THESIS

THE EVALUATION OF MYOFIBER REMODELING AND SKELETAL MUSCLE INFLAMMAGING USING A NOVEL GUINEA PIG MODEL

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ABSTRACT

THE EVALUATION OF MYOFIBER REMODELING AND SKELETAL MUSCLE INFLAMMAGING USING A NOVEL GUINEA PIG MODEL

Approximately 40% of total body mass is accounted for by the musculoskeletal system and thus, when it becomes dysfunctional it strongly influences whole body function. Sarcopenia is one facet of musculoskeletal aging and contributes to other age-related chronic diseases. Aging is a major risk factor for osteoarthritis, which is characterized by a concomitant loss of skeletal muscle, further contributing to decreased mobility. Age-related increases in low-grade inflammation and oxidative stress, referred to as the “inflammaging” phenotype, is common to both osteoarthritis and sarcopenia. While we have begun to understand the underlying pathology of sarcopenia, treatments are still lacking. One barrier to progress in identifying treatments is lack of a preclinical model that recapitulates the human skeletal muscle aging phenotype. Dunkin Hartley guinea pigs rapidly and spontaneously develop primary osteoarthritis beginning at about 4 months of age. The purpose of these studies was to determine if the Dunkin Hartley guinea pig can serve as a model to understand human skeletal muscle aging. Thus, we speculate that the Dunkin Hartley guinea pig may also be a valuable model of myofiber remodeling and skeletal muscle inflammaging. We compared skeletal muscle myofiber properties of the gastrocnemius and soleus from 5, 9, and 15-month Dunkin Hartley guinea pigs. We also compared these changes to a strain of guinea pig, Strain 13, that does not develop knee osteoarthritis at an early age. Additionally, in a second study, we assessed markers of skeletal muscle inflammation, oxidatively modified proteins, and redox signaling in 5 and 15-month Dunkin Hartley guinea pigs. The Dunkin Hartley guinea pig showed evidence of skeletal muscle aging including declines in gastrocnemius density and a shift in myofiber size

distribution to encompass a greater percentage of smaller myofibers in both the gastrocnemius and soleus. Male Dunkin Hartley guinea pigs experience a trend to decrease Nrf2 protein content from 5 to 15-months implying altered redox signaling, while female Dunkin Hartley guinea pigs experienced a significant increase from 5 to 15-months. Skeletal muscle myofiber remodeling, a component of musculoskeletal aging, influences both muscle function and quality of life. Based on these analyses, Dunkin Hartley guinea pigs appear to be a potentially valuable model of musculoskeletal aging.

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CHAPTER 1 – INTRODUCTION

By 2035, the number of people over the age of 65 will exceed the number of people under the age of 18 for the first time in history.[1] While humans are living longer compared to past decades, these extra years of life tend to be burdened with four or more chronic diseases.[2] Caring for a population with multiple chronic diseases places an enormous burden on the healthcare system as age-related diseases have both precipitous and expensive health outcomes. Knowing the number of elderly individuals is expected to increase in the subsequent years, there will be a larger percent of the population comprising older adults and, therefore, more individuals with age-related chronic diseases.

Healthspan, a subset of lifespan, is the period spent free from an age-related chronic disease (e.g., cardiovascular disease, cancer, Alzheimer's, sarcopenia, etc.).[3] The primary goal of our laboratory is to identify approaches to increase human healthspan. The trans-NIH Geroscience Interest Group established seven interrelated pillars of aging that provide a roadmap for integrative aging research. The pillars include macromolecular damage, inflammation, metabolism, proteostasis, adaptation to stress, epigenetics, and stem cell regeneration.[4] The interconnectedness of these pillars means that impairments to one pillar have implications for decrements in others.[5] One example of this is "inflammaging," a common geroscience term that combines an age-related chronic low-grade systemic inflammation with increases in oxidative stress (i.e., macromolecular damage).[6] The inflammaging phenotype arises from cellular and molecular impairments due to cellular senescence, mitochondrial dysfunction, and inflammasome activation.[7] Additional contributors to the inflammaging phenotype include sterile inflammation (i.e., the term given to impaired immune responses that occurs with age) and dysregulated redox signaling.[8,9] Together, alterations of immune and redox pathways yield net increases in pro-inflammatory molecules and reactive oxygen species

(ROS). In attempt to extend healthspan, preventing and/or minimizing the inflammaging phenotype is a logical therapeutic target.

One group of age-related changes driven by the pillars of aging is musculoskeletal decline, which encompasses decrements in skeletal muscle, bone, articular cartilage, and associated connective tissues. Commonly termed musculoskeletal aging, this phenotype includes multiple age-related chronic diseases, functional impairments, and declines in quality of life.[10] The structural interconnectedness of skeletal muscle, bone, articular cartilage, and their associated connective tissues, in turn, contributes to mobility. Therefore, it is difficult to delineate a single driver, but multiple pillars of aging are implicated in musculoskeletal decline.

Sarcopenia, defined as the age-related loss of muscle mass and function is one age-related chronic disease included in musculoskeletal aging phenotype.[11,12] Initially, the definition of sarcopenia only included declines in skeletal muscle mass but has since encompassed alterations in muscle function.[13,14] Specific skeletal muscle myofiber changes not only drive the sarcopenia phenotype in humans but they also influence the muscle's ability to produce force. One myofiber change is a decline in skeletal muscle density and concomitant increase in inter and intra-muscular fat deposition.[15–17] Additionally, older adults have a greater percentage of smaller myofibers coupled with an increase of type I fibers but a decrease in type II fibers.[18,19] Older adults compared to younger adults also experience a decrease in pennation angle in older adults as defined as the angle of a fiber from the muscle's line of action.[20,21] Collectively, these myofiber changes contribute to the sarcopenia phenotype in humans and influence metrics of skeletal muscle function.

While much progress has been made to understand the underlying etiology of sarcopenia, critical barriers impede successful translation to human health, including an incomplete comprehension of underlying etiology and lack of preclinical models that recapitulate the human muscle aging phenotype. One barrier to full understanding of the underlying etiology is the multifactorial mechanistic contributors to sarcopenia. Evidence suggests that increases in

macromolecular damage, increases in inflammation, alterations in metabolism, and protein dyshomeostasis— all pillars of aging— contribute to the myofiber remodeling that is characteristic of sarcopenia in humans.[18,22,23] Additionally, the lack of an effective preclinical model that closely resembles human musculoskeletal aging is a significant roadblock to both basic discovery and translation of promising treatments.

Rodent models provide insights into the underlying mechanisms encompassed in sarcopenia. However, rodent models currently used to study musculoskeletal aging, including genetic models and hindlimb unloading/immobilization, present additional co-morbidities that are not present in the human sarcopenic phenotype. For example, the PolyA mouse has a mitochondrial DNA mutation that results in an aged skeletal muscle phenotype. However, it involves additional co-morbidities absent in older adults (e.g., decreases in subcutaneous fat).[24,25] Additionally, the PolyA model lacks the declines in muscle function that are present in human sarcopenia.[26] Finally, the mitochondrial DNA mutation mainly occurs during embryonic development and does not follow the linear increases in mitochondrial DNA mutations that occur with increasing age in older adults.[27] The Cu/Zn superoxide dismutase knock-out mice (i.e., Sod1KO) is another model of both sarcopenia and frailty.[28] Sod1, an antioxidant that converts potent superoxide into hydrogen peroxide, plays a significant role in redox homeostasis.[29] This whole-body knock-out has helped implicate mitochondrial dysfunction, neuromuscular degradation, and increases in reactive oxygen species as mechanisms contributing to sarcopenia.[30] However, like the PolyA mouse, the whole-body Sod1 knock-out demonstrates other co-morbidities not present in human sarcopenia including infertility, a 30% decrease in lifespan, liver hyperplasia, and hepatocellular carcinoma.[31,32] Finally, skeletal muscle hindlimb unloading, denervation, and immobilization are also utilized to yield skeletal muscle atrophy.[33] Skeletal muscle mass in all three of these interventions quickly and rapidly declines within two weeks and does not mimic the slow progressive rate observed in human aging.[34] Collectively, these models allow researchers to elucidate some drivers of sarcopenia (i.e., the pillars of

aging). However, identifying a preclinical model that closely mimics overall human musculoskeletal aging, without the use of genetic or mechanical manipulation and associated comorbidities, would help overcome barriers impeding both basic discovery and successful translation to human health.

Osteoarthritis is another age-related pathology encompassed in the human musculoskeletal aging phenotype that, like sarcopenia, has inflammaging characteristics in its etiology.[35] Furthermore, those with osteoarthritis experience declines in skeletal muscle mass while those with sarcopenia are at increased risk of osteoarthritis.[36,37] In a recent review of the relationship between osteoarthritis and sarcopenia, authors support the need for mechanisms to be elucidated of the bidirectional relationship between sarcopenia and osteoarthritis.[38] Therefore, since skeletal muscle, bone, articular cartilage, and associated connective tissues are all negatively impacted by aging, a model that mimics skeletal muscle aging within a more comprehensive model of musculoskeletal decline would be valuable in making progress to identify mechanisms and develop effective treatments for humans.

The Dunkin Hartley guinea pig is a well-characterized model of idiopathic primary osteoarthritis with a similar pathophysiology to humans.[39–41] Beginning at 4 months of age, Dunkin Hartley guinea pigs have evidence of joint degradation. By 9 months, gait is altered and by 16 months of age, mobility is severely constrained.[41] As joint degeneration is one component of musculoskeletal aging, an ongoing agenda in our laboratory is to characterize the skeletal muscle changes in these guinea pigs to more comprehensively identify their value as a model of overall musculoskeletal aging. Our preliminary data suggest that Dunkin Hartley guinea pigs experience skeletal muscle age-related changes including declines in muscle protein synthesis, fiber type alterations, and declines in muscle mitochondrial respiration (Musci and Walsh et al., unpublished; Musci, et al., unpublished). Specifically, rates of myofibrillar, mitochondrial, cytosolic, and collagen protein synthesis decline between 5 and 15 months of age (Musci and Walsh et al., unpublished). The declines in protein synthesis are similar to that seen in humans

as they occur at a slower, more progressive rate compared to other rodent models.[34] Also like humans, Dunkin-Hartley guinea pigs have a decline in the percentage of type II fibers and concomitant increase in type I fibers as from 5 to 15 months (Musci and Walsh et al., unpublished)[18]. Finally, skeletal muscle submaximal ADP stimulated, and uncoupled mitochondrial respiration are decreased in 15-month compared to 5-month animals (Musci et al., unpublished).

Dunkin Hartley guinea pigs also display increases in the local inflammatory mediator IL-1β in the knee joint along with increases in C3, a marker of low-grade systemic inflammation (Radakovich et al., unpublished)[40,42]. Musculoskeletal functional changes also exist in the Dunkin Hartley guinea pig as they display decrements in gait by 16 months of age.[41] Declines in protein synthesis, shifts in skeletal muscle fiber type, declines in mitochondrial function, increases in inflammatory mediators, and mobility impairments are common in humans with osteoarthritis and sarcopenia, two diseases encompassed in musculoskeletal decline.[43] Therefore, we hypothesize that the Dunkin Hartley guinea pig may also serve as a more comprehensive model of musculoskeletal aging compared to the currently used models. However, there are numerous additional characteristics of skeletal muscle aging (i.e., density, myofiber distribution, pennation angle, and collagen content) that need to be assessed in order to support our working hypothesis. Moreover, the skeletal muscle inflammaging phenotype should also be characterized.

GAP IN KNOWLEDGE AND STATEMENT OF THE PROBLEM

Both aims seek to contribute to characterization of the Dunkin Hartley guinea pig as a model of musculoskeletal aging. The lack of preclinical models that mimic human musculoskeletal aging is a major barrier to developing potential treatments specifically for sarcopenia. It is noted that the Dunkin Hartley guinea pig appears to experience musculoskeletal decline at a very young age, but it is unknown if their myofiber remodeling mimics the human sarcopenic phenotype. Therefore, the purpose of this study was to evaluate skeletal muscle mass, density, myofiber distribution,

pennation angle, and collagen content (aim #1), as well as skeletal muscle inflammaging characteristics (aim #2), in ages of guinea pigs that encompass the onset and progression of osteoarthritis.

SPECIFIC AIMS AND HYPOTHESES

Specific aim #1: To evaluate age-related skeletal muscle myofiber changes including muscle mass, density, myofiber distribution, pennation angle, and collagen content in 5, 9, and 15-month male Dunkin Hartley guinea pigs.

Hypothesis #1: Compared to the 5-month Dunkin Hartley guinea pigs, 15-month guinea pigs will have greater evidence of age-related skeletal muscle myofiber changes (i.e., decreased density, a shifted myofiber size distribution, a decreased pennation angle, and increased collagen content) relative to the age-matched control Strain 13 guinea pig.

Specific aim #2: To evaluate age-related skeletal muscle inflammaging including inflammatory changes, redox signaling, and oxidatively modified proteins in 5 and 15-month Dunkin Hartley guinea pigs of both sexes.

Hypothesis #2: Compared to the 5-month Dunkin Hartley guinea pigs, 15-month Dunkin Hartley guinea pigs will have greater evidence of skeletal muscle inflammation, impaired markers of redox signaling, and greater accumulation of oxidatively modified proteins.

CHAPTER 2 – REVIEW OF LITERATURE

MUSCULOSKELETAL AGING

Musculoskeletal aging is a collection of age-related diseases all affecting the musculoskeletal system due to declines in cellular function. Decrements in skeletal muscle, bone, articular cartilage, and associated connective tissues are encompassed within musculoskeletal aging.[10] Therefore, older adults with musculoskeletal decline are at greater risk of age-related chronic diseases and injuries including sarcopenia, osteoporosis/osteopenia, osteoarthritis, and tendon and ligament tears.[10] Moreover, some of these musculoskeletal age-related diseases are combined demonstrating the interconnected nature of age-related declines of the musculoskeletal system.[44]

SARCOPENIA

Definition and evolution

Sarcopenia (from the Greek *sarco* – flesh and *penia* – loss) was first described in 1989 by Rosenberg as the age-related loss of lean body mass.[12] Thereafter, Baumgarter detailed sarcopenia as two standard deviations below the mean of a young reference group using a DEXA scan.[11] Additionally, as of 2016, sarcopenia now has an ICD-10MC Code.[45] Declines in muscle mass can occur as early as the fourth decade of life wherein individuals lose 1-2% of muscle per year.[34] However, declines in muscle mass are estimated to increase to 3% per year beginning in the sixth decade of life.[46] In the last 30 years, the definition of sarcopenia evolved to encompass more than just loss of skeletal muscle mass and now also includes skeletal muscle dysfunction.[47] While the European Working Group on Sarcopenia in Older People (EWGSOP) now includes dysfunction in their definition there is still not a consensus for an international definition of sarcopenia.[48,49]

Declines in skeletal muscle function are not synonymous with declines in skeletal muscle mass nor do they always occur in tandem. An example that depicts the differences between skeletal muscle mass and function is that changes in muscle cross-sectional area only account for approximately 50% of the variability in age-related changes in muscle function as assessed using a 1 repetition max leg press.[50] A recent longitudinal study including over 1,300 men and women examined lean mass, muscle function, and physical function. It was not until after age 70 that changes in whole body lean mass characteristics were observed, but as early as 50 years of age decrements of power (i.e., a metric of muscle function) occurred.[51,52] Muscle function is also one component of gait and balance. Thus, it is not surprising that those with sarcopenia have a 50% greater fall risk compared to those without sarcopenia.[52] While sarcopenia alone does not dictate falls, it is a contributor along with the other age-related aspects of musculoskeletal decline including joint pain, physical inactivity, and poor balance.[52–54] Therefore, it is important to examine the role of muscle function in sarcopenia as skeletal muscle function is linked with fall risk, reduced quality of life, and mortality in older adults[55].

Diagnostic criteria for sarcopenia often vary and include measurements of lean mass (e.g. using a DEXA scan), functional assessments (e.g. hand grip or a 6-minute walk test), and most recently the use of a short questionnaire.[56] Regardless of the approach for diagnostic assessment, estimates of the prevalence vary. The gold standards for quantification of skeletal muscle mass are computed tomography or magnetic resonance imaging, yet both are very expensive and primarily utilized in the research setting.[57] Comparing adjusted skeletal muscle mass to height against a criterion value is a common metric for sarcopenia diagnosis.[58] Using this criterion comparison of skeletal muscle mass adjusted to height, sarcopenia affects 75% of men and 35% of women over the age of 60 and rises to 88% of men and 53% of women over the age of 80.[59] While diagnostic criteria for sarcopenia varies, hospitalizations and increased healthcare costs associated with the syndrome are growing.[60]

Economic Cost

The economic cost of sarcopenia was recently assessed for the first time in almost twenty years.[60] Hospitalizations equated to approximately $40.4 billion in those with sarcopenia. For those over 65 years of age, the cost per person per year was higher than those without sarcopenia.[60] Specifically, the odds of being hospitalized were two times greater in those with sarcopenia compared to those without sarcopenia, translating to an additional $2300 per year of healthcare costs due to the increased hospital stays.[60] Additionally, the cost of hospitalization for those with sarcopenia is substantially higher than the $18.5 billion from a 2002 estimate of sarcopenia associated disability.[61] With the predicted growing aging population, the number of individuals with sarcopenic associated healthcare costs is projected to increase.

Co-morbidities

Like many age-related chronic diseases, sarcopenia has numerous co-morbidities. Namely, there are strong associations between impairments in muscle mass and strength and cardiovascular risk, metabolic syndrome, and immunosuppression.[62] Additionally, other age-related chronic diseases predispose older adults to sarcopenia (i.e., secondary sarcopenia).[56] Specifically, those with cardiovascular disease (31.4%), dementia (26.4%), diabetes (31.1%), and respiratory diseases (26.8%) have secondary sarcopenia.[63] Finally, declines in mass and function increase risk of mortality by 3.7-fold.[63–65] Sarcopenia has also recently been combined with other age-related pathologies conveying the idea that sarcopenia and other diseases do not occur in silos but share overarching mechanisms.[44,66].

