The Journal of Physiology

Fibre Type and Localisation-Specific Muscle Glycogen Utilisation during Repeated High-Intensity Intermittent Exercise

Jeppe Foged Vigh-Larsen, Niels Ørtenblad, Ole Emil Andersen, Hallur Thorsteinsson, Thea Holm Kristiansen, Stine Bilde, Mads Sloth Mikkelsen, Joachim Nielsen, Magni Mohr, and Kristian Overgaard
DOI: 10.1113/JP283225

Corresponding author(s): Jeppe Foged Vigh-Larsen (jeppefoged@ph.au.dk)

The referees have opted to remain anonymous.

Review Timeline:

Submission Date:	21-Apr-2022
Editorial Decision:	24-May-2022
Revision Received:	28-Jun-2022
Editorial Decision:	20-Jul-2022
Revision Received:	09-Aug-2022
Accepted:	24-Aug-2022

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.)

Dear Mr Vigh-Larsen,

Re: JP-RP-2022-283225 "Fibre Type and Localisation-Specific Muscle Glycogen Utilisation during Repeated High-Intensity Intermittent Exercise" by Jeppe Foged Vigh-Larsen, Niels Ørtenblad, Ole Emil Andersen, Hallur Thorsteinsson, Thea Holm Kristiansen, Stine Bilde, Mads Sloth Mikkelsen, Joachim Nielsen, Magni Mohr, and Kristian Overgaard

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

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

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.

Authors are asked to use The Journal's premium BioRender (https://biorender.com/) account to create/redraw their Abstract Figures. Information on how to access The Journal's premium BioRender account is here: https://physoc.onlinelibrary.wiley.com/journal/14697793/biorender-access and authors are expected to use this service. This will enable Authors to download high-resolution versions of their figures. The link provided should only be used for the purposes of this submission. Authors will be charged for figures created on this premium BioRender account if they are not related to this manuscript submission.

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)

- Abstract figure file (see above)

- Statistical Summary Document

- 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

REQUIRED ITEMS:

- Author photo and profile. First (or joint first) authors are asked to provide a short biography (no more than 100 words for one author or 150 words in total for joint first authors) and a portrait photograph. These should be uploaded and clearly labelled with the revised version of the manuscript. See Information for Authors for further details.

- The contact information provided for the person responsible for 'Research Governance' at your institution is an author on this paper. Please provide an alternative contact who is not an author on this paper or confirm that the author whose email was provided has sole responsibility for research governance. This is the person who is responsible for regulations, principles and standards of good practice in research carried out at the institution, for instance the ethical treatment of animals, the keeping of proper experimental records or the reporting of results.

- The Journal of Physiology funds authors of provisionally accepted papers to use the premium BioRender site to create high resolution schematic figures. Follow this link and enter your details and the manuscript number to create and download figures. Upload these as the figure files for your revised submission. If you choose not to take up this offer we require figures to be of similar quality and resolution. If you are opting out of this service to authors, state this in the Comments section on the Detailed Information page of the submission form. The link provided should only be used for the purposes of this submission. Authors will be charged for figures created on this premium BioRender account if they are not related to this manuscript submission.

- Please upload separate high-quality figure files via the submission form.

- Please ensure that any tables are in Word format and are, wherever possible, embedded in the article file itself.

- Please ensure that the Article File you upload is a Word file.

- A Statistical Summary Document, summarising the statistics presented in the manuscript, is required upon revision. It must be on the Journal's template, which can be downloaded from the link in the Statistical Summary Document section here: https://jp.msubmit.net/cgi-bin/main.plex?form_type=display_requirements#statistics.

- Papers must comply with the Statistics Policy - https://jp.msubmit.net/cgi-bin/main.plex?form_type=display_requirements#statistics.

In summary:

- If n {less than or equal to} 30, all data points must be plotted in the figure in a way that reveals their range and distribution. A bar graph with data points overlaid, a box and whisker plot or a violin plot (preferably with data points included) are acceptable formats.

- 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.

- Statistics Summary Document completed appropriately upon revision.

- A Data Availability Statement is required for all papers reporting original data. This must be in the Additional Information section of the manuscript itself. It must have the paragraph heading "Data Availability Statement". All data supporting the results in the paper must be either: in the paper itself; uploaded as Supporting Information for Online Publication; or archived in an appropriate public repository. The statement needs to describe the availability or the absence of shared data. Authors must include in their Statement: a link to the repository they have used, or a statement that it is available as Supporting Information; reference the data in the appropriate sections(s) of their manuscript; and cite the data they have shared in the References section. Whenever possible the scripts and other artefacts used to generate the analyses presented in the paper should also be publicly archived. If sharing data compromises ethical standards or legal requirements then authors are not expected to share it, but must note this in their Statement. For more information, see our Statistics Policy.

- Please include an Abstract Figure. 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:

This manuscript has been considered by two expert reviewers in the field of muscle metabolism. Both see merit in the work and acknowledge the significant amount of work that has gone into generating the paper. However, both also see a number of issues with the paper in its current form, some of which are major. Indeed, it would appear that additional analysis is merited to enable the authors to elaborate more specifically on the potential role of the initial muscle glycogen content on their study outcomes particularly as the variation between volunteers is considerable, i.e. did the initial muscle glycogen content influence SS, IMF, and intra glycogen use in the type I and type II fibres?

REFEREE COMMENTS

Referee #1:

First of all this referee wants to congratulate the authors with their excellent work. The amount of work done is impressive, and in general the manuscript reads very well and delivers interesting novel findings, for sure. However, due to the exercise protocol used, in combination with the extreme variation of initial muscle glycogen contents between subjects, this referee is sometimes unsure about the interpretation of the data. Precisely, what observations are due to the very specific exercise protocol used (EX1-2-3 + RSA testing), and what is the possible confounding role of initial glycogen contents in the outcomes of the study? This referee in perceives the discussion in places to be very speculative and lacking some strong conclusions about the specific role of subcellular glycogen fractions in muscle energy provision during high-intensity intermittent exercise + its potential role in the development of muscle fatigue.

MAJOR

(1) Figure 4 shows that there was a very large range in the initial muscle glycogen concentrations. Values actually range between ~350 and 650 mmol per kg dw, which represents low physiological up to muscle glycogen contents. To the opinion of this referee, this wide range requires some explanation because subjects were 'well-trained', which is hardly compatible with muscle glycogen contents as low as 350 mmol per kg dm. Therefore, how was carbohydrate intake and exercise controlled in the 48 hours before the experiments?

The results of the study also clearly show that during this type of intermittent exercise the active muscles apparently select the fibres where in glycogen fuel is still available. Thus, initial muscle fibre content probably is a primary determinant of the output of the current study? Therefore, it may be essential to know whether SS, IMF, and intra glycogen levels in the type I and type II fibres were dependent on the initial muscle glycogen content, and how this possibly impacted net glycogen breakdown during the intermittent exercise protocol?

Along the same line: it is clear that EX1 largely depleted IMF and intra glycogen in the type II fibres. But, how should this be

interpreted? Was IMF and intra glycogen only completely degraded in the individuals starting with (very) low muscle glycogen, whilst individuals with high initial contents were able to continue using IMF and intra glycogen throughout EX2 and EX3? Must individuals with too low muscle glycogen contents not be excluded from the study?

Additional analyses to look at the potential role of initial glycogen content could significantly add to the importance of the paper because it could improve the understanding of the role of muscle glycogen loading in endurance exercise performance

(2) The authors use 20% of the baseline geometric mean as an arbitrary cut-off level for glycogen depletion. This referee doubts whether such approach is physiologically relevant. The cut-off value must probably be an absolute value (~100 mmol per kg dw?) below which phosphorylase activity in a muscle fiber is probably turned down because of lack of glycogen substrate? Setting an absolute value for 'glycogen depletion' could change the interpretation of figure 7.

(3) The exercise protocol chosen is quite complex because sprint-interval exercise (RSA) was alternated with intermittent high-intensity endurance training. Why such protocol was chosen? It is clear from the introduction that the study focusses on fibre-specific glycogen utilization during EX1-EX2-EX3. But, what was the potential impact of the RSA exercise on glycogen utilization during EX1-EX2-EX3? The authors in fact discuss the results as if the RSA was not part of the exercise protocol. Or was RSA only used to assess muscle fatigue in type II fibers? But neither the RSA results nor the EX results are correlated with the depletion of subcellular glycogen depots

MINOR

Lines 119-122 - It is well established that metabolic features of type I and type II muscle fibres can largely differ between arm and leg muscles, because training status between the two sites in most individuals is very different, with leg muscles being much better trained than arm muscles (Ørtenblad et al, 2018 and others)

Lines 157-165 - please add whether the subjects were endurance trained resistance trained, sprint trained? Important to know what type of training they were involved in.

