EFFECTS OF EXTRACELLULAR MATRIX STIFFNESS ON MUSCLE FIBER MECHANOTRANSDUCTION SIGNALING IN AGED RATS

By

Matthew Salzano

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Director of Thesis: Zachary J. Domire, PhD

Major Department: Kinesiology

The loss of muscle mass with age, termed sarcopenia, is a common problem among the over 60 years-old population. Resistance training is often prescribed as treatment, but older adults have shown a reduced response to exercise. Muscle stiffness is shown to increase with age due to increased glycation. Increased stiffness will cause muscle cells to experience less strain for any given load. Strain is known to be the mechanical signal for a muscle's response to exercise, which leads us to hypothesize that response to exercise is impaired because of increased stiffness.

Nineteen rats were split into young (12 months) and old (32-33 months) groups. Each rat underwent 3 sets of 10 maximum eccentric dorsiflexions. Following sacrifice, the tibialis anterior (TA) of both legs and extensor digitorum longus (EDL) of the non-exercised leg harvested. The EDL underwent stiffness testing to determine the Young's modulus of the muscle. Focal adhesison kinase (FAK) is a protein that is phosphorylated with stretch, making it a good indicator of exercise response. The TAs were used in immunoblotting analysis to determine the ratio of FAK that was phosphorylated.

Our study showed decreases in muscle size and increases in muscle stiffness with age, with more variability occurring in older rats. A negative relationship was also seen between

muscle stiffness and size. The main findings of our study showed that FAK activity decreases are related to muscle stiffness.

The results of this study show that aging is similar between rats and humans, in regards to muscle size and stiffness, and suggest sarcopenic muscle is stiffer than healthy muscle. The results also suggest old muscle loses the capacity to hypertrophy in response to exercise and that increased muscle stiffness is responsible for the decreased response to exercise. This study implicates that reduced response to exercise due to increased muscle stiffness is a possible mechanism behind the development of sarcopenia.

Effects of Extracellular Matrix Stiffness on Muscle Fiber Mechanotransduction Signaling in Aged Rats

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Matthew Salzano

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by

Matthew Salzano

APPROVED BY:

DIRECTOR OF
THESIS: _____
(Zachary Domire, PhD)

COMMITTEE MEMBER: _____
(Terry Jones, PhD)

COMMITTEE MEMBER: _____
(Anthony Kulas, PhD)

COMMITTEE MEMBER: _____
(Katsu Funai, PhD)

CHAIR OF THE DEPARTMENT
OF KINESIOLOGY: _____
(Stacey Altman, JD)

DEAN OF THE
GRADUATE SCHOOL: _____
Paul J. Gemperline, PhD

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Chapter 1 – Introduction

The population is growing older and with that comes degenerative diseases like sarcopenia, the loss of skeletal muscle with aging. The post-war baby boom generation, those born between the end of World War II and about 1960, have already increased the population of those in the 60-plus-year-old category by 11.5 million in the United States since 2010 (US Administration on Aging, 2011). As this generation continues to age, the population of 70-plus- and 80-plus-year-olds will also dramatically increase. Modern medicine and knowledge of health has kept people alive well into their 70's and 80's, but living longer allows people to be more easily susceptible to degenerative diseases like sarcopenia.

Though no set definition exists, researchers typically define classes of sarcopenia as standard deviations from normal, healthy muscle mass. One standard deviation classifies as moderate sarcopenia while two or more standard deviations is classified as severe sarcopenia. The prevalence of moderate sarcopenia in this age range is anywhere up to 70% among men and 60% in women (Janssen, Heymsfield, & Ross, 2002; Janssen, 2006). Severe sarcopenia affects up to 17% of men and 10% of women (Janssen et al., 2002; Janssen, 2006). The prevalence of sarcopenia is important because sarcopenic-related disabilities start to arise in moderate sarcopenic adults (Janssen et al., 2002) but a significant risk for disability occurs in severe sarcopenic adults (Janssen, 2006).

Sarcopenia's effect on the 60-plus-years-old population results in decreased quality of life and increased costs associated with disability. The loss of muscle mass associated with sarcopenia causes a strength deficit, resulting in disabilities and functional impairments (Ozcan, Donat, Gelecek, Ozdirenc, & Karadibak, 2005; Rantanen et al., 1999). These disabilities affect

the ability to perform activities of daily living, such as getting up out of a chair, carrying groceries, getting something down from a high-reaching position, etc. Sarcopenia-related functional impairments leading to assistance with daily activities and coverage of medical bills due to falling place a significant financial burden on the health care system (Janssen, Shepard, Katzmarzyk, & Roubenoff, 2004). Because of its impact on the quality of individuals' lives, and further economic impact, it is important to find a cause for sarcopenia so a treatment can be developed.

Sarcopenia, despite affecting a large number of elderly persons, is an age-related disease instead of a result of aging. Because it is a disease, it is important to investigate the mechanism by which this disease occurs. Although heavily researched, the cause for the development of sarcopenia remains unknown despite many theories. It is widely accepted, however, that the cause of sarcopenia is multifaceted instead of a single cause (Morley, Baumgartner, Roubenoff, Mayer, & Nair, 2001; Roubenoff, 2001). Physical inactivity is a common idea since activity levels of adults are known to decrease. Davis' Law, a corollary to Wolff's Law, supports this by saying soft tissue adapts to the demands imposed upon it. So it stands to reason that less physical activity reduces the demand placed on the muscles, and as a result, the muscles shrink in size. However, Roubenoff (2001) downplays physical inactivity as a cause because masters-level athletes also develop sarcopenia. This brings up the question: why does sarcopenia still occur even with physical activity? It is possible that despite demands still being placed on the muscles, the muscles in sarcopenic adults are not responding in the manner of a non-sarcopenic adult's muscles. Though the mechanism behind sarcopenia may be multifaceted, it seems that this reduced response to exercise seems to be a major factor.

A reduced response to exercise would result in reduced strength and muscle size, which could help explain the onset of sarcopenia. This includes the body's response to activities of daily living by maintaining the muscle mass and strength needed to live an independent lifestyle. Older adults have been shown to respond to exercise, but the extent of their adaptations is less than that of younger people (Raue, Slivka, Minchev, & Trappe, 2009). Despite participating in identical exercise protocols, muscle cross-sectional area (CSA) did not increase in older adults while muscle CSA increased significantly in younger adults (Raue et al., 2009). Strength gains in the older adults were also far less than those in young adults (Raue et al., 2009).

A reduced response to acute exercise has also been established. Altered expression levels of muscle remodeling and growth genes in response to exercise or stretch have already been shown in older adults post-exercise (Dennis et al., 2008; Hameed, Orrell, Cobbold, Goldspink, & Harridge, 2003; Owino, Yang, & Goldspink, 2001; Yang, Alnaqeeb, Simpson, & Goldspink, 1996). Reduced expression of the genes could result in the inability of muscle to grow and also result in muscle breakdown, which together could result in sarcopenia. Exercise-induced gene expression occurs via mechanotransduction, which is the process by which a mechanical signal, like stretch, is converted into a biological event (Hornberger & Esser, 2004).

Mechanotransduction makes sense to investigate because it is the first step in a cell's response to load-induced stress. The phosphorylation of focal adhesion kinase (FAK), an integrin-bound protein, is thought to start the signaling cascade within the cell after stress is induced. FAK phosphorylation contributes to the load-induced hypertrophy response of muscle through activation of the p70S6K, independent of the mTOR-Akt pathway (Klossner, Durieux, Freyssenet, & Flueck, 2009). Impairment of mechanotransduction means the cells are not

responding to or are having a reduced response to a mechanical signal, which causes an impairment of the signaling cascade leading to a reduction in gene expression. Rice et al. showed that this occurs in rat aortas when they found that intracellular protein responses to pressure were reduced in older rats (2007). This reduced response to the mechanical signal of pressure suggests that mechanotransduction is impaired with age. It stands to reason that this impaired mechanotransduction also occurs in skeletal muscle. This would result in decreased protein synthesis, since mechanotransduction has been implicated in protein synthesis regulation (Hornberger & Esser, 2004).

The reason for why mechanotransduction is impaired with age is unknown. A possible mechanism for this impairment is increased stiffness of the extracellular matrix (ECM), which would stiffen the muscle cell and blunt its response to stretch (Pauwels, Dowling, Okafor, Breighner, & Domire, 2012b). ECM stiffness has been attributed to advanced glycation end products (AGEs) that accumulate on collagen fibers with age (Haus, Carrithers, Trappe, & Trappe, 2007; Reddy, 2004). Precise identification of the relationship between mechanotransduction and muscle stiffness is an important step towards discovery of sarcopenia causes and treatments.

Hypothesis

It is hypothesized that increased muscle stiffness with age impairs the mechanotransduction signaling of muscle cells in response to stretch.

Purpose

The purpose this study is to investigate the effects of muscle stiffness on the response to exercise in rats.

Significance of Study

A decrease in FAK phosphorylation levels in stiffer muscles would indicate mechanotransduction is impaired with increasing muscle stiffness. The next step in the process would then be to determine the cause of muscle stiffness. One theory on the cause of muscle stiffness is the accumulation of AGEs due to glycation within the muscle (Haus et al., 2007; Reddy, 2004), but further studies are needed to determine the exact cause. A method to reduce glycation within muscle will then need to be found to allow mechanotransduction to return to normal levels. Mechanotransduction returning to normal levels would be the first step in getting elderly to respond to exercise and even activities of daily living. This would result in muscle mass that is maintained at a healthy level, thus reducing the effects of sarcopenia and allowing for a better quality of life for the older population.

Delimitations

- 19 mature, male Fischer 344 x Brown Norway Hybrid rats will be used as subjects in this study.
- Each rat will complete only one session of the resistance exercise before sacrifice.

Limitations

- A correlation between two or more variables in this study will only reflect an association but will not imply causation.
- Since animal models are not a perfect fit for humans, the results of this study may not be applicable to human subjects.
- The differences in aging rate between rats and humans could result in different stiffening processes between two species.

Chapter 2 – Literature Review

The purpose of this study is to investigate the effect of skeletal muscle stiffness on mechanotransduction signaling within the muscle. This review of literature will focus on: sarcopenia and its impact, possible reasons for sarcopenia (specifically a reduced response to exercise), and impaired mechanotransduction as a reason for reduced exercise response.

Sarcopenia

The United States Administration on Aging (AOA) and the World Health Organization (WHO) present retrospective and prospective statistics that show increases in the number of adults over the age of sixty. These statistics show that the significant growth in the number 60-plus-year-olds over the last decade will likely continue in the future. The U.S. AOA reported that from the 2000 to 2010 censuses, the population of adults over 60 years-old rose from 16.3% of the population to 18.5%, an increase of almost 11.5 million people (2011). This coincides with the start of the "baby boomer" generation entering the later stages of life. Because of this, the percentage of adults over 60 years old in the U.S. will more than likely continue to rise significantly. But, it is not just the U.S. that is growing older. The WHO reported that between 2000 and 2050, the percentage of adults over 60 years of age is supposed to increase from 11% to 22% (2012). The total number of 60-plus-year-olds will be close to 2 billion, compared to the roughly 605 million currently in the world (World Health Organization, 2012). With aging comes conditions like sarcopenia and a growing population only adds to numbers of those affected.

Muscle loss attributed to aging, termed sarcopenia, is prevalent among the older population. However, there is no set percentage of muscle loss to define sarcopenia, so researchers have use their best judgement to define it when conducting studies. Janssen, et al. (2002) defined class I sarcopenia as between 1-2 standard deviations below young adult values (31.5-37% skeletal muscle mass) and class II sarcopenia as greater than 2 standard deviations below young adult values (<31.5% skeletal muscle mass). They found that the prevalence of class I sarcopenia to be 59% in women and 45% in men, while presence of class II sarcopenia was 10% in women and 7% in men over the age of 60 years old (Janssen et al., 2002). Janssen, et al. (2006), used skeletal muscle mass normalized to height to define sarcopenia, with moderate referring to 8.51-10.75 kg/m^2 for men and 5.76-6.75 kg/m^2 for women, while severe sarcopenia was defined as <8.50 kg/m^2 and <5.75 kg/m^2 for men and women, respectively. Utilizing 5,036 participants in the Cardiovascular Health Study (CHS), they found the prevalence of moderate sarcopenia in persons 65 years old or older to be 70.7% in men and 41.9% in women, while those with severe sarcopenia accounted for 17.1% of men and 10.7% of women (Janssen, 2006) Although the studies by the Janssen group offer different views on which gender is more greatly vulnerable to sarcopenia, they collectively show that sarcopenia affects a large percentage of older adults. Combine this with the population as a whole growing older, and the amount of sarcopenic adults is expected to dramatically increase. A growing sarcopenic population means that more money will be needed for help and treatment of these adults.

Sarcopenia is a growing public health concern that is putting an economic burden on the United States. Janssen, et al. (2004) reported that in 2000, the United States spent $18.5 billion in healthcare expenses for direct sarcopenia-related issues. These sarcopenia-related burdens include requiring help for personal and routine needs and hospital expenditures (Janssen et al.,

2004). The authors suggest this could a low estimate, as it does not include secondary issues like productivity loss (Janssen et al., 2004). They did mention that a sensitivity analysis put the range from as low as $11.8 billion to as high as $26.2 billion (Janssen et al., 2004). They put this into perspective by citing an earlier study that reported osteoporosis, another common public health concern, healthcare expenditures reached about $13.8 billion, before inflation, in 1995 (Ray, Chan, Thamer, & Melton, 1997). This shows that osteoporosis and sarcopenia account for a similar percentage of healthcare costs (Janssen et al., 2004). Janssen, et al. also proclaimed that if sarcopenia were eliminated, 85.6% and 26.0% of disabilities (also know as population-attributed risk scores) for men and women, respectively, would also be eliminated, thus taking a significant burden off of the U.S. economy. These healthcare costs due to help and injury treatment can be attributed to the decrease in quality of life caused by sarcopenia.

