Transcriptomic, proteomic and phosphoproteomic underpinnings of daily exercise performance and Zeitgeber activity of training in mouse muscle

Geraldine Maier, Julien Delezie, Pål O. Westermark, Gesa Santos, Danilo Ritz, and Christoph Handschin
DOI: 10.1113/JP281535

Corresponding author(s): Christoph Handschin (christoph.handschin@unibas.ch)

The following individual(s) involved in review of this submission have agreed to reveal their identity: Brendan Gabriel (Referee #1); Jose R Pauli (Referee #2)

Review Timeline:

| Event                          | Date       |
|-------------------------------|------------|
| Submission Date               | 09-Mar-2021|
| Editorial Decision            | 26-Mar-2021|
| Revision Received             | 07-Jun-2021|
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Reviewing Editor: Audrey Bergouignan

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 Professor Handschin,

Re: JP-RP-2021-281535 “Transcriptomic, proteomic and phosphoproteomic underpinnings of daily exercise performance and Zeitgeber activity of endurance training” by Geraldine Maier, Julien Delezie, Pål O. Westermark, Gesa Santos, Danilo Ritz, and Christoph Handschin

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.

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.

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I look forward to receiving your revised submission.
If you have any queries please reply to this email and staff will be happy to assist.

Yours sincerely,

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EDITOR COMMENTS

Reviewing Editor:

The statistical Summary document is missing.

I also would like to commend the authors for this excellent work and the quality of the figures presented in the manuscript. However, like Referee 1, I would like to invite the authors to indicate in the title the work was performed in mice and to tone down their conclusions on the clinical implications of their work.

Please take into account the suggestions of Referee 1 to improve the text.

Adding a figure to summarize the key findings could be helpful for the reader.

The Summary Statement document is missing.
REFEREE COMMENTS

Referee #1:

I commend the authors on the large array of quality data they have collected in an extremely intensive and robust study protocol. I hope the authors did not disturb their circadian rhythms too much in the process.

The ‘omics’ data in this study are extremely comprehensive and will aid future researchers significantly when assessing this field. As with many circadian biology studies, feeding is a confounder in the acute exercise protocol in this study, but the authors do cover this limitation sufficiently in the discussion.

My main suggestions for improvement relate to the presentation of the study in text. The authors should carefully revise this based on the suggestions below. In general the authors should be mindful that there have been several studies assessing similar non-‘omic’, circadian biology outcomes in their study, in humans and mice, and be sure to cite and discuss these satisfactorily. Additionally, the authors have (understandably) relatively low n in many of these experiments, and it should be trivial to present the individual values in most figure panels.

General:

Where possible, show individual data-points rather than ‘plunger’ bar charts or single-line time-course.

The authors use different terminology throughout the manuscript for the ZT at which exercise was performed (e.g. ‘early morning’ at one point and ‘light’ in another). These should be defined early (in abstract if possible) and be consistent throughout the manuscript.

Title: please make the readers of the title aware that this study was conducted in mice.

Abstract: Although this is a comprehensive and well-conducted study, the lack of human data
renders the sentence "These results are of high importance to understand circadian aspects of training design in athletes and the application of chrono-exercise-based interventions in patients." a slight over-reach in my opinion. The authors should temper this sentence, bearing in mind the study was conducted in mice.

Key points: "Maximal endurance performance is greater in the early morning", I don't find the terminology 'morning' helpful here, perhaps the authors could clarify this statement. E.g. 'late active phase' or a statement along those lines.

"Training exerts poor Zeitgeber activity on the muscle and liver clocks". Could this statement be written with more clarity for a general audience?

Line 83: "Inversely, a potential Zeitgeber activity of training also is unclear." This sentence should be clarified.

Line 86: "Therefore, whether the enhanced effects of timed exercise training on health parameters in both clinical and preclinical contexts [16, 17] depend on the Zeitgeber properties of exercise remains to be investigated [18, 19]." This line is difficult to read and could be clarified. E.g. I presume the authors mean 'the putative enhanced effects of timed exercise'.

