**Response to Review:**

We thank the reviewers for their thoughtful and helpful comments, which we have addressed throughout the manuscript and in this reply point by point. We would first like to address the main concern of the statistical analysis – While our point was to not bias our analysis with large numbers of samples we approached the analysis this way because we intended to account for any variability in specific sections of muscle (note: 4 distinct sections) by not ‘grouping’ everything into one average per sample. However, as suggested by the reviewers we grouped all measures into a single mean value per muscle sample (young; n=9, old; 8) and the main statistical findings were unchanged (Please see the statistical printout below). Values provided in the text are Mean±SD and figures percent difference of the means and %SEM. Furthermore, please consider this is a summer student project, which is part of a much larger overarching project including the mechanical data reviewers touched on. Thus, no values for mechanical data are included in the revised manuscript.

The statistical analysis was performed on averaged data using a one way ANOVA: Sarcomere length (P=0.049), Sarcomere number (P=0.016), Fascicle Length (P=0.003). Therefore, interpretation of results are unchanged.

**SARCOMERE LENGTH**

One Way Analysis of Variance Friday, September 05, 2014, 11:35:48 AM

Data source: Data 1 in Notebook1

Normality Test (Shapiro-Wilk) Passed (P = 0.746)

Equal Variance Test: Passed (P = 0.114)

Group Name  N  Missing Mean Std Dev SEM

Young 9 0 2.310 0.0507 0.0169

Old 8 0 2.223 0.110 0.0388

Source of Variation  DF   SS   MS    F     P

Between Groups 1 0.0321 0.0321 4.596 0.049

Residual 15 0.105 0.00699

Total 16 0.137

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference  (P = 0.049).

Power of performed test with alpha = 0.050: 0.416

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor:

Comparison Diff of Means p q P P<0.050

Young vs. Old 0.0871 2 3.032 0.049 Yes

**SARCOMERE NUMBER**

One Way Analysis of Variance Friday, September 05, 2014, 11:35:10 AM

Data source: Data 1 in Notebook1

Normality Test (Shapiro-Wilk) Passed (P = 0.174)

Equal Variance Test: Passed (P = 0.919)

Group Name N Missing Mean Std Dev SEM

Young 9 0 5684.828 440.404 146.801

Old 8 0 5087.630 465.711 164.654

Source of Variation DF SS MS  F   P

Between Groups 1 1510497.691 1510497.691 7.381 0.016

Residual 15 3069850.988 204656.733

Total 16 4580348.679

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference  (P = 0.016).

Power of performed test with alpha = 0.050: 0.659

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor:

Comparison Diff of Means p q P P<0.050

Young vs. Old 597.198 2 3.842 0.016 Yes

**FASCICLE LENGTH**

One Way Analysis of Variance Friday, September 05, 2014, 11:34:35 AM

Data source: Data 1 in Notebook1

Normality Test (Shapiro-Wilk) Passed (P = 0.435)

Equal Variance Test: Passed (P = 0.998)

Group Name N Missing Mean Std Dev SEM

Young 9 0 13.131 1.142 0.381

Old 8 0 11.291 1.027 0.363

Source of Variation DF SS MS   F   P

Between Groups 1 14.340 14.340 12.070 0.003

Residual 15 17.822 1.188

Total 16 32.162

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference  (P = 0.003).

Power of performed test with alpha = 0.050: 0.889

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor:

Comparison Diff of Means p q P P<0.050

Young vs. Old 1.840 2 4.913 0.004 Yes

**Reviewer A:**

Article Commentary:

**R: An interesting study on the effects of aging on muscle ultrastructure that may contribute to increased stiffness in aged muscle.**

Thank you

**R: Was muscle stiffness measured? Was muscle stiffness greater in the muscles from old animals?  If not, then this data would be difficult to interpret in that context.**

The muscle stiffness was not measured. These data are part of a larger project looking at exactly that point.