Lines 191-192 - agree that this 'pre'-exercises may not have significantly altered muscle glycogen content. Yet such pre-exercise could alter the activation of glycogen phosphorylase and PFK in EX? And if so, this may alter the results of the study?

Line 221 - did the authors consider a potential role of cycling cadence in muscle fiber recruitment (Dantas et al., 2009)?

Lines 326-328 - for this type of study it is probably more relevant just to look at the p-value of the calculated regression rather than using the Hopkins criteria?

Lines 337-338 - the degree of fatigue for this type of intermittent exercise is surprisingly low, isn't it? Was the workload underestimated, which means that none of the EX bouts was nearly maximal? Maybe it would be interesting to clearly explain in the methods section whether the exercise protocol aimed to induce a substantial degree of muscle fatigue, or the intention was rather to maintain constant exercise loads throughout EX1-2-3?

Figure 4 - the figure clearly shows that some subjects had depleted nearly all muscle glycogen by the end of EX1. Can you really use these subjects for the purpose of this study? Because when glycogen is 'out', it is difficult to further evaluate the breakdown in EX2 and EX3.

Figure 7 - this referee tried to clearly understand and interpret these figures, yet after multiple attempts it is not yet clear what is the relevance of this way of presenting the data.

Table 1 - the PCr, lactate, and pH data shown in this table clearly show that metabolism shifted from largely anaerobic in EX1, to predominantly oxidative in EX2+EX3.

Figure 8 - one should probably not draw a regression line on a non-significant correlation (panel B)? Furthermore, when calculating a pearson correlation the data points used must represent independent measures? This is not the case in these figures because values measured after EX3 obviously depend on the values measured after EX1 in the same individual.

Lines 484-488 - these conclusions are a bit vague and therefore somewhat disappointing to this referee."Collectively, this suggests that the utilisation of subcellular glycogen fluctuates both temporally and spatially" and "In association with the heterogeneity in single-fibre depletion patterns this may constitute an important link between reductions in muscle glycogen content and fatigue". Furthermore, I do not really see how the latter conclusion is supported by the data shown in the manuscript because (1) the too low initial glycogen contents in some subjects + (2) the degree of fatigue induced by EX1-2-3 is really marginal (3%, on average, which means that some subjects probably did not exhibit any fatigue at all?).

Lines 502-503 - 'reduced glycolytic flux coupled with fatigue development in type 2 fibres'. What is the meaning of 'coupled with' here? Is the fatigue due to reduced glycolytic flux? Or does the fatigue result from other metabolic factors which in turn suppress glycolytic flux?

Lines 520-523 - if anything glucose-6-phosphate is probably elevated at the end of EX1, but no more after EX2 and EX3? It would be interesting to measure G6P in the residual muscle samples to support this statement.

Lines 545-546 - isn't it a bridge too far to state that subcellular depletion pattern may be independent of exercise intensity? Such statement in fact also implies that subcellular depletion pattern is independent on the contribution of glycolytic versus oxidative glycogen breakdown in energy provision? (see also lines 629-634 in this regard). If subcellular depletion is independent of exercise intensity and glycolytic versus oxidative glycogen breakdown, why do we need different subcellular fractions in muscle, especially IMF versus intra?

586-587 - this referee is wondering which precise data in the manuscript really provide strong direct support for the statement that intra glycogen may be implicated in muscle fatigue in type II fibers?

Referee #2:

In this manuscript fibre type and subcellular localisation of glycogen and glycogen utilisation was explored. The manuscript is well written and provides a significant and impactful contribution to the literature, by recognised researchers in the field who lead they way in providing original research in this area. Our understanding of glycogen metabolism is limited when subcellular compartments are considered, and yet over the years of research it has been shown that these are important. This manuscript addresses those and contributes to insights into mechanisms. The study and experimental designs are robust. There are a few comments that should be addressed to tighten the conclusion. The major concern being the use of relative terms when reporting and then discussing glycogen utilisation in the three fractions. Given the much larger proportion of SS and IMF glycogen, the decline in the intra is perhaps high as a %, this is very low in absolute amounts. Please include in the discussion the subcellular depletion levels in relation to total glycogen pools. This is important, because the increase in metabolic rate is differentially distributed through the muscle fibres, as the authors introduce. This is particularly important to include in the final conclusions.

Also, inclusion of Casey et al, AJP 1996 is necessary. That paper shows fibre dependent glycogen utilisation after two exercise bouts, also adenine nucleotide metabolism. Please include in introduction and discussion.

Minor comments

Throughout, IMF seems to be used in text, but in graphs this is intermyofibrillar. Please be consistent throughout. E.g. some plots have the latter and it is not an obvious transition between the two. I'd be comfortable with intermyofibrillar or inter being used throughout. Then the difference between intra and inter can be visualised better.

Line 52: add glycogen: a higher 'glycogen' breakdown....

Line 66: remove 'Thus'

Line 96: reword "and/or potential unique roles of each glycogen fraction.."

Line 145: it seems odd to hypothesise that there will be large heterogeneity in something. The inclusion of this hypothesis does not appear warranted.

Lines 262-264: the way this is written implies that there were always n=3 type I and type II fibres and n=4 intermediated fibres that we discarded. Please reword to explain what happens.

Line 265: units should be included and what 't' is, defined.

Line 285: add ~10 mg 'dry weight'. Provide the concentration of $HClO_4$ used.

Line 302: it is unclear when 'the other leg' was biopsied. Please provide this information in the appropriate section.

Line 308: include that plasma was removed and stored

Line 315: how is a 2 point calibration curve valid? The slope of the line is only dependent on those two points and will not necessarily be indicative of the relationship between amount of insulin and sample.

Line 316: describe the enzymatic assay

Line 323: please provide detail in appropriate figure legends when the data were log transformed.

Lines 381-392: it is unclear form the statistics shown in the figure that this is the case.

Line 400-408: give the differences in the contribution of each of the glycogen subcellular regions, it is a little misleading to talk about % declines between the different regions in the same section. This would be more clear to be in absolute values - as well as the %. For example, whilst intra is down ~51%, this is 2 um3um 103 compared with 14 and 20 2 um3um 103 IMF and SS, respectively.

Line 409. I don't believe the statistics show this.

Line 413-426: please include the detection limit of glycogen granule size.

Line 461: Rather than 'stable' it would be better to say 'at this elevated level'

Line 481: the statement "fibre types were characterized by pronounced heterogeneity..." is not something that can be concluded from the data presented, aside from there being large SDs. This would be more evident by including individual points, which I realise will be a very busy graph, but possibly worthwhile.

Line 508: ".... And IMF glycogen fractions,...." This was missing from the results, please include in that section also.

Line 518: please include that this refers to cardiac muscle

Line 560: 'has' should be 'have'

Line 572: 'at' should be 'under'

Line 585: 'part' might be better as 'proportion'

Line 615: please include somewhere in discussion the limitation of the smallest granules that can be detected and how that affects the findings and discussion.

Fig 2: how is it ascertained that all panels are type II fibres? Provide how this was determined. Were all fibres shown from the same individual? Assume that is the case, so say so. If not, then they should be.

Please include all TEM images as supp data.

Figure 6: the statistics shown are unclear. Please describe the post-hoc test used here. How can only the two points show interaction?

Figure 7: define the solid and dotted lines. Break N=102 down into n=3 type I, n=3 type II from each of N=17 subjects.

Figure 8B: remove the solid line as that implies significance. Why is only the lower end of the glycogen shown (up to 200 mmol/kg dw) - but in Fig 8A the full extent is shown (ie up to 700 mmol/kg dw). From which samples - pre and post? State details.

Table 1: define (*). Include a line that shows TCr (similar to TAN).

