Functional reserve and sex differences during exercise to exhaustion revealed by post-exercise ischaemia and repeated supramaximal exercise

Marcos Martin Rincon, Miriam Gelabert-Rebato, Mario Perez-Valera, Victor Galvan-Alvarez, David Morales-Alamo, Cecilia Dorado, Robert Boushel, Jostein Hallén, and Jose A. L. Calbet
DOI: 10.1113/JP281293

Corresponding author(s): Jose Calbet (lopezcalbet@gmail.com)

The referees have opted to remain anonymous.

Review Timeline:

Submission Date:	23-Dec-2020
Editorial Decision:	22-Feb-2021
Revision Received:	19-Apr-2021
Editorial Decision:	27-May-2021
Revision Received:	07-Jun-2021
Accepted:	17-Jun-2021

Senior Editor: Michael Hogan

Reviewing Editor: Paul Greenhaff

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 22-Feb-2021

Dear Professor Calbet,

Re: JP-RP-2020-281293 "Functional reserve and sex differences during exercise to exhaustion revealed by post-exercise ischaemia and repeated supramaximal exercise" by Jose A. L. Calbet, Marcos Martin Rincon, Miriam Gelabert-Rebato, Mario Perez-Valera, Victor Galvan-Garcia, David Morales-Alamo, Cecilia Dorado, Robert Boushel, and Jostein Hallén

Thank you for submitting your manuscript to The Journal of Physiology. It has been assessed by a Reviewing Editor and by 3 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.

The reports are copied at the end of this email. Please address all of the points and incorporate all requested revisions, or explain in your Response to Referees why a change has not been made.

I hope you will find the comments helpful and have no difficulty returning your revisions within 4 weeks.

Your revised manuscript should be submitted online using the links in Author Tasks Link Not Available.

Any image files uploaded with the previous version are retained on the system. Please ensure you replace or remove all files that have been revised.

REVISION CHECKLIST:

- Article file, including any tables and figure legends, must be in an editable format (eg Word)

- Upload each figure as a separate high quality file

- Upload a full Response to Referees, including a response to any Senior and Reviewing Editor Comments;

- Upload a copy of the manuscript with the changes highlighted.

You may also upload:

- A potential 'Cover Art' file for consideration as the Issue's cover image;

- Appropriate Supporting Information (Video, audio or data set https://jp.msubmit.net/cgi-bin/main.plex?form_type=display_requirements#supp).

To create your 'Response to Referees' copy all the reports, including any comments from the Senior and Reviewing Editors, into a Word, or similar, file and respond to each point in colour or CAPITALS and upload this when you submit your revision.

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,

Michael C. Hogan
Senior Editor
The Journal of Physiology
https://jp.msubmit.net
http://jp.physoc.org
The Physiological Society
Hodgkin Huxley House
30 Farringdon Lane
London, EC1R 3AW
UK
http://www.physoc.org
http://journals.physoc.org

EDITOR COMMENTS:

Raw data is not included in Figs or as additional information.

No statistical summary document supplied as far as I can see, but this is the initial version.

This manuscript has been reviewed by three expert reviewers who have different opinions regarding the merits of the research. However, the majority opinion is that the findings could be quite influential and therefore the work could be potentially interesting to the readership of JP. Importantly, all reviewers have raised a number of major concerns that the authors need to consider, including the indirect nature of several of the methodological approaches employed, the manner in which the data have been presented (needs to comply with JP guidelines) and whether the study design/power is sufficient to evaluate the main hypothesis related to potential sex-based differences. These concerns must be fully addressed by the authors.

Senior Editor Comments:

Need SD and data points.

REFEREE COMMENTS

Referee 1:

Here authors ascertain task failure during high intensity supra-maximal exercise in male and female subjects. The authors aim to explain mechanisms of task failure and possible sex differences herein. The importance of the study is twofold: 1) understanding fatigue mechanisms with whole body high intensity exercise and 2) understanding probable fundamental sex differences in anaerobic capacity and task failure with supramaximal exercise. The group of investigators have clear experience with exercise protocols in a human integrative setting and use sophisticated well-chosen methods and design in order to pursue the aims. Here they use repeated sprint exercise (120% VO2max) in a cross-over design, with and with-out blood flow occlusion in recovery periods between bouts. There were no differences between male and female performance during the first bout exercise, however, metabolic determinants differed in a number of parameters. Also, its concluded that a large functional reserve remains at task failure with the exercise at 120% VO2max , in both sexes. This is an elaborate study with an important physiological question addressed and the conclusions represent an important advance in our knowledge. The manuscript is well written paper with several significant findings, the design is original and data collection is sound. However, there are a number of issues and interpretations which needs the authors consideration.

Major:

Data presentation. In order to present data that allow inspection, not concealment, of the nature of the distribution of the data, please present data according to the guidelines for The Journal of Physiology. Thus, it is strongly recommended that all data points must be plotted within the figure of the manuscript, e.g. a bar graph with data points overlaid. Herein possibly link data points for each person for each of the three timepoints. Please use a box and whisker plot to present data.

Study overview. Although relatively straight forward and clear design, the study procedure appears difficult to grasp in the Methods section. Ill suggest that authors start the section (line 161), with a study overview and referring to figure 1. As is in present manuscript, the overview Fig. 1 is not denoted to before late in Methods. I acknowledge that there is a large number om careful considerations and methods used, but this is not easy to grasp, when design is a little unclear for the reader. Herein also section clearly the pre-test and experimental tests.

Although I agree on the very interesting and well argued conclusion, that task failure with the exercise is "not caused by the achievement of a limiting glycolytic rate" (e.g. lines 473-474), nor "anaerobic capacity" (e.g. paragraph starting line 416), one cannot exclude these factors at the intracellular myofiber level. Thus, local cellular compartments may be exhausted, or glycolytic rate limited. Although this may be speculative its still plausible and deserves an opening in the discussion. With this discussion on limiting factors, you here state that " .. task failure is likely linked to a direct effect of the metabolites and ion disturbances associated with substrate-level phosphorylation" (lines 477-478). How, would this "disturbance" be alleviated during occlude recovery? As you argue that aerobic metabolism will be very limited, i.e. H+, ADP, AMP, Pi etc. would still be present.

Lines 403-405. Its stated here, and throughout the manuscript, that men and women, ...should have exhausted the anaerobic capacity .... How is this justified? Although possible, the anaerobic capacity was not estimated and a such, a clear explanation for the statement is needed. If anaerobic capacity was exhausted, then how can you state that "Neither in men nor in women was task failure caused by exhaustion of anaerobic capacity? (e.g. lines 424-425).

Discussion. This is a very extensive Discussion, which although interesting, at times is unnecessary long. Data and main conclusions are diluted by the at times unfocused discussion. Please shorten and focus on discussion of main data and conclusions drawn by present study. Herewith, paragraph at page 26 could possibly be deleted.

Minor:

Why were the subjects instructed to pedal unloaded during resting phase between bouts? This was not done during occlusion.

The relevant and logic calculation of lactate production rate per LLM (lines 450-452) should preferably be explained explicitly.

Power calculation line 161-164. Please move to statistics. Its stated that a sample size of 12 was required to detect sex differences. Which main parameter was used for this calculation (power, anaerobic capacity or other)?

Cited reviews on muscle (fibre) fatigue mechanisms. Please use more recent and up to date references, i.e. Allen, Lamb and Westerblad, Physiol Rev 2008 or JAP 2008).

Line 43. Here you introduce the term "functional reserve", please define for the reader the concept of functional reserve.

Line 123, aims. You aim to determine "ascertain what mechanisms may explain between-sex differences in ..." Do you mean " ..possible between-sex differences ...".

Paragraph starting at line 274, O2 demand during supramaximal exercise. Here one can only assume a constant efficiency, which may be valid. However, you use this assumption after exhaustive exercise, and occlude recovery. Please discuss if this assumption still is valid and state this assumption clearly.

Line 303. Non-significant sex differences. This should be no difference.

Line 306-307. Its not clear to me how the RER values you use are measured, i.e. for which period and as this is non-steady state exercise how would you justify the interpretations of the use of RER.

Line 313. Add "were on average" after bouts.

Table 3. Recovery with free circulation 1 - should it be 1 and 2?