SARCOPENIA ETIOLOGY

The Geroscience Interest Group coined the seven pillars of aging (i.e., macromolecular damage, inflammation, metabolism, proteostasis, adaptation to stress, epigenetics, and stem cell regeneration) that are common drivers of age-related chronic diseases.[4] These pillars are highly interconnected and an alteration in one pillar will lead to modifications to others.[5] For example, increases in mitochondrial ROS inducing macromolecular damage also drive

increases in pro-inflammatory mediators.[67,68] Highly implicated in human sarcopenia are the interconnected pillars of macromolecular damage, inflammation, proteostasis, and metabolism.

Macromolecular damage

Macromolecular damage broadly encompasses increases in production and activity of deleterious molecules including ROS. Among the mechanisms underlying increases in macromolecular damage are declines in the balance of oxidants to antioxidants and impairments in redox sensitive signaling.[8] Redox signaling is the collective term given to the biochemical reactions that involve the transfer of electrons (i.e., oxidation-reduction reactions) or interactions between a sensor protein and second messenger (i.e., covalent adduct formation).[69] Scientists have done a remarkable job theoretically modeling alterations in redox signaling although it is difficult to make these assessments in humans. One example is the Keap1-Nrf2 redox signaling axis. Nrf2 is a transcription factor that regulates the transcriptional activation of over 400 genes involved in cytoprotection.[70,71] Under unstressed conditions Nrf2 is bound to Keap1 in the cytoplasm and targeted for degradation. Thus, Keap1 is a repressor of Nrf2 activation.[72] Furthermore, the Nrf2 pathway is susceptible to impairments and is associated with the sarcopenic phenotype in both animals and humans[73,74]. Specifically, older sarcopenic adults demonstrate higher 4-HNE a marker of macromolecular damage and impaired glutathione cycling which is influenced by Nrf2.[75,76]

Older adults also experience age-related increases in ROS generation that contributes to macromolecular damage in skeletal muscle. One metric of this is the observation of an increased ratio of electron leak to hydrogen peroxide emissions at both submaximal and maximal concentrations of ADP in older men.[18] This observation additionally resulted in increased lipid peroxidation. Frail women additionally experience significant increases in another metric of mitochondrial ROS production compared young females.[77] The men and women in these studies also experienced myofiber remodeling influencing the sarcopenic phenotype of older adults.[18,77]

Inflammation

Increases in chronic low-grade inflammation and ROS (i.e., inflammaging) is extensively implicated in numerous age-related chronic diseases, including sarcopenia.[6,78,79] Mechanisms behind the contribution of increased pro-inflammatory states to skeletal muscle mass and functional losses include NFkB activation and apoptotic signaling through increases in cytokines like TNF.[80] In skeletal muscle, TNF is primarily secreted by myeloid cells including M1 macrophages. Only one study has assessed TNF in human skeletal muscle. As suspected, TNF both at the mRNA and protein level was higher in older adults compared to younger adults.[81] TNF reduces myogenic differentiation through the NFkB pathway and decreases the stability of MyoD.[82] Specifically, TNF appears to inhibit myogenesis through the reduction on muscle cell fusion.[80] Thus, this cellular increase in inflammatory pathways results in cascades ultimately reducing skeletal muscle remodeling.

Increases in inflammation additionally increase apoptotic pathways that contributes to sarcopenia. Specifically, TNF receptor binding can activate the caspase cascade and induce cell death.[22] Further, type II fibers are more susceptible to this TNF inducible apoptotic signal thereby also supporting the posit that fast-twitch fibers are more susceptible to age-related changes.[83] Therefore, while there is not a single molecular driver of inflammatory-mediated muscle atrophy TNF appears to be implicated in multiple pathways.

Activated pro-inflammatory cytokines are known to impair muscle protein synthesis while simultaneously activating degradation pathways contributing to skeletal muscle protein dyshomeostasis characteristic of sarcopenia.[84] For example, potent pro-inflammatory cytokines IL-6 and TNF reportedly upregulate the ubiquitin proteasome system through activation of FOXO3a.[85] Moreover, TNF and IL-6 negatively correlate with declines in skeletal muscle mass.[86,87] Decrements in muscle function in older adults, are also associated with increased circulating pro-inflammatory cytokines as lower peak knee extension torque is correlated to greater IL-1β in older men compared to younger men.[88] Inflammation thus not only influences

sarcopenia etiology at cellular, tissue, and organ levels but also affects the whole organism through declines in mobility and quality of life.

Inflammaging

There is a positive feedback cycle between oxidative stress and inflammation that likely contributes to the inflammaging phenotype. “Inflammaging” is the term given to the age-related increase in chronic low-grade systemic inflammation coupled with increases in oxidative stress.[6] Further inflammaging supports the notion of the interconnectedness in the pillars of aging. Specifically, ROS are thought to contribute to age-related skeletal muscle loss either through altered endogenous defense mechanisms, increased damage to macromolecular structures and impaired redox signaling.[8,89,90] Additionally, ROS is canonical signal for inflammation but, conversely, inflammation augments ROS production.[67] This identifies a “chicken or the egg” phenomena between the two pillars and while it is challenging to establish a cause and effect relationship between the two it is well-accepted that both contribute to the etiology of sarcopenia.

Proteostasis

Protein homeostasis (i.e., proteostasis) is maintained by a complex network of cellular processes that encompasses protein synthesis, folding, post-translational modifications, targeting, and degradation.[91] Proteostatic mechanisms are impaired as a consequence of age.[92] While not reported by all studies, protein synthesis tends to decline with age.[93,94] There are dysregulations in protein degradation systems including the ubiquitin-proteasomal system and autophagy-lysosomal system that result in increases in protein aggregation and oxidative stress.[92] For example, the autophagy marker LC3-I is negatively correlated with muscle strength in older adults, suggesting that the maintenance of skeletal muscle quality control imparts changes in functional assessments.[23] In support of this, another study demonstrated a negative correlation between skeletal muscle cross-sectional area and LC3-I.[23] The notion of autophagy contributing to age-related proteostatic declines is further bolstered by observed decrements in

specific muscle fiber force when autophagy is inhibited via Atg7 knock-out model.[95] The impaired processes of protein degradation collectively result in muscle degradation and drive the sarcopenic phenotype.

Proteostatic impairments are also observed in older adults when challenged with a stimulus to increase protein synthesis and explained by the concept of anabolic resistance. Specifically, anabolic resistance is defined as the reduced skeletal muscle protein synthetic response to protein intake or contraction.[96,97] Basal rates of skeletal muscle protein synthesis often remain unchanged with age[98]. However, in response to a known stimulus to increase skeletal muscle protein synthesis, like resistance exercise, there is a divergence in results between older versus younger adults. Brook et al. reported that older adults experienced a blunted hypertrophic response likely due to lower rates of skeletal muscle protein synthesis compared to their younger counterparts after 6 weeks of resistance training. This blunted increase in protein synthesis was also coupled with smaller improvements in 1-repetition maximum for knee extension and a non-significant improvement in maximal voluntary contraction.[98] Thus, treatments to improve mechanisms of proteostasis by circumventing anabolic resistant pathways might improve metrics of skeletal muscle mass and function.

Metabolism

Mitochondrial metabolism is an all-encompassing term including the chemical reactions that convert macronutrients into usable cellular energy in the form of ATP using the processes of oxidative phosphorylation. Skeletal muscle mitochondrial respiration declines with age and is significantly correlated with markers of skeletal muscle function including cardiorespiratory fitness, grip strength, leg strength, and gait speed.[99]

Mitochondrial dysfunction further drives impairments in the connections between the nervous system and skeletal muscle.[100] Denervation is described by a collection of structural changes occurring at the neuromuscular junction. These changes include a decrease in the number of motor neurons in the spinal cord and degradation of neuromuscular junction

connections which drives skeletal muscle fiber atrophy.[101–103] Another age-related change in metabolism that potentially helps explain human sarcopenia is the ability to oxidize fat. Lipotoxicity or unwanted lipid spillover of lipids to organs (e.g., liver, heart, and skeletal muscle).[104] This spillover is a consequence of decreases in the ability to metabolize fats. Specifically, in a skeletal muscle injury-induced rodent model leading to lipotoxicity, termed myosteatosis in skeletal muscle, there were declines in fatty acid oxidation, increases in fat deposition, and decreases in muscle fiber cross-sectional area.[105] Increased skeletal muscle fat seems to induce a metabolic phenotype characterized by both increased intermuscular triglycerides (IMTG) and intra-muscular adipose tissue (IMAT).[106,107] Additionally, the increases in IMAT have been negatively correlated with normalized peak exercise workload and normalized maximal skeletal muscle power production in older adults.[107] Collectively, mitochondrial metabolism is implicated in human sarcopenia, therefore improving mitochondrial energetics might be one logical therapeutic target to improve skeletal muscle structure and function.

SARCOPENIA TREATMENTS

Current treatments for sarcopenia include exercise and increased protein intake.[56] While these treatments have yielded positive benefits, what has yet to be identified is effective treatments for older adults.[55] In other words, with age treatment effectiveness decreases, and this is likely due to the concept of anabolic resistance.

Exercise has demonstrated significant positive results in improving skeletal muscle function outcomes.[108,109] Specifically, older adults who underwent 12 weeks of aerobic exercise training experienced significant improvements in skeletal muscle cross-sectional area as well as strength. [110] However, there are other studies that demonstrate when a young reference group is included the magnitude of change is age-dependent. Older women after 12-weeks of resistance training, experienced blunted hypertrophic responses compared to a young reference group, likely explained by anabolic resistance.[111] Additionally, this same observation has

occurred in older men after 6 weeks of hypertrophic resistance training where they experienced blunted improvements is skeletal muscle protein synthesis and metrics of muscle function compared to younger men.[98] Moreover, exercise has its limitations for implementation. It is well accepted that sarcopenia leads to exercise intolerance and so the ability for older adults to implement exercise programs is minimal.[112] Also, older adults often present with multiple barriers like decreased self-efficacy, lack of social support, and fear of injury that result in effective exercise prescription and completion.[113–115]

Another proposed treatment is increased protein intake to augment skeletal muscle protein synthesis. However, older adults demonstrate anabolic resistance where they experience a blunted response from protein feeding.[116] Specifically, after 3 months of leucine protein feeding in older adults there were no positive benefits in skeletal muscle mass or function.[117] In another study with increased protein intake for 8 months authors observed increases muscle function in the absence of increased mass.[118]

Since exercise and increased protein intake yield some positive results scientists have additionally tested the combination therapy of the two combined. Despite this combination, which results in promising results in young healthy adults the inquiries in older adults is equivocal.[119] Specifically, older sarcopenic women did experience an additive effect of exercise and increased protein intake as the combination resulted in increases in muscle mass and strength compared to the individual treatments alone.[120] However, in older men when protein intake was increased both before and after exercise there were no synergistic effects observed.[121] While each of these aforementioned therapeutics provide some positive results, it is still necessary to develop effective treatments for sarcopenia that can be easily adhered to in older adults.

TRANSLATIONAL MODELS FOR SARCOPENIA

A major barrier to translating basic discoveries into effective therapeutics for preventing or treating sarcopenia in humans is a lack of preclinical models that closely mimic

musculoskeletal decline in older adults. Current preclinical models present limitations including the requirement for mechanical injury or genetic manipulation to model the disease. For example, the PolyA mouse has a mitochondrial DNA mutation that results in an aged skeletal muscle phenotype. However, this model also includes co-morbidities absent in older humans, such as decreases in subcutaneous fat.[24,25] Importantly, the PolyA mouse model lacks the declines in muscle function that are indeed present in aging humans[26]. Finally, the mitochondrial DNA mutations mainly occur during embryonic development and do not follow the linear increases in mitochondrial DNA mutations that occur with increasing age in older adults.[27] The Cu/Zn superoxide dismutase knock-out mouse (i.e., Sod1KO) is a more recently developed model of both sarcopenia and frailty.[28] Sod1 is an endogenous antioxidant that converts potent superoxide into hydrogen peroxide and plays a significant role in redox homeostasis.[29] Thus, by knocking it down there is potential to elucidate redox mechanisms implicated in numerous age-related diseases including sarcopenia. However, like the PolyA mouse, the whole-body Sod1KO mouse demonstrates other physiological alterations not present in aging humans with sarcopenia including infertility, a 30% decrease in lifespan, liver hyperplasia, and hepatocellular carcinoma.[31,32] The skeletal muscle specific Sod1KO yields a partial sarcopenic phenotype that does not include neuromuscular function impairments.[122] Skeletal muscle hindlimb unloading, denervation, and immobilization are frequently used to yield skeletal muscle atrophy.[33] However, skeletal muscle mass in all three of these interventions quickly and rapidly declines within two weeks and does not mimic the slow progressive rate observed in older adults.[34] While these rodent models contribute to current understanding of aging biology, they do not comprehensively model sarcopenia or more broadly musculoskeletal aging in a timeline that is useful for investigating mechanisms of age-related musculoskeletal decline or efficacy of preclinical interventions. Thus, establishing a preclinical model that recapitulates the spontaneous onset and insidious progression of sarcopenia, particularly in the broader setting of whole musculoskeletal aging, in a short timeline is critical for success in accomplishing these

long-term goals of developing effective therapeutics.

Age-related skeletal muscle mass and functional decline does not occur independent of changes in other components of the musculoskeletal system. For example, osteoarthritis is another age-related pathology encompassed in the human musculoskeletal aging phenotype. Osteoarthritis, like sarcopenia, has inflammaging characteristics in its etiology.[123] Furthermore, those with osteoarthritis experience declines in skeletal muscle mass while those with sarcopenia further are at increased risk of developing osteoarthritis.[36,37] In a recent review examining the relationship between osteoarthritis and sarcopenia, authors support the need for mechanisms to be elucidated of this potential bidirectional relationship.[38] In addition, they highlight the lack of treatments for osteoarthritis as well. Therefore, since skeletal muscle, bone, articular cartilage, and associated connective tissues are all affected with aging, a model that mimics decline in all components would be valuable.

DUNKIN HARTLEY GUINEA PIG

The Dunkin Hartley guinea pig is a non-transgenic outbred strain of guinea pig that spontaneously develops primary joint degeneration beginning at 4 months of age in a similar pathophysiology to that of humans.[39,41,42] Our laboratory has been evaluating the skeletal muscle aging phenotype throughout the development of osteoarthritis in Dunkin Hartley guinea pigs to identify if they can also serve as a more comprehensive model of human musculoskeletal aging that overcomes the limitations of existing models.

Because increases in oxidative stress and inflammation are associated with both osteoarthritis and sarcopenia in humans, we speculate that the Dunkin Hartley guinea pig might be a valuable model of overall musculoskeletal aging. Our preliminary and published data suggest Dunkin Hartley guinea pigs also display decrements in the pillars of aging implicated in human sarcopenia, including inflammation, metabolism, and proteostasis. For example, these guinea pigs present with age-related increases in low-grade systemic inflammation as assessed by serum compliment C3, and increased joint inflammation evidenced by greater IL-1β

expression (Radakovich et al., unpublished;)[42]. Evidence of skeletal muscle mitochondrial dysfunction includes a significant age-related decline in submaximal ADP stimulated respiration and uncoupled respiration, and a decrease in ADP sensitivity (Musci et al., unpublished). Dunkin Hartley guinea pigs additionally show significant age-related declines in long-term rates of skeletal muscle protein synthesis in four subcellular fractions of mitochondrial, myofibrillar, cytosolic, and collagen proteins (Musci and Walsh et al., unpublished). Finally, our recent data document a skeletal muscle fiber type shift that mimics muscle in aging humans, with a lower percentage of type II fibers and larger percentage of type I fibers in the gastrocnemius from 5 to 15-months of age (Musci and Walsh et al., unpublished). Therefore, we have some preliminary evidence that the Dunkin Hartley guinea pig mimics the myofiber remodeling observed in older adults.

Osteoarthritis predisposes older adults to sarcopenia and those with sarcopenia are at an increased risk of osteoarthritis. It is hard to delineate the cause-and-effect relationship, however both are implicated in the collective age-related musculoskeletal decline.[38] The Dunkin Hartley guinea pig may serve as a model to study age-related skeletal muscle changes in the context of the more comprehensive model of musculoskeletal decline we observe in humans. Moreover, these skeletal muscle age-related changes, indicative of myofiber remodeling, are concurrent with age-related declines in gait in both the Dunkin Hartley guinea pig and in humans[51]. Gait is a metric of whole-body function and is influenced by multiple variables including joint pain, visual acuity, and muscle strength.[124] A final strength of this model is that these observed musculoskeletal deficits are evident beginning at an early age and follow a predictable progression culminating in dysfunction at an age corresponding to approximately 10% of the species maximal predicted lifespan of 12 years.[125] This is in comparison to other rodent models that do not display an aging skeletal muscle phenotype until much later in their maximal predicted lifespans (approximately 50%), and without concomitant declines in other components of the musculoskeletal system.[126,127] However, there are still gaps in knowledge to

comprehensively establish the Dunkin Hartley guinea pig as a model of musculoskeletal aging. Additional characteristics of myofibrillar remodeling (i.e., density, myofiber distribution, pennation angle, and collagen content) coupled with age-related changes in skeletal muscle inflammation, redox signaling, and accumulation of oxidatively modified proteins are lacking.

SUMMARY

Musculoskeletal aging is comprised of age-related changes in skeletal muscle, bone, articular cartilage, and associated connective tissues. Age-related decrements in each of these locations contribute to the development of age-related chronic diseases and are collectively driven by the pillars of aging. Therefore, we believe it is necessary to examine skeletal muscle aging properties in a comprehensive model of musculoskeletal aging. However, current preclinical models of sarcopenia are influenced by genetic and mechanical manipulations and do not recapitulate human skeletal muscle aging within the musculoskeletal aging paradigm. The Dunkin Hartley guinea pig is a well-established model of osteoarthritis and we speculate might also be a more comprehensive model of musculoskeletal aging.

CHAPTER III – METHODS

Study 1 addressed aim 1 through assessing the age-related changes in myofibrillar remodeling whereas study 2 addressed aim 2 through assessing the age-related changes in skeletal muscle inflammation, redox signaling, and accumulation of oxidatively modified proteins. Each study used a separate cohort of guinea pigs.