END OF COMMENTS

Confidential Review **21-Apr-2022**

Response to reviewers:

Manuscript - JP-RP-2022-283225 "Fibre Type and Localisation-Specific Muscle Glycogen Utilisation during Repeated High-Intensity Intermittent Exercise" by Jeppe Foged Vigh-Larsen, Niels Ørtenblad, Ole Emil Andersen, Hallur Thorsteinsson, Thea Holm Kristiansen, Stine Bilde, Mads Sloth Mikkelsen, Joachim Nielsen, Magni Mohr, and Kristian Overgaard

---

EDITOR COMMENTS

Reviewing Editor:

This manuscript has been considered by two expert reviewers in the field of muscle metabolism. Both see merit in the work and acknowledge the significant amount of work that has gone into generating the paper. However, both also see a number of issues with the paper in its current form, some of which are major. Indeed, it would appear that additional analysis is merited to enable the authors to elaborate more specifically on the potential role of the initial muscle glycogen content on their study outcomes particularly as the variation between volunteers is considerable, i.e. did the initial muscle glycogen content influence SS, IMF, and intra glycogen use in the type I and type II1 fibres?

We thank the editor for the assessment and consideration of our work, which is highly appreciated. Indeed, we agree that the different initial glycogen contents could potentially be of importance for the utilisation patterns observed. Accordingly, we have performed further analyses to substantiate these findings and responded accordingly to the reviewer comments and included further details in the discussion section. Furthermore, we have critically revised the remaining manuscript in accordance with the constructive and helpful comments provided by the expert reviewers and believe that this has substantially helped improve the clarity and quality of the paper. Moreover, a mistake in the calculation of percentage changes has been corrected in the text body to reflect the changes in geometric means (rather than aritmethic means) as already correctly presented in the initial figures.

REFEREE COMMENTS

Referee #1:

First of all this referee wants to congratulate the authors with their excellent work. The amount of work done is impressive, and in general the manuscript reads very well and delivers interesting novel findings, for sure. However, due to the exercise protocol used, in combination with the extreme variation of initial muscle glycogen contents between subjects, this referee is sometimes unsure about the interpretation of the data. Precisely, what observations are due to the very specific exercise protocol used (EX1-2-3 + RSA testing), and what is the possible confounding role of initial glycogen contents in the outcomes of the study? This referee in

perceives the discussion in places to be very speculative and lacking some strong conclusions about the specific role of subcellular glycogen fractions in muscle energy provision during high-intensity intermittent exercise + its potential role in the development of muscle fatigue.

We thank the reviewer for the positive and constructive feedback, which we have addressed as described in the point-by-point response below. Firstly, we acknowledge the overall comments on the discussion section and conclusions provided, and have reduced the length of some of the more speculative sections of the discussion accordingly and more strongly outlined the conclusions that we deem substantiated by the findings of the present manuscript.

MAJOR

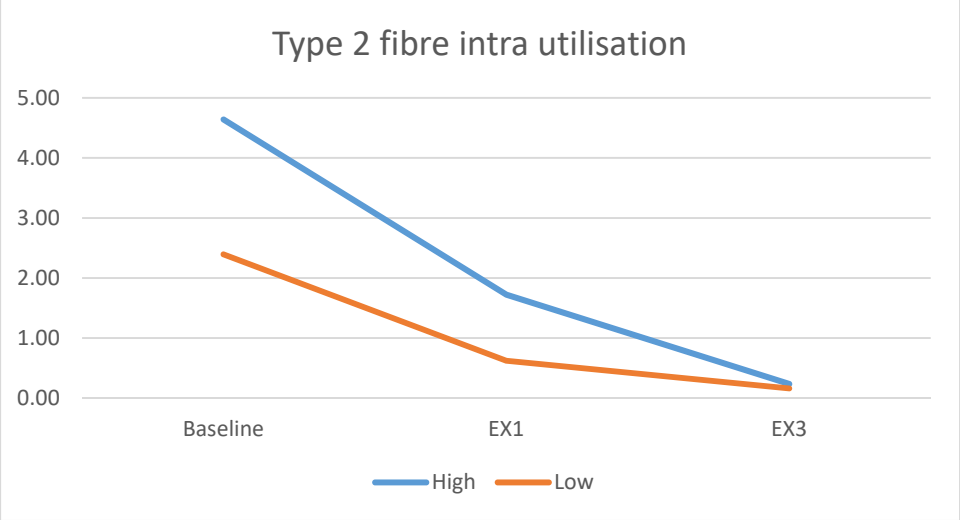
(1) Figure 4 shows that there was a very large range in the initial muscle glycogen concentrations. Values actually range between ~350 and 650 mmol per kg dw, which represents low physiological up to muscle glycogen contents. To the opinion of this referee, this wide range requires some explanation because subjects were 'well-trained', which is hardly compatible with muscle glycogen contents as low as 350 mmol per kg dm. Therefore, how was carbohydrate intake and exercise controlled in the 48 hours before the experiments?

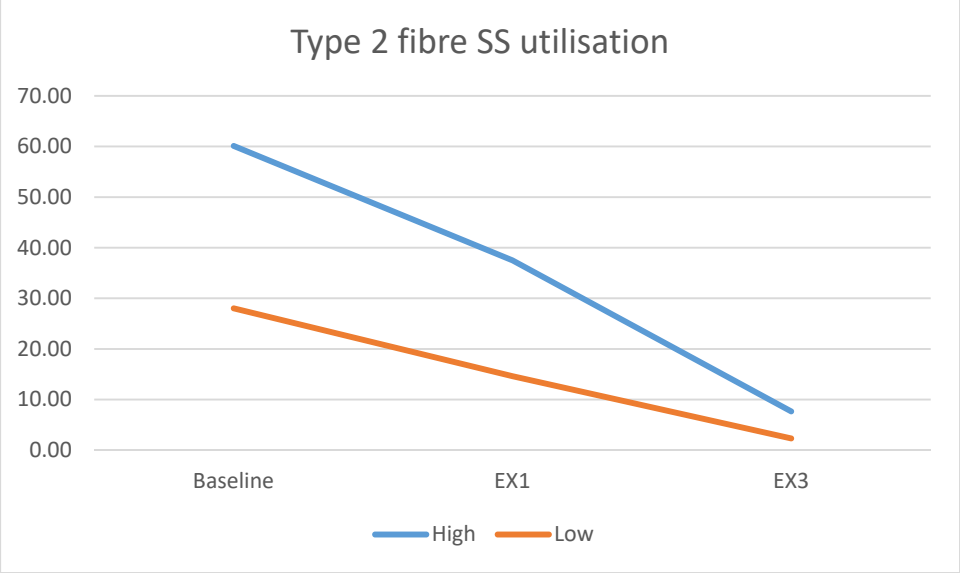
We acknowledge that the variation in initial glycogen is somewhat higher than expected. The subjects were defined as well-trained based on a required minimal VO_{2max} of 50 and an average VO_{2max} of 57 +/- 5 ml/kg/min though this exact statement is somewhat subjective and no exact cut-off values (to the best of our knowledge) are firmly established in the literature. However, given the variation in VO_{2max} between subjects these could likely more appropriately be defined as “moderately to well-trained” which has now been corrected throughout the manuscript and which could explain a major part of the variation in initial glycogen content since the training status indeed was significantly associated with initial glycogen content (r^2=0.27, P=0.027). The participants were instructed not to perform any strenuous exercise in the two days leading up to the experimental day and were provided a standardized meal on the morning of the experimental day in order to ensure the same “acute” substrate availability (blood glucose, FFA etc.). We did not control for the diet in the days preceding the experimental days, although this would likely have reduced the variability in initial glycogen further.

The results of the study also clearly show that during this type of intermittent exercise the active muscles apparently select the fibres where in glycogen fuel is still available. Thus, initial muscle fibre content probably is a primary determinant of the output of the current study? Therefore, it may be essential to know whether SS, IMF, and intra glycogen levels in the type I and type II fibres were dependent on the initial muscle glycogen content, and how this possibly impacted net glycogen breakdown during the intermittent exercise protocol?

This is a relevant proposal. We have studied this further by grouping our subjects in tertiles based on initial whole muscle glycogen content. At baseline no differences were apparent between the highest and lowest tertiles in the relative distribution of IMF, intra and SS glycogen (Low vs. high tertile: 77, 11 and 12% vs. 77, 12 and 12% in type 1 fibres and 81, 11 and 8% vs. 80, 12 and 9% in type 2 fibres), whereas the absolute levels in each subfraction differed as expected. However, although the absolute levels seemed to decrease slightly more in the high

tertile (not statistically different), the relative utilisation of glycogen was not different between each fraction in the highest and lowest tertiles (again based on initial total glycogen) meaning that a higher utilisation of glycogen in the participants with high compared to low initial glycogen levels was similarly distributed across subfractions in relative terms. Accordingly, when for example the intra glycogen fraction was highly used during EX1 this was the case for both the high and low tertile and likewise there was a slowed utilisation in the subsequent bouts for both tertiles as these approached very low post-exercise levels (see figure below), although this reduced utilisation appeared to be slightly more augmented in the low tertile. Thus, based on these patterns we believe that the variation in initial glycogen content do not materially alter the pattern of subcellular glycogen use in the type of exercise used here and therefore, this analysis does not alter the overall results/interpretation of our study. We have incorporated these relevant considerations in the discussion section. Adding these analyses in the results section was considered but since we do not have sufficient power to confidently compare the changes between tertiles statistically we found it most suitable to include in the discussion section.





