THE ROYAL SOCIETY PUBLISHING

PROCEEDINGS B

Allometry of mitochondrial efficiency is set by metabolic intensity

Boël Mélanie, Romestaing Caroline, Voituron Yann and Roussel Damien

Article citation details
Proc. R. Soc. B **286**: 20191693.
http://dx.doi.org/10.1098/rspb.2019.1693

Review timeline

Original submission:	2 May 2019
1st revised submission:	19 July 2019
2nd revised submission:	5 September 2019
Final acceptance:	5 September 2019

Note: Reports are unedited and appear as submitted by the referee. The review history appears in chronological order.

Review History

RSPB-2019-1025.R0 (Original submission)

Review form: Reviewer 1 (Pierre U. Blier)

Recommendation
Accept with minor revision (please list in comments)

Scientific importance: Is the manuscript an original and important contribution to its field?
Excellent

General interest: Is the paper of sufficient general interest?
Excellent

Quality of the paper: Is the overall quality of the paper suitable?
Good

Is the length of the paper justified?
Yes

Should the paper be seen by a specialist statistical reviewer?
Yes

Do you have any concerns about statistical analyses in this paper? If so, please specify them explicitly in your report.
No

It is a condition of publication that authors make their supporting data, code and materials available - either as supplementary material or hosted in an external repository. Please rate, if applicable, the supporting data on the following criteria.

Is it accessible?
Yes

Is it clear?
Yes

Is it adequate?
Yes

Do you have any ethical concerns with this paper?
No

Comments to the Author
This manuscript, scrutinizing the allometry of energetic efficiency of muscle mitochondria from different species of mammals of body mass, may represent a major contribution to relate the mitochondrial functions to the scaling of metabolism in animals. It will also be of interest to anybody involved in the field of evolution of mitochondrial function. Three original findings definitely deserve to be broadly published: 1. The efficiency of oxidative phosphorylation increase at higher respiration rates. 2. The rate of increase of efficiency with increase in the rate of oxidative phosphorylation is mass dependent 3. Mitochondrial efficiency increases with body mass when mitochondria are close to the basal metabolic rate, but is independent of body mass at the maximum metabolic rate.

These results are of importance but seems to results mostly from one trait of mitochondrial function which diverge significantly according to mass. This trait is seen in fig1A and is represented as the intercept of the abscissa and likely magnify the divergences among species (and according to mass) of the LEAK state of respiration. In figure 1A, if the slopes are linear, the essential of divergences in the slope of efficiency (ATP/O) over ATP synthesis (Figure 2 A) should mostly be explained by the intercept on the abscissa axis. At low respiration rates, the ATP synthesis is closer to zero as ATP/O ratio. It therefore appears that the key trait delineating divergences in efficiency kinetic according to body mass is LEAK state of respiration. These considerations do not diminish the values of the analysis but might somewhat reorient the interpretation (obviously if I am right). Furthermore, defining the "resting" state of mitochondria as the intercept of the slope (fig 1 A) may be more physiologically relevant than the standard "state3/(state 4 or state 2)" .

Another important point that has partly been eluded in the discussion is the linearity of the slope of fig 1A. This suggests that the changes of ATP synthesis per unit of changes of O2 consumption are constant and therefore the leak reflected by the intercept should not vary with the rates of O2 consumption or ATP synthesis.

I have some concerns about the methodological approach that could probably be answered

easily. The authors expressed the ATP synthesis rates as well as the oxygen consumption rates per units of mitochondrial proteins. They therefore assume low level of contamination by non-mitochondrial proteins and if ever there is any, they assume, I guess, that contamination is independent of body mass. Knowing, however, that the mitochondrial content of muscle varies negatively with body mass, we could suspect that the relative contamination by non-mitochondrial protein content could also be related to body mass. If this is the case, it would induce a bias in the relation of ATP synthesis or oxygen consumption with body mass when express per protein content (fig 1B). In any case, differential contamination by non-mitochondrial protein would not affect the conclusion on efficiency since it is defined by ATP/O and is not dependent of protein content measurement.
The authors mention they control for the potential non-mitochondrial ATP synthesis by measuring ATP production in presence of oligomycin. Could residual ATPase activities also induce a bias in the measurements of ATP synthesis (for example by competing with the hexokinase of the reaction medium)?

Minor Comments

Lines 55-58. All these explanations are still debated and therefore suggesting that they can determine the power law of metabolic scaling could be interpreted as overemphasis. I would therefore suggest presenting them as hypotheses.

Line 105. Why two different procedures to purify mitochondria?
Line 109. How was the RCR calculated?
Lines 121-132. Some explanations are missing. How long have you sample the 100 μ l aliquots and why? It is explained in Teulier et al. (2010) but it would be relevant to remind it here.
Lines 161-162. This is true if we consider that the LEAK state of respiration is constant and maintained at every state of oxidative phosphorylation, but we could also suspect that this leak is dependent of the state of respiration. ie Highest rate of ATP synthesis will impact redox status of the ETS and might insure a highest proportion of electrons diverted to ETS and O2 through cytochrome oxidase as well as a lower quantity of H+ channeled through the membrane leak. But see my previous comments on the linearity of variation of ATP synthesis according to oxygen consumption.

Lines 164-165. "ATP synthesis rates shows that mitochondrial coupling efficiencies had a positive dependence on body mass". Except at maximal rates of ATP synthesis and oxygen consumption.