"depend on the Zeitgeber properties of exercise remains to be investigated [18, 19]." I am not clear why citations 18,19 are relevant to this statement or sentence?

Although the authors cite 19 in the intro, these findings should be expanded upon as they are pertinent to the current study.

Line 99: "Differences in low- and moderate-intensity treadmill exercise performance were reported within the active phase of wild-type mice [12]." This should state 'previously reported'

Line 109: remove 'drastic'

Line 125: "Altogether, these data demonstrate that mice are surprisingly better at performing a maximal running test in the early light phase of the 24-h LD cycle." Given this would correspond with evening in humans, I don't find this surprising.

Line 314: "Scheduled daytime wheel-running activity in mice exposed to a skeleton photoperiod" please give an indication of what these results show in the title.

Line 418: "Thus, a strong mutual interaction between skeletal muscle, the circadian timing system, and exercise has been suspected [5]." I would suggest it has been more than suspected e.g. PMID: 28683290 demonstrates this.

Line 425: "time of day on muscle cellular responses and performance" "remain poorly described" I assume the authors are referring to exercise (or possibly muscle) performance here. If so, exercise performance (also muscle performance) has actually been intensively studied in regards to diurnal fluctuations. E.g. PMID: 8000814, PMID: 8877122, PMID: 11023216, PMID: 9459533, PMID: 20030545, PMID: 20524806, PMID: 25289885, PMID: 20524806 (not an exhaustive list of studies in the field). I suggest the authors clarify what they mean in this sentence, and temper their implication of novelty accordingly.

Line 420: "First, the daily variations in metabolic and functional properties, ultimately controlled by the circadian clock, will affect endurance and strength capacities." This sentence is unclear, do the authors mean that it has been postulated from the literature that variations in metabolic
properties will affect endurance and strength. It is well-known that human strength capacity fluctuates in a diurnal manner, and it is unclear in this sentence if the authors are stating that as an unknown?

Line 429: "collectively, our results strongly suggest that training has very poor Zeitgeber activity on the skeletal muscle clock" the authors should specify which time of training they are referring to. Also please see remarks about Zeitgeber below.

Line 432: "daytime training leads" it is unclear how this differs from the previous sentence mentioning 'training'

Line 483: "Future experiments with inducible, skeletal muscle specific knockout models for clock components might thus help to clarify the contribution of this system to muscle metabolism a" PMID: 28683290 should be cited and discussed here.

Line 503: "Thus, athletes will have to be assessed individually ("personalized training")." There has already been some research into the individual chronotypes etc of athletes, perhaps the authors could cite the relevant research here e.g. PMID: 30357501 etc. Also the authors should either elaborate on this statement, or remove it, as it is not supported by the surrounding text at present.

Line 506: “Lastly, our data indicate that training is not only not a strong Zeitgeber in a healthy mouse muscle, but, if chronically performed at the wrong time of the day, might lead to perturbations of the muscle clock which resemble perturbations of other peripheral clocks in pathological states and in aging, characterized by a general dampening of oscillation amplitudes" One may argue that an extrinsic stimulus leading to 'perturbations of the muscle clock' would in fact precisely fit the definition of a Zeitgeber. I suggest the authors define what they mean by Zeitgeber early on in the manuscript, as it appears to differ from some forms of usage the literature. Also, please clarify this passage of text.

Line 513: “Regardless of the outcome of future studies in such a direction, however, the negative results of our studies in regard to the effect of daytime activity in mice as Zeitgeber of the muscle clock should not be interpreted to discount training interventions" As a conclusion, and in mind of the general reader, I find this opaque. I think what the authors mean by Zeitgeber is something which entrains homeostatic rhythms, whereas it is frequently used in the literature to denote an extrinsic stimuli that can 're-set' the molecular clock. In most cases I think it would be clearer for the reader if the authors 'spelled-out' what they are trying to say when they use the word Zeitgeber. Additionally, I do not understand why the results are 'negative'? Please clarify.

