Preserved skeletal muscle oxidative capacity in older adults despite decreased cardiorespiratory fitness with aging

Xiaoyan Zhang, Hawley E. Kunz, Kevin Gries, Corey R. Hart, Eric C. Polley, and Ian Lanza
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The following individual(s) involved in review of this submission have agreed to reveal their identity: Andrew Philp (Referee #1); Gilles Gouspillou (Referee #2)

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Reviewing Editor: Karyn Hamilton

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision 19-Apr-2021

Dear Dr Lanza,

Re: JP-RP-2021-281691 "Preserved skeletal muscle oxidative capacity in older adults despite decreased cardiorespiratory fitness with aging" by Xiaoyan Zhang, Hawley E. Kunz, Kevin Gries, Corey R. Hart, Eric C. Polley, and Ian Lanza

Thank you for submitting your manuscript to The Journal of Physiology. It has been assessed by a Reviewing Editor and by 2 expert Referees and I am pleased to tell you that it is considered to be acceptable for publication following satisfactory revision.

Please advise your co-authors of this decision as soon as possible.

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EDITOR COMMENTS

Reviewing Editor:

Methods Details:
Some details about assessment of mitochondrial quality are missing.

Thank you for submitting your original research manuscript for consideration by The Journal of Physiology. We recruited two expert Referees to participate in the peer review process. As you will see, both are complimentary about the manuscript, indicating that it is well written and addresses an important intersection of aging and muscle physiology. However, you will also see that there are also some serious concerns brought to light. For example, the novelty is in question because a very similar hypothesis was tested in a study published in 2018; it is not clear what your manuscript adds to the existing body of literature. There are also some necessary analyses of mitochondrial quality that are absent from the results, in addition to some muscle analyses that would help support your findings, and add information to the existing published literature on this topic. The Referees took great care in providing detailed feedback and we do hope that this provides you with guidance as you move forward with the project.

Senior Editor:

Thank you for submitting your work to the Journal of Physiology. Your report has been carefully considered by two expert reviewers and a review editor (RE) that are experts in the field. While all three referees find your work interesting, both reviewers and the RE have raised several important questions that must be satisfactorily addressed in the revised report.

REFEREE COMMENTS

Referee #1:

Zhang et al provide a detailed investigation into the role of ageing and habitual physical fitness on oxidative capacity in skeletal muscle. Their main finding suggests that skeletal muscle oxidative capacity is maintained during ageing in physically active individuals, and that this process is consistent in male and female cohorts. Overall I enjoyed reading he manuscript and think that the results will be well received by the journal's readers. The experiments performed were appropriate and the authors interpretation logical. I have some minor comments/questions that I hope will help with the clarity of the story presented:

1. A strength of the study is the relatively large sample size and mix of male and female participants. As such, I would suggest that the authors include additional graphs presenting separate male and female datasets, in addition to the merged figure already provided for figures 1-3.

2. The authors refer to the cohort as 'healthy older adults' but don't really state how they define this category? Further, there doesn't appear to be any direct assessment of sarcopenia, so it is unclear how the participants would sit in this category. In this context, it seems to be an oversight from the authors that they didn't attempt to assess sarcopenia in these individuals (i.e. grip strength, SARC-F, SPPB etc)? Based on the data they do have, are the authors able to comment on this and whether the older participants had any hallmarks of sarcopenia?

3. The authors show quite clearly that mitochondrial respiration is maintained across the cohort, but it is unclear if mitochondrial content changes with age (again another topic debated in the

literature). It would therefore seem logical that the authors use the muscle they have collected and stored at -80 to address this issue. There are numerous static markers of mitochondrial content that can be used for this measurement (see Larsen et al PMID: 22586215) which could easily be used to address whether mitochondrial content tracks the functional data the authors report.

Referee #2:

In their manuscript, Dr. Zhang et al. assessed physical activity, VO2peak, mitochondrial respiration and H2O2 emission and in young and old men and women. They report that while older participants display lower VO2peak values vs their younger counterparts, they do not show sign of reduced mitochondrial respiration or increased H2O2 emission. They also report that while moderate-to- vigorous physical activity correlates with VO2peak, it cannot entirely explain the age-related reduction in cardiorespiratory fitness.

Overall, this manuscript is well written and provides interesting data for the muscle aging and muscle physiology research fields. However, I have several comments and concerns that need to be addressed.