Review form: Reviewer 2

Recommendation
Major revision is needed (please make suggestions in comments)

Scientific importance: Is the manuscript an original and important contribution to its field?
Good

General interest: Is the paper of sufficient general interest?
Good

Quality of the paper: Is the overall quality of the paper suitable?
Marginal

Is the length of the paper justified?
Yes

Should the paper be seen by a specialist statistical reviewer?
No

Do you have any concerns about statistical analyses in this paper? If so, please specify them explicitly in your report.
Yes

It is a condition of publication that authors make their supporting data, code and materials available - either as supplementary material or hosted in an external repository. Please rate, if applicable, the supporting data on the following criteria.

Is it accessible?
No

Is it clear?
N/A

Is it adequate?
N/A

Do you have any ethical concerns with this paper?
No

Comments to the Author
This ms reports the mass-dependence of mitochondrial efficiency in mammals. I found the data interesting, but I feel that a solid background in mitochondrial bioenergetics is necessary to understand the importance of the study. In other words, the manuscript is not written for a general audience. The importance of the questions asked and the knowledge gaps are not well developed in the Introduction. There are no stated aims or hypotheses in the Introduction, and throughout the manuscript should provide more explanation of the methodology and findings to make it accessible to the broad audience of the Proceedings. Also, the manuscript should be proofread and edited for English grammar and spelling.

Specific Comments

17 the definite article before "metabolic activity" should be deleted

19 do you mean that all studies investigating mass effects on metabolism used oxygen consumption? I don't know whether that is correct, but it is certainly not correct as a general case so the statement needs to be qualified at least.

21 the meaning of the secondary clause (.., which implicitly..) is not quite clear, please rewrite

22-23 this is a truism: any effect modifying the efficiency of ATP production will affect energy management. Also, why would there be an effect of body mass?

27-28 'activation-state dependent flexibility' is not quite clear: better to use simple language that is more explicit. Same with 'dynamic transition of mitochondrial efficiency'

34 re-write 'mitochondria allows the generation of' to 'mitochondria generate'

35 please see my comment above

33-42 this significance statement is not easy to penetrate, especially if it is meant for the general reader, and I would recommend re-writing it

50 'they are' = 'it is'

64 reference needed after 'inner membrane'

67 replace "These reactions.." with 'Proton leak has been found to correlate negatively...'

69 insert 'increasing' before "body mass"

69-71 this sentence is important to set-up the study, but it says little about the questions that are still unknown and their importance. References 19-22 are experimental studies so that it is not clear how "experimental validation is still lacking" and it is not clear what the "intensity of the relationship" refers to.

The aims of the study should be stated in the context of the literature background.

75 I recommend to finish the Introduction with clearly stated hypotheses

79-90 how and why were these species chosen? What is their phylogenetic relationship?

81, 83 which laboratories?

93 here and below, please specify which muscle was used

96 if it is a standard protocol, please provide a reference. It may be standard for researchers working with mitochondria but not for a more general readership.

108-112 this section should be included in the paragraph describing respiration assays and maybe even results because RCRs are more than just a quality test

116 please describe the equipment used - Oroboros?

119 'state' should be plural

125 please provide a reference for this method to determine ATP. Some more explanation in the text would also be helpful.

132 how was efficiency actually determined from these measurements?

136 please provide more details about the use of the "Phylogenetic Independent Contrast model". I am familiar with Felsenstein's paper, but the phylogenies and the procedures used to correct or test for phylogenetic relationships should be described explicitly.

138 here and below "have been" = 'were'

141 what is a "risk factor" - probability? false discovery rate?

146-150 this section should be at the end of the Introduction or start of the Methods

151 "differ" = 'differs'

183-184 please provide more information (and reference if available) of the "channeling factor" - is it equivalent to the slope or the linearised curve? Fig. 3 just shows mtEC and it is not clear what the importance of the exponential function is or where it is shown.

299 here and below: what is (n)?

303 indicate that these rates are maximal in the y-axis label

322 what do the different lines indicate in the panels?

Decision letter (RSPB-2019-1025.R0)

04-Jun-2019

Dear Dr Roussel:

I am writing to inform you that your manuscript RSPB-2019-1025 entitled "Allometry of mitochondrial efficiency is set by metabolic intensity" has, in its current form, been rejected for publication in Proceedings B.

This action has been taken on the advice of referees, who have recommended that substantial revisions are necessary. With this in mind we would be willing to consider a resubmission, provided the comments of the referees are fully addressed, and the paper is presented in a way that makes it accessible to abroad audience and not just those with interests in metabolic physiology. It is important to note that this is not a provisional acceptance, and the resubmission will be treated as a new manuscript. We may approach the same reviewers if they are available and it is deemed appropriate to do so by the Associate Editor, but we may also solicit reviews from new reviewers.

Please note that resubmissions must be submitted within six months of the date of this email. In exceptional circumstances, extensions may be possible if agreed with the Editorial Office. Manuscripts submitted after this date will be automatically rejected.

Please find below the comments made by the referees, not including confidential reports to the Editor, which I hope you will find useful. If you do choose to resubmit your manuscript, please upload the following:

1) A 'response to referees' document including details of how you have responded to the comments, and the adjustments you have made.
2) A clean copy of the manuscript and one with 'tracked changes' indicating your 'response to referees' comments document.
3) Line numbers in your main document.

To upload a resubmitted manuscript, log into http://mc.manuscriptcentral.com/prsb and enter your Author Centre, where you will find your manuscript title listed under "Manuscripts with

Decisions." Under "Actions," click on "Create a Resubmission." Please be sure to indicate in your cover letter that it is a resubmission, and supply the previous reference number.

