Supplementary Table I provides the levels of significance associated with comparisons made in each trial separately and combined. Consistent with a previous study, a progressive decline in flight performance was observed with age.[52] Significant differences in flight ability of the total populations of MB10 and MB00 groups were found during weeks 3, 4, and 6. Flies raised in 10 mg/ml HMB had a more robust ability to fly when compared to flies in normal food, with flight scores of 5.0±0.1 vs 4.3±0.2 during week 3 ($p<0.01$), 3.7 ± 0.2 vs 2.0 ± 0.2 during

week 4 ($p<0.0001$), and 0.38 ± 0.1 v 0.0±0.0 during week 6 ($p<0.001$) (Figure 2.2A, Supplementary Table I).

When separated by gender, differences in flight ability between MB10 and MB00 flies were detected in males during weeks 4 (4.4 ± 0.4 vs 3.2 ± 0.3; $p<0.01$) and 6 (0.7± 0.2 vs 0.0± 0.0; $p<0.01$) (Figure 2.2B, Supplementary Table I), and in females during weeks 1 through 5 (5.7 ± 0.1 vs 5.4 ± 0.2 during week 1 ($p<0.05$), 4.5 ± 0.2 vs 5.4 ± 0.1 during week 2 ($p<0.01$), 5.1 ± 0.1 vs 4.4 ± 0.3 during week 3 ($p<0.05$), 2.9 ± 0.2 vs 0.9 ± 0.2 during week 4 ($p<0.0001$), and 0.5 ± 0.2 vs 0.0 ± 0.0 during week 5 ($p<0.0001$), for MB10 and MB00 flies, respectively) (Figure 2.2C, Supplementary Table I). Not only did male and female middle aged flies fed 10mg/ml HMB have enhanced flight ability, but they were able to retain flight ability for one week longer than their respective controls (Figure 2.2).

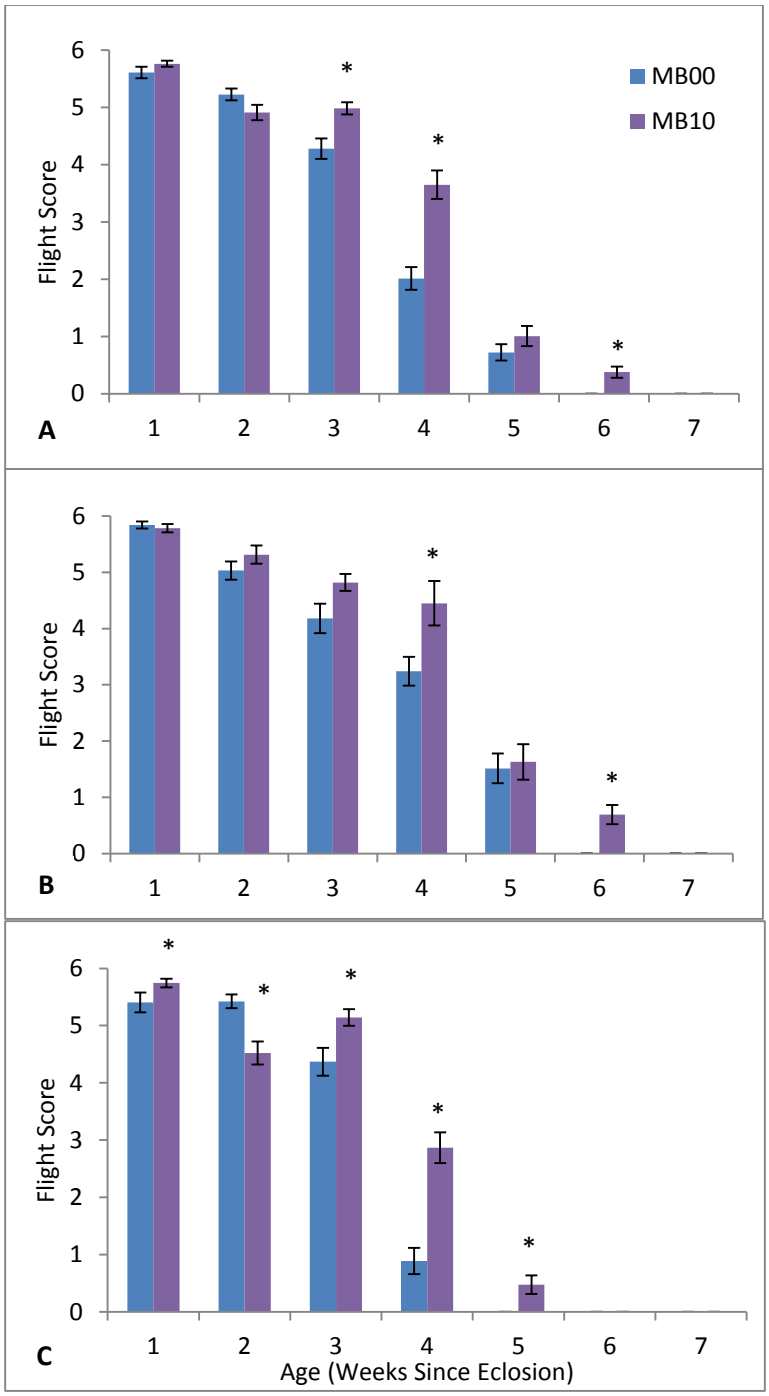

Figure 2.2. Flight Study IA: Effect of HMB high dose on flight ability. Trials 1 and 2 combined. Average flight scores of MB00 and MB10 treatment groups per week since eclosion. (A) Significant differences in flight ability between the total populations of MB00 and MB10 flies were found in weeks 3 ($p = 0.0025$), 4 ($p < 0.0001$), and 6 ($p = 0.0008$). (B) Significant differences in flight ability between MB00 and MB10 male flies were found in weeks 4 ($p = 0.0045$), and 6 ($p = 0.0020$). (C) Significant differences in flight ability between MB00 and MB10 female flies were found in weeks 1 ($p = 0.0454$), 2 ($p = 0.0020$), 3 ($p = 0.0178$) and 4 ($p < 0.0001$), and 5 ($p < 0.0001$).

Part B of Flight Study I compared the flight ability of MB01 and MB05 (medium dose) treatment groups. Significant differences in flight ability were observed in the combined trials between the male populations in weeks 1 (5.9±0.1 vs 5.5±0.1 for MB01 and MB05 respectively; $p<0.001$), 2 (5.6±0.1 vs 4.8±0.3 for MB01 and MB05 respectively; $p<0.01$), and 6 (1.2±0.2 vs 0.3±0.1 for MB05 and MB01, respectively; $p<0.0001$). Young males fed the MB01 dose had better flight indexes than males fed the MB05 dose during weeks 1 and 2 (Figure 2.3). However, the higher MB05 dose was better at ameliorating the decline in flight ability with age, as evidenced by the significant difference in flight ability observed during week 6, and a better, though not statistically different, flight ability in week 5 (Figure 2.3). No difference in flight ability was observed when comparing total and female populations (data not shown).

Figure 2.4 represents the results from Flight Study II, showing the effect of a high dose of HMB on flight ability when supplemented from median age. The group that was switched to food containing HMB at middle age retained flight ability to a greater extent than the control group at week 5 (1.3±0.1 vs 0.8±0.3) and at week 6 (0.5±0.1 vs 0.3±0.1). However, these differences were not statistically significant. Significant differences observed in weeks 1 and 3, before HMB was supplemented to one of the two groups, suggests that population differences may be masking an HMB effect.

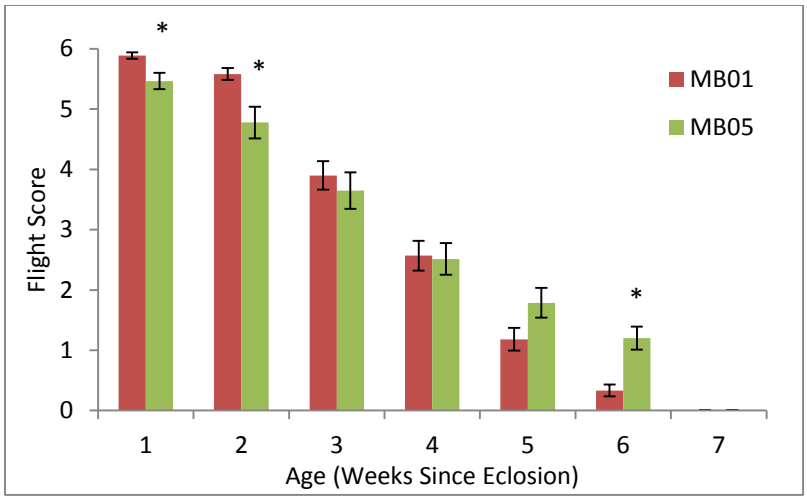

Figure 2.3. Flight Study IB: Effect of HMB medium dose on flight ability. Trials 1 and 2 combined. Average flight scores of MB01 and MB05 males per week since eclosion. Significant differences in flight ability between MB01 and MB05 male flies were found in weeks 1 ($p=0.0005$), 2 ($p=0.0016$), and 6 ($p=0.0001$). No significant differences were found between the female populations.

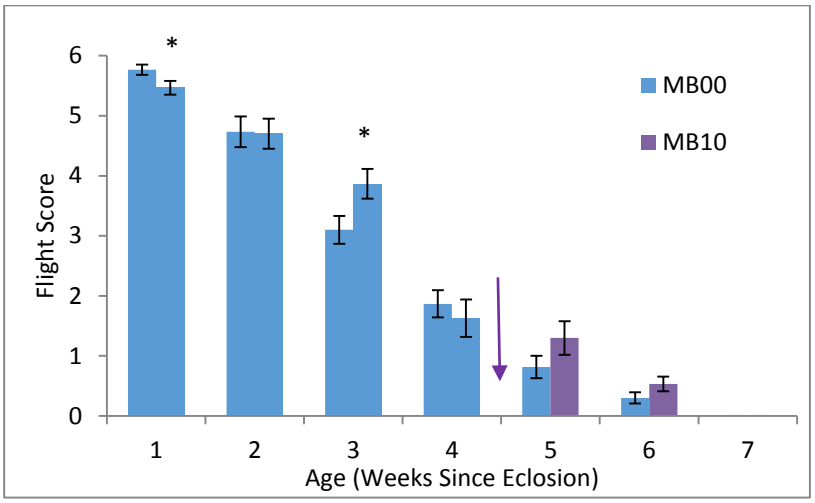

Figure 2.4: Flight Study II—Feeding HMB after middle age has no effect on flight ability. Average flight scores of MB00 and MB10 per week since eclosion. MB10 treatment group food supplementation with HMB at day 34 (purple arrow). No significant difference in flight ability was found between the groups after HMB supplementation from week 5 to death.

2.4.2. Effect of HMB supplementation on lifespan

Figure 2.5A shows the average data from two separate trials comparing the lifespan of the MB00 and MB10 groups. There was a significant difference in the median lifespan between the two groups (39 days for MB00 vs 40 days for MB10, $p<0.05$; Table 2.1), but no difference in the survival distribution, as determined by the logrank test (Figure 2.5B). Significant differences in overall survival between MB00 and MB10 male populations (Figure 2.5C) and total populations (Figure 2.5D) ($p<0.05$) were observed in one trial. Separating the groups by sex revealed a significant difference in median age among males ($p<0.05$), but not females (data not shown), for the two trials combined.

Supplementation with HMB at 10 mg/ml increased maximum lifespan by nearly 15% (77 ± 1 days MB10 vs 66 ± 1 days MB00; Figure 2.5A and Table 2.1). Extended longevity was seen in females (+8 days, or ~12% lifespan extension) and more prominently in males (+11 days, or ~17% lifespan extension; Table 2.1).

Part B of Study I compared flies maintained in food supplemented with HMB at 1 mg/ml (MB01) and flies maintained in food supplemented with HMB at 5 mg/ml (MB05). The median lifespan was similar for the two groups while the maximum lifespan was greater for both male and female flies in the MB05 group (53± 1 days for MB01 vs 58± 1 days for MB05; Table 2.2). The higher HMB dose extended maximum lifespan by ~9% when compared to the lower HMB dose. A comparison of the lifespan curves by

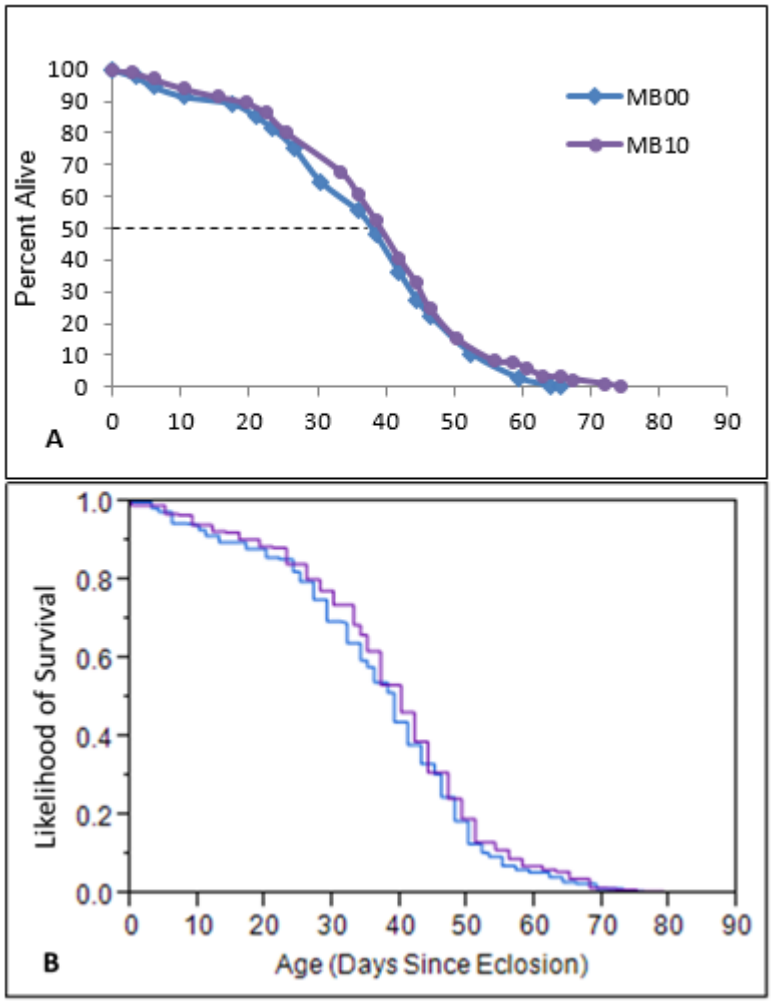

Figure 2.5. Lifespan Study IA—Effect of HMB high dose on lifespan. Average of Trials 1 and 2. (A) Survival curves of total population of MB00 and MB10 treated flies. The median age of the MB00 treatment group was 39±1days; its maximum lifespan was 66±1days. The MB10 treatment group had a median age of 40±1days and a maximum lifespan of 77 ±1days. (B) No difference in overall survival was observed between total populations.

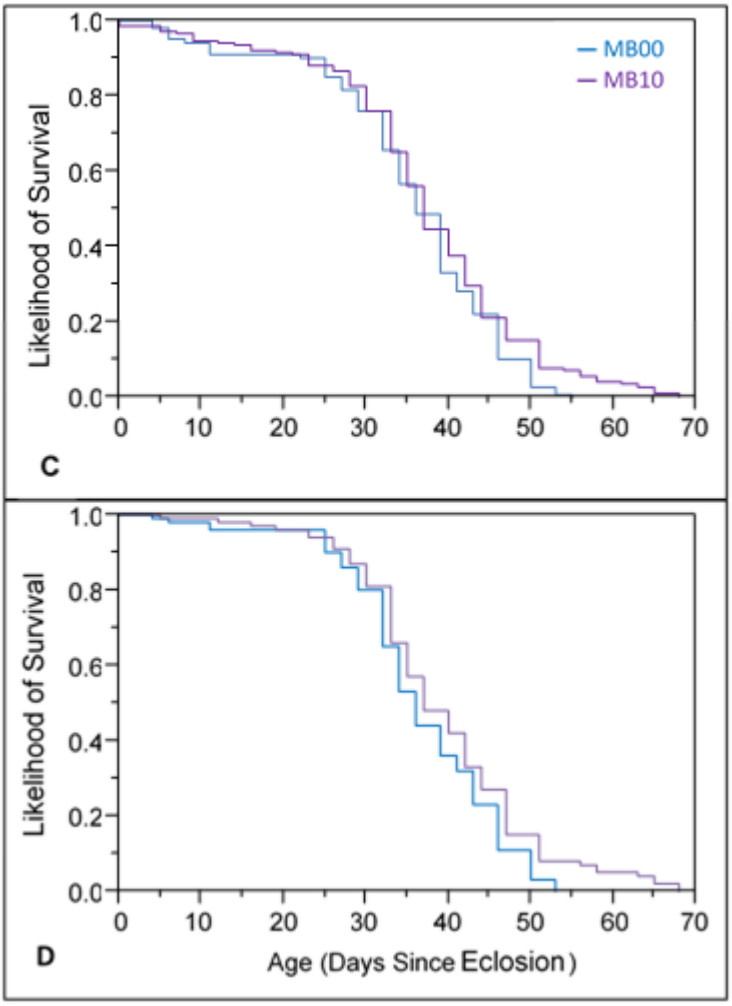

Figure 2.5 (continued): Lifespan Study IA—Effect of HMB high dose on lifespan. Trial 2. (C) Survival plot comparing total populations of MB00 and MB10 treatment groups. The log-rank test revealed that a significant difference in survival existed between MB00 and MB10 treatment groups (p=0.0099). (D) Survival plot comparing male populations of MB00 and MB10 treatment groups. The log-rank test determined that a significant difference in survival existed between MB00 and MB10 males (p=0.0196).