Fig. 2, 3 and 4. Why do you use SEM, when stated in Stats that you use SD unless otherwise stated? Where do you use SD? Se also above on recommended figure outline.

Fig. 2. Please state clearly what 100% is percentage of, i.e. anaerobic yield is percentage of whole energy expenditure. What about Work (%), Accumulated O2, and oxygen deficit?

Suppl. Line 13. .. the first bout was associated with ... . neg or pos associated?

Referee 2:

General:

This paper attempts to assess various metabolic, physiologic and performance responses to repeated bout of supramaximal exercise in men and women using solely noninvasive techniques. There are, however, several questions regarding validation of techniques as well as appropriate control issues (see below).

Specific:

1 Line 67-73 - Indicating that AMP, ADP, lactate and H+ are not responsible for task failure and then using the Sahlin and Ren (1989) reference to support this statement, when only the latter two were addressed in the paper, is not appropriate scientific citation. And why is Pi not discussed in this context? 4 pages of introduction is a little too much, in my opinion. You should be able to justify performing the study with less.

2 Line 158-163 - Since one of earlier studies (Phillips et al 1996) showing that menstrual cycle markedly affected force generation, many have followed with mixed results (ie, negative or positive), depending on experimental conditions. Moreover, the cycle also affects metabolism (eg, insulin sensitivity and carbohydrate metabolism), which was not controlled for in the present study. So unless you demonstrate that the cycle is not important using the protocols used in the present study, one has to consider a potential confounding effect of the cycle. One should have, at the very least, documented where in the cycle the women were at the time of study.

3 Line 201-203 - Eating any kind of meal one h before the intense protocol used is likely to create gastric discomfort with the protocol used (if not overt vomiting). Moreover, the lack of standardization of diet between subjects as well as time of ingestion leads to further problems regarding effects on outcome. Finally, the common approach to avoid such problems is simply to study everyone after an overnight fast.

4 Line 215-216 - Why did the subjects in free circulation cycle at 20 rpm whereas in occlusion nothing was done? Can this have affected recovery in any way (ie, enhancing blood flow)?

5 Line 253-256 - This is a very critical validation. The fact that the results are not published/presented and the conditions under which the comparisons are performed are not described does not reinforce acceptance of the statement.

6 Line 265-266 - To what extent did pain after occlusion affect exercise performance in the following bout? To my understanding, exercise following fatigue and then occlusion is quite painful. I did not see such results presented.

7 Line 306-307 - Using RER to reflect anaerobic metabolism under the conditions of study is not convincing. The authors will need to cite a study demonstrating that this is valid. I am less than convinced.

8 Fig 3A-C - Why are work, accumulated O2 and O2 deficit significantly higher in the men in the first bout in the open state? As I understood, the first bout should result in similar performance (as in the women), since the first occlusion occurred after the first bout. How is it that the VO2 (Fig 3D) and anaerobic energy yield (Fig 2D) are the same when more work was performed?

Referee 3:

This is a comprehensive study from an experienced group of investigators who are well versed in the physiology from a theoretical and technical/measurement perspective. My main overarching comments relate to the research design and presentation of results.

Major:

Ln 141-161, Subjects. Please clarify the nature of the sample size estimation using G*Power, i.e., type of power analysis (specific test selected) and other input parameters. Also, what was the basis for using an effect size of 0.6 for the primary outcome measure, which was presumably a specific measure of functional reserve? Was there any a priori consideration as to what constituted a meaningful difference (i.e., between males/men and females/women), given the inherent measurement variability, i.e. what was the "signal to noise" ratio?

In recruiting participants, was there any a priori consideration given, or steps taken, to "match" the men and women - either in pairs or as a group - on any variable. For example, matching participants on VO2 per fat free mass is often recommended as best practice in the design of studies that investigate biological sex-based differences. The two groups seemingly did not differ on this parameter (Table 1), but it unclear if this was by design or the result of chance?

Ln 286-290, Statistics. Please clarify the nature of the statistical analyses. Specifically, the reference to a "two-way repeated measures ANOVA... with sex as a between-subjects factor" is confusing to this reviewer. How was the potential effect of sex/gender (i.e., the primary focus) specifically evaluated? Tables 2 and 3 seemingly imply that a 3-factor ANOVA was used (i.e., "Oc x Sex x Bout" column), but the textual presentation and explanation of results is lacking.

Ln 300 and general presentation of results. Related to the above comment regarding the nature of the statistical analyses (and specific type of ANOVA performed), please clarify the basis for reporting specific differences as significant or not, and associated P values. For example, Ln 313: "Time to exhaustion in the second and third bouts (sic) 43.7% lower in the bouts preceded by occlusions, with this effect being similar in men and women (P=0.54) (Table 2)". What does this mean? Especially in the text, please clarify whether P values reported are based on interactions or specific main effects or post-hoc comparisons.

Figures. Mindful of a recent Editorial in the journal and efforts to generally enhance data presentation (https://doi.org/10.1113/JP277519), please show individual data as much as possible (i.e., to convey distribution) as opposed to "dynamite plunger plots". Please also depict SD (which conveys variability regarding the sample) as opposed to SEM, and consider confidence intervals.

Other specific:

Ln 43. Given the title and overarching focus, it would be useful to explicitly define "functional reserve" at the outset.

Ln 65. Perhaps "energy contribution from" rather than "recruitment of" (given the usual association of the latter term with motor unit activation")?

Ln 79. While the gender of each participant was self-reported in the present study for the purpose of the two groupings (Ln 156-158), would it be more appropriate to refer to "males" and "females" when generally referring to potential (biological) sex-based differences?

Ln 122. Perhaps "following" rather than "during"?

Results, general: Please consider significant digits when reporting results.

END OF COMMENTS

EDITOR COMMENTS:

Raw data is not included in Figs or as additional information.

No statistical summary document supplied as far as I can see, but this is the initial version.

This manuscript has been reviewed by three expert reviewers who have different opinions regarding the merits of the research. However, the majority opinion is that the findings could be quite influential and therefore the work could be potentially interesting to the readership of JP. Importantly, all reviewers have raised a number of major concerns that the authors need to consider, including the indirect nature of several of the methodological approaches employed, the manner in which the data have been presented (needs to comply with JP guidelines) and whether the study design/power is sufficient to evaluate the main hypothesis related to potential sex-based differences. These concerns must be fully addressed by the authors.

We appreciate indeed the opportunity to resubmit a revised version. We acknowledge the effort put in by the reviewers and are grateful for the insightful comments.
We have included a statistical summary document.
We have redone all the figures to comply with JP guidelines.
The statistical power was sufficient according to a *priory* analysis; we have added more information in this regard.
We have also included an appendix with the validation study of the TOI values used to assess O_2 extraction, as well as the TOI O_2 extraction index utilized to check on sex differences in O_2 extraction.

Senior Editor Comments:

Need SD and data points.
Data points have been included in all figures. SD are used when vertical bars are reported.

REFEREE COMMENTS

Referee 1:

Here authors ascertain task failure during high intensity supra-maximal exercise in male and female subjects. The authors aim to explain mechanisms of task failure and possible sex differences herein. The importance of the study is twofold: 1) understanding fatigue mechanisms with whole body high intensity exercise and 2) understanding probable fundamental sex differences in anaerobic capacity and task failure with supramaximal exercise. The group of investigators have clear experience with exercise protocols in a human integrative setting and use sophisticated well-

chosen methods and design in order to pursue the aims. Here they use repeated sprint exercise (120% VO2max) in a cross-over design, with and with-out blood flow occlusion in recovery periods between bouts. There were no differences between male and female performance during the first bout exercise, however, metabolic determinants differed in a number of parameters. Also, its concluded that a large functional reserve remains at task failure with the exercise at 120% VO2max , in both sexes. This is an elaborate study with an important physiological question addressed and the conclusions represent an important advance in our knowledge. The manuscript is well written paper with several significant findings, the design is original and data collection is sound. However, there are a number of issues and interpretations which needs the authors consideration.

Thank you.