Along the same line: it is clear that EX1 largely depleted IMF and intra glycogen in the type II fibres. But, how should this be interpreted? Was IMF and intra glycogen only completely degraded in the individuals starting with (very) low muscle glycogen, whilst individuals with high initial contents were able to continue using IMF and intra glycogen throughout EX2 and EX3? Must individuals with too low muscle glycogen contents not be excluded from the study?

Again a valid point, that requires further analysis. During EX1 there was a large reduction in IMF and intra glycogen in type II fibres as stated by the reviewer. In relative terms this reduction was similar between tertiles (~60-70% reductions). Accordingly, low absolute levels were available in both tertiles after EX1 (1.72 and 0.62 um^3 um^{-3} 10^3 compared to baseline levels of 4.64 and 2.39 um^3 um^{-3} 10^3) – see figure above. From this point there was a continuous reduction in both fractions to 0.24 and 0.16 um^3 um^{-3} 10^3 in the high and low tertile, respectively, meaning that in absolute levels the utilisation appeared to be somewhat higher in the high tertile (not statistically significant), but that the relative utilisation (expressed relative to the initial baseline level) during EX2+EX3 was only slightly different between tertiles (~20 and ~30% reductions during EX2+EX3, respectively). Thus, it appears that although there may have been a slightly more pronounced slowing of utilisation in the low tertile, then the pattern was comparable between tertiles in relative terms. Importantly, the pattern in relation to the SS fractions were identical for both tertiles as in contrast the SS fraction in type 2 fibres was used much more homogenously during EX1 and EX2+EX3 (~40-50% reductions during both periods in both tertiles).

Although we would have liked to start with a more homogenous group it would likely be inappropriate to exclude participants retrospectively. However, we have in the manuscript included a further elaboration of the results in relation to the points above in the discussion section.

Additional analyses to look at the potential role of initial glycogen content could significantly add to the importance of the paper because it could improve the understanding of the role of muscle glycogen loading in endurance exercise performance

This has been outlined above and included in the manuscript discussion.

(2) The authors use 20% of the baseline geometric mean as an arbitrary cut-off level for glycogen depletion. This referee doubts whether such approach is physiologically relevant. The cut-off value must probably be an absolute value (~100 mmol per kg dw?) below which phosphorylase activity in a muscle fiber is probably turned down because of lack of glycogen substrate? Setting an absolute value for 'glycogen depletion' could change the interpretation of figure 7.

The 20% value was used in order to be able to compare the degree of depletion between subcellular fractions with markedly different initial absolute levels (e.g. ~75-85% of the total glycogen stored in the IMF fraction). Thus, if absolute levels are to be used we would have to decide on a specific absolute threshold in each fraction which in our view is difficult to determine. This could be approached based on single-fiber studies investigating reductions in

function below different glycogen levels in each fraction, but the data is currently not sufficient to do this confidently. Moreover, glycogen phosphorylase acts on each individual granule meaning that instead of distinct absolute glycogen levels it could perhaps be more relevant to do this based on the individual granule sizes. However, again this will similarly become somewhat arbitrary. Thus, if the initial point of view is that we have stored a given amount of glycogen in each subcellular fraction in proportion to the local subcellular demands in association with the supply from other energy systems, then our contention is that it is relevant to express the depletion in relative terms within each subcellular fraction. On the contrary, we would agree with the reviewer that if for example an inferior amount of intra glycogen is stored compared to the relative demands then we may underestimate the depletion if expressed in relative terms. We have included a brief discussion of this in the discussion section.

(3) The exercise protocol chosen is quite complex because sprint-interval exercise (RSA) was alternated with intermittent high-intensity endurance training. Why such protocol was chosen? It is clear from the introduction that the study focusses on fibre-specific glycogen utilization during EX1-EX2-EX3. But, what was the potential impact of the RSA exercise on glycogen utilization during EX1-EX2-EX3? The authors in fact discuss the results as if the RSA was not part of the exercise protocol. Or was RSA only used to assess muscle fatigue in type II fibers? But neither the RSA results nor the EX results are correlated with the depletion of subcellular glycogen depots

As stated by the reviewer the glycogen utilisation clearly depends on both the repeated sprint tests and the high-intensity intermittent exercise bouts. This has now been more clearly emphasized throughout the manuscript. The repeated maximal sprint tests were included to assess the decline in exercise capacity during the protocol and to more closely reflect the demands during high-intensity intermittent sports where high-intense bouts are commonly interspersed by brief maximal actions. Importantly, one repeated sprint test and one period of high-intensity exercise was framed by the baseline and post EX1 biopsy, whereas two repeated sprints tests and two periods of high-intensity exercise were framed by the post EX1 biopsy and post EX3 biopsy meaning that the impact of the RSA tests was evenly balanced. Thus, the final (4^{th}) repeated sprint test was performed instantly after the last muscle biopsy,

Moreover, the repeated sprints were included to induce a more substantial glycogen breakdown to clearly elucidate any potential differences in glycogen utilisation between subcellular fractions (the reasoning being that if a different utilisation occurs between fractions this will be most clearly elucidated if the breakdown of glycogen and thus separation between fractions is high – although this did not appear to be the case).

MINOR

Lines 119-122 - It is well established that metabolic features of type I and type II muscle fibres can largely differ between arm and leg muscles, because training status between the two sites in most individuals is very different, with leg muscles being much better trained than arm muscles (Ørtenblad et al, 2018 and others).

This is a good point raised by the reviewer which further highlights the novelty of the present study (obtaining leg biopsies during high-intensity exercise) as the only prior quantitative study investigating subcellular muscle glycogen metabolism during high-intensity exercise used triceps biopsies. This point has been included in the introduction.

Lines 157-165 - please add whether the subjects were endurance trained resistance trained, sprint trained? Important to know what type of training they were involved in.

The subjects included were all accustomed to intermittent exercise incorporating elements of high-intense exercise and were not included if only performing endurance exercise training or being mainly resistance trained. As such, the study participants were involved in different intermittent sport activities including soccer, team handball, ultimate frees bee, basketball and/or intermittent running. Moreover, all were accustomed with regular cycling exercise.

Lines 191-192 - agree that this 'pre'-exercises may not have significantly altered muscle glycogen content. Yet such pre-exercise could alter the activation of glycogen phosphorylase and PFK in EX? And if so, this may alter the results of the study?

Glycogen phosphorylase (and similarly PFK) activity is mainly regulated acutely by the energetic state of the muscle e.g. increase in AMP/ATP ratio and/or Ca^{2+} transients and catecholamines and its activity rapidly and markedly increased during intense exercise (and immediately downturned when the resting levels of muscle energy homeostasis are restored (Katz et al. 2022). As such, it is our conception that the activation by the “pre” exercises would be quickly downturned and/or that the repeated sprint test and high-intense intermittent exercise protocols would instantly override any potential baseline alterations in the activity of these enzymes. Moreover, it is common to perform exercise following a warm up such that the current setting represents a relevant exercise scenario.

Line 221 - did the authors consider a potential role of cycling cadence in muscle fiber recruitment (Dantas et al., 2009)?