Table 2.1. Summary of Median and Maximum Lifespan – Study IA						
	Median Age (Interquartile range) (± 1 Day)			Maximum Age (± 1 Day)		
Test Group	Male	Female	Total	Male	Female	Total
MB00	38 (19.5)	39 (17.5)	39 (17)	66	64	66
MB10	42 (16)	37 (19)	40 (17)	77	72	77

Table 2.2. Summary of Median and Maximum Lifespan – Study IB						
	Median Age (Interquartile range) (± 1 Day)			Maximum Age (± 1 Day)		
Test Group	Male	Female	Total	Male	Female	Total
MB01	32 (16)	32 (23)	32 (19)	53	53	53
MB05	34 (12)	32 (16)	32 (16)	58	58	58

the logrank test found differences for the total population ($p<0.001$) (Figure 2.6A) and for the male population ($p<0.05$) (Figure 2.6B) but not for the female population (results not shown).

Study II examined the effect of HMB administered at approximately median age and throughout the remainder of lifespan. A subset of flies was switched from a normal diet to a diet supplemented with 10mg/ml HMB at day 34, near the median age determined in Study I (Table 2.1). As expected, the median age of both groups was the same, 36±1 days for the control group MB00 and 35±1 days for the experimental group MB10. There was no difference in maximum lifespan between the two groups (52±1day for MB00 vs 53.5± 1 days for HMB10). No difference in median age or maximum lifespan was detected when the groups were separated by sex (data not shown).

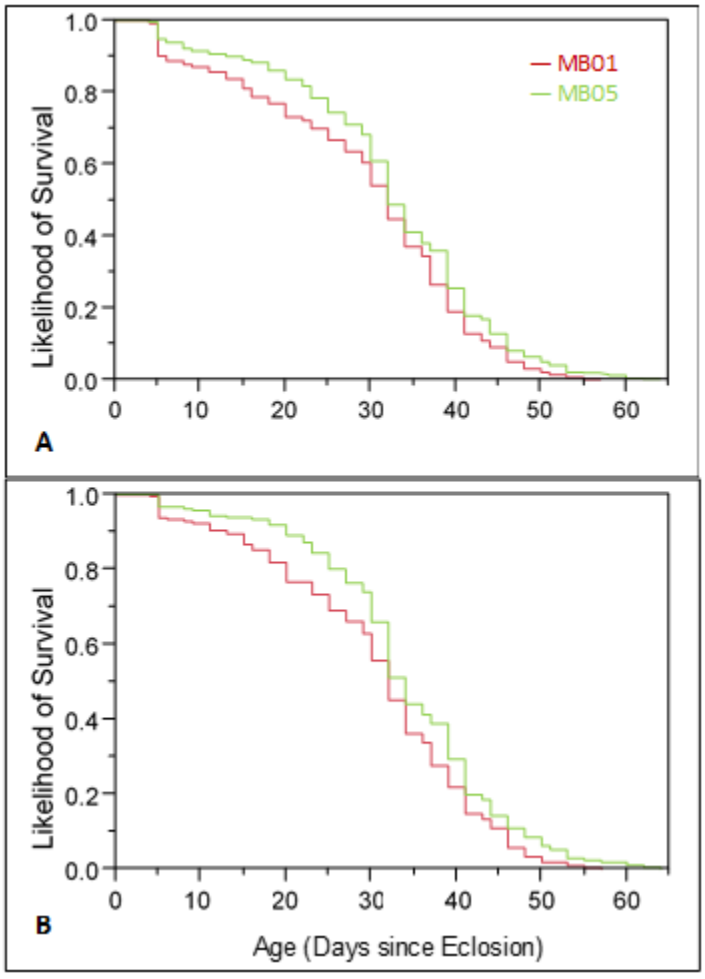

Figure 2.6. Lifespan Study IB—Effect of HMB medium dose on lifespan. Average of trials 1 and 2. (A) Survival plot comparing total populations (male and female combined) of MB01 and MB05 treatment groups. The log-rank test showed that a significant difference in survival was observed between the total populations of MB01 and MB05 treatment groups (p=0.0014). (B) Survival plot comparing MB01 and MB05 male treatment groups. The log-rank test showed that a significant difference in survival exists between MB01 and MB05 males (p=0.0056).

2.5. Discussion

In this study, we described for the first time, the use of *Drosophila melanogaster* to study the effects of an HMB supplemented diet on muscle function and aging. We found that HMB supplementation better retains flight ability in aging flies when compared to a normal diet, and unexpectedly, extends lifespan in a dose and sex-dependent manner when supplied from eclosion to death. Retention of flight ability and lifespan extension of aged flies requires HMB supplementation from the onset of adult life.

The effects of HMB on flight ability in both males and females was most pronounced when supplied at the high dose of 10mg/mL. Additionally, old males (> 4 weeks) responded more strongly to HMB supplementation than old females, especially at the medium dose of 5 mg/ml.

Conversely, an enhanced flight phenotype was observed before median age (< 4weeks) in females supplemented with 10mg/mL HMB. These gender differences could be the result of different feeding habits. Young mated females are known to consume more food than males, suggesting that the effect seen at median age may result from a greater intake of HMB.

Whether these sex-specific differences are caused by differential, sex and age specific mechanisms, or whether these effects are simply due differential HMB consumption by the two sexes, remains unclear. Future studies will monitor food and HMB consumption of the flies.

Drosophila now joins a group of organisms, including humans, for which HMB has been shown to have a positive effect on muscle function. The decline in flight ability with age in *Drosophila* has been shown to correspond with changes in flight muscle structure and contractile properties.[52] Thus, improved flight ability of old flies fed HMB may result from improvements in muscle function. This, combined with the fact that the structure and metabolic mechanisms of *Drosophila* indirect flight muscle are largely conserved with the structure and metabolic mechanisms of muscle in other organisms, including human skeletal muscle, allows the findings of this study to be compared to others that have shown HMB to have a positive effect on muscle of other species. This provides us with a means to investigate whether HMB yields similar effects in *Drosophila* muscle as it does in the muscle of other organisms, namely in humans.

The finding that 10mg/mL of HMB supplied from eclosion through adulthood enhances and prolongs flight ability in *D. melanogaster* corresponds with findings from several studies in rats and humans that have shown improvements in muscle health parameters with HMB supplementation. Hao *et al* suspended the hind limbs of rats to induce muscle atrophy and found that HMB was able to diminish the extent of muscle fiber loss and myonuclear apoptosis in these atrophied rats.[44] Similarly, Wilson *et al* found that HMB was able to ameliorate losses in strength and myofiber dimension in aging rats.[46]

In human trials, HMB has been able to prevent decreases in lean body mass associated with 10 days of bed rest.[34] It has been able to prevent muscle damage induced by long runs[16] and has been shown to increase lean body mass and decrease percent body fat of elderly people (70+ years) participating in a strengthening program.[28] However,

other studies have found HMB to have no effect on muscle mass and strength in human trials[24] which calls for the development of additional, long term studies to accurately elucidate the biological effect of HMB.[66] The results presented here, highlighting that HMB extends flight performance in aging flies, provide evidence for the use of *Drosophila* in future studies attempting to understand the biological effect of HMB.

Currently, there are several hypotheses regarding the molecular mechanism through which HMB supports the functional preservation phenotypes previously described in muscle. Of these hypotheses, the most supported involves signaling through the TOR pathway. TOR is a serine/threonine kinase that is part of the larger protein complex, TORC1, which acts as a central nutrient signaling hub downstream of the insulin receptor.[11] TORC1 regulates many cellular processes, including protein synthesis, autophagy, cell growth, proliferation, survival, energy metabolism, lipid synthesis, lysosome biogenesis, and cytoskeletal structure.[11] An up-regulation of TORC1 results in an increase in protein synthesis and a decrease in autophagy. These anabolic and anti-catabolic signals may result in net muscle gains, which may translate to improved muscle functionality.

An opposing hypothesis is that HMB supports proteostasis in aging muscle, promoting a balance between muscle protein synthesis and protein degradation to generate enhanced muscle integrity and performance later in life. Demontis *et al* has shown that the decline in aging muscle function in *Drosophila* is tied to an accumulation of protein aggregates in the muscle with age, and that FOXO-mediated autophagy in the muscle not only maintains aged muscle integrity by decreasing protein aggregation locally, but helps delay the accumulation of proteins in other tissues as well via decreased

insulin signaling.[7] In this sense, muscle proteostasis may dictate systemic aging and age-impaired functionality.

In this study, HMB may influence metabolism by improving lifetime muscle proteostasis, yielding improved functionality in aging muscle; HMB's flight-enhancing effect may be mediated by an increase in autophagy and decrease protein aggregate accumulation in aging muscle tissue, possibly due to increased FOXO signaling or decreased TOR signaling.

While many studies support the efficacy of HMB supplementation in promoting gains in LBM and muscle strength, no studies have reported an effect on lifespan. This study found that higher concentrations of HMB not only prolong the flight ability of aged flies, but surprisingly, increase the lifespan of *Drosophila melanogaster* when supplied from eclosion through adulthood as well.

The criteria of the log rank and median tests were met in the lifespan studies, that is, data were independent, the distributions of data of each treatment group had similar shapes, censoring in the log rank test was not dependent on the treatment, and all groups had an equally likely chance of dying. However, several discrepancies between log-rank and median test results were found. While the median ages of MB00 and MB10 groups of the combined trials were significantly different, the log-rank test reported no difference in overall survival. Conversely, while the median ages of the MB01 and MB05 groups of the combined trial were not significantly different, the log-rank test revealed differences in overall survival. These divergent results are explained by the fact that median and maximum ages are not the only parameters that can characterize a survival

curve; other fluctuations in the survival curve, non-evident in reported median and maximum ages, may contribute to differences observed in overall survival.

A comparison of maximum lifespan showed more consistent results than the logrank and median tests. The following comparisons are observational, as there is no known method to statistically compare maximum lifespans. Maximum age followed a similar trend in each trial, only when HMB was supplemented from eclosion. This trend was maintained when the two trials were combined: the MB10 treatment group lived 15% longer than the control group and the MB05 group lived 9% longer than the MB01 group, suggesting that higher concentrations of HMB may more effectively prolong lifespan than lower concentrations (Table 2.1 and 2.2).

In humans, the most commonly administered daily dose of HMB is 3g/day.[14] A couple studies examined the effects of 6 g HMB/day, however, no additional effects on LBM, FM or strength were reported when compared to a dose of 3g/day.[17,23] The 3g HMB dose is equivalent to the amount of HMB that would be produced through the metabolism of about 60 g of leucine, which is about 10 times the amount leucine consumed in a normal diet.[12] It is estimated that flies eat 1-2ul of food/day,[67] and therefore, consume about 0.01-0.02 mg HMB/day when fed a diet supplemented with a high dose of HMB. This high dose of HMB supplementation (10mg/ml) is equivalent to the breakdown of about 20 times the normal amount of leucine present in standard fly food. Relatively, the high dose of HMB in this experiment is double the dose commonly provided in human studies. It appears that high doses of HMB in both humans and *Drosophila*, though optimally different, enhance muscle health.

With this, it can be concluded that HMB, only when supplied in standard fly food from eclosion throughout adulthood, has an effect on longevity and flight phenotypes. In particular, a concentration of 10mg/mL HMB has a more distinct effect on longevity and flight phenotypes than any other concentration of HMB tested in Studies I and II. 10mg/mL HMB increases longevity for both male and female flies, and appears to enhance flight ability in both males and females around median age, with an effect observed before median age in females only. Thus, it appears that HMB supplementation impacts both lifespan and flight ability of *D. melanogaster* in a dose and sex-dependent manner.

This extended lifespan phenotype was unexpected, especially given that HMB was found to help flies retain flight ability as well; an increase in muscle anabolism and flight ability would presumably require an increase in metabolic activity, while an increase in longevity would presumably require a decrease in metabolism. Because muscle anabolism and longevity are the result of opposing signals in the TOR pathway, how could HMB simultaneously enhance both muscle function and longevity if it is working through the TOR pathway? One hypothesis is that HMB's flight-enhancing and lifespan prolonging effect is mediated by an increase in autophagy and decrease protein aggregate accumulation in aging muscle tissue, due to increased FOXO signaling and/or decreased TOR signaling.[7,11] In this case, both the healthspan and lifespan of flies may be positively regulated through improved muscle proteostasis.

If the enhanced flight and longevity phenotypes observed in this study were the result of increased autophagy and enhanced muscle proteostasis via decreased TOR

signaling, then perhaps the HMB supplemented flies experienced a form of dietary restriction, or decreased caloric intake without malnutrition[68-71]. This limitation of nutrients is thought to cause a shift in metabolism to ensure survival, redirecting the use of energy from growth and reproduction, to general maintenance of somatic cells. The decrease in metabolism may have life-preserving and life-prolonging effects.[70] While there is still much to learn of the mechanistic basis of dietary restriction, it is currently thought to be mediated by the insulin/TOR signaling pathway in many species, including yeast, worms, flies and mice.[69,72,73]

Because TORC1 is energy and nutrient sensitive, dietary restriction may decrease TORC1 signaling to produce a lifespan extension phenotype. Kapahi *et al* found that overexpression of the TORC1inhibitor, TSC1/TSC2, as well as expression of TOR and S6K dominant negative alleles, resulted in lifespan extension in a nutrient dependent manner.[68] Interpreting these studies, it is possible that a decrease in TOR signaling through dietary restriction may both increase autophagy to preserve muscle proteostasis, and increase lifespan.

Although it is uncertain whether HMB supplementation induces dietary restriction (directly or indirectly), one possibility is that HMB is distasteful to the flies, causing MB10 and MB05 flies to eat less than the MB00 and MB01 flies they were respectively compared to. In such a case, MB10 and MB05 flies may take in less nutrients, decreasing metabolic activation via the insulin signaling/TOR pathway, increasing lifespan and increasing flight ability by enhancing muscle proteostasis, relative to MB00 and MB01 flies. To address this question, follow-up studies should monitor food intake by one of several quantitative assays.[67] Other factors that have been shown to directly influence longevity and muscle

function, e.g. exercise and genetic factors, would also need to be controlled for in future studies that examine the effect of HMB in *Drosophila.*

The study discussed in this paper had several limitations. There was no way to monitor the amount of food that flies were eating, which would have been useful to know in order to more precisely compare the effects observed in treatment groups, i.e. to discern whether one group ate more than another, and whether dietary restriction is an applicable theory in these studies. Additionally, the fly lines were not isogenized, which may have contributed to some variation in phenotype. Finally, because regular exercise is a factor of muscle strength and functionality, the fact that there was no way to control the exercise levels of the flies in this preliminary study may have been limiting. Follow-up studies are addressing these limitations.

Because the molecular mechanism of muscle anabolism through HMB supplementation remains controversial, and because this is the first study to have shown an increase in flight ability and longevity with HMB supplementation in *D. melanogaster*, the question of how HMB exerts its effects at the molecular level is one that remains to be answered through future experimentation.

As HMB has an effect on *D. melanogaster,* this study sets the stage for future studies to assess the genetic basis of HMB action by using a cost-effective and genetically manipulable test organism. This study adds to others that have examined the effect of nutritional supplementation on Drosophila lifespan and health[1], namely studies that examined the effects of anti-oxidizing, or anti-oxidizing enhancing, dietary supplements, such as Carnosine[55], Creatine[56], Curcumin[57], Epicatechin[58], and even

blueberry extract[59], on processes of aging. All studies found that supplementation was able to mediate negative effects of oxidative stress, to some extent, in *D. melanogaster*.

Such results encourage the use of *D. melanogaster* in future nutritional supplementation and age-related studies. In the context of HMB research, such future studies may use *D. melanogaster* to address the questions of what relationship, if any, HMB has with dietary restriction, and what metabolic pathway HMB acts through to provide the enhanced longevity and flight phenotypes observed in this study. Effective findings in *D. melanogaster* may be translated to human studies to yield products or treatments that promise to enhance the quality of an aging-life.