Major comments:

1- Although very interesting, the finding that old individuals can display preserved maximal mitochondrial respiration despite reduction in VO2peak is not novel. Indeed, Distefano et al. (PMID: 29368427) already reported that active individuals display a decline in VO2 peak without change in mitochondrial respiration. The study from Distefano et al. should at the very least be acknowledged.

2- Although it is unlikely to affect the outcome of this study, the authors should also normalize their VO2 data to lean mass. Indeed, fat mass can be considered as "dead weight" when performing a VO2max test. Such normalization might even strengthen the main message of this manuscript.

3- The authors do not provide any information allowing readers to assess the quality of their mitochondrial preparations. They should at the very least provide the ACR values (state 3 / state 2 respiration rates). If mitochondrial membrane integrity tests were performed (with cytochrome C and/or NADH), it should be clearly stated.

4- Based on the data provided in Figure 1C and E, it seems that the ATP/O ratio for all participants would be close to 1 (if we divide data in Fig 1C by data in Fig 1E). This is very far from theoretical values and from what has been previously measured in permeabilized myofibers (Lark et al., Am J Physiol Cell Physiol 311: C239-C245, 2016). How can the authors explain such

low ATP/O ratios?

5- Figure 1G and H: the absolute H2O2 emission rates should be provided. This will allow comparisons with previously published data.

6- Figure 3: For all correlations provided in Figure 3, the authors should consider providing, on top of the information they already provide, the R2 values and p-values for young and old participants separately (i.e. do these correlations hold when only young or old participants are considered).

7- One major limitation of this study is that it "only" focused on one physiological factor contributing to VO2 (i.e. mitochondrial respiration). This study would have been strengthened if several other physiological factors contributing to VO2 were assessed such as heart rate, stroke volume, cardiac output and muscle capillarization for instance. While I understand that the authors likely won't be able to provide data on heart physiology, I am wondering whether they could assess muscle capillarization using the biopsy samples they collected. If they prepared histology blocs for all of their participants, this could be done by staining for CD31 or even using a lead-ATPase stain.

8- The reference number of the approval obtained from the Mayo Foundation Institutional Review Board should be provided.

Minor comments:

1- On page 4, the authors wrote that "[...] other painstakingly controlled studies have not revealed any age-related impairments in skeletal muscle mitochondrial function using similar methodologies". Caution should be taken with the use of "mitochondrial function" as the literature cited only investigated some aspects of mitochondrial function (namely respiration and H2O2 emission). Just as an example, it was shown that while mitochondria from active older men do not show sign of decline in maximal oxygen consumption and H2O2 emission, they do display significant impairment in their mPTP function and calcium retention capacity (Gouspillou et al., FASEB J, 2014; PMID: 24371120). This comment applies to many sections in the manuscript where the authors use the term "mitochondrial function" too loosely.

2- It is stated in the section statistical analyses that "Subject characteristics, body composition, and metabolic parameters were compared between the young and old groups using unpaired student t-tests." Please specify whether these t-test were uni- or bi-lateral t-tests.

END OF COMMENTS

Referee #1:

Zhang et al provide a detailed investigation into the role of ageing and habitual physical fitness on oxidative capacity in skeletal muscle. Their main finding suggests that skeletal muscle oxidative capacity is maintained during ageing in physically active individuals, and that this process is consistent in male and female cohorts. Overall I enjoyed reading he manuscript and think that the results will be well received by the journal's readers. The experiments performed were appropriate and the authors interpretation logical. I have some minor comments/questions that I hope will help with the clarity of the story presented:

1. A strength of the study is the relatively large sample size and mix of male and female participants. As such, I would suggest that the authors include additional graphs presenting separate male and female datasets, in addition to the merged figure already provided for figures 1-3.
   RESPONSE: We agree that presenting male and female datasets in addition to the merged data is an important opportunity to highlight potential sex differences in this large cohort. In response to this suggestion, we modified Figures 1, 2, and 3 to include regression lines with confidence intervals for males and females separately in addition to the overall regression for the combined cohort. We also incorporated color into these figures to help draw attention to sex differences (males = blue, females = red). The results and discussion section now includes additional text related to these sex-specific analyses.