Figure legends: it should be clarified what the light and dark shading refers to in the figures.

"The data have been deposited in GEO (accession numbers XXX)." I assume the authors will update this upon acceptance for publication.
Referee #2:

The authors evaluated the effects of timed exercise at the systemic and muscle cellular levels by assessing maximal treadmill exercise capacity across the 24-h light-dark (LD) cycle. The authors also dissected the transcriptome and (phospho-)proteome responses of working muscles at two distinct phases of the LD cycle. In addition, to investigate the potential Zeitgeber activity of exercise was used a skeleton photoperiod (SPP) in combination with restricted wheel-running access to interrogate the consequences of scheduled daytime voluntary training on skeletal muscle gene and protein regulation. The study is very interesting and was very well conducted by the authors. The results are quite broad and fill an important gap in science. Some concerns and suggestions are addressed to the authors below.

1. In the materials and methods section, the information should be included if the mice remained alone or in groups in the cages;

2. Why was the inclination used to perform the maximum running test on a treadmill? Any special reason?

3. Is it possible to include information if the food intake in the night cycle (which is higher) happens more intensely at the beginning, middle, or end of the night cycle?

4. It was mentioned in the study that the lactate collection at the end of the exercise occurred within a time of up to 1 min. However, was a single collection performed? There is a significant variation in the lactate values between the times 0 to 10 minutes after the end of the exercise. In addition, the lactate collection in these times (0, 3, 5, 7, and 10 min) can help to identify the highest lactate value after exercise.

5. In addition to the total distance covered, it is relevant to include the maximum speed and total time data obtained by the animals in the running test. Thus, the authors must consider the inclusion of these data in the manuscript or as complementary material;

6. The authors may consider changing the color of the blue rectangles used in the light and dark cycle in the figures. Perhaps the change can help to better understand the results found in the light and dark cycle in the study;

7. Was the forced performance test of high-intensity exercises throughout the day carried out with the animals fasting? This information can be made clearer in the text.

8. Although the text is quite explanatory. A schematic figure with the description of the steps, especially related to the moments when the exercises were performed, tissue collection, would be valid to facilitate the understanding of the reader;

9. The authors very well wrote the discussion section. Information on limitations, future prospects, and differences between rodents and humans have been included. As a single observation, the authors could consider including a schematic figure at the end with the description of some of the most important points found in the light and dark cycle and, above all, of the performance in these different periods of the day.
END OF COMMENTS
EDITOR COMMENTS

Reviewing Editor:

The statistical Summary document is missing.

I also would like to commend the authors for this excellent work and the quality of the figures presented in the manuscript. However, like Referee 1, I would like to invite the authors to indicate in the title the work was performed in mice and to tone down their conclusions on the clinical implications of their work.

Please take into account the suggestions of Referee 1 to improve the text.

Adding a figure to summarize the key findings could be helpful for the reader.

The Summary Statement document is missing.

Senior Editor:

Thank you for allowing us to revise our manuscript. The different suggestions from both Reviewers have substantially improved our paper. Below is our detailed responses to all of their comments (in blue).

We particularly clarified any confusion about the use of the term ‘Zeitgeber’ in our study and tone down our statements as suggested by Reviewer #1. We likewise now provide a ‘graphical résumé’, the ‘statistical summary’ document, and deposited our sequencing and proteomics data to GEO and ProteomeXchange, respectively.

For all the other points raised by both Reviewers, we are providing some additional elements of discussion and further clarified the different sections of our manuscript.

Please note that any change applied in this revised version are highlighted in yellow.
REFEREE COMMENTS

We deeply thank both Referees for their interest in our work and for their helpful comments that will improve our manuscript. We have tried to do our best to respond to the points raised and we appreciate the opportunity to clarify results. As indicated below, we have checked all the comments provided by both Reviewers and have made necessary changes accordingly.