STUDY 1 – AIM #1

Husbandry, euthanasia, and tissue acquisition

All procedures were approved by the Colorado State University Institutional Animal Care and Use Committee (Protocol #16-6755AA; renewed as 19-9129A) and were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Male Dunkin-Hartley guinea pigs (n=18) were obtained from Charles River Laboratories (Wilmington, MA, USA). Male strain 13 guinea pigs (n=18) were obtained from the US Army Medical Research Institute of Infectious Diseases (Fort Detrick, MD, USA). Strain 13 guinea pigs were selected as a comparison to Dunkin Hartley guinea pigs because they are a strain of guinea pig that is not prone to developing idiopathic osteoarthritis at a young age. Three ages, 3.5, 7.5, and 13.5 months, of each strain were obtained 6 weeks prior to necropsy. Animals were maintained at Colorado State University's Laboratory Animal Resources housing facilities and were monitored daily by a veterinarian. All guinea pigs were singly housed in solid bottom cages, maintained on a 12-12-hour light-dark cycle, and provided ad libitum access to food and water. At the time of tissue harvest, the guinea pigs were 5, 9, or 15 months of age (n=6/group). In accordance with the standards of the American Veterinary Medical Association, animals were anesthetized with a mixture of isoflurane and oxygen; thoracic cavities were opened, and blood was collected via direct cardiac puncture. Immediately afterward, the anesthetized animals were transferred to a chamber filled with carbon dioxide for euthanasia.

Skeletal muscle mass, volume, and density and tibia length

Magnetic resonance imaging (MRI) was used to obtain volume in the soleus and both heads of the gastrocnemius, which had been formalin fixed with the knee and ankle joints at 90° prior to being individually dissected at their attachments to the bone. MRI scans were performed at Rocky Mountain Magnetic Resonance of Colorado State University. MRI measurements were performed with a 2.3 T Bruker BioSpec, equipped with a 20.5 cm, 100 mT/m gradient system, using a custom built 3.4 cm internal diameter, single channel RF Litz coil (Doty Scientific Inc, Columbia, SC, USA) tuned to detect ^{1}H at 100.3 MHz. The excised muscle tissue was first weighed and then imaged in groups of 8. In the T1- weighted gradient echo images, a fast low-angle shot sequence was used to acquire volumetric images resolved with 0.5 mm isotropic resolution in three-dimensions: echo time = 4.73 ms; repetition time = 15 ms; field of view = 96.0 x 33.5 x 29.5 mm^3; matrix size = 192 x 67 x 59.

The volumetric images were exported as DICOM and Analyze 11® was used for segmentation and ROI analysis of total volume, muscle volume, and non-muscle volume which primarily consists of tendon. Results included the percent of muscle and tendon within each image. Muscle mass and volume were utilized to calculate density – mass divided by volume (mg/mm^3).

Tibial length was determined using calibrated digital calipers. Measurements were collected on the posterior/caudal aspect of the bone from the intercondylar eminence to the articular surface of the medial malleolus. Measurements were taken in triplicate with the mean recorded.

Myofiber size distribution

We employed immunohistochemistry to measure myofiber size distribution in the gastrocnemius and soleus muscles. During tissue harvest, portions of both the soleus and gastrocnemius were embedded in OCT and frozen in isopentane cooled by liquid nitrogen. We then mounted 5µm skeletal muscle cryosections on microscope slides, allowed them to air dry for 10min, fixed them in -20° C acetone for 10min, and rehydrated them in 1X phosphate buffered saline (PBS). We then blocked the samples in 10% normal goat serum (NGS) for 1hr and rinsed them in 1X PBS

for 30sec. Samples were incubated in the following primary antibodies diluted in 10% NGS for 2hr at room temperature protected from the light: Laminin: Abcam 11576, 1:500; MyHC I: DHSB BA-F8, 1:50; MyHC IIB: DHSB 10F5, 1:50; MyHC IIA: DHSB 2F7 1:50. Following 3, 5min rinses in 1X PBS, we incubated the cross sections with secondary antibodies also in 10% NGS for 1hr (ThermoFisher AlexaFluor 350 A21093; 647 A21242; 555 A21426; 488 A21121; concentration: 1:500), applied an anti-fade reagent (Prolong Gold Antifade, ThermoFisher), and adhered cover slips to the microscope slides.

The slides were imaged by the Center of Muscle Biology at the University of Kentucky as described.[128] Briefly, images were acquired using an upright microscope at 20x magnification (AxioImager M1, Zen2.3 Imaging Software; Zeiss, Göttingen, Germany), which automatically acquires consecutive fields in multiple channels. These fields were stitched together in a mosaic image. The different fiber types were visually identified based on color differences in the merged image. The merged images were then analyzed using MyoVision, software developed by the Center for Muscle Biology at the University of Kentucky. The software used the anti-laminin immunofluorescence to establish line and edge structures, generating fiber outlines to provide the cross-sectional area of each fiber. Within each fiber, the software then qualified the fiber type based on the fluorescence. Type I fibers were fluorescent at 647nm, Type IIA fibers at 488nm, Type IIB fibers at 555nm. Fibers that were negative under all channels were classified as Type IIX. An average of over 1000 myofibers were analyzed per animal for each muscle. To analyze fiber size distribution, fibers were categorized into 250μm^2 bins. The number of fibers in each bin was then divided by the total number of fibers analyzed to determine the percent distribution of each bin.

Pennation angle

Formalin-fixed gastrocnemius was stained with India ink, and images captured with a Motic microscope. Pennation angle (θ), defined as the angle of the fiber from the muscle's line of action, was measured using ImageJ. Measurements were made in four different regions of the

muscle and an average angle was recorded. This technique allows for unbiasing regions of muscle due to the heterogeneity of the gastrocnemius and has been previously performed in other rodent muscle.[129] Additionally, muscle width was quantified (mm) to calculate the ratio (θ/mm) of pennation angle to width given other metrics of muscle size (i.e., muscle mass and volume) were different between strains. Measurements were quantified in a blinded and randomized fashion by a single observer. Repeatability testing was completed by performing the measurements twice (r^2 = 0.5953).

Collagen content

Formalin fixed gastrocnemius and soleus were paraffin embedded, cross sectioned, and stained with Masson's Trichrome staining following a protocol established at the Colorado State University Diagnostic Medicine Center. Stained cross sections were imaged using an upright microscope at 10x magnification. We then used ImageJ to determine the percentage of area stained blue to reflect the proportion of tissue that is collagen. To do this, a single reviewer color split the channels within ImageJ into red, green, and blue. The red channel was selected and then a similar size region within the image was cropped for each animal. Then, the reviewer set a threshold to quantify the amount of blue stain indicative of collagen deposition was present in each region of the cross section.

STUDY 2 – AIM #2

Husbandry, euthanasia, and tissue acquisition

Twenty-eight male and 28 female Dunkin Hartley guinea pigs were obtained from Charles River Laboratories (Wilmington, MA, USA) at 1 and 4 mo of age. Animals were maintained at Colorado State University's Laboratory Animal Resources housing facilities and were monitored daily by a veterinarian. All guinea pigs were singly housed in solid bottom cages, maintained on a 12-12-hour light-dark cycle, and provided ad libitum access to food and water. At the time of tissue harvest, the guinea pigs were 5 or 15-months of age (n=14/group/sex). In accordance with the standards of the American Veterinary Medical Association, animals were anesthetized

with a mixture of isoflurane and oxygen; thoracic cavities were opened, and blood was collected via direct cardiac puncture. Immediately afterward, the anesthetized animals were transferred to a chamber filled with carbon dioxide for euthanasia. The project was approved by Colorado State University IACUC, Protocol #16-6755A.

Anthropometrics

Body mass was recorded weekly and immediately prior to euthanasia. For the guinea pigs that were sacrificed at 5-months of age measurements began at 4 weeks of age and for the guinea pigs that were sacrificed at 15-months of age measurements began at 16 weeks of age. Tibia length was also measured by calibrated digital calipers to track growth. For the guinea pigs that were sacrificed at 5-months of age measurements began at 1.5 months of age and recorded bimonthly and for the guinea pigs that were sacrificed at 15-months of age measurements began at 5 months of age and recorded monthly. At necropsy limb muscles (e.g. gastrocnemius, soleus, tibialis anterior, and plantaris) were excised, placed in PBS, trimmed free of connective tissue, and wet weight was recorded. Then, they were immediately flash frozen in liquid nitrogen for further analysis.

Protein content

Western blot methods were used to identify markers of skeletal muscle inflammaging in the gastrocnemius. Depending on the marker of interest between 3 and 6 animals were used per sex per age. Markers of interest include tumor necrosis factor (TNF; for assessment of inflammation), 4-Hydroxynonenal (4-HNE; for assessment of oxidatively modified proteins), and nuclear factor erythroid 2-related factor 2 (Nrf2) and glutathione-s-transferase (GST) (for assessment of redox signaling). The gastrocnemius was powdered under liquid nitrogen and 50-70mg was weighed and placed in an Eppendorf tube. Following powdering, samples were placed in a Bullet Blender for 3min at speed 9 twice with 50-60mg of zirconium beads and 1.0mL of Radioimmunoprecipitation assay *(*RIPA) buffer (150mM NaCl, 0.1mM EDTA, 50mM Tris, 0.1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, final pH = 7.50) with HALT

protease inhibitors. Then samples were diluted to 1µg/ml with BioRad Laemmli Sample Buffer (950µl) and B-Mercaptoethanol (50µl) as a reducing agent. Samples were then boiled at 50°C for 10min to denature proteins. Then 10µl of each sample was loaded on to a 4%–20% criterion pre-cast gel. The gel was run at 120V for 120min and then transferred to PVDF membrane. Subsequently membranes were ponceau stained for 5min at room temperature on a rocker, imaged on FluorChem E chemiluminescence imager (Protein Simple), and then rinsed with 0.1M NaOH and DI water to remove the ponceau stain. Membranes were blocked at room temperature on a rocker for 1h in 5% BSA and TBST. After blocking membranes, were rinsed 3 x 10min with TBST. Membranes were probed with primary antibodies against TNF (Abcam-1793), 4-HNE (Abcam-46545), Nrf2 (Santa Cruz 13032), and GST (Abcam- 53942) diluted to 1:500 in PBS and placed on a shaker overnight at 4°C. Then, membranes were rinsed with TBST 3 x 10min and incubated with horse-radish peroxidase-conjugated goat anti-rabbit IgG antibody for 4-HNE (Santa Cruz 2004) or horse-radish peroxidase-conjugated goat anti-mouse for TNF, Nrf2, and GST (Santa Cruz 2005) diluted to 1:10,000 for 45min. Membranes were rinsed with TBST 3 x 10min and incubated for 5:00min in the dark with SuperSignal West Dura Extended Duration Substrate (Thermo 34075). Bands were imaged on FluorChem E chemiluminescence imager (Protein Simple). Analysis was completed using AlphaView SA Software and individual bands of interest were normalized to the ponceau stain of the entire lane. For 4-HNE quantification both prominent bands (n=5) were quantified individually as well as the entire lane.

Power calculations

Aim 1: The animals for this aim were a part of a pilot and feasibility grant for proof of concept work, therefore it was not powered to detect differences.

Aim 2: The larger study from which the muscle tissues were collected for aim #2 was powered based on the primary gait outcome of stride length. Changes in stride length with a treatment in a previous study had an average effect size of 2 cm and a standard deviation of 0.9 cm. As a

more conservative estimate to capture the decline in gait—an integrative outcome related to musculoskeletal health—with age only (independent of any treatment) we set an effect size of 1 cm for the power calculation, a power of 0.80 and significance (chance of Type I error) set at p=0.05 (Lenth power calculator). Based on these values, we calculated an n=13 animals/group, and increased the number to n=14/group to account for unanticipated losses. However, for some of the outcomes in study 2 only used a subset of these and the n-values are noted.

Data Analysis

Data are represented as mean ± standard error. Statistical analysis included a repeated measures 2-way ANOVA (age x strain) for study 1. Additionally, a 1-way ANVOA was used to calculate differences in myofiber size distribution at each respective bin. For study 2 independent t-tests were used to compare age-related changes from 5 to 15-months in Dunkin Hartley guinea pigs. Then, 2-way ANOVAs (age x sex) were used to examine sex differences for study 2. When necessary Tukey post-hoc analyses were used. Significance was set at $p<0.05$. However, trends $p<0.11$ were also reported as some analysis were not appropriately. GraphPad Prism 8.0 was used for all analyses.

CHAPTER IV – RESULTS

STUDY 1 – AIM #1 RESULTS

Body mass: There were overall effects of both guinea pig strain and age on body mass. Both 5 and 15-mo Dunkin Hartley (DH) guinea pigs were significantly larger than Strain 13 (S13) guinea pigs (5-mo DH: 901.78 ± 31.12g; 5-mo S13: 730.35 ± 31.30g; $p<0.05$) (15-mo DH: 1015.85 ± 39.97g; 15-mo S13: 858.28 ± 45.13g; $p<0.05$). Additionally, 9-mo S13 guinea pigs were significantly larger than 5-mo S13 guinea pigs (5-mo: 730.35 ± 31.30g; 9-mo: 914.23 ± 20.79g; $p<0.05$). (Figure 1A)

Tibia length: There were overall effects of both strain and age on tibia length. The tibias of 5-mo DH guinea pigs were significantly longer than 5-mo S13 guinea pigs (5-mo DH: 48.89 ± 31.3mm; 5-mo S13: 46.01 ± 0.32mm; $p<0.05$). Both 9 and 15-mo S13 guinea pigs had significantly longer tibias compared to 5-mo S13 guinea pigs (5-mo S13: 46.01 ± 0.32mm; 9-mo S13: 48.61 ± 0.25mm; 15-mo S13: 49.01 ± 0.26mm; $p<0.05$). (Figure 1B)

Muscle mass: For the gastrocnemius there was an overall significant effect of strain with DH guinea pigs being larger mass (Figure 2A). For the soleus at every age DH guinea pigs' solei were larger than their age-matched S13 counterparts (5-mo DH: 212.35 ± 12.23mg; 9-mo DH: 253.92 ± 11.57mg; 15-mo DH: 274.85 ± 16.43mg; 5-mo S13: 139.23 ± 5.89mg; 9-mo S13: 183.70 ± 10.52mg; 15-mo S13: 192.08 ± 8.47mg; $p<0.05$). Additionally, both 15-mo DH guinea pigs and 15-mo S13 guinea pigs were significantly larger than their 5-mo strain matched counterparts (5-mo DH: 212.35 ± 12.23mg; 15-mo DH: 274.85 ± 16.43mg; 5-mo S13: 139.23 ± 5.89mg; 15-mo S13: 192.08 ± 8.47mg; $p<0.05$) (Figure 2D).

Muscle volume: For the gastrocnemius there was an overall significant effect of both age and strain on muscle volume. There was a significant increase in gastrocnemius volume from 5 to 15-mo in the DH guinea pigs only (5-mo DH: 1529.39 ± 84.53mm^3; 15-mo DH: 1956.06 ± 60.39mm^3; $p<0.05$) (Figure 2B). For the soleus, there was also an overall significant effect of both age and strain on muscle volume. There was a significant increase in soleus volume from

5 to 15-mo in the DH guinea pigs only (5-mo DH: 225.22 ± 12.05mm^3; 15-mo DH: 303.56 ± 10.19mm^3; $p<0.05$). Additionally, 15-mo DH guinea pigs had significantly larger soleus volume than 15-mo S13 guinea pigs (15-mo DH: 303.56 ± 10.19mm^3; 15-mo S13: 226.69 ± 5.15mm^3; $p<0.05$) (Figure 2E).

Density: The DH guinea pigs had a significant age-related decline in gastrocnemius density from 5 to 15-mo that was absent in S13 guinea pigs (5-mo DH: 1.19 ± 0.05mg/mm^3; 15-mo DH: 1.02 ± 0.02mg/mm^3; $p<0.05$) (Figure 2C). However, age-related decreases in density were absent in the soleus other than and overall significant effect of age where in the DH guinea pig experienced significant increases in soleus density at 9 and 15-mo compared to 5-mo (Figure 2F).

Muscle mass relative to body mass and tibia length: For the gastrocnemius there was an overall effect for both strain and age when muscle mass was expressed relative to body mass. Additionally, there was a significant decrease in the gastrocnemius ratio in the S13 guinea pigs from 5 to 9-mo (5-mo S13: 2.21 ± 0.05mg/g; 9-mo S13: 1.94 ± 0.03mg/g; $p<0.05$) (Figure 3A). For the soleus there was an overall effect for both strain and age when muscle mass was made relative to body mass (Figure 3C). When expressed relative to tibia length, there was an overall significant effect of strain in the gastrocnemius mass (Figure 3B). In the soleus there was a significant effect of age on muscle mass relative to tibia length. Additionally, in the DH guinea pigs the ratio increased from 5 to 15-mo (5-mo DH: 4.34 ± 0.24mg/mm; 15-mo DH: 5.60 ± 0.33mg/mm; $p<0.05$) (Figure 3D).

Non-muscle volume: There were no differences in gastrocnemius non-muscle volume in either strain or across ages which is primarily compromised of tendon (Figure 4A). Additionally, there was only an effect of strain, not age, on soleus non-muscle volume (Figure 4B).

Myofiber size distribution: In the gastrocnemius there was a shift toward a larger percentage of smaller myofibers as a consequence of age in the DH guinea pigs; this shift was not present in the S13 guinea pigs (Figure 5A). This same strain-specific observation was also present in the

soleus although the shift was less dramatic (Figure 5B). Specifically, 5-mo Dunkin Hartley guinea pigs had a greater percentage of larger fibers ($p<0.05$ for a diameter of 2750 μm^2) while 15-mo Dunkin Hartley guinea pigs had tended to have a greater percentage of smaller myofibers ($p=0.1057$ for a diameter of 1750 μm^2). In the soleus, only 5-mo DH guinea pigs displayed significantly greater percentages of larger fibers at 3750μm^2, 4250μm^2, 4500μm^2, 4750μm^2, and 5000μm^2 compared to old 15-mo DH guinea pigs. However, the 15-mo DH guinea pigs had a significantly greater percentage of smaller myofibers at 2000μm^2 compared to the young 5-mo DH guinea pigs. All age-related changes were absent in the S13 guinea pigs.

