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Resistance Training Diminishes Mitochondrial Adaptations to Subsequent Endurance Training in Healthy Untrained Men

Paulo H.C. Mesquita, Joshua S Godwin, Bradley Ruple, Casey Sexton, Mason C. McIntosh, Breanna J. Mueller, Shelby Osburn, Christopher Brooks Mobley, Cleiton Augusto Libardi, Kaelin C. Young, L. Bruce Gladden, Michael Roberts, and Andreas N Kavazis
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The following individual(s) involved in review of this submission have agreed to reveal their identity: Tim Snijders (Referee #1); Simone Porcelli (Referee #2)

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Dear Dr. Kavazis,

Re: JP-RP-2023-284822 "Resistance Training Diminishes Mitochondrial Adaptations to Subsequent Endurance Training" by Paulo H.C. Mesquita, Joshua S Godwin, Bradley Ruple, Casey Sexton, Mason C. McIntosh, Breanna J. Mueller, Shelby Osburn, Christopher Brooks Mobley, Cleiton Augusto Libardi, Kaelin C. Young, L. Bruce Gladden, Michael Roberts, and Andreas Kavazis

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If you have any queries, please reply to this email and we will be pleased to advise.

Yours sincerely,

Michael C. Hogan
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- If n > 30, then the entire raw dataset must be made available either as supporting information, or hosted on a not-for-profit repository e.g. FigShare, with access details provided in the manuscript.

- 'n' clearly defined (e.g. x cells from y slices in z animals) in the Methods. Authors should be mindful of pseudoreplication.

- All relevant 'n' values must be clearly stated in the main text, figures and tables, and the Statistical Summary Document (required upon revision).

- The most appropriate summary statistic (e.g. mean or median and standard deviation) must be used. Standard Error of the Mean (SEM) alone is not permitted.

- Exact p values must be stated. Authors must not use 'greater than' or 'less than'. Exact p values must be stated to three significant figures even when 'no statistical significance' is claimed.

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- Please include an Abstract Figure file, as well as the figure legend text within the main article file. The Abstract Figure is a piece of artwork designed to give readers an immediate understanding of the research and should summarise the main conclusions. If possible, the image should be easily 'readable' from left to right or top to bottom. It should show the physiological relevance of the manuscript so readers can assess the importance and content of its findings. Abstract Figures should not merely recapitulate other figures in the manuscript. Please try to keep the diagram as simple as possible and without superfluous information that may distract from the main conclusion(s). Abstract Figures must be provided by authors no later than the revised manuscript stage and should be uploaded as a separate file during online submission labelled as File Type 'Abstract Figure'. Please ensure that you include the figure legend in the main article file. All Abstract Figures should be created using BioRender. Authors should use The Journal's premium BioRender account to export high-resolution images. Details on how to use and access the premium account are included as part of this email.

EDITOR COMMENTS

Reviewing Editor:

The study investigated in humans the potential carry-over effect of a first resistance training intervention on a subsequent endurance training intervention. The study is of interest, considering the substantial molecular and functional interplays between hypertrophic and mitochondrial responses to training. Both reviewers consider the study of interest and with potential impact on the field. The data are novel. Both reviewers raise well-detailed concerns about study design and data interpretation, which should be convincingly addressed by the authors.

REFEREE COMMENTS

Referee #1:

The study by Mesquita et al. investigated the effect of prior resistance training on adaptations to subsequent endurance training. The authors show that prior resistance training did not further improve endurance performance and blunted most mitochondrial adaptations to the endurance training. The study appears to be well-performed, is novel and reports a great amount of data. Below a few suggestions for the authors to consider.

1. The rationale/hypothesis could be formulated more clearly in the introduction. The rationale is mainly based on an animal study showing that prior resistance training enhances subsequent resistance training adaptations after a period of detraining, which is suggested to be a result of an increased myonuclear number per muscle fiber. In the discussion, the authors more clearly state the hypothesis that the increased myonuclear number per fiber would increase both the transcriptional and translational capacity, allowing for enhanced mitochondrial adaptations (line 681-683). The latter should also be reflected in the hypothesis formulated in the introduction.

2. If the premise is that resistance exercise training increases myonuclear content, and thereby improves transcriptional and translational capacity to augment subsequent ET training adaptations (line 681-683), then its strange that the changes in myonuclear domain size observed are not discussed in more depth in the manuscript. The current study shows a decline in type I muscle fiber myonuclear domain size in response to the initial 7 weeks of resistance exercise training. This would mean that RT+ET group has greater type I muscle fiber transcriptional and translational capacity a the start of the ET compare with the ET-only group. The observation that myonuclear domain is specifically decreased in type I muscle fiber is of particular relevance as the subsequent exercise training is aerobic in nature, targeting this muscle fiber type. Yet, it did augment mitochondrial adaptation during ET in the RT+ET group. This disconnect warrants a more detailed discussion as this goes directly against the formulated hypothesis.

3. How confident are the authors that an accurate assessment of myonuclear content and domain size has been performed. 1. Myonuclear domain size observed is much greater (2 fold) then typically reported by other human studies. 2. Whereas changes in type I myonuclear domain size in response RT is observed using cross-sections this is not case with the single fiber approach. How can this be explained.

Minor comments:

1. Abstract: please include p-values to indicate significance for the results reported in line 51-56.

2. Methods section line 127-129: These sentences are better suited in the results section of the manuscript.

3. VO2max and VO2peak are used interchangeably throughout the manuscript. Please use one consistently. In addition, description of VO2peak in table 1 is incorrect.

4. Line 297-368: please include the average number of muscle fibers that were evaluated per cross-section per outcome. In addition, how was the fiber cross-sectional orientation verified for the assessment of muscle fiber size?

5. Figure 1; abbreviations used in the figure are not listed in the legend.

6. In line 648, the authors state that previous studies show no differences in satellite cell content following endurance training, but the reference that is used (ref 37) does show an increase in satellite cell content.