2. The authors refer to the cohort as 'healthy older adults' but don't really state how they define this category? Further, there doesn't appear to be any direct assessment of sarcopenia, so it is unclear how the participants would sit in this category. In this context, it seems to be an oversight from the authors that they didn't attempt to assess sarcopenia in these individuals (i.e. grip strength, SARC-F, SPPB etc)? Based on the data they do have, are the authors able to comment on this and whether the older participants had any hallmarks of sarcopenia?
   RESPONSE: We thank the reviewer for raising this key point, and we acknowledge that our initial version of the manuscript did not adequately discuss what we meant by "healthy older adults." The revised manuscript better describes the characteristics of the participants and our efforts to include independent-living older adults without mobility impairments and free from major age-related chronic diseases (diagnosed diabetes, cardiovascular disease, cancer, etc). The reviewer's suggestion to assess sarcopenia by grip strength, SARC-F, or SPPB is excellent. Although we did not incorporate any of these specific measurements into the study design, we have body composition measured by DEXA. Using appendicular lean mass measurements, we calculated the appendicular skeletal muscle index (ASMI), which has been used as an index for sarcopenia (PMID 9554417). We find that ASMI was not statistically different between young and older adults (young = 7.5 kg/m^2, older = 7.0 kg/m^2, P=0.08). We used the sex-specific cutoffs identified by Baumgartner (PMID 9554417) (men: 7.26 kg/m^2, women: 5.45 kg/m^2) and found that 10 of the 52 older participants (5 men, 5 women) fell below the cutoffs associated with sarcopenia. The ASMI data is now included in table 1. We also

include unilateral leg extension 1-repetition maximum measurements in table 1, which demonstrate the anticipated reductions in maximal strength of the leg extensor muscles with age (young: 65.5 kg, older: 44 kg, P<0.001). We believe that these additional data to some extent address the reviewer's question about whether older participants exhibited any hallmarks of sarcopenia. Our revised manuscript provides additional discussion / context related to the ASMI and muscle strength data, pointing out that although we observe lower muscle strength in older adults, there were more modest differences in ASMI between young and older adults, with 10 individuals falling below published cutoffs associated with sarcopenia.

3. The authors show quite clearly that mitochondrial respiration is maintained across the cohort, but it is unclear if mitochondrial content changes with age (again another topic debated in the literature). It would therefore seem logical that the authors use the muscle they have collected and stored at -80 to address this issue. There are numerous static markers of mitochondrial content that can be used for this measurement (see Larsen et al PMID: 22586215) which could easily be used to address whether mitochondrial content tracks the functional data the authors report.
RESPONSE: We wholeheartedly agree with the reviewer's comment. Measurements of mitochondrial content in skeletal muscle samples would provide additional insight beyond the functional assays that we performed in permeabilized muscle fibers. We would typically approach this question from several angles, with complementary measurements of citrate synthase activity, expression of respiratory chain subunits, mtDNA abundance, and electron microscopy of muscle biopsy tissue. Although this reviewer's request is quite logical and reasonable, we find ourselves in a position where we are not currently able to provide the additional data. The main reason is that the data contained within this manuscript are part of a larger project that includes a 6 month intervention that is ongoing. Here we performed a cross-sectional analysis of "baseline" data from a subset of young and older adults, focusing on outcomes measured in real-time that are not susceptible to drift or batch effects. Many analyses, including molecular measurements of muscle mitochondrial mass, cannot be completed until the conclusion of the larger trial, which is nearly 2 years in the future. We regret that we cannot address this reviewer's comment and hope that the reviewer agrees that our manuscript will still provide meaningful insights and will move the field forward. In our revised discussion section, we dedicate some new text to discuss muscle mitochondrial content changes with age and the limitation of not including data to directly address that possibility.

Referee #2:

In their manuscript, Dr. Zhang et al. assessed physical activity, VO2peak, mitochondrial respiration and H2O2 emission and in young and old men and women. They report that while older participants display lower VO2peak values vs their younger counterparts, they do not show sign of reduced mitochondrial respiration or increased H2O2 emission. They also report that while moderate-to- vigorous physical activity correlates with VO2peak, it cannot entirely explain the age-related reduction in cardiorespiratory fitness. Overall, this manuscript is well written and provides interesting data for the muscle aging and muscle physiology research fields. However, I have several comments and concerns that need to be addressed.