Sarcopenia can interfere with one's ability to complete everyday tasks, like walking, climbing stairs, and lifting everyday objects. This inability to complete everyday tasks can lead to a cycle of decreased physical activity (Rantanen et al., 1999). Class I sarcopenia has been shown to slightly increase the likelihood of physical functional impairment and disability compared to those with normal muscle mass, while class II sarcopenia has been shown to increase the likelihood by 2-3 fold (Janssen et al., 2002). However, when other variables (age, sex, health behaviors, etc.) were adjusted for, class II sarcopenia remained associated with increased likelihood of functional impairment and disability while class I sarcopenia no longer had a clear association (Janssen et al., 2002). The functional impairment is likely caused by the decrease of a person's strength below the reserve capacity to perform a task (Rantanen et al., 1999). Once this happens, the person has difficulty performing the task because it takes their full capacity to perform said task (Rantanen et al., 1999). Muscle strength was found to inversely

correlate with both age and motor disability in women (Rantanen et al., 1999). These results suggest age plays a factor in quality of life, though Ozcan, et al. found no correlation between age and quality of life (2005). They did, however, suggest age affects muscle strength and functional ability, which they reported to be correlated with lower reported qualities of life (Ozcan et al., 2005). This makes sense because strength is needed for everyday activities like to getting up out of chairs, moving about the world, and picking objects up. Any loss of strength affects a person's ability to perform everyday activities, especially if it falls below the minimum level of strength to perform the tasks. This is supported by the vicious cycle Rantanen, et al. proposed where growing older leads to decreases in physical activity, resulting in decreased muscle strength, which impairs motor function, causing physical activity to decrease even more (Rantanen et al., 1999). Rantanen, in her review of muscle strength in relation to disability and mortality, also concluded that muscle strength is a powerful risk factor for functional limitations, disability, and mortality in old age (2003). Because of this, it is important that a cause for sarcopenia be found so a possible treatment can be developed.

Mechanisms for Sarcopenia

Despite being well studied, the mechanisms that result in sarcopenia are still not understood. They are, however, widely accepted to be multifactorial, which means it is thought there is no single cause for sarcopenia (Morley et al., 2001; Roubenoff, 2001). Figures 1 and 2 below show several of the factors thought to cause sarcopenia: decreased physical activity, nutrition, anabolism/catabolism factors, and decreases in motor unit numbers and activation (Morley et al., 2001; Roubenoff, 2001). A decrease in testosterone, an anabolic hormone, has been shown to occur at a rate of 1.3-2.0% (95% CI) after the age of 40 years old (Feldman et al.,

2002). Harman et al. (2001) found that incidences of low testosterone levels occurred in roughly 30% and 50% of men in the 70-79 and 80+ age range, respectively. However, if low testosterone was the main factor in sarcopenia, it seems like it would be an easy fix by providing testosterone supplementation to these individuals. Morley, et al. stated in their review of other studies that catabolic inflammatory cytokines possess the capability to cause sarcopenia, but that those results do not prove their role in it (2001).

Reviews on sarcopenia mechanisms have suggested that physiologic anorexia, or decreased essential nutritional intake, is a possible cause for sarcopenia as insufficient protein results in a negative nitrogen balance and muscle breakdown (Greenlund & Nair, 2003; Morley et al., 2001). The muscle breakdown from insufficient nutrition would be the result of decreased protein synthesis due to lack of proteins needed to build muscle. Protein supplementation is thought to reverse muscle breakdown, however studies have not shown consistent effects on muscle mass (Hickson, 2015)

Impaired protein synthesis, however, could still be a possible mechanism behind sarcopenia. Larsson & Ramamurthy (2000), along with Greenlund & Nair (2003)suggest protein synthesis rates play a role in the onset of sarcopenia, with protein synthesis rates decreasing and protein degradation rates remaining the same. Data from Balogopal et al. (1997) support this by showing that fractional synthesis rates of myosin heavy chains decrease significantly with age. A decrease in myosin heavy chains, a contractile protein, will affect the size and force producing ability of muscle.

Delbono et al. (1995) suggest that other factors than decreased contractile protein amount are responsible for decreased muscle weakness, and subsequently found decreased activity with

age in the protein that helps regulate the excitation-coupling mechanism in muscle fibers, which could decrease force production with age. Strength losses with age can also be the result of loss of motor units with age, which decrease by about one-half in the seventh decade of life (Doherty, Vandervoort, Taylor, & Brown, 1993).

Another common idea is that decreased physical activity is a major cause of sarcopenia, but Roubenoff (2001) and Siparsky et al.(2014) disagree with this idea by mentioning that even masters-level athletes still become sarcopenic. These two studies, however, do not provide sources for those statements so their validity is in question. Other studies (Faulkner, Davis, Mendias, & Brooks, 2008; Wiswell et al., 2001) show strength still decreases with advancing age in masters athletes, which backs the claim of Roubenoff and Siparsky et al. since muscle strength and size are known to be related. Assuming masters athletes can become sarcopenic, the theory that decreased physical activity causes sarcopenia is discredited, but also introduces a new question and a possible new theory.

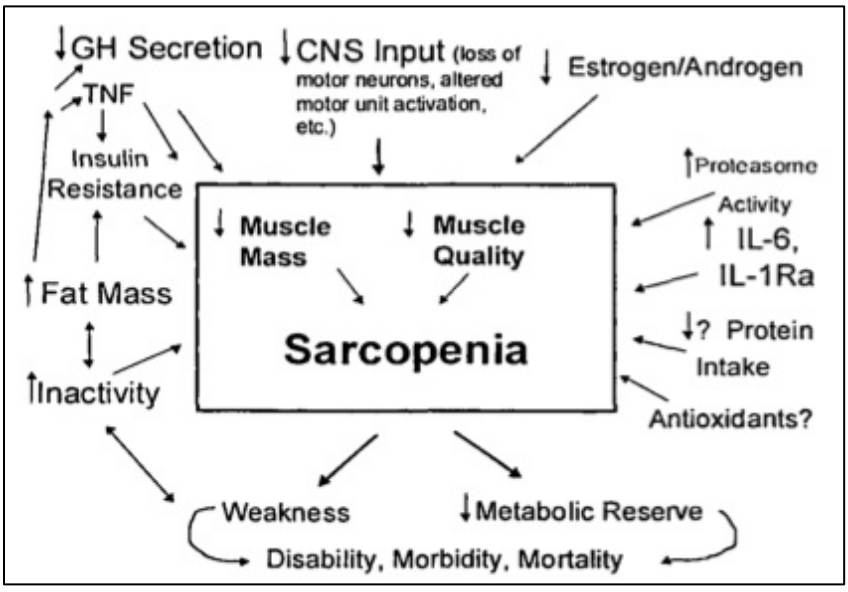


Figure 1. Multifactorial origin of sarcopenia (Roubenoff, 2001)

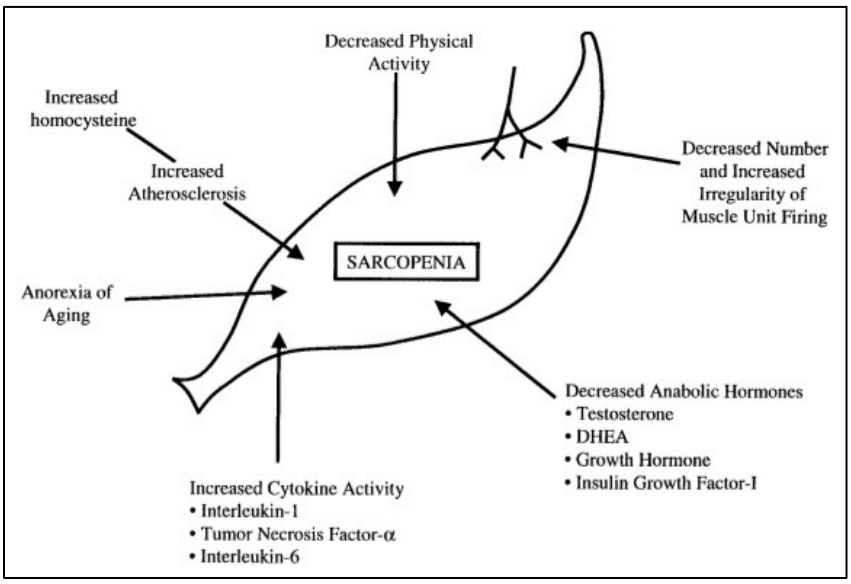


Figure 2. Multifactorial origin of sarcopenia (Morley et al., 2001)

If masters-level athletes, old adults who still train regularly, and adults who are still active are still diagnosed with sarcopenia, then the question on why this occurs arises. One possibility is a reduced response to physical activity, whether through exercise or activities of daily living, in older adults. Wolff's Law, which states that bone undergoes adaptations when placed under stress, has a corollary called Davis' Law that applies to soft tissue. Davis' Law states that muscle will adapt to the demands it experiences, like those from physical activity. In healthy adults, muscle will grow with exercise, be maintained through activities of daily living, or shrink with disuse. So why is muscle still shrinking with use in these physically active adults? It is possible that the muscles of these adults are either not recognizing the stress of physical activity, so they are not responding how healthy muscles should respond. This reduced response would then result in smaller muscles, which results in loss of strength. So while sarcopenia may be caused by several factors, a reduced response to exercise seems to be a possible major factor.

A reduction in the ability to gain strength in response to resistance training with age has been established. A review of 3 different studies that each compared the responses of old women to young women to a resistance-training program found that old women had a reduced capacity to increase strength (Greig et al., 2011; Raue et al., 2009; Roth et al., 2000). These results are consolidated and presented above in Table 1. LaRoche et al. found similar results when comparing young vs. elderly women after a 3x/week, 8-week resistance training program (2008). Trained young women increased their peak torque, rate of torque development, and impulse by 16%, 34%, and 53%, respectively, while the trained older women only showed increases of 7%, 9.2%, and 12%, respectively (LaRoche et al., 2008). The peak torque findings, however, were not significant but approached significance with a p-value of 0.06 (rate of torque and impulse were significant with p-values of 0.002 and 0.001, respectively) (LaRoche et al., 2008). The parity in strength gains between old and young adults seems to be related to an attenuated

Table 1: Strength Results of Young vs. Old Women in 3 Training Programs

Study	Subjects	Training Program	Baseline Results	Post-Training Results
Roth et al. (2000)	10 young (26 ± 1 yr); 10 old (67 ± 3 yr)	Unilateral knee extensions; 55 repetitions per session; 3 days/week; 9 weeks	Old 31% weaker*	Young: 37% increase*; Old: 22% increase*; Old significantly weaker*
Raue et al. (2009)	9 young (21 ± 2 yr); 6 old (85 ± 1 yr)	Bilateral leg extensions 3 sets of 10 repetitions; 3 days/week; 12 weeks	Old 36% weaker than young*	Young: 36% increase* Old: 26% increase*
Greig et al. (2011)	16 young (19-30 yr); 9 old (76-82 yr)	Unilateral, isometric knee extension; 4 sets of 15 repetitions; 3 days/week; 12 weeks	Old 30% weaker than young*	Young: 27% increase*; Old: 16% increase*; Significant difference in increases between ages*
*** $p < 0.05$**				

hypertrophic response in older adults.

Evidence shows there is a reduced hypertrophic response in older adults to resistance training, which suggests any strength gains made are due to adaptations other than increased muscle size. After a 3x/week, 16-week resistance training program, old adults were found to have less muscle fiber hypertrophy (none in type I, 23% in type II) than young adults (18% in type I, 32% in type II) (Kosek, Kim, Petrella, Cross, & Bamman, 2006). But, relative strength gains were found to be similar across age groups. The authors then concluded that the strength gains were due to neural adaptations instead of hypertrophy. Raue et al. also reported 23% smaller thigh muscle cross-sectional areas (CSA) from pre-training measurements in old women compared to young women (2009). They reported no change in CSA in old women post-treatment while the younger women significantly increased their thigh CSA (Raue et al., 2009). Adding this to their strength results above, it seems that the strength gained by the older women was due to factors other than hypertrophy, such as neural gains. This agrees with the conclusions that Roth et al. made in their study. These neural adaptations also seem to play a role in detraining and post-training maintenance prescriptions.

When the effects of training and detraining were investigated, old adults had significantly less increases in strength during training and significantly more strength loss during detraining (Lemmer et al., 2000). In relation to detraining, Bickel et al. sought to determine the minimum amount of resistance training required to retain gains made during a 3x/week, 16-week resistance training program (2011). They found that in older adults, muscle size was not maintained in either post-training prescription (1 day/week, either $1/3^{rd}$ or $1/9^{th}$ of 16 week program volume) but strength gains were retained, while young adults retained muscle size and strength regardless

of post-training prescription (Bickel et al., 2011). These results show that older adults have a reduced capacity to maintain muscle hypertrophy and need more resistance training to retain gains made during a resistance training program (Bickel et al., 2011). The greater strength losses in detraining and reduction in muscle size during post-training prescriptions seem to support the idea of neural adaptations as the main player in strength gains in older adults. The attenuated hypertrophy seen in training and maintenance training in older adults must be a result of the muscle's impaired cellular response that results in muscle growth.

An attenuated muscular response to chronic exercise has also been seen within animal models. Cutlip, et al. (2006) conducted a study where results of subjecting young and old rats to a hypertrophy-inducing 4.5-week stretch-shortening cycle protocol were compared. Though the young and old rats had similar force levels before the study, the young rats increased their isometric and peak eccentric forces 25% and 27.9%, respectively, while the old rats had a 34% and 22.4% decrease in the isometric and peak eccentric forces. The wet masses of the exercised dorsiflexors were also compared to the wet masses of the unexercised, contralateral muscles, where the young, exercised muscles were significantly bigger than young, unexercised muscles and old, exercised muscles were not significantly different than old, unexercised muscles. In another studym rats were subjected to 8 weeks of mechanical overload following bilateral surgical ablation of the gastrocnemius, and atrophic muscles in very old rats were found to have lost their capacity to grow in response to overload (Blough & Linderman, 2000).

A reduction in muscle modeling gene expression in response to acute exercise has also been shown to occur older adults. Hwee & Bodine suggest that a delay in Akt activation and reduced activation of mTOR downstream targets (a pathway responsible for protein synthesis) is

responsible for altered growth response in old rats (2009). Genes potentially involved in muscle remodeling and growth have been shown to be differentially expressed between young adults and healthy, but weaker, older adults (Dennis et al., 2008). Mechanosensitive growth factor, or MGF, is one of these genes that is differentially altered (Hameed et al., 2003; Owino et al., 2001). MGF is an insulin-like growth factor isoform (IGF-1Eb in rabbits; IGF-1Ec in humans) that is up-regulated in response to stretch and eccentric exercise in rabbit and human muscles, respectively (Yang et al., 1996). In one study, MGF mRNA was shown to significantly increase in all ages during post-synergistic ablation mechanical overload in plantaris muscles of rats, but the degree of increase was noticeably less in the old rats (Owino et al., 2001). In another study, where young and old men completed 10 sets of 6 repetitions of single legged knee extension at 80% of their one repetition maximum on one leg, MGF expression increased significantly in the exercised leg of young men compared to the unexercised leg (Hameed et al., 2003). However, no significant difference in MGF expression was seen between the legs of the old men (Hameed et al., 2003). Though these two studies have slightly different results, both show that MGF expression is slightly altered in response to exercise in older individuals.

With decreased anabolic and remodeling gene expression comes decreased manufacturing of proteins to build and repair muscle. If muscles are externally loaded through exercise, but the normal result of gene expression is altered, then it stands to reason there is impairment in the pathway from recognition of stress to gene expression. It also makes sense to study mechanotransduction, the conversion of stress to cellular response, since it is the beginning of the pathway, and any impairment would alter the cellular events downstream of it.