Major:

Data presentation. In order to present data that allow inspection, not concealment, of the nature of the distribution of the data, please present data according to the guidelines for The Journal of Physiology. Thus, it is strongly recommended that all data points must be plotted within the figure of the manuscript, e.g. a bar graph with data points overlaid. Herein possibly link data points for each person for each of the three timepoints. Please use a box and whisker plot to present data.
All data are included in the figures and box and whisker plots used. We had in total 36 subjects, and each subject underwent 3 bouts of exercise. Connecting all individual data points results in almost impossible-to-read figures and, therefore, we believe that just presenting the individual data may be clearer.

Study overview. Although relatively straight forward and clear design, the study procedure appears difficult to grasp in the Methods section. Ill suggest that authors start the section (line 161), with a study overview and referring to figure 1. As is in present manuscript, the overview Fig. 1 is not denoted to before late in Methods. I acknowledge that there is a large number of careful considerations and methods used, but this is not easy to grasp, when design is a little unclear for the reader. Herein also section clearly the pre-test and experimental tests.
We have better structured the methods' section and introduced the protocol figure earlier, as suggested. We have explained how we converted the O_2 deficit values into lactate.

Although I agree on the very interesting and well argued conclusion, that task failure with the exercise is "not caused by the achievement of a limiting glycolytic rate" (e.g. lines 473-474), nor "anaerobic capacity" (e.g. paragraph starting line 416), one cannot exclude these factors at the intracellular myofiber level. Thus, local cellular compartments may be exhausted, or glycolytic rate limited. Although this may be speculative its still plausible and deserves an opening in the discussion. With this discussion on limiting factors, you here state that ".. task failure is likely linked to a direct effect of the metabolites and ion disturbances associated with substrate-level phosphorylation" (lines 477-478). How, would this "disturbance" be alleviated during

occlude recovery? As you argue that aerobic metabolism will be very limited, i.e. H+, ADP, AMP, Pi etc. would still be present.

These are fascinating points, which should be addressed with invasive procedures. We have conducted a follow-up study using a similar protocol with muscle biopsies and catheterisation of the femoral artery and vein. In this new study, we are analysing metabolites and using electron microscopy to test whether depletion of specific myofibrillar glycogen stores could contribute to task failure. These analyses require a lot of time and are integrated into a different project, in which the first results will be available in 1.5 years or so.

We agree with the reviewer that the critical question to answer is by which mechanisms does recovery occur during the occlusions. The traditional approach would be to consider that the ion pumps and the antioxidant systems remove part of the impediments during occluded recovery. For example, we have recently shown that some antioxidant enzymes may be acutely up-regulated (Gallego-Selles *et al.*, 2020). In theory, ADP could be partly removed, although some recent studies *in vitro* indicate that is not as critical to elicit muscle fatigue as previously thought. Since the total amount of P_i cannot change (P_i is not excreted to the extracellular space) there are only two possibilities to diminish the concentration of intracellular P_i. One is to resynthesise PCr, ATP and other nucleotides. However, as explained, during ischaemia applied at exhaustion there is no PCr resynthesis. Alternatively, P_i could be sequestered in intracellular compartments, as for example the sarcoendoplasmic reticulum (Ferreira *et al.*, 2021) or the mitochondrial matrix (Carafoli, 2012). In the case of the sarcoendoplasmic reticulum, this process may be facilitated by the SERCA activity during the recovery. The ATP needed for SERCA activity is produced by glycolysis, resulting in further lactate accumulation during ischaemic recovery, as previously shown (Morales-Alamo *et al.*, 2015). If we are certain, the functional reserve should be linked to the total amount of sarcoendoplasmic reticulum and SERCA protein and factors that regulate the activity of the SERCA. We are targeting this in our ongoing projects. Nevertheless, a hypothesis putting too much emphasis on P_i would have to deal with the observation made by Bruton et al. (2003) in the CK -/- mice. CK-/- mice fibres were more fatigue resistant than wildtype fibres during a bout of repeated tetanic contractions (Bruton *et al.*, 2003) but produced less force.

In the revised version of the manuscript, some of these ideas are mentioned, but not all, because some are speculative and beyond the current paper's scope.

As a related point, we have calculated the glycolytic rates during the bouts performed after ischaemic recovery and compared them to the maximal glycolytic rates achieved during a Wingate test.

Lines 403-405. Its stated here, and throughout the manuscript, that men and women, ...should have exhausted the anaerobic capacity .... How is this justified? Although possible, the anaerobic capacity was not estimated and a such, a clear explanation for the statement is needed. If anaerobic capacity was exhausted, then how can you state that "Neither in men nor in women was task failure caused by exhaustion of anaerobic capacity? (e.g. lines 424-425).

According to classical studies, most subjects should be using the totality of their anaerobic capacity when performing constant -intensity exercise at 120% of the

VO_2max on the cycle ergometer. According to Medbo et al. (1993) 94% of the anaerobic capacity is already used in all-out effort lasting 60 s. Our experiments indicate that not all the anaerobic capacity is exhausted during supramaximal exercise to exhaustion at 120% of VO_2max. Accordingly, we modified the affected sentence (Line 468, first paragraph of the discussion to "Although men and women performed the exercise at an intensity **which should have exhausted** the anaerobic capacity". In this finding resides part of the novelty of the present study. We have added references to the studies that support this conditional statement.

Discussion. This is a very extensive Discussion, which although interesting, at times is unnecessary long. Data and main conclusions are diluted by the at times unfocused discussion. Please shorten and focus on discussion of main data and conclusions drawn by present study. Herewith, paragraph at page 26 could possibly be deleted.
We understand the criticism. We have deleted the section on efficiency (page 26 of the previous version). We have also made some minor changes to other parts of the discussion to convey a more explicit message. Nevertheless, we have added additional information regarding the glycolytic rates.

Minor:

Why were the subjects instructed to pedal unloaded during resting phase between bouts? This was not done during occlusion.
To avoid postexercise orthostatic hypotension. Active recovery is not necessary during the occlusion since blood pressure remains elevated by two mechanisms. First, because the blood cannot pool in the legs due to the occlusion and second, by the activation of the metaboreflex, which help maintaining the blood pressure elevated. Besides, exercising with the occlusion applied in already exhausted muscle is almost impossible.

The relevant and logic calculation of lactate production rate per LLM (lines 450-452) should preferably be explained explicitly.
Thank you for bringing this point up. Since the myoglobin O_2 stores are depleted during the occlusions and PCr is not resynthesized (Morales-Alamo *et al.*, 2015), the totality of the O_2 deficit measured during the subsequent bout corresponds to the energy supplied by the glycolytic component of substrate-level phosphorylation. This O_2 deficit was converted to moles of ATP assuming a volume of 22.4 litres per mole of oxygen (STPD), a muscle temperature of 38.5 ºC and a phosphorus-to-O_2 ratio (P/O) of 2.5 (Hinkle *et al.*, 1991). Lactate production was obtained knowing that 1.5 moles of ATP are produced per mole of lactate. For this purpose, we assumed a muscle temperature of 38.5 °C but assuming 37.5 °C increases the value by 0.5%. We added a paragraph in the results section with the mean values and SD and calculated the corresponding glycolytic rates. In the discussion, we are comparing these rates to published data for the Wingate test. Our calculated rates represent 17-20% of the rate achieved during a Wingate test by men.

Power calculation line 161-164. Please move to statistics. Its stated that a sample size of 12 was required to detect sex differences. Which main parameter was used for this calculation (power, anaerobic capacity or other)?
This part has been rewritten, adding the information requested.

Cited reviews on muscle (fibre) fatigue mechanisms. Please use more recent and up to date references, i.e. Allen, Lamb and Westerblad, Physiol Rev 2008 or JAP 2008).
Done

Line 43. Here you introduce the term "functional reserve", please define for the reader the concept of functional reserve.
Done

Line 123, aims. You aim to determine "ascertain what mechanisms may explain between-sex differences in ..." Do you mean"..possible between-sex differences ...".
Yes, you are right. Thank you for pointing this out. The sentence has been corrected.