Again, we would like to thank both Reviewers for their suggestions that we believe not only help us to clarify but also strengthen our findings.

Referee #1:

I commend the authors on the large array of quality data they have collected in an extremely intensive and robust study protocol. I hope the authors did not disturb their circadian rhythms too much in the process.

The 'omics' data in this study are extremely comprehensive and will aid future researchers significantly when assessing this field. As with many circadian biology studies, feeding is a confounder in the acute exercise protocol in this study, but the authors do cover this limitation sufficiently in the discussion.

My main suggestions for improvement relate to the presentation of the study in text. The authors should carefully revise this based on the suggestions below. In general the authors should be mindful that there have been several studies assessing similar non-'omic', circadian biology outcomes in their study, in humans and mice, and be sure to cite and discuss these satisfactorily. Additionally, the authors have (understandably) relatively low n in many of these experiments, and it should be trivial to present the individual values in most figure panels.

General:

Where possible, show individual data-points rather than 'plunger' bar charts or single-line time-course.

We now provide individual data point for all data depicted in bar graphs in our manuscript; unfortunately, for those data depicted in 'line graphs' our attempts to produce something easily readable, not overfilled with symbols, have failed. This concerns datasets depicted in panels 8C, D, F, G and Figure 9 and 10. We, however, now show SD instead of SEM in all data panels, provide a full statistical report, and added a statement in the Method and Data availability sections.
indicating that all data supporting the findings of this study are available from the corresponding author upon request.

The authors use different terminology throughout the manuscript for the ZT at which exercise was performed (e.g. 'early morning' at one point and 'light' in another). These should be defined early (in abstract if possible) and be consistent throughout the manuscript.

Thank you for this suggestion – this is now clarified early in the Results section.

Title: please make the readers of the title aware that this study was conducted in mice.

This has now been changed as suggested.

Abstract: Although this is a comprehensive and well-conducted study, the lack of human data renders the sentence "These results are of high importance to understand circadian aspects of training design in athletes and the application of chrono-exercise-based interventions in patients." a slight over-reach in my opinion. The authors should temper this sentence, bearing in mind the study was conducted in mice.

We agree with the reviewer and have accordingly changed this sentence.

Key points: "Maximal endurance performance is greater in the early morning", I don't find the terminology 'morning' helpful here, perhaps the authors could clarify this statement. E.g.. 'late active phase' or a statement along those lines.

This is now changed to 'early daytime’ across the manuscript.

"Training exerts poor Zeitgeber activity on the muscle and liver clocks". Could this statement be written with more clarity for a general audience?

Thank you for this suggestion – this key point has now been simplified.

Line 83: "Inversely, a potential Zeitgeber activity of training also is unclear." This sentence should be clarified.

This is now clarified with the addition of the following: ‘i.e. whether repeated bouts of exercise affect phase and amplitude of the molecular clock in muscle or other tissues’.

Line 86: "Therefore, whether the enhanced effects of timed exercise training on health parameters in both clinical and preclinical contexts [16, 17] depend on the Zeitgeber properties of exercise remains to be investigated [18, 19]." This line is difficult to read
and could be clarified. E.g. I presume the authors mean 'the putative enhanced effects of timed exercise'.

'Putative' is now added to the text.

"depend on the Zeitgeber properties of exercise remains to be investigated [18, 19]."I am not clear why citations 18,19 are relevant to this statement or sentence?

Both citations have now been removed.

Although the authors cite 19 in the intro, these findings should be expanded upon as they are pertinent to the current study.

Despite the use of treadmill exercise at similar phases of the daily cycle, contrary to Sato et al., our mice were pushed to run until exhaustion and did not have access to food after the treadmill exercise sessions, making the comparison of our respective gene expression studies somewhat difficult. Moreover, we only focused on the acute response to treadmill exercise (at +0h and +3h) and did not evaluate daily rhythmicity in gene or protein expression subsequent to an acute bout of exercise.