2.6. Conclusions

We report that, consistent with studies in vertebrates, HMB supplementation in *Drosophila* attenuates the decline in muscle function with age. We also report the novel finding that HMB supplementation enhances lifespan. *Drosophila* are commonly used to test hypothesized pharmacological treatments prior to their testing in more costly and time-consuming mammalian models, to ultimately identify pharmacological treatment in humans. As this project identified *D. melanogaster* as an appropriate model organism to explore the effects of HMB on age-related muscle deterioration, future studies may use *D. melanogaster* to help uncover the biological mechanisms of such observations, as well as potential treatments for age-associated muscle dysfunction and disease.

2.7. Acknowledgments

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Chapter 3. The effect of dietary restriction and HMB supplementation on the flight ability and survivorship of *Drosophila melanogaster*

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3.1. Abstract

Previous studies in the Vigoreaux lab showed that adult lifetime supplementation with 10mg/mL HMB attenuates the decline in flight ability and increases survivorship in *Drosophila melanogaster.* To further discern the dietary optimization of HMB supplementation, the following studies examined the effect of 10mg/mL HMB supplementation from the larval stages throughout adulthood under various dietary conditions. As dietary restriction without malnutrition and HMB supplementation independently increase longevity in flies, the following studies examined the relationship between dietary restriction and HMB supplementation. Flies were subject to non-restricted, reduced yeast, or intermittently cycled feeding regimens, with or without HMB. The flight ability of flies was monitored weekly and the number of flies dead per group was recorded daily in the cycled group, or every other day in the non-restricted and reduced yeast groups. Both modes of dietary restriction improved flight ability during or after week 3, while only cycling flies on and off food increased lifespan and improved survivorship of males (19% relative to the reduced yeast group; 11% relative to the non-restricted group) and females (16% relative to the reduced yeast group; 20% relative to the non-restricted group). HMB supplementation increased flight ability during or after week 6 in all groups, increased lifespan of reduced yeast females by10%, decreased lifespan of non-restricted males by 31%, and decreased lifespan of cycled males and females by 16% and 19%, respectively. These results suggest that HMB may act via different pathways to influence fly flight and survivorship and that different mechanisms may operate under various dietary conditions, in different sexes. Because HMB supplemented dietary restriction had different effects on flight ability and survival than

dietary restriction alone, HMB likely acts via a different mechanism than dietary restriction.

3.2. Introduction

As muscle function declines with age, so too does overall quality of life. Beta-hydroxy-beta-methylbutyrate (HMB) has been shown to enhance muscle performance and attenuate the progression of muscle wasting in mice and humans, however, the mechanism through this occurs remains controversial. For decades, *Drosophila melanogaster* has served as a powerful model organism for uncovering the genetic basis of aging,[74] muscle function,[75] and human diseases,[53] including neuropathies, myopathies and motor neuron diseases,[76] obesity,[77] heart disease,[78] and cancer.[79] Large brood numbers combined with its short lifespan (5 to 10 weeks) makes *Drosophila* an ideal model organism for statistically powerful, rapid and inexpensive testing of medical nutritional ingredients.

Two studies by the Vigoreaux lab (Study I and II, detailed in Chapter 2), showed that 10mg/mL HMB supplementation in food, when supplied from eclosion through adulthood to death, attenuates age-related decreases in flight performance and, surprisingly, significantly prolongs lifespan of *D. melanogaster*. These results encouraged the use of *D. melanogaster* in HMB research, allowing additional studies, questioning the optimal timing of HMB supplementation and the mechanism of HMB's effect on muscle function and longevity, to be pursued. Subsequent studies in the Vigoreaux lab (Studies III and IV, detailed in this chapter) addressed two questions that arose from the first two studies.

Study III further addressed the question of the dietary optimization of HMB supplementation in *Drosophila.* From Study I and II, 10mg/mL HMB was defined as the optimal dosage. It was determined that supplementation with HMB after median age to death has no effect, but supplementation with HMB from eclosion to death influences flight ability and lifespan of flies. Because larvae eat more than adults, lifetime consumption of HMB will be greater in flies supplemented with HMB as larvae than in flies not supplemented as larvae. It was hypothesized that, when supplied from the time eggs hatch into larvae, throughout adulthood to death, 10mg/mL HMB would enhance flight performance before mid-life, attenuate mid-life decline in flight performance, and prolong lifespan of flies. On another note, larvae eat more than adults and larval stages are characterized by periods of growth. This means that larvae may have very different metabolic demands than adults. Additionally, most larval tissues do not make it to adulthood, thus, it is quite possible that HMB supplementation from the larval stages through adulthood would have no additional effect on flight ability and survivorship.

Study IV began to address the question of how HMB may extend the lifespan of *Drosophila* populations. One possibility is that HMB's lifespan prolonging effect is mediated via dietary restriction. Dietary restriction without malnutrition has been documented to extend lifespan in various organisms including worms, flies and vertebrates.[69] Another possibility is that its lifespan-prolonging effect is not mediated through dietary restriction, but through mechanisms that induce similar effects as dietary restriction.

In flies, the two main approaches that have attempted to induce lifespan extension via dietary restriction involve diluting food concentration or depriving

flies of food in cycles. The food dilution approach is much more commonly employed. Before diluting the food, it must be determined whether the total caloric availability will be diluted (also known as caloric restriction), or whether a specific nutrient will be diluted. In *Drosophila*, restriction of a specific nutrient (ex: yeast/protein/amino acid and/or sugar/carbohydrate) appears to extend lifespan, and the optimal sugar:yeast (carb:protein) ratio for lifespan extension is different in males and females.[80,81] Because of this, one experiment of Study IV reduced the yeast content of standard fly food by 75% in an attempt to mimic dietary restriction conditions and produce an enhanced lifespan.

One drawback to this approach is that flies may engage in compensatory feeding; they may eat more food to achieve the same nutrient intake as they would normally.[82] For this reason, it is important to have a way of knowing how much flies are eating and what nutrients flies are taking in per meal. Various methodologies have tried to make accurate measurements of food consumption by measuring dye uptake via spectrophotometry,[83,84] fecal deposits, radiolabels in food media[82], body weight, number of proboscis extensions,[85] and loss of food from a capillary tube (CAFE assay).[86] It may also be interesting to measure metabolic rate, perhaps by measuring $C0_2$ production or the rate of female egg laying, as metabolic rate influences aging and longevity,[3] and may provide insight into the mechanistic basis of dietary restriction.

Intermittent/cyclical feeding does not seem to be a common way of restricting fly diet, which means that it is either an opportunity for further investigation or a dead end, assuming that it is unpopular in the literature for a reason; only a couple of older studies used intermittent feeding regimens in flies, but none extended lifespan.[87,88] In mammals,

while a disruption of circadian feeding patterns can lead to cancer, obesity and a reduction in lifespan, a resetting of circadian patterns can have beneficial effects on health and longevity.[89] Although the few studies that have been done did not show lifespan extension in intermittently fed flies, it is still possible that intermittent feeding may be a reasonable method to enhance lifespan, as the few studies that have been done in *Drosophila* may have disrupted, rather than reset, the circadian patterns in the flies.

It is known that 24 hours of starvation is too much; most flies died early on during an intermittent cycling study (24h on food/24h off food) performed by the Vigoreaux lab. It is known that flies maintained on a 12h/12h light/dark cycle feed primarily in the morning (Z0-Z2), less so in the evening (Z8-10), and very little throughout the night.[90] With this, another experiment of Study IV cycled flies 8 hours on, 16 hours off food every day to determine whether dietary restriction via a cycled feeding regimen could influence the healthspan and lifespan of the flies.

If HMB induces a form of dietary restriction in flies, HMB-fed flies would likely consume less food than normally fed flies and/or reset circadian patterns to live longer. If HMB has a more direct effect, it is possible that HMB fed flies will consume the same amount of food as normally fed flies but will live longer as HMB activates metabolic pathways that mimic those under conditions of dietary restriction. In Study IV, the healthspan (flight ability) and lifespan of a restricted diet supplemented with HMB was measured to determine whether HMB could extend lifespan beyond what was expected via dietary restriction alone.

3.3. Methods

3.3.1. Fly Stocks

The laboratory strain Oregon R (OR) of the common fruit fly *Drosophila melanogaster* was used throughout this study. Flies were raised in 200 ml plastic bottles containing standard cornmeal fly food consisting of 5.4 g/L agar, 12.8 g/L yeast, 60.9 g/L cornmeal, 8% (v/v) molasses, and 1.3% (v/v) propionic acid. The food was prepared every seven days and used within one week of preparation. Flies were maintained at 20°C, 70% humidity, and a 12/12 h light/dark cycle throughout the course of the study.

3.3.2. Study Design

Before collecting flies for Studies III and IV, ten bottles of regular and ten bottles of 10mg/ml HMB supplemented food were seeded with approximately fifty OR adult flies (2 to 5 days old) per bottle. Once populated, twenty (10 males and 10 females) newly eclosed (0-1 day old) flies were transferred from these brood bottles to each of 20 35mL vials of each regular or HMB supplemented diet. For each diet administered (detailed in the next sections), ten vials contained flies whose flight performance was tested once a week (labeled as flight test flies: "FT"), while the remaining ten vials contained flies whose lifespan was monitored (labeled as lifespan flies: "LS").

<u>Study III: Larval Supplementation Experiments</u>

Two different diets were administered in this experiment: (i) standard fly food and (ii) standard fly food supplemented with CaHMB (Lonza) at a concentration of 10 mg/mL. Each group was maintained on the same diet through larval and adult stages.

Study IV: Dietary Restriction Experiments

Two different Dietary Restriction Experiments were conducted in adults: the Reduced Yeast Experiment and the Cycled Food Deprivation Experiment. A non-dietetically restricted experiment was run in tandem to the cycled food and reduced yeast experiments, which served as an experiment itself, and as a control for the other two experiments (Study IV A). All flies were reared as larvae in food with or without HMB. HMB supplementation continued throughout the adult life of flies.

The Reduced Yeast Experiment (Study IV B) reduced the nutritional content of the available food, as detailed below. During the Cycled Food Deprivation Experiment (Study IV C), flies were cycled between standard fly food and a non-nutritional 1% agar gel; they were restricted (put on the non-nutritional media) from 6pm-10am, or in zeitgeber time, ZT10-ZT2 (two hours before "lights off" to two hours after "lights on"). Rather than reducing the nutritional content of the fly food to dietetically restrict the flies, as done in the Reduced Yeast experiment, the Cycled Food Deprivation Experiment restricted feeding to certain times of the day.

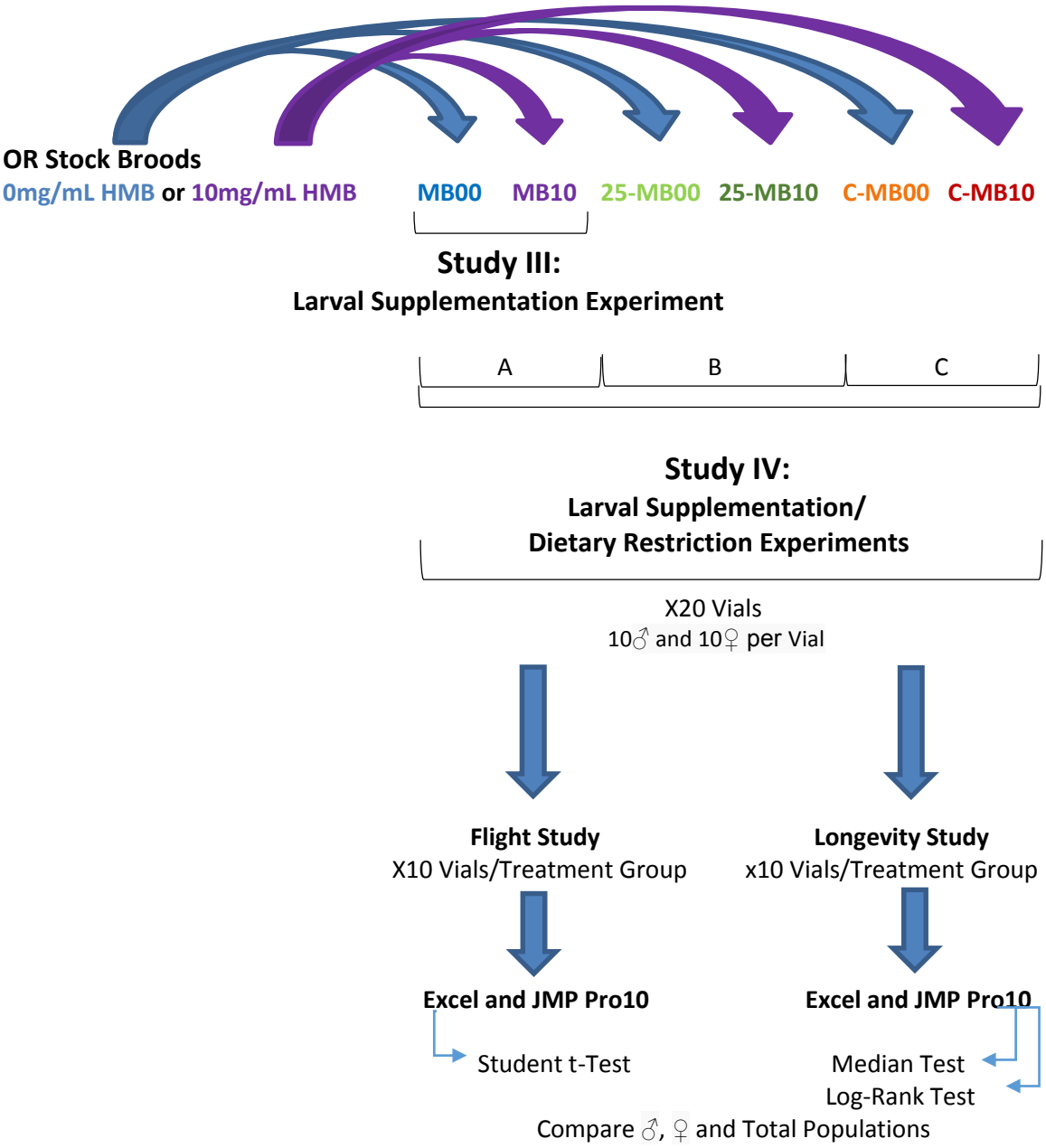

Figure 3.1. Experimental outline of Study III and IV. During Studies III and IV, CaHMB feeding began during the larval stages. In Study III, MB00 and MB10 treatment groups were tested together and statistically compared. In Study IV, Experiment A compared non-dietetically restricted treatment groups (MB00 v MB10), Experiment B compared the Reduced Yeast treatment groups (25-MB00 v 25-MB10), and Experiment C compared the treatment groups cycled on and off food (C-MB00 v C-MB10).

Non-Dietary Restriction Experiment

Two different diets were administered in this experiment: (i) standard fly food and (ii) standard fly food supplemented with CaHMB (Lonza) at a concentration of 10 mg/mL.

Reduced Yeast Experiment

Two different diets were administered in this experiment: (i) standard fly food with a 75% reduction in yeast and (ii) standard fly food with a 75% reduction in yeast supplemented with 10mg/mL of CaHMB.

Cycled Food Deprivation Experiment

Two different diets were administered in this experiment: a standard agar-based nutritional media (control; C-MB00), standard agar-based nutritional media supplemented with CaHMB (Lonza) at a concentration of 10 mg/mL (experimental; C-MB10).

3.3.3. Blinding

Once lifespan and flight test flies were distributed to vials of both control and HMB food for each experiment, the vials of Study III were blinded as "A" or "B", and the vials of Study IV were blinded as "D" and "E" in the Reduced Yeast experiment, "C" and "A" in the Cycled Food Deprivation experiment, and "B" and "F" in the non-dietetically restricted experiment.

3.3.4. Flight Performance

When the flight test flies of each diet were between 2 and 5 days old, their flight ability was tested mid-day. The day prior to testing, 10 flies (5 male, 5 female) are anesthetized with CO_2, checked under a dissecting microscope for wing damage, and placed in individual food vials overnight. Only flies with intact wings were tested. Flight

measurements were performed at room temperature (22°C) and relative humidity of ~20%. Temperature, humidity, and sex and age of the fly were recorded. Individual flies were released from the center of a Plexiglas flight chamber with a light source at the top, and their flight path scored as up (U), horizontal (H), down (D), or not at all (N). Flight index was determined using the formula:

Flight index = 6 x U/T + 4 x H/T + 2 x D/T + 0 x N/T, where U, H, D, and N are the number of flights in each category and T is the total number of flies tested. The flight score for each fly is the average of six attempts. At least ten flies (or the total number of survivors for advanced aged time points if less than ten) were tested for each group at each time point.

Flight tests were conducted at day 3 (baseline) and approximately every seven days thereafter. All flies that were tested each week (5 male, 5 female) were stored in individual Eppendorf tubes at -20ºC to be used in future studies (e.g., analysis of protein expression or HMB levels).