Major comments:

1- Although very interesting, the finding that old individuals can display preserved maximal mitochondrial respiration despite reduction in VO2peak is not novel. Indeed, Distefano et al. (PMID: 29368427) already reported that active individuals display a decline in VO2 peak without change in mitochondrial respiration. The study from Distefano et al. should at the very least be acknowledged.
RESPONSE: We are very familiar with the Distefano paper that the reviewer mentions. It is an excellent paper, and the main reason why we did not cite it in the original manuscript is because they specifically focused on highly-active young and older adults (runners, cyclists, swimmers) compared to sedentary older adults. Here we intentionally excluded highly-active young and older adults to allow us to specifically focus on how variability at the lower range of habitual physical activity can explain age-related changes in VO2 peak and mitochondrial physiology. Although Distefano et al. nicely show that high levels of physical activity in older adults maintain muscle mitochondrial function and muscle quality/function, we thought it would be difficult to draw comparisons between that study and ours. In hindsight, the reviewer is correct; the prior finding that VO2 peak is lower in highly active older adults vs. highly active young adults despite similar muscle oxidative capacity is squarely in line with what we observe here at the other end of the physical activity spectrum. We fully agree that the Distefano paper should be acknowledged, and we have expanded the discussion section to accommodate appropriate discussion of our results in the context of the important earlier work (Discussion, page 13).

2- Although it is unlikely to affect the outcome of this study, the authors should also normalize their VO2 data to lean mass. Indeed, fat mass can be considered as "dead weight" when performing a VO2max test. Such normalization might even strengthen the main message of this manuscript.
RESPONSE: We appreciate this helpful suggestion and agree that normalizing to lean mass is a logical complement to simply using total body mass as a denominator. In response to this suggestion, we added VO2 peak normalized to total lean mass from DEXA to Table 1. This did not affect the outcome of the study since older adults, on average, showed about 35% lower VO2 peak than young when normalized to total body weight and 32% lower VO2 peak when normalized to lean

mass. We also added a new set of figures to Figure 1 to include VO2 peak normalized to lean mass for regression analysis (Figure 1C) and also the age-by-sex group analysis (Figure 1D). A new Figure 3F provides regression analysis between VO2 peak normalized to lean mass and muscle oxidative capacity, which is also largely unaffected by normalizing to lean mass vs. total body weight.

3- The authors do not provide any information allowing readers to assess the quality of their mitochondrial preparations. They should at the very least provide the ACR values (state 3 / state 2 respiration rates). If mitochondrial membrane integrity tests were performed (with cytochrome C and/or NADH), it should be clearly stated.
RESPONSE: We added acceptor control ratios (ACR, state 3/state 2) and respiratory control ratios (RCR, state 3/state 4) to the results section of the manuscript. The ACR and RCR values were approximately 5, and did not differ between young and older adults. We also added a brief statement to the manuscript indicating that we did not perform any measurements of mitochondrial membrane integrity as our usual cytochrome c test for membrane integrity interferes with the multiplexed ROS production measurements (Methods, page 9 and Results page 10-11).

4- Based on the data provided in Figure 1C and E, it seems that the ATP/O ratio for all participants would be close to 1 (if we divide data in Fig 1C by data in Fig 1E). This is very far from theoretical values and from what has been previously measured in permeabilized myofibers (Lark et al., Am J Physiol Cell Physiol 311: C239-C245, 2016). How can the authors explain such low ATP/O ratios?
RESPONSE: We thank the reviewer for their insight into the dataset and how the ATP production and O_2 consumption data could be merged to provide a window into mitochondrial coupling efficiency (ATP/O ratio); an important element of mitochondrial physiology that we do not address in the manuscript. The papers out of the Neufer Lab describe *simultaneous* measurements of J_{ATP} and J_{O2} using an Oroboros oxygraphy retrofitted with a fiber optic cable connected to a spectrofluorometer. Although we also measured J_{ATP} and J_{O2} using nearly identical methodologies, we did not perform them simultaneously in the same set of muscle fibers. We used separate fiber bundles for the ATP production assay and respiration measurements. Without the ability to measure both rates simultaneously in the same sample preparation, we do not feel confident making comparisons of ATP-to-O ratios from this study with those reported previously.

5- Figure 1G and H: the absolute H2O2 emission rates should be provided. This will allow comparisons with previously published data.
RESPONSE: Figures 1I and 1J (formerly 1G and H) were modified to include absolute H_2O_2 emission rates as suggested by the reviewer. The conclusions from our analyses are unchanged.