Mechanotransduction as a Cause of Reduced Exercise Response

Mechanotransduction is an important pathway in the adaptation of muscle in response to exercise. It is defined as the process of converting mechanical signals into biological events (Hornberger & Esser, 2004). This occurs by interconnected structural elements that span the cell membrane transmitting force from the outside of the cell to the inside (Wang, Butler, & Ingber, 1993). These structural elements, called integrins, are transmembrane proteins that connect the extracellular matrix (ECM) to the cytoskeleton (CSK) (Wang et al., 1993). Changes in the CSK brought about integrin changes induce molecular signaling within in the cell (Wang et al., 1993). In their review, Hornberger & Esser refer to a previous study (not published at the time of the review) they conducted with a few colleagues in which uniaxial and multiaxial stretches resulted in different cell responses (2004). Data shows that the differentiation by mechanical stimuli is required to activate skeletal myogenesis pathways (Zhang, Truskey, & Kraus, 2007). Hornberger & Esser also suggest that protein synthesis can be regulated by mechanotransduction and is only activated by specific stretches (2004). For example, MGF mRNA expression was shown to be more sensitive to strain rate than type of loading (Cheema et al., 2005). It was also suggested that mechanically induced protein synthesis pathways are not known but that it is possible these pathways are also involved in protein synthesis via mitogens and nutrients (Hornberger & Esser, 2004). The involvement of mechanotransduction in protein synthesis shows that any impairment will have costly physical effects.

The signaling for protein synthesis in response to exercise occurs through the phosphorylation of a integrin-bound proteins, called focal adhesion kinases (FAK), via signals from integrins that sense ECM changes. Focal adhesions are molecular bridges within the cell

that connect integrins to CSK actin filaments, allowing for the transmission of force from the ECM to CSK (Wang et al., 1993). Focal adhesion kinase phosphorylation was shown to increase in response to cyclical stretch in murine myoblasts (Zhang et al., 2007). FAK has also been associated with the regulation of mitogen activated protein kinase (MAPK), which has been implicated as an in important regulator of protein synthesis and load-induced protein expression (as cited by (Rice et al., 2007)). In relation, research shows that p70S6K, a load-modulated signaling pathway of protein synthesis independent of the mTOR-Akt pathway, is influenced by FAK phosphorylation (Klossner et al., 2009). This influence on p70S6K implies FAK contributes to the modulation of the load-induced hypertrophy response of muscle (Klossner et al., 2009). Klossner et al.'s results (2009) also support previous studies suggesting FAK's role in protein synthesis and cell size regulation. Phosphorylation of FAK occurs at the beginning of a cell's response to stretch, so it makes sense for it to be the focus of investigation. Studying actions downstream of the signaling cascade only shows that action is impaired but does not show if anything occurring before it is affected.

Though not heavily researched, there is evidence that FAK phosphorylation is changed in older individuals. In their study, Rice, et al. showed that pressure loaded responses of FAK in rat aortas decrease with increasing age (2007). FAK phosphorylation in skeletal muscle was shown to decrease with disuse, and has been implicated in the loss of muscle mass (de Boer et al., 2007). Decreased FAK phosphorylation means that FAK is receiving fewer or weaker signals from the integrin.

Decreased FAK phosphorylation is possibly a result of increased ECM stiffness. An increase in ECM stiffness means that a muscle's stretch response under a given load will be

reduced. Reduced stretch would result in decreased mechanosensing of the integrin, which cause a decrease in FAK phosphorylation. Less FAK phosphorylation would then result in less cell signaling of the muscle in response to stretch, thereby causing impaired protein synthesis.

There is evidence to suggest muscle stiffness increases with age, and that this stiffness could impair muscle function. Older rats were reported as having significantly stiffer tibialis anterior epimysiums than young rats (Gao, Kostrominova, Faulkner, & Wineman, 2008). Passive elastic stiffness was also found to be significantly greater in the calf muscle tendon units of older women vs. younger women (Gajdosik et al., 2005). Increasing muscle stiffness with advancing age may affect mechanotransduction, thus making it a possible contributor to sarcopenia.

The material properties of a muscle cell's environment can cause changes to its ability to detect and respond to mechanical stimuli. Engler, et al.'s *in vitro* (2004)study showed that cell differentiation is affected by the substrates the cells are grown on, and that substrate stiffness could affect mechanically induced signaling responsible for cell differentiation. This means that the stiff ECM, a substrate on which the muscle grows in the body, would result in stiff muscle during regeneration and remodeling, which could then affect mechanotransduction. Pauwels and colleagues' study of caloric restricted vs. *ad libitum* fed rats (Pauwels et al., 2012b) showed a negative correlation between Young's Modulus, a measure of material stiffness, and muscle CSA, regardless of diet. The authors then went on to suggest this muscle stiffness is a contributor to sarcopenia by impairing skeletal muscle's ability to grow or maintain size in response to activities of daily living. The ECM is the main contributor to the Young's modulus

of muscle, and over the course of an individual's life, its physical properties are altered in way that increases muscle stiffness.

The physical properties of collagen in the ECM also leave it open to posttranslational modifications, such as glycation, resulting in increased stiffness. It is a posttranslational modification of proteins that occurs when a nonezymatic Maillard reaction occurs between a reducing sugar and primary amino acid to form reversible Schiff bases, which can then undergo further Amadori rearrangements to form irreversible advanced glycation end products (AGEs) (Haus et al., 2007; Ramamurthy, Hook, Jones, & Larsson, 2001; Ramamurthy & Larsson, 2013; Reddy, 2004; Snow, Fugere, & Thompson, 2007; Verzijl et al., 2000). These cross-links between collagen fibers can be defined as either enzymatic or nonezymatic. Enzymatic cross-links are soluble and continually broken down, while nonenzymatic cross-links and insoluble and not able to be broken down. Insoluble collagen cross-link numbers were increased by about 200% from young age to old age in humans (Haus et al., 2007). The percentage of myofibers showing AGE was nearly ten times higher in very old rats than in young rats (Snow et al., 2007). Haus, et al. suggested that increased AGE formation with increasing age results in muscle connective tissue protein stiffness, which could contribute to impaired muscle function (2007).

Several studies of glycation and stiffness back up Haus et al.'s claim of a glycation-stiffness association. Young's Modulus was found to be significantly higher in nonenzymatic glycated rabbit Achilles tendons compared to nonglycated tendon (Reddy, 2004). Reddy found an inverse correlation between soluble collagen to stiffness in rabbit Achilles tendons. Soluble collagen was also found to be lower in glycated rabbit Achilles tendons compared to nonglycated tendons. Collagen turnover rate has been shown to be a strong determinant of its vulnerability to

glycation (Verzijl et al., 2000). Haus et al.'s study of human skeletal muscle found no difference in collagen concentration and enzymatically mediated, or soluble, cross-link numbers between young and old persons, suggesting that these components are tightly regulated and do not contribute to stiffness (Haus et al., 2007). They did find that nonezymatic cross-links were significantly increased in older adults and suggest that they play a role in increased muscle stiffness (Haus et al., 2007). The increased stiffness of collagen via nonezymatic glycation implicates its importance to the stiffening of muscles. Increased stiffness would impair muscle function by not allowing the muscle to respond to stretches at a cellular level. This impaired muscle function would likely result in sarcopenia from impaired ability to increase muscle strength and mass.

Summary

Sarcopenia affects a large percentage of older adults, resulting in a loss of quality of life and economic concerns. With the population growing older, the impact will be greater with more money being spent to assist these people with activities of daily living. Because of this, it is important to investigate the causes of sarcopenia in hopes of treating, or even eliminating, it. This is a tough task as it is accepted that sarcopenia is the result of a variety of age-related changes. Though this study has decided to focus on the reduced response to exercise, especially regarding mechanotransduction. Older adults have shown to not respond to exercise as well as younger adults, and that muscle remodeling and growth genes, like MGF, are expressed differently in older adults.

Altered MGF expression is possibly due to mechanotransduction impairment. Mechanotransduction impairment could result in a decreased protein synthesis rate, which would

cause muscle breakdown and loss if below protein degradation rate. Possible reason for impaired mechanotransduction is increased muscle stiffness by glycation. Glycation of muscular collagen has shown to increase with age and be directed correlated with stiffer muscles. Muscle stiffness was also found to negatively correlate with muscle CSA, which is a component of sarcopenia. Because of this, it is important to study the effects of muscle stiffness on mechanotransduction in hopes of learning more about possible causes of sarcopenia.

Chapter 3 – Methods

Research Animals

Nineteen Fischer 344 x Brown Norway hybrid (FBN) rats were used for this study. Twenty rats were originally acquired through the National Institute of Aging, but a 32 month-old rat passed away during transport. The rats were split into two groups: young (12 months; n = 10) and old (32-33 months; n = 9). The purpose of this study is to study the effects of muscle stiffness on exercise response, so varying degrees of muscle stiffness were needed. This was achieved by using young and old rats, since muscle stiffness has been shown to increase with age (Gao et al., 2008). The ages of these rats equates to roughly 30 years-old and 75-90 years-old, respectively, in humans (Sengupta, 2013) (Figure 3). Only male rats were used in this study to control for any possible sex differences. Upon arrival, the rats were kept in a standard wire cage, fed food and water *ad libitum*, and kept on a standard 12-hour light/dark cycle. The rats were given an acclimation period of 2 weeks between arrival and testing.

Rat age versus human age: Social maturity phase

Rat age (years)	Human age (years)
6 months (0.5)	18
12 months (1.0)	30
18 months (1.5)	45
24 months (2.0)	60
30 months (2.5)	75
36 months (3.0)	90
42 months (3.5)	105
45 months (3.75)	113
48 months (4.0)	120

Figure 3. Rat-human age equivalency (Sengupta, 2013)

Instrumentation

A custom-built rat dynamometer (shown below), based on the one used by Cutlip, et al. (2006), was used for exercise testing. The dynamometer featured a load cell attached to a sleeve on a footplate, which allowed for the recording of muscular force as the footplate was moved through a range of motion. The dynamometer also had moveable table, on which the rat was placed in a supine position with its foot through the foot sleeve. The dynamometer was run by a LabView computer programs (National Instruments, Austin, TX) and connected to a data acquisition board. The LabView program allowed for automaticity and consistency in the exercise to eliminate any human error, as well as the recording and storage of data. The program for the dynamometer automatically started each set of exercise and moved the foot through a 65-degree range of motion.

To perform the eccentric exercise, the rats were stimulated with a Grass Medical S88 muscle stimulator and Grass Medical F-E2 electrodes (Warwick, RI). The S88 stimulator is a "general purpose stimulator for nerve and muscle stimulation procedures" that is compatible and can be synchronized with computers (Natus Neurology, 2015). The F-E2 model of electrodes utilize a precision sharpened monopolar platinum needle for use in subdermal stimulation (Natus Neurology, 2014). The stimulator and electrodes were also connected to the LabView program for the dynamometer. Pairing the stimulator with the dynamometer in the same program allowed for the initial stimulation signal to coincide with the beginning of the eccentric phase of the contraction. After the initial signal, the program maintained a sustained signal throughout the entire eccentric contraction.

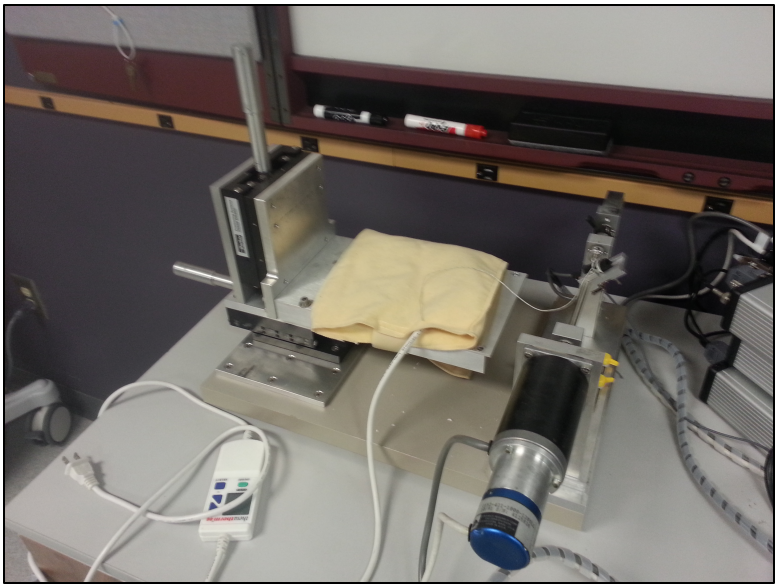


Figure 4. Custom-built rat dynamometer based on the one used in Cutlip et al. (2006)

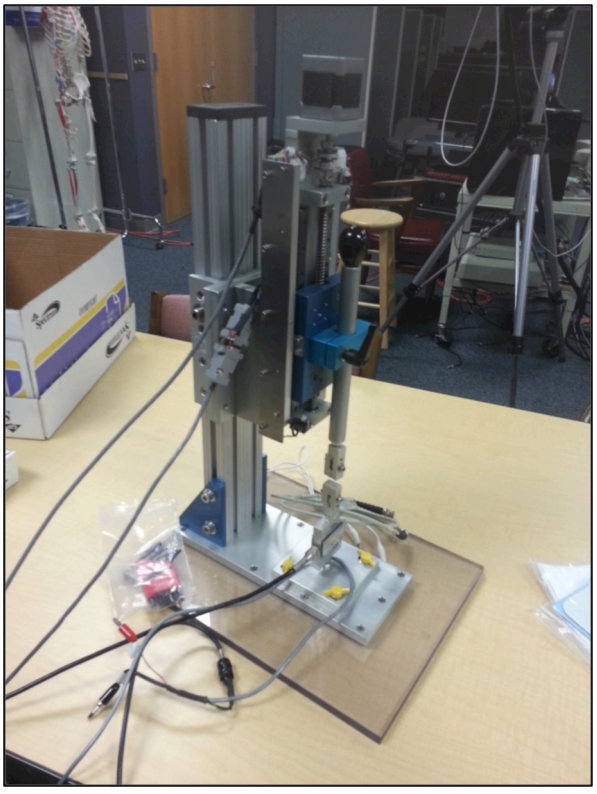


Figure 5. Custom-built tensile tester based on the one used in Pauwels et al. (2012a; 2012b)

A custom-built tensile tester (shown below), based on the one used by Pauwels et al. (2012a; 2012b), was used to measure muscle stiffness. The tensile tester featured two clamps that held the excised TA in place. The lower clamp was attached to a load cell, which recorded force. The upper clamp was connected to an arm, which moved up and down in a linear fashion, with a potentiometer, which measured linear displacement. The tensile tester was also connected to a data acquisition board and run by a LabView program. The program for the tensile tester moved the upper clamp in a linear fashion at a rate of 2 cm per minute while recording force and linear displacement.