3.3.5. Lifespan

For the larval supplementation experiment, the non-dietetically restricted experiment and the reduced yeast experiment, lifespan flies were transferred to new food vials every other day, and the number living, the number dead, and the sex of dead flies was recorded. Flies were monitored until all the flies died. Median and maximum lifespan were reported as ± 1 day to account for flies that died on days between transfers. For the cycled food deprivation experiment, this process was repeated every day, as dictated by the

cycling, until all flies were dead; median and maximum ages were reported to the day. Graphical representations of survival were generated through JMP (version 10.0).

3.3.6. Statistical Analyses

The median, interquartile range (IQR), and maximum lifespan were determined from longevity data. Differences between median were determined by a nonparametric median test and differences between the survival curves were determined by the log-rank test. Flies that were recorded as "escaped" or "stuck alive in food" were censored in the log-rank test (Supplementary Table III). All flight data were recorded as a mean ± SE. Student's t-tests were used to determine flight differences between and within the treatments of each experiment. These tests were performed using JMP (version 10.0). Results were considered significant at $p < 0.05$.

3.4. Results

3.4.1. Study III: Larval Supplementation Experiment

Effect of HMB supplementation on flight performance

Flies lose their flight ability with age due to increased muscle damage.[52] To determine if HMB had any effect on delaying the decline in flight performance when supplied during the larval stages through death, we measured flight ability in flies maintained in food with and without HMB. In this assay, flight scores range from 0 (flightless) to 6 (upward flight). The average flight scores ± SE per week of male, female and total populations of MB00 and MB10 Groups are shown in Figure 3.2. Significant differences between and within MB00 and MB10 flies are displayed in Tables I and II.

The significant differences between groups (Figure 3.2; Table 3.1) did not appear until mid-life when flight ability of flies began to decline. MB10 flies maintain flight

ability to a greater extent later in life than control flies, as indicated by higher scores for a greater number of weeks in Figure 3.2.

In the total population, significant differences between MB00 and MB10 flies were found in weeks 5 (4.97±0.23 v 5.85±0.7; p=0.0006), 6 (3.67±0.30 v 5.35±0.2; p=0.0001), 8 (1.13±0.31 v 2.63±0.39; p=0.0039) and 9 (0.0±0.0 v 2.44±0.44; p=0.0001). The differences in weeks 8 and 9 found in the total populations were caused by differences in the female populations, as no males could fly during those weeks.

MB10 male flies had a significantly greater average flight score than MB00 during weeks 5 (5.86±0.10 v 5.2±0.23; p=0.0192) and 6 (4.83±0.36 v 2.13±0.37; p=0.0001), while MB00 flies had an average flight score that was significantly greater than MB10 flies only in week 7 (0.93±0.36 v 0.0 ±0.0; p=0.0222), by which time, MB10 males lost flight ability. By week 8, MB00 males lost all flight ability as well.

MB10 females had a significantly greater average flight score than MB00 females later in life, during weeks 4 (6.0 ± 0.0 v 5.67±0.14; p=0.0192), 5 (5.83±0.09 v 4.78±0.38; p=0.0081), 7 (4.69±0.33 v 3.2±0.51; p=0.0143), 8 (2.63±0.39 v 1.13±0.31; p=0.0039) and 9 (2.44±0.44 v 0.0±0.0; p=0.0001). In contrast to the trend found in the male population, all control females lost flight ability by week 9—one week sooner than HMB supplemented flies. HMB supplemented females lost flight ability by week 10. HMB supplemented females maintained flight ability for 3 weeks longer than HMB supplemented males. Control females maintained flight ability for one week longer than control males.

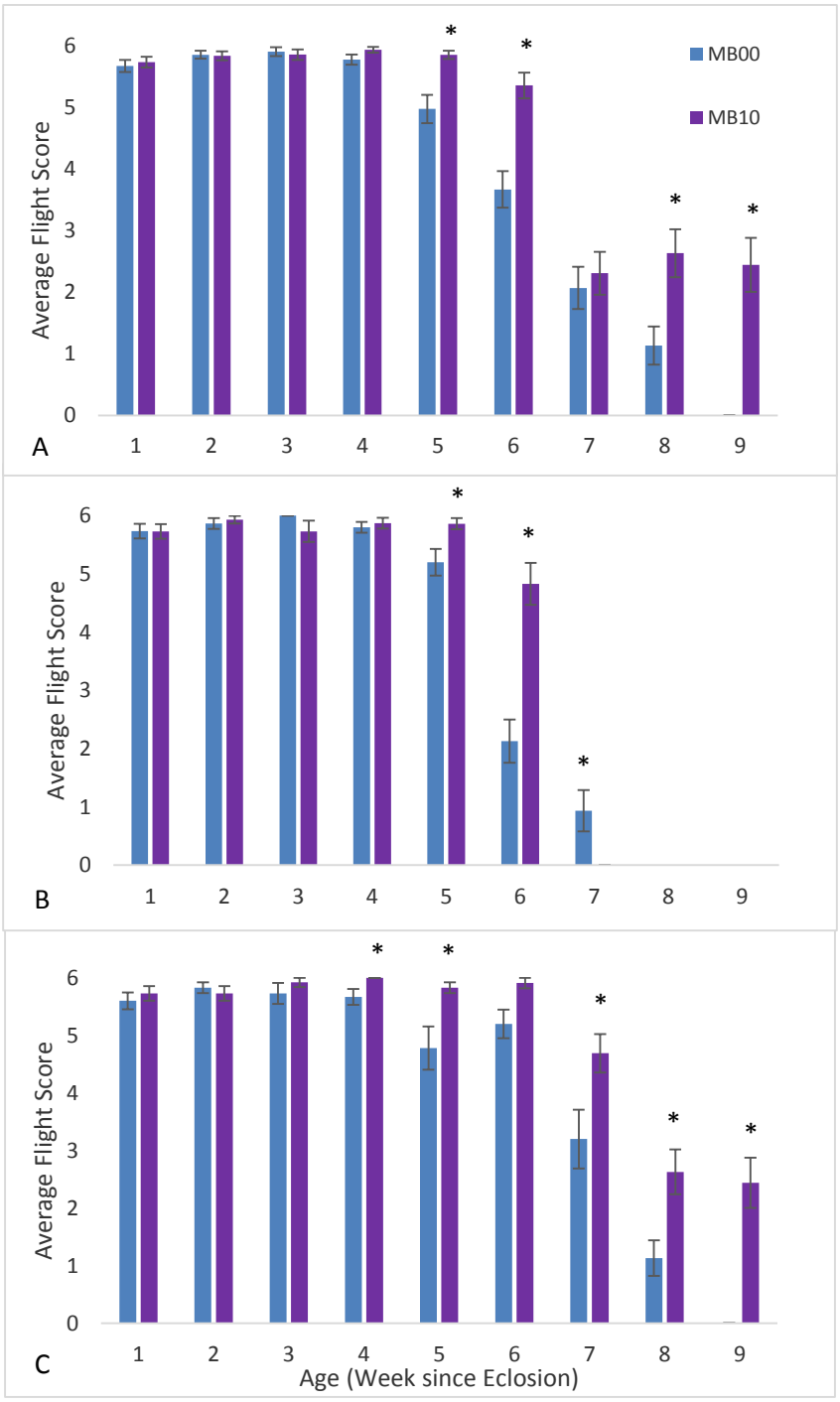

Figure 3.2: Flight Study III— Effect of larval HMB supplementation on flight ability. Average Flight Scores of (A) total populations (B) male populations and (C) female populations of MB00 and MB10 treatment groups. **(*)** indicates a significant difference between groups (p-value<0.05).

Table 3.1: Comparison of Average Flight Scores Between Groups MB00 and MB10 of Study III			
	p-value		
Week	**Total**	**Male**	**Female**
1	0.6127	1.000	0.4968
2	1.000	0.5614	0.6936
3	0.6699	0.0692	0.2899
4	0.0844	1.000	**0.0192***
5	**0.0006***	**0.0192***	**0.0081***
6	**0.0001***	**0.0001***	0.1217
7	0.4542	**0.0222***	**0.0143***
8	**0.0001***	**All dead**	**0.0039***
9	**0.0001***	**All dead**	**0.0039***
10	NO FLIGHT	**All dead**	NO FLIGHT
11	NO FLIGHT	**All dead**	NO FLIGHT
12	NO FLIGHT	**All dead**	NO FLIGHT
13	NO FLIGHT	**All dead**	NO FLIGHT
*p-value<0.05 indicates a significant difference between groups			

Table 3.2: Within group comparison of Average Flight Scores of Study III			
Group	**Population**	**Compared Weeks**	**p-Value**
MB00	**Total**	**4/5**	**0.0096**
		5/6	**< 0.0001**
		6/7	**< 0.0001**
		7/8	**0.0055**
		8/9	**0.0028**
	Males	**5/6**	**< 0.0001**
		6/7	**< 0.0001**
	Females	**4/5**	**0.0338**
		6/7	**< 0.0001**
		7/8	**< 0.0001**
		8/9	**0.0036**
MB10	**Total**	**6/7**	**< 0.0001**
	Males	**5/6**	**< 0.0001**
		6/7	**< 0.0001**
	Females	**6/7**	**0.0374**
		7/8	**< 0.0001**
p-value<0.05 indicates a significant difference between groups			

As with differences between groups, significant differences from week to week within groups (Table 3.2) only started to appear after the 4^{th} or 5^{th} week, when flight ability began to decline. The total population of control flies experienced a significant decline in flight ability from week 4 to week 5 ($p=0.0096$), from week 5 to week 6 ($p<0.0001$), from week 6 to week 7 ($p<0.0001$), from week 7 to week 8 ($p=0.0055$) and from week 8 to week 9 ($p=0.0028$). HMB supplemented flies, however, only experienced a significant decrease in flight ability from week 6 to week 7 ($p<0.0001$). Because the total population of control flies experienced multiple significant declines in flight ability from week to week, while the total population of HMB supplemented flies only experienced one significant decline from week 6 to 7, it seems that MB10 flies experienced more moderate changes from week to week than non-supplemented flies.

When we look at these declines in flight ability by sex, both MB00 and MB10 males declined from week 5 to week 6 ($p<0.0001$), and week 6 to week 7 ($p<0.0001$) (Table 3.2). Thus, there does not appear to be any differences in the trends of flight performance decline from week to week for males.

While control females experienced multiple significant declines, from week 4 to week 5 ($p=0.0338$), from week 6 to week 7 ($p<0.0001$), from week 7 to week 8 ($p<0.0001$), and from week 8 to week 9 ($p=0.0036$), MB10 females only experienced declines from week 6 to week 7 ($p=0.0374$) and from week 7 to week 8 ($p<0.0001$) (Table 3.2). From this, we see that the onset of decline in flight ability is earlier, and the rate of decline is faster in unsupplemented females than in supplemented females.

Effect of HMB supplementation on lifespan

The lifespan curves for MB00 and MB10 total, male, and female populations are represented in Figure 3.3. The male MB00 and MB10 flies reached their maximum lifespans (52 ± 1 days and 59± 1 days respectively) and median ages (40 (IQR:5) ± 1 days and 45 (IQR:10) ± 1 days, respectively) much sooner than the females (Table 3.3), whose maximum lifespans were reached at 101 ± 1 days and 99 ± 1 days, and whose median lifespans were 44 (IQR:16) ± 1 days and 52 (IQR:45) ± 1 days, respectively.

HMB fed males had a greater median age and displayed greater overall survival than control males (p=0.0001), as determined by the median and log rank test (Table 3.3 and Figure 3.3). Although the maximum age of unsupplemented females was 2 days greater than the maximum age of supplemented females, HMB fed females had significantly greater median age (p=0.0136) and likelihood of overall survival (p=0.0222) than control females, a trend not unlike that found in the total population (p=0.0001 for both median and log rank test, Table 3.3).

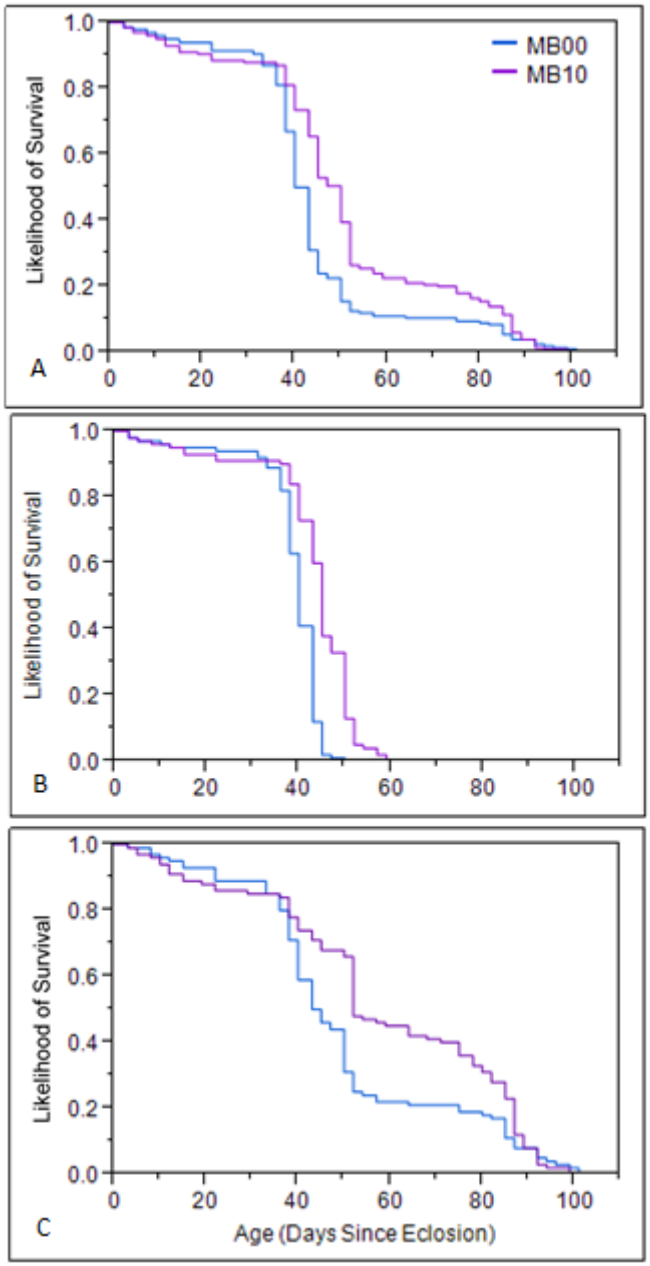

Figure 3.3: Lifespan Study III—Effect of larval HMB supplementation on lifespan. Survival curves comparing (A) total (B) male (C) female populations of MB00 and MB10 treatment groups. Median ages and likelihood of overall survival are significantly different in all populations ($p<0.05$) (Table 3.1).

Table 3.3: Summary of Median and Maximum Lifespan –Study III						
	Median Age (IQR)			Maximum Age		
	Male	Female	Total	Male	Female	Total
MB00	40 (5)	44 (16)	42 (7)	52	101	101
MB10	45 (10)	52 (45)	50 (17)	59	99	99
	Median Test significant (*) at p-value<0.05			Log-rank Test significant (*) at p-value<0.05		
	Male	Female	Total	Male	Female	Total
MB00 v MB10	**0.0001***	**0.0136***	**0.0001***	**0.0001***	**0.0222***	**0.0001***
±1 day for median and maximum age						

3.4.2. Study IV: Dietary Restriction Experiments

Effect of HMB supplementation on flight ability

Flight Study IVA compared the flight ability of unsupplemented flies to flies supplemented with HMB from larvae throughout adulthood. These flies were not subject to any form of dietary restriction, and served as controls for the reduced yeast and cycled experiments. As shown in Figure 3.4, significant differences in flight ability between MB00 and MB10 treatment groups were found in total, male and female populations later in life.

In the total populations, MB10 flies outperformed MB00 flies during weeks 6 ($p=0.0196$), 8 ($p<0.0001$) and 9 ($p=0.0056$) (Figure 3.4A, Supplementary Table IV). In the male populations, MB10 flies outperformed MB00 flies during weeks 8 ($p<0.0001$) and 9 ($p=0.0303$), while MB00 flies outperformed MB10 flies during week 5 ($p=0.0106$) (Figure 3.4B, Supplementary Table IV). In the female populations, MB10 flies had greater flight scores during week 6 ($p=0.0214$) than MB00 flies (Figure 3.4C, Supplementary Table IV).

Figure 3.5 shows the average flight scores of flies in Study IVB, fed a reduced yeast diet with or without HMB. Flies fed a reduced yeast diet supplemented with HMB had higher flight scores in weeks 1 ($p=0.0002$), 2 ($p<0.0001$), 6 ($p=0.0289$), 7 ($p=0.0341$), 8 ($p<0.0001$) and 9 ($p<0.0001$) than flies fed an unsupplemented reduced yeast diet (Figure 3.5A, Supplementary Table IV). In the male populations, differences in flight ability were found in weeks 1 ($p=0.0028$), 2 ($p<0.0001$), 7 ($p<0.0230$), 8 ($p<0.0001$) and 9 ($p<0.0001$) (Figure 3.5B, Supplementary Table IV). In the female populations, differences were found in weeks 1 ($p=0.0190$), 2 ($p<0.0001$) and 8 ($p<0.0001$) (Figure 3.5C, Supplementary Table IV).