6- Figure 3: For all correlations provided in Figure 3, the authors should consider providing, on top of the information they already provide, the R2 values and p-values for young and

old participants separately (i.e. do these correlations hold when only young or old participants are considered).
RESPONSE: We added additional regression lines, R^2, and p-values for men and women separately in addition to the overall regression of the combined cohort in response to Reviewer 1's first comment. This reviewer's suggestion to include additional regression analyses for young and older groups separately is valuable, we are concerned that this will result in a cluttered set of figures that may distract from the main messages of the study. For that reason, we placed a higher priority on highlighting potential sex differences and age-by sex interactions in the data.

7- One major limitation of this study is that it "only" focused on one physiological factor contributing to VO2 (i.e. mitochondrial respiration). This study would have been strengthened if several other physiological factors contributing to VO2 were assessed such as heart rate, stroke volume, cardiac output and muscle capillarization for instance. While I understand that the authors likely won't be able to provide data on heart physiology, I am wondering whether they could assess muscle capillarization using the biopsy samples they collected. If they prepared histology blocs for all of their participants, this could be done by staining for CD31 or even using a lead-ATPase stain.
RESPONSE: We very much appreciate the reviewer's expert insight and constructive comment. While we cannot provide any additional data related to cardiac physiology or muscle histology, we value this comment and will definitely keep these additional outcomes in mind for the future.

8- The reference number of the approval obtained from the Mayo Foundation Institutional Review Board should be provided.
RESPONSE: We revised the text to include the IRB reference number as well as the ClinicalTrials.gov registration number for this study.

Minor comments:

1- On page 4, the authors wrote that "[...] other painstakingly controlled studies have not revealed any age-related impairments in skeletal muscle mitochondrial function using similar methodologies". Caution should be taken with the use of "mitochondrial function" as the literature cited only investigated some aspects of mitochondrial function (namely respiration and H2O2 emission). Just as an example, it was shown that while mitochondria from active older men do not show sign of decline in maximal oxygen consumption and H2O2 emission, they do display significant impairment in their mPTP function and calcium retention capacity (Gouspillou et al., FASEB J, 2014; PMID: 24371120). This comment applies to many sections in the manuscript where the authors use the term "mitochondrial function" too loosely.
RESPONSE: The reviewer makes an important point that the terms "mitochondrial function / dysfunction" are used loosely and out of convenience to avoid otherwise cumbersome wording. Nevertheless, the reviewer's comment is important, and we made greater efforts throughout the revised manuscript to avoid using the blanket terms.

2- It is stated in the section statistical analyses that "Subject characteristics, body composition, and metabolic parameters were compared between the young and old groups using unpaired student t-tests." Please specify whether these t-test were uni- or bi-lateral t-tests.
RESPONSE: We added additional clarification that we used 2-tailed t-tests.

1st Revision - Editorial Decision 12-May-2021

Dear Dr Lanza,

Re: JP-RP-2021-281691R1 "Preserved skeletal muscle oxidative capacity in older adults despite decreased cardiorespiratory fitness with aging" by Xiaoyan Zhang, Hawley E. Kunz, Kevin Gries, Corey R. Hart, Eric C. Polley, and Ian Lanza

Thank you for submitting your manuscript to The Journal of Physiology. It has been assessed by a Reviewing Editor and by 2 expert Referees and I am pleased to tell you that it is considered to be acceptable for publication following satisfactory revision.

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I look forward to receiving your revised submission.

If you have any queries please reply to this email and staff will be happy to assist.

Yours sincerely,

Scott K. Powers
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EDITOR COMMENTS

Reviewing Editor:

Thank you for the careful revisions you made in response to the Referees' concerns. The manuscript is much improved as a result and one of the Referees is fully satisfied with your revisions. However, there is still one Referee question requiring a response, as well as a few suggested minor edits to improve clarity for readers.

Senior Editor:

Thank you for submitting your work to the Journal of Physiology for publication. Your manuscript has undergone a second review; both referees comments that the paper is improved but referee #2 has raised additional questions that require attention before a final decision on publication can be rendered. Therefore,in the next revision of your report, please pay close attention to the issues raised by reviewer #2. We look forward to receiving your revised manuscript.

REFEREE COMMENTS

Referee #1:

The authors have addressed all of my concerns/queries from the first round of revision. I have nothing further to add other than to congratulate them on a very interesting study!