Study Design

This was a quasi-experimental study, with relative FAK phosphorylation acting as the dependent variable. The phosphorylation of FAK has been suggested as in integral, beginning step of the mechanotransduction-signaling cascade (Hornberger & Esser, 2004). FAK is phosphorylated when the muscle cell membrane undergoes a mechanical stretch, making it a good indicator of response to exercise (Zhang et al., 2007). It is our idea that if the muscle is undergoing sub-normal stretches to normal loads due to increased muscle stiffness, then FAK phosphorylation levels will be below normal as well. Age has already shown to have a positive relationship with stiffness (Gajdosik et al., 2005; Gao et al., 2008) and a negative relationship with pressure-loaded FAK phosphorylation (Rice et al., 2007). However, it is not known if muscle stiffness plays a role in impairing the phosphorylation of FAK in response to mechanical loads or stretch.

Age was one the independent variables of this study, and was directly manipulated by using rats of different ages. Muscle stiffness was used as both a dependent variable and a second independent variable in this study. However, muscle stiffness cannot be directly manipulated in

an easy manner. Since muscle stiffness has been shown to correlate with age (Gao et al., 2008), using different aged rats gave us varying levels of muscle stiffness. The purpose of this study was to determine if age and/or muscle stiffness affects the response to exercise, as measured by FAK phosphorylation.

Exercise Protocol

The rats were first placed in an induction chamber and anesthetized with isoflurane gas at 5 L/min. When paw pinch reflex was lost, the isoflurane was lowered to 3 L/min and the rat was placed on the dynamometer table in a supine position. The dynamometer table was covered with a heating pad set to 100 °F so the rats were kept warm under anesthesia. The left hind limb of each rat was then placed in the foot sleeve attached to the footplate of the dynamometer. Stimulator electrodes were then placed subcutaneously just inferior to the knee so as to span the peroneal nerve (Figure 6). This allowed for isolated contraction of the dorsiflexor mucles. The rats were stimulated with a 120 Hz square-wave pulse with a duration of 0.2 ms. Voltage used for the stimulation was different for each rat. To find the voltage used, voltage was incrementally increased from 4 volts by 1 volt until isometric force, as measured by a LabView program, no longer increased. The rats then performed three sets of ten maximal eccentric contractions of the dorsiflexor muscles. The rats were moved through the range of motion at 30 degrees per second. Each stimulation, automatically signaled by a LabView program, occurred the beginning of the eccentric portion of each repetition and lasted through the entire portion of the repetition. There was a 10-second rest period between each repetition and a 1-minute rest period between each set.

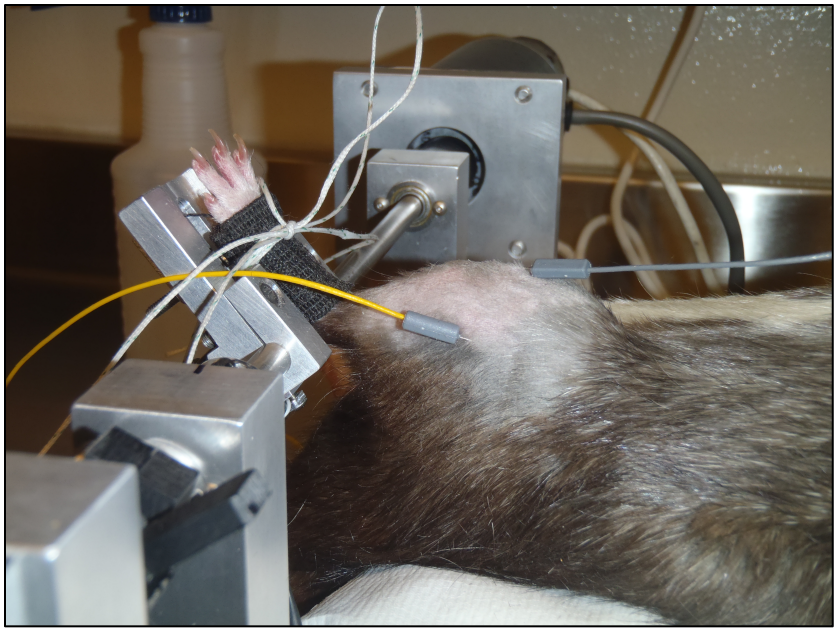


Figure 6. Electrode placement spanning the peroneal nerve just inferior to the knee.

Tissue Harvesting

After the exercise testing, rats were immediately gassed with an overdose of isoflurane gas, for at least 20 minutes. After the overdose of isoflurane, a pneumothorax was performed to ensure death. This immediate death is sufficient time for FAK phosphorylation to occur, as showed by Pauwels et al. (Pauwels et al., 2012a).

The extensor digitorum longus (EDL) of the non-exercised leg and the tibialis anterior (TA) of both legs were extracted following sacrifice. The EDL was used for stiffness testing, while the TAs were used to test for FAK phosphorylation. The non-exercised EDL was used to avoid any possible damage done by the eccentric exercise. Stiffness testing occurred within 30 minutes of sacrifice to avoid the effects of rigor mortis (Pauwels et al., 2012a). The TAs were

sectioned 3 mm below the proximal tendon. The TA sections were then flash frozen with liquid nitrogen and stored at -80 °C to be used later for FAK phosphorylation analysis.

Muscle Stiffness Testing

Muscle stiffness of the EDL was tested similar to Pauwels et al's (2012a; 2012b) testing protocol. Once extracted, the EDL was placed within the clamps so that approximately 25% of the muscle belly was positioned within each clamp. This allowed for force production to occur through the belly of the muscle instead of the tendons. The muscle sample was lengthened until no slack was left and a resistive force was developed. The distance between the clamps was measured, and this was defined at the muscle's initial length (L_0). The width and thickness of the muscle, used for the calculation of CSA, was measured while the muscle was in the clamps. The muscle was lengthened to 10% past L_0 10 times for preconditioning. The preconditioning protocol accounts for changes in muscle with stretch due to its viscoelastic nature (Taylor, Dalton, Seaber, & Garrett, 1990). Taylor et al. (Taylor et al., 1990) found a decrease in peak tension with each pull of the muscle, but that the difference in peak tension was not significant between pulls 7 and 10. After preconditioning, L_0 was once again measured in case this changed during preconditioning. The muscle was then stretched from L_0 at a rate of 2 cm/min until failure.

In this study, stiffness was defined as the elastic modulus, or Young's modulus, of the muscle. Young's modulus is defined as the slope of the linear, also called elastic, region of a stress-strain curve (Figure 7). A stress-strain curve is similar to a force-deformation curve, which shows a relationship between how much force (F) is needed to deform a material. A stress-strain curve, however, normalizes force and deformation into stress and strain, respectively. The normalization of force and deformation into stress and strain allows for the

quality of the material to be assessed without the influence of the material's size. Stress (σ), the normalization of force to the material's size, is found by the equation:

$$\sigma = \frac{F}{CSA}.$$

Using the assumption that muscle is an ellipse, CSA was found with the following equation:

$$CSA = \frac{\pi(width)(thickness)}{4}.$$

Strain (ε), the normalization of change in length (ΔL) to the material's initial length, is found by the equation:

$$\varepsilon = \frac{\Delta L}{L_0}.$$

The plotting of stress and strain gives linear and nonlinear regions. The linear region is defined as the region where the material will return to its original length when stretched. The nonlinear, or plastic, region is the region past the linear region where any stretch applied to the material will cause it damage and not allow the material to return to its original length. The region of interest of this study was the linear region, whose slope was found using a regression analysis. Stiffness data was processed in MatLab (Mathworks, Natick, MA).

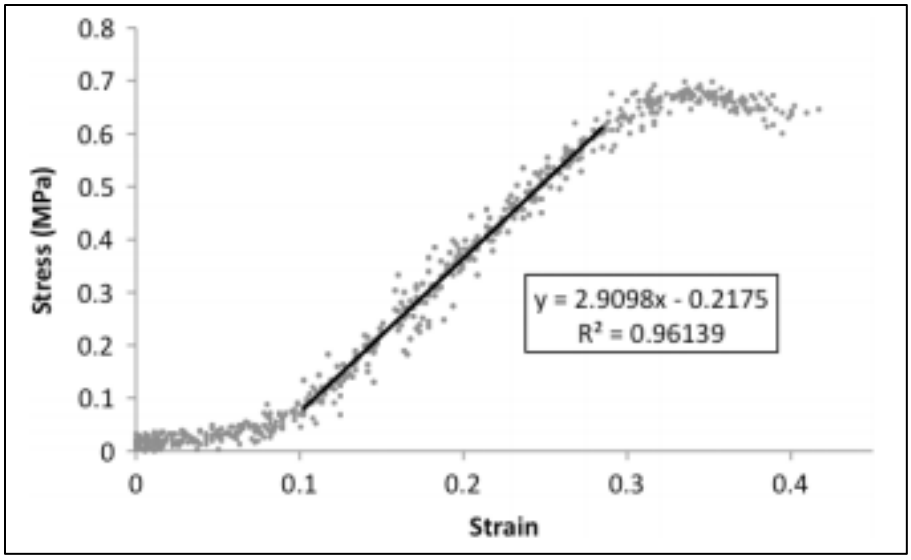


Figure 7. Sample stress-strain curve (Pauwels et al., 2012b). Linear region indicated by black line. Equation for black line given, in which slope of line is equal to the Young's modulus.

Western Blot Analysis

Before running Western blots on muscle samples, a positive control of 3T3/A31 cell lysate (Millipore, Temecula, CA) was used to determine if antibody would recognize FAK. To prepare muscles for gel electrophoresis, muscle samples were homogenized using a ground glass homogenizer (Glas-Col, Terre Haute, IN) in the following buffer: 10 mM HEPES (pH 7.4), 125 mM sucrose, 1 mM EDTA, 10 mM $Na_4P_2O_7 \bullet 10H_2O$, 10 mM β-glycerophosphate, 2 mM NaF, 1 mM Na_3VO_4, and commercially prepared protease inhibitor cocktail (Sigma, St. Louis, MO). Protease inhibitor was included to deactivate any proteases from degrading the FAK. Homogenizer tube was placed in a beaker of ice during homogenization to keep homogenate from overheating and proteins from becoming denatured. Total protein concentration of each homogenate was determined using a BCA protein assay kit (Pierce, Rockford, IL) (Gordon, Fluck, & Booth, 2001). Protein homogenates were diluted to in a loading buffer (62.5 mM Tris-

HCl (pH 6.8), 2% SDS, 10% glycerol, 100 mM DTT, 0.02% bromophenol blue). Homogenates in loading buffer were heated at 100° C for 3 minutes.

Proteins were separated using electrophoresis by loading 50 µg of protein from each homogenate and a protein homogenate used as a standard into an 8% sodium doecyl sulfate-polyacrylamide gel. The 8% SDS-polyacrylamide gel has been shown to be effective in separating out FAK (Gordon et al., 2001). Proteins were transferred to a polyvinylidene fluoride (PVDF) membrane for 4 hours at a temperature of 4° C in a transfer buffer consisting of 25 mM Tris-base, 192 mM glycine, and 10% methanol. PVDF membranes were used because of their ability to be reprobed (EMD Millipore, 2015).

To probe for phosphorylated FAK, membranes were blocked in 5% nonfat dry milk in TBS-T (10 mM Tris-HCl (pH 7.3), 150 mM NaCl, 0.05% Tween 20 at room temperature for 1 hour. Blocking ensures that that antibody used will only stick to the desired protein and not the membrane. After blocking, membranes were incubated in anti-phospho-FAK (Tyr397) (Millipore) diluted in 5% milk in TBS-T at 4° C overnight. After washes in TBS-T, membranes underwent a 1-hour incubation in anti-rabbit secondary antibody conjugated with horseradish peroxidase (HRP) at room temperature. HRP activity was visualized using enhanced chemiluminescence solution (GE Healthcare, Piscataway, NJ) and exposure to autoradiographic film (Blue Devil Film, Genesee Scientific, San Diego, CA). The procedure of measuring chemiluminescent light from HRP activity allows for the reprobing of membranes since the light produced is not bound to the membrane (Thermo FIsher Scientific, 2015).

To probe for total FAK, the membrane was stripped in 62.5 mM Tris-HCl (pH 6.7), 2% SDS and 100 mM 2-mercaptoethanol (reducing agent) at 60° C for 30 minutes to remove anti-

phospho-FAK antibody. Membranes were once again blocked in 5% nonfat dry milk in TBS-T at room temperature for 1 hour. After blocking, membranes were incubated in anti-FAK (Millipore) diluted in 5% milk in TBS-T at 4° C overnight. After washes in TBS-T, membranes underwent a 1-hour incubation in anti-mouse secondary antibody conjugated with HRP at room temperature. HRP activity was visualized using enhanced chemiluminescence solution and exposure to autoradiographic film. Films for both phosphorylated FAK and total FAK were imaged via transmissive scanning with a HP ScanJet G4050 (Hewlett-Packard, Palo Alto, CA). Films were then analyzed with SigmaScan Pro 5.0 (Jandel Scientific, Systat, Point Richmond, CA).

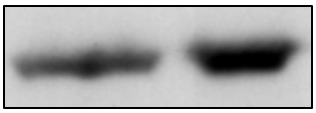


Figure 8. Example of FAK bands (mass: ~125 kD) in Western blot comparing right (unstimulated) and left (stimulated) legs. Unstimulated leg is the first band shown followed by the stimulated leg. Bands were traced analyzed with SigmaScan Pro 5.0.

Data Analysis

Analyzed film data was normalized to the standard (rat gastrocnemius) on each respective gel to eliminate any differences between gels. This data was then normalized to the young, unstimulated means of the pFAK and total FAK so that differences could be compared to the young, unstimulated control. The amount of total FAK was then divided by the amount of pFAK to determine relative FAK phosphorylation, or FAK activity as it will be referred to for the rest of this paper. The difference in FAK activity between legs was taken to determine any differences between stimulation and no stimulation. This difference in FAK activity between legs will be referred to as FPR (FAK phosphorylation response) for the rest of the paper. FPR was analyzed to determine FAK activity in response to a stimulus compared to basal levels.

Modulus, muscle CSA, FAK activity (pFAK:Total FAK ratio), and FPR are presented as means ± standard deviation. Independent samples *t*-tests were used to compare the mean stiffness and the mean muscle CSA of the young and old groups. Regression analyses were used to determine any relationships between the following: FAK activity vs. stiffness, FPR vs. stiffness, FAK activity vs. muscle CSA, and muscle CSA vs. stiffness. Pearson correlation coefficient, r, is presented for each relationship. A 2 x 2 ANOVA was performed to determine the effects of age and stimulation on relative phosphorylation. An alpha level of 0.05 was set to reduce the chance of committing a type I error. All statistical analyses were performed with SPSS Statistics 20 (IBM, Armonk, NY).