Paragraph starting at line 274, O2 demand during supramaximal exercise. Here one can only assume a constant efficiency, which may be valid. However, you use this assumption after exhaustive exercise, and occlude recovery. Please discuss if this assumption still is valid and state this assumption clearly.
To deal with this critical point, we have included a limitations section. Our previous study used 10 s and 60 s long occlusions after incremental exercise to exhaustion (Morales-Alamo *et al.*, 2015). Although the level of metabolic disturbance was greater after 60 than 10 s occlusions, the VO_2 per power output was lower after 60 s ischemia than after 10 s. This finding is not compatible with a marked reduction of muscle efficiency during supramaximal exercise under severe fatigue conditions. Besides, some researchers think that efficiency may even increase with fatigue (Myburgh, 2004).

Line 303. Non-significant sex differences. This should be no difference.
Corrected

Line 306-307. Its not clear to me how the RER values you use are measured, i.e. for which period and as this is non-steady state exercise how would you justify the interpretations of the use of RER.
RER was measured as VCO_2/VO_2. RER was not used in calculations of substrate oxidation during exercise at 120% of VO_2max. As the reviewer mentions, this would be incorrect due to the lack of a steady-state and the presence of hyperventilation. Men had a greater RER during the first bout to exhaustion at 120% of VO_2max. Women produce less energy through substrate-level phosphorylation than men but had a somewhat similar pulmonary ventilation response. This caused a lower $P_{ET}CO2$ in women, which can explain the lower brain oxygenation detected with NIRS in women. Thus, the RER is reported because it was significantly different between sexes, reflecting relative greater hyperventilation levels (i.e., more pulmonary ventilation for a given CO_2 production) in women.

Line 313. Add "were on average" after bouts.
Thank you, we have corrected the mistake.

Table 3. Recovery with free circulation 1 - should it be 1 and 2?
Yes, we have corrected the mistake.

Fig. 2, 3 and 4. Why do you use SEM, when stated in Stats that you use SD unless otherwise stated? Where do you use SD? Se also above on recommended figure outline.
SEMs have been replaced by SD in the vertical bars. In the revised version, we have replaced SEMs in figures by SD. In tables, we already showed SDs.

Fig. 2. Please state clearly what 100% is percentage of, i.e. anaerobic yield is percentage of whole energy expenditure. What about Work (%), Accumulated O2, and oxygen deficit?
We apologise for the confusion. The appropriate information has been included in the legend.

Suppl. Line 13. .. the first bout was associated with ... . neg or pos associated?
We are reporting the Pearson correlation coefficient's value (when negative, the corresponding "-"precedes the value, otherwise the association is positive).

Referee 2:

General:

This paper attempts to assess various metabolic, physiologic and performance responses to repeated bout of supramaximal exercise in men and women using solely noninvasive techniques. There are, however, several questions regarding validation of techniques as well as appropriate control issues (see below).
Thank you. Although we used only non-invasive procedures in this study, we validated our NIRS measurements with invasive techniques. The validation study is reported as an Appendix (after consultation with the editorial office) at the end of the manuscript.

Specific:

1 Line 67-73 - Indicating that AMP, ADP, lactate and H+ are not responsible for task failure and then using the Sahlin and Ren (1989) reference to support this statement, when only the latter two were addressed in the paper, is not appropriate scientific citation. And why is Pi not discussed in this context? 4 pages of introduction is a little too much, in my opinion. You should be able to justify performing the study with less.
Sorry for the confusion created. We have re-written this paragraph to mention Pi and to express better what has been previously done. We have removed contents from the

introduction (almost one page). However, we have defined the term "functional reserve" better, adding few lines to this purpose.

2 Line 158-163 - Since one of earlier studies (Phillips et al 1996) showing that menstrual cycle markedly affected force generation, many have followed with mixed results (ie, negative or positive), depending on experimental conditions. Moreover, the cycle also affects metabolism (eg, insulin sensitivity and carbohydrate metabolism), which was not controlled for in the present study. So unless you demonstrate that the cycle is not important using the protocols used in the present study, one has to consider a potential confounding effect of the cycle. One should have, at the very least, documented where in the cycle the women were at the time of study.
Whether and how much the menstrual cycle affects force generation remains controversial, as the reviewer points out. Our subjects were healthy, physically active young humans without medical conditions. No study so far has demonstrated that insulin sensitivity has any effect on supramaximal exercise performance. During this type of exercise, the main energy substrate is muscle glycogen. Some years ago, we did an experiment studying the impact of 75 g of glucose ingested 1 hour before Wingate tests in 15 men and determined effects on performance, muscle signalling, and metabolic responses (Guerra *et al.*, 2010). Glucose supplementation before supramaximal exercise had no impact on performance (Guerra *et al.*, 2010). There are some additional studies with similar findings.

Regarding the menstrual cycle phase, most studies have shown no impact of the menstrual cycle on high-intensity exercise performance (Botcazou *et al.*, 2006; Bushman *et al.*, 2006; Wiecek *et al.*, 2016). Interestingly, no impact of menstrual cycle on power output nor oxygen deficit during exercise at 120% of VO_2max has been reported (Shaharudin *et al.*, 2011). Given these antecedents, we did not consider it necessary to control for the menstrual cycle phase. Nonetheless, we have mentioned this in the limitations' section.

3 Line 201-203 - Eating any kind of meal one h before the intense protocol used is likely to create gastric discomfort with the protocol used (if not overt vomiting). Moreover, the lack of standardisation of diet between subjects as well as time of ingestion leads to further problems regarding effects on outcome. Finally, the common approach to avoid such problems is simply to study everyone after an overnight fast.
Subjects were recommended to ingest a light breakfast and always the same breakfast during experimental days. Moreover, from the arrival to the laboratory until the start of the high-intensity bouts, at least another hour elapsed, in part because we scheduled the subjects 30 min ahead of time to avoid undue delays and because we needed 30-40 min to fully prepare the subjects for the experiments. All subjects were familiarised with the protocol. No subject had problems complying with the exercise protocol.

4 Line 215-216 - Why did the subjects in free circulation cycle at 20 rpm whereas in occlusion nothing was done? Can this have affected recovery in any way (ie, enhancing blood flow)?
To avoid postexercise symptomatic orthostatic hypotension. Active recovery is not necessary during the occlusion since blood pressure remains elevated by two

mechanisms. First, because the blood cannot pool in the legs due to the occlusion and second, by the activation of the metaboreflex, which help maintaining the blood pressure elevated. Besides, exercising with the occlusion applied in already exhausted muscle is almost impossible.

Pedalling at 20 rpm during 20s recovery can hardly affect recovery (leg blood flow during the first 20 s after all-out exercise is already elevated due to postexercise hyperaemia, even without pedalling). Moreover, any impact would have minimal and similar for men and women.

5 Line 253-256 - This is a very critical validation. The fact that the results are not published/presented and the conditions under which the comparisons are performed are not described does not reinforce acceptance of the statement.
The data regarding the validation of this assessment has been added as an appendix at the end of the manuscript.

6 Line 265-266 - To what extent did pain after occlusion affect exercise performance in the following bout? To my understanding, exercise following fatigue and then occlusion is quite painful. I did not see such results presented.
At the start of exercise, the cuff is released, and the pain disappears instantaneously. Thus, during the following exercise, there was no pain. During the occlusions the level of pain was moderate and similar in men and women. These results were reported in lines 396-397 of the original manuscript. Additionally we can add that in the first bout, the pain was rated 6.6 ±2.2 and 5.8±2.3 A.U., by men and women, respectively P = 0.31), and in the second bout, 7.6±2.4 and 7.8±1.5 A.U., by men and women, respectively P = 0.72.
We asked specifically to the subjects about their feelings during dynamic exercise at the exit of the occlusions and they reported that there was no pain as soon as the blood flow was restored.

7 Line 306-307 - Using RER to reflect anaerobic metabolism under the conditions of study is not convincing. The authors will need to cite a study demonstrating that this is valid. I am less than convinced.
The recruitment of anaerobic metabolism during high-intensity exercise is accompanied by increased CO_2 production. H^+ is equimolarly produced with lactate. Part of the protons produced during high-intensity exercise reacts with bicarbonate to form carbonic acid (H_2CO_3), which under catalysis by carbonic anhydrase is transformed in $CO_2 + H_2O$. This CO_2 adds to the CO_2 produced in the mitochondria by substrate oxidation. The CO_2 stimulates ventilation (as well as the H^+, the increased temperature, and other factors). The outcome is that during high-intensity exercise, the RER (VCO_2/VO_2) increases above 1.00. There are many studies presenting this type of results. See an example in Table 1 of the study published in 2015 in J Physiol (Calbet *et al.*, 2015). Nevertheless, we wrote, "This increased reliance on anaerobic metabolism in men compared with women was associated with a higher respiratory exchange ratio (RER)", which seems correct to us.