Fibrosis: There was an overall effect of age in collagen content in either the gastrocnemius (Figure 6A). However, there was no age or strain related changes in collagen content in soleus (Figure 6B).

Pennation Angle: There were no significant effects of age or strain on pennation angle or muscle width in the gastrocnemius muscle (Figure 7A and 7B). The ratio of pennation angle to muscle width however demonstrated a significant effect of age on pennation angle: muscle width, with 15-mo S13 guinea pigs having a greater ratio compared to 5-mo S13. (5-mo S13: 0.021 ± 0.001°/mm; 15-mo S13: 0.027 ± 0.002°/mm; $p<0.05$) (Figure 7C).

STUDY 1 – AIM #1 FIGURES

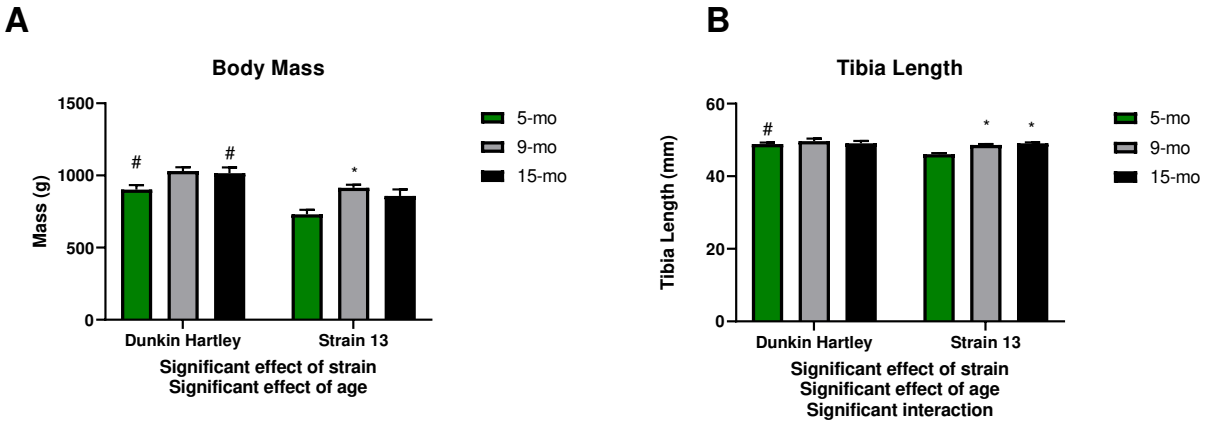


Figure 1. Body mass (g) (A) and tibia length (mm) (B) of 5, 9, and 15-mo Dunkin Hartley and Strain 13 guinea pigs. * denotes significantly different from 5-mo within the same strain; # denotes significantly different from Strain 13 of the same age

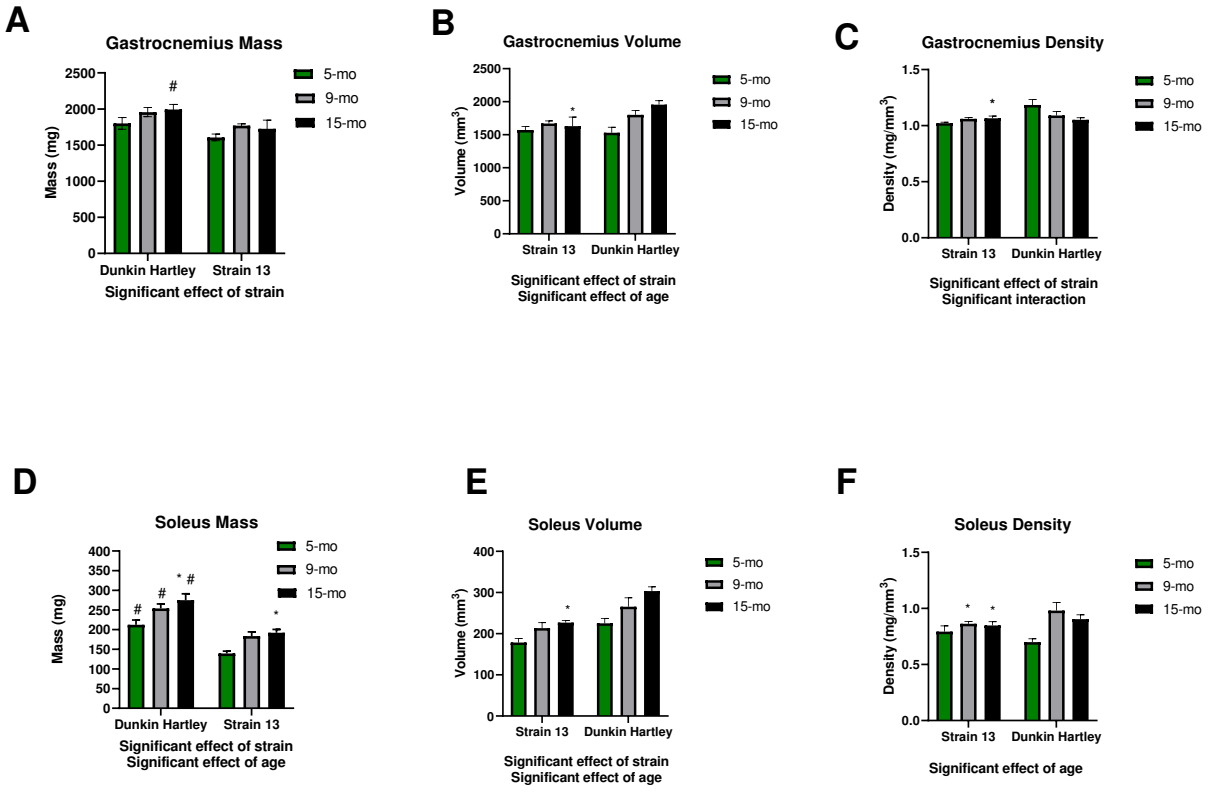


Figure 2. Muscle mass (mg), muscle volume (mm^3), and muscle density (mg/mm^3) for gastrocnemius (A, B, and C) and soleus (D, E, and F), respectively of 5, 9, and 15-mo Dunkin Hartley and Strain 13 guinea pigs. * denotes significantly different from 5-mo within the same strain; # denotes significantly different from Strain 13 of the same age

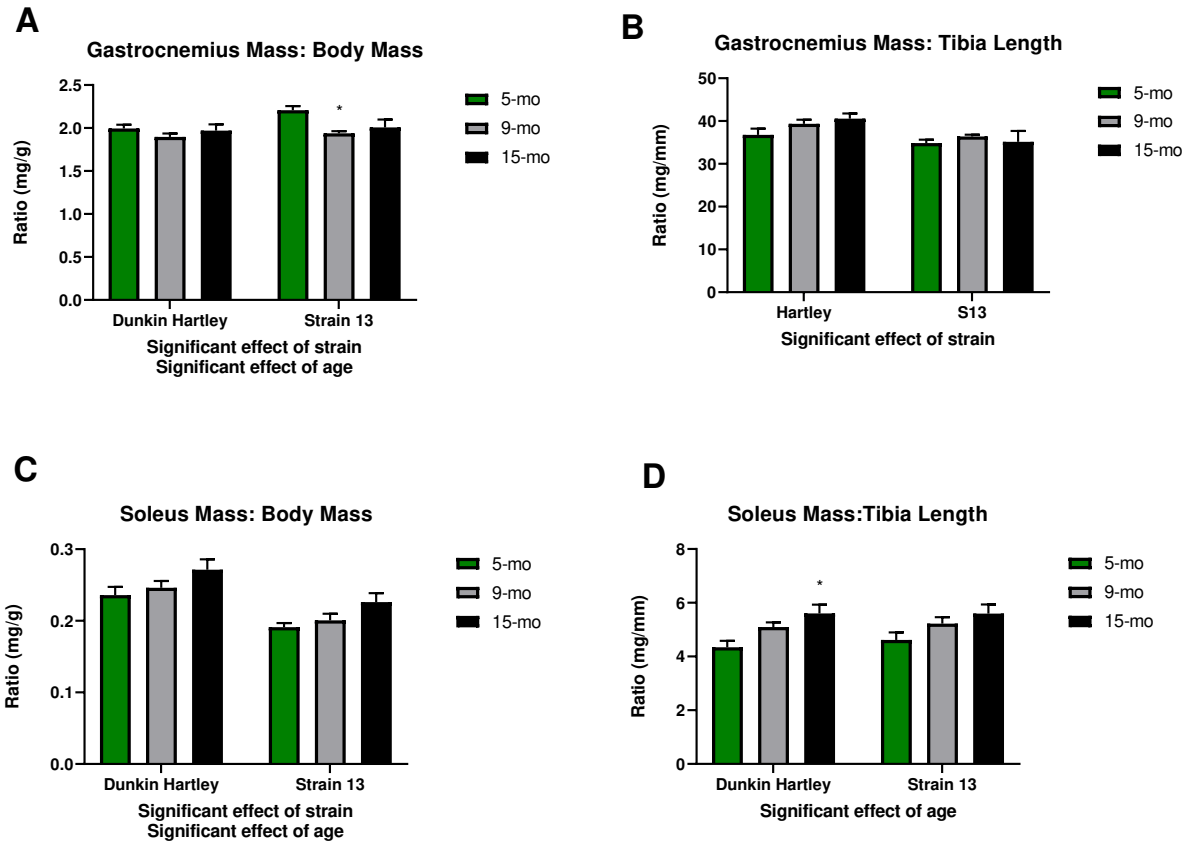


Figure 3. Muscle Mass relative to body mass (mg/g) and tibia length (mg/mm) for the gastrocnemius (A and B) and soleus (C and D), respectively of 5, 9, and 15-mo Dunkin Hartley and Strain 13 guinea pigs. * denotes significantly different from 5-mo within the same strain; # denotes significantly different from Strain 13 of the same age

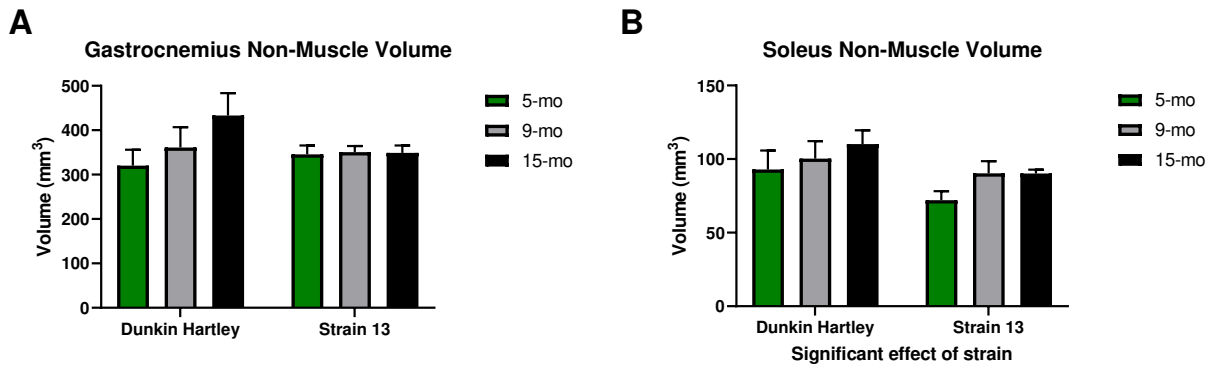


Figure 4. Non-muscle volume (mm^3) for the gastrocnemius (A) and soleus (B) of 5, 9, and 15-mo Dunkin Hartley and Strain 13 guinea pigs.

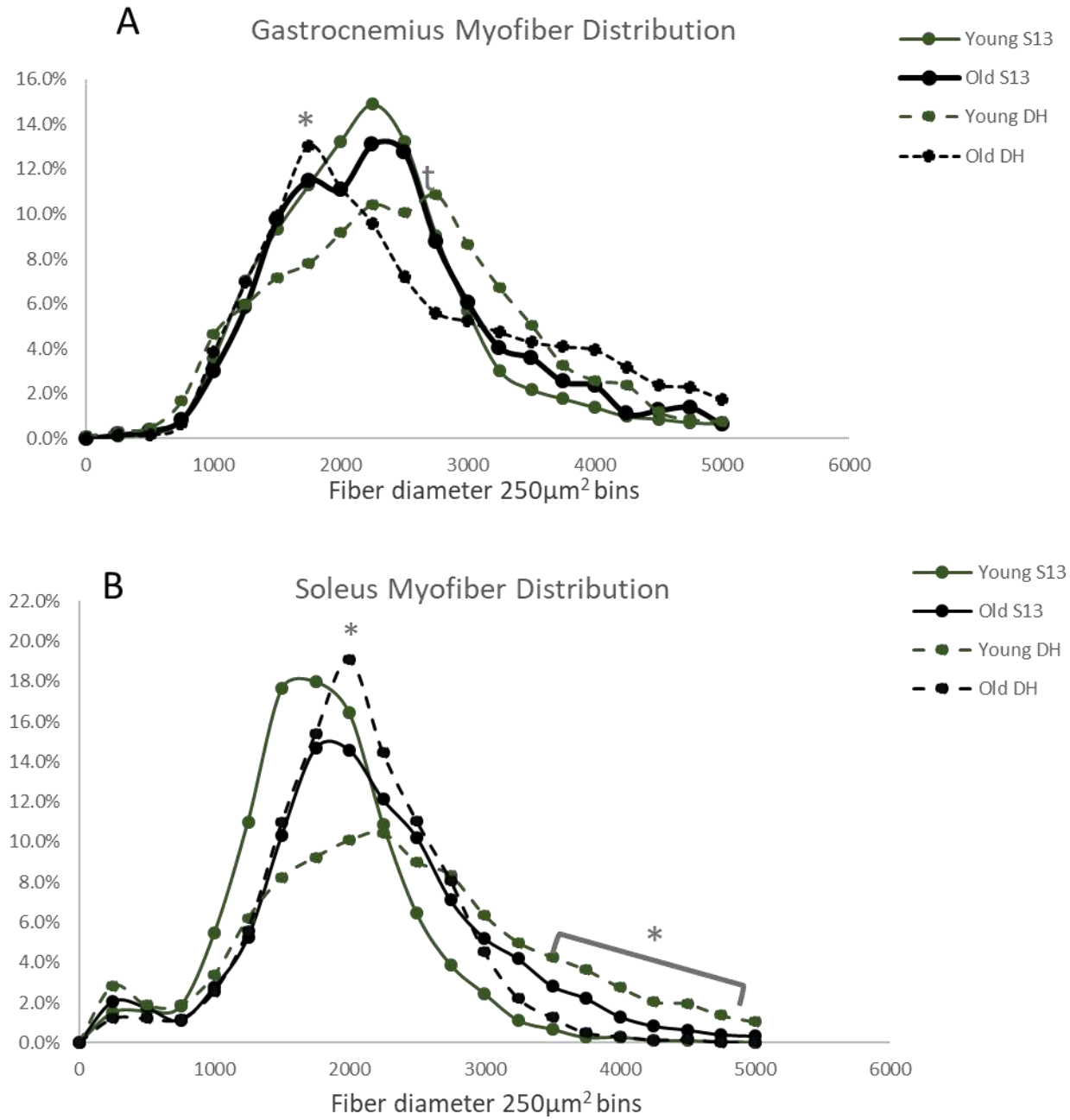


Figure 5. Gastrocnemius myofiber distribution (%) of an average of 100 myofibers in 250µm^2 bins for 5 and 15-mo Dunkin Hartley and Strain 13 guinea pigs (A). Significant difference between 5-mo Dunkin Hartley and 15-mo Dunkin Hartley at 2500 µm^2 and trend t ($p = 0.1057$) between 5-mo Dunkin Hartley and 15-mo Dunkin Hartley at 1750 µm^2. Soleus myofiber distribution (%) of an average of 100 myofibers in 250µm^2 bins for 5 and old 15-mo Dunkin Hartley and Strain 13 guinea pigs (B). Significant difference between 5-mo Dunkin Hartley and 15-mo Dunkin Hartley at 2000, 3750, 4250, 4500, 4750, and 5000 µm^2.