Chapter 4 – Results

Data for each rat is presented, as well as the means and standard deviations for each age group, in Table 2. Stiffness data for one old rat and phosphorylation data for three old rats and one young rat were not collected. The three old rats had health problems (2 with mammary tumors, 1 with a possible spinal cord injury), so their FAK data was thrown out. The missing stiffness for one old rat is a result of experimental error. Problems with stimulation, possibly caused by an anatomical variance, in the young rat caused us to throw out its FAK data as well. Maximal stimulation in this rat was not achieved after several electrode placements and voltage changes. The other data collected for these five rats were still used for analyses.

Muscle Size

Muscle CSA, however, was found to significantly decrease with age (7.75 ± 0.99 vs. 5.72 ± 2.10 mm^2; p = 0.04) (Figure 9). The young rats exhibited a smaller range than the old (2.89 vs. 5.57 mm^2, respectively). The higher SD in the old group shows greater variability in muscle size than the rats in the young group. Of note are the 5 old rats that fell above the line showing 2 SD below the young mean. These 5 rats exhibited muscle sizes similar to those of their young counterpart, with one old rat have the 3rd largest muscle CSA of all rats.

Muscle Stiffness

Young's modulus trends towards increase with age, from 2.01 (± 0.61) MPa in the young rats to 3.38 (± 2.01) MPa in the old rats (p = 0.057, Cohen *d* effect size: 0.9) (Figure 10). The SDs for each group show old rats had greater variability in stiffness levels than the young rats.

				pFAK : Total FAK ratio		*Ratio Difference*	*Normalized pFAK amount*		*Normalized Total FAK amount*	
	Age (months)	**Muscle CSA**	**Modulus (MPa)**	**Stim**	**No-Stim**	**Stim - No Stim**	**Stim**	**No-Stim**	**Stim**	**No-Stim**
OR1	33	5.89	1.50	-	-	-	-	-	-	-
OR2	33	6.28	3.33	-	-	-	-	-	-	-
OR3	33	4.71	6.37	-	-	-	-	-	-	-
OR4	34	6.28	0.79	0.80	0.47	0.33	1.21	1.08	1.52	2.32
OR5	34	3.27	5.77	0.38	0.81	-0.43	0.72	1.31	1.86	1.61
OR6	34	3.88	-	0.68	0.30	0.38	0.86	0.43	1.27	1.43
OR7	34	4.95	4.30	0.39	0.35	0.04	0.76	0.49	1.93	1.42
OR8	33	8.84	1.85	1.02	0.53	0.49	0.75	0.69	0.73	1.30
OR9	33	7.10	3.10	0.54	0.76	-0.22	0.96	1.18	1.77	1.54
YR1	13	8.10	2.28	1.86	1.31	0.55	0.98	1.50	0.52	1.14
YR2	13	7.93	2.54	-	-	-	-	-	-	-
YR3	13	9.13	0.93	1.28	1.51	-0.23	0.92	1.25	0.72	0.83
YR4	13	6.59	2.32	1.53	0.89	0.64	1.24	0.78	0.81	0.88
YR5	13	7.65	2.08	0.53	0.63	-0.10	0.81	0.74	1.52	1.17
YR6	13	7.37	2.26	0.77	0.91	-0.14	0.77	0.60	1.00	0.65
YR7	13	7.15	2.81	0.71	1.06	-0.35	0.84	1.24	1.19	1.17
YR8	13	9.48	1.33	1.16	0.89	0.27	0.78	1.01	0.67	1.13
YR9	13	6.89	1.32	1.09	0.80	0.29	0.90	0.78	0.82	0.97
YR10	13	7.35	2.22	1.18	1.05	0.13	1.18	1.10	1.00	1.05
	Old Average	5.72	3.38	0.64	0.54	0.10	0.88	0.86	1.51	1.60
	Old SD	2.10	2.01	0.25	0.21	0.37	0.19	0.37	0.45	0.37
	Young Average	7.75	2.01	1.12	1.01	0.12	0.94	1.00	0.92	1.00
	Young SD	0.99	0.61	0.42	0.27	0.35	0.17	0.30	0.30	0.18

Table 2. Age, muscle, CSA, Young's Modulus, relative phosphorylation, relative phosphorylation difference between stimulated and unstimulated legs, and amounts of pFAK and Total FAK for each rat. Means and standard deviations (SD) for each age group are also presented. OR: old rat, YR: young rat.

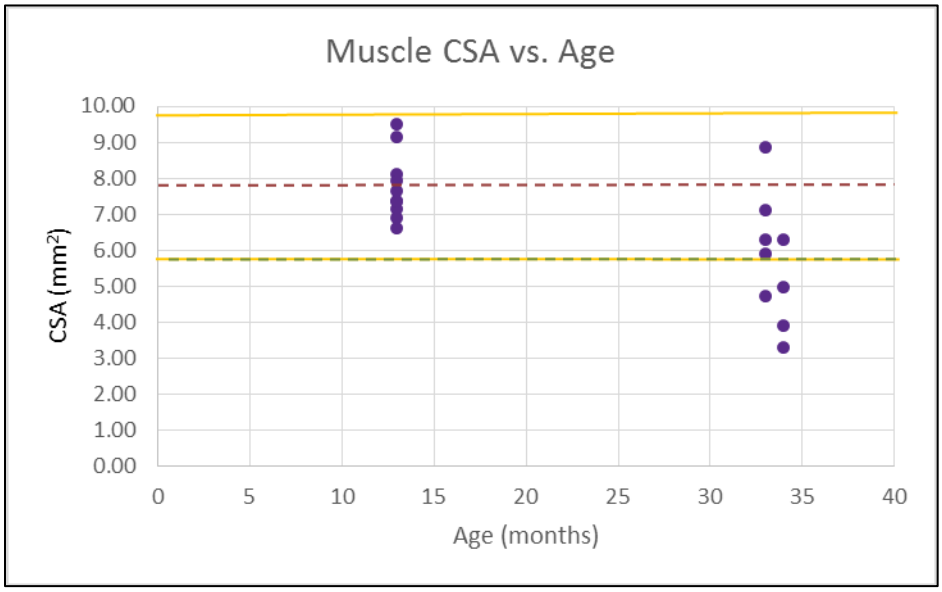


Figure 9. Muscle CSA vs. Age. Means: YR: 7.75 ± 0.99 mm^2, OR: 5.72 ± 2.10 mm^2. Dashed red line indicates young mean (7.75 mm^2). Solid gold lines indicate 2 SD above and below young mean (9.73 & 5.77 mm^2, respectively). Dashed green line indicates old mean (5.72 mm^2).

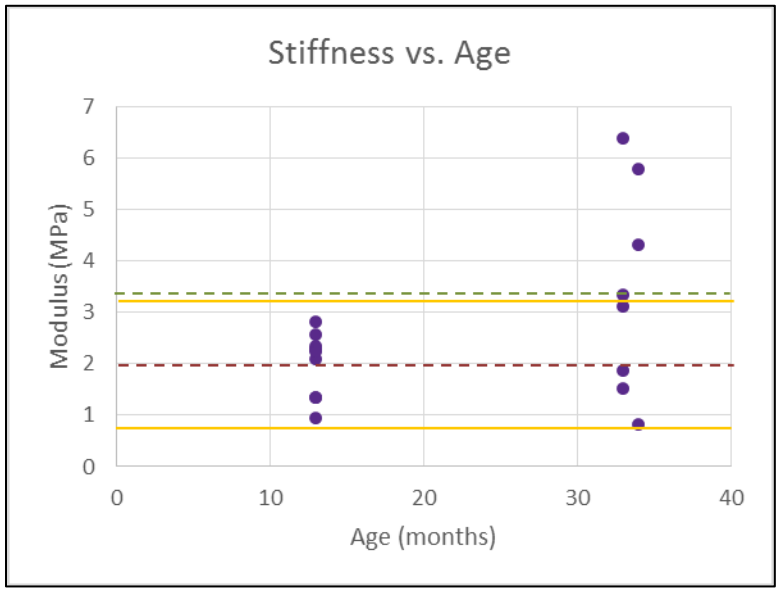
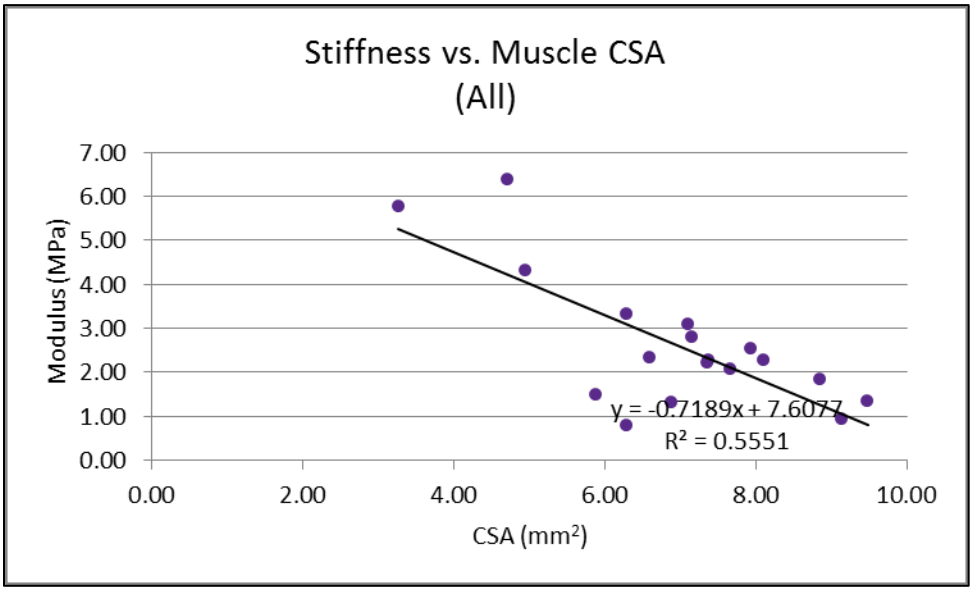
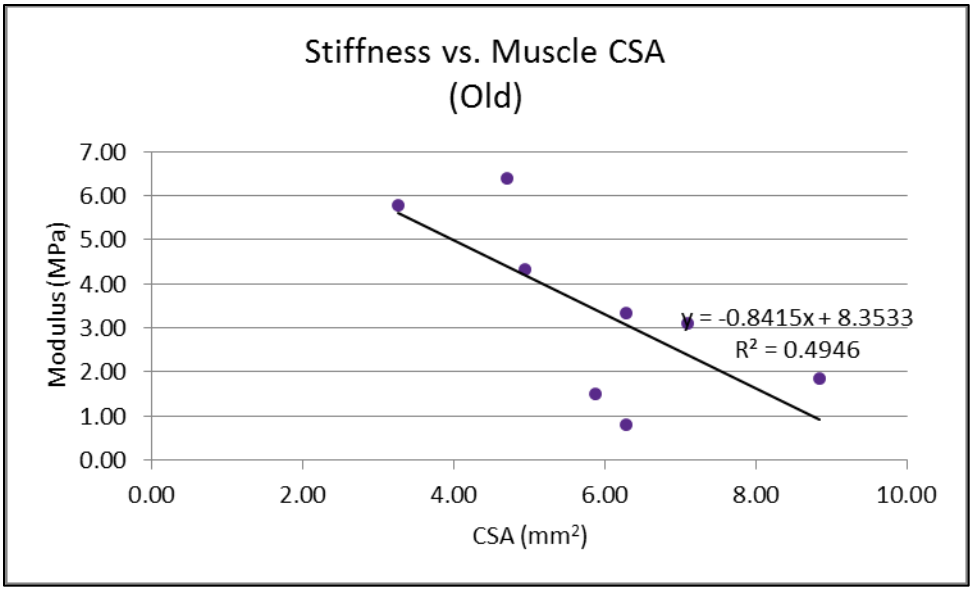


Figure 10. Stiffness vs. Age. Means: YR: 2.01 ± 0.61 MPa, OR: 3.38 ± 2.01 MPa. Dashed red line indicates young mean (2.01 MPa). Solid gold lines indicate 2 SD above and below young mean (3.23 & 0.79, respectively MPa). Dashed green line indicates old mean (3.38 MPa).

Although, half of the old rats did fall below the line of 2 SD above the young mean, resulting in muscle stiffness levels close to the young rats. Although, half of the old rats did fall below the line of 2 SD above the young mean, resulting in muscle stiffness levels close to the young rats.

Stiffness vs. muscle size results are presented in Figures 11a-c. For all rats, the relationship between Young's modulus and muscle CSA was significant, strong, and negative (slope = -0.720, r = -0.745, p = 0.00). This relationship was similar in just the old rats (slope = -0.843, r = -0.704, p = 0.026), but weaker the in young rats (slope = -0.372, r = -0.572, p = 0.042). It is noteworthy that most of the rats, for both age groups, were clustered together and fell within a range of roughly 2.5 MPa for stiffness, and roughly 3.75 mm^2 for muscle CSA. For the 3 rats not a part of the cluster, both their muscle CSAs and Young's moduli fell out of the respective ranges.





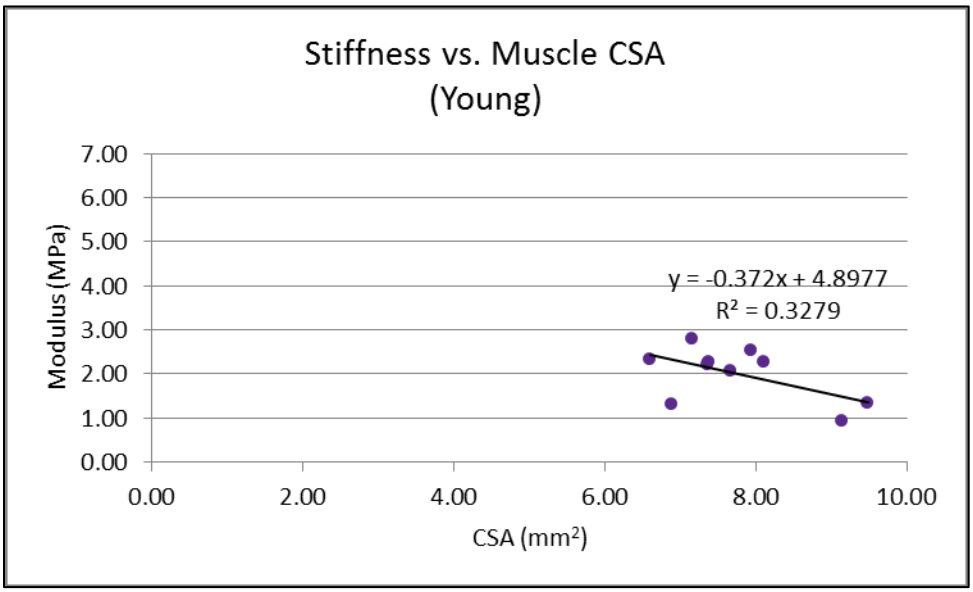


Figure 11. Relationship of Young's modulus to muscle CSA in all rats. Shown regression lines indicate significant relationship. **(A, top)**, slope = -0.720, r = -0.745, p = 0.000; in old rats **(B, middle)**, slope = -0.843, r = -0.704, p = 0.026; in young rats **(C, bottom)**, slope = -0.372, r = -0.572, p = 0.042.