***

Referee #2:

In literature, a huge number of studies investigated the functional and molecular adaptations to either resistance or endurance training interventions. Similarly, many research studies have also investigated the effects of combined endurance and resistance training (namely concurrent training) in both sedentary and well-trained athletes. However, the carry-over effects of a "priming" training intervention on a subsequent one are less known and they are raising a lot of interest, since the discovery of the complexity of the molecular mechanisms shared between the "hypertrophic" and "mitochondrial" pathways.

The authors carried out an interesting investigation aimed to evaluate the effects of 7 weeks of resistance training followed by 7 weeks of endurance training, compared to a 7-week endurance training intervention alone.

They recruited twenty-five healthy young male participants, separated in RT+ET and ET. Resistance training was performed twice weekly by the RT+ET group only, and consisted of leg press, bench press, leg extension, cable pull-down, and leg curls exercises from 70 to 95%1RM. Seven weeks of endurance training were prescribed as high-intensity interval training (HIIT) and participants trained on a motorized treadmill by intervals (1 minute sprints) at 80-100%VO2max. Before and after each training intervention, each participant performed evaluations on two nonconsecutive days. During the first day, anthropometric measurements were performed and muscle structure was evaluated by ultrasound. A muscle biopsy from the right VL was also collected. Immunohistochemistry was performed to determine fiber cross-sectional area (fCSA), myonuclear content, myonuclear domain size, satellite cell number, and mitochondrial content. Western blots were used to quantify markers of mitochondrial biogenesis and dynamics (I-V complexes, PGC-1α, NRF1, TFAM, MFN1, MFN2, DRP1, PINK1, and PARKIN). Citrate synthase activity and markers of ribosome content were also investigated. On the second day, participants underwent to a maximal cardiorespiratory test fro VO2max and OBLA determination.

RT elicited adaptations commonly reported in the literature: body composition imporved; strength and VL thickness increased; a higher content of mixed and type II fCSA, myonucleai, markers of ribosome, and satellite cells were reported. ET improved VO2max and the speed at OBLA in both groups. However, there were no differences between RT+ET vs ET. Additionally, seven weeks of RT performed prior to seven weeks of ET blunted most mitochondrial adaptations to ET showed interfere with mitochondrial adaptations.

According to their results, the authors concluded that prior RT had no additional benefits on performance adaptations to ET and several molecular adaptations to ET were blunted. The authors speculated that performing RT before ET may results in an enhanced catabolic/proteolytic state.

The manuscript has many strengths, for which the authors should be congratulated. Nevertheless, I think the study has some limitations in the study design and data interpretation.

Major comments:

STUDY DESIGN - OVERTRAINING

In their study, Mesquita et al. compared 14 weeks of training (7 weeks of RT + 7 weeks of ET) to 7 weeks of ET alone. As such, training volume in RT+ET is doubled compared to ET, being RT+RT participants not allowed to recovery from their first weeks of training. Thus, in RT+ET we can appreciate that HIIT intervention was initiated without allowing the body to fully adapt to the previous interventions whereas ET were completely at rest before RT. How can the authors attribute difference between RT+ET and ET to RT, excluding the blunted response to ET in RT+ET was determined by the different training volume between the two groups? How can the authors exclude that an intervention ET+ET would have resulted in similar results? I would suggest the authors to discuss this issue, also taking into consideration recent findings from Flockhart et al. (doi: 10.1016/j.cmet.2021.02.017) who investigated mitochondrial function and mitochondrial dynamics after excessive endurance training.

Moreover, it is known that during repeated exercise bouts skeletal muscle cells tend to remove damage proteins and

mitochondria and replace them with more efficient and functional ones. These processes usually need an adequate recovery period. If this is not the case, do the authors have data that can help the readers to exclude RT+ET overtraining?

GLYCOGEN STORES

Although intensity and duration of exercise appear to be the most important determinants of induced changes in mitochondrial content and function, recent studies (DOI:10.1007/s40279-018-0867-7.; DOI:10.1152/japplphysiol.00060) have suggested that initiating some training sessions with reduced muscle glycogen stores can better improve training-induced adaptations in markers of oxidative metabolism when compared with starting all training sessions with normal muscle glycogen stores. It would be interesting to know whether the RT training modified glycogen content in the skeletal muscle cells. This would help to clarify the role of glycogen stores in modulating the mitochondrial adaptations to endurance training.

EXERCISE INTENSITY OF RESISTANCE TRAINING

In the literature there are contrasting findings about the effects of resistance training on mitochondria and the modulating effects may depen on training status and exercise principles employed. In the present study, participants of RT+ET group trained at high-load during RT phase, 2 time/week. However, results remain more equivocal for the effects of high-load resistance training on mitochondrial adaptations (DOI: 10.3389/fphys.2017.00713). The authors should justify the rationale for selecting this training intensity instead of a low-load resistance training and they should discuss this issue in the manuscript.

PREVIOUS LITERATURE

It has been shown (DOI: 10.1152/japplphysiol.00914.2010) that 25 sessions of isometric NMES of the quadriceps muscle over an 8-wk period can induce changes in myofibrillar proteins (a fast-to-slow phenotype shift) and energy production systems (a glycolytic-to-oxidative shift). How do the authors consider these results in relation to those presented in the present study?

Minor comments:

Lines 64-66: The role of skeletal muscle oxidative function in determining endurance performance is debated. It may be worthy to rephrase the sentence, taking into consideration other references (e.g. DOI: 10.1113/JP271229).

Line 76: The authors may evaluate to reconsider this statement taking into consideration some studies (DOI: 10.1152/japplphysiol.01627.2011; doi: 10.1152/japplphysiol.00883.2012) where functional indexes of muscle oxidative metabolism were evaluated after RT.