Sincerely,
Victoria Braithwaite

==========
Professor V A Braithwaite
mailto: proceedingsb@royalsociety.org
==========

Associate Editor, Comments to Author:
This looks to be an important paper to the field of evolutionary physiology, contending that the allometry of mitochondrial efficiency with body mass depends upon metabolic intensity in mammals. This links in to general research into metabolic scaling, but advances upon previous studies because the authors measure the efficiency of various mammalian species to convert oxygen to ATP, whereas previous studies have made assumptions on efficiency and assumed it was constant across species. While interesting, this will be a difficult paper for the broader audience of Proceedings B to digest and appreciate -- a point that Referee 2 has also noted. The authors need to carefully revise the Introduction and Discussion in light of this referees comments in order to increase the general acccessibility of, and ultimate impact, of this paper. This includes clarification of the hypotheses and aims of the study.

Referee 1 has made some intriguing technical insights that the authors need carefully consider. The referee also raises some methodological concerns that each require attention.

Please carefully attend to all of the comments of the referees.

====
Reviewers' Comments to Author:

Referee: 1

This manuscript, scrutinizing the allometry of energetic efficiency of muscle mitochondria from different species of mammals of body mass, may represent a major contribution to relate the mitochondrial functions to the scaling of metabolism in animals. It will also be of interest to anybody involved in the field of evolution of mitochondrial function. Three original findings definitely deserve to be broadly published: 1. The efficiency of oxidative phosphorylation increase at higher respiration rates. 2. The rate of increase of efficiency with increase in the rate of oxidative phosphorylation is mass dependent 3. Mitochondrial efficiency increases with body mass when mitochondria are close to the basal metabolic rate, but is independent of body mass at the maximum metabolic rate.

These results are of importance but seems to results mostly from one trait of mitochondrial function which diverge significantly according to mass. This trait is seen in fig1A and is represented as the intercept of the abscissa and likely magnify the divergences among species (and according to mass) of the LEAK state of respiration. In figure 1A, if the slopes are linear, the essential of divergences in the slope of efficiency (ATP/O) over ATP synthesis (Figure 2 A) should mostly be explained by the intercept on the abscissa axis. At low respiration rates, the ATP synthesis is closer to zero as ATP/O ratio. It therefore appears that the key trait delineating divergences in efficiency kinetic according to body mass is LEAK state of respiration. These considerations do not diminish the values of the analysis but might somewhat reorient the interpretation (obviously if I am right). Furthermore, defining the “resting” state of mitochondria

as the intercept of the slope (fig 1 A) may be more physiologically relevant than the standard "state3/(state 4 or state 2)" .

Another important point that has partly been eluded in the discussion is the linearity of the slope of fig 1A. This suggests that the changes of ATP synthesis per unit of changes of O2 consumption are constant and therefore the leak reflected by the intercept should not vary with the rates of O2 consumption or ATP synthesis.

I have some concerns about the methodological approach that could probably be answered easily. The authors expressed the ATP synthesis rates as well as the oxygen consumption rates per units of mitochondrial proteins. They therefore assume low level of contamination by non-mitochondrial proteins and if ever there is any, they assume, I guess, that contamination is independent of body mass. Knowing, however, that the mitochondrial content of muscle varies negatively with body mass, we could suspect that the relative contamination by non-mitochondrial protein content could also be related to body mass. If this is the case, it would induce a bias in the relation of ATP synthesis or oxygen consumption with body mass when express per protein content (fig 1B). In any case, differential contamination by non-mitochondrial protein would not affect the conclusion on efficiency since it is defined by ATP/O and is not dependent of protein content measurement.
The authors mention they control for the potential non-mitochondrial ATP synthesis by measuring ATP production in presence of oligomycin. Could residual ATPase activities also induce a bias in the measurements of ATP synthesis (for example by competing with the hexokinase of the reaction medium)?

Minor Comments

Lines 55-58. All these explanations are still debated and therefore suggesting that they can determine the power law of metabolic scaling could be interpreted as overemphasis. I would therefore suggest presenting them as hypotheses.

Line 105. Why two different procedures to purify mitochondria?
Line 109. How was the RCR calculated?
Lines 121-132. Some explanations are missing. How long have you sample the 100 μ l aliquots and why? It is explained in Teulier et al. (2010) but it would be relevant to remind it here.
Lines 161-162. This is true if we consider that the LEAK state of respiration is constant and maintained at every state of oxidative phosphorylation, but we could also suspect that this leak is dependent of the state of respiration. ie Highest rate of ATP synthesis will impact redox status of the ETS and might insure a highest proportion of electrons diverted to ETS and O2 through cytochrome oxidase as well as a lower quantity of H+ channeled through the membrane leak. But see my previous comments on the linearity of variation of ATP synthesis according to oxygen consumption.

Lines 164-165. "ATP synthesis rates shows that mitochondrial coupling efficiencies had a positive dependence on body mass" . Except at maximal rates of ATP synthesis and oxygen consumption.

==

Referee: 2

This ms reports the mass-dependence of mitochondrial efficiency in mammals. I found the data

interesting, but I feel that a solid background in mitochondrial bioenergetics is necessary to understand the importance of the study. In other words, the manuscript is not written for a general audience. The importance of the questions asked and the knowledge gaps are not well developed in the Introduction. There are no stated aims or hypotheses in the Introduction, and throughout the manuscript should provide more explanation of the methodology and findings to make it accessible to the broad audience of the Proceedings. Also, the manuscript should be proofread and edited for English grammar and spelling.