FAK Activity

Figure 12 shows the FAK activity results of both stimulated and unstimulated legs for both age groups. The 2 x 2 ANOVA showed that there was a main effect for age on FAK activity ($p = 0.000$), but not for stimulation ($p = 0.357$), and that there was no Age x Stimulation interaction ($p = 0.933$). Though not significant, there does appear to be a small increase in FAK as a result of stimulation in both the young and old rats, but this increase seems to be similar in both groups. Also, basal FAK activity, as noted by the unstimulated legs, is larger in the young rats than old rats.

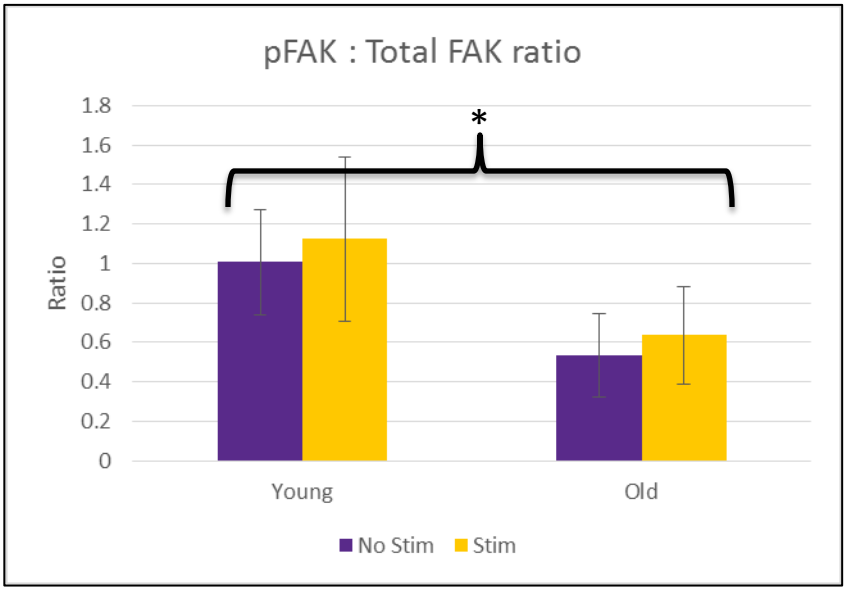
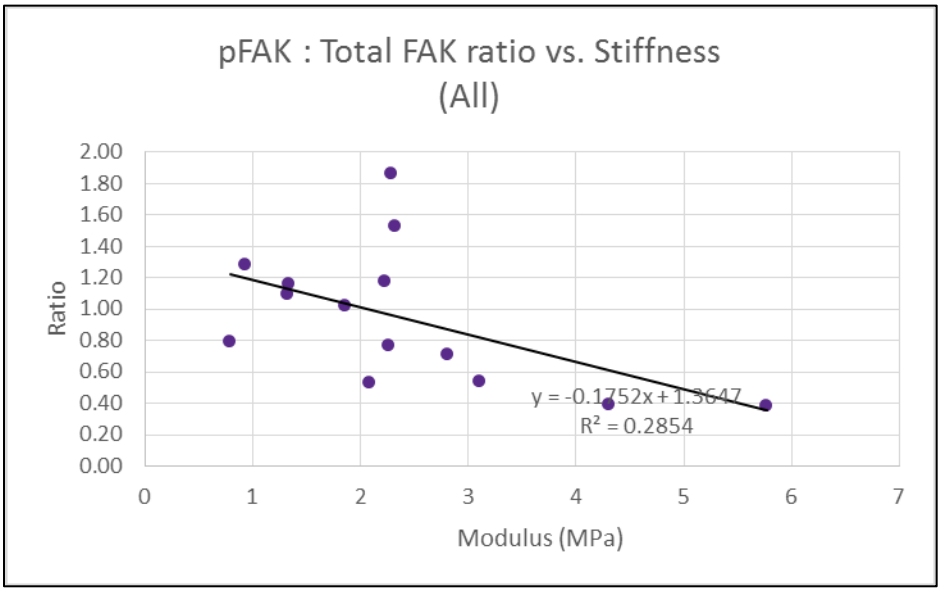
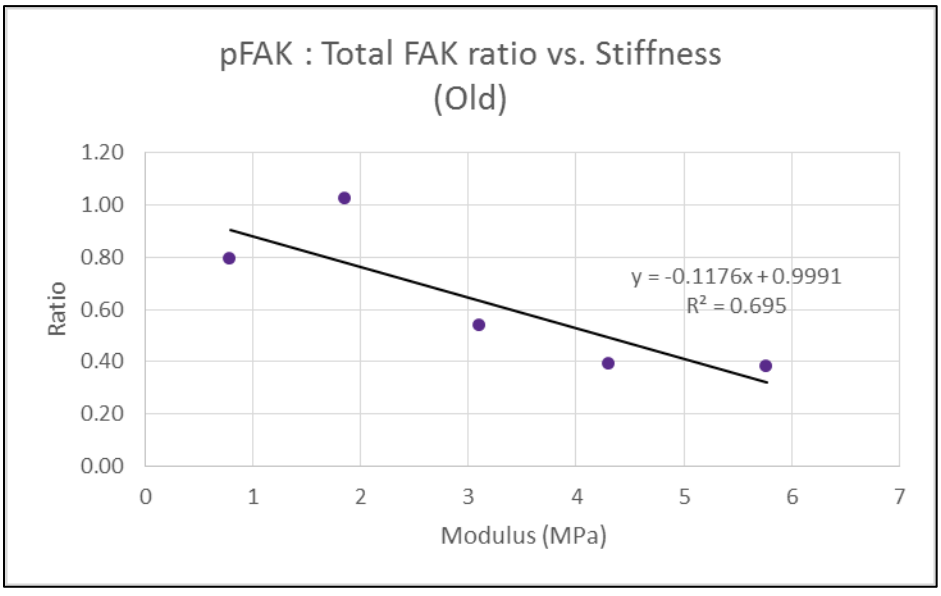


Figure 12. FAK activity in stimulated leg means in both stimulated and unstimulated legs for each age group. * indicated significance ($p = 0.000$)

In the stimulated legs for all rats, FAK activity was shown to have significant, moderately negative correlation with Young's modulus (slope = -0.176, r = -0.535, p = 0.024) (Figure 13a). However, when this was divided into age groups, the negative correlation was much stronger in

the old rats (slope = -0.119, r = -0.839, p = 0.038) and absent in the young rats (slope = -0.098, r = -0.145, p = 0.355), as shown in Figures 13b and 13c. Furthermore, 3 of the old rats showed similar FAK activity-to-muscle stiffness ratios as the young rats. The 2 old rats with muscle stiffness levels way above the young rats also had FAK activity that dipped well below the young range.





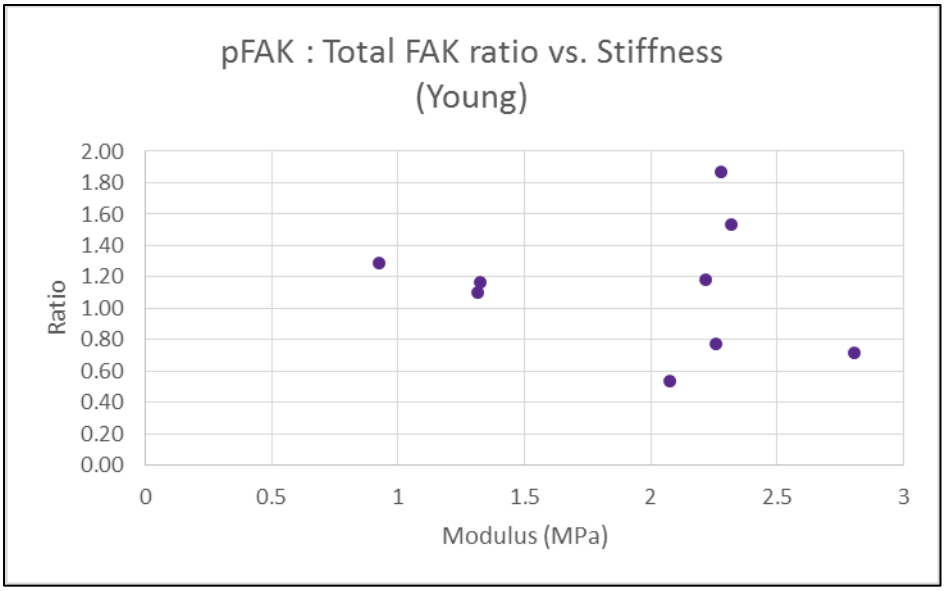
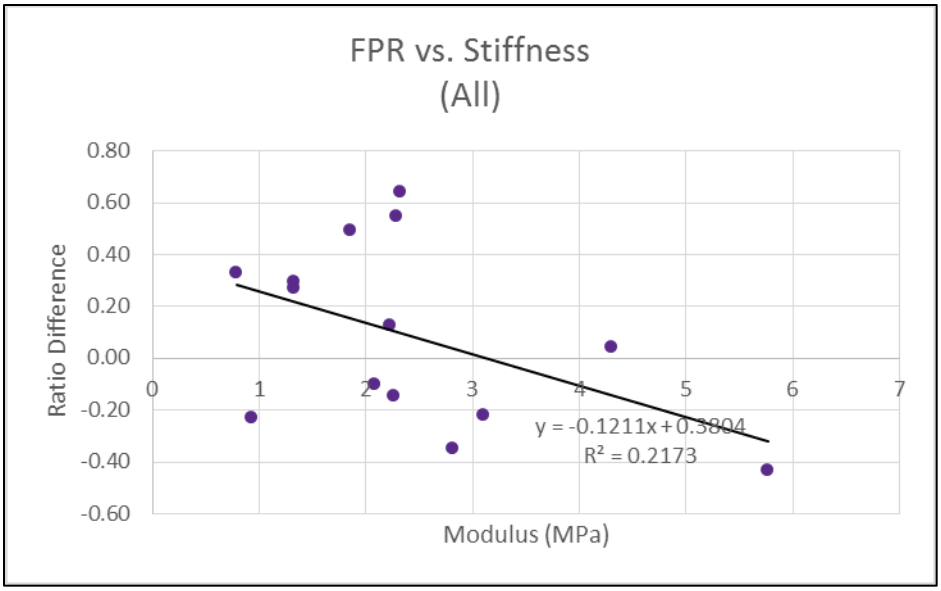
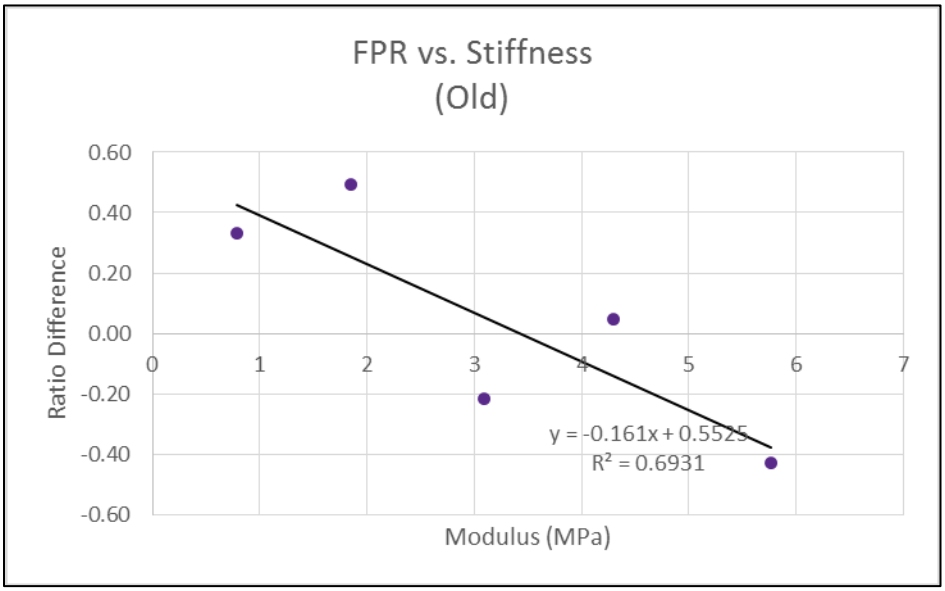


Figure 13. Relationship of FAK activity in stimulated legs and stiffness in all rats. Shown regression lines indicate significant relationship. **(A, top)**, slope = - slope = -0.176, r = -0.535, p = 0.024; in old rats **(B, middle)**, slope = 0.119, r = -0.839, p = 0.038; in young rats **(C, bottom)**, slope = -0.098, r = -0.145, p = 0.355.

A similar pattern is seen when difference in FAK activity between the stimulated and unstimulated legs is compared to Young's modulus (Figures 14a-c). For all rats, there is a significant, moderately negative correlation (slope = -0.121, r = -0.466, p = 0.046) that becomes stronger in old rats (slope = -0.161, r = -0.833, p = 0.04) and weaker and non-significant in young rats (slope = -0.009, r = -0.015, p = 0.485). About half of the rats for each group had lower FAK activity in the stimulated leg vs. the unstimulated leg (noted by the negative ratio). However, in the young rats, this lower FAK activity spanned the range of stiffness levels while in the old rats, this occurred in the rats with a higher Young's modulus.