Lines 85-86: In this study, the participants underwent two weeks of resting between aerobic conditioning and resistance training. Thus, they probably tried to avoid potential negative effects of intensified training. Please consider this issue in the discussion (see also general comments above).

Line 113: Two minutes may not be sufficient to reach steady state in lactate production/utilization, particularly in untrained subjects. Why did the authors decide to utilize this protocol? The author should comment on this and take into consideration the effects of this exercise protocol on VO2max and OBLA results.

Line 135: If I am not wrong, the participants performed the 3RM test after the maximal cardiorespiratory test. How many minutes were the subjects allowed to recovery? Are the authors sure the results were not underestimated due to post-exertional fatigue?

Line 154: please include details about the site where the muscle biopsies were sampled? Was the same in the two/three time points or incision was performed few centimeters away from the previous one?

Lines 176-177: I was not able to find details about the results from the validation test. Please include information.

Line 635: "mitochondrial network". I don't think that changes in TOM20 may be sufficient to draw conclusions on mitochondrial network. Did the authors measure other markers of mitochondrial content (e.g. SIRT1)? Did they collect muscle samples for TEM analyses?

Lines 693-696: The hypothesis suggested by the authors is interesting but it is not supported by the data presented. The authors should try to elucidate whether an enhanced catabolic/proteolytic state can explain the blunted response to ET in RT+ET group. What about the role of ROS/antioxidant systems balance? Do the author think that this mechanism may have contributed to the results?

END OF COMMENTS

EDITOR COMMENTS

Reviewing Editor:

The study investigated in humans the potential carry-over effect of a first resistance training intervention on a subsequent endurance training intervention. The study is of interest, considering the substantial molecular and functional interplays between hypertrophic and mitochondrial responses to training. Both reviewers consider the study of interest and with potential impact on the field. The data are novel. Both reviewers raise well-detailed concerns about study design and data interpretation, which should be convincingly addressed by the authors.

We appreciate the editor's time and effort to revise our manuscript. We have addressed all reviewers' comments to the best of our ability.

REFEREE COMMENTS

Referee #1:

The study by Mesquita et al. investigated the effect of prior resistance training on adaptations to subsequent endurance training. The authors show that prior resistance training did not further

improve endurance performance and blunted most mitochondrial adaptations to the endurance training. The study appears to be well-performed, is novel and reports a great amount of data. Below a few suggestions for the authors to consider.

1. The rationale/hypothesis could be formulated more clearly in the introduction. The rationale is mainly based on an animal study showing that prior resistance training enhances subsequent resistance training adaptations after a period of detraining, which is suggested to be a result of an increased myonuclear number per muscle fiber. In the discussion, the authors more clearly state the hypothesis that the increased myonuclear number per fiber would increase both the transcriptional and translational capacity, allowing for enhanced mitochondrial adaptations (line 681-683). The latter should also be reflected in the hypothesis formulated in the introduction.

Thanks for your comment. We have rearranged the introduction to better formulate our rationale and hypothesis. The last paragraphs now read (**changes are bold and underlined**):

"The enhancement of endurance performance through RT may affect attributes other than running economy. Different studies have shown that RT may also lead to positive mitochondrial adaptations (Groennebaek *et al.*, 2018; Lim *et al.*, 2019; Mesquita *et al.*, 2020; Ruple *et al.*, 2021a). However, endurance performance was not investigated in these studies. Interestingly, a study conducted by Lee et al. (2018) in rats found that RT promoted enhanced mitochondrial adaptations to a subsequent block of RT, which seemed to be related to increased myonuclear number per myofiber achieved in the first block of training. **A greater myonuclei content would augment the transcriptional capacity of the skeletal muscle. In addition, even though not investigated by the authors, RT is also known to increase ribosome content (Figueiredo *et al.*, 2021a; Figueiredo *et al.*, 2021b), which in turn increases translational capacity. Since most mitochondrial proteins are encoded by the nuclear genome (Ryan & Hoogenraad, 2007) and are synthesized by cytosolic ribosomes, the combination of increased myonuclei and ribosome content could explain the enhanced mitochondrial adaptations in the second block of training.** While these data are promising, it is currently unknown whether RT enhances

mitochondrial adaptations to subsequent endurance training (ET) in humans and whether the enhanced mitochondrial adaptations would lead to better endurance performance. In addition, while several studies have investigated the effects of concurrent training, when both RT and ET are combined within the same training session or program, no study to date has employed a design that investigated RT-only followed by ET-only. This is especially relevant considering a recent study reported that prior ET facilitated adaptations to a subsequent period of RT (Thomas *et al.*, 2022).

Therefore, the purpose of this study was to investigate the effects of prior RT on the molecular and performance adaptations to subsequent ET in humans. We hypothesized that RT prior to ET would increase **myonuclei and ribosome content in skeletal muscle, increasing its transcriptional and translational capacity, which would in turn enhance** mitochondrial adaptations to ET, ultimately leading to improved endurance performance."

2. If the premise is that resistance exercise training increases myonuclear content, and thereby improves transcriptional and translational capacity to augment subsequent ET training adaptations (line 681-683), then its strange that the changes in myonuclear domain size observed are not discussed in more depth in the manuscript. The current study shows a decline in type I muscle fiber myonuclear domain size in response to the initial 7 weeks of resistance exercise training. This would mean that RT+ET group has greater type I muscle fiber transcriptional and translational capacity a the start of the ET compare with the ET-only group. The observation that myonuclear domain is specifically decreased in type I muscle fiber is of particular relevance as the subsequent exercise training is aerobic in nature, targeting this muscle fiber type. Yet, it did augment mitochondrial adaptation during ET in the RT+ET group. This disconnect warrants a more detailed discussion as this goes directly against the formulated hypothesis.