The average flight scores of the flies of Study IVC are shown in Figure 3.6. The total population of flies that were supplemented with HMB in addition to being cycled on and off food had greater flight ability during weeks 6 ($p<0.0001$), 7 ($p=0.0326$), and 10 ($p=0.0389$) when compared to unsupplemented flies that were cycled on and off food (Figure 3.6A, Supplementary

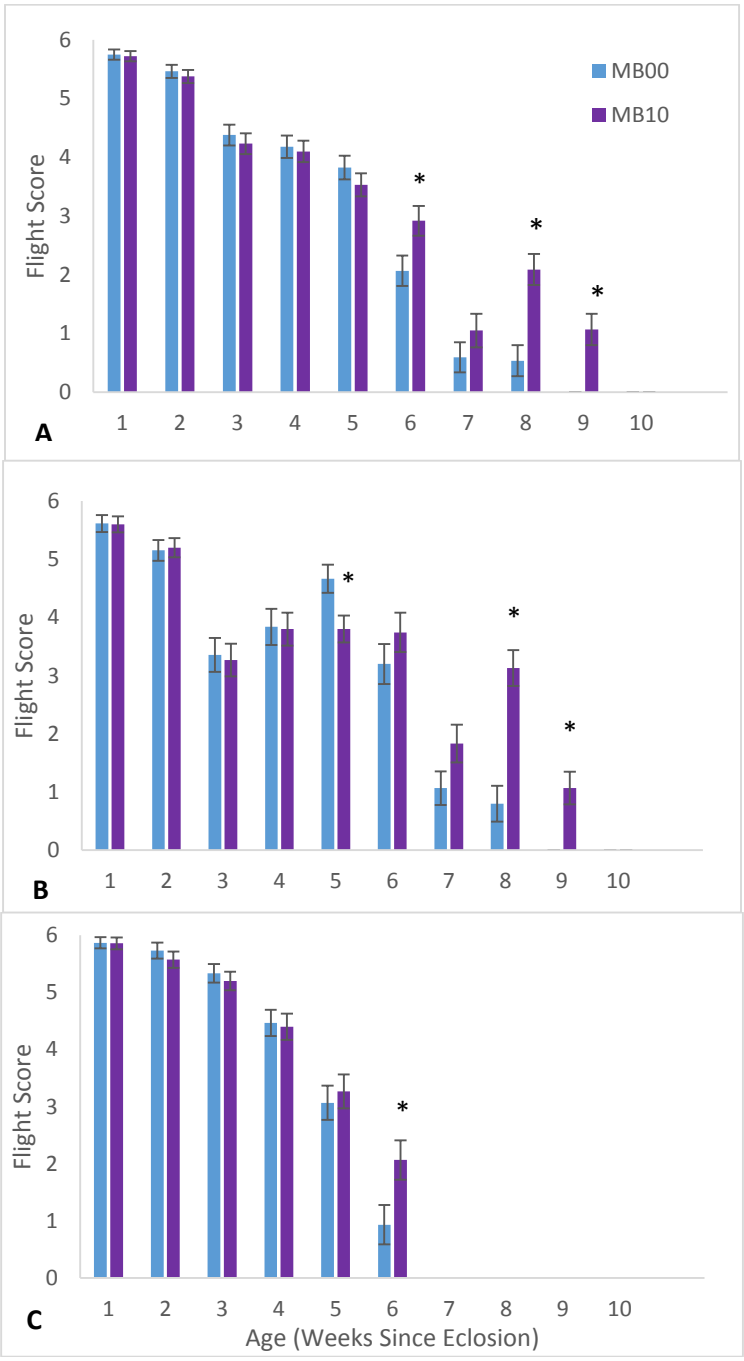

Figure 3.4: Flight Study IVA— Effect of HMB on flight ability under a non-restricted feeding regimen. Average Flight Scores of (A) total populations (B) male populations and (C) female populations of MB00 and MB10 treatment groups. **(*)** indicates a significant difference between groups (p-value<0.05).

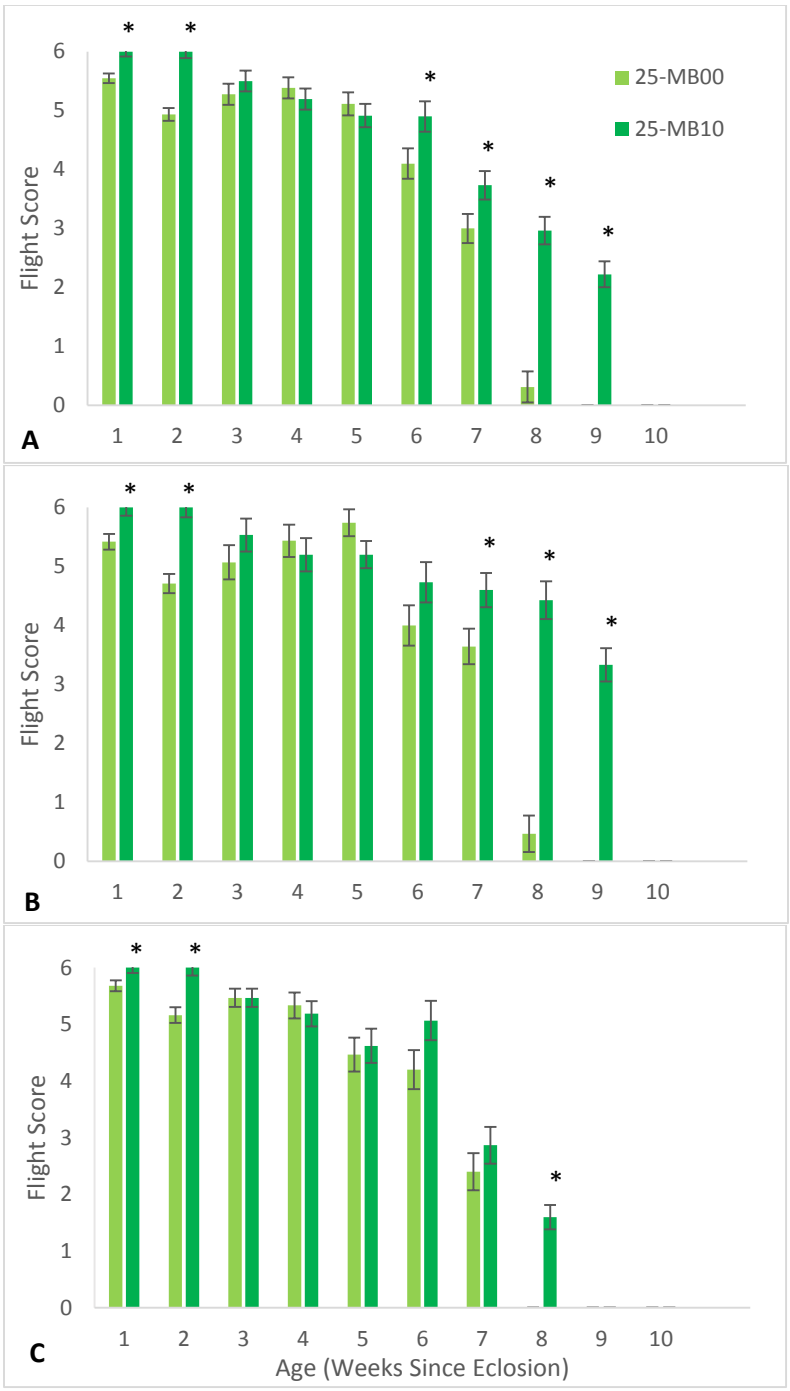

Figure 3.5: Flight Study IVB— Effect of HMB on flight ability under a reduced yeast feeding regimen. Average Flight Scores of (A) total populations (B) male populations and (C) female populations of 25-MB00 and 25-MB10 treatment groups. **(*)** indicates a significant difference between groups (p-value<0.05).

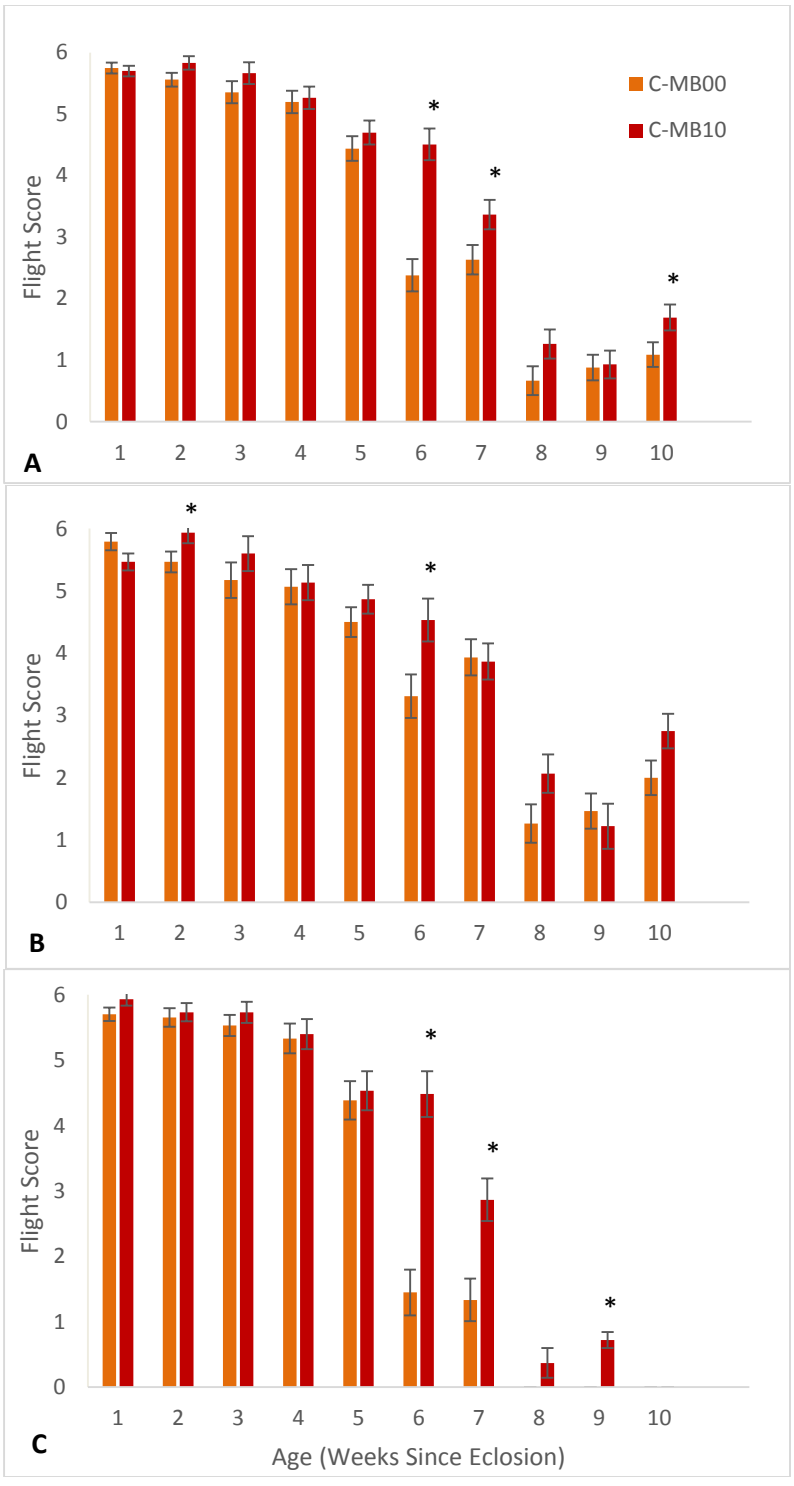

Figure 3.6: Flight Study IVC—Effect of HMB on flight ability under a cycled feeding regimen. Average Flight Scores of (A) total populations (B) male populations and (C) female populations of C-MB00 and C-MB10 treatment groups. **(*)** indicates a significant difference between groups (p-value<0.05).

Table IV). Cycled male flies supplemented with HMB outperformed unsupplemented cycled male flies in weeks 2 ($p=0.0489$) and 6 ($p=0.0135$) (Figure 3.6B, Supplementary Table IV), and cycled female flies outperformed unsupplemented cycled female flies in weeks 6 ($p<0.0001$), 7 ($p=0.0011$) and 9 ($p<0.0001$) (Figure 3.6C, Supplementary Table IV).

Results varied when the flight abilities of flies within each group from week to week were compared (Supplementary Table V). The onset of the decline in flight ability occurred in week 3 for the non-restricted males of both MB00 and MB10 groups ($p=0.0001$). Flight ability did not decline again until week 5 in the MB00 males ($p=0.0006$), and until week 7 in the MB10 males ($p=0.0002$), suggesting that HMB supplementation may help slow the decline in flight ability associated with age in males. The females of both groups began to lose flight ability by week 4 ($p=0.0065$, $p=0.0395$ for MB00 and MB10, respectively) (Supplementary Table V).

The onset of the decline in flight ability of reduced yeast MB00 males occurred in week 2 ($p=0.0220$). These flies did not experience another decline in flight ability until week 6 ($p=0.0001$). Reduced yeast MB10 males did not experience a decline in flight ability until week 9 ($p=0.0005$). The flight ability of female reduced yeast MB00 flies began to decline in week 5 ($p=0.0045$), while the flight ability of female reduced yeast MB10 flies did not begin to decline until week 7 ($p=0.0001$). HMB supplementation may help slow the age-associated decline in flight ability in both male and females on a reduced yeast diet (Supplementary Table V).

Finally, the onset of the decline in flight ability in the intermittently fed MB00 males occurred in week 6 ($p=0.0013$), while it did not occur until week 7 in the MB10 group ($p=0.0477$). The flight ability of females began to decline in week 5 in both groups ($p=0.0016$, $p=0.0037$ for C-MB00 and C-MB10 groups, respectively). As seen in the non-restricted group, HMB appears to slow the decline in flight ability in males (Supplementary Table V).

A comparison of the controls of Study IV A, B and C show that dietary restriction alone, either by reducing yeast intake or by restricting food availability to certain times of the day, is

able to enhance and prolong the flight ability of flies (Figure 3.7, Supplementary Table VI). The total population of flies from both the reduced yeast group and the cycled group had greater flight ability than flies of the non-dietetically restricted group during weeks 3, 4, 5, and 7. This was true of males during weeks 3, 4 and 7, and of females during weeks 4, 5, and 7 (Supplementary Table VI). Flies that were cycled on and off food maintained flight ability for two weeks longer than flies of the reduced yeast and non-restricted groups (Figure 3.7).

Effect of HMB supplementation on lifespan

Survival curves for the groups of Lifespan Study IVA are shown in Figure 3.8. When comparing the total populations, MB00 flies had a median age of 50 (IQR: 21) ± 1 days, while MB10 flies had a median age of 41 (IQR: 24) ± 1 days. MB00 flies had a 10% maximum age of 73 ± 1 days and an absolute maximum age of 89 ± 1days, while MB10 flies had a 10% maximum age 64 ± 1 days and an absolute maximum age of 73 ± 1days (Figure 3.8A, Table 3.4).

When comparing the male populations, MB00 males had a median age of 49 (IQR: 26) ± 1 days, a 10%maximum of 73 ± 1 days and an absolute maximum of 89 ± 1 days. MB10 males had a median age of 39 (IQR: 22) ± 1 days, a 10%maximum of 54± 1 days and an absolute maximum of 68 ± 1 days (Figure 3.8B, Table 3.4).