In the present study high and varying cycling cadences (90-108 RPM during the 45 s bouts and up to 120 RPM during the maximal sprints) were used to reflect the pedaling frequencies utilised when producing high power outputs in cycling races, at least during flat stages (Lucia et al. 2004). According to Dantas et al. 2009 this approach is the most efficient. Additionally, these frequencies may resemble high-frequency movements in other sports. Others (Lucia et al. 2004, Deschenes et al. 2000) have shown similarly that efficiency is improved at high cadences and speculated that this is due to an elevated recruitment of type 1 fibers (due to the lower force requirements during each pedaling stroke). This has been shown prior to this by Ahlquist et al. 1992 at least when comparing cadences of 50 or 100 RPM at a fixed intensity of 85% VO2max, where the low cadence resulted in greater type II fiber depletion. Contrarily, Gollnick et al. 1974 observed no difference in fiber type depletion at supramaximal work loads (120-150% VO2max) when altering the pedaling frequency (30-120 RPM) though these findings may be flawed by the use of an insensitive approach for determining fiber type specific glycogen utilistation (subjective PAS ratings compared to quantitative approach by Ahlquist et al. using microphotometric techniques). Thus, during supramaximal exercise it may be that type 1 and 2

fibers are highly recruited irrespective of pedaling frequency – and with a greater turnover in type 2 fibers due to the distinct metabolic properties – or if we accept the results by Ahlquist et al. that a high cadence facilitates the contribution from type I fibers in addition to type II fibers.

Lines 326-328 - for this type of study it is probably more relevant just to look at the p-value of the calculated regression rather than using the Hopkins criteria?

We agree that the p-values are probably more relevant. For simplicity we refer to the correlations presented using the Hopkins criteria and only for the significant relationships.

Lines 337-338 - the degree of fatigue for this type of intermittent exercise is surprisingly low, isn't it? Was the workload underestimated, which means that none of the EX bouts was nearly maximal? Maybe it would be interesting to clearly explain in the methods section whether the exercise protocol aimed to induce a substantial degree of muscle fatigue, or the intention was rather to maintain constant exercise loads throughout EX1-2-3?

As indicated by the reviewer the intention with the high-intensity intermittent exercise protocol was to instigate marked alterations in muscle homeostasis and induce a high reliance on muscle glycogen metabolism by performing exercise at the highest intensity possible which was sustainable (or almost sustainable..) throughout the protocol. As such the 45-s bouts were performed at ~105% W_{max} (corresponding to around 110% of VO_{2max}) meaning that this was high-intense but not maximal exercise. The subjects were able to complete the protocol with only minor load reductions as intended (<5%) but with marked increases in ratings of perceived exertion to maximal levels (the subjects only barely being able to complete the final bouts). On the contrary, the repeated sprint testing, being a maximal test, revealed a ~17% drop in repeated sprint performance after the protocol clearly demonstrative of substantial fatigue development. This has been further elaborated in the methods section as suggested by the reviewer.

Figure 4 - the figure clearly shows that some subjects had depleted nearly all muscle glycogen by the end of EX1. Can you really use these subjects for the purpose of this study? Because when glycogen is 'out', it is difficult to further evaluate the breakdown in EX2 and EX3.

As highlighted by the reviewer some subjects had low glycogen levels already after EX1 (three subjects below ~150 mmol kg/dw) which means that a relatively low amount of glycogen was available for the remaining exercise periods. This degree of depletion already after EX1 was somewhat higher than expected. However, with our sample size of 17 participants, several participants with larger amounts were available and analyses has now been performed and included in the discussion section on the impact of initial glycogen content on subcellular breakdown.

We would like to emphasize though, that even with substantial reductions in glycogen content during EX1 in some participants, it is still in our conception noteworthy to study the subsequent utilisation pattern during EX2+EX3. Here, comparable low levels were reached within each three subcellular fractions which could not necessarily have been predicted beforehand. Contrarily it could have been the case that one specific fraction was continuously utilised until complete depletion and an inability to continue exercising and that in contrast more glycogen was still

remaining in other fractions, which was not the case. So, despite a marked reduction in glycogen content initially in some subjects this does not preclude their depletion patterns from being of interest, although as addressed in previous comments and incorporated in the discussion section, the divergence between participants with different initial glycogen contents is of course important to be aware of.

Figure 7 - this referee tried to clearly understand and interpret these figures, yet after multiple attempts it is not yet clear what is the relevance of this way of presenting the data.

This figure contains quite a large amount of information. The purpose of figure 7 is to show the depletion of subcellular glycogen and the heterogeneity at the single-fiber level in relation to the gradually lowered muscle glycogen concentrations. As an alternative these could have been plotted as a function of the time point e.g. baseline, post EX1 and post EX3 but due to the variability in whole-muscle glycogen concentrations at each time point this would not provide the same level of information. As can be seen from this figure, the heterogeneity decreases as depletion progresses, while severely emptied single-fibers (here defined as below 20% of the baseline geometric mean as an arbitrary reference point - we have inserted a shaded area in the figure to more clearly show the threshold area) start to present at the total single-fiber level, as well as in the IMF and SS fraction in both fiber types when the whole-muscle glycogen concentrations are reduced somewhere below 200 mmol/kg dw. In contrast, especially intra glycogen in type 2 single-fibers is depleted earlier (see open dots –around 300-400 mmol/kg) in fig. 7E) in accordance with the accelerated breakdown mainly of type 2 fiber intra glycogen. This may indeed be an important part of the explanation for impaired muscle function (e.g. Ca2+ kinetics (Ørtenblad et al. 2011, Duhamel et al. 2007, Gejl 2014) and performance (Vigh-Larsen et al. 2021, Balsom et al. 1999, Bangsbo 1992) previously reported at glycogen concentrations below ~250-300 mmol/kg dw when severe single-fiber depletion can be expected (we do not have many biopsies at this level (250-300 mmol/kg dw) so we can mainly see the associations just above and below this point). In addition, we have incorporated an additional bar graph next to each single-fibre figure to summarize one of the main points with regards to the proportion of depleted fibres (<20%) at each glycogen concentration (0-200, 200-400 and >400).

We chose to express these in percentage in order to be able to compare the level of depletion between each subcellular fraction which are otherwise characterized by largely different initial contents and which are assumed to be stored in quantities in proportion to the physiological demands (unless these are abnormal as in the present study, as interpreted by the authors). Furthermore, the fractions are expressed in different units due to the nature of the volume fraction estimations and thus not directly comparable in absolute units. As such, the intermyofibrillar store is expressed relative to the whole myofibrillar area (and not just the interspace where it is stored because this space is plastic based on the non-static presence and size of mitochondria, lipid droplets, amount of glycogen stored etc). In contrast, intra glycogen is expressed relative to the intramyofibrillar space and subsarcolemmal glycogen relative to the fibre surface length, which is the established methodology applied in the previous literature. This has been more clearly explained in the methods section.

Table 1 - the PCr, lactate, and pH data shown in this table clearly show that metabolism shifted from largely anaerobic in EX1, to predominantly oxidative in EX2+EX3.

We agree with this point which is also discussed briefly in the discussion section line 500-504 and line 629-634. We have changed the wording slightly to more clearly emphasize this point.

Figure 8 - one should probably not draw a regression line on a non-significant correlation (panel B)? Furthermore, when calculating a pearson correlation the data points used must represent independent measures? This is not the case in these figures because values measured after EX3 obviously depend on the values measured after EX1 in the same individual.

We thank the reviewer for pointing this out which is a clear mistake (the regression line on panel B). This has now been removed

We included the values for both EX1 and EX3 for the TAN correlation to show, that the association seem to be clearly augmented below 200 mmol/kg-1 dw. However, we acknowledge that the data points for EX1 and EX3 are not independent and have therefore plotted the regression for EX1 and EX3 separately.

Lines 484-488 - these conclusions are a bit vague and therefore somewhat disappointing to this referee."Collectively, this suggests that the utilisation of subcellular glycogen fluctuates both temporally and spatially" and "In association with the heterogeneity in single-fibre depletion patterns this may constitute an important link between reductions in muscle glycogen content and fatigue". Furthermore, I do not really see how the latter conclusion is supported by the data shown in the manuscript because (1) the too low initial glycogen contents in some subjects + (2) the degree of fatigue induced by EX1-2-3 is really marginal (3%, on average, which means that some subjects probably did not exhibit any fatigue at all?).