8 Fig 3A-C - Why are work, accumulated O2 and O2 deficit significantly higher in the men in the first bout in the open state? As I understood, the first bout should result in

similar performance (as in the women), since the first occlusion occurred after the first bout. How is it that the VO2 (Fig 3D) and anaerobic energy yield (Fig 2D) are the same when more work was performed?

This finding was consistent, i.e., it was observed for the three variables. We can only speculate with the possibilities of a nocebo effect or random variation. This effect reached a statistical significance in men, but the similar trend observed in women did not reach statistical significance. The first time the subjects did the experiment they did not know whether there would be occlusion or not after the first bout (they were always prepared in the same way to avoid a nocebo effect). Nevertheless, in the second experiment, they could have guessed that there will be no occlusion if they had occlusion in the first experiment. Nonetheless, the conditions were counterbalanced (this means that half of the subjects could have guessed that there will be an occlusion after the first bout).

In Figure 2D, the rate of oxygen consumption (VO_2, that is mL per min) is illustrated, i.e., the mean rate at which O_2 was consumed during the whole first bout was similar. As endurance time was greater for men in the open circulation trial, this resulted in more time, i.e., more work performed, and a greater accumulated VO_2 (Fig 3B). In figure 3D, the percentage of the total energy yield produced by the anaerobic metabolism is depicted. This proportion was similar, despite minor differences in endurance time. (Please, note that we have exchange the order of figures and Figure 2 is now Figure 3, and vice versa the old Figure 3 is figure 2 in the revised version.

Referee 3:

This is a comprehensive study from an experienced group of investigators who are well versed in the physiology from a theoretical and technical/measurement perspective. My main overarching comments relate to the research design and presentation of results.

We appreciate indeed the insightful comments and suggestions.

Major:

Ln 141-161, Subjects. Please clarify the nature of the sample size estimation using G*Power, i.e., type of power analysis (specific test selected) and other input parameters. Also, what was the basis for using an effect size of 0.6 for the primary outcome measure, which was presumably a specific measure of functional reserve? Was there any a priori consideration as to what constituted a meaningful difference (i.e., between males/men and females/women), given the inherent measurement variability, i.e. what was the "signal to noise" ratio?

The type of analysis selected was ANOVA for repeated measures with exercise bout (three levels for the exercise variables and two levels when focussing on recovery) as within-subject factor and sex (two levels) as between-subjects factor. We explain in more detail how we calculated the sample size using G-Power using data from our previous study (Morales-Alamo *et al.*, 2015). We aimed at detecting at least a 20% difference between men and women since sex differences in mean power output

during supramaximal exercise and VO2max range usually between 20 and 30%. Nevertheless, the actual sample size was 36; thus, the final sample size allowed detecting between sex differences of 13-16%, depending on the level of correlation assumed between repeated measurements. We could not determine the signal-to-noise ratio for the functional reserve a priori; however, the CV for repeated measurements of power output and VO2 during supramaximal exercise in well-familiarised subjects and with state-of-the-art equipment is 3-6%. It is essential to highlight that the sample size required to determine a functional reserve is minimal since this can be tested for each subject individually. If the subject can perform exercise after the occlusion, it has a functional reserve. This was the case for all subjects in the two bouts of exercise.

Please, have in mind that this study is among the studies including more subjects to test between-sex differences in performance-related variables.

In recruiting participants, was there any a priori consideration given, or steps taken, to "match" the men and women - either in pairs or as a group - on any variable. For example, matching participants on VO2 per fat free mass is often recommended as best practice in the design of studies that investigate biological sex-based differences. The two groups seemingly did not differ on this parameter (Table 1), but it unclear if this was by design or the result of chance?

Subjects were recruited as they volunteered without any other restriction than the inclusion criteria. By including many subjects, we expected that VO_2 per fat-free mass would be matched, as it happened in a previous study we did more than 10 years ago, also looking at between sex-differences, but in sprint performance (Perez-Gomez *et al.*, 2008). No attempt was made to match men and women for any specific variable. We relied on sampling randomly and admitting only healthy, physically active subjects with availability for many tests required. Most of them were students with a high interest in exercise physiology.

Ln 286-290, Statistics. Please clarify the nature of the statistical analyses. Specifically, the reference to a "two-way repeated measures ANOVA... with sex as a between-subjects factor" is confusing to this reviewer. How was the potential effect of sex/gender (i.e., the primary focus) specifically evaluated? Tables 2 and 3 seemingly imply that a 3-factor ANOVA was used (i.e., "Oc x Sex x Bout" column), but the textual presentation and explanation of results is lacking.

The statistics section has been revised. A three-way ANOVA was applied, as reported in the tables. The text has been corrected to reflect this better. Sex differences were tested with the sex contrast included in the ANOVAs but also using unpaired t-tests at some specific time points. Besides, the following interactions were evaluated: occlusion by sex (to determine whether men and women responded differently to the occlusions), bout by sex (to find out whether the repetition of bouts elicited different responses in men and women); and occlusion by bout interaction (to test whether there was a different response over time in the bouts with occlusion compared with open circulation recovery).

Ln 300 and general presentation of results. Related to the above comment regarding the nature of the statistical analyses (and specific type of ANOVA performed), please

clarify the basis for reporting specific differences as significant or not, and associated P values. For example, Ln 313: "Time to exhaustion in the second and third bouts (sic) 43.7% lower in the bouts preceded by occlusions, with this effect being similar in men and women (P=0.54) (Table 2)". What does this mean? Especially in the text, please clarify whether P values reported are based on interactions or specific main effects or post-hoc comparisons.
When p-values are reported in tables, these are omitted in the text to improve readability. We have revised the full results section, which we have re-organized to convey a more clear message and eliminate ambiguity. We also explicitly mention the type of statistical analysis after the p-values.

Figures. Mindful of a recent Editorial in the journal and efforts to generally enhance data presentation (https://doi.org/10.1113/JP277519), please show individual data as much as possible (i.e., to convey distribution) as opposed to "dynamite plunger plots". Please also depict SD (which conveys variability regarding the sample) as opposed to SEM, and consider confidence intervals.
All figures have been redone to show all data points. The vertical bar figures are summary figures reporting the average of the last two bouts with error bars representing the SD.

Other specific:

Ln 43. Given the title and overarching focus, it would be useful to explicitly define "functional reserve" at the outset.
Done

Ln 65. Perhaps "energy contribution from" rather than "recruitment of" (given the usual association of the latter term with motor unit activation")?
We have replaced the term "recruitment"

Ln 79. While the gender of each participant was self-reported in the present study for the purpose of the two groupings (Ln 156-158), would it be more appropriate to refer to "males" and "females" when generally referring to potential (biological) sex-based differences?
The paragraph's only aim is to clarify that gender (what subjects feel/consider themselves) coincided with their biological birth sex in accordance with granting agency guidelines on sex and gender. This allows us to use the term women to refer to human females and men to refer to human males, terms that we like more when referring to human beings.

Ln 122. Perhaps "following" rather than "during"?
You are right, changed as suggested.

Results, general: Please consider significant digits when reporting results.
Done. All percentage values are reported with one significant decimal. P values are reported with three significant figures when needed (i.e., for P values < 0.001 and

when the P values is close to 0.05, as for example P=0.045, P=0.051, P=0.027 or P=0.067).

References

Botcazou M, Gratas-Delamarche A, Allain S, Jacob C, Bentue-Ferrer D, Delamarche P & Zouhal H. (2006). [Influence of menstrual cycle phase on catecholamine response to sprint exercise in the woman]. *Appl Physiol Nutr Metab* **31,** 604-611.

Bruton JD, Dahlstedt AJ, Abbate F & Westerblad H. (2003). Mitochondrial function in intact skeletal muscle fibres of creatine kinase deficient mice. *J Physiol* **552,** 393-402.

Bushman B, Masterson G & Nelsen J. (2006). Anaerobic power performance and the menstrual cycle: eumenorrheic and oral contraceptive users. *J Sports Med Phys Fitness* **46,** 132-137.