Specific Comments

17 the definite article before "metabolic activity" should be deleted

19 do you mean that all studies investigating mass effects on metabolism used oxygen consumption? I don't know whether that is correct, but it is certainly not correct as a general case so the statement needs to be qualified at least.

21 the meaning of the secondary clause (.., which implicitly..) is not quite clear, please rewrite

22-23 this is a truism: any effect modifying the efficiency of ATP production will affect energy management. Also, why would there be an effect of body mass?

27-28 'activation-state dependent flexibility' is not quite clear: better to use simple language that is more explicit. Same with 'dynamic transition of mitochondrial efficiency'

34 re-write 'mitochondria allows the generation of' to 'mitochondria generate'

35 please see my comment above

33-42 this significance statement is not easy to penetrate, especially if it is meant for the general reader, and I would recommend re-writing it

50 'they are' = 'it is'

64 reference needed after 'inner membrane'

67 replace "These reactions.." with 'Proton leak has been found to correlate negatively...'

69 insert 'increasing' before "body mass"

69-71 this sentence is important to set-up the study, but it says little about the questions that are still unknown and their importance. References 19-22 are experimental studies so that it is not clear how "experimental validation is still lacking" and it is not clear what the "intensity of the relationship" refers to.

The aims of the study should be stated in the context of the literature background.

75 I recommend to finish the Introduction with clearly stated hypotheses

79-90 how and why were these species chosen? What is their phylogenetic relationship?

81, 83 which laboratories?

93 here and below, please specify which muscle was used

96 if it is a standard protocol, please provide a reference. It may be standard for researchers working with mitochondria but not for a more general readership.

108-112 this section should be included in the paragraph describing respiration assays and maybe even results because RCRs are more than just a quality test

116 please describe the equipment used - Oroboros?

119 'state' should be plural

125 please provide a reference for this method to determine ATP. Some more explanation in the text would also be helpful.

132 how was efficiency actually determined from these measurements?

136 please provide more details about the use of the "Phylogenetic Independent Contrast model". I am familiar with Felsenstein's paper, but the phylogenies and the procedures used to correct or test for phylogenetic relationships should be described explicitly.

138 here and below "have been" = 'were'

141 what is a "risk factor" - probability? false discovery rate?

146-150 this section should be at the end of the Introduction or start of the Methods

151 "differ" = 'differs'

183-184 please provide more information (and reference if available) of the "channeling factor" - is it equivalent to the slope or the linearised curve? Fig. 3 just shows mtEC and it is not clear what the importance of the exponential function is or where it is shown.

299 here and below: what is (n)?

303 indicate that these rates are maximal in the y-axis label

322 what do the different lines indicate in the panels?

Author's Response to Decision Letter for (RSPB-2019-1025.R0)

See Appendix A.

RSPB-2019-1693.R0

Review form: Reviewer 1 (Pierre U. Blier)

Recommendation
Accept with minor revision (please list in comments)

Scientific importance: Is the manuscript an original and important contribution to its field?
Excellent

General interest: Is the paper of sufficient general interest?
Good

Quality of the paper: Is the overall quality of the paper suitable?
Good

Is the length of the paper justified?
Yes

Should the paper be seen by a specialist statistical reviewer?
No

Do you have any concerns about statistical analyses in this paper? If so, please specify them explicitly in your report.
No

It is a condition of publication that authors make their supporting data, code and materials available - either as supplementary material or hosted in an external repository. Please rate, if applicable, the supporting data on the following criteria.

Is it accessible?
Yes

Is it clear?
Yes

Is it adequate?
Yes

Do you have any ethical concerns with this paper?
No

Comments to the Author
The authors properly answered questions and comments.

Here are my remaining minor comments.

Lines 58 to 69. I am missing the logical link between these two propositions. The fact that 90% of oxygen consumption is done by mitochondria does not necessarily suggest that most of the energy needs are supported by mitochondria.

Lines 84-85. Awkward formulation of the sentence.

Line 106. Fresh tissue or « Fresh tissues »?

Line 119. Which protease?

Lines 198-200. This seems in contradiction with lines 187 -189

Decision letter (RSPB-2019-1693.R0)

02-Sep-2019

Dear Dr Roussel

I am pleased to inform you that your manuscript RSPB-2019-1693 entitled "Allometry of mitochondrial efficiency is set by metabolic intensity" has been accepted for publication in Proceedings B.

The referee has recommended publication, but also suggests some minor revisions to your manuscript. Therefore, I invite you to respond to the referee's comments and revise your manuscript. Because the schedule for publication is very tight, it is a condition of publication that you submit the revised version of your manuscript within 7 days. If you do not think you will be able to meet this date please let us know.

To revise your manuscript, log into https://mc.manuscriptcentral.com/prsb and enter your Author Centre, where you will find your manuscript title listed under "Manuscripts with Decisions." Under "Actions," click on "Create a Revision." Your manuscript number has been appended to denote a revision. You will be unable to make your revisions on the originally submitted version of the manuscript. Instead, revise your manuscript and upload a new version through your Author Centre.

When submitting your revised manuscript, you will be able to respond to the comments made by the referee and upload a file "Response to Referees". You can use this to document any changes you make to the original manuscript. We require a copy of the manuscript with revisions made since the previous version marked as ‘tracked changes’ to be included in the ‘response to referees’ document.

Before uploading your revised files please make sure that you have:

1) A text file of the manuscript (doc, txt, rtf or tex), including the references, tables (including captions) and figure captions. Please remove any tracked changes from the text before submission. PDF files are not an accepted format for the "Main Document".

2) A separate electronic file of each figure (tiff, EPS or print-quality PDF preferred). The format should be produced directly from original creation package, or original software format. PowerPoint files are not accepted.