**R: Some clarification in the description of statistical testing is needed, as the authors report numbers of measures that suggest pseudosampling.  Were the individual measures of fascicle length, sarcomere length, and sarcomere number treated as independent samples (i.e. n=180 for young and n=160 for old for fasicle length/sarc number measurements, and n=900 for young and n=800 for old for measures of sarcomere length)?  If yes, this would be pseudo sampling and would be statistically incorrect.  If so, the values obtained for each individual rat should be averaged to produce a single value for each individual (n=9 for young and n=8 for old), and these values compared in the statistical tests.**

The reviewer is correct. In light of this concern the statistical analysis was performed on the means of each sample as suggested. The final outcome did not change. Please see the above statistical report.

**Reviewer B:**

**General comments:**

**R: This paper examines age-dependent differences in sarcomere and fascicle lengths as well as sarcomere count in series as a potential contributor to age-related increases in muscle stiffness. The authors report highly significant differences in all three of the quantified variables, with reductions in all 3 with age. The research question is appealing, and the methods are germane to the research question. I commend the author’s efforts in this work. However, there are serious concerns regarding the statistical analysis that profoundly affect the interpretation of the data (see below). Unfortunately, large changes to the manuscript will be required, however, I believe that the paper has the potential to be a fine addition to JURA if these concerns are addressed. I would be happy to review this paper in a revised form upon resubmission.**

**Major Concerns:**

**There are some serious issues with the statistics that need to be resolved. The incredibly high levels of significance led me to believe something is awry. Using the reported means, N-values and either SEM or standard deviations, the p value of a t-test can be calculated. I have done this and posted the values in the chart below (calculated values). Accordingly, the reported p values cannot possibly be correct.**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | **Reported Value** | **Reported Value** | **Reported****Value** | **Calculated Value** | **Calculated Value** |
| **Variable of Interest** | **Young** | **Old** | **P Value** | **P Value if 2nd value is SEM** | **P Value if 2nd values is Standard Deviation** |
| **Fascicle Length** | **13.1±2;n=9** | **11.3±2;n=8** | **P<0.001** | **P=0.54** | **P=0.08** |
| **Sarcomere Length** | **2.31±0.08;n=9** | **2.22±0.12;n=8** | **P<0.001** | **P=0.54** | **P=0.09** |
| **Sarcomeres in Series** | **5683±846;n=9** | **5092±902;n=8** | **P<0.001** | **P=0.64** | **P=0.18** |

**I believe that these author performed these tests using the total of “20 fascicle length and 100 sarcomere length measurements were obtained from each muscle, which resulted in a total of 340 fascicle length and 1700 sarcomere length measurements.” These values should have been averaged into a single value for each variable for each muscle, and the t-tests performed on the resultant averaged values. Depending on whether the values are expressed as Mean ± SEM or Mean ± Standard deviation, this means, respectively, that there are either no effects of age on any of the quantitative variables, or there are statistical trends that would justify expanding the study with increased numbers in attempt to achieve statistical significance.**

**The data presentation in Figure 2 is also concerning. The error bars are far too small. Based on the values reported in the results, the chart should appear like one of the charts below, again depending on whether the reported values are followed by the SEM or SD. If SEM, were these values calculated with the 100s of values, or is it a simple oversight, and they are simply not expressed as a percentage of mean value?**

**I would like to see the raw data used in the calculations appended to the resubmission as supplemental material to be used only for review purposes.**

We appreciate the reviewers concern and have addressed this above and throughout the manuscript.

**Other Comments:**

**R: Title: “Loss” at the end implies that aging reduces the numbers of serial sarcomeres lost.**

We have removed ‘loss’ from the title

**R: Short title: Too broad. Change to something more specific.**

Changed from “Age Related Changes to Muscle” to “Age Related Changes to Muscle Structure”

**Abstract Introduction:**

**R: “Aging is associated with decreased active force production leading to muscle weakness and subsequently decreased muscle performance.” The wording here suggests a time-dependent causality which is not necessarily true – each is a symptom of the other. Suggest changing to “Aging is associated with muscle weakness and decreased muscle performance due, in part, to impaired active force production.” This also makes a better lead into your next sentence on the contribution of passive stiffness. See also next point, and the abstract discussion point.**

We have made this change as suggested.

**R: The connection between the increased passive force and muscle weakness is not clear. The link between the two should be established (and subsequently discussed), or the first sentence should be deleted as it does not affect the remainder of the abstract**.

A sentence was added regarding how passive force goes in the opposite direction with aging. “Aging also affects the muscle’s passive force properties, but in the opposite manner.”