We agree that our statements here can be considered too conservative and vague and have revised these accordingly to more clearly specify the study conclusions. The purpose with the high-intensity intermittent exercise bouts (3 periods of 10 x 45 s work) was to exercise the participants at the highest possible sustainable (or almost sustainable) intensity and not intended as a measure of fatigue (although ratings of perceived exertion were recorded throughout and increased markedly). Instead the repeated sprint tests incorporated were maximal and intended to measure exercise tolerance directly. During these, the subjects did indeed exhibit significant fatigue development reflected by a ~10% drop in repeated sprint ability after EX1 and EX2 and a further drop (total of 17%) in repeated sprint ability post EX3 compared to baseline levels (all repeated sprint tests performed after ~2.5 min rest to allow for muscle biopsy sampling, thus with more severe fatigue most likely present instantly post-exercise). Thus, we do believe that the present data provide a possible explanation for reductions in muscle function/performance which is observed in relation to moderately lowered muscle glycogen levels (see for example Vigh-Larsen et al. 2021) as a result of pronounced single-fiber depletion prior to depletion of the whole-muscle stores, and in particular specific depletion in this specific exercise scenario of the intramyofibrillar fraction in type 2 fibers. However, this is difficult to test statistically in the given design since all subjects exhibited a pronounced decrease in glycogen (and intra glycogen) during the exercise protocol and since the individual variation in the decline in repeated sprint ability is sensitive to the degree of fatigue induced directly in relation to the given work load prescribed (which is difficult to balance due to inter-individual differences in the capacity for

intermittent work at a certain percentage of W_{max} and is therefore better tested in a cross over design with the same participant exercising at the exact same intensity across interventions), coupled with the complexity of fatigue development during high-intensity exercise. However, we have altered our wording as suggested by the reviewer.

Lines 502-503 - 'reduced glycolytic flux coupled with fatigue development in type 2 fibres'. What is the meaning of 'coupled with' here? Is the fatigue due to reduced glycolytic flux? Or does the fatigue result from other metabolic factors which in turn suppress glycolytic flux?

This is a good point by the reviewer but a difficult question to answer as in this specific scenario it could be both. So either metabolic perturbations e.g. accumulation of ADP, Pi (and decreases in ATP) and/or other alterations of the muscle metabolic homeostasis (pH, ionic perturbations, ROS, others) which could downturn excitation-contraction coupling processes and hence glycolytix flux independent of glycogen concentrations. Could also be directly related to glycogen depletion (in part) reducing the glycolytic flux and exacerbating the metabolic perturbations, e.g. decreased Pi buffering, accelerated PCr degradation and impaired ATP resynthesis through glycolysis (and potentially through reductions in tricarboxylic acid intermediates).

We have altered this wording to account for both options.

Lines 520-523 - if anything glucose-6-phosphate is probably elevated at the end of EX1, but no more after EX2 and EX3? It would be interesting to measure G6P in the residual muscle samples to support this statement.

We agree that it would have been an interesting addition to also measure G6P which was the intention but had to be omitted due to insufficient tissue yield – measuring G6P, G1P, free glucose etc. requires a large amount of tissue due to the low resting levels in the muscle. However, we believe that it is reasonable to suggest that G-6-P accumulation likely would be lower as exercise progressed and the glycolytic flux through type 2 fibers decreased concomitant with an increased contribution from oxidative phosphorylation as has for example been shown by Gaitanos et al. 1993 or Parolin et al. 1999 after repeated intensive high-intensity exercise.

Lines 545-546 - isn't it a bridge too far to state that subcellular depletion pattern may be independent of exercise intensity? Such statement in fact also implies that subcellular depletion pattern is independent on the contribution of glycolytic versus oxidative glycogen breakdown in energy provision? (see also lines 629-634 in this regard). If subcellular depletion is independent of exercise intensity and glycolytic versus oxidative glycogen breakdown, why do we need different subcellular fractions in muscle, especially IMF versus intra?

The statement is put forward to highlight that in type 1 fibers we do not see a preferential depletion of intra or IMF glycogen, but instead a balanced depletion across fractions despite the fact that the energy turnover (glycogen breakdown rate) is also elevated in type 1 fibers in comparison with previous studies where preferential depletion of intra glycogen in type 1 fibers was observed. Thus, this is somewhat contrary to the perception that exercise intensity is the

(only) driver of specific depletion of intra glycogen. However, we acknowledge the reviewer´s point and have accordingly removed this statement.

586-587 - this referee is wondering which precise data in the manuscript really provide strong direct support for the statement that intra glycogen may be implicated in muscle fatigue in type II fibers?

We agree that we have no data to firmly establish that type II fibers are fatigued in relation to intra depletion although we see substantial specific type 2 fibre intra glycogen depletion concomitant with significant impairments in repeated sprint ability. Instead we can surmise, as stated, that because of the specific depletion of intra glycogen in type 2 single-fibers this could potentially lead to muscle fatigue as we refer to the previously suggested important role of intra glycogen in muscle fatigue (Ørtenblad et al. 2011, Nielsen et al. 2009; 2014, Jensen et al. 2021.

Referee #2:

In this manuscript fibre type and subcellular localisation of glycogen and glycogen utilisation was explored. The manuscript is well written and provides a significant and impactful contribution to the literature, by recognised researchers in the field who lead they way in providing original research in this area. Our understanding of glycogen metabolism is limited when subcellular compartments are considered, and yet over the years of research it has been shown that these are important. This manuscript addresses those and contributes to insights into mechanisms. The study and experimental designs are robust. There are a few comments that should be addressed to tighten the conclusion. The major concern being the use of relative terms when reporting and then discussing glycogen utilisation in the three fractions. Given the much larger proportion of SS and IMF glycogen, the decline in the intra is perhaps high as a %, this is very low in absolute amounts. Please include in the discussion the subcellular depletion levels in relation to total glycogen pools. This is important, because the increase in metabolic rate is differentially distributed through the muscle fibres, as the authors introduce. This is particularly important to include in the final conclusions.

We thank the reviewer for the positive comments and for the helpful suggestions for improving the clarity of the paper. We acknowledge the point about also highlighting the large difference in glycogen content between fractions (and thereby utilisation in absolute terms) and have emphasized this more clearly now in the discussion and conclusion section.

Also, inclusion of Casey et al, AJP 1996 is necessary. That paper shows fibre dependent glycogen utilisation after two exercise bouts, also adenine nucleotide metabolism. Please include in introduction and discussion.

We thank the reviewer for highlighting the interesting paper by Casey et al. which has now included in the introduction and discussion section.

Minor comments

Throughout, IMF seems to be used in text, but in graphs this is intermyofibrillar. Please be

consistent throughout. E.g. some plots have the latter and it is not an obvious transition between the two. I'd be comfortable with intermyofibrillar or inter being used throughout. Then the difference between intra and inter can be visualised better.

Thanks for noticing this inconsistency. We have now corrected the figures to state IMF, SS and intra glycogen in concordance with the text body, as these abbreviations are the ones established and used in the previous literature.

Line 52: add glycogen: a higher 'glycogen' breakdown....

This has now been added, as suggested.

Line 66: remove 'Thus'

This has been removed.

Line 96: reword "and/or potential unique roles of each glycogen fraction.."

This specific sentence has been reworded as suggested.

Line 145: it seems odd to hypothesise that there will be large heterogeneity in something. The inclusion of this hypothesis does not appear warranted.

The hypothesis is based on previous studies showing that glycogen is stored and utilised heterogeneously between fibres (see for example Vøllested 1985 or Essen et al. 1974; 1975). This implies that the point of glycogen depletion at the single fiber level (for some fibers) may be reached much earlier than the point of depletion at the whole-muscle level. A main aim was to asses at which point a substantial proportion of single-fibers started to reach depleted levels as this has been speculated to drive impairments in muscle function (prior to complete depletion of whole-muscle glycogen). Moreover, we were interested to see whether this was augmented at the subcellular level and in specific subcellular regions, e.g. even if a single-fiber was not depleted at the total glycogen level, distinct subcellular regions could be depleted (as it seems to be the case for the intramyofibrillar region in type 2 fibers). We have reworded this to include a hypothesis on accelerated single-fiber depletion rather than just heterogeneity.

Lines 262-264: the way this is written implies that there were always n=3 type I and type II fibres and n=4 intermediated fibres that we discarded. Please reword to explain what happens.

This has now been reworded to describe the fibre-typing and fibre inclusion process in closer detail.

Line 265: units should be included and what 't' is, defined.

t is the section thickness and is defined in line 266. Units has now been added as suggested.

Line 285: add ~10 mg 'dry weight'. Provide the concentration of $HClO_4$ used.

The information on dry weight has been added. The concentration was 0.5 M which has also been added to the section.

Line 302: it is unclear when 'the other leg' was biopsied. Please provide this information in the appropriate section.

This information was mistakenly missing and has now been included in the appropriate methods section. We thank the reviewer for highlighting this. The baseline biopsiy was obtained from the right leg and the two exercise biopsies from the left leg. This selection was based on previous results by Hultman et al. 1967 and Costill et al. 1988 showing no difference in resting content or utilisation between the dominant and non-dominant leg, whereas repeated biopsy effects (e.g. impaired glycogen resynthesis) has been shown if biopsies are obtained in too close proximity. Thus, if all biopsies were to be obtained from the same leg, this would result in a large differentiation between sites (up to 15 cm) if an appropriate distance between sampling sites were to be maintained.