3) Electronic supplementary material: this should be contained in a separate file and where possible, all ESM should be combined into a single file. All supplementary materials accompanying an accepted article will be treated as in their final form. They will be published alongside the paper on the journal website and posted on the online figshare repository. Files on

figshare will be made available approximately one week before the accompanying article so that the supplementary material can be attributed a unique DOI.

Online supplementary material will also carry the title and description provided during submission, so please ensure these are accurate and informative. Note that the Royal Society will not edit or typeset supplementary material and it will be hosted as provided. Please ensure that the supplementary material includes the paper details (authors, title, journal name, article DOI). Your article DOI will be 10.1098/rspb.[paper ID in form xxxx.xxxx e.g. 10.1098/rspb.2016.0049].

4) A media summary: a short non-technical summary (up to 100 words) of the key findings/importance of your manuscript.

5) Data accessibility section and data citation
It is a condition of publication that data supporting your paper are made available either in the electronic supplementary material or through an appropriate repository.

In order to ensure effective and robust dissemination and appropriate credit to authors the dataset(s) used should be fully cited. To ensure archived data are available to readers, authors should include a 'data accessibility' section immediately after the acknowledgements section. This should list the database and accession number for all data from the article that has been made publicly available, for instance:
• DNA sequences: Genbank accessions F234391-F234402
• Phylogenetic data: TreeBASE accession number S9123
• Final DNA sequence assembly uploaded as online supplemental material
• Climate data and MaxEnt input files: Dryad doi:10.5521/dryad.12311
NB. From April 1 2013, peer reviewed articles based on research funded wholly or partly by RCUK must include, if applicable, a statement on how the underlying research materials – such as data, samples or models – can be accessed. This statement should be included in the data accessibility section.

If you wish to submit your data to Dryad (http://datadryad.org/) and have not already done so you can submit your data via this link
http://datadryad.org/submit?journalID=RSPB&manu=(Document not available) which will take you to your unique entry in the Dryad repository. If you have already submitted your data to dryad you can make any necessary revisions to your dataset by following the above link.
Please see https://royalsociety.org/journals/ethics-policies/data-sharing-mining/ for more details.

6) For more information on our Licence to Publish, Open Access, Cover images and Media summaries, please visit https://royalsociety.org/journals/authors/author-guidelines/.

Once again, thank you for submitting your manuscript to Proceedings B and I look forward to receiving your revision. If you have any questions at all, please do not hesitate to get in touch.

Sincerely,
Victoria Braithwaite

Professor V A Braithwaite
mailto: proceedingsb@royalsociety.org

Associate Editor, Comments to Author:

Dear Dr Roussel,

Thank you for addressing the referees' comments within your revision of this manuscript. I think you have done a good job of pitching the study to a broader audience (and thus will be of greater interest and accessibility to readers of Proceedings B). You have also adequately responded to the technical questions of the referees, and Referee 1 has reviewed this revision and thought the responses appropriate. Notwithstanding, Referee 1 has asked you to clarify a few more technical issues before the paper is good to publish.

Line 190: Check: do you mean the slopes were also independent of body mass (since p value here is not statistically significant -- also, would be more informative to provide the true p value rather than report "n.s."). [you have said the slopes were also not independent].

Overall I think this will be an excellent addition to the literature -- it's an excellent study, well done.

==========

Reviewer Comments to Author:

Referee: 1

The authors properly answered questions and comments.

Here are my remaining minor comments.

Lines 58 to 69. I am missing the logical link between these two propositions. The fact that 90% of oxygen consumption is done by mitochondria does not necessarily suggest that most of the energy needs are supported by mitochondria.

Lines 84-85. Awkward formulation of the sentence.

Line 106. Fresh tissue or « Fresh tissues »?

Line 119. Which protease?

Lines 198-200. This seems in contradiction with lines 187 -189

Author's Response to Decision Letter for (RSPB-2019-1693.R0)

See Appendix B.

Decision letter (RSPB-2019-1693.R1)

05-Sep-2019

Dear Dr Roussel

I am pleased to inform you that your manuscript entitled "Allometry of mitochondrial efficiency is set by metabolic intensity" has been accepted for publication in Proceedings B.

You can expect to receive a proof of your article from our Production office in due course, please check your spam filter if you do not receive it. PLEASE NOTE: you will be given the exact page length of your paper which may be different from the estimation from Editorial and you may be asked to reduce your paper if it goes over the 10 page limit.

If you are likely to be away from e-mail contact please let us know. Due to rapid publication and an extremely tight schedule, if comments are not received, we may publish the paper as it stands.

If you have any queries regarding the production of your final article or the publication date please contact procb_proofs@royalsociety.org

Your article has been estimated as being 6 pages long. Our Production Office will be able to confirm the exact length at proof stage.

Open Access
You are invited to opt for Open Access, making your freely available to all as soon as it is ready for publication under a CCBY licence. Our article processing charge for Open Access is £1700. Corresponding authors from member institutions (http://royalsocietypublishing.org/site/librarians/allmembers.xhtml) receive a 25% discount to these charges. For more information please visit http://royalsocietypublishing.org/open-access.

Paper charges
An e-mail request for payment of any related charges will be sent out shortly. The preferred payment method is by credit card; however, other payment options are available.

Electronic supplementary material:
All supplementary materials accompanying an accepted article will be treated as in their final form. They will be published alongside the paper on the journal website and posted on the online figshare repository. Files on figshare will be made available approximately one week before the accompanying article so that the supplementary material can be attributed a unique DOI.