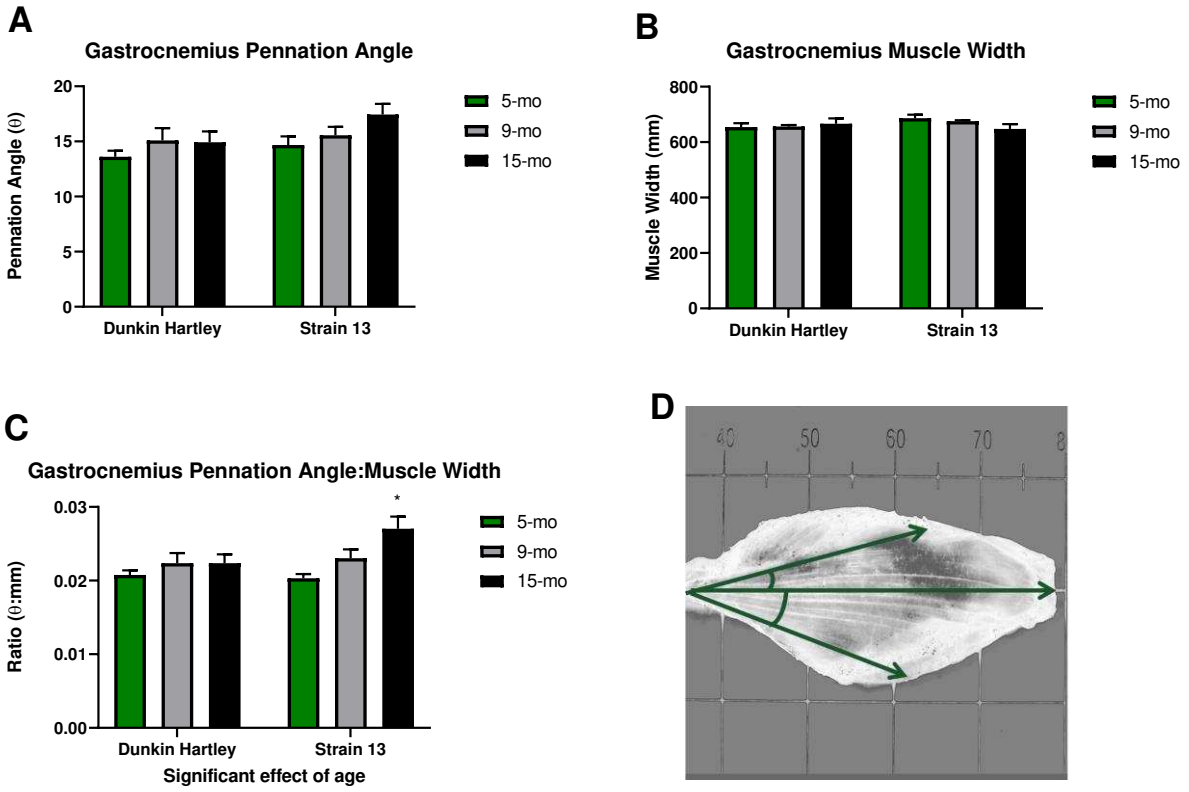


Figure 6. Pennation angle (θ) (A), muscle width (mm) (B), and pennation angle relative to muscle width (θ/mm) (C) for the gastrocnemius of 5, 9, and 15-mo Dunkin Hartley and Strain 13 guinea pigs. Panel D is a representative image of a gastrocnemius pennation angle. * denotes significantly different from 5-mo within the same strain

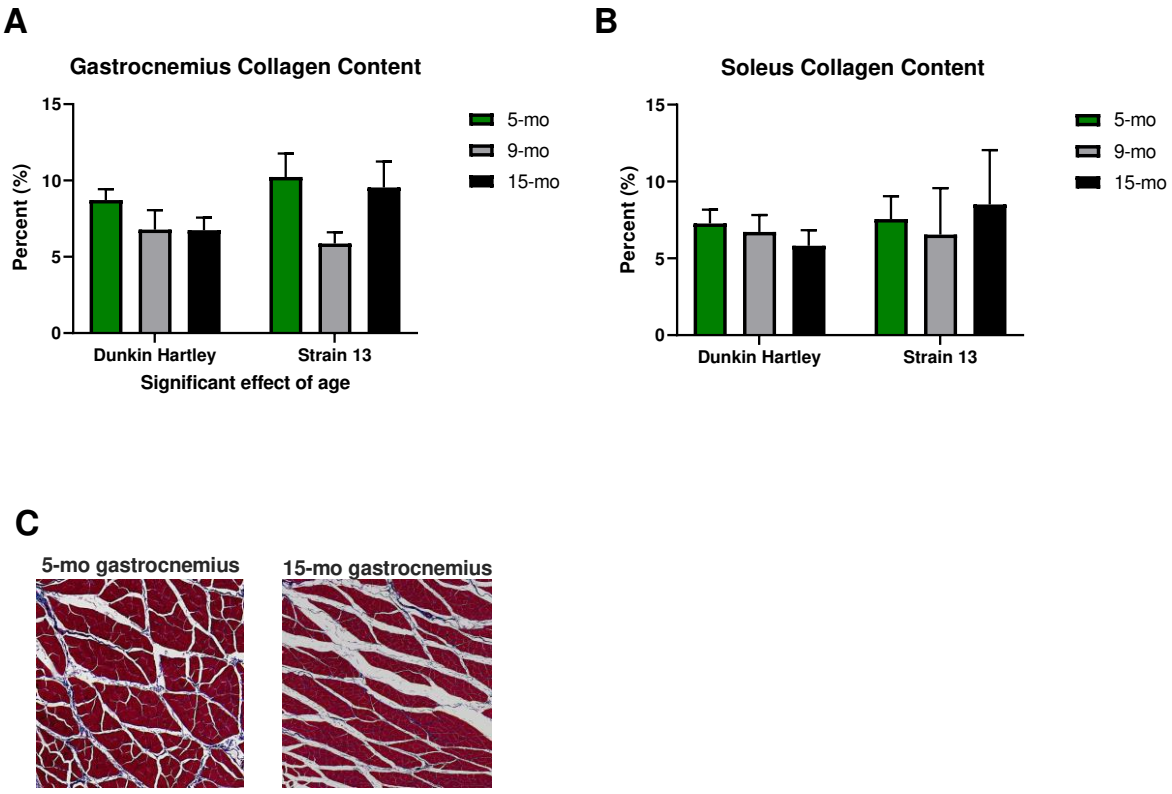


Figure 7. Collagen content (%) in the gastrocnemius (A) and soleus (B) of 5, 9, and 15-mo Dunkin Hartley and Strain 13 guinea pigs. Panel C is a representative image of a stained gastrocnemius cross-section from a 5 and 15-mo Dunkin Hartley guinea pigs.

STUDY 2 – AIM #2 RESULTS

Three guinea pigs were lost due to premature morbidity/euthanasia or mortality, so final sample sizes were n=13 for 5-mo males, n=14 for 5-mo female, n = 14 for 15-mo males, and n = 12 for 15-mo females.

Body mass: Body mass for both the young and old DH guinea pigs were tracked weekly over time (Figures 8A and 8B). Changes in body mass over time were also recorded (Figures 8E and 8F). At necropsy there was a significant effect of age on body mass (Figure 9A) and an additional sex-specific effect with larger mass in both 5 and 15-mo males compared to 5 and 15-mo female DH guinea pig (Figures 9C).

Tibia length: Tibia length was measured monthly for both the 5 and 15-mo guinea pigs (Figures 8C and 8D). Change in tibia length was also recorded (Figures 8G and 8H). Based on the last measurement made prior to euthanasia, there was a significant increase in tibia length from 5 to 15-mo DH guinea pigs (Figure 9B). Tibia length was also significantly greater in the male DH guinea pigs compared to female guinea pigs (Figure 9D).

Muscle mass: For all limb muscles (gastrocnemius, soleus, plantaris, and tibialis anterior) there was a significant of age on muscle mass (Figure 10 A-D). This finding was consistent when muscle masses were expressed relative to either body mass (Figure 11 A-D) or tibia length (Figure 12 A-D). When examining sex-specific effects in the 5-mo and 15-mo guinea pigs, males had significantly larger masses for the gastrocnemius, soleus, plantaris, and tibialis anterior muscles compared to female 5-mo and 15-mo guinea pigs (Figure 10 E-H). When muscle mass was expressed relative to body mass, there was only an overall significant effect of sex in the gastrocnemius and tibialis anterior where male DH guinea pigs has larger muscles (Figure 11 E-H). Finally, when muscle mass was expressed relative to tibia length, there was a significant effect of sex for all limb muscles (Figure 12 E-H).

Protein content: TNF

There were no overall effects of age or sex on TNF protein content for either the TNF homotrimer (at 52kDa) or the glycosylated form of TNF (28-37kDa) (Figure 13 A and B). Although there were no overall effects of age, we chose to make multiple comparisons between ages within each sex. The rationale for this was based on observed sex-differences in other measures related to the inflammaging phenotype in this same cohort of guinea pigs (unpublished). Here we observed an overall significant interaction for the glycosylated form of TNF, wherein females had an increase from 5 to 15-months whereas males had a decrease in glycosylated TNF from 5 to 15 months (Figure 13D).

Protein content: 4-HNE

We assessed modification of proteins via 4-HNE-protein adducts that form as a consequence of lipid peroxidation. We analyzed a total of 5 prominent bands at 100, 50, 37, 25, and 15kDa and additionally analyzed the entire lane for each gastrocnemius sample. There was an age-related decrease in 4-HNE present in 4 (Band 2 50kDa, Band 3 37kDa, Band 4 25 kDa, and Band 5 15 kDa) of the 5 bands (Figure 14 A-E). Additionally, when the entire lane was quantified there was an age-related decrease in 4-HNE protein content from 5 to 15-months of age. Similar to our observations with TNF, this effect of age was driven by the females (Figure 14F and M). In other prominent bands of 4-HNE, there was not a sex dimorphic effect (Figure 14 H-L).

Protein content: Nrf2

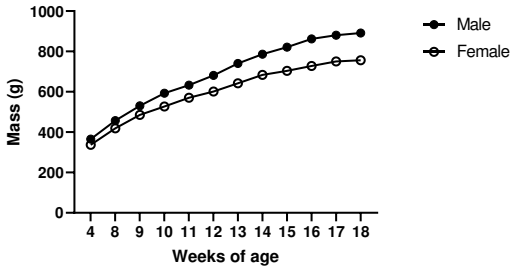
There was no overall age-related difference in Nrf2 content (Figure 15A). However, there was a sex dimorphism in age-related changes in Nrf2, where males had a trend for lower Nrf2 protein content with age ($p=0.1081$) and females had significantly greater Nrf2 protein content with age (Figure 15B).

Protein content: Glutathione-s-transferase

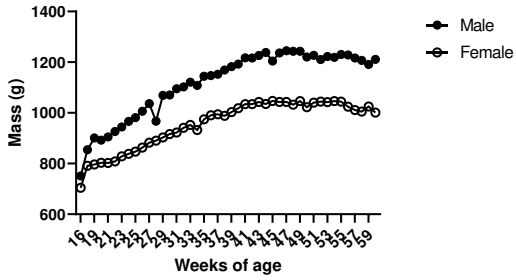
Consistent with the observed an age-related decrease in 4-HNE, there was an age-related increase in glutathione-s-transferase (Figure 16A). Finally, when analyzed by sex, females tended to drive the overall significant increase from 5 to 15-months ($p=0.0611$).

STUDY 2 – AIM #2 FIGURES

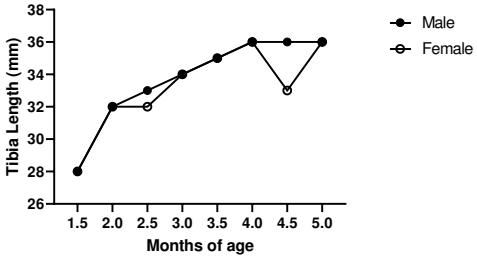
A



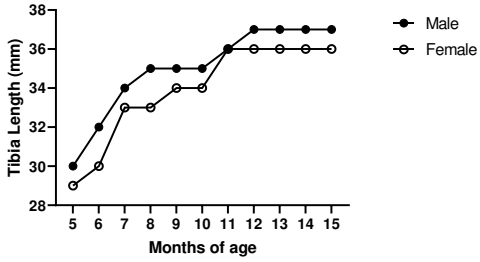
B



C



D



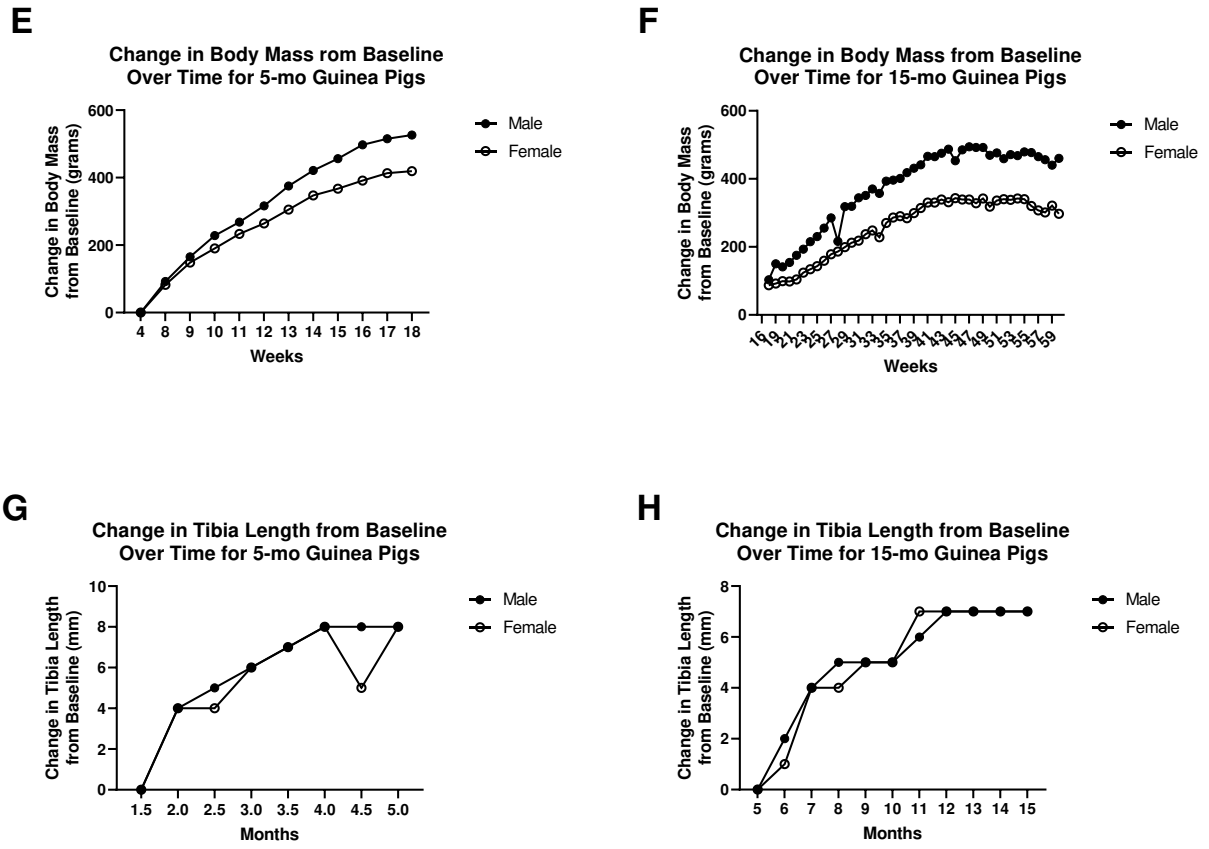


Figure 8. Growth curve of body mass (g) and tibia length (mm) over time for 5-mo (A and C) and 15-mo (B and D) for Dunkin Hartley guinea pigs, respectively. Change in body mass (g) and tibia length (mm) from baseline over time for 5-mo (E and G) and 15-mo (F and H) for Dunkin Hartley guinea pigs, respectively.

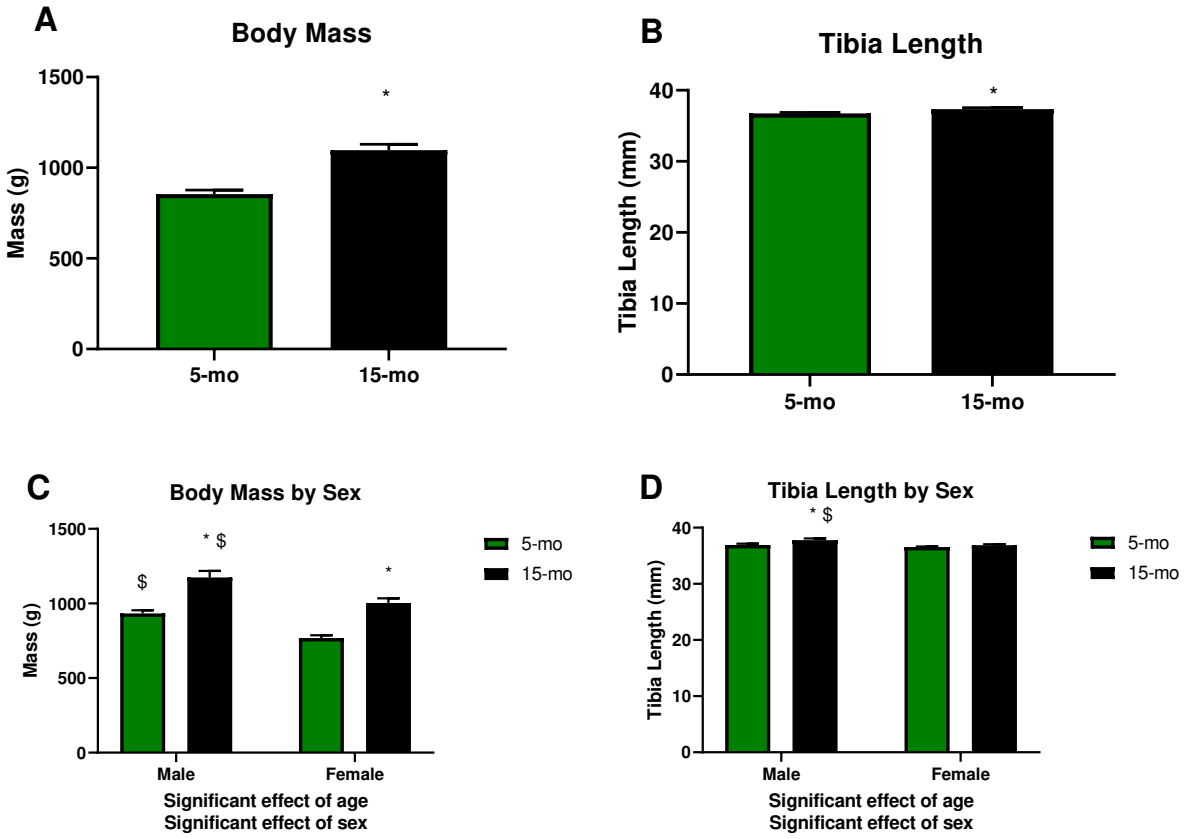
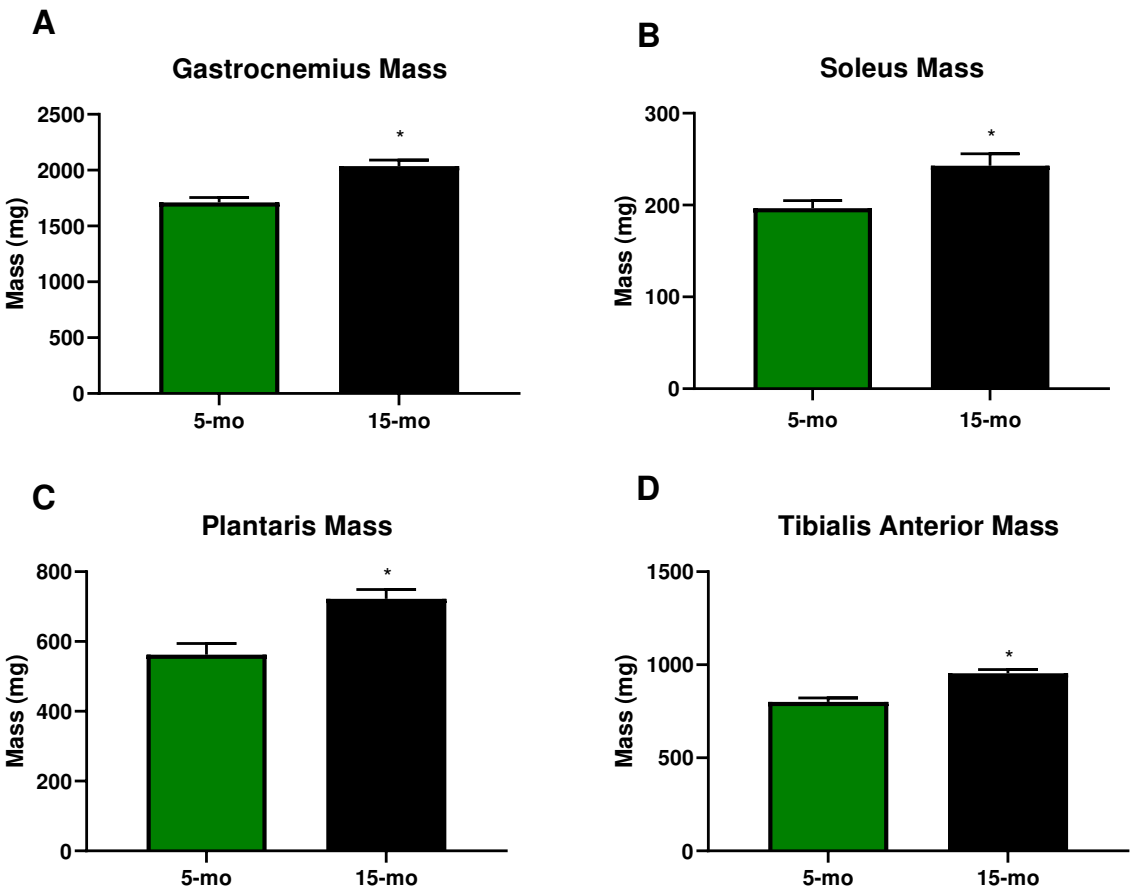