Referee #2:

The authors have done an excellent job answering most of my comments and concerns. However, my comment #4 on the seemingly low ATP/O ratios was not addressed. I first want to state that I fully understand the hesitancy of the authors to present such ATP/O ratios and I want to make clear that I am not recommending them to do so. Their reasons for not including such data (i.e. non-simultaneous measurement of O2 consumption and ATP production rates)

are perfectly valid. However, these ratios should still be close to theoretical and previously published values. Indeed, the authors report oxygen consumption and ATP production in absolute values (both in pmol/s/mg). As such, dividing the ATP production rate by the oxygen consumption rate should retrieve ATP/O values close to 2 since a combo of complex I and II substrates was used. The fact that the ATP/O values that can be inferred from figure 1 are very low (<1; ATP/O = JATP / [JO2x2]) is concerning and leads me to believe that either the ATP production rates or the oxygen consumption rates are inaccurate. The ACR and RCR data provided by the authors clearly demonstrate that the quality of their permeabilized myofiber preparations was good. Since the oxygen consumption data appear in line with the available literature, I therefore suspect that an error might have occurred in the calculation of the ATP production rates. As such, and while data between groups are still likely comparable, I suspect that the ATP production rates are erroneous. Again, and to summarize my comment/concern, if data in Figure 1 are actually expressed in pmol/s/mg, then the ATP/O ratios must be close to 2 regardless of whether ATP production and oxygen consumption measurements were performed on disctinct permeabilized myofiber bundles.

Additional minor comments and suggestions:

- While I understand the explanation provided for not including the R2 values and p-values for young and old participants separately in all correlations, I still believe that this information could be valuable to readers of the Journal of Physiology. A way to achieve it without cluttering all figures could be to present these R2 values and p-values in a separate table. However, this is only a suggestion, and I let the authors decide whether to follow it or not.

- The following sentence should be removed on page 17: "The authors of this manuscript certify that they comply with the ethical guidelines for authorship and publishing in the Journal of Cachexia, Sarcopenia and Muscle [50]"

- The order of the figure appears shuffled in this revised version (i.e. previous figure 1 is now in 3rd position while previous figure 3 is now in 1rst position).

- There is a minor formatting issue with the legend of table 1 (the legend starts on the right side of table 1).

END OF COMMENTS

We thank the reviewers and editor for their thoughtful comments on the revision of our manuscript for consideration by the Journal of Physiology. We are encouraged by their positive remarks and have made additional revisions to the manuscript in response to Reviewer 2 and as indicated below.

Referee #1:

The authors have addressed all of my concerns/queries from the first round of revision. I have nothing further to add other than to congratulate them on a very interesting study!
RESPONSE: Thank you for the positive comment and for your time and energy in reviewing the manuscript.

Referee #2:

The authors have done an excellent job answering most of my comments and concerns. However, my comment #4 on the seemingly low ATP/O ratios was not addressed. I first want to state that I fully understand the hesitancy of the authors to present such ATP/O ratios and I want to make clear that I am not recommending them to do so. Their reasons for not including such data (i.e. non-simultaneous measurement of O2 consumption and ATP production rates) are perfectly valid. However, these ratios should still be close to theoretical and previously published values. Indeed, the authors report oxygen consumption and ATP production in absolute values (both in pmol/s/mg). As such, dividing the ATP production rate by the oxygen consumption rate should retrieve ATP/O values close to 2 since a combo of complex I and II substrates was used. The fact that the ATP/O values that can be inferred from figure 1 are very low (<1; ATP/O = JATP / [JO2x2]) is concerning and leads me to believe that either the ATP production rates or the oxygen consumption rates are inaccurate. The ACR and RCR data provided by the authors clearly demonstrate that the quality of their permeabilized myofiber preparations was good. Since the oxygen consumption data appear in line with the available literature, I therefore suspect that an error might have occurred in the calculation of the ATP production rates. As such, and while data between groups are still likely comparable, I suspect that the ATP production rates are erroneous. Again, and to summarize my comment/concern, if data in Figure 1 are actually expressed in pmol/s/mg, then the ATP/O ratios must be close to 2 regardless of whether ATP production and oxygen consumption measurements were performed on disctinct permeabilized myofiber bundles.
RESPONSE: We appreciate this reviewer's continued insight into the data, and their patient approach to thoughtfully re-articulating the issue that we did not adequately address in our earlier revision. We better understand the underlying issue; that quantitative measurements of J_{O2} and J_{ATP}, even if performed in distinct fiber bundles, should approach the canonical ATP/O ratio. The fact that J_{O2} appears to track well with literature values suggests that J_{ATP} may be incorrect. Seeing the reviewer's point through a new lens, we puzzled over this and were unable to identify any technical issues or errors in data analysis. However, we believe that there is one fundamental difference between respiration measurements and ATP production measurements that may explain low J_{ATP}/J_{O2}. For respiration measurements, permeabilized muscle fibers were evaluated in hyper-oxygenated media whereby the oxygen concentration of the media was increased to ~400uM and maintained above 250uM throughout the experiment. This is done to avoid