Thank you for your fine contribution. On behalf of the Editors of the Proceedings B, we look forward to your continued contributions to the Journal.

Sincerely,

Proceedings B
mailto: proceedingsb@royalsociety.org

Appendix A

Dr. Damien Roussel
LEHNA, UMR5023, CNRS, Université Claude Bernard Lyon1
Bâtiment Charles Darwin C, F-69622 Villeurbanne cedex, France
e-mail : damien.roussel@univ-lyon1.fr

Lyon, 19th of July 2019

Resubmission of manuscript: RSPB-2019-1025

Dear Victoria Braithwaite,

We acknowledge the receipt of your letter and the referees' comments concerning our manuscript entitled "Allometry of mitochondrial efficiency is set by metabolic intensity" by Boël et al.

Please find enclosed a revised version of our manuscript. A response to reviewers is appended below and carefully addresses, point-by-point, all of the issues raised in the reviewers' comments and describes the corresponding changes to the manuscript.

Here are the major changes made as suggested by the two reviewers:

1- We have rewritten the Introduction and Discussion sections in order to increase the general accessibility of our paper (Reviewers 1 and 2), to clarify the aim and hypotheses of the present work (Reviewer 2), and to better explain the importance of leak state of respiration as an explicative mechanism of the divergences in the coupling efficiency among species (Reviewer 1).
2- We have provided answers to the methodological concerns raises by the Reviewer 1. We have also added information and details in the Methods section on our experimental procedures (Reviewers 1 and 2).
3- We carefully attend and respond to all of the comments of the reviewers.
4- The manuscript has been proofread and edited for English grammar and spelling.

Yours sincerely,

Damien Roussel

Associate Editor, Comments to Author:

This looks to be an important paper to the field of evolutionary physiology, contending that the allometry of mitochondrial efficiency with body mass depends upon metabolic intensity in mammals. This links in to general research into metabolic scaling, but advances upon previous studies because the authors measure the efficiency of various mammalian species to convert oxygen to ATP, whereas previous studies have made assumptions on efficiency and assumed it was constant across species. While interesting, this will be a difficult paper for the broader audience of Proceedings B to digest and appreciate -- a point that Referee 2 has also noted. The authors need to carefully revise the Introduction and Discussion in light of this referees comments in order to increase the general acccessibility of, and ultimate impact, of this paper. This includes clarification of the hypotheses and aims of the study.

Referee 1 has made some intriguing technical insights that the authors need carefully consider. The referee also raises some methodological concerns that each require attention.

Please carefully attend to all of the comments of the referees.

Response: We have carefully addressed, point-by-point, all of the issues raised in the reviewers' comments (see our responses below). Here are the major changes made as suggested by the two reviewers

1- We have rewritten the Introduction and Discussion sections in order to increase the general accessibility of our paper (Reviewers 1 and 2);

2- We have clarified the aim and hypotheses of the present work (Reviewer 2);

3- The importance of leak state of respiration as an explicative mechanism of the divergences in the coupling efficiency among species is now better explain in the revised manuscript (Reviewer 1).

4- We have provided answers to the methodological concerns raises by the Reviewer 1.

5- We have also added details in the Methods section on our experimental procedures (Reviewers 1 and 2).

6- We carefully attend and respond to all of the comments raised by the reviewers.

7- The manuscript has been proofread and edited for English grammar and spelling.

Referee: 1

This manuscript, scrutinizing the allometry of energetic efficiency of muscle mitochondria from different species of mammals of body mass, may represent a major contribution to relate the mitochondrial functions to the scaling of metabolism in animals. It will also be of interest to anybody involved in the field of evolution of mitochondrial function. Three original findings definitely deserve to be broadly published:

1. The efficiency of oxidative phosphorylation increase at higher respiration rates.

2. The rate of increase of efficiency with increase in the rate of oxidative phosphorylation is mass dependent

3. Mitochondrial efficiency increases with body mass when mitochondria are close to the basal metabolic rate, but is independent of body mass at the maximum metabolic rate.

These results are of importance but seems to results mostly from one trait of mitochondrial function which diverge significantly according to mass. This trait is seen in fig1A and is represented as the intercept of the abscissa and likely magnify the divergences among species (and according to mass) of the LEAK state of respiration. In figure 1A, if the slopes are linear, the essential of divergences in the slope of efficiency (ATP/O) over ATP synthesis (Figure 2 A) should mostly be explained by the intercept on the abscissa axis. At low respiration rates, the ATP synthesis is closer to zero as ATP/O ratio. It therefore appears that the key trait delineating divergences in efficiency kinetic according to body mass is LEAK state of respiration. These considerations do not diminish the values of the analysis but might somewhat reorient the interpretation (obviously if I am right). Furthermore, defining the "resting" state of mitochondria as the intercept of the slope (fig 1 A) may be more physiologically relevant than the standard "state3/(state 4 or state 2)".

Another important point that has partly been eluded in the discussion is the linearity of the slope of fig 1A. This suggests that the changes of ATP synthesis per unit of changes of O2 consumption are constant and therefore the leak reflected by the intercept should not vary with the rates of O2 consumption or ATP synthesis.

Response: We agree with the reviewer's comments. **We have rewritten the "results and discussion" section to clearly discuss this point: the fact that most of the divergences between species could be explained by one key trait of mitochondrial function, i.e. the leak state of respiration**. Pages 10, lines 209-220.