Calbet JA, Losa-Reyna J, Torres-Peralta R, Rasmussen P, Ponce-Gonzalez JG, Sheel AW, de la Calle-Herrero J, Guadalupe-Grau A, Morales-Alamo D, Fuentes T, Rodriguez-Garcia L, Siebenmann C, Boushel R & Lundby C. (2015). Limitations to oxygen transport and utilization during sprint exercise in humans: evidence for a functional reserve in muscle O_2 diffusing capacity. *J Physiol* **593,** 4649-4664.

Carafoli E. (2012). The interplay of mitochondria with calcium: an historical appraisal. *Cell Calcium* **52,** 1-8.

Ferreira JJ, Pequera G, Launikonis BS, Rios E & Brum G. (2021). A chloride channel blocker prevents the suppression by inorganic phosphate of the cytosolic calcium signals that control muscle contraction. *J Physiol* **599,** 157-170.

Gallego-Selles A, Martin-Rincon M, Martinez-Canton M, Perez-Valera M, Martin-Rodriguez S, Gelabert-Rebato M, Santana A, Morales-Alamo D, Dorado C & Calbet JAL. (2020). Regulation of Nrf2/Keap1 signalling in human skeletal muscle during exercise to exhaustion in normoxia, severe acute hypoxia and post-exercise ischaemia: Influence of metabolite accumulation and oxygenation. *Redox Biol* **36,** 101627.

Guerra B, Guadalupe-Grau A, Fuentes T, Ponce-Gonzalez JG, Morales-Alamo D, Olmedillas H, Guillen-Salgado J, Santana A & Calbet JA. (2010). SIRT1, AMP-activated protein kinase phosphorylation and downstream kinases in response to a single bout of sprint exercise: influence of glucose ingestion. *Eur J Appl Physiol* **109,** 731-743.

Hinkle PC, Kumar MA, Resetar A & Harris DL. (1991). Mechanistic stoichiometry of mitochondrial oxidative phosphorylation. *Biochemistry* **30,** 3576-3582.

Medbo JI & Tabata I. (1993). Anaerobic energy release in working muscle during 30 s to 3 min of exhausting bicycling. *J Appl Physiol* **75,** 1654-1660.

Morales-Alamo D, Losa-Reyna J, Torres-Peralta R, Martin-Rincon M, Perez-Valera M, Curtelin D, Ponce-Gonzalez JG, Santana A & Calbet JA. (2015). What limits performance during whole-body incremental exercise to exhaustion in humans? *J Physiol* **593,** 4631-4648.

Myburgh KH. (2004). Can any metabolites partially alleviate fatigue manifestations at the cross-bridge? *Med Sci Sports Exerc* **36,** 20-27.

Perez-Gomez J, Rodriguez GV, Ara I, Olmedillas H, Chavarren J, Gonzalez-Henriquez JJ, Dorado C & Calbet JA. (2008). Role of muscle mass on sprint performance: gender differences? *Eur J Appl Physiol* **102,** 685-694.

Shaharudin S, Ghosh AK & Ismail AA. (2011). Anaerobic capacity of physically active eumenorrheic females at mid-luteal and mid-follicular phases of ovarian cycle. *J Sports Med Phys Fitness* **51,** 576-582.

Wiecek M, Szymura J, Maciejczyk M, Cempla J & Szygula Z. (2016). Effect of sex and menstrual cycle in women on starting speed, anaerobic endurance and muscle power. *Physiol Int* **103,** 127-132.

1st Revision - Editorial Decision 27-May-2021

Dear Professor Calbet,

Re: JP-RP-2021-281293R1 "Functional reserve and sex differences during exercise to exhaustion revealed by post-exercise ischaemia and repeated supramaximal exercise" by Marcos Martin Rincon, Miriam Gelabert-Rebato, Mario Perez-Valera, Victor Galvan-Alvarez, David Morales-Alamo, Cecilia Dorado, Robert Boushel, Jostein Hallén, and Jose A. L. Calbet

Thank you for submitting your manuscript to The Journal of Physiology. It has been assessed by a Reviewing Editor and by 3 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.

The reports are copied at the end of this email. Please address all of the points and incorporate all requested revisions, or explain in your Response to Referees why a change has not been made.

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.

I hope you will find the comments helpful and have no difficulty returning your revisions within 4 weeks.

Your revised manuscript should be submitted online using the links in Author Tasks Link Not Available.

Any image files uploaded with the previous version are retained on the system. Please ensure you replace or remove all files that have been revised.

REVISION CHECKLIST:

- Article file, including any tables and figure legends, must be in an editable format (eg Word)

- Upload each figure as a separate high quality file

- Upload a full Response to Referees, including a response to any Senior and Reviewing Editor Comments;

- Upload a copy of the manuscript with the changes highlighted.

You may also upload:

- A potential 'Cover Art' file for consideration as the Issue's cover image;

- Appropriate Supporting Information (Video, audio or data set https://jp.msubmit.net/cgi-

bin/main.plex?form_type=display_requirements#supp).

To create your 'Response to Referees' copy all the reports, including any comments from the Senior and Reviewing Editors, into a Word, or similar, file and respond to each point in colour or CAPITALS and upload this when you submit your revision.

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,

Michael C. Hogan
Senior Editor
The Journal of Physiology
https://jp.msubmit.net
http://jp.physoc.org
The Physiological Society
Hodgkin Huxley House
30 Farringdon Lane
London, EC1R 3AW
UK
http://www.physoc.org
http://journals.physoc.org

EDITOR COMMENTS

Reviewing Editor:

Thank you for revising the manuscript. Reviewer 1 and Reviewer 3 believe there are merits in the work and that it could be a quite influential paper if published. Both have also provided comments that they feel will improve the manuscript and the authors are recommended to consider these comments in detail when undertaking any revisions.

REFEREE COMMENTS

Referee #1:

The authors have in the revised manuscript and responses, addressed my main concerns with editorial revisions. This revised manuscript now appears much stronger. The Discussion is still long and exhaustive, which is understandable given the large data set and important physiological question addressed. Congratulations with the study and manuscript.

Referee #2:

The referees have addressed several of my previous concerns in a satisfactory manner, but several other concerns remain (see below).

1. Lines 251-253 - You have assumed that the TOI during ischaemia represents the maximal levels of deoxygenation reachable. I assume that you are referring to the 20 s ischemic recovery between bouts. However, the units you present are on the order of 50 AU (sometimes you use % and sometimes AU, which is confusing). In your earlier publication (Morales-Alamo et al 2015) you showed clearly that 10 min of ischaemia brought TOI from a basal level of about 75% (similar to basal in present study) down to a stable level of about 30%. With intense exercise (even more intense than current study), the values were generally on the order of 50-60%, as in the present study. So how can one accept that your assumption is correct?

2. Lines 283-285 - In this context, the references you cite for complete oxygen depletion during ischemia are not relevant for your conditions of study. Some of those papers induced oxygen depletion during ischemic isometric contraction (>65% MVC force, which will certainly deplete the local oxygen store within a few s) followed by circulatory occlusion over several min. Under those conditions there is certainly no oxygen in the muscle. But that is not at all clear from your design. And it is highly unlikely that oxygen will be depleted during 20 s of ischemia in a resting muscle (ie during recovery), as the metabolic demand is very low.

3. Lines 285-290 - Keeping the above in mind, the use of O2 deficit estimates under the current conditions of study raise questions regarding quantitative estimates of lactate production in active muscle. It would be of interest to see what sort of values you would get with McArdles patients (who cannot produce lactate) using your approach.

4. Regarding your response to my previous criticism of using RER under the current conditions to reflect anaerobic metabolism, I am less than convinced. Your suggestion that the increased CO2 and H+, consequent to lactic acid production, will drive increases in RER to exceed 1.00 is questionable. I refer you to the elegant studies by Hagberg et al. clearly dissociating CO2 and H+ in driving ventilation, as studied in McArdles patients (JAP 52:991, 1982; JAP 68:1393, 1990). Moreover, ventilation during exercise is likely to be mostly dependent on central neural mechanisms (Science 211:844, 1981).