Thanks for your comment. This is an excellent observation. We have tried to address these findings in the discussion (lines 767-801). The paragraph now reads: (**changes are bold and underlined**):

“Most studies investigating molecular adaptations in response to ET have focused on mitochondrial variables due to their importance in oxidative metabolism. Several studies have shown increased mitochondrial content and function in response to various forms of ET (Jacobs *et al.*, 2013; MacInnis & Gibala, 2017; Granata *et al.*, 2018). Considering that approximately 98% of the proteins that make up mitochondria are encoded by the nuclear genome (Ryan & Hoogenraad, 2007), we hypothesized that RT-mediated increases in myonuclei and ribosomes would increase both the transcriptional and translational capacity of myofibers, allowing for enhanced mitochondrial adaptations. In fact, Lee and collaborators (Lee *et al.*, 2018) reported that prior RT facilitated mitochondrial adaptations to a subsequent block of RT in rats. Using both rodent and cell models, these authors also demonstrated that higher myonuclear number was related to a greater expression of mitochondrial genes and proteins in response to exercise. However, even though RT led to increased myonuclei and ribosome content in the current study, most mitochondrial adaptations to subsequent ET were blunted. For example, the protein levels of mitochondrial complexes I-IV in the ET-only group showed increases from 32% to 66%, while the RT+ET group only increased from 1% to 11%. Moreover, mixed fiber relative mitochondrial content increased 15% in the ET-only group but decreased 13% in the RT+ET group. **In addition, the lack of changes in type I fiber mitochondrial content was especially surprising. Type I myonuclear domain size significantly decreased in response to RT, which suggests type I fibers had higher transcriptional capacity following RT. However, contrary to our initial hypothesis whereby we thought this phenomenon would facilitate mitochondrial expansion, mitochondrial content in type I fibers remained unaltered after RT or RT+ET. One possible explanation for these findings is that while myonuclei are involved in protein synthesis and cellular processes, the direct impact on mitochondrial content may be influenced by other regulatory mechanisms. For example, mitochondrial content can be regulated by proteins such as peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), NRF2, and TFAM (Gureev *et al.*, 2019). PGC-1α expression can be influenced by various factors such as exercise, metabolic state, and hormonal signaling. Therefore, changes in mitochondrial content may depend on the activation and regulation of these pathways, rather than solely on the number of myonuclei. In addition, a previous study investigating fiber type-specific adaptations to ET found that sprint interval training selectively stimulated increases in mitochondrial content**

in type II fibers, while a MICT increased mitochondrial content similarly in type I and type II fibers (Skelly *et al.*, 2021). Therefore, it is possible that even though type I fibers had greater transcriptional capacity following RT, our HIIT protocol did not stimulate mitochondrial adaptations in type I fibers. Future studies should further investigate the differences between ET protocols.

3. How confident are the authors that an accurate assessment of myonuclear content and domain size has been performed. 1. Myonuclear domain size observed is much greater (2 fold) then typically reported by other human studies. 2. Whereas changes in type I myonuclear domain size in response RT is observed using cross-sections this is not case with the single fiber approach. How can this be explained.

1. Indeed, our myonuclei number seems to be lower than what is usually reported in the literature, which caused the greater myonuclear domain size. Fiber cross-sectional area and myonuclei number were determined through MyoVision, which has been shown to have an accuracy of ~ 94% (PMID: 28982947). We believe that performing the automated analysis through MyoVison eliminates examiner bias and maintains consistency throughout all time-points. In addition, the participants included in the current study had not performed RT in the last 3 years, which may partly explain the low number of myonuclei.

2.Single fiber analysis of myonuclei number and myonuclear domain size are still not widely adopted in the field of exercise training, which makes it hard to explain the differences between single fiber and cross-sectional analysis. However, such discrepancies have been reported previously (Moro et al., 2021; PMID: 32134710). We believe that as the single fiber analysis becomes more common and more results are available, the field will be able to elucidate the differences and similarities between the single fiber and cross-sectional techniques.

Minor comments:

1. Abstract: please include p-values to indicate significance for the results reported in line 51-56.

Due to length limitation (250 words), we were not able to include all specific p-values. We tried to indicate significance "generally" for main results including $p<0.050$ and hope that it suffices.

2. Methods section line 127-129: These sentences are better suited in the results section of the manuscript.

Thanks for the suggestion. We have move it to the results session (lines 428-430).

3. VO2max and VO2peak are used interchangeably throughout the manuscript. Please use one consistently. In addition, description of VO2peak in table 1 is incorrect.

We use VO2peak to refer to the test performed during familiarization session. On the other hand, we use VO2max to refer to the test performed during the testing sessions (T1, T2, T3), which had an extra validation step to confirm whether participants achieved maximal oxygen consumption. Such validation step was not performed during the familiarization session, hence we used the term VO2peak. We have changed the description of VO2peak in table 1 it to "peak oxygen uptake".

4. Line 297-368: please include the average number of muscle fibers that were evaluated per cross-section per outcome. In addition, how was the fiber cross-sectional orientation verified for the assessment of muscle fiber size?

We have added average number of fibers analyzed for each outcome. Fiber orientation was

verified using a light microscope at the time of sectioning, repositioning the samples when fibers seemed to be incorrectly oriented. Moreover, a detection range of 500–15,000 μm^2 was used to ensure that artifacts and incorrectly orientated (oblong) fibers were removed.

5. Figure 1; abbreviations used in the figure are not listed in the legend.

We have added the abbreviations that were missing.

6. In line 648, the authors state that previous studies show no differences in satellite cell content following endurance training, but the reference that is used (ref 37) does show an increase in satellite cell content.

Thanks for your comment. However, ref 37 (Joanisse et al., 2013; PMID: 23928822) showed no significant change in satellite cell associated with type I or type II fibers. The only significant increase observed was in hybrid fibers. Since we didn't investigate hybrid fibers and we were specific in the discussion: "Our results agree with previous studies that showed **no changes in type I and II satellite cell** or myonuclear number after different forms of ET…", we believe our statement is correct and hope that the reviewer agrees.