**R: The purpose statement suggests a breadth of study that is not present. Please revise to more specifically address the properties measured.**

The purpose was changed from “The purpose of this study was to investigate potential structural property changes that occur in aged muscle that may contribute to increased passive force..” To the more specific “The purpose of this study was to investigate age related sarcomere length, sarcomere number and fascicle length changes that may contribute to increased passive force.”

**R: What is your hypothesis?**

A hyposthesis was added to the abstract. “It was hypothesized that fascicle length in old age

would be shorter compared with young, however, the sarcomere length would remain unaltered.

**Abstract Methods:**

**R: FL abbreviation is only used once and does not save any words, and can thus be removed from the abstract.**

FL was removed.

**R: Optimal length is typically abbreviated as Lo rather than L0.**

Corrected throughout paper

**R: “The muscle length where peak force occurred (i.e. plateau of the force-length relationship (FL); L0)” – Redundant.**

Deleted “(i.e. plateau of the force-length relationship (FL)”

**R: “Muscles were fixed at L0 in 10% formalin, fascicle length, sarcomere number and the sarcomere length were compared at L0.” Additional separation is needed between formalin and fascicle length. Consider splitting in 2 parts.**

Split the sentence and added more detail. “The MG was fixed at Lo in 10% formalin, digested in nitric acid and individual fascicles were isolated. Fascicle length, sarcomere number and the sarcomere length were then compared at Lo….”

**R: “old rats (*n =* 8) rats” Delete first instance of rats.**

Deleted

**R: MG is only used once, use it in place of muscle later in the abstract. Alternatively delete the abbreviation. The former is probably preferable as it is more specific.**

Replaced the word “muscle” with MG thoughout abstract.

**R: Abstract Results: Precede the sentence with “In comparison to the muscle of young rats.” Needs to reflect the statistical findings upon reanalysis.**

We have made this change as suggested.

**R: Abstract Discussion: Will need to address the new results**

Following the new analysis, the results are the same as before.

**R: Key words: Keywords should not include elements of the title. The key words are used to aid search engines in locating articles relevant to specific topics, and are thus used to broaded the base of search terms. Suggest the following ~~Aging, fascicle, sarcomere,~~ passive force, skeletal muscle, rat, laser diffraction, medial gastrocnemius**

We have made this change as suggested.

**Introduction:**

**R: “On the other hand, older adults maintain force production during lenthening contractions better (i.e. eccentric contraction) relative to other contraction modes and compared with young [1]” This sentence needs to be edited for clarity. Does this imply that older adults have higher eccentric contraction force than young adults?**

Removed comparision to “young”, and left the comparison to other contraction types. Changed from “On the other hand, older adults maintain force production during lenthening contractions better (i.e. eccentric contraction) relative to other contraction modes and compared with young [1].” To “On the other hand, older adults maintain force production during lenthening contractions (i.e. eccentric contraction) relative to other contraction modes [1].”

**R: “The age-related maintenance of eccentric strength is evident in humans as well as in reduced muscle preparations” The meaning is not clear regarding reduced muscle preparations. The wording suggests that reduced prepartations are perhaps an animal model, but I believe the author is trying to indicate that some subset of whole organisms, isolated muscles, skinned and intact muscle fibers, and myofibrils exhibit these changes. Please clarify.**

“Muscle preperations” has been replaced with “Whole muscle and skined single fibre preperations”

**R: “In addidion to many of the mechanisms proposed for the preservation of eccentric strength, alterations to the structural properties of the muscle fascicle leading to elevated passive force have not been investigated [4, 5].” This sentence implies that few of the mechanisms proposed are tested. Is this correct? Perhaps mention a few of them.**

The mechanisms of the maintance of ECC strength in older adults is beyond the scope of this manuscript. We have provided references for the statement in the text.

**R: “Thus for a given amount of stretch, the fascicles and sarcomeres of muscles from old and young may experience divergent length changes for a given displacement or joint angular rotation.” Append “resulting in increased stiffness in the aged muscles.”**

We have made this change as suggested.

**R: In the purpose, add serial prior to sarcomere number, change skeletal muscles to medial gastrocnemius and delete the medial gastrocnemius section from the end.**

We have made these change as suggested.