Line 99: "Differences in low- and moderate-intensity treadmill exercise performance were reported within the active phase of wild-type mice [12]." This should state 'previously reported'

This has now been changed as suggested.

Line 109: remove 'drastic'

This has now been removed.

Line 125: "Altogether, these data demonstrate that mice are surprisingly better at performing a maximal running test in the early light phase of the 24-h LD cycle." Given this would correspond with evening in humans, I don't find this surprising.

'Surprisingly' has now been removed.

Line: 314: "Scheduled daytime wheel-running activity in mice exposed to a skeleton photoperiod" please give an indication of what these results show in the title.

This subtitle has now been changed to ‘Mice with restricted wheel access spontaneously run during daytime under a skeleton photoperiod’ as suggested.
Line 418: "Thus, a strong mutual interaction between skeletal muscle, the circadian timing system, and exercise has been suspected [5]." I would suggest it has been more than suspected e.g. PMID: 28683290 demonstrates this.

We find the work by Jordan et al. very interesting, yet results obtained with global germline inactivation of clock gene should be interpreted with caution (PMID: 26843191; PMID: 27752300). For instance, altered skeletal muscle function and/or exercise capacity as seen in the CRY1/CRY2 double whole-body KO mice could be secondary to the systemic consequences caused by the disruption of molecular clock in other peripheral and central tissues, and/or due to the noncircadian function of both CRY early in development. We nevertheless changed “suspected” to “proposed”.

Line 425: "time of day on muscle cellular responses and performance" "remain poorly described" I assume the authors are referring to exercise (or possibly muscle) performance here. If so, exercise performance (also muscle performance) has actually been intensively studied in regards to diurnal fluctuations. E.g. PMID: 8000814, PMID: 8877122, PMID: 11023216, PMID: 9459533, PMID: 20030545, PMID: 20524806, PMID: 25289885, PMID: 20524806 (not an exhaustive list of studies in the field). I suggest the authors clarify what they mean in this sentence, and temper their implication of novelty accordingly.

Thank you for bringing these studies to our attention. We now have amended the text as suggested and clarified that our study was performed in the mouse.

Line 420: "First, the daily variations in metabolic and functional properties, ultimately controlled by the circadian clock, will affect endurance and strength capacities." This sentence is unclear, do the authors mean that it has been postulated from the literature that variations in metabolic properties will affect endurance and strength. It is well-known that human strength capacity fluctuates in a diurnal manner, and it is unclear in this sentence if the authors are stating that as an unknown?

We apologize for the lack of clarity and have now clarified our view in the discussion part.

Line 429: "collectively, our results strongly suggest that training has very poor Zeitgeber activity on the skeletal muscle clock" the authors should specify which time of training they are referring to. Also please see remarks about Zeitgeber below.

'Early daytime voluntary wheel running' now replaces ‘training' to clarify this point.

Line 432: "daytime training leads" it is unclear how this differs from the previous sentence mentioning ‘training'
See point above – we hope that with this change, the meaning should be clear now.

Line 483: "Future experiments with inducible, skeletal muscle specific knockout models for clock components might thus help to clarify the contribution of this system to muscle metabolism a" PMID: 28683290 should be cited and discussed here.

In line with the above comment, we do not think that the work of Jordan et al. supports our recommendation. Jordan et al. state in their work that:

‘While enhanced PPARδ-driven fatty acid oxidation in muscles likely contributes to enhanced exercise performance in dKO mice, other effects of global CRY1 and CRY2 deficiency throughout development may also contribute to this improvement in fitness. Further investigation, including examination of animals lacking CRY1 and CRY2 in specific anatomic locations, is needed to determine the relative contributions of various underlying mechanisms to modulation of exercise physiology by cryptochromes.’