I have some concerns about the methodological approach that could probably be answered easily. The authors expressed the ATP synthesis rates as well as the oxygen consumption rates per units of mitochondrial proteins. They therefore assume low level of contamination by non-mitochondrial proteins and if ever there is any, they assume, I guess, that contamination is independent of body mass. Knowing, however, that the mitochondrial content of muscle varies negatively with body mass, we could suspect that the relative contamination by non-mitochondrial protein content could also be related to body mass. If this is the case, it would induce a bias in the relation of ATP synthesis or oxygen consumption with body mass when express per protein content (fig 1B). In any case, differential contamination by non-mitochondrial protein would not affect the conclusion on efficiency since it is defined by ATP/O and is not dependent of protein content measurement.

Response: The reviewer is right, volume density of mitochondria in skeletal muscle varies slightly but not significantly with body mass in mammals (see for instance Hoppeler and Flück, 2002; Else and Hulbert, 1985). In one hand, these works suggest that the relative contamination

by non-mitochondrial protein content could be related to body mass, with higher contamination in large mammals than in small species. In the other hand, the lack of statistical significance also suggest that such contamination would be minimal, and so the bias on mitochondrial fluxes. **Most of all**, as state by the reviewer, **such differential contamination does not affect the main conclusion of the paper that is on mitochondrial efficiency, the calculation of which is independent of protein content determination**. For these reasons **we assume** that the possible differential contamination of our mitochondrial preparation **is negligible regarding the main topic and conclusion of the paper** on mitochondrial efficiency.

The authors mention they control for the potential non-mitochondrial ATP synthesis by measuring ATP production in presence of oligomycin. Could residual ATPase activities also induce a bias in the measurements of ATP synthesis (for example by competing with the hexokinase of the reaction medium)?

Response: By constantly regenerating the added ADP**, the excess of hexokinase/glucose ensure that the ADP/ATP ratio remain high which in turn would limit most of the residual ATPase activities**. Noted that the absence of sodium and calcium in our respiratory buffer must prevent some ATPase activities such as Na/K-ATPase and Ca-ATPase. On the contrary, there is a non-mitochondrial ATP synthesis which would be supported mostly by adenylate kinase activity. This non-mitochondrial ATP synthesis is only measurable at high concentration of ADP (**this is now clearly specify in the "methods" section page 8, lines 157-162**). At these high concentrations of ADP, the adenylate kinase would rather produce ATP than consume it, inducing negligible competition with hexokinase for ATP.

Minor Comments

Lines 55-58. All these explanations are still debated and therefore suggesting that they can determine the power law of metabolic scaling could be interpreted as overemphasis. I would therefore suggest presenting them as hypotheses. **Changed accordingly (page 4 – line 54-56)**

Line 105. Why two different procedures to purify mitochondria?

Response: The modified procedure in small mammals was used to limit the loss of mitochondrial materials**. We added text in "Methods" section to clarify this point (page 7, lines 125-126).**

Line 109. How was the RCR calculated? **We clarify the calculation (page 8, lines 161-165).**

Lines 121-132. Some explanations are missing. How long have you sample the 100µl aliquots and why? It is explained in Teulier et al. (2010) but it would be relevant to remind it here. **We added details and explanations about our experimental protocol.**

Lines 161-162. This is true if we consider that the LEAK state of respiration is constant and maintained at every state of oxidative phosphorylation, but we could also suspect that this leak is dependent of the state of respiration. ie Highest rate of ATP synthesis will impact redox status of the ETS and might insure a highest proportion of electrons diverted to ETS and O2 through cytochrome oxidase as well as a lower quantity of H+ channeled through the membrane leak.

But see my previous comments on the linearity of variation of ATP synthesis according to oxygen consumption.

Response: The leak state of respiration sharply decreases with the increase in ATP synthesis rate. **We have rewritten the "Introduction" section** (page 5, lines 78-90) and **added text in the "Results and Discussion" section** (page 10, lines 213-220) **to clearly introduce this fundamental property of mitochondrial bioenergetics**.

Lines 164-165. "ATP synthesis rates shows that mitochondrial coupling efficiencies had a positive dependence on body mass". Except at maximal rates of ATP synthesis and oxygen consumption.

Response: Mitochondrial coupling efficiencies had a positive dependence on body mass only when it is calculated at the same ATP synthesis rates, i.e. at the same metabolic activity. This is clarify **page 10 lines 202-207**.

Referee: 2

This ms reports the mass-dependence of mitochondrial efficiency in mammals. I found the data interesting, but I feel that a solid background in mitochondrial bioenergetics is necessary to understand the importance of the study. In other words, the manuscript is not written for a general audience. The importance of the questions asked and the knowledge gaps are not well developed in the Introduction. There are no stated aims or hypotheses in the Introduction, and throughout the manuscript should provide more explanation of the methodology and findings to make it accessible to the broad audience of the Proceedings. Also, the manuscript should be proofread and edited for English grammar and spelling.

Response: **We have added aim and hypotheses in the "Introduction" section. Throughout the manuscript, we have rewritten/added text in all sections of the paper to provide more explanation of the methodology and findings and to make,** we hope so**, the present work more accessible to the broad audience of the Proceeding**. The manuscript has been proofread and edited for English grammar and spelling.

Specific Comments

17 the definite article before "metabolic activity" should be deleted

19 do you mean that all studies investigating mass effects on metabolism used oxygen consumption? I don't know whether that is correct, but it is certainly not correct as a general case so the statement needs to be qualified at least.

21 the meaning of the secondary clause (.., which implicitly..) is not quite clear, please rewrite

22-23 this is a truism: any effect modifying the efficiency of ATP production will affect energy management. Also, why would there be an effect of body mass?