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In literature, a huge number of studies investigated the functional and molecular adaptations to either resistance or endurance training interventions. Similarly, many research studies have also

investigated the effects of combined endurance and resistance training (namely concurrent training) in both sedentary and well-trained athletes. However, the carry-over effects of a "priming" training intervention on a subsequent one are less known and they are raising a lot of interest, since the discovery of the complexity of the molecular mechanisms shared between the "hypertrophic" and "mitochondrial" pathways.

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They recruited twenty-five healthy young male participants, separated in RT+ET and ET. Resistance training was performed twice weekly by the RT+ET group only, and consisted of leg press, bench press, leg extension, cable pull-down, and leg curls exercises from 70 to 95%1RM. Seven weeks of endurance training were prescribed as high-intensity interval training (HIIT) and participants trained on a motorized treadmill by intervals (1 minute sprints) at 80-100%VO2max. Before and after each training intervention, each participant performed evaluations on two nonconsecutive days. During the first day, anthropometric measurements were performed and muscle structure was evaluated by ultrasound. A muscle biopsy from the right VL was also collected. Immunohistochemistry was performed to determine fiber cross-sectional area (fCSA), myonuclear content, myonuclear domain size, satellite cell number, and mitochondrial content. Western blots were used to quantify markers of mitochondrial biogenesis and dynamics (I-V complexes, PGC-1α, NRF1, TFAM, MFN1, MFN2, DRP1, PINK1, and PARKIN). Citrate synthase activity and markers of ribosome content were also investigated. On the second day,

participants underwent to a maximal cardiorespiratory test fro VO2max and OBLA determination.

RT elicited adaptations commonly reported in the literature: body composition imporved; strength and VL thickness increased; a higher content of mixed and type II fCSA, myonucleai, markers of ribosome, and satellite cells were reported. ET improved VO2max and the speed at OBLA in both groups. However, there were no differences between RT+ET vs ET. Additionally, seven weeks of RT performed prior to seven weeks of ET blunted most mitochondrial adaptations to ET showed interfere with mitochondrial adaptations.

According to their results, the authors concluded that prior RT had no additional benefits on performance adaptations to ET and several molecular adaptations to ET were blunted. The authors speculated that performing RT before ET may results in an enhanced catabolic/proteolytic state.

The manuscript has many strengths, for which the authors should be congratulated. Nevertheless, I think the study has some limitations in the study design and data interpretation.

Major comments:

STUDY DESIGN - OVERTRAINING

In their study, Mesquita et al. compared 14 weeks of training (7 weeks of RT + 7 weeks of ET)

to 7 weeks of ET alone. As such, training volume in RT+ET is doubled compared to ET, being RT+RT participants not allowed to recovery from their first weeks of training. Thus, in RT+ET we can appreciate that HIIT intervention was initiated without allowing the body to fully adapt to the previous interventions whereas ET were completely at rest before RT. How can the authors attribute difference between RT+ET and ET to RT, excluding the blunted response to ET in RT+ET was determined by the different training volume between the two groups? How can the authors exclude that an intervention ET+ET would have resulted in similar results? I would suggest the authors to discuss this issue, also taking into consideration recent findings from Flockhart et al. (doi: 10.1016/j.cmet.2021.02.017) who investigated mitochondrial function and mitochondrial dynamics after excessive endurance training.

Moreover, it is known that during repeated exercise bouts skeletal muscle cells tend to remove damage proteins and mitochondria and replace them with more efficient and functional ones. These processes usually need an adequate recovery period. If this is not the case, do the authors have data that can help the readers to exclude RT+ET overtraining?

Thanks for your comment. We believe that overtraining occurrence was very unlikely in our study for several reasons, including:

1. It is unlikely that overtraining happened with RT over only 7 weeks of training. Even though there are not many high-quality studies investigating overtraining with RT, most studies failed to detect decrements in performance (Grandou et al., 2020; PMID: 31820373). Furthermore, participants significantly increased strength and body composition, and showed no changes in aerobic performance or mitochondrial variables in response to RT, which does not suggest overtraining happened.

2. It is unlikely that RT-induced mitochondrial adaptations blunted mitochondrial adaptations to ET, as most variables remained unaltered after RT. Therefore, there was no "overtaxing" of mitochondria during the first training block.

3. Even though the study by Flockhart et al. is interesting, it is hard to compare their results with our study (or many other HIIT studies). They used an extremely high-volume of HIIT over a very short time. In the 1st week of training (considered light training), their participants were already performing 40min of high-intensity intervals, with a total duration of 45 minutes per training session. In the 3rd week of training, participants performed 5 sessions: 3 sessions had a total duration of 65 minutes, 45 minutes being at high-intensity; 2 sessions had a total duration of 45 minutes, 20 minutes being at high-intensity. In our study, the total time at high intensity was 10min at most. Flockhart's training configuration is unusual, presented an extremely high training volume, and had a fast load progression. Although important to understand the effects of overtraining, its comparability to other HIIT studies, or clinical/sports settings, is limited.

4. The fact that participants in the RT+ET improved endurance performance to a similar extent compared to ET-only group also suggests that overtraining did not happen, as a decrement in performance is considered an important aspect of overtraining.

5. Furthermore, we collected ratings of perceived exertion (RPE) after each high-intensity interval for every session. The RPE after the first high-intensity interval of the first training session was (mean ± SD) 2±1 for both groups ($p=0.617$). A higher RPE after the 1st interval could indicate insufficient recovery from the previous training block. In addition, the mean final RPE for all training sessions was 8±1 for both groups ($p=0.979$), indicating a similar perception of exertion between groups.