limitations in respiration due to oxygen diffusion in permeabilized muscle fibers. The closed-chamber system of the Oroboros Oxygraph allows this control. In contrast, ATP production measurements were performed in media at ambient air O_2 saturation. The spectrofluorometer is an open cuvette system that does not allow us to titrate oxygen tension in media and maintain it in the same way as the Oroboros system. Inasmuch, the rates of ATP production at state 3 respiration are likely to be limited by oxygen diffusion in this open system. We believe that this is the most likely explanation for lower than expected J_{ATP} for the prevailing level of J_{O2}. In the revised manuscript we carefully point out that the measurements of ATP production capacity are likely limited by O_2 diffusion and should be interpreted cautiously as they likely underestimate the true J_{ATP}.

Additional minor comments and suggestions:

- While I understand the explanation provided for not including the R2 values and p-values for young and old participants separately in all correlations, I still believe that this information could be valuable to readers of the Journal of Physiology. A way to achieve it without cluttering all figures could be to present these R2 values and p-values in a separate table. However, this is only a suggestion, and I let the authors decide whether to follow it or not.
RESPONSE: We thank the reviewer for the suggestion and agree that the separate R2 and P-values for young and older adults separately would be interesting to some readers. We prefer not to include these additional correlations because we worry that it will make than manuscript cumbersome, particularly with the additional regression analyses of males and females separately and the comparatively smaller sample size of young vs. older participants.

- The following sentence should be removed on page 17: "The authors of this manuscript certify that they comply with the ethical guidelines for authorship and publishing in the Journal of Cachexia, Sarcopenia and Muscle [50]"
RESPONSE: Thank you for noticing this! We removed the irrelevant sentence from the manuscript.

- The order of the figure appears shuffled in this revised version (i.e. previous figure 1 is now in 3rd position while previous figure 3 is now in 1rst position).
RESPONSE: We believe that the order of the figures was inadvertently switched when uploaded to the manuscript submission site and we have corrected this.

- There is a minor formatting issue with the legend of table 1 (the legend starts on the right side of table 1).
RESPONSE: We corrected this formatting issue.

2nd Revision - Editorial Decision 20-May-2021

Dear Dr Lanza,

Re: JP-RP-2021-281691R2 "Preserved skeletal muscle oxidative capacity in older adults despite decreased cardiorespiratory fitness with aging" by Xiaoyan Zhang, Hawley E. Kunz, Kevin Gries, Corey R. Hart, Eric C. Polley, and Ian Lanza

I am pleased to tell you that your paper has been accepted for publication in The Journal of Physiology.

NEW POLICY: In order to improve the transparency of its peer review process The Journal of Physiology publishes online as supporting information the peer review history of all articles accepted for publication. Readers will have access to decision letters, including all Editors' comments and referee reports, for each version of the manuscript and any author responses to peer review comments. Referees can decide whether or not they wish to be named on the peer review history document.

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Authors should note that it is too late at this point to offer corrections prior to proofing. The accepted version will be published online, ahead of the copy edited and typeset version being made available. Major corrections at proof stage, such as changes to figures, will be referred to the Reviewing Editor for approval before they can be incorporated. Only minor changes, such as to style and consistency, should be made a proof stage. Changes that need to be made after proof stage will usually require a formal correction notice.

All queries at proof stage should be sent to TJP@wiley.com

Yours sincerely,

Scott K. Powers
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EDITOR COMMENTS

Reviewing Editor:

Thank you for your careful consideration of the Referee's concerns. Your responses were thoughtful and the manuscript is improved because of this peer review process. Nicely done.

Senior Editor:

Comments to the Author:

Thank you for submitting your work to the journal of Physiology. Your work has been carefully analyzed by exert reviewer and review editor. All parties are not happy with your revised report and I share their enthusiasm for the report. Congtrations on the completion of a nice study.

REFEREE COMMENTS

Referee #2:

The authors have satisfactorily addressed my comments and concerns.

END OF COMMENTS