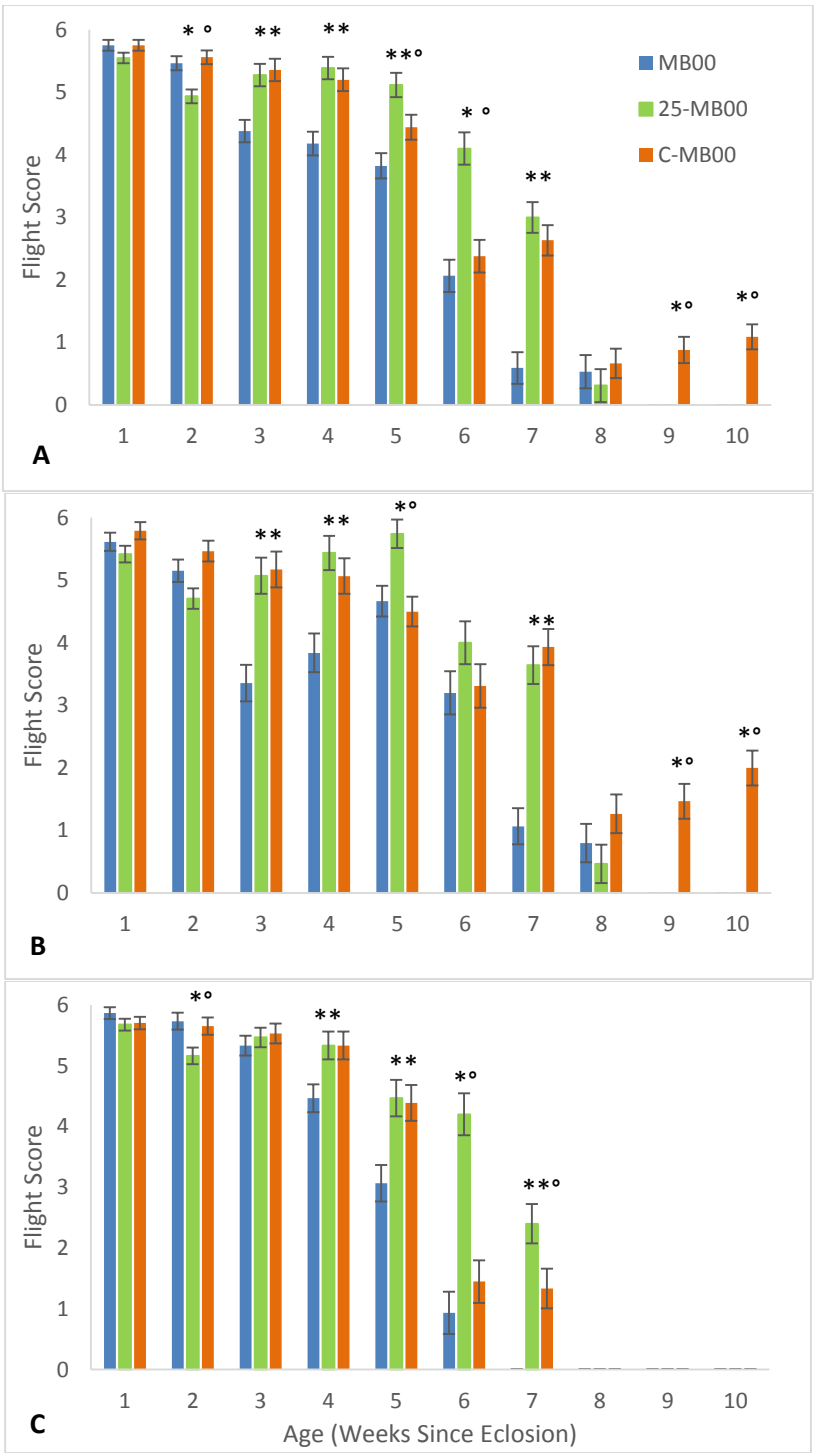

Figure 3.7: Flight Study IV—Effect of dietary restriction on flight ability. Average Flight Scores of (A) total populations (B) male populations and (C) female populations of MB00, 25-MB00, and C-MB10 treatment groups. (*) indicates a significant difference from MB00 (p-value<0.05); (°) indicates a significant difference from 25-MB00 (p-value<0.05).

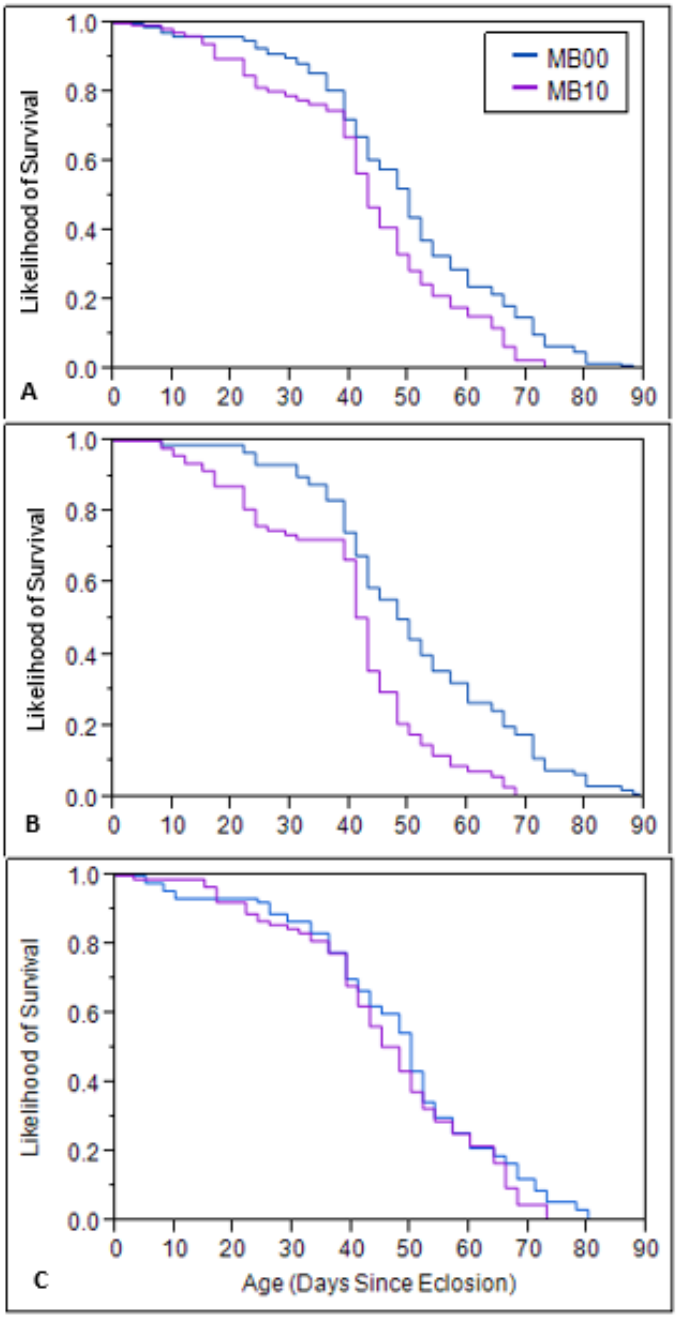

Figure 3.8: Lifespan Study IVA— Effect of HMB on lifespan under a non-restricted feeding regimen. Survival curves comparing (A) total (B) male (C) female populations of MB00 and MB10 treatment groups. Median ages and likelihood of overall survival are significantly different in total and male populations, but not in female populations. Median: T ($p<0.0001$) M ($p<0.0001$) F ($p=0.0664$). Logrank: T ($p<0.0001$) M ($p<0.0001$) F ($p=0.1937$).

Table 3.4. Summary of Median and Maximum Lifespan—Study IV					
	25%	**Median (IQR)**	**75%**	**90%**	**Maximum**
Total Populations					
MB00	39	50 (21)	60	73	89
MB10	26	41 (24)	50	64	73
25-MB00	48	58 (16)	64	66	83
25-MB10	48	58 (20)	68	73	91
C-MB00	55	63 (25)	80	90	99
C-MB10	52	56 (10)	62	70	85
Male Populations					
MB00	39	49 (26)	65	73	89
MB10	22	39 (22)	44	54	68
25-MB00	49	58 (15)	64	69	83
25-MB10	48	55 (16)	64	69	79
C-MB00	55	63 (24)	79	90	99
C-MB10	54	57 (10)	64	73	85
Female Populations					
MB00	39	50 (21)	60	71	80
MB10	36	45 (21)	57	66	73
25-MB00	43	55 (21)	64	66	83
25-MB10	52	64 (19)	71	78	91
C-MB00	54	63 (26)	80	90	96
C-MB10	51	56 (10)	61	64	81
± 1 day for MB00, MB10, 25-MB00 and 25-MB10 only					

Table 3.5: p-values of Median and Log rank tests—Study IV						
	Median Test			Log rank Test		
Compared Groups	Male	Female	Total	Male	Female	Total
MB00 v MB10	**<0.0001**	0.0664	**<0.0001**	<**0.0001**	0.1937	<**0.0001**
25-MB00 v 25-MB10	0.0606	**0.0482**	0.9604	0.3516	**<0.0001**	**0.0288**
C-MB00 v C-MB10	0.0518	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**

MB00 females had a median age of 50 (IQR: 21) ± 1 days, a 10%maximum of 71± 1 days and an absolute maximum of 80 ± 1 days, while MB10 females had a median age of 45 (IQR: 21) ± 1days, a 10% maximum of 66 ± 1 days and an absolute maximum of 73 ± 1 days (Figure 3.8C, Table 3.4).

The median ages and likelihoods of overall survival of MB00 and MB10 groups were significantly different in the total ($p<0.0001$, $p<0.0001$) and male ($p<0.0001$, $p<0.0001$) populations, but not in female populations ($p=0.0664$, $p=0.1937$) (Table 3.5).

Figure 3.9 shows the survival curves of Lifespan Study IVB. In the total population, both 25-MB00 and 25-MB10 flies had a median age of 58 ± 1 days (IQR: 16 and 20, respectively). 25-MB00 flies had a 10%maximum of 66 ± 1 days and an absolute maximum of 83 ± 1 days, while 25-MB10 flies had a10%maximum of 73 ± 1 days and an absolute maximum of 91 ± 1 days (Figure 3.9A, Table 3.4).

The lifespan curves of male 25-MB00 and 25-MB10 flies were similar: 25-MB00 flies had a median age of 58 (IQR:15) ± 1 days, a 10% maximum of 69 ± 1 days and an absolute maximum of 83 ± 1 days, while male 25-MB10 flies had a median age of 55 (IQR:16) ± 1, a 10% maximum of 69 ± 1 days and an absolute maximum age of 79 ± 1 days (Figure 3.9B, Table 3.4).

The lifespan curves of female 25-MB00 and 25-MB10 flies differed: 25-MB00 flies had a median age of 55 (IQR: 21) ± 1 days, a 10% maximum of 66 ± 1 days and an absolute maximum of 83± 1 days, while 25-MB10 flies had a median age of 64 (IQR: 19) ± 1, a 10% maximum of 78± 1 days and an absolute maximum age of 91 ± 1 days (Figure 3.9C, Table 3.4).

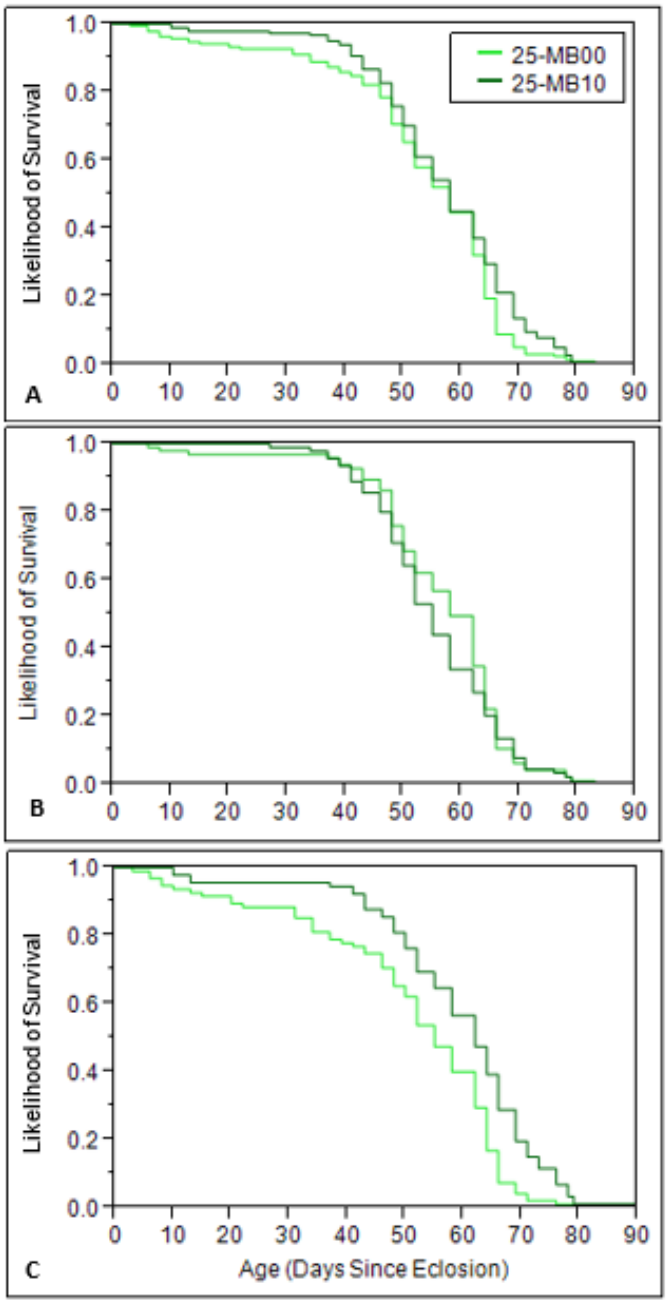

Figure 3.9: Lifespan Study IVB— Effect of HMB on lifespan under a reduced yeast feeding regimen. Survival curves comparing (A) total (B) male (C) female populations of 25-MB00 and 25-MB10 treatment groups. Median ages differ in the female populations only ($p<0.05$). The likelihood of overall survival significantly differs in the total and female populations, but not the male populations ($p<0.05$). Median: T ($p=0.9604$) M (0.0606) F ($p=0.0482$). Logrank: T ($p<0.0288$) M ($p=0.3516$) F ($p<0.0001$).

Median ages differed in the female populations only (p=0.0482, Table 3.5). The likelihood of overall survival significantly differed in the total (p=0.0288) and female populations (p<0.0001), but not the male populations (p=0.3516, Table 3.5).

Survival curves of Lifespan Study IVC are shown in Figure 3.10. The total population of C-MB00 flies had a median age of 63 (IQR:25) days, a 10% maximum of 90 days and an absolute maximum of 99 days, while C-MB10 flies had a median age of 56 (IQR: 10), a 10% maximum of 70 days and an absolute maximum age of 85 days (Figure 3.10A, Table 3.4).

The male C-MB00 population's median age was 63 (IQR: 24) days, 10% maximum was 90 days, and absolute maximum was 99 days. The male C-MB10 population had a median age of 57 (IQR: 10) days, a 10% maximum of 73 days and an absolute maximum of 85 days (Figure 3.10B, Table 3.4). The median age of the C-MB00 females was 63 (IQR: 26) days, the 10% maximum was 90 days and the absolute maximum was 96 days, while the females of the C-MB10 population had a median age of 56 (IQR: 10) days, a 10%maximum of 64 days and an absolute maximum of 81 days (Figure 3.10C, Table 3.4)

Median ages differed in the total (p<0.0001) and female (p<0.0001) populations of C-MB00 and C-MB10 groups, while the likelihood of overall survival significantly differed in all populations (p<0.0001, Table 3.5).

A separate study subject flies to 24 hours of starvation every other day (24h on food/24h off food) to determine if a cycling protocol with longer food deprivation would enhance observed the lifespan extension phenotype. This level of starvation, however, was too great for the flies, especially for the males; all males died by day 25 (>250%

decrease from the maximum age of flies on a 8h/16h on/off food cycle) and females died by day 42 (>100% decrease from the maximum age of flies on a 8h/16h on/off food cycle), in both supplemented and unsupplemented groups, during this intermittent cycling study.

Figure 3.11 compares the survival curves of the control groups of Study IV. Median age and likelihood of overall survival were greater in total ($p<0.0001$, $p<0.0001$), male ($p<0.0001$, $p<0.0001$), and female ($p=0.0002$, $p<0.0001$) populations of the cycled group than in the non-restricted group (Supplementary Table VII). Only median age was greater in the reduced yeast group in male ($p=0.0011$), female ($p=0.0062$), and total ($p<0.0001$) populations when compared to the non-restricted group (Supplementary Table VII). Cycled males and females had significantly greater overall survival than reduced yeast flies ($p<0.0001$), however, only cycled females had greater median age than reduced yeast females ($p=0.0082$). While median ages were greater in both dietary restriction experiments relative to the non-restricted experiment, maximum age was only greater in the cycling experiment.