6.To be able to complete testing in all participants, there was a one week-break from the last session of RT to the start of ET, which potentially minimized the occurrence of overtraining. We have added this information to the “Experimental design” session.

GLYCOGEN STORES

Although intensity and duration of exercise appear to be the most important determinants of induced changes in mitochondrial content and function, recent studies (DOI:10.1007/s40279-018-0867-7.; DOI:10.1152/japplphysiol.00060) have suggested that initiating some training sessions with reduced muscle glycogen stores can better improve training-induced adaptations in markers of oxidative metabolism when compared with starting all training sessions with normal muscle glycogen stores. It would be interesting to know whether the RT training modified glycogen content in the skeletal muscle cells. This would help to clarify the role of glycogen stores in modulating the mitochondrial adaptations to endurance training.

This is a good point. Unfortunately, we do not have muscle samples left to conduct extra analysis. To the best of our knowledge, there are very few studies investigating the effects of chronic RT on muscle glycogen stores. A study conducted by Lundberg et al. (doi:10.1152/japplphysiol.01082.2013) found that 5 weeks of RT did not alter muscle glycogen stores. Therefore, we do not currently have enough evidence to suggest the muscle glycogen stores at the beginning of the ET were different between groups.

EXERCISE INTENSITY OF RESISTANCE TRAINING

In the literature there are contrasting findings about the effects of resistance training on mitochondria and the modulating effects may depen on training status and exercise principles employed. In the present study, participants of RT+ET group trained at high-load during RT phase, 2 time/week. However, results remain more equivocal for the effects of high-load resistance training on mitochondrial adaptations (DOI: 10.3389/fphys.2017.00713). The authors should justify the rationale for selecting this training intensity instead of a low-load resistance training and they should discuss this issue in the manuscript.

It is important to note that the goal was not to promote mitochondrial adaptations with RT. The hypothesis was that the well-known adaptations to RT, such as increased myonuclei and ribosome content (rationale is further discussed in the manuscript), could enhance mitochondrial adaptations to **ET**. Therefore, we specifically chose a high-load protocol to avoid mitochondrial adaptations with RT, as we believed that mitochondrial adaptations to ET could be impaired if participants were to start ET with higher mitochondrial content.

PREVIOUS LITERATURE

It has been shown (DOI: 10.1152/japplphysiol.00914.2010) that 25 sessions of isometric NMES of the quadriceps muscle over an 8-wk period can induce changes in myofibrillar proteins (a fast-to-slow phenotype shift) and energy production systems (a glycolytic-to-oxidative shift). How do the authors consider these results in relation to those presented in the present study?

It is very difficult to compare the results of the study suggested by the reviewer with those of the present study. In the study by Gondin et al. (2011) almost twice as many training sessions were performed than in the present study. Furthermore, the study cited by the reviewer used electrical

stimulations, which result in recruitment patterns and adaptations that differ from traditional RT (DOI 10.1007/s00421-011-2128-4; DOI 10.1007/s00421-011-2012-2). After the reviewer's comment, we conducted an analysis of the fiber type changes in the current study and found that there was no significant change in fiber type I % in response to RT (T1: 39 ± 16; T2: 39 ± 11; p=0.961). In addition, there was no significant difference in fiber type I % between groups at T2 (RT+ET: 39 ± 11; ET-only: 36 ± 15; p=0.601). We've included this information in the manuscript to provide more information to readers.

Minor comments:

Lines 64-66: The role of skeletal muscle oxidative function in determining endurance performance is debated. It may be worthy to rephrase the sentence, taking into consideration other references (e.g. DOI: 10.1113/JP271229).

Respectfully, we think that important distinctions must be made. Even though VO_{2max} is an important aspect of endurance performance, it is not the only one (and may not be the most important). The fact that mitochondrial function may not be a limiting factor to VO_{2max} in some cases (as indicated in the study mentioned by the reviewer), does not mean that it is not a limiting factor to endurance performance. In addition, maximal mitochondrial respiration (measured in the cited study) is only one variable of mitochondrial function. Other variables, such as the mitochondrial sensitivity to ADP, may be more important to VO_{2max} and/or endurance performance. Therefore, we respectfully believe that our statement that skeletal muscle oxidative phosphorylation capacity is considered a strong predictor of endurance performance is a fair statement and hope that the reviewer agrees.

Line 76: The authors may evaluate to reconsider this statement taking into consideration some studies (DOI: 10.1152/japplphysiol.01627.2011; doi: 10.1152/japplphysiol.00883.2012) where functional indexes of muscle oxidative metabolism were evaluated after RT.

Thanks for your comment. The goal of the paragraph mentioned by the reviewer is to show that RT can also cause positive mitochondrial adaptations, which could lead to increased endurance performance. We believe that the 1st paper suggested does not fit the idea of paragraph since they did not find significant changes in any of the variables investigated. However, we have included the findings of the 2nd study mentioned (lines 89-92).

Lines 85-86: In this study, the participants underwent two weeks of resting between aerobic conditioning and resistance training. Thus, they probably tried to avoid potential negative effects of intensified training. Please consider this issue in the discussion (see also general comments above).

Please, see previous replies.

Line 113: Two minutes may not be sufficient to reach steady state in lactate production/utilization, particularly in untrained subjects. Why did the authors decide to utilize this protocol? The author should comment on this and take into consideration the effects of this exercise protocol on VO2max and OBLA results.

Indeed, we recognize that the protocol adopted isn't the best option to measure lactate-related variables. Due to logistics, it was not feasible to include a third testing session to perform a specific test to determine lactate threshold, hence our decision to measure lactate during the VO_2max test. We have added this as a limitation in the "Experimental considerations" session. In

addition, most VO_2max tests cited in the HIIT literature either utilize cycle ergometers or their duration are substantially longer than the recommended. We considered the following aspects to choose our protocol:

1. The protocol should be conducted on a treadmill, so we could prescribe the HIIT sessions based on the VO_{2max} variables.