Therefore, as stated in our discussion, we strongly encourage studies with time- and tissue-specific control of clock gene deletion in mouse skeletal muscle which may circumvent current problems and limitations inherent to the use of germline models of clock gene deletion (PMID: 26843191; PMID: 27752300 already cited in the text).

Line 503: "Thus, athletes will have to be assessed individually ("personalized training")." There has already been some research into the individual chronotypes etc of athletes, perhaps the authors could cite the relevant research here e.g. PMID: 30357501 etc. Also the authors should either elaborate on this statement, or remove it, as it is not supported by the surrounding text at present.

We removed this sentence and cited the suggested research.

Line 506: "Lastly, our data indicate that training is not only not a strong Zeitgeber in a healthy mouse muscle, but, if chronically performed at the wrong time of the day, might lead to perturbations of the muscle clock which resemble perturbations of other peripheral clocks in pathological states and in aging, characterized by a general dampening of oscillation amplitudes" One may argue that an extrinsic stimulus leading to 'perturbations of the muscle clock' would in fact precisely fit the definition of a Zeitgeber. I suggest the authors define what they mean by Zeitgeber early on in the manuscript, as it appears to differ from some forms of usage the literature. Also, please clarify this passage of text.

Thank you for this comment. We now clarified the use of ‘Zeitgeber’ in the text and in the context of our study.

Line 513: "Regardless of the outcome of future studies in such a direction, however, the negative results of our studies in regard to the effect of daytime activity in mice as
Zeitgeber of the muscle clock should not be interpreted to discount training interventions" As a conclusion, and in mind of the general reader, I find this opaque. I think what the authors mean by Zeitgeber is something which entrains homeostatic rhythms, whereas it is frequently used in the literature to denote an extrinsic stimuli that can 're-set' the molecular clock. In most cases I think it would be clearer for the reader if the authors 'spelled-out' what they are trying to say when they use the word Zeitgeber. Additionally, I do not understand why the results are 'negative”? Please clarify.

We agree with the reviewer and have now removed this last paragraph from the manuscript.

Figure legends: it should be clarified what the light and dark shading refers to in the figures.

This clarification has now been added in the figure legend text.

"The data have been deposited in GEO (accession numbers XXX)." I assume the authors will update this upon acceptance for publication.

The data have been deposited and the GEO accession numbers will be added upon acceptance of the manuscript (data have been deposited and we are currently waiting for the accession number).
The authors evaluated the effects of timed exercise at the systemic and muscle cellular levels by assessing maximal treadmill exercise capacity across the 24-h light-dark (LD) cycle. The authors also dissected the transcriptome and (phospho-)proteome responses of working muscles at two distinct phases of the LD cycle. In addition, to investigate the potential Zeitgeber activity of exercise was used a skeleton photoperiod (SPP) in combination with restricted wheel-running access to interrogate the consequences of scheduled daytime voluntary training on skeletal muscle gene and protein regulation. The study is very interesting and was very well conducted by the authors. The results are quite broad and fill an important gap in science. Some concerns and suggestions are addressed to the authors below.

1. In the materials and methods section, the information should be included if the mice remained alone or in groups in the cages;

We apologize for the lack of clarify. For the ‘Daytime scheduled wheel-running activity’ experiment, mice were single-housed in standard cages, within an environment-controlled cabinet (UniProtect Air Flow Cabinet, Bioscape) as indicated in the method. For the ‘Forced high-intensity exercise performance across the day’ experiment, we have now indicated in the Method part that mice where group-housed.