Line 308: include that plasma was removed and stored

This has now been included.

Line 315: how is a 2 point calibration curve valid? The slope of the line is only dependent on those two points and will not necessarily be indicative of the relationship between amount of insulin and sample.

The 2-point calibration statement is a simplification of the actual method which includes a 2-point calibration or adjustment as stated but which is compared to a master curve through data in the reagentbar code. Thus, the calibration is done with 6-8 calibrators by Roche and is then adjusted on the given apparatus by 2 calibrators.

We have removed the wording of 2-point calibration accordingly.

Line 316: describe the enzymatic assay

A more detailed description has now been included in the section as suggested.

Line 323: please provide detail in appropriate figure legends when the data were log transformed.

No log-transformed data are presented in the figures where geometric means are presented to account for differences in normal distribution.

Lines 381-392: it is unclear form the statistics shown in the figure that this is the case.

The figure shows the time x fibre type interactions e.g. when the utilisation rate of subcellular glycogen was higher in type 2 compared to type 1 fibres. Additional information has been added in the figure legend.

Line 400-408: give the differences in the contribution of each of the glycogen subcellular regions, it is a little misleading to talk about % declines between the different regions in the same section. This would be more clear to be in absolute values - as well as the %. For example, whilst intra is down ~51%, this is 2 um3um 103 compared with 14 and 20 2 um3um 103 IMF and SS, respectively.

The declines in subcellular muscle glycogen content are expressed in relative terms because the three different volume fractions are not quantified and reported in the same units because of the unique features of each storage location. As such, the intermyofibrillar store is expressed relative to the whole myofibrillar area (and not just the interspace where it is stored because this space is plastic based on the presence of mitochondria, lipid droplets, amount of glycogen stored etc). In contrast, intra glycogen is expressed relative to the intramyofibrillar space and subsarcolemmal glycogen relative to the fibre surface which is the established methodology applied in the previous literature. This means that the size of the three fractions are not immediately comparable. When the distribution of glycogen in IMF, Intra and SS fractions is expressed relative to the total content, the fibres were assumed to be of cylindrical shape with a diameter of 80 µm. Since IMF and Intra glycogen are expressed as volume densities and SS glycogen as volume per fibre surface area, total values were obtained by recalculating the SS glycogen to a volume density by the formula: volume beneath the surface area of a cylindrically shaped fibre (Vb)=R×0.5×A, where R is fibre radius and A is the fibre surface area in line with the previous literature.

We agree with the point raised by the reviewer though of emphasizing the clear differences in glycogen content stored and utilised in each fraction in absolute terms which has now been more thoroughly highlighted in the discussion section.

Line 409. I don't believe the statistics show this.

This is depicted in figure 5 where the relative distribution of each subcellular fraction at each time point (in each fibre type) is plotted. Here, the distribution of glycogen in each fraction is not different after EX3 compared to baseline. The * symbol in the SS fraction of type 2 fibres reflects a change compared to the post EX1 time point.

Line 413-426: please include the detection limit of glycogen granule size.

The detection limit of glycogen granules is around ~10 nm. However, because the small glycogen granules that are potentially undetected contribute with very little glycogen this does not significantly alter the estimation of the total volume fraction (as clearly seen from the very strong correlation between whole-muscle and subcellular glycogen also at the low absolute levels). A note of the detection limit has been included in the suggested section.

Line 461: Rather than 'stable' it would be better to say 'at this elevated level'

The wording has been changed to improve the clarity as suggested.

Line 481: the statement "fibre types were characterized by pronounced heterogeneity..." is not something that can be concluded from the data presented, aside from there being large SDs. This would be more evident by including individual points, which I realise will be a very busy graph, but possibly worthwhile.

We are not completely certain if the reviewer refers to figure 6 or 7 in relation to this statement, but in line with the reviewer suggestion we agree that individual values in figure 6 would be informative (if this is the figure referred to) but probably not feasible due to the many data points and overlay between type 1 and 2 fibres. The main point of figure 6 is to show the average difference in glycogen breakdown of each fraction between fibre types and not to show the heterogeneity. We have attempted to include individual values in this plot but decided not to keep these based on a resulting lack of clarity (it becomes very busy and masks the other main points). Instead we have shown individual values in figure 7 where single-fiber values from each subject are presented in relation to the whole-muscle glycogen concentration. It is our conception that this graph clearly depicts large heterogeneity in single-fiber levels for each given subject at the respective glycogen concentrations. Moreover, it is evident from the figure that for example single-fiber levels of intra glycogen in type 2 fibers are low compared to single-fiber values of type 1 fibres at specific total glycogen concentrations and also that the disparity within fibre types in each sample is large.

Line 508: ".... And IMF glycogen fractions,...." This was missing from the results, please include in that section also.

This is described in the result section in the first 5-6 lines under the heading “Utilisation of subcellular glycogen in type 1 and 2 fibres during EX1”.

Line 518: please include that this refers to cardiac muscle

This section has been removed in order to remove/reduce some of the more speculate parts of the discussion.

Line 560: 'has' should be 'have'

This has been corrected.

Line 572: 'at' should be 'under'

This has been corrected.

Line 585: 'part' might be better as 'proportion'

We agree, this has been corrected.

Line 615: please include somewhere in discussion the limitation of the smallest granules that can be detected and how that affects the findings and discussion.

A discussion on this relevant aspect has now been included in the highlighted section.

Fig 2: how is it ascertained that all panels are type II fibres? Provide how this was determined. Were all fibres shown from the same individual? Assume that is the case, so say so. If not, then they should be.

All fibres were type II fibres based on z-disk width measurements and from the same individual. This has now been clarified in the legend.

Please include all TEM images as supp data.

We have a total of 7344 TEM images (>40 GB) included in the manuscript which we can make accessible for inspection upon request. However providing all these images as supplemental material would require that the journal can provide a feasible storage form. On the other hand, we have still ongoing analyses of these images in relation to mitochondria morphology changes, lipid droplets etc. which are not yet published.

Figure 6: the statistics shown are unclear. Please describe the post-hoc test used here. How can only the two points show interaction?

The differences in rate of decline is based on the mixed model analyses (time x fibre type interactions) and not obtained from a post hoc test. The focus of the analyses were on rates of utilisation from pre to EX1 and from EX1 to EX3. We have only depicted the fibre type differences in utilisation for these comparisons. Here, a difference is present for utilisation of glycogen between fibre types (for the intra and IMF fractions) only for baseline to EX1. There are time effects for all time points in each fraction and fibre types and also overall time x type interactions for baseline to post EX2+EX3 which are not shown. We have provided further information in the figure legend.

Figure 7: define the solid and dotted lines. Break N=102 down into n=3 type I, n=3 type II from each of N=17 subjects.

These suggestions have now been included to clarify the figure legend further.

Figure 8B: remove the solid line as that implies significance. Why is only the lower end of the glycogen shown (up to 200 mmol/kg dw) - but in Fig 8A the full extent is shown (ie up to 700 mmol/kg dw). From which samples - pre and post? State details.

The solid line has been removed as suggested. The TAN figure included both the post EX1 and post EX3 values to show that the reduction seem to be augmented below ~200 mmol kg/dw of glycogen. However, since the values are not independent the figure has now been changed to show these relationships independently. For phosphocreatine the post exercise values were only shown for post EX3 in relation to muscle glycogen contents at the point where these levels were

most severely lowered and we would anticipate potential accelerations in PCr degradation. For clarity we have also included the post EX1 values in a separate regression and added further details in the figure legend.

Table 1: define (*). Include a line that shows TCr (similar to TAN).

The trend (*) has been removed. Information on total creatine has been added as suggested.

END OF COMMENTS

Dear Mr Vigh-Larsen,

Re: JP-RP-2022-283225R1 "Fibre Type and Localisation-Specific Muscle Glycogen Utilisation during Repeated High-Intensity Intermittent Exercise" by Jeppe Foged Vigh-Larsen, Niels Ørtenblad, Ole Emil Andersen, Hallur Thorsteinsson, Thea Holm Kristiansen, Stine Bilde, Mads Sloth Mikkelsen, Joachim Nielsen, Magni Mohr, and Kristian Overgaard

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

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

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.