2. It should include increments in both speed and inclination. Because of our strict inclusion criteria regarding training history, our participants were very untrained. Protocols that have increments in speed only usually achieve very high speeds, which may not be safe for individuals unaccustomed to running on a treadmill.

3. In addition, we performed a verification step to further validate the values achieved during the graded maximal testing.

Line 135: If I am not wrong, the participants performed the 3RM test after the maximal cardiorespiratory test. How many minutes were the subjects allowed to recovery? Are the authors sure the results were not underestimated due to post-exertional fatigue?

That is correct. The participants performed the 3RM test after the VO_{2max} test. This occurred due to logistical constraints. It was not feasible to have each test performed on separate days. With that in mind, considering that VO_{2max} was more important than 3RM (3RM were mainly meant to show that RT worked, leading to increases in strength) for the current study, we decided to perform the VO_{2max} first, so that it would not be influenced by the 3RM test. The participants had approximately 10-15min rest between tests. In addition, even if 3RM values were underestimated, such effect was consistent throughout all time-points.

Line 154: please include details about the site where the muscle biopsies were sampled? Was the same in the two/three time points or incision was performed few centimeters away from the previous one?

The biopsies at T2 and T3 were obtained ~2 cm proximal of each preceding biopsy scar. We have included this information in the methods.

Lines 176-177: I was not able to find details about the results from the validation test. Please include information.

The information is described in the following sentences (lines 207-210).

"After completing the test, participants rested for 10 minutes, were connected to the metabolic cart again and ran for as long they could at a speed and inclination corresponding to the stage following the stage they stopped during the test. This step was included as a verification method to ensure that participants reached maximal oxygen consumption (VO_2max)."

Line 635: "mitochondrial network". I don't think that changes in TOM20 may be sufficient to draw conclusions on mitochondrial network. Did the authors measure other markers of mitochondrial content (e.g. SIRT1)? Did they collect muscle samples for TEM analyses?

Unfortunately, we did not measure SIRT1 or performed TEM analyses, but included other estimates of mitochondrial content (e.g., mitochondrial complexes protein content and CS activity). We recognize that the term "mitochondrial network" may be misleading and changed it to "mitochondrial content".

Lines 693-696: The hypothesis suggested by the authors is interesting but it is not supported by

the data presented. The authors should try to elucidate whether an enhanced catabolic/proteolytic state can explain the blunted response to ET in RT+ET group. What about the role of ROS/antioxidant systems balance? Do the author think that this mechanism may have contributed to the results?

We recognize that our data do not support this hypothesis. However, determining the catabolic/proteolytic state was not one of the goals of the present study, and this hypothesis was generated a posteriori. We believe we have made that clear in manuscript when we state that this was a speculation and included as a limitation in the "Experimental considerations" session. Regarding the role of ROS/antioxidant system balance, we are unsure of how it would contribute to the results found. However, as RT has been shown to improve redox state and redox state is closely related to mitochondrial function, future studies should further investigate this issue.

END OF COMMENTS

Dear Dr. Kavazis,

Re: JP-RP-2023-284822R1 "Resistance Training Diminishes Mitochondrial Adaptations to Subsequent Endurance Training" by Paulo H.C. Mesquita, Joshua S Godwin, Bradley Ruple, Casey Sexton, Mason C. McIntosh, Breanna J. Mueller, Shelby Osburn, Christopher Brooks Mobley, Cleiton Augusto Libardi, Kaelin C. Young, L. Bruce Gladden, Michael Roberts, and Andreas Kavazis

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EDITOR COMMENTS

Reviewing Editor:

The referees are substantially satisfied with the revision. Both have only relatively minor suggestions for changes, which the authors should take into consideration.

REFEREE COMMENTS

Referee #1:

The authors have made considerable improvements to the manuscript based on comments raised. This reviewer only has some minor suggestions for the authors to consider.

General suggestion: As scientific studies are frequently (incorrectly) extrapolated to elite athletes it is key that readers are aware that the current study was performed in untrained healthy young men. Whether similar observation would be made in trained/elite runners who would switch to a block of resistance exercise training followed by running again, remains unknown. Hence, it would be good to add to the title " ... in healthy untrained men". In addition, add "untrained" to line 826 to "..only younger adult men were examined herein."

Line 731: the authors refer to the study of Joanisse et al. to support the statement that satellite cell content is not changing in type I and Type II muscle fibers following ET. Its true that Joanisse et al. shows an increase in satellite cells in hybrids fibers only following ET/HIIT . But the premise in this study is that following HIIT a type I or type II fibers is transitioning towards a different fiber type in which satellite cells are "assisting" in this transition. Hence, it would be strange to use that as a reference to support that ET does not change satellite cell content per se. A better reference here would be Snijders et al. 2011 (PMID: 21321955) that shows no change in type I en type II satellite cell content following prolonged endurance exercise training.

Line 786: here the authors speculate that changes mitochondrial content may be more likely to be influenced by other proteins like PGC1a, NRF2 and TFAM rather than an increased myonuclear content relative to muscle fiber size. Instead of speculating that these changes in these proteins may explain the discrepancy observed on the decline in type I muscle fiber myonuclear domain size, it would even been better to measure these protein in the current study, to show whether there is actual evidence to support this.

***

Referee #2:

I would like to thank the authors for the work in revising their manuscript. Thank you for taking into consideration my

comments and for your replies. I think the manuscript has significantly improved and I have no major comments to raise.

Minor comments

Lines 66-67: You may consider of adding "cells" after "satellite"

Lines 130-132: It may be useful to add the text "RT, Resistance Training; ET, Endurance Training" as done in the figure 1 legend.

Lines 183-184: The amount of lidocaine is quite low in the experience of this reviewer. Did the subjects report pain during the procedure? Did the authors utilize local anesthetic cream before the procedure?