Referee #3:

I appreciate the authors' efforts to substantively revise their manuscript in responses to the reviewers' comments. My one remaining comment extends from my previous major comment regarding the sample size estimation. The authors have provided additional context regarding the estimation for the effect size used (0.5; revised from the original submission), as well as standard inputs for α error probability (0.05) and power (0.80) and assumed correlation (0.5). The specific type of test used in G*Power is still unclear (i.e., t-test, F-test, and specific type, e.g., between factors?) - this can obviously have a tremendous impact on the resulting output. The sample size seems low given the design, at least from a purely statistical perspective. In saying this, I appreciate that the sample size is on the larger side for a mechanistic physiological study,

as well as the overall amount of work (and cost) involved in an undertaking of this sort. While not meaning to belabor the point unnecessarily - or hold the authors to an unfair standard that is higher than for other submissions in the current environment - I am nonetheless mindful of the renewed efforts by the Journal to enhance and justify the nature of statistical reporting as articulated in multiple editorials and other venues over the last several years, including recently PMID: 33507571 (DOI: 10.1113/JP281360). I would encourage the authors to further reflect on this point and include a brief section in the discussion that considers the potential limitations of the design from a statistical perspective - perhaps expanding on the present limitations section. Last, the authors may wish to consider reporting effect sizes and confidence intervals, at least for major variables of interest. This would of value to readers, both to put the magnitude of changes in better context, and assist in the estimation of sample sizes for other studies on related topics.

END OF COMMENTS

EDITOR COMMENTS

Reviewing Editor:

Thank you for revising the manuscript. Reviewer 1 and Reviewer 3 believe there are merits in the work and that it could be a quite influential paper if published. Both have also provided comments that they feel will improve the manuscript and the authors are recommended to consider these comments in detail when undertaking any revisions.
Thank you. We have carefully considered the new comments.

REFEREE COMMENTS

Referee #1:

The authors have in the revised manuscript and responses, addressed my main concerns with editorial revisions. This revised manuscript now appears much stronger. The Discussion is still long and exhaustive, which is understandable given the large data set and important physiological question addressed. Congratulations with the study and manuscript.
We appreciate and acknowledge your contribution to improve this manuscript.
Thanks, indeed.

Referee #2:

The referees have addressed several of my previous concerns in a satisfactory manner, but several other concerns remain (see below).

1. Lines 251-253 - You have assumed that the TOI during ischaemia represents the maximal levels of deoxygenation reachable. I assume that you are referring to the 20 s ischemic recovery between bouts. However, the units you present are on the order of 50 AU (sometimes you use % and sometimes AU, which is confusing). In your earlier publication (Morales-Alamo *et al.*, 2015) you showed clearly that 10 min of ischaemia brought TOI from a basal level of about 75% (similar to basal in present study) down to a stable level of about 30%. With intense exercise (even more intense than current study), the values were generally on the order of 50-60%, as in the present study. So how can one accept that your assumption is correct?

Thank you for pointing this out. We have corrected this mistake in the revised version since all reported TOI values should be in TOI units (i.e., arbitrary units) throughout the manuscript.
Our present results concur with our previous study. We used the same device and male subjects, which were similar in physical characteristics and training status to the male subject participating in the present investigation. Therefore, resting TOI values, as pointed out by the reviewer, were similar in the two studies. Ischaemic TOI values were also similar in the two studies. In Morales-Alamo et al. 2015 we also determined the TOI values after 10 min of ischaemia at rest in the supine position with leg raised. In that study, the TOI stabilized after 8 min at lower levels than when ischaemia was applied at exhaustion. In follow-up experiments with post-exhaustion ischaemia periods up to 90-115 s, we have confirmed that the TOI drops very fast at exhaustion stabilizing, as seen in Fig. 4 of Morales-Alamo et al. (2015) at the beginning of the ischaemia. This is interpreted by the reviewer as an indication of incomplete deoxygenation when ischaemia is applied at exhaustion. However, the latter would not be compatible with the high energy turnover measured during the ischaemia (32% of leg VO_2peak from the 10th to the 60th second of recovery in Morales-Alamo et al. (2015). So then why is the TOI plateauing at a lower level after 8-10 min of ischaemia applied in the rested state? The most plausible explanation is related to differences in skin blood flow and the amount of blood trapped in the leg when ischaemia is applied. Before the start of the 10 min ischaemia in Morales-Alamo et al. (2015), the subjects were in the supine position, and the leg was raised for several minutes to reduce as much as possible the amount of blood in the leg to minimize the risk of thrombosis, as done before applying cuffs before knee surgery.
In contrast, when the occlusion is swiftly applied at the end of an incremental exercise to exhaustion, the subjects are in the upright position, skin blood flow is increased, and leg blood flow is 20-fold higher, i.e., a larger amount of blood is trapped in the legs during the post-exercise occlusions. Part of this blood is in the skin and subcutaneous adipose tissue, while the major part in the veins draining the leg muscles. Therefore, the value of TOI corresponding to zero oxygenation depends on the amount of blood trapped in the system and its distribution between the different tissues and vascular beds contributing to TOI. In other words, the TOI measured with the leg exsanguinated cannot be compared with the TOI measured during ischaemia at the end of exercise (TOI_{OBV}). This interpretation is supported by the fact that the TOI stops dropping much earlier (within seconds) and at a higher value during post-exercise ischaemia than when the occlusion is applied at rest. Finally, if we calculate a common regression equation using the values of TOI and actual fractional O_2 extraction (measured with catheters and reported in the appendix), the calculated 100% extraction value corresponds to a zero difference between TOI_{OBV} – TOI_{MIN}. The latter implies that the TOI_{MIN} corresponds to the zero-oxygenation level in terms of O_2 extraction. This does not negate the possibility of some oxygen remaining in the venules and veins draining the muscles, which cannot diffuse backwards to the capillaries and muscle mitochondria.

2. Lines 283-285 - In this context, the references you cite for complete oxygen depletion during ischemia are not relevant for your conditions of study. Some of those papers induced oxygen depletion during ischemic isometric contraction (>65% MVC

force, which will certainly deplete the local oxygen store within a few s) followed by circulatory occlusion over several min. Under those conditions there is certainly no oxygen in the muscle. But that is not at all clear from your design. And it is highly unlikely that oxygen will be depleted during 20 s of ischemia in a resting muscle (ie during recovery), as the metabolic demand is very low.

We due respect, we disagree with the interpretation made by the reviewer. First, we measured the energy turnover during postexercise ischaemia in Morales-Alamo et al., and it was 20 times the resting energy expenditure (Morales-Alamo et al. pp 4643). The latter means that if O_2 is available and the time required to reach zero oxygenation is 8 min at rest (480 s), the expected time would be 24 s with this metabolic rate equivalent to 32% leg VO_2peak. However, the actual time is much less because at the start of the post-exercise occlusion, myoglobin is partly deoxygenated, and the mean capillary O_2 content much lower, and the metabolic rate much higher. In summary, our interpretation is supported by robust experimental data.

3. Lines 285-290 - Keeping the above in mind, the use of O_2 deficit estimates under the current conditions of study raise questions regarding quantitative estimates of lactate production in active muscle. It would be of interest to see what sort of values you would get with McArdles patients (who cannot produce lactate) using your approach.

Our O_2 deficit estimates could be minimally affected by any O_2 remaining in the muscle at the start of the recovery period. However, we were careful to use post-exercise occlusions long enough to allow for almost complete depletion of O_2 stores during the post-exercise ischaemic recovery. MacArdles' disease patients would not tolerate our exercise model since they would be cramping during post-exercise ischaemia. Nevertheless, this could be a good idea for future studies using small muscle mass exercise models.

4. Regarding your response to my previous criticism of using RER under the current conditions to reflect anaerobic metabolism, I am less than convinced. Your suggestion that the increased CO2 and H+, consequent to lactic acid production, will drive increases in RER to exceed 1.00 is questionable. I refer you to the elegant studies by Hagberg et al. clearly dissociating CO2 and H+ in driving ventilation, as studied in McArdles patients (JAP 52:991, 1982; JAP 68:1393, 1990). Moreover, ventilation during exercise is likely to be mostly dependent on central neural mechanisms (Science 211:844, 1981).