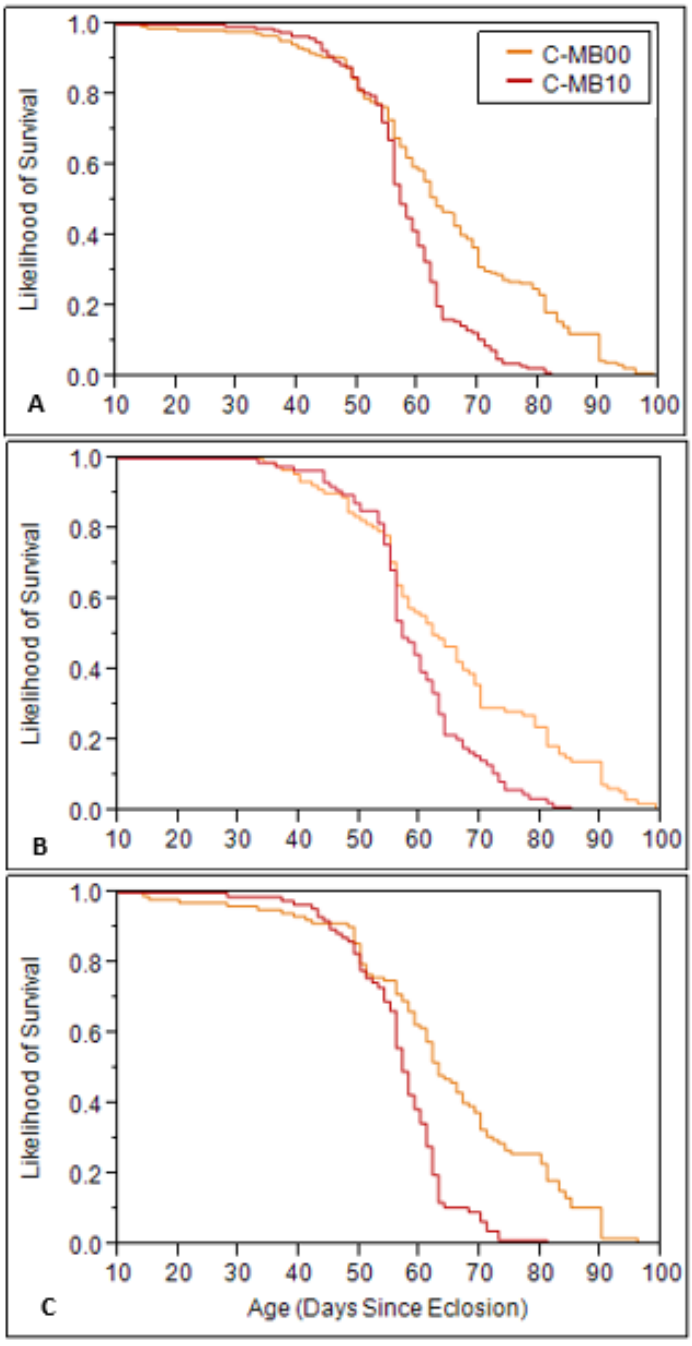

Figure 3.10: Lifespan Study IVC—Effect of HMB on lifespan under a cycled feeding regimen. Survival curves comparing (A) total (B) male (C) female populations of C-MB00 and C-MB10 treatment groups. Median ages differ in the total and female populations, while the likelihood of overall survival significantly differs in all populations. Median: T ($p<0.0001$) M ($p=0.0518$) F($p<0.0001$). Logrank: T($p<0.0001$), M ($p<0.0001$), F($p<0.0001$).

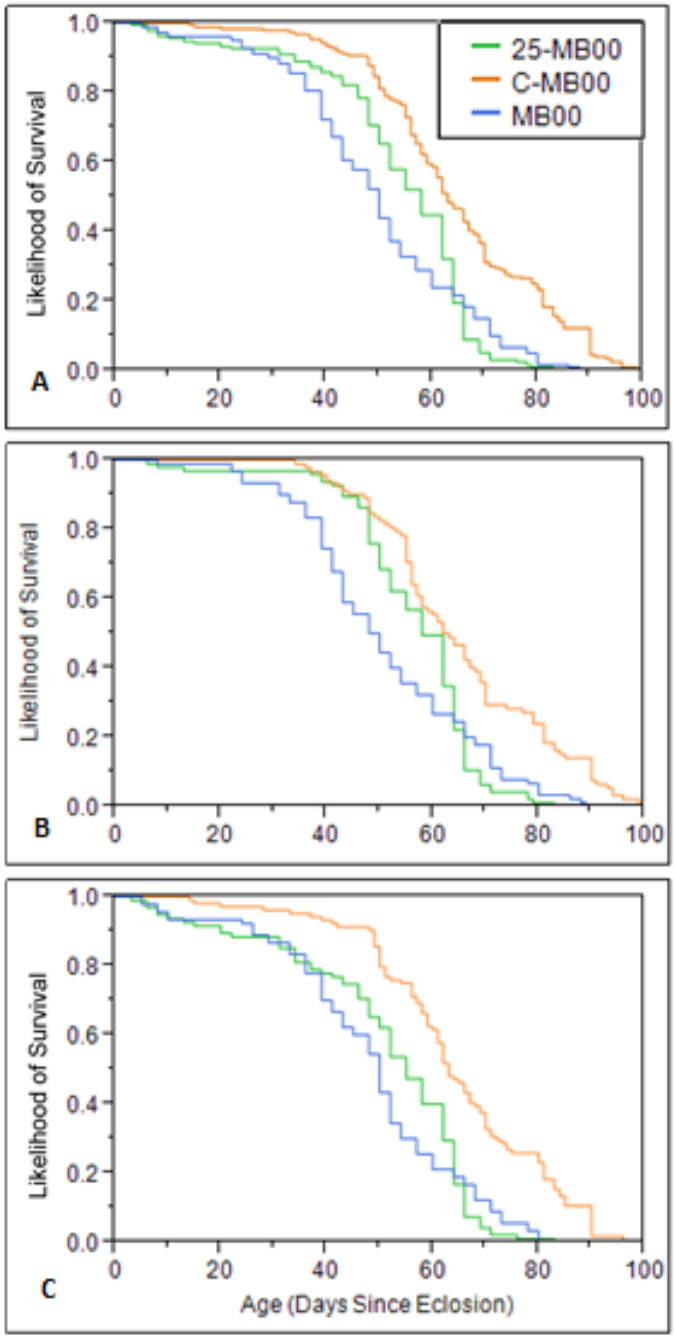

Figure 3.11: Lifespan Study IV—Effect of dietary restriction on lifespan. Survival curves of (A) total populations (B) male populations and (C) female populations of MB00, 25-MB00, and C-MB00 treatment groups. Median ages and likelihood of overall survival significantly differ in all three populations ($p<0.001$).

3.5. Discussion

The two studies detailed in this chapter further investigated the dietary optimization of HMB by examining the effect of larval HMB supplementation, under various dietary conditions, on the flight ability and survival of *D. melanogaster*.

Larval HMB supplementation in Study III seemed to affect flight ability and survivorship to a similar, if not greater, extent than supplementation from eclosion. Whether there is a statistical difference between these two supplementation methodologies, however, remains unclear. A future study may attempt to compare the two methods.

Additionally, because Study III and Study IVA had the same experimental design, yet produced different longevity results, a third trial is necessary to elucidate the true effects of larval HMB supplementation on lifespan. It is possible that this discrepancy was a consequence of censoring during the logrank test. A substantial number of HMB supplemented flies of the non-restricted group were lost and censored throughout Study IV; about 25% of HMB supplemented males got stuck alive in food (Supplementary Table III), which is a greater percentage of flies stuck alive than found in any other group. This loss of flies shifted the survivorship curve left, which may have caused the logrank test to detect a decrease in survivorship when such a decrease may not have existed had those flies not been stuck alive. Again, a third trial is needed to determine the effect of HMB, supplied to larvae throughout adulthood, on longevity under non-restricted conditions.

Study IV found that HMB supplementation decreased survivorship in non-restricted males and cycled males and females, increased survivorship in reduced yeast

females, and prevented the decline in flight ability later in life in all groups and both sexes. While the longevity results of Study IV varied, the flight test results corresponded with the findings of previous studies in the Vigoreaux lab (Study IA and B detailed in Chapter 2), as well as with several HMB studies in humans that reported improvements in aged muscle strength and function with HMB supplementation (Table 1.3).[28-35]

These improvements in flight ability with HMB supplementation varied depending on sex. Although females lost flight ability sooner than males in all three experiments of Study IV, HMB still enhanced the flight ability of females later in life. It is possible that these sex differences in flight ability were caused by the different nutrient and energy demands of males and females. Females require a greater amount of energy than males to compensate for egg production. For this reason, perhaps females do not have as much energy to expend on flying, thus, may lose flight ability sooner than males.

Improvements in flight ability with HMB supplementation also varied depending on feeding regimen. HMB enhanced flight ability of the females in the cycled group one week longer than the females in the reduced yeast group, which had enhanced flight ability for one week longer than the non-restricted group. Conversely, HMB enhanced flight ability of the males in the reduced yeast group to a greater extent than the males in the cycled group and non-restricted group. Thus, it seems that the timing of HMB feeding more strongly dictated flight ability in the female population, while the yeast (protein) levels in food more strongly influenced the HMB-induced flight response in the male population. No studies have yet compared the effects of HMB in males versus females, let alone the optimization of HMB supplementation in males versus females. Because HMB produced differential effects on flight ability, it may act via a different metabolic

pathway, or at least to a different extent on the same metabolic pathway, depending on sex and feeding regimen.

Similarly, the differential effects of HMB on longevity between the two dietary restriction feeding regimens and between the sexes suggest that HMB may act on different metabolic pathways to influence survival in different conditions and populations. To recount, HMB decreased the survivorship of males of the non-restricted group, increased survivorship of females of the reduced yeast group, and decreased survivorship of both males and females of the cycled group. Additionally, there was a sex specific effect in the survivorship of HMB populations in the non-restricted and reduced yeast groups; HMB fed females consistently had better survivorship than HMB fed males.

It is possible that these sex differences in survivorship were caused by the different nutrient and energy demands of males and females.[80,91] With regard to the non-restricted and reduced yeast HMB fed flies, it is possible that females were more selective, consumed less food than males, and therefore lived longer than males. Under the reduced yeast conditions, it is possible that because females require a greater amount of energy than males to compensate for egg production,[92] the effect dietary restriction was more pronounced in the female populations, and so too, was the effect of HMB. Finally, because females lost flight ability sooner than males, and because flying requires a huge amount of energy, perhaps female energy expenditure was lower than males, allowing female HMB fed flies to live longer than male HMB fed flies in non-restricted and reduced yeast conditions.

Although no studies have shown HMB to have different effects in males versus females, and no studies have yet examined the effect of HMB in dietetically restricted flies, the differential longevity effects observed between feeding regimens and between sexes correspond to the findings of one study by Magwere et al.[80] This study showed that dietary restriction decreased mortality rates and caused a lifespan extension in both male and female flies, however, female flies had a greater optimal response, at a higher concentration of food, than males.[80] Thus, the rate of mortality depends on both the sex and the feeding regimen of the flies.

Despite the fact that HMB decreased the survivorship of non-restricted and cycled flies, the flies supplemented with HMB were still able to maintain flight ability to a greater extent than unsupplemented flies. This decrease in lifespan and increase in flight ability corresponds to what would be expected if the TOR pathway were activated by HMB. Kapahi *et al* showed that an increase in TOR activity was tied to a decrease in lifespan, while a decrease in TOR was tied to an increase in lifespan in *Drosophila.* [68] An increase in TOR would also result in an increase in protein synthesis, which may support an enhanced flight phenotype.

The fact that the increase in flight ability was not met with a proportional increase in lifespan in all cases suggests that HMB may have different mechanisms of action on fly flight and longevity depending on feeding composition and pattern. For example, while the HMB supplemented females of the reduced yeast group experienced an increase in lifespan and an increase in flight ability, cycled HMB supplemented flies experienced a decrease in lifespan and an increase in flight ability. As previously described, the results of the cycled flies corresponds to what would be expected with an

upregulation of the TOR pathway by HMB. This hypothesis, however, does not explain the results of the reduced yeast females. The results of the reduced yeast females are similar to those of Study I and III. In these cases, it was hypothesized that HMB, like dietary restriction, may decrease TOR signaling, which would both increase autophagy to preserve muscle proteostasis and increase lifespan. While this is possible, i.e. that HMB may act on the same pathway as, though to a different extent than, dietary restriction, it is also possible that HMB acts independently of dietary restriction.

For example, in the case of the reduced yeast females, while dietary restriction may have down-regulated TOR pathway signaling to increase lifespan and potentially enhance muscle proteostasis through increased autophagy, perhaps HMB acted independently via the FOXO pathway to restore muscle proteostasis and improve organismal aging.[7] The additive effects of two independent pathways may have allowed for the observed improvement in flight ability and longevity with HMB supplementation, beyond that of dietary restriction alone, in the reduced yeast females.

When comparing the controls of Study IV, dietary restriction alone improved flight ability of the flies. A study by Bazzel *et al* found that dietary composition impacts various physiological indices, including fatigue tolerance, climbing speed, cardiac performance, lipid storage, and autophagy.[93] Additionally, this study found that flies on a reduced yeast diet had greater endurance than flies on a balanced sugar/yeast diet.[93] Consistent with these notions of enhanced performance with dietary restriction, we showed that dietary restriction was able to enhance and prolong flight ability in flies.

Cycling flies on and off food increased maximum age and survivorship relative to both the non-restricted group and the reduced yeast group, while the reduced yeast group

only showed increased median age relative to the non-restricted group. Thus, timing of feeding and composition of food are important components of longevity, however, it seems that timing of feeding alone has a greater effect on survivorship than reducing food composition.

This was surprising to learn; while studies in *Drosophila* have shown increases in lifespan with a reduction in yeast content, none have shown a lifespan extension with an intermittent feeding regimen in *Drosophila*.[87,88] It is possible that because disrupting or resetting circadian patterns can influence health and lifespan,[89] restricting feeding to certain times of the day may have reset the circadian patterns of the flies in the cycled experiment, allowing for the observed lifespan extension.

One of the major limitations of this study was that nutrient and caloric intake was not measured. Because of this, it remains unclear how restricted the flies were in each study. It is possible that flies employed some form of compensatory eating action which would have nullified the premise of assumed dietary restriction. Additionally, HMB intake was not measured, nor was the body mass of the flies. This limited our understanding of the effect of HMB in different treatment groups; it is still unknown what the proper dosage of HMB is relative to body mass and food intake in flies. Future studies will monitor food intake using a dye and spectrophotometry, and will measure HMB levels of weighed flies via HPLC.

Another limitation lies in the fact that the cycling study altered the temporal circadian eating patterns of flies. Even when flies have unlimited access to food, they eat in patterns. Fruit flies consume a large breakfast from ZT0-ZT2 during a 12h/12h light/dark cycle, a much smaller dinner from ZT8-ZT10, and consume small amounts of

food between meals[90]. The cycled feeding assay that we performed likely disrupted the flies' normal feeding behavior; flies were restricted from ZT0-ZT2, presumably forcing them to change their feeding habits in the morning. This change in eating pattern may have impacted lifespan changes to a greater extent than dietary restriction alone. This ambiguity makes it difficult to conclude whether the outcome of the intervention was caused by mechanisms of dietary restriction alone or, through the (added) disruption of circadian patterns. A future study may measure levels of various circadian proteins, such as CLOCK or timeless, under various forms of dietary restriction and HMB supplementation.

Because HMB has different effects under different dietary conditions, future studies may try to determine a feeding schedule (timing and dosage) that maximizes the effect of HMB. Future studies may also examine the tissue distribution and accumulation of HMB under various conditions. This could be done by measuring the amount of HMB in various parts of the fly, i.e. the head, the thorax and abdomen of flies, using HPLC. Muscle fibers and fat bodies of HMB fed flies could be imaged and compared to normally fed flies, and mitochondria, nuclei, and lysosomes of muscle fibers of HMB fed flies could be stained, imaged and compared as well. GAL4/UAS constructs could be expressed in *Drosophila* to locate the tissue distribution of up or down regulated genes in response to HMB supplementation, thus, serving as a means to investigate the molecular mechanism of HMB as well.

3.6. Concluding Remarks

A summary of Studies I-IV is provided in Table 3.6. From Studies I and II, we found that HMB, supplied from eclosion through adulthood at a concentration of 10mg/ml, had the most pronounced effect on both the flight ability and longevity of male and female flies. Supplementation with 5mg/ml HMB produced similar effects in males, but not in females, suggesting that females may need a larger dosage difference than the difference between 1 and 5 mg/ml, before an effect may be observed.

From Study III, we found that larval HMB supplementation yielded similar, if not improved, outcomes in flight ability and survivorship as supplementation from eclosion. However, the improvements in longevity observed in Study III were not observed in the equivalent experiment run in Study IV; rather than increase longevity and survivorship as in Study III, HMB supplementation decreased lifespan and survivorship in the comparable experiment of Study IV, suggesting that another trial should be run to determine the true effect of larval HMB supplementation on lifespan and survival.