27-28 'activation-state dependent flexibility' is not quite clear: better to use simple language that is more explicit. Same with 'dynamic transition of mitochondrial efficiency'

Responses: **We have rewritten the "summary" section taking into account all of the above recommendations**.

34 re-write 'mitochondria allows the generation of' to 'mitochondria generate'

35 please see my comment above

33-42 this significance statement is not easy to penetrate, especially if it is meant for the general reader, and I would recommend re-writing it

Responses: **We have rewritten the "statement" section taking into account all of the above recommendations**.

50 'they are' = 'it is' **Changed accordingly**

64 *reference needed after 'inner membrane'* **We have added one reference.**

67 replace "These reactions.." with 'Proton leak has been found to correlate negatively...' **Changed accordingly**

69 insert 'increasing' before "body mass" **Changed accordingly**

69-71 *this sentence is important to set-up the study, but it says little about the questions that are still unknown and their importance. References 19-22 are experimental studies so that it is not clear how "experimental validation is still lacking" and it is not clear what the "intensity of the relationship" refers to.*

The aims of the study should be stated in the context of the literature background.

75 *I recommend to finish the Introduction with clearly stated hypotheses.*

Responses: **We have rewriting the introduction section in order clarify the questions** and **the aim of the present study** (**page 5, lines 78-90**).

79-90 how and why were these species chosen? What is their phylogenetic relationship?

Response: **We added one supplemental figure to show the phylogenetic relationship** between the species used in the present study. We also added text in "Statistical analyses" paragraph of the "Methods" section to clarify the statistical analyses.

81, 83 which laboratories? **We have added names and locations of laboratories.**

93 here and below, please specify which muscle was used. **We have specify which muscle was used (page 6, line 117).**

96 if it is a standard protocol, please provide a reference. It may be standard for researchers working with mitochondria but not for a more general readership. **We have added a reference.**

108-112 this section should be included in the paragraph describing respiration assays and maybe even results because RCRs are more than just a quality test. **We have moved and completed this paragraph in the "Methods" section (page 8, lines 161-165).**

116 please describe the equipment used - Oroboros? **Added accordingly (page 7, lines 137-138).**

119 'state' should be plural **Changed accordingly.**

125 please provide a reference for this method to determine ATP. Some more explanation in the text would also be helpful.

132 how was efficiency actually determined from these measurements?

Response: **We added details in the "Methods section"** to clarify the method used, providing more explanation in throughout this section and in particular to describe how mitochondrial ATP synthesis was determined and corrected from oligomycin-insensitive ATP synthesis (**Page 8, lines 155-160**).

136 please provide more details about the use of the "Phylogenetic Independent Contrast model". I am familiar with Felsenstein's paper, but the phylogenies and the procedures used to correct or test for phylogenetic relationships should be described explicitly.

Response: W**e have rewritten the "statistical analysis" section** to explicitly describe the procedures used to correct and test for phylogenetic relationships.

138 here and below "have been" = 'were' **Corrected accordingly**

141 what is a "risk factor" - probability? false discovery rate?

Response: W**e have rewritten the "statistical analysis" section** to clarify the statistical procedures we used.

146-150 this section should be at the end of the Introduction or start of the Methods **This section has been moved at the start of the Methods.**

151 "differ" = 'differs' **Corrected accordingly**

183-184 please provide more information (and reference if available) of the "channeling factor" - is it equivalent to the slope or the linearised curve? Fig. 3 just shows mtEC and it is not clear what the importance of the exponential function is or where it is shown.

Response: **We have rewritten this paragraph** to better explain the meaning of the "channeling factor" (**page 11, lines 232-239**).

299 here and below: what is (n)? **Response**: **(n) is given alongside the name of species**. We have added this information in the Figure's legends.

303 *indicate that these rates are maximal in the y-axis label.* **We prefer to keep this notion of "maximal" in the legend of the figure instead of in the y-axis because not all rates shown in this figure are maximal rates, e.g. the basal oxygen consumption rate measured in the presence of oligomycin**.

322 what do the different lines indicate in the panels? **This has been clarified in the text (page 10 lines 204-207)**.

Appendix B

Dr. Damien Roussel
LEHNA, UMR5023, CNRS, Université Claude Bernard Lyon1
Bâtiment Charles Darwin C, F-69622 Villeurbanne cedex, France
e-mail : damien.roussel@univ-lyon1.fr

Lyon, 5th of August 2019

Manuscript: RSPB-2019-1693

All of the remaining minor comments have been addressed in the present version of the manuscript:

Associate editor: Line 190: we have corrected the sentence: "the slopes were also independent of body mass" and we have reported the p value in the text.

Reviewer 1:

1- Lines 58-69: According to the reviewer's comment we have removed the first of the sentence. The sentence is: "*Mitochondria significantly contribute to metabolism in aerobic eukaryotic organisms by providing most of the cellular energy needs in the form of ATP (17).*"
2- Lines 84-85: the formulation of the sentence has changed in: "*Whether such dynamic functioning of mitochondrial bioenergetics depends upon body mass has not been tested and quantified yet.*"
3- Line 106: Fresh tissue has been changed in Fresh tissues.
4- Line 119: the name of protease (subtilisin A) has been added in the text.
5- Lines 198-200: This section is not in contradiction with lines 187-189. In lines 187-189 the efficiency is calculated at maximal ATP synthesis rate, and the maximum is different between species. Whereas in lines 198-200, mitochondrial efficiency is calculate at the same ATP synthesis rate. This notion is explicitly shown in Fig. 2A and explicitly described in the corresponding text, the following sentence in lines 200-201.