FPR vs. Stiffness
(All)
Ratio Difference
Modulus (MPa)
y = -0.1211x + 0.3804
R² = 0.2173
0.80
0.60
0.40
0.20
0.00
-0.20
-0.40
-0.60
0
1
2
3
4
5
6
7


FPR vs. Stiffness
(Old)
Ratio Difference
Modulus (MPa)
y = -0.161x + 0.5525
R² = 0.6931
0.60
0.40
0.20
0.00
-0.20
-0.40
-0.60
0
1
2
3
4
5
6
7

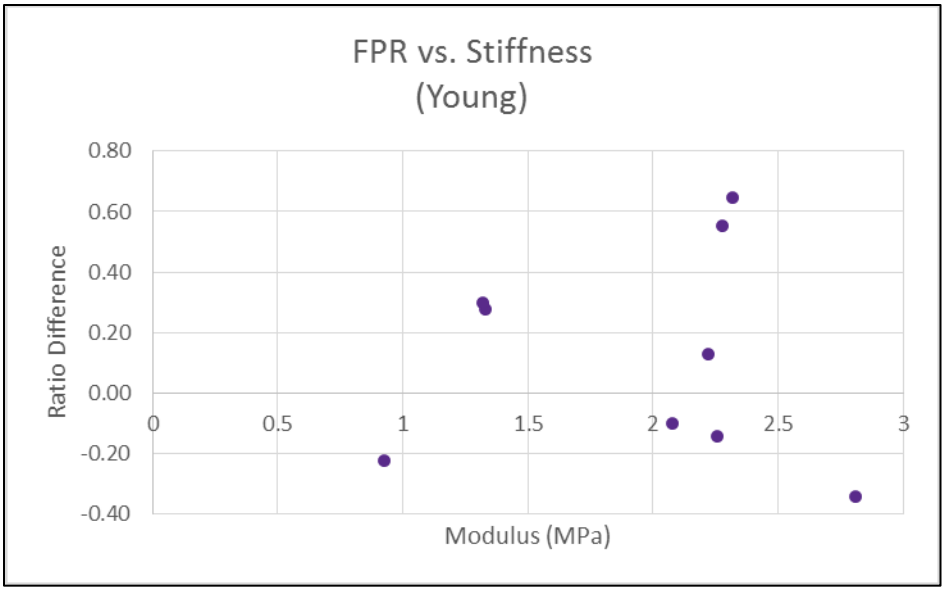


Figure 14. Relationship of relative phosphorylation difference between stimulated and nonstimulated legs and stiffness in all rats. Shown regression lines indicate significant relationship. **(A, top)**, slope = -0.121, r = -0.466, p = 0.046; in old rats **(B, middle)**, slope = -0.161, r = -0.833, p = 0.04; in young rats **(C, bottom)**, slope = -0.009, r = -0.015, p = 0.485.

Chapter 5 – Discussion

Partial results of this study showed that muscle properties in the old rat population are significantly different, but also more variable, than muscle in young rat population. Young rats showed, on average, a bigger muscle than the old rats, but about half of the old rats had muscle sizes similar to the young rats. A similar pattern was also seen comparing stiffness across ages, with young rats having a lower average, but some old rats having values similar to young rats. A negative relationship between stiffness and muscle size was seen across all age groups, but was more prominent in the old group than the young group.

The purpose of this study was to examine FAK with regards to age and aging-related changes of muscle. FAK activity was shown to decrease with age, but amount of increase seen with stimulation was no different between age groups. A negative relationship between FAK activity and stiffness was strong in the old group and weak in the young group. A similar pattern was also seen when comparing FAK phosphorylation response. These results support our hypothesis that phosphorylation of FAK is impaired with increased muscle stiffness.

The results in the comparison of muscle size with age show that sarcopenia is prevalent in rats and similar to sarcopenia in humans. The decrease in muscle size with age shows that older rats are more likely to have smaller muscles than young rats, but that muscle size in an older rat population is a lot more variable. When comparing ages, young rats had, on average, about a 35% larger muscle CSA than the young rats. However, upon closer examination (Figure 9), 5 of the 9 older rats fell within 2 standard deviations below the young average (mean: 7.75 mm^2, 2 SD below: 5.77 mm^2), with one older rat having the 3rd largest muscle in the study. These numbers seem very similar to numbers seen in human adults. Since the definition of sarcopenia is arbitrary but usually based off of standard deviations from a young control (Janssen

et al., 2002; Janssen, 2006), we can define it in this study as 2 SD below the young average. Using this definition, we find that roughly 45% of our older rats were sarcopenic, a similar number seen in the male, over 60 years-old population in humans (Janssen et al., 2002). These similar sarcopenia numbers suggest that aging, with regards to muscle, is very similar between rats and humans, and that rats can be used as good aging model.

When comparing muscle stiffness across age groups, we see a similar pattern to muscle size arise: significant differences between old and young with more variability in old population. The old rats had a mean Young's modulus that was about 70% higher than the young rats. This increase in stiffness has previously been seen in the epimysium of rats (Gao et al., 2008). However, in our study, the standard deviation in the old rats was also over three times as high as the standard deviation in the young rats, indicating greater variability amongst the old rats. This increase in variability of stiffness is similar to what has been seen in the muscles of older adult humans (Domire, McCullough, Chen, & An, 2009) (Figure 15). About half of the old rats fell within the range of 2 standard deviations above the young mean (Figure 10), showing they had similar muscle quality as their young counterparts.

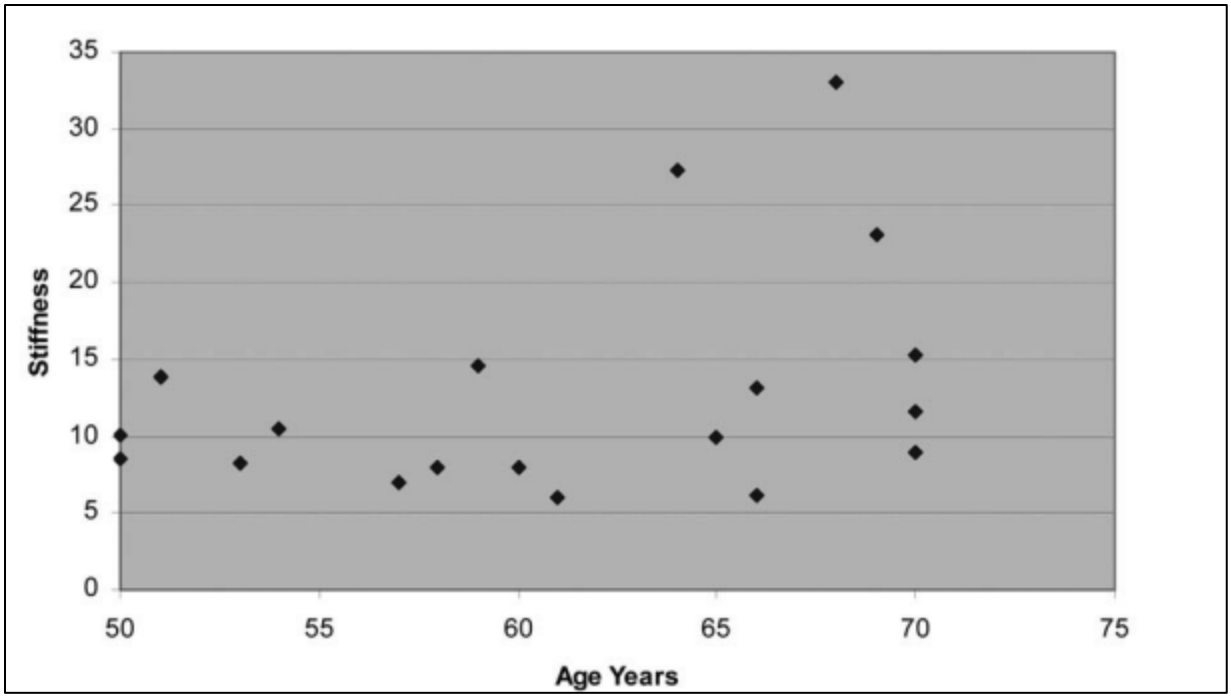


Figure 15. Muscle stiffness vs. Age (Domire et al., 2009).

Our hypothesis of impaired FAK phosphorylation with increased stiffness implies that stiff muscles will also be smaller. We saw that across age groups, a strong, negative relationship occurred, with smaller muscles having increased stiffness. This relationship has previously been identified in a study comparing caloric restricted and *ad libitum* rats (Pauwels et al., 2012b). When splitting the rats into age groups, the relationship was strong in the old rats and moderate in the young rats. A closer look at the relationship (Figure 11) shows that stiffness levels of both and young rat muscles above the sarcopenia cut-off mentioned previously was quite variable. The muscle sizes of the old rats that fell into the sarcopenia category had stiffness levels above the rest of the group. These results help to support the implication of our hypothesis that sarcopenic muscles are stiffer than healthy muscles.

To our knowledge, we are the first study to examine the relationship of FAK phosphorylation with age in skeletal muscle. However, this has previously been studied in the

smooth muscle of rat aortas (Rice et al., 2007). Our results show that FAK activity of skeletal muscle, in general, is significantly decreased with age. This suggests that as it ages, muscle loses its capacity to phosphorylate FAK, which could possibly impair the protein synthesis for hypertrophy (Klossner et al., 2009). If this holds true, then decreased FAK activity with age could be a possible contributing mechanism for sarcopenia. It is important to note that the muscles of the healthy older rats had FAK activity levels similar to the young rats. It was the muscles of the unhealthy old rats with decreased levels of FAK activity. However, this does not discredit decreased FAK activity as a mechanism for sarcopenia since not all older adults are sarcopenic (Janssen et al., 2002; Janssen, 2006).

Since FAK is known to phosphorylate with stretch or exercise (Zhang et al., 2007), we thought it was important to compare the FAK activity between the non-stimulated and stimulated (exercised) legs. In this study, we found only a small increase in FAK activity from the non-stimulated legs to the stimulated legs. This increase was small and very similar between both age groups. This disagrees with what has been seen in smooth muscle, where phosphorylation in response to a stimulus increased in young rats and decreased in old rats (Rice et al., 2007) (shown in Figure 16). Our results do agree with Gordon et al. (2001) (Figure 17), found FAK activity to increase in the skeletal muscles of rats (ages unknown) after a chronic overload protocol.

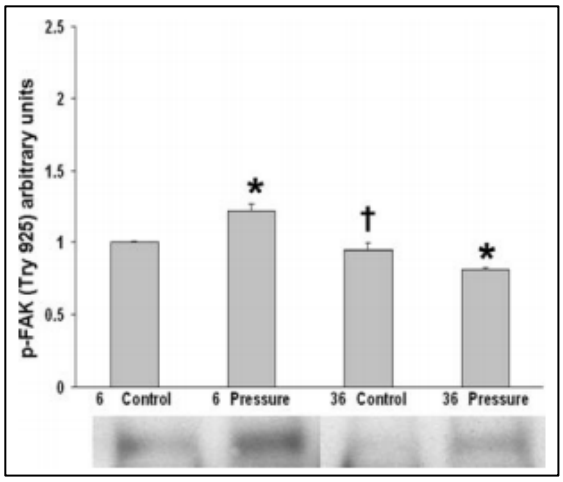


Figure 16. FAK phosphorylation and response to stimulus (young: left 2 bars; old: right 2 bars) from Rice et al. (2007).

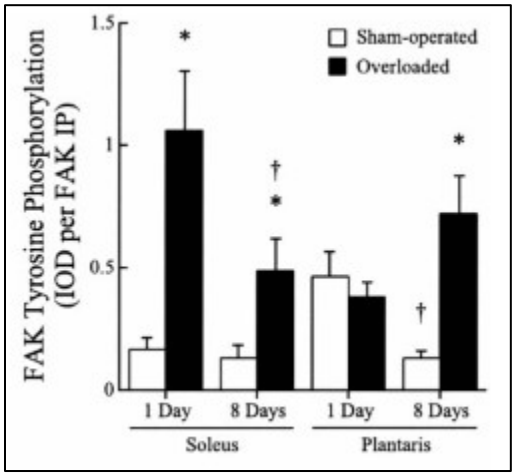


Figure 17. Increase in FAK activity after overload protocol (Gordon et al., 2001).

The reason for such a small increase in our study can either be attributed to high basal levels or low response to stimulation. If the rats were physically active prior to testing, then FAK activity would be increased and not true basal levels. It is also possible that isoflurane could have an effect on FAK, but this has not been studied exclusively. However, it has been shown that isoflurane increases phosphorylation ERK1 and ERK2, extracellular signal-regulated kinases, in smooth muscle (Zhong & Su, 2002).

It is also possible that levels of phosphorylation in response to stimulation are low. One reason could be that the exercise protocol was not sufficient enough to induce a response, but this seems unlikely since it was an effective protocol in a previous study (Pauwels et al., 2012a). Another reason could be the prolonged time-to-sacrifice, which could have allowed FAK to return to near basal levels. Most of the rats had a time-to-sacrifice of over 20 minutes, but Lehoux et al. (2005) showed FAK levels return to baseline levels after 20 minutes. Future studies could use a different method of sacrifice so that the time-to-sacrifice is more immediate and true pFAK levels can be determined.

The main purpose of this study was to examine the relationship between FAK activity and muscle stiffness. Since a strong, negative relationship was seen in the older rats, our results support our hypothesis that FAK activity in response to exercise would decrease with increasing muscle stiffness. Across both age groups, however, the relationship is only moderate. This relationship is very strong within the old population but does not exist in the young population. Similar to the relationship of stiffness and muscle size, these results suggest that there is a possible healthy range of muscle stiffness where FAK activity is variable, but as stiffness levels go out of that range, FAK activity levels start to decrease. This negative relationship, especially in the old rats, suggests that muscle stiffness possibly has an effect on FAK activity. This makes sense knowing that FAK is phosphorylated with stretch (Zhang et al., 2007) and is bound to integrins, which sense stretch of the ECM (Wang et al., 1993). If stiffness of a muscle is increased, then the stretch the muscle experiences under a given load will be decreased. If the integrins sense less stretch, they may not signal as much phosphorylation of FAK, which will result in less cell signaling for hypertrophy (Klossner et al., 2009). While decreased FAK

activity with age is a suggested mechanism for sarcopenia, it could be the influence of increased muscle stiffness on FAK activity that is one of the actual mechanisms behind sarcopenia.

Our results also show that total amounts of total FAK and pFAK are probably irrelevant. Examination of the non-stimulated control leg shows that total FAK amount increased with age while pFAK stayed relatively similar (Figure 18). This increase in total FAK with no increase in pFAK explains why FAK activity was so low in the old adults when compared to the young rats. The increase of total FAK with age has been seen in smooth muscle (Rice et al., 2007), however further research is needed to determine why total FAK increases and pFAK stays relatively the same. Total FAK showed no relationship with muscle size within each age group (Figures 19a and 19b). The reason for raised amounts of FAK in smaller muscles is unknown, but future research could study why it occurs. Even though FAK is increased is smaller and aged muscles, it seems to be how much of the FAK is actually being phosphorylated (FAK activity) that seems to be more important.