Hagberg et al. (1982; Hagberg *et al.*, 1990) studied MacArdles' patients. MacArdle patients performed incremental exercise to exhaustion and reached exhaustion at a VO_2max of 1.2 L/min, 50% below the 2.6 L/min value observed in the same study in healthy controls of similar age. In agreement with our interpretation, the maximal exercise mean RER value of the MacArdles' patients was 1.05 in one study (Hagberg *et al.*, 1982) and 1.02 in the other (Hagberg *et al.*, 1990), i.e., far below that observed in healthy subjects. Nevertheless, this low RER in MacArdles' disease patients is not caused by insufficient hyperventilation, as pointed out by the reviewer. One of the best indicators of hyperventilation is $P_{ET}CO_2$, and these patients reached lower values of $P_{ET}CO_2$ (around 24 mmHg at exhaustion) than the control subjects. No doubt that central neural mechanisms play an essential role in regulating exercise ventilation, but

recent studies have demonstrated that muscle afferent also contributes to exercise hyperventilation. Finally, the study by Nielsen et al. (2002) demonstrated that acidification plays a role in the regulation of pulmonary ventilation during supramaximal exercise. In this study, Nielsen et al. infused saline or bicarbonate into a central vein during supramaximal exercise. Without bicarbonate, the arterial pH dropped from 7.4 at rest to 7.05 at exhaustion. With bicarbonate, the arterial pH was maintained close to 7.4 throughout the exercise; consequently, pulmonary ventilation was lower during the supramaximal exercise with bicarbonate infusion. Interestingly, bicarbonate infusion was associated with much larger lactate accumulation in blood and greater CO_2 production due to H+ buffering by bicarbonate and, hence, a much larger RER. Thus, both types of studies (Hagberg et al. and Nielsen et al.) agree with our current interpretation: the lower RER during the first supramaximal bout at 120% of VO_2max in our female subjects is compatible with a lower energy production through the glycolysis.

Therefore, we briefly mention Hagberg's studies in the discussion to reinforce our interpretation of RER and glycolytic energy production.

We want to express our gratitude for the effort put in reviewing our manuscript and the insightful comments provided by the reviewer.

Referee #3:

I appreciate the authors' efforts to substantively revise their manuscript in responses to the reviewers' comments. My one remaining comment extends from my previous major comment regarding the sample size estimation. The authors have provided additional context regarding the estimation for the effect size used (0.5; revised from the original submission), as well as standard inputs for α error probability (0.05) and power (0.80) and assumed correlation (0.5). The specific type of test used in G*Power is still unclear (i.e., t-test, F-test, and specific type, e.g., between factors?) - this can obviously have a tremendous impact on the resulting output. The sample size seems low given the design, at least from a purely statistical perspective. In saying this, I appreciate that the sample size is on the larger side for a mechanistic physiological study, as well as the overall amount of work (and cost) involved in an undertaking of this sort. While not meaning to belabor the point unnecessarily - or hold the authors to an unfair standard that is higher than for other submissions in the current environment - I am nonetheless mindful of the renewed efforts by the Journal to enhance and justify the nature of statistical reporting as articulated in multiple editorials and other venues over the last several years, including recently PMID: 33507571 (DOI: 10.1113/JP281360). I would encourage the authors to further reflect on this point and include a brief section in the discussion that considers the potential limitations of the design from a statistical perspective - perhaps expanding on the present limitations section. Last, the authors may wish to consider reporting effect sizes and confidence intervals, at least for major variables of interest. This would of value to readers, both to put the magnitude of changes in better context, and assist in the estimation of sample sizes for other studies on related topics.

We appreciate the expert comments made by the reviewer. We have included in the manuscript information regarding the type of test used in G*Power (F-tests, ANOVA for repeated measures between factors). We have calculated effect sizes for each

between sex comparison and reported the values together with the 95% confidence interval. We have added in the limitations section a comment regarding the relevance of the sample size, highlighting some of the comparisons for which we cannot rule out a type 2 error.
We want to express our gratitude for your expert advice.

References

Hagberg JM, Coyle EF, Carroll JE, Miller JM, Martin WH & Brooke MH. (1982). Exercise hyperventilation in patients with McArdle's disease. *Journal of applied physiology: respiratory, environmental and exercise physiology* **52,** 991-994.

Hagberg JM, King DS, Rogers MA, Montain SJ, Jilka SM, Kohrt WM & Heller SL. (1990). Exercise and recovery ventilatory and VO2 responses of patients with McArdle's disease. *J Appl Physiol (1985)* **68,** 1393-1398.

Morales-Alamo D, Losa-Reyna J, Torres-Peralta R, Martin-Rincon M, Perez-Valera M, Curtelin D, Ponce-Gonzalez JG, Santana A & Calbet JA. (2015). What limits performance during whole-body incremental exercise to exhaustion in humans? *J Physiol* **593,** 4631-4648.

Nielsen HB, Bredmose PP, Stromstad M, Volianitis S, Quistorff B & Secher NH. (2002). Bicarbonate attenuates arterial desaturation during maximal exercise in humans. *J Appl Physiol* **93,** 724-731.

2nd Revision - Editorial Decision 17-Jun-2021

Dear Dr Calbet,

Re: JP-RP-2021-281293R2 "Functional reserve and sex differences during exercise to exhaustion revealed by post-exercise ischaemia and repeated supramaximal exercise" by Marcos Martin Rincon, Miriam Gelabert-Rebato, Mario Perez-Valera, Victor Galvan-Alvarez, David Morales-Alamo, Cecilia Dorado, Robert Boushel, Jostein Hallén, and Jose A. L. Calbet

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.

The last Word version of the paper submitted will be used by the Production Editors to prepare your proof. When this is ready you will receive an email containing a link to Wiley's Online Proofing System. The proof should be checked and corrected as quickly as possible.

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

Are you on Twitter? Once your paper is online, why not share your achievement with your followers. Please tag The Journal (@jphysiol) in any tweets and we will share your accepted paper with our 23,000+ followers!

Yours sincerely,

Michael C. Hogan
Senior Editor
The Journal of Physiology
https://jp.msubmit.net
http://jp.physoc.org
The Physiological Society
Hodgkin Huxley House
30 Farringdon Lane
London, EC1R 3AW
UK
http://www.physoc.org
http://journals.physoc.org

P.S. - You can help your research get the attention it deserves! Check out Wiley's free Promotion Guide for best-practice recommendations for promoting your work at www.wileyauthors.com/eeo/guide. And learn more about Wiley Editing Services which offers professional video, design, and writing services to create shareable video abstracts, infographics, conference posters, lay summaries, and research news stories for your research at www.wileyauthors.com/eeo/promotion.

* IMPORTANT NOTICE ABOUT OPEN ACCESS *

Information about Open Access policies can be found here
https://physoc.onlinelibrary.wiley.com/hub/access-policies

To assist authors whose funding agencies mandate public access to published research findings sooner than 12 months after publication The Journal of Physiology allows authors to pay an open access (OA) fee to have their papers made freely available immediately on publication.

You will receive an email from Wiley with details on how to register or log-in to Wiley Authors Services where you will be able to place an OnlineOpen order.

You can check if you funder or institution has a Wiley Open Access Account here
https://authorservices.wiley.com/author-resources/Journal-Authors/licensing-and-open-access/open-access/author-compliance-tool.html

Your article will be made Open Access upon publication, or as soon as payment is received.

If you wish to put your paper on an OA website such as PMC or UKPMC or your institutional repository within 12 months of publication you must pay the open access fee, which covers the cost of publication.

OnlineOpen articles are deposited in PubMed Central (PMC) and PMC mirror sites. Authors of OnlineOpen articles are permitted to post the final, published PDF of their article on a website, institutional repository, or other free public server, immediately on publication.

Note to NIH-funded authors: The Journal of Physiology is published on PMC 12 months after publication, NIH-funded authors DO NOT NEED to pay to publish and DO NOT NEED to post their accepted papers on PMC.

EDITOR COMMENTS

Reviewing Editor:

Thank you for making the changes to the revised version. Both reviewers are happy with these revisions.

REFEREE COMMENTS

Referee #1:

Accept

Referee #3:

I appreciate the authors' considerate efforts to further revise their manuscript and have no further comments.

****