2. Why was the inclination used to perform the maximum running test on a treadmill? Any special reason?

The determination of ‘true exhaustion’ is a difficult task; nonetheless, running capacity is generally evaluated with an uphill angle from 5 to 25% in healthy, young adult rodents (PMID: 12235029, PMID: 11179077). Prior to this work, we tested different running conditions by challenging mice in a closed metabolic treadmill allowing us to measure maximum oxygen consumption and evaluate the respiratory exchange ratio, alongside blood lactate level—as a proxy for fatigue. We found that a 15% positive inclination was well tolerated by young adult mice and allowed us to consistently observe a peak/plateau of VO2 values, RER values close or above 1, and blood lactate ≥ 5 mmol/L. We thus have used the exact same protocol in this study using an ‘open’ treadmill.

3. Is it possible to include information if the food intake in the night cycle (which is higher) happens more intensely at the beginning, middle, or end of the night cycle?

Figure 8G is showing food intake at two different time periods across the night.

4. It was mentioned in the study that the lactate collection at the end of the exercise occurred within a time of up to 1 min. However, was a single collection performed?
There is a significant variation in the lactate values between the times 0 to 10 minutes after the end of the exercise. In addition, the lactate collection in these times (0, 3, 5, 7, and 10 min) can help to identify the highest lactate value after exercise.

**We agree that post-exercise blood lactate time curves are of high interest and relevance. However, in relation to point 2 and in the context of another study, we evaluated blood lactate at two different times post-exhaustion and observed only a slight reduction of blood lactate values between 1 and 5 min after exercise as depicted in the graph below. We thus decided to evaluate blood glucose within 1 min upon exhaustion in this study.**

![Graph showing blood glucose and lactate levels](image)

- Two mouse groups (series 1; 2) were challenged with treadmill in the early daytime. Blood glucose (BG) and lactate (BL) were measured 1 minute (+1) and 5 minutes (+5) after exhaustion was reached. Data not published.

5. In addition to the total distance covered, it is relevant to include the maximum speed and total time data obtained by the animals in the running test. Thus, the authors must consider the inclusion of these data in the manuscript or as complementary material;

**Total time is provided in Fig. 2A. We did not include maximum speed as it is a much less accurate parameter—in a speed increment protocol—to precisely compare the performance of different mice.**

6. The authors may consider changing the color of the blue rectangles used in the light and dark cycle in the figures. Perhaps the change can help to better understand the results found in the light and dark cycle in the study;

**Gray rectangles are now consistently used to indicate the nighttime, dark period.**

7. Was the forced performance test of high-intensity exercises throughout the day carried out with the animals fasting? this information can be made clearer in the text.

**We added the following to the method section: ‘Importantly, all mice had a free access to food prior to an exercise bout, that is, were not fasted’.

8. Although the text is quite explanatory. A schematic figure with the description of the steps, especially related to the moments when the exercises were performed, tissue collection, would be valid to facilitate the understanding of the reader;
We have tried to clarify the exercise protocols in Figs. 1A and 8A, the corresponding figure captions and/or method. We hope that this helps to better understand the experimental setup.

9. The authors very well wrote the discussion section. Information on limitations, future prospects, and differences between rodents and humans have been included. As a single observation, the authors could consider including a schematic figure at the end with the description of some of the most important points found in the light and dark cycle and, above all, of the performance in these different periods of the day.

We appreciate this comment and thank you for this excellent suggestion. We have now added a ‘schematic figure’ (Fig. 13).
Dear Dr Handschin,

Re: JP-RP-2021-281535R1 "Transcriptomic, proteomic and phosphoproteomic underpinnings of daily exercise performance and Zeitgeber activity of training in mouse muscle" by Geraldine Maier, Julien Delezie, Pål O. Westermark, Gesa Santos, Danilo Ritz, and Christoph Handschin

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

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

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Yours sincerely,

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EDITOR COMMENTS

Reviewing Editor:

All the comments have been well addressed. The authors must be commended for their great work.

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REFEREE COMMENTS

Referee #1:

The authors have satisfactorily responded to my comments. I commend them on a great
manuscript that will enhance the field of study.

Referee #2:

I agree with the changes and additions made by the authors to the manuscript.