Authors are asked to use The Journal's premium BioRender (https://biorender.com/) account to create/redraw their Abstract Figures. Information on how to access The Journal's premium BioRender account is here: https://physoc.onlinelibrary.wiley.com/journal/14697793/biorender-access and authors are expected to use this service. This will enable Authors to download high-resolution versions of their figures. The link provided should only be used for the purposes of this submission. Authors will be charged for figures created on this premium BioRender account if they are not related to this manuscript submission.

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)

- Abstract figure file (see above)

- Statistical Summary Document

- 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:

This revised manuscript has been reconsidered by the same reviewers that handled the initial submission. Both reviewers were positive about the revised manuscript. However, Reviewer 1 has concerns remaining regarding the interpretation of the initial muscle glycogen data. Specifically, the point of whether the initial absolute muscle glycogen content influenced the rate of glycogen degradation during exercise, and in different sub-fractions. It seems including absolute muscle glycogen data in the manuscript (or supplementary data) would also be useful to the reader, as would be some discussion of absolute rather than percentage degradation.

REFEREE COMMENTS

Referee #1:

This reviewer thanks the authors for the excellent revision of the manuscript, including the additional analyses done to evaluate the potential role of initial glycogen content. Once again this referee congratulates the authors for this excellent work. However, there are some remaining concerns with regard to the interpretation of the initial glycogen data. It is a fact that there is very high variation of initial glycogen contents between subjects, and it is important to know whether this has played a role in the data generated.

First, in their reply the authors show the results from the analyses on intra glycogen and SS glycogen, but not for intramyofibrillar. This is a bit bizarre because nearly 80% of the total muscle glycogen content is composed of intramyofibrillar glycogen. Thus, if one fraction is to be carefully looked at, it probably is the IMF fraction?

In the interpretation of these analyses the authors also indicate that the difference between the low and high tertiles are not statistically significant. However, when looking at the figures, it is quite obvious that the absence of significance most probably is due to insufficient number of observations (~low statistical power). Along the same line, in the discussion the authors write "although in absolute levels the glycogen breakdown rate appeared to be slightly higher in the high tertile". In this regard it must be mentioned that from a perspective of metabolic regulation absolute concentrations are pivotal, and relative concentrations are largely irrelevant. For the above reasons, to the opinion of this referee the additional analyses performed by the authors do not at all excluded that initial glycogen is an important regulator of net glycogen breakdown during high-intensity intermittent exercise in the different muscle glycogen fractions, indeed?

Having said this, to the opinion of this referee it is important to show the data either in the manuscript, or as supplemental online data. In addition, in the discussion the authors for this specific issue should probably rather focus on absolute changes and not on relative changes? Because 50% breakdown of a high initial glycogen content yields much more ATP than 50% of a low content, of course.

No additional minor comments.

Referee #2:

The authors have done a very good job at addressing each of my concerns and providing the necessary clarity.

END OF COMMENTS

1st Confidential Review **28-Jun-2022**

Response to reviewers:

Manuscript - JP-RP-2022-283225 "Fibre Type and Localisation-Specific Muscle Glycogen Utilisation during Repeated High-Intensity Intermittent Exercise" by Jeppe Foged Vigh-Larsen, Niels Ørtenblad, Ole Emil Andersen, Hallur Thorsteinsson, Thea Holm Kristiansen, Stine Bilde, Mads Sloth Mikkelsen, Joachim Nielsen, Magni Mohr, and Kristian Overgaard

---

EDITOR COMMENTS

Reviewing Editor:

This revised manuscript has been reconsidered by the same reviewers that handled the initial submission. Both reviewers were positive about the revised manuscript. However, Reviewer 1 has concerns remaining regarding the interpretation of the initial muscle glycogen data. Specifically, the point of whether the initial absolute muscle glycogen content influenced the rate of glycogen degradation during exercise, and in different sub-fractions. It seems including absolute muscle glycogen data in the manuscript (or supplementary data) would also be useful to the reader, as would be some discussion of absolute rather than percentage degradation.

We highly appreciate the thorough review and positive and constructive comments provided by the reviewers. A figure depicting the muscle glycogen utilisation in each subfraction for the participants divided in tertiles based on the initial glycogen content has been included as supplementary data and additional comments incorporated in the discussion section to address the relevant points raised by the reviewer.

REFEREE COMMENTS

Referee #1:

This reviewer thanks the authors for the excellent revision of the manuscript, including the additional analyses done to evaluate the potential role of initial glycogen content. Once again this referee congratulates the authors for this excellent work. However, there are some remaining concerns with regard to the interpretation of the initial glycogen data. It is a fact that there is very high variation of initial glycogen contents between subjects, and it is important to know whether this has played a role in the data generated.

First, in their reply the authors show the results from the analyses on intra glycogen and SS glycogen, but not for intramyofibrillar. This is a bit bizarre because nearly 80% of the total muscle glycogen content is composed of intramyofibrillar glycogen. Thus, if one fraction is to be carefully looked at, it probably is the IMF fraction?

In the interpretation of these analyses the authors also indicate that the difference between the low and high tertiles are not statistically significant. However, when looking at the figures, it is quite obvious that the absence of significance most probably is due to insufficient number of observations (~low statistical power). Along the same line, in the discussion the authors write "although in absolute levels the glycogen breakdown rate appeared to be slightly higher in the high tertile". In this regard it must be mentioned that from a perspective of metabolic regulation absolute concentrations are pivotal, and relative concentrations are largely irrelevant. For the above reasons, to the opinion of this referee the additional analyses performed by the authors do not at all excluded that initial glycogen is an important regulator of net glycogen breakdown during high-intensity intermittent exercise in the different muscle glycogen fractions, indeed?

Having said this, to the opinion of this referee it is important to show the data either in the manuscript, or as supplemental online data. In addition, in the discussion the authors for this specific issue should probably rather focus on absolute changes and not on relative changes? Because 50% breakdown of a high initial glycogen content yields much more ATP than 50% of a low content, of course.

No additional minor comments.

We thank the reviewer for these very relevant suggestions and have included a supplementary figure depicting the subcellular glycogen breakdown for the participants divided in tertiles based on the initial glycogen content. Moreover, we have modified our interpretation of the data in accordance with the good points raised by the reviewer, highlighting the potential influence of the variation in glycogen content and refraining from excessive focus on relative changes when discussing the high and low tertile responses.

Referee #2:

The authors have done a very good job at addressing each of my concerns and providing the necessary clarity.

We thank the reviewer once again for the examination of our work and are pleased that the revisions provided sufficiently address the initial concerns.

Dear Dr Vigh-Larsen,

Re: JP-RP-2022-283225R2 "Fibre Type and Localisation-Specific Muscle Glycogen Utilisation during Repeated High-Intensity Intermittent Exercise" by Jeppe Foged Vigh-Larsen, Niels Ørtenblad, Ole Emil Andersen, Hallur Thorsteinsson, Thea Holm Kristiansen, Stine Bilde, Mads Sloth Mikkelsen, Joachim Nielsen, Magni Mohr, and Kristian Overgaard

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 *

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.

EDITOR COMMENTS

Reviewing Editor:

Thanks for the work in addressing the concerns of Reviewer 1 about the previously revised manuscript. Both reviewers are happy with the modifications made and believe the manuscript will be quite influential. As an afterthought, Reviewer 2 has raised the point that it was previously requested that all TEM images be included as supplementary data, which the authors pointed out would be >7000 images that are being further analysed for other work. However, in an effort to help the reader appreciate the TEM data used for the analyses in the manuscript, Reviewer 2 has requested some raw data be made available, i.e. please prepare a subset of samples to show the repeatability of regions (e.g. 12 images) of the same muscle fibres at the same time points. Hopefully the authors will be willing to do this as it will improve the quality of the manuscript a little further, but it is not essential for acceptance.

Some Figures (3, 6 and some of 7) do not contain data points. Just means and SD, which does not comply with JP guidelines.

REFEREE COMMENTS

Referee #1:

This referee thanks the authors for the additional revisions. No further comments, and congratulations with this nice piece of work.

Referee #2:

It was previously requested that all TEM images be included as supp data, which the authors pointed out would be >7000 images and these are being further analysed for other work. Even so, in order to appreciate the TEM data that are used for the analyses, more raw data should be made available, so please prepare a subset of samples to show the repeatability of regions (e.g. 12 images) of the same muscle fibres at the same time points.

2nd Confidential Review 09-Aug-2022