Figure 9. Body mass (g) (A) and tibia length (mm) (B) of 5 and 15-mo Dunkin Hartley guinea pigs with sexes combined. Body mass (g) (C) and tibia length (mm) (D) split by sex. * denotes significantly different from 5-mo; $ denotes significantly different from female guinea pigs of the same age


A
Gastrocnemius Mass
Mass (mg)
2500
2000
1500
1000
500
0
*
5-mo
15-mo
B
Soleus Mass
Mass (mg)
300
200
100
0
*
5-mo
15-mo
C
Plantaris Mass
Mass (mg)
800
600
400
200
0
*
5-mo
15-mo
D
Tibialis Anterior Mass
Mass (mg)
1500
1000
500
0
*
5-mo
15-mo

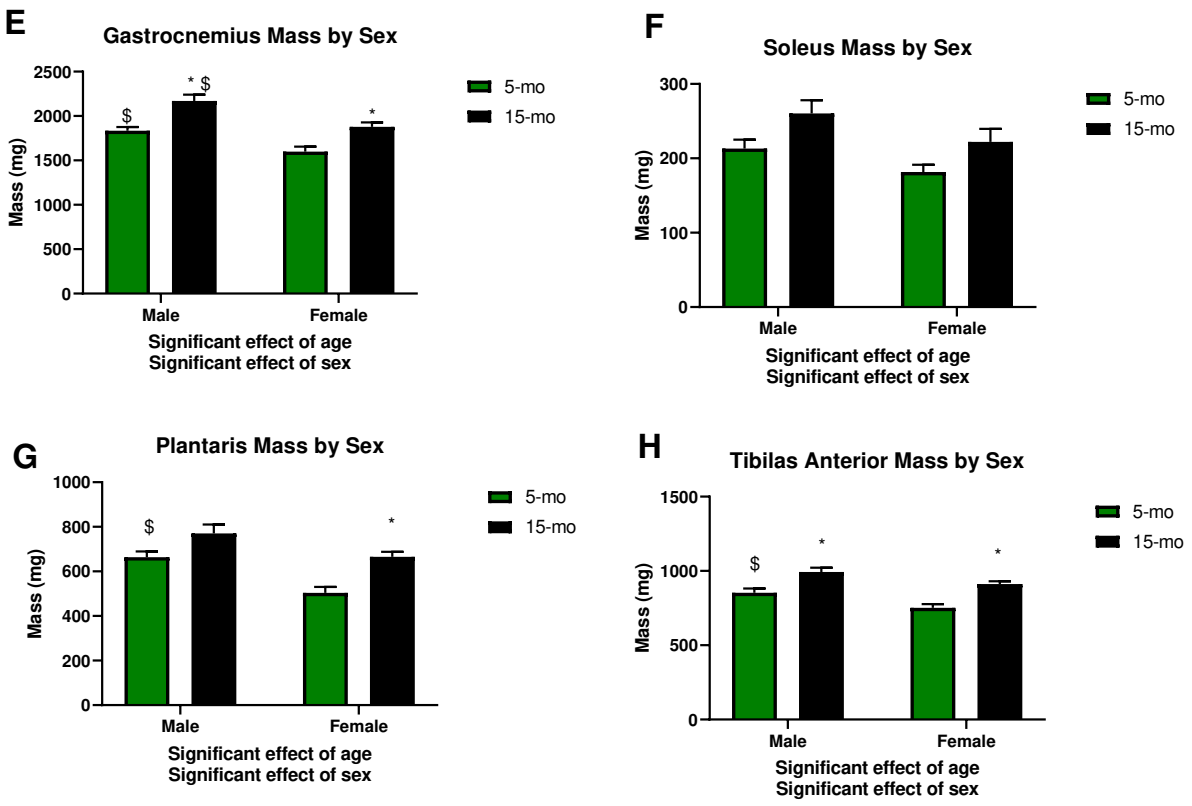
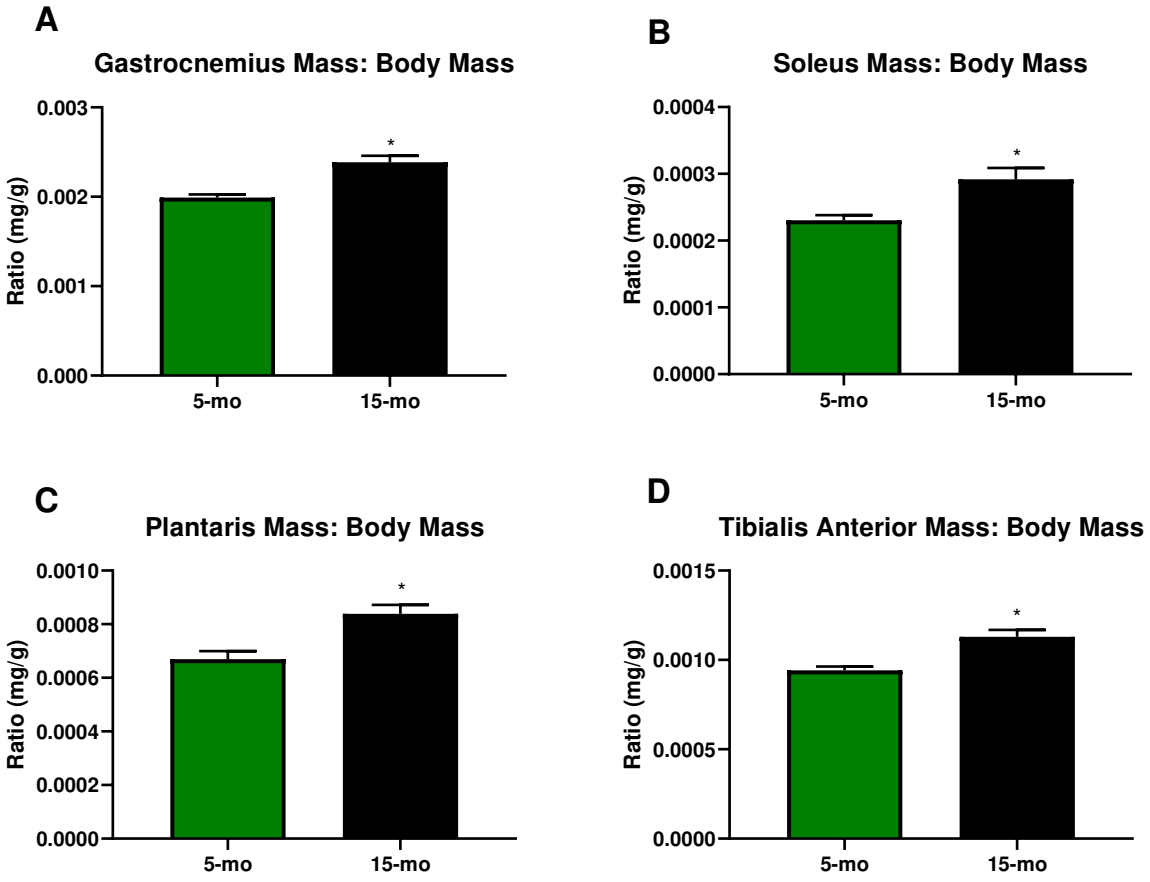


Figure 10. Muscle mass (mg) of gastrocnemius (A), soleus (B), plantaris (C), and tibialis anterior (D) of 5 and 15-mo Dunkin Hartley guinea pigs with sexes combined. Muscle mass (mg) of gastrocnemius (E), soleus (F), plantaris (G), and tibialis anterior (H) split by sex. * denotes significantly different from 5-mo; $ denotes significantly different from female guinea pigs of the same age


A
Gastrocnemius Mass: Body Mass
Ratio (mg/g)
0.003
0.002
0.001
0.000
*
5-mo
15-mo
B
Soleus Mass: Body Mass
Ratio (mg/g)
0.0004
0.0003
0.0002
0.0001
0.0000
*
5-mo
15-mo
C
Plantaris Mass: Body Mass
Ratio (mg/g)
0.0010
0.0008
0.0006
0.0004
0.0002
0.0000
*
5-mo
15-mo
D
Tibialis Anterior Mass: Body Mass
Ratio (mg/g)
0.0015
0.0010
0.0005
0.0000
*
5-mo
15-mo

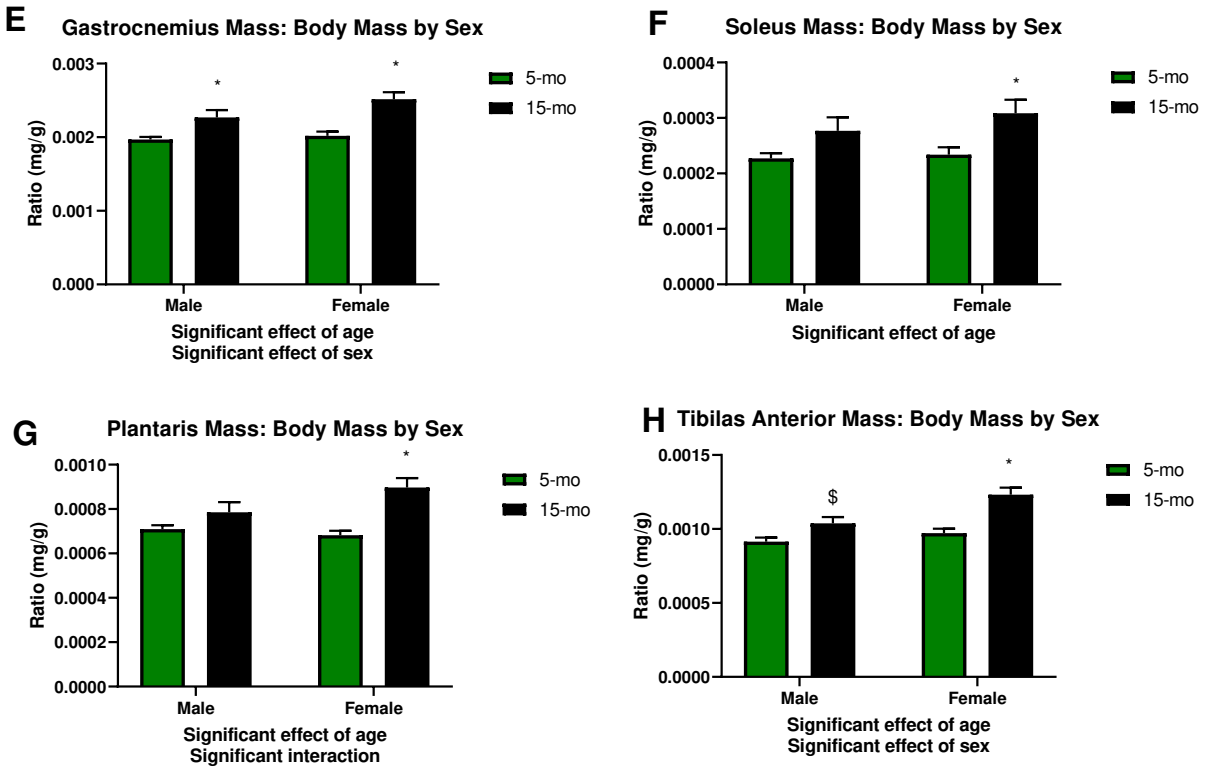
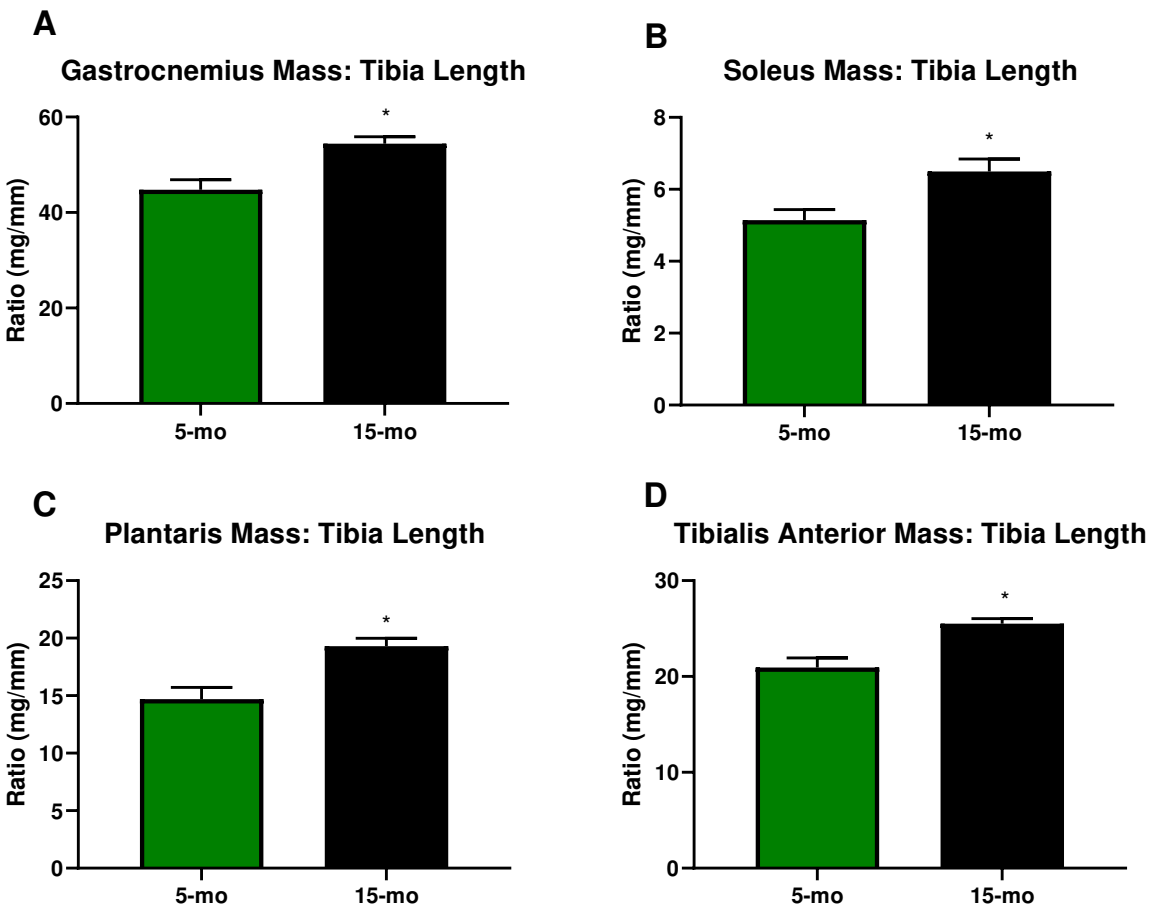


Figure 11. Muscle relative to body mass (mg/g) of gastrocnemius (A), soleus (B), plantaris (C), and tibialis anterior (D) of 5 and 15-mo Dunkin Hartley guinea pigs with sexes combined. Muscle mass relative to body mass (mg/g) of gastrocnemius (E), soleus (F), plantaris (G), and tibialis anterior (H) split by sex. * denotes significantly different from 5-mo; $ denotes significantly different from female guinea pigs of the same age


A
Gastrocnemius Mass: Tibia Length
Ratio (mg/mm)
60
40
20
0
*
5-mo
15-mo
B
Soleus Mass: Tibia Length
Ratio (mg/mm)
8
6
4
2
0
*
5-mo
15-mo
C
Plantaris Mass: Tibia Length
Ratio (mg/mm)
25
20
15
10
5
0
*
5-mo
15-mo
D
Tibialis Anterior Mass: Tibia Length
Ratio (mg/mm)
30
20
10
0
*
5-mo
15-mo