From Study IV, we found that dietary restriction, especially by restricting food to certain times of the day, improved both the healthspan and survivorship of male and female flies. HMB supplementation in *Drosophila* attenuated the decline in muscle function with age in both non-restricted and dietetically restricted conditions. HMB decreased survivorship of non-restricted and cycled flies, yet further improved the survivorship of reduced yeast females. Because HMB influenced flight ability and longevity to different extents depending on feeding regimen and sex, HMB may affect flight independently of longevity in certain dietary conditions and populations. Furthermore, HMB may affect longevity through different pathways than dietary

Table 3.6. Summary of the Results of Studies I-IV					
	Group	**Flight Ability (>median age)**	**Survivorship***	**Lifespan**	**Median Age**
STUDY I: HMB Supplementation from Eclosion					
Males					
Relative to Respective Controls	MB10	↑	----	↑	↑
	MB05	↑	↑	↑	----
Females					
Relative to Respective Controls	MB10	↑	----	↑	----
	MB05	----	----	↑	----
STUDY II: HMB Supplementation from Median Age					
Males					
Relative to MB00	MB10	----	----	↑	----
Females					
Relative to MB00	MB10	----	----	↑	----
STUDY III: HMB Supplementation from the Larval Stages					
Males					
Relative to MB00	MB10	↑	↑	↑	↑
Females					
Relative to MB00	MB10	↑	↑	↓	↑
STUDY IV: HMB Supplementation from the Larval Stages in Various Dietary Conditions					
Males					
Relative to Respective Controls	MB10	↑	↓	↓	↓
	25-MB10	↑	----	↓	----
	C-MB10	↑	↓	↓	----
Relative to MB00	25-MB00	↑	----	↓	↑
	C-MB00	↑	↑	↑	↑
Relative to 25-MB00	C-MB00	↑	↑	↑	↑
Females					
Relative to Respective Controls	MB10	↑	----	↓	----
	25-MB10	↑	↑	↑	↑
	C-MB10	↑	↓	↓	↓
Relative to MB00	25-MB00	↑	----	↑	↑
	C-MB00	↑	↑	↑	↑
Relative to 25-MB00	C-MB00	↓	↑	↑	↑
(*) Determined by the logrank test; (----) No difference between groups					

restriction as HMB supplementation affected longevity to different extents than dietary restriction alone. The differences observed between male and female populations in response to dietary restriction and HMB supplementation suggest that male and female populations have different biological aging mechanisms that future studies should take into consideration.

The findings of this project encourage the use of *D. melanogaster* to investigate the complex relationships between nutrition, age-related muscle deterioration, and longevity. Future studies may use *D. melanogaster* to help uncover the biological mechanisms of such relationships, as well as potential treatments for age-associated muscle dysfunction and disease.

3.7. Acknowledgements

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Supplementary Material

Supplementary Table I: Comparison of Flight Ability by Trials and Groups of Study I					
Week	**Compared Treatment Groups**		**p-value**		
			Males	**Females**	**Total**
Trial 1					
1	MB00	MB10	0.7363	0.0086*	0.0348*
	MB01	MB05	0.0070*	0.4034	0.5316
2	MB00	MB10	0.6382	0.0641	0.2545
	MB01	MB05	<0.0001*	0.0982	0.1125
3	MB00	MB10	0.1087	0.8562	0.3618
	MB01	MB05	0.0684	1.0000	0.1794
4	MB00	MB10	<0.0001*	<0.0001*	<0.0001*
	MB01	MB05	0.2880	0.2136	0.0627
5	MB00	MB10	0.5245	<0.0001*	0.1047
	MB01	MB05	0.0336*	1.0000	0.0666
6	MB00	MB10	0.0725	1.0000	0.0303*
	MB01	MB05	0.0005*	1.0000	0.0023*
Trial 2					
1	MB00	MB10	0.6754	0.5519	0.4687
	MB01	MB05	0.0232*	0.8727	0.1362
2	MB00	MB10	0.2422	0.0005*	0.2186
	MB01	MB05	0.4183	0.4354	0.8748
3	MB00	MB10	0.2528	<0.0001*	0.0002*
	MB01	MB05	0.3905	0.4207	1.0000
4	MB00	MB10	0.0764	0.0068*	0.6832
	MB01	MB05	0.4059	<0.0001*	0.0280*
5	MB00	MB10	0.5821	1.000	0.9062
	MB01	MB05	1.000	0.0180*	0.6863
6	MB00	MB10	0.0039*	1.0000	0.0091*
	MB01	MB05	0.0325*	1.0000	0.0430*
Trials 1 and 2 Combined					
1	MB00	MB10	0.6151	0.0454*	0.1430
	MB01	MB05	0.0005*	0.5025	0.2008
2	MB00	MB10	0.2908	0.0020*	0.1180
	MB01	MB05	0.0016*	0.2961	0.1567
3	MB00	MB10	0.0570	0.0178*	0.0025*
	MB01	MB05	0.4746	0.5768	0.3679
4	MB00	MB10	0.0045*	<0.0001*	<0.0001*
	MB01	MB05	0.8957	0.3019	07352
5	MB00	MB10	0.7556	<0.0001*	0.1660
	MB01	MB05	0.0833	0.4267	0.0827
6	MB00	MB10	0.0015*	1.0000	0.0008*
	MB01	MB05	<0.0001*	1.0000	0.0003*
(*) indicates a significant difference between groups ($p<0.05$)					

Supplementary Table II: Comparison of Flight Ability Between Groups of Study IA and Groups of Study IB, Trials Combined					
Week	**Compared Treatment Groups**		**p-value**		
			Males	**Females**	**Total**
1	MB10	MB05	0.008*	0.371	0.036*
	MB10	MB01	0.352	0.200	0.411
	MB00	MB05	0.002*	0.274	0.491
	MB00	MB01	0.664	0.685	0.533
2	MB10	MB05	0.042*	0.049*	0.936
	MB10	MB01	0.301	0.361	0.145
	MB00	MB05	0.331	0.228	0.127
	MB00	MB01	0.034*	0.002*	0.901
3	MB10	MB05	0.001*	<0.0001*	<0.0001*
	MB10	MB01	0.008*	<0.0001*	<0.0001*
	MB00	MB05	0.120	0.006*	0.002*
	MB00	MB01	0.409	0.031*	0.032*
4	MB10	MB05	<0.0001*	0.001*	<0.0001*
	MB10	MB01	<0.0001*	<0.0001*	<0.0001*
	MB00	MB05	0.086	0.030*	0.832
	MB00	MB01	0.105	0.282	0.897
5	MB10	MB05	0.667	0.001*	0.738
	MB10	MB01	0.224	<0.0001*	0.044*
	MB00	MB05	0.340	0.417	0.279
	MB00	MB01	0.435	1.0000	0.500
6	MB10	MB05	0.013*	1.0000	0.048*
	MB10	MB01	0.076	N/A	0.073
	MB00	MB05	<0.0001*	N/A	<0.0001*
	MB00	MB01	0.138	N/A	0.131
(*) indicates a significant difference between groups (p<0.05)					

Supplementary Table III: Percent Censored flies in Study IV			
	Male	**Female**	**Total**
MB00	0.0%	1.1%	0.5%
MB10	24.5%	6.6%	15.9%
25-MB00	1.0%	0.0%	0.5%
25-MB10	3.3%	4.3%	3.8%
C-MB00	0.0%	0.0%	0.0%
C-MB10	5.6%	12.5%	9.1%

Supplementary Table IV: Intra-Experimental Comparison of flight ability in total, male and female populations of Study IV

Groups Compared		Week									
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Total Population											
MB00	**MB10**	0.8341	0.5949	0.5622	0.7564	0.3017	**0.0196**	0.2381	**<0.0001**	**0.0056**	**1**
25-MB00	**25-MB10**	**0.0002**	**<0.0001**	0.3737	0.4457	0.4734	**0.0289**	**0.0341**	**<.0001**	**<0.0001**	**1**
C-MB00	**C-MB10**	0.6831	0.0804	0.2158	0.796	0.3535	**<0.0001**	**0.0326**	0.0738	0.8702	**0.0389**
Male Population											
MB00	**MB10**	0.9389	0.8502	0.8225	0.9243	**0.0106**	0.2624	0.0796	**<0.0001**	**0.0303**	**1**
25-MB00	**25-MB10**	**0.0028**	**<0.0001**	0.253	0.548	0.0962	0.1331	**0.0230**	**<0.0001**	**<0.0001**	**1**
C-MB00	**C-MB10**	0.0956	**0.0489**	0.2855	0.8681	0.2715	**0.0135**	0.871	0.0682	0.5954	0.0601
Female Population											
MB00	**MB10**	0.9462	0.4219	0.5624	0.8375	0.6369	**0.0214**	1	1	1	
25-MB00	**25-MB10**	**0.019**	**<0.0001**	1	0.6486	0.7185	0.0776	0.3127	**<0.0001**	1	
C-MB00	**C-MB10**	0.1083	0.6954	0.3852	0.8375	0.7278	**<0.0001**	**0.0011**	0.2514	**0.0001**	

p-value<0.05 indicates a significant difference between groups

Supplementary Table V: Within Group Comparison of Flight Ability in Study IV			
Group	Compared Weeks	Male	Female
MB00	2/3	**0.0001**	
	3/4		**0.0065**
	4/5	**0.0006**	**0.0001**
	5/6	**0.0001**	**0.0001**
	6/7		**0.0058**
	8/9	**0.0001**	
MB10	2/3	**0.0001**	
	3/4		**0.0395**
	4/5		**0.0037**
	5/6		**0.0021**
	6/7	**0.0002**	**0.0001**
	7/8	**0.0115**	
	8/9	**0.0001**	
	9/10	**0.0001**	
25-MB00	1/2	**0.0220**	
	4/5		**0.0045**
	5/6	**0.0001**	
	6/7		**0.0001**
	7/**8**	**0.0001**	**0.0001**
25-MB10	6/7		**0.0001**
	7/8		**0.0003**
	8/9	**0.0005**	**0.0002**
	9/10	**0.0001**	
C-MB00	4/5		**0.0016**
	5/6	**0.0013**	**0.0001**
	7/8	**0.0001**	**0.0001**
	10/11	**0.0001**	
C-MB10	4/5		**0.0037**
	6/7	**0.0477**	**0.0001**
	7/8	**0.0001**	**0.0001**
	8/9	**0.0300**	
	9/10	**0.0002**	
	10/11	**0.0001**	
p-value<0.05 indicates a significant difference between groups Colored weeks indicate last week of flight for males (blue) and females (red)			

Supplementary Table VI: Inter-Experimental Comparison of Flight Ability of Groups in Study IV

Compared Groups		Week									
		1	2	3	4	5	6	7	8	9	10
Total Population											
MB00	25-MB00	0.0977	**0.0008**	**0.0005**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	0.553	1	1
MB00	25-MB10	**0.041**	**0.0008**	**<.0001**	**0.0001**	**0.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	1
MB00	C-MB00	1	0.5504	**0.0001**	**0.0001**	**0.03**	0.3957	**<.0001**	0.7066	**0.0105**	**0.0017**
MB00	C-MB10	0.6831	**0.0203**	**<.0001**	**<.0001**	**0.002**	**<.0001**	**<.0001**	**0.0401**	**0.0086**	**<.0001**
MB10	25-MB00	0.145	0.0046	**<.0001**	**<.0001**	**<.0001**	**0.0012**	**<.0001**	**<.0001**	**0.0056**	1
MB10	25-MB10	**0.023**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**0.0134**	**0.001**	1
MB10	C-MB00	0.8341	0.2541	**<.0001**	**<.0001**	**0.0013**	0.1422	**<.0001**	**<.0001**	0.5845	**0.0017**
MB10	C-MB10	0.8424	**0.0041**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**0.0203**	0.698	**<.0001**
C-MB00	25-MB00	0.0977	**<.0001**	0.7515	0.4647	**0.0158**	**<.0001**	0.2881	0.3159	**0.0105**	**0.0024**
C-MB00	25-MB10	**0.041**	**0.0049**	0.5657	0.9799	0.0913	**<.0001**	**0.0014**	**<.0001**	**<.0001**	**0.0003**
C-MB10	25-MB00	0.2046	**<.0001**	0.1213	0.6378	0.1349	0.2653	0.2881	**0.0075**	**0.0086**	**<.0001**
C-MB10	25-MB10	**0.0127**	0.2829	0.5046	0.775	0.4411	0.2856	0.284	**<.0001**	**<.0001**	**<.0001**
Male Population											
MB00	25-MB00	0.3258	0.0685	**<.0001**	**0.0002**	**0.0015**	0.1015	**<.0001**	0.4455	1	1
MB00	25-MB10	0.0567	**0.0007**	**<.0001**	**0.0015**	0.1138	**0.0019**	**<.0001**	**<.0001**	**<.0001**	1
MB00	C-MB00	0.3803	0.2017	**<.0001**	**0.004**	0.6258	0.8221	**<.0001**	0.2859	**0.0031**	**0.0002**
MB00	C-MB10	0.4591	**0.0017**	**<.0001**	**0.0024**	0.5519	**0.0067**	**<.0001**	**0.0041**	**0.0248**	**<.0001**
MB10	25-MB00	0.3471	**0.0371**	**<.0001**	**<.0001**	**<.0001**	0.593	**<.0001**	**<.0001**	**0.0303**	1
MB10	25-MB10	**0.0399**	**0.0008**	**<.0001**	**0.0006**	**<.0001**	**0.0412**	**<.0001**	**0.004**	**<.0001**	1
MB10	C-MB00	0.323	0.2585	**<.0001**	**0.0019**	**0.0367**	0.3759	**<.0001**	**<.0001**	0.3163	**0.0002**
MB10	C-MB10	0.4909	**0.0021**	**<.0001**	**0.0011**	**0.0013**	0.1024	**<.0001**	**0.0154**	0.7353	**<.0001**
C-MB00	25-MB00	0.0548	**0.0014**	0.8039	0.3486	**0.0002**	0.1612	0.4871	0.0682	**0.0031**	**<.0001**
C-MB00	25-MB10	0.2897	**0.0246**	0.3671	0.7398	**0.0367**	**0.0042**	0.1057	**<.0001**	**<.0001**	**<.0001**
C-MB10	25-MB00	0.8052	**<.0001**	0.1911	0.4418	**0.0076**	0.2739	0.5922	**0.0003**	**0.0248**	**<.0001**
C-MB10	25-MB10	**0.0064**	0.7772	0.8664	0.8681	0.3089	0.6811	0.0754	**<.0001**	**<.0001**	**<.0001**
Female Population											
MB00	25-MB00	0.17	**0.004**	0.5624	**0.0083**	**0.0011**	**<.0001**	**<.0001**	1	1	
MB00	25-MB10	0.333	0.1755	0.5624	**0.0253**	**0.0004**	**<.0001**	**<.0001**	**<.0001**	1	
MB00	C-MB00	0.2535	0.6954	0.3852	**0.0083**	**0.0019**	0.2971	**0.0071**	1	1	
MB00	C-MB10	0.6308	1	0.0834	**0.0045**	**0.0007**	**<.0001**	**<.0001**	0.332	**0.0005**	
MB10	25-MB00	0.2003	**0.0413**	0.2473	**0.0045**	**0.0051**	**<.0001**	**<.0001**	1	**1**	
MB10	25-MB10	0.3083	**0.0331**	0.2473	**0.0146**	**0.0018**	**<.0001**	**<.0001**	**<.0001**	1	
MB10	C-MB00	0.2902	0.6801	0.1486	**0.0045**	**0.0083**	0.2108	**0.0132**	1	**1**	
MB10	C-MB10	0.5894	0.4219	**0.0214**	**0.0024**	**0.0031**	**<.0001**	**<.0001**	0.332	**0.0005**	
C-MB00	25-MB00	0.8525	**0.0134**	0.772	1	0.8498	**<.0001**	**0.0219**	1	1	
C-MB00	25-MB10	**0.0373**	0.0829	0.772	0.6486	0.5816	**<.0001**	**0.0011**	**<.0001**	1	
C-MB10	25-MB00	**0.0641**	**0.004**	0.2473	0.8375	0.8749	0.5665	0.3127	0.332	**0.0005**	
C-MB10	25-MB10	0.628	0.1755	0.2473	0.5068	0.838	0.2373	1	**0.0001**	**0.0005**	

p-value<0.05 indicates a significant difference between groups

Supplementary Table VII: Inter-Experimental Comparison of Median and Log rank Test p-values of Study IV

Compared Groups		Median Test			Log rank Test		
		Male	Female	Total	Male	Female	Total
MB00	25-MB00	**0.0011**	**0.0062**	**<0.0001**	0.4226	0.5209	0.3284
MB00	25-MB10	**0.0389**	**<0.0001**	**<0.0001**	0.8234	**0.0004**	**0.0067**
MB00	C-MB00	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
MB00	C-MB10	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**0.0494**	**0.0097**
MB10	25-MB00	**<0.0001**	**0.0002**	**<0.0001**	**<0.0001**	0.0887	**<0.0001**
MB10	25-MB10	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
MB10	C-MB00	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
MB10	C-MB10	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**0.0057**	**<0.0001**
C-MB00	25-MB00	0.4323	**0.0082**	**0.0250**	**<0.0001**	**<0.0001**	**<0.0001**
C-MB00	25-MB10	**0.0053**	0.3625	**0.0115**	**<0.0001**	**0.0015**	**<0.0001**
C-MB10	25-MB00	0.2761	0.4526	0.4302	0.4647	0.9055	0.5009
C-MB10	25-MB10	**0.0384**	**0.0076**	0.4260	0.0507	**0.0011**	0.3554

p-value<0.05 indicates a significant difference between groups

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