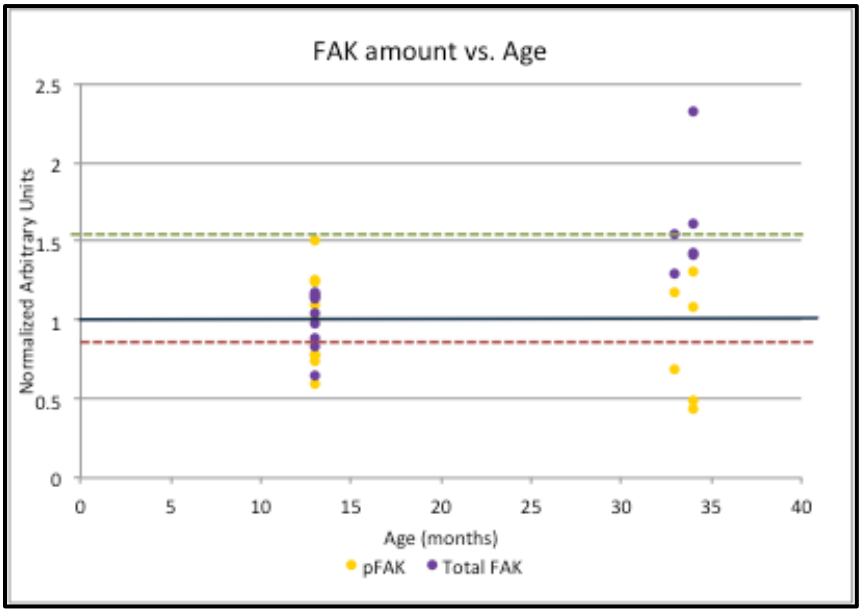


Figure 18. Relationship of Total FAK and pFAK amounts with age. (Total FAK: $p = 0.000$; pFAK: $p = 0.222$). Solid blue line indicates young mean for total FAK and pFAK. Dashed lines indicate old mean (green: Total FAK; red: pFAK).

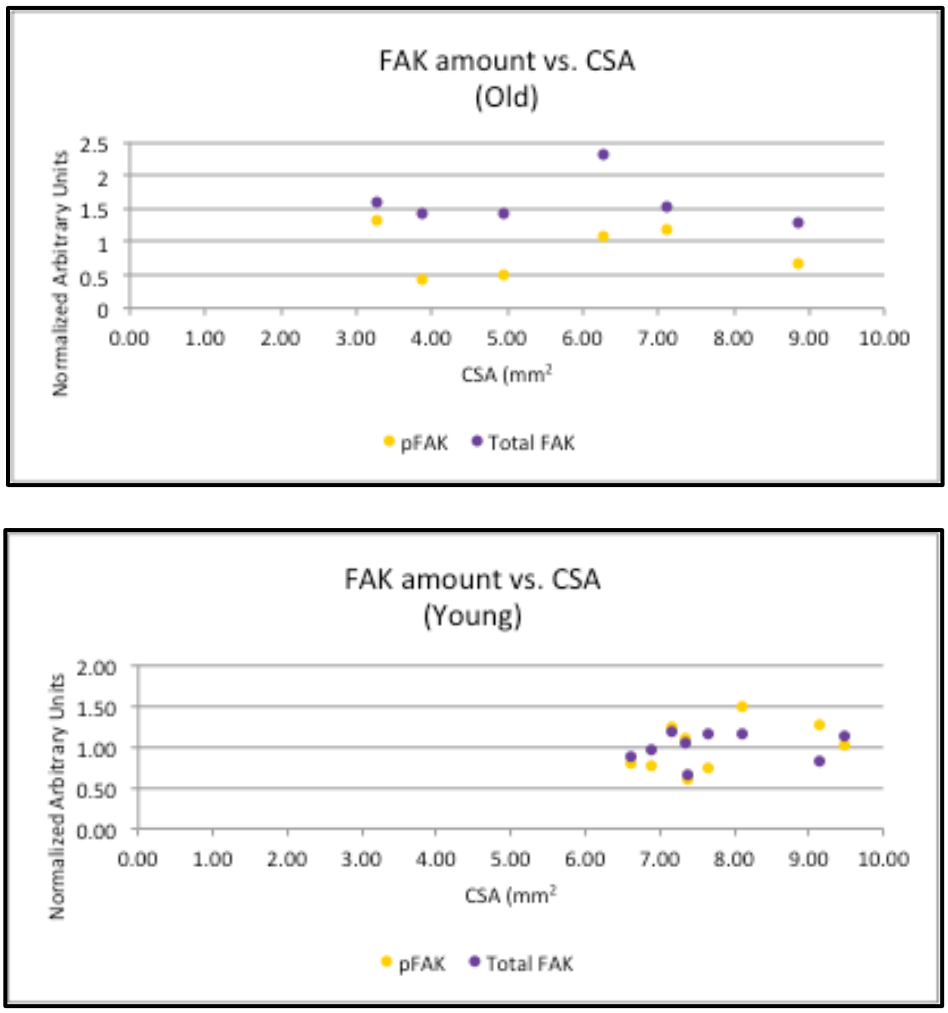
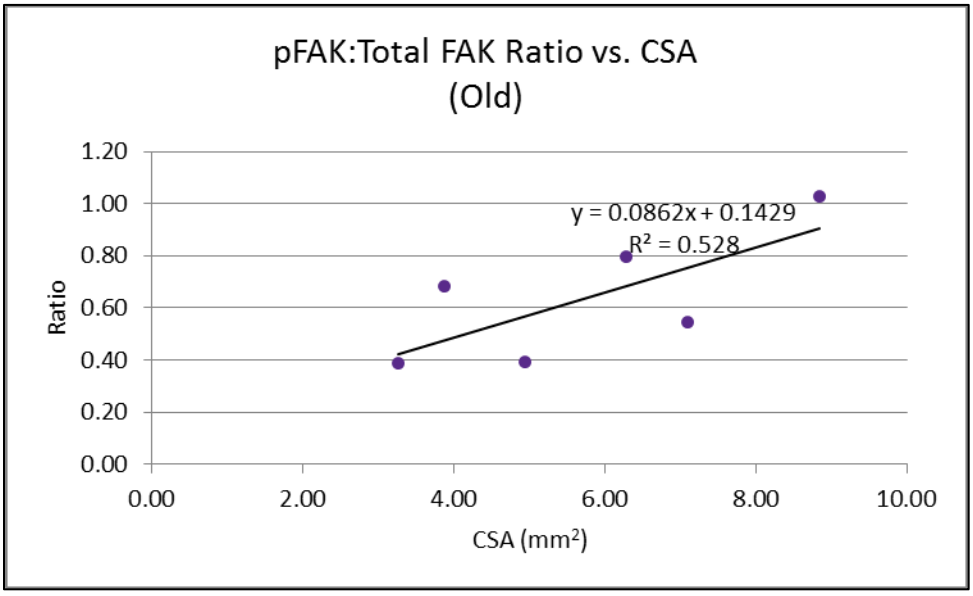


Figure 19. Relationship of Total FAK and pFAK amounts with muscle size. **(A, top)** Within old group (Total FAK: $p = 0.493$; pFAK: $p = 0.498$). **(B, bottom)** Within young group (Total FAK: $p = 0.361$; pFAK: $p = 0.132$).

A pattern in the relationship between FPR and stiffness arises that is similar to the pattern between FAK activity and stiffness. Our results show a moderate relationship across age groups, a strong relationship in just the old rats, and no relationship in just the young rats. These results help support our hypothesis that FAK activity decreases with increasing stiffness. In the FPR results, we see that about half of the rats in each group had a negative difference, meaning more

FAK activity occurred in the non-stimulated leg. Why this occurs is unknown, but is discussed previously in the FAK activity vs. age paragraph. In the young rats, these negative FPRs are spread out across the range of stiffness levels, but in the old rats, negative FPRs are seen in the rats with the higher stiffness levels. This helps support the idea that, in old muscle, FAK activity is influenced by muscle stiffness.

It has been established that muscle size decreases with age and stiffness, and that our results suggest decreased FAK activity, whether as a result of age or muscle stiffness, could be the mechanism behind this decreased muscle size. As shown in Figure 20a and 20b, FAK activity has trends towards a strong positive relationship with muscle size in older rats, but no relationship in young rats. This bolsters support for the idea that FAK activity is at least part of the mechanism for sarcopenia. Results from this study show muscle stiffness influences FAK activity and is another part in the mechanism. The missing part is why stiffness is increasing with age, but this is thought to be due to the build-up of nonenzymatic AGEs (Haus et al., 2007; Snow et al., 2007).



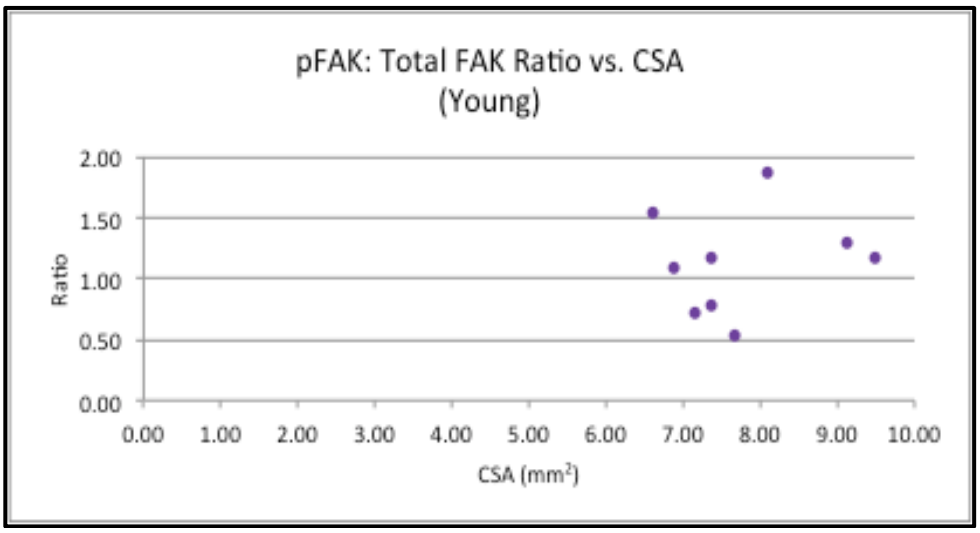


Figure 20. FAK activity relationship to old rats **(A, top)**, slope = 0.086, r = 0.724, p = 0.052, Cohen's *d* effect size: 3.4); and young rats **(B, bottom)**, slope = 0.066, r = 0.156, p = 0.344. Regression line indicates trend.

There are several limitations and delimitations with this study. First off, the sample size, especially for the old group, was small due not being able to use all of the rats. While significance was found in the old group, this may change if a larger sample group is tested. This study was also performed in rats, so it is unwise to assume these results will be similar in humans even though our results suggest similar aging between the two species. As previously mentioned, the stimulation protocol could have been ineffective in producing enough of a phosphorylation response, but this is unlikely since it is the same protocol described by Pauwels et al. (2012a), in which FAK phosphorylation was seen. The small increase in phosphorylation seen between stimulated and non-stimulated legs is likely due to the time period between exercise and sacrifice, which was discussed earlier. Future studies would need to sacrifice the rats after a shorter time period, perhaps immediately, to measure the full FAK response.

This study has helped lay the groundwork for future research focusing on FAK and skeletal muscle. First off, a study could look at the effect of anesthetics on FAK activity. Rats could be separated into two groups of sacrifice method (decapitation and isoflurane overdose) and FAK activity levels could be compared between the groups. Rats could also be put through a similar exercise protocol as ours but splits rats into groups using different anesthetics. It is important to know if anesthetics affect FAK activity so future studies can know which anesthetics to avoid. Also, figuring out the time course of FAK activation levels is important to the overall understanding of the protein. Using a similar exercise protocol as ours, a study could sacrifice rats at different time points to determine when FAK activity is greatest and when it starts to decline.

Our results of FAK activity decreases with muscle stiffness indicate the need for future research in humans and sarcopenia. Though aging between rats and humans seems similar, our results need to be confirmed in humans. A current study in our lab is testing this idea by non-invasively measuring muscle stiffness and putting subjects through a unilateral exercise protocol followed by bilateral muscle biopsies. Assuming this study confirms our results, the next step is to research ways to manipulate stiffness. Since muscle stiffness is thought to increase as a result of AGEs and increased collagen cross-linking (Haus et al., 2007; Snow et al., 2007). Another current study in our lab is using a stretching protocol in older adults to determine if muscle stiffness will decrease. The idea is that stretching is a mechanical way of breaking the collagen cross-links. A drug, Alagebrium, was developed several years ago as a way to chemically remove collagen cross-links and AGEs to reduce stiffness in the cardiovascular system (Alteon, 2007). Though the drug had no effect on exercise capacity in older adults, it did moderately reduced ventricular stiffening (Fujimoto et al., 2013). Future studies in this area could re-modify

Alagebrium or develop a new drug to break collagen cross-links and reduce stiffness in skeletal muscle. If effective methods of manipulating stiffness are found, these will be useful for experimental and clinical purposes. The direct manipulation of muscle stiffness would allow for better observation of its effect on cellular processes. The ability to manipulate stiffness would also present a potential treatment option for sarcopenia by reducing muscle stiffness and reversing FAK activity impairment.

From this study, we can conclude the FAK activity is impaired with age, and it is likely tbat increased muscle stiffness is a major contributor to this impairment. This study also supports the literature that says muscle size is decreased with age and muscle stiffness is increased with age. These results implicate muscle stiffness as a major factor in the development of sarcopenia.

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Appendix: AUP Approval Letter

East Carolina University

Animal Care and Use Commitee
212 Ed Warren Life Sciences Building
East Carolina University
Greenville, NC 27834

252-744-2436 office
252-744-2355 fax

July 10, 2013

Zachary Domire, Ph.D.
Department of Kinesiology
Ward Sports Medicine Bldg.
ECU School of Medicine

Dear Dr. Domire:

Your Animal Use Protocol entitled, "A Pilot Investigation of Variable Response to Exercise within an Aged Muscle" (AUP #P080) was reviewed by this institution's Animal Care and Use Committee on 7/10/13. The following action was taken by the Committee:

"Approved as submitted"

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies.

Sincerely yours,

Susan McRae, Ph.D.
Chair, Animal Care and Use Committee

SM/jd

enclosure

Appendix: AUP Amendment Approval Form

East Carolina University

Animal Care and Use Commitee
212 Ed Warren Life Sciences Building
East Carolina University
Greenville, NC 27834

252-744-2436 office
252-744-2355 fax

March 13, 2015

Zachary Domire, Ph.D.
Department of Kinesiology
Ward Sports Medicine Building
East Carolina University

Dear Dr. Domire:

The Amendment to your Animal Use Protocol entitled, "A Pilot Investigation of Variable Response to Exercise within an Aged Muscle", (AUP #P080) was reviewed by this institution's Animal Care and Use Committee on 3/13/15. The following action was taken by the Committee:

"Approved as amended"

****Please contact Dale Aycock prior to any hazard use**

A copy of the Amendment is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies. **Please ensure that all personnel associated with this protocol have access to this approved copy of the AUP/Amendment and are familiar with its contents.**

Sincerely yours,

Susan McRae, Ph.D.
Chair, Animal Care and Use Committee

SM/jd

enclosure