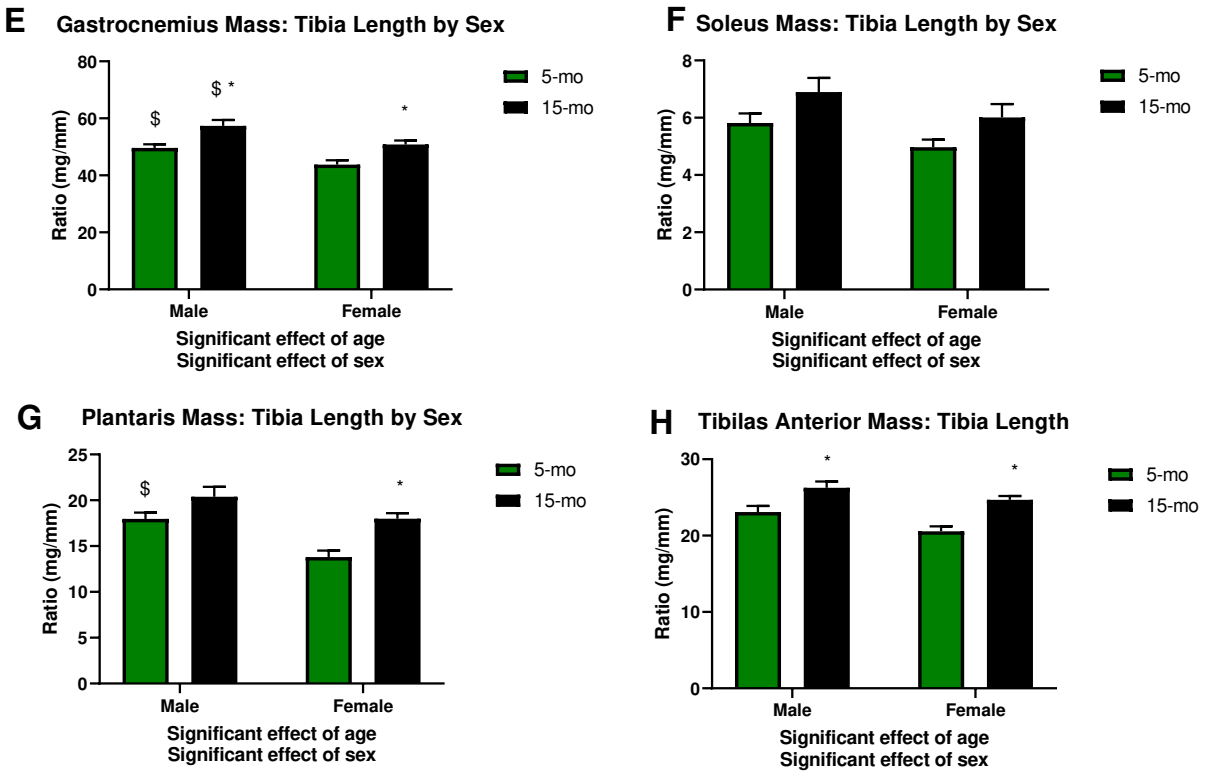


Figure 12. Muscle relative to tibia length (mg/mm) of gastrocnemius (A), soleus (B), plantaris (C), and tibialis anterior (D) of 5 and 15-mo Dunkin Hartley guinea pigs with sexes combined. Muscle mass relative to tibia length (mg/mm) of gastrocnemius (E), soleus (F), plantaris (G), and tibialis anterior (H) split by sex.* denotes significantly different from 5-mo; $ denotes significantly different from female guinea pigs of the same age

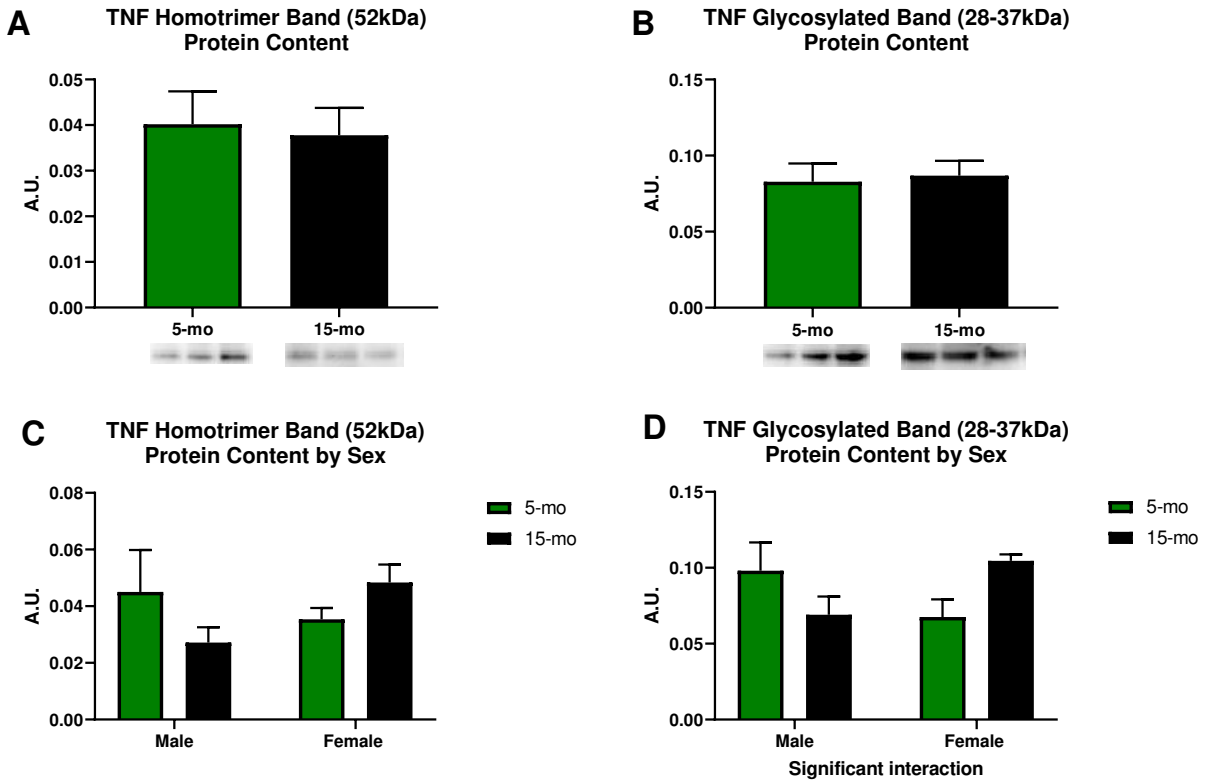
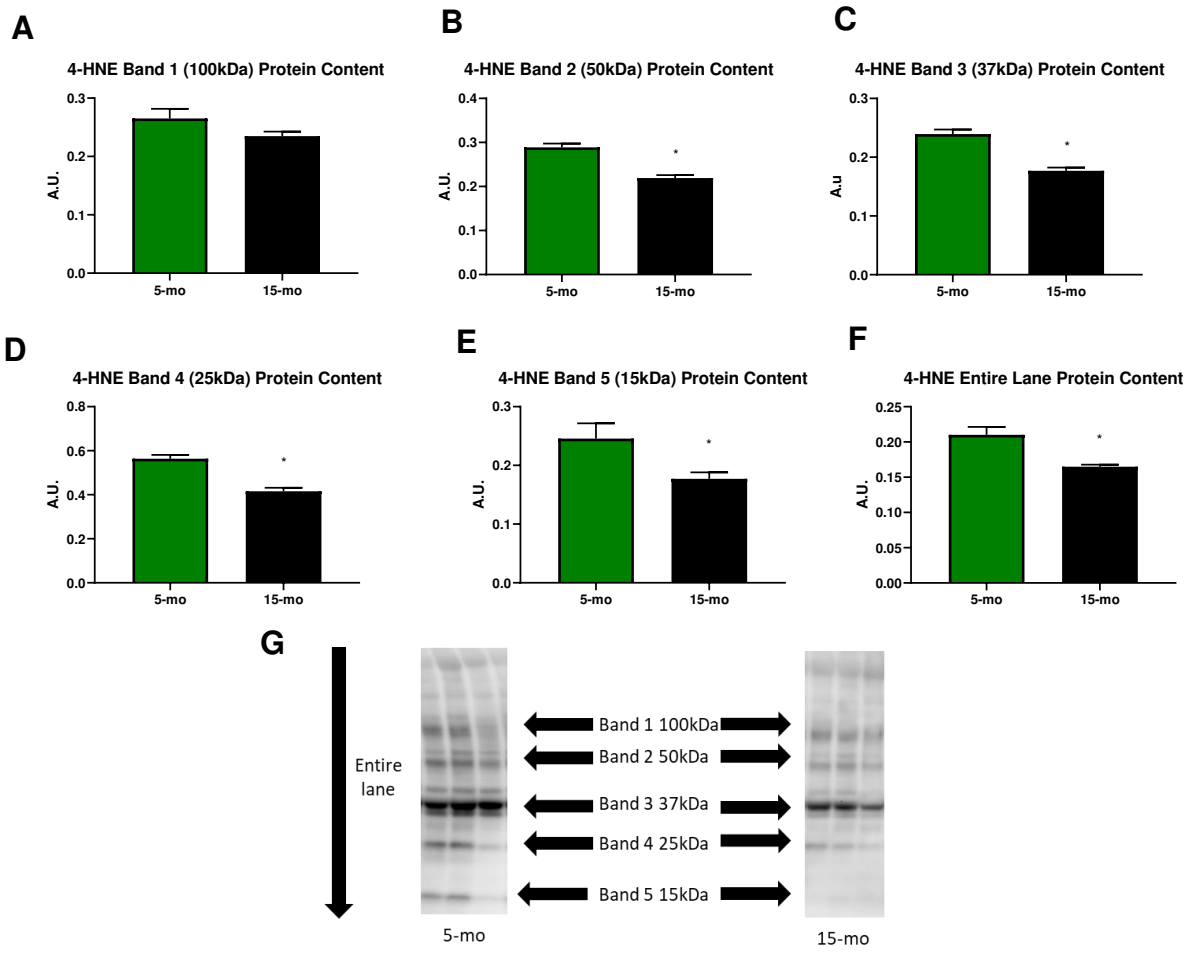


Figure 13. TNF protein content (A.U.) of the gastrocnemius of 5 and 15-mo male and female Dunkin Hartley guinea pigs with sexes combined of the homotrimer band at 52kDa (A) and glycosylated band at 28-37kDa (B), respectively. Results were then broken up by sex for the the homotrimer band at 52kDa (C) and glycosylated band at 28-37kDa (D), respectively. n = 3 per age per sex


A
4-HNE Band 1 (100kDa) Protein Content
A.U.
0.3
0.2
0.1
0.0
5-mo
15-mo
B
4-HNE Band 2 (50kDa) Protein Content
A.U.
0.4
0.3
0.2
0.1
0.0
*
5-mo
15-mo
C
4-HNE Band 3 (37kDa) Protein Content
A.u
0.3
0.2
0.1
0.0
*
5-mo
15-mo
D
4-HNE Band 4 (25kDa) Protein Content
A.U.
0.8
0.6
0.4
0.2
0.0
*
5-mo
15-mo
E
4-HNE Band 5 (15kDa) Protein Content
A.U.
0.3
0.2
0.1
0.0
*
5-mo
15-mo
F
4-HNE Entire Lane Protein Content
A.U.
0.25
0.20
0.15
0.10
0.05
0.00
*
5-mo
15-mo
G
Entire lane
Band 1 100kDa
Band 2 50kDa
Band 3 37kDa
Band 4 25kDa
Band 5 15kDa
5-mo
15-mo

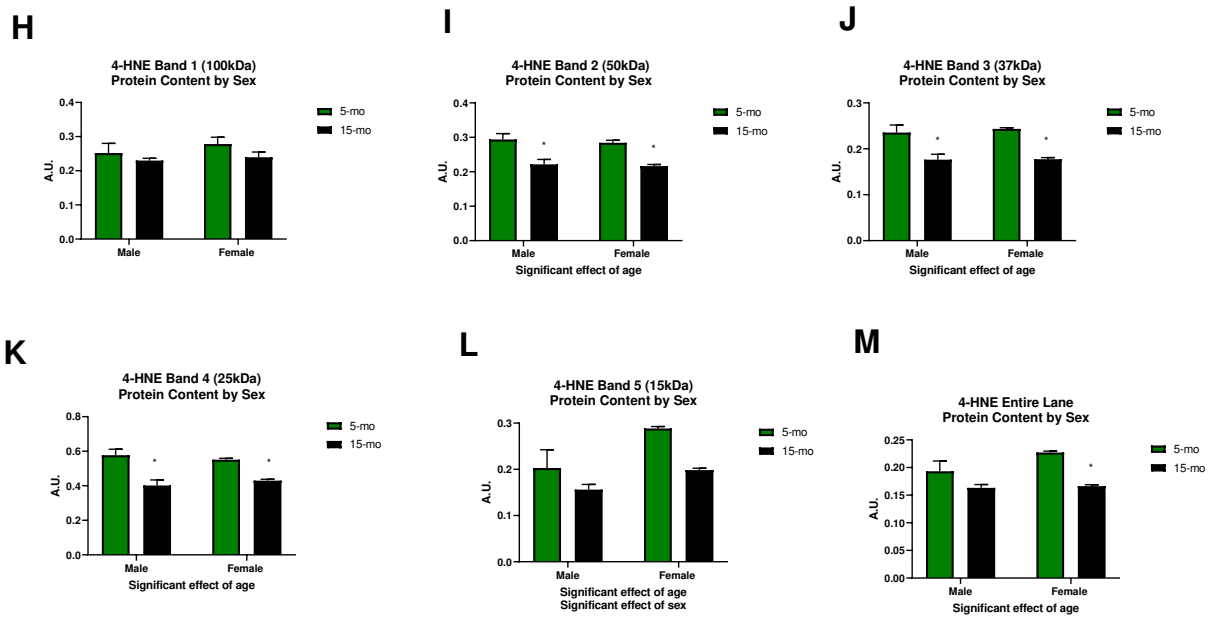


Figure 14. 4-HNE protein content (A.U.) of the gastrocnemius of 5 and 15-mo both male and female Dunkin Hartley guinea pigs with sexes combines with Band 1 at 100kDa (A), Band 2 at 50kDa (B), Band 3 at 37kDa (C), Band 4 at 35kDa (D), Band 5 at 15kDa (E), and then the entire lane (F) quantified. A representative blot is shown in panel G. Results were broken up by sex and depicted for the 5 bands and sum of the lane in panels H-M, respectively. *p<0.05 compared to 5-mo; n = 3 per age per sex

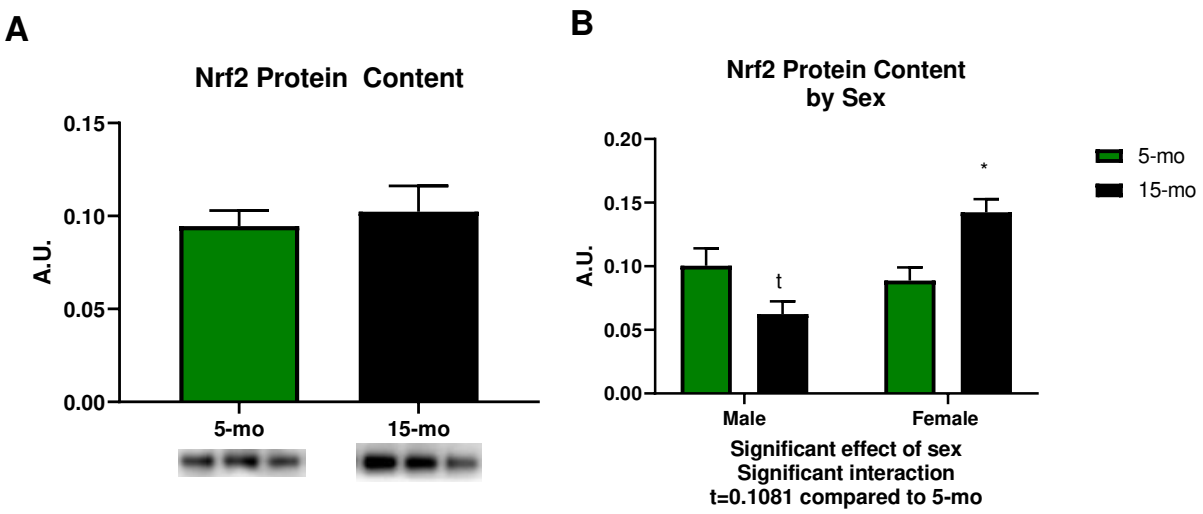


Figure 15. Nrf2 protein content (A.U.) of the gastrocnemius of 5 and 15-mo male and female Dunkin Hartley guinea pigs with sexes combined (A). Results were then broken up by sex (B). *denotes statistically different from 5-mo; t denotes p=0.10811 between 5-mo and 15-mo male Dunkin Hartley guinea pigs; n = 6 per age per sex

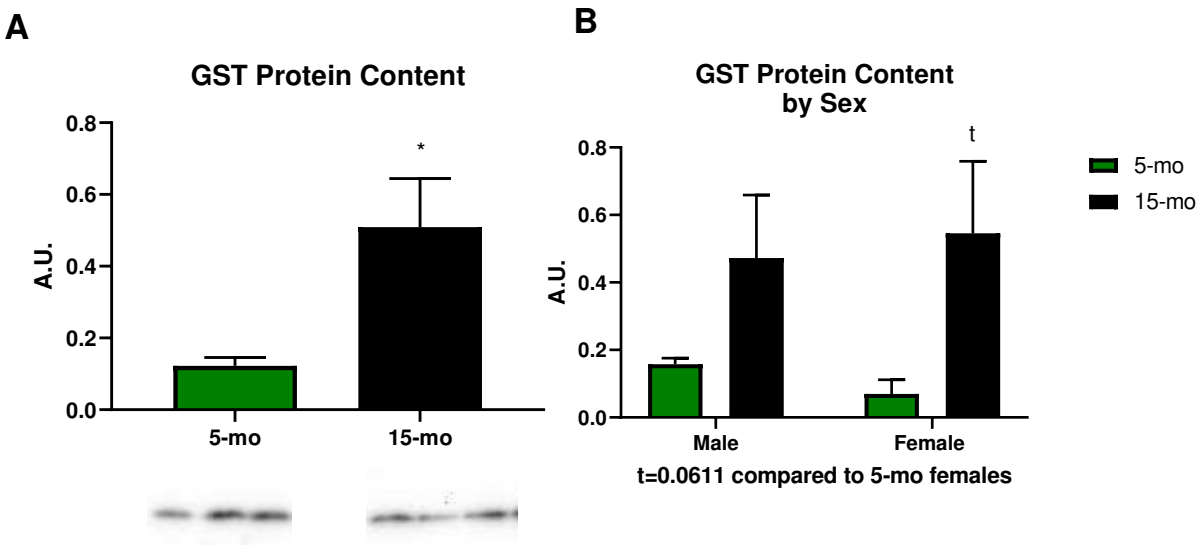


Figure 16. GST protein content (A.U.) of the gastrocnemius of 5 and 15-mo male and female Dunkin Hartley guinea pigs with sexes combined (A). Results were then broken up by sex (B). *p<0.05 compared to 5-mo; t denotes p=0.0611 between 5-mo and 15-mo female Dunkin Hartley guinea pigs; n = 6 per age per sex

CHAPTER V – DISCUSSION

PRINCIPAL OUTCOMES

Musculoskeletal aging encompasses the decrements in bone, skeletal muscle, articular cartilage, and associated connective tissues and contributes to the development of age-related chronic diseases including sarcopenia and osteoarthritis.[10] The Dunkin Hartley guinea pig is a well-characterized model of osteoarthritis and thus we believe it was warranted to additionally characterize the skeletal muscle.[39,41,42] The purpose of these studies was two-fold. The purpose of the first study was to assess myofibrillar age-related changes (i.e., skeletal muscle density, myofiber distribution, pennation angle, and collagen content) in male 5, 9, and 15- month Dunkin Hartley guinea pigs, compared to the same ages of Strain 13 guinea pigs, not prone to osteoarthritis at a young age. The purpose of the second study was to begin identifying if there was evidence of a skeletal muscle inflammaging phenotype (i.e., evidence of skeletal muscle inflammation, greater accumulation of oxidatively modified proteins, and impaired markers of redox signaling) in both male and female 5 and 15-month Dunkin Hartley guinea pigs. The ages were selected to encompass the time period from onset of joint degeneration through advanced disease. Overall findings support the posit that Dunkin Hartley guinea pigs undergo skeletal muscle remodeling that mimics some aspects of human skeletal muscle aging. Specifically, we observed declines in muscle density, shifts in myofiber size distribution, and sex-specific alterations in redox signaling from 5 to 15-months of age in the Dunkin Hartley guinea pig. Additional findings included the absence of any age-related changes in muscle mass, non-muscle volume, pennation angle, collagen content, or a marker of muscle inflammation. Finally, the accumulation of oxidatively modified proteins, contrary to our hypothesis, was decreased in 15-month Dunkin Hartley guinea pigs compared to 5-month guinea pigs.