**R: The adjectives “young” and “old” are often left hanging. You should either define your groups as being named Young and Old, or add a noun for them to modify.**

We have made this change as suggested by adding the noun “rats” where appropriate.

**Methods**

**R: “increments in muscle length from -4 mm to +4 mm.” The meaning of this range is unclear. Did you begin at a slack length or just taut length and increase muscle length by 1 mm increments for 9 mm?**

The force length plateau was determined in 1mm length steps from a length of near zero force. This was added to methods.

**R: Who is the supplier of the formalin fixative?**

Added supplier VWR 10% Neutral Buffered Formalin.

**R: What is the model #and who manufactured the force transducer and muscle puller?**

The aparattus was custom made. Please consider this is a summer student project on a small portion of a larger overarching project. The mechanical data is not included.

**R: How long was nitric acid digestion?**

This information was added (4 hr digestion)

**R: Just to check, did you create the left panel of figure 1? If so, fantastic job! If not, the original source should be cited, and you might need to get permission to reprint (ask the Editors).**

The image has been cited and editors have been contacted to ask permission to print. “Image adapted from “Science and Engineering Education”, University of Wisconsin, 2008, http://education.mrsec.wisc.edu/supplies/OTK/index.html.”

**R: What software was used in conjunction with the Matrox Inspector Camera?**

The software and camera are: Matrox imaging software and camera

**R: What equation did you use to calculate sarcomere length?**

Added

Sarcomere Length Equation (Ls)



Ls: distance between dark and light bands (aka average sarcomere length) (μm)

λ: wavelength (converted to μm)

x: distance between point and first order diffraction (mm)

h: distance from fascicle to diffraction recording station (mm)

**Results:**

**R: Are all values expressed as Mean±SEM?**

Mean SD was used in text and percentage difference in the Means and %SEM in Figures

**R: Did you record joint angles at optimal length? This would be a very interesting variable to include if you have it.**

As this summer project is part of a larger overarching project, mechanical data is not included.

**Discussion:**

**R: This will be kept brief as it is liable to change based on the new analysis.**

The results are the same as the initial analysis

**There are several places in which references should be provided.**

References added in discussion, please see manuscript changes.

**R: Regarding the limitation of Lo measurements during contraction and fixation is done passively: The author’s point is not clear. Is the following what you are trying to argue? As an alternative to the potential changes in thick & thin filament length, the shorter length in old muscle could be due to stiffer series elasticity. When the muscle with the stiffer series elasticity contracts, relative to the muscle with a less stiff series elasticity,there is less slack to taken up before the muscle reaches full force. These differences could contribute to the reduced sarcomere length seen in the aged muscles when they are fixed passively... The increased tendon compliance with age suggests this is not the case.**

The point has more to do with what happens after the active contraction for young and old muscle. A sentence was added for clarity. “The shorter sarcomeres in old muscle could therefore be due to factors such as the series elastic property differences in young and old muscles, which play a role in passive length. Therefore, when the muscle is not longer actively contracted and enters a passive state, the stiffer series elasticity in old muscle would keep it at a shorter length than the young muscle [12].”

**R:** **“Since there is not a single sarcomere length of maximal force production... coverin approximately 7% of optimal SL” This appears to contradict itself. Does the author mean 7% of typical muscle excursion rather than optimal SL?**

The 7% refers to the percent size of the plateau at the top of the force length curve. Peak force can be generated in a range of 7% of total sarcomere length. Edited for clarity: “Since, on the force-length curve plateau, there is not a single sarcomere length for maximal force production, but a range of lengths spanning ~7% of the force length curve. Therefore, the ~ 4% range in this experiment could be all be found on the plateau region of the sarcomere FL relationship [9].”

**R: Assuming this effect occurs in all muscles, how would increased stiffness in opposing muscle groups factor into the mix?**

Given the muscle preparation there was no antagonist stiffness. The muscle was surgerically isolated and tendon and was inseries with the muscle puller.

**Conclusions:**

**R: Change hypothesis to hypotheses.**

Changed

**R: Add a brief comment regarding the functional consqeuences.**

Added: “The consequences of these age related muscle changes is potentially a less functional range of motion for older adults and less force throughout that range of motion to perform tasks of daily living.”