Line 213: "3RM" or "3 RM"? Please decide which is the abbreviation you prefer and modify it accordingly throughout the entire manuscript.

Line 220-223: Please include few words about the reason of including exercises which are not addressed to quadriceps muscle.

Line 414: Please consider adding here the two factors (time (T) x group. (G)) which were considered for the two-way analysis of variance (ANOVA).

Line 461: It may be worthy to report how many subjects reached a VO2 value in the verification trial higher than the VO2 value at exhaustion in the cardio-pulmonary test. I tried to address this issue in one of the comments of phase 1 but I probably lacked clarity.

END OF COMMENTS

1st Confidential Review **05-Jun-2023**

EDITOR COMMENTS

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healthy untrained men". In addition, add "untrained" to line 826 to "..only younger adult men were examined herein."

Thanks for your comment. We completely agree and therefore, have included the suggestions in the manuscript.

Line 731: the authors refer to the study of Joanisse et al. to support the statement that satellite cell content is not changing in type I and Type II muscle fibers following ET. Its true that Joanisse et al. shows an increase in satellite cells in hybrids fibers only following ET/HIIT . But the premise in this study is that following HIIT a type I or type II fibers is transitioning towards a different fiber type in which satellite cells are "assisting" in this transition. Hence, it would be strange to use that as a reference to support that ET does not change satellite cell content per se. A better reference here would be Snijders et al. 2011 (PMID: 21321955) that shows no change in type I en type II satellite cell content following prolonged endurance exercise training.

Thanks for your suggestion. We have changed the references in the manuscript.

Line 786: here the authors speculate that changes mitochondrial content may be more likely to be influenced by other proteins like PGC1a, NRF2 and TFAM rather than an increased myonuclear content relative to muscle fiber size. Instead of speculating that these changes in these proteins may explain the discrepancy observed on the decline in type I muscle fiber myonuclear domain size, it would even been better to measure these protein in the current study, to show whether there is actual evidence to support this.

We appreciate your comment and suggestion. We measured PGC-1a, NRF1, and TFAM protein levels (presented in the manuscript). Our intent with those sentences in the discussion was to show the reader that mitochondrial biogenesis is a complex process, influenced by several factors, including NRF2, which is less explored in the literature. However, in view of our results and all the variables investigated, we respectfully think that measuring NRF2 protein levels would add little to the current manuscript. In addition, due to sample limitation, we wouldn't be able to determine NRF2 protein levels in all participants.

***

Referee #2:

I would like to thank the authors for the work in revising their manuscript. Thank you for taking into consideration my comments and for your replies. I think the manuscript has significantly improved and I have no major comments to raise.

Minor comments

Lines 66-67: You may consider of adding "cells" after "satellite"

Thanks for catching this. We have added it to the manuscript.

Lines 130-132: It may be useful to add the text "RT, Resistance Training; ET, Endurance Training" as done in the figure 1 legend.

Thanks for your suggestion. We have added it to the manuscript.

Lines 183-184: The amount of lidocaine is quite low in the experience of this reviewer. Did the subjects report pain during the procedure? Did the authors utilize local anesthetic cream before the procedure?

No abnormal pain was reported by the participants besides the “high-pressure feeling” of the biopsy procedure itself. We did not use local anesthetic cream before the procedure. This amount of lidocaine has been commonly used in our laboratory without major complaints. Also, participants were not able to feel the pilot incision made after lidocaine injection, confirming the anesthetic effect of the lidocaine.

Line 213: "3RM" or "3 RM"? Please decide which is the abbreviation you prefer and modify it accordingly throughout the entire manuscript.

Thanks for your comment. We’ve made it consistently “3 RM”.

Line 220-223: Please include few words about the reason of including exercises which are not addressed to quadriceps muscle.

We have included the following to the manuscript:

Lines 222-224: “Exercises not targeting quadriceps were included as an attempt to increase adherence to training, avoid potential muscle imbalances, and to provide a more complete workout, developing the major muscle groups.”

Line 414: Please consider adding here the two factors (time (T) x group. (G)) which were considered for the two-way analysis of variance (ANOVA).

Thanks for your suggestion. We've included it in the manuscript.

Line 461: It may be worthy to report how many subjects reached a VO2 value in the verification trial higher than the VO2 value at exhaustion in the cardio-pulmonary test. I tried to address this issue in one of the comments of phase 1 but I probably lacked clarity.

We've included the following to the manuscript to try to convey that information:

Lines 468-470: "At T1, 6 participants achieved greater VO_2 values in the verification step of the maximal cardiorespiratory test, ranging from 0.24 to 1.50 ml/kg/min."

Lines 479-483: "At T2, 3 participants from RT+ET and 7 from ET-only group achieved greater VO_2 values in the verification step of the maximal cardiorespiratory test, ranging from 0.21 to 3.59 ml/kg/min. At T3, 2 participants from RT+ET and 2 from ET-only group achieved greater VO_2 values in the verification step of the maximal cardiorespiratory test, ranging from 0.03 to 1.81 ml/kg/min."

END OF COMMENTS

Dear Dr Kavazis,

Re: JP-RP-2023-284822R2 "Resistance Training Diminishes Mitochondrial Adaptations to Subsequent Endurance Training in Healthy Untrained Men" by Paulo H.C. Mesquita, Joshua S Godwin, Bradley Ruple, Casey Sexton, Mason C. McIntosh, Breanna J. Mueller, Shelby Osburn, Christopher Brooks Mobley, Cleiton Augusto Libardi, Kaelin C. Young, L. Bruce Gladden, Michael Roberts, and Andreas N Kavazis

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EDITOR COMMENTS

Reviewing Editor:

The authors have responded to the last minor issues raised by the Reviewers. Congratulations for an excellent study.

Senior Editor:

Congratulations!

2nd Confidential Review **28-Jun-2023**