SIMILARITIES AND DISSIMILARITIES BETWEEN SKELETAL MUSCLE REMODELING IN DUNKIN HARTLEY GUINEA PIGS AND AGING HUMANS

Sarcopenia includes declines in skeletal muscle mass and function. While we did not observe a decline in skeletal muscle mass, others have reported this same finding.[42] The age range studied was chosen to encompass the early through late stages of joint degeneration but additionally included a period of growth as evidenced by an increase in body mass and tibia length in both studies. Despite the absence of differences in skeletal muscle mass cross-sectionally, the 15-month Dunkin Hartley guinea pigs had lower muscle density compared to the 5-month Dunkin Hartley guinea pigs. An increase in fat deposition likely explains, at least in part, the decline in skeletal muscle density given that fat has a lower density than skeletal muscle. This increase in skeletal muscle fat deposition, termed myosteatosis, occurs with aging, disease states (e.g. type 2 diabetes mellitus), and injuries (e.g., hip fractures).[130–132] Density decreased in the gastrocnemius, comprised of mixed fiber types, but not in the soleus which is primarily type I fibers. This observation is consistent with a recent report that type II fibers are more susceptible to lipid spillover and fat deposition in muscle.[104] Recently, using a rotator cuff injury rodent model inducing myosteatosis, authors identified the underlying mechanisms contributing to this fat accumulation. Specifically, increases in fat accumulation in skeletal muscle created a lipid induced pro-inflammatory environment and decreased fatty acid transport and oxidation.[105] Our recent analyses indeed suggest that along with lower muscle density suggestive of lipid deposition, Dunkin Hartley guinea pigs have increased circulating pro-inflammatory mediators (Radakovich et al., unpublished) and decreased muscle mitochondrial respiration in response to fatty acids substrates (Musci et al., unpublished).

In humans, declines in density are explained by accumulation of inter-muscular and intra-muscular fat (IMAT) in locomotor muscle, which correspond to decreases in mobility.[15–17] These increases in IMAT are also significantly correlated with an increase in the circulating pro-inflammatory mediator c-reactive protein.[15,16] While we did not assess skeletal muscle function in the current study, we hypothesize that skeletal muscle force production would decrease with greater lipid deposition.

Myofiber size distribution is another characteristic of myofiber remodeling. While per cross-sectional area fast twitch and slow twitch can create the same peak force the distribution of myofibers in older adults tends to have a greater percentage of smaller myofibers.[19,133] Consistent with this, we observed a quantitative shift in the gastrocnemius and soleus myofiber size distribution in the 15-month compared to 5-month Dunkin-Hartley guinea pigs. However, this shift was absent in the Strain 13 guinea pigs. This histomorphologic observation of alterations in myofiber size distribution in other rodent models of sarcopenia is coupled with an increase in angular skeletal muscle fibers implicated by denervation.[19,102,134] As denervation is implicated in human sarcopenia, we additionally hypothesize the Dunkin Hartley guinea pigs may show evidence of denervation from 5 to 15-months of age contributing to its skeletal muscle myofiber remodeling.[102,134]

While both skeletal muscle density and myofiber size distribution in the current study mirror what is observed in the human skeletal muscle aging phenotype, a number of findings did not (i.e., pennation angle, non-muscle volume, and collagen content). Pennation angle, a metric of architectural myofiber remodeling defined as the angle of the individual muscle fiber from the line of action of the muscle, is a determinant of force generation along its line of action.[21]. In humans, both gastrocnemius and soleus pennation angles decrease with age due to decreases in the number of sarcomeres both in series and in parallel.[20] Contrary to our hypothesis, we did not observe an age-related decrease in pennation angle. When pennation angle was normalized to muscle width there was an overall significant effect of age and a significant increase from 5 to 15-months in the Strain 13 guinea pigs that was absent in the Dunkin Hartley guinea pigs. Since both Dunkin Hartley and Strain 13 guinea pigs are still growing during the timeframe of this study, as evidenced by the increasing body and muscle masses, muscle fiber pennation angle reflected the greater muscle size from 5 to 15 months of age. Our cross-sectional measures are the first in Dunkin Hartley or strain 13 guinea pigs and the gastrocnemius muscle fiber angles quantified are similar to those previously measured in a

different strain of guinea pigs.[129] Given differences in quantification methods in our study versus others and the paucity of longitudinal data, potential relationships between pennation angle and force production, and how they change with age in both in humans and preclinical models should be addressed in future studies.[20,135]

Quantification methods and paucity of longitudinal data in human subjects are also worth noting when discussing our collagen findings. Skeletal muscle collagen deposition is believed to increase or remain unchanged with age in humans as a consequence of declines in collagen turnover.[136,137] To elaborate, both synthesis and degradation rates of collagen proteins decline with age but degradation declines to an even greater extent, thereby increasing accumulation of collagen proteins[136,138]. Contrary to the work of Haus et. al and Kragstrup et. al, we observed a non-significant decrease in skeletal muscle collagen content despite significant declines in long-term rates of fractional collagen protein synthesis. Our lab has additionally used a spectrophotometric method to quantify relative collagen content in tissue using Sirius Red and Fast Green assay in which we observed significant declines in collagen content from 5 to 15-months in the Dunkin Hartley guinea pig (Musci and Walsh et al., unpublished). Thus, our observations to date show that the Dunkin Hartley guinea pig does not mimic what is believed to be an age-related increase in muscle collagen deposition, based on limited observations seen in humans. Since post-translational modifications to collagen impact its degradation and function measuring these alterations may provide insight into why collagen did not increase in our Dunkin Hartley guinea pigs.

We did not observe any age-related changes in non-muscle volume which is primarily compromised of tendon volume. In humans, metrics of tendon stiffness is more commonly assessed than non-muscle volume. For example, age-related decreases in tendon stiffness reportedly impose a greater injury risk.[126] Tendon stiffness additionally contributes to musculoskeletal function as decreased stiffness was correlated with the 6-minute walk test in

older adults.[139] In future studies it is important to examine parameters of tendon architecture and function as potential contributors to musculoskeletal decline in this novel guinea pig model.

POTENTIAL MECHANISMS UNDERLYING SKELETAL MUSCLE REMODELING IN DUNKIN HARTLEY GUINEA PIGS

Given that the Dunkin Hartley guinea pig displayed some skeletal muscle myofiber remodeling similarities to aging humans, we next decided to explore if this remodeling was accompanied by increased inflammation and oxidative stress. Increases in chronic low-grade inflammation and oxidative stress, termed Inflammaging, are implicated in age-related chronic diseases including sarcopenia.[6,78,79] In humans, increases in serum TNF, IL-6, and IL-1β are positively correlated to declines in skeletal muscle mass and function.[86–88]

TNF is therefore one molecule contributing to increased inflammation in age-related skeletal muscle dysfunction through impaired regeneration and increased apoptosis. There has been one study in humans that has examined TNF in skeletal muscle where it was decreased at both the transcriptional and translational level in older adults compared to younger adults.[81] Specifically, TNF reduces myogenic differentiation through the NFkB pathway and decreases stability of MyoD, impairing differentiation of myoblasts into myotubes during skeletal muscle regeneration.[82] TNF additionally activates apoptotic pathways like caspase 8 that increases skeletal muscle wasting in older rats.[139] To our knowledge the current study is the first to assess skeletal muscle TNF protein content in a non-transgenic rodent model of musculoskeletal decline. It appears that Dunkin Hartley guinea pigs display similar bands of TNF both as a homotrimer and as a glycosylated form. The homotrimer is the soluble form of TNF known to increase proinflammatory processes while glycosylated appears to decrease apoptosis and secretion of IL-6.[140] There is also a monomer of TNF in skeletal muscle of other rodents, but this band was undetected in our guinea pigs [141].

Contrary to our hypothesis we observed no significant differences in gastrocnemius TNF protein content between 5 and 15-months of age in either the homotrimer band or glycosylated

TNF band. Since we have observed sex differences in other analyses within this cohort of guinea pigs (Musci et al., unpublished; Andrie et al., unpublished), here we analyzed sex differences in TNF content. We observed a significant interaction between age and sex in the band corresponding with glycosylated TNF, where females had a higher level of TNF from 5 to 15-months and males had a lower level of TNF from 5 to 15-months. This interaction implies that, during progression of osteoarthritis and myofiber remodeling, changes in inflammatory markers are occurring at different trajectories in male and female guinea pigs. Moreover, at the 15-month timepoint the severity of osteoarthritis is different between males and females. Specifically, the osteoarthritis OARSI histology score is significantly lower indicating better joint quality, in the 15-month female guinea pigs compared to the 15-month male guinea pigs (Andrie et al., unpublished). TNF mRNA counts in the cartilage were not different between 5 and 15-months of age. However, C3 mRNA in the articular cartilage was significantly lower in 15-month males compared to 5-month males but significantly greater in 15-month females compared to 5-month female Dunkin Hartley guinea pigs (Andrie et al., unpublished). While these mRNA measures were made in articular cartilage there is evidence of complement-dependent TNF release[142]. We hypothesize that male Dunkin Hartley guinea pigs might display impairments in the ability to respond to acute cellular stressors, resulting in a blunted musculoskeletal inflammatory response as a consequence of age and progression of musculoskeletal decline.

Age-related increases in oxidative stress are part of the skeletal muscle inflammaging phenotype.[6] These increases in oxidative stress through mitochondrial ROS generation are implicated in multiple age-related chronic diseases including sarcopenia.[6,18] 4-HNE, a byproduct of lipid peroxidation, is one common marker of increases in oxidative stress. 4-HNE can oxidatively modify macromolecular cellular components by forming adducts with proteins and DNA, thereby contributing to age-related diseases.[143] Holloway et. al hypothesized that increased skeletal muscle levels of 4-HNE in older adults are a consequence of increased mitochondrial ROS as a fraction of electron leak.[18] Contrary to those findings and our

hypothesis, we observed significant age-related decreases in 4-HNE protein content from 5 to 15-months (Figure 14). While the decline in oxidatively modified proteins did not support our hypothesis, other experiments using these same guinea pigs confirmed that skeletal muscle mitochondrial hydrogen peroxide emissions were not different between 5 and 15-months (Musci et al., unpublished). As hydrogen peroxide and 4-HNE are only two approaches for identifying oxidative stress it is important in future studies to address other metrics of macromolecular damage.

In addition to oxidatively modified muscle proteins, we assessed Nrf2, a redox-sensitive transcription factor regulating a cytoprotective gene program.[70,71] Skeletal muscle Nrf2 content was increased in female Dunkin Hartley guinea pigs with age but tended to decrease with age in the males. This interesting sex dichotomy may imply that male and female guinea pigs are responding to their increasing disease states differently. Although we did not see an increase in oxidatively modified proteins we know that these animals are still relatively young and are undergoing myofiber remodeling with concomitant increased severity of osteoarthritis beyond 15-months of age. Together these two multifactorial events likely lead to increased stimuli including increased intracellular ROS from the progressing osteoarthritis severity in these guinea pigs that are known to activate Nrf2[144]. In the female Dunkin Hartley guinea pigs, these disease stressors seem to effectively activate Nrf2. However, the males do not appear to have a similar robust increase in Nrf2 in response to the increasing stress of disease progression. The mechanism for this sex difference is not known but may provide insights into the need to develop different treatments between males and females. Furthermore, we have also observed sex-differences in Keap1 articular cartilage mRNA which is a negative regulator of Nrf2 wherein only females experienced a significant decrease implying greater Nrf2 activation from 5 to 15-months of age (Andrie et al., unpublished).

Interestingly, in skeletal muscle of older sedentary adults with osteoarthritis, there is reportedly a blunted ability to activate Nrf2 compared to younger adults and older active

adults.[74] While this study included both males and females it did not delineate sex-differences. Therefore, because the Nrf2 activation might be impaired in the 15-month male Dunkin Hartley guinea pigs it could be a potential therapeutic target to mitigate age-related declines in redox responsiveness.

Nrf2 regulates expression of glutathione-s-transferases[72], an important family of enzymes that catalyzes detoxification of xenobiotic substrates including 4-HNE by conjugation with reduced glutathione.[72,145–147] We observed greater GST content in 15 compared to 5-month animals when males and females were combined. When examining the multiple comparison data female had a trend p=0.0611 to increase GST protein content from 5 to 15-months of age but this multiple comparison was not significant in the males. Nrf2 activation is not requisite for GST transcription and translation as other transcription factors including AP-1 and GATA-1 influence GST.[148,149] Thus, it would be important to examine other transcriptional and translational regulators in future studies along with other downstream targets of Nrf2.

To summarize, the Dunkin Hartley guinea pigs have age-related and sex-specific changes in markers of inflammaging. There was a significant interaction in that males decreased TNF and Nrf2 content from 5 to 15-months of age while females increased TNF and Nrf2 content. Interestingly, both males and females had lower 4-HNE content and higher GST content at 15-months of age compared to 5-months. These observed changes are in conjunction with increasing osteoarthritis severity which is known to be mediated by increases in inflammation with concomitant increases antioxidant defenses, likely an adaptive response to increased oxidative stress in the knee joint (Radakovich et al., unpublished)[42]. Therefore, we hypothesize that there are additional alterations in other modulators of the inflammaging pathways including transcriptional suppression and decreases in energetic efficiency that occur in these guinea pigs as their skeletal muscle myofiber remodeling progresses beyond the 15-month time point in which we studied them. Thus, therapies that target redox and inflammatory pathways like Nrf2 might be efficacious in attenuating this musculoskeletal decline.

LIMITATIONS AND CONSIDERATIONS

While we assessed a number of well-recognized aspects of skeletal muscle remodeling and skeletal muscle inflammaging associated with human aging, there are limitations that warrant mention. One limitation is that study 1 only included male guinea pigs. Another significant limitation in both study 1 and 2 was the lack of a skeletal muscle specific functional outcome. As part of another project, we are assessing gait, a metric of whole-body function, in these guinea pigs. However, a more skeletal muscle-centric functional assessment will be necessary to evaluate the contribution of the observed myofiber remodeling to skeletal muscle function. Additionally, while the Strain 13 guinea pigs have some value as a control comparison, there are numerous limitations that make them less than an ideal comparison. For example, Strain 13 guinea pigs are inbred while Dunkin Hartleys are outbred, and these differences in genetic heterogeneity limit strain comparisons. Therefore, we have begun to characterize the outbred pigmented guinea pig strain as a potentially superior control strain for comparisons to Dunkin Hartleys. Preliminary evidence suggests these pigmented guinea pigs are not prone to musculoskeletal decline in the same age range as Dunkin Hartley guinea pigs. While a comprehensive musculoskeletal profile on the pigmented guinea pigs is in progress, they may provide a more robust control strain for future studies.

CONCLUSIONS AND FUTURE DIRECTIONS

Musculoskeletal aging is a collection of age-related decrements in skeletal muscle, bone, articular cartilage, and their associated connective tissues driven by the pillars of aging. One facet of this age-related phenotype is sarcopenia. While progress has been made in understanding the underlying etiologies encompassed in musculoskeletal aging the lack of a preclinical model that mimics human conditions is a barrier to fully elucidating the multifactorial mechanisms and making progress in developing effective therapies. The Dunkin Hartley guinea pig, a well-characterized model of osteoarthritis, one pathology encompassed in musculoskeletal aging additionally appears to also display age-related changes in skeletal

myofibrillar remodeling and redox signaling. The Dunkin Hartley guinea pig circumvents limitations of current preclinical models (e.g., genetic and mechanical manipulation) and displays these age-related musculoskeletal changes very early on in its maximal predicted lifespan. Given decrements is skeletal muscle and articular cartilage occur in tandem the Dunkin Hartley guinea pig may serve as a model of musculoskeletal aging.

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