

Sleep in unnatural times: illuminated night negatively affects sleep and associated hypothalamic gene expressions in diurnal zebra finches

Twinkle Batra, Indu Malik, Abhilash Prabhat, Sanjay Kumar Bhardwaj and Vinod Kumar

Article citation details

Proc. R. Soc. B **287**: 20192952.

<http://dx.doi.org/10.1098/rspb.2019.2952>

Review timeline

Original submission: 19 December 2019

1st revised submission: 15 April 2020

2nd revised submission: 12 May 2020

Final acceptance: 12 May 2020

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

Review History

RSPB-2019-2952.R0 (Original submission)

Review form: Reviewer 1

Recommendation

Reject – article is scientifically unsound

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

Acceptable

General interest: Is the paper of sufficient general interest?

Good

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

Acceptable

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

Title: Sleep in unnatural times: Illuminated night negatively affects sleep and associated hypothalamic gene expressions in diurnal zebra finches

Note: there were no line numbers in the text so my comments generally refer to the page numbers and rough paragraphs.

The authors exposed female zebra finches to dim light at night and checked *bmal1* and *per2* pacemakers in the hypothalamus at 6 time points, oxalate and sleep behaviour of a subset of the birds (9 for oxalate, 6 for sleep) at one time point and only over 2 days (not all 3 weeks), and sleep regulatory pathways at day and night. While the questions are interesting, the various (non-agreeing) sample sizes used during the experiment is potentially problematic. There are no reports of daytime activity so diurnal differences in *achm3* levels are difficult to interpret. Lastly, the authors only picked two pacemaker genes for study. While there is significant change of *per2* in LD and dLAN, for *bmal1* there is no difference. Without testing more genes such as *Cry* in the repression loop or *Clk* in the activation loop, it is difficult to draw any conclusion whether this effect is specific or not.

Abstract: please add how long birds were exposed and state in the abstract this was just performed on females

Introduction:

P4: There is some evidence from Dominoni group recent publication and following citation that oxalate may not be representative for birds:

Raap, T., Pinxten, R., & Eens, M. (2018). Artificial light at night causes an unexpected increase in oxalate in developing male songbirds. *Conservation Physiology*, 6(1). doi: 10.1093/conphys/coy005

P4-on: For all cytokine work, authors should consider this recent publication: Mishra, I., Knerr, R. M., Stewart, A. A., Payette, W. I., Richter, M. M., & Ashley, N. T. (2019). Light at night disrupts diel patterns of cytokine gene expression and endocrine profiles in zebra finch (*Taeniopygia guttata*). *Scientific Reports*, 9(1), 15833. doi: 10.1038/s41598-019-51791-9

P4-on: Authors should consider clock gene recent work on dim light and urban/rural areas: Renthlei, Z., & Trivedi, A. K. (2019). Effect of urban environment on pineal machinery and clock genes expression of tree sparrow (*Passer montanus*). *Environmental Pollution*, 255, 113278. <https://doi.org/10.1016/j.envpol.2019.113278>

Methods:

P6: It would be interesting to have measured males especially for sleep reducing reasons

P6: state what types of light were used, spectra and radiance

Why did the authors pick only *per2* and *bmal1* and not other circadian genes? It's not clear from

the discussion or introduction.

P6: only 6 birds were videographed. There is ample evidence from Niels Rattenborg's group that there is large intra- and inter-individual variation in sleep behaviours. There were 72 birds total but effective ample size for behaviours is 6 per group. Authors mention video limitations but videos could be cycled during last week of the experiment, assuming authors had 12 cameras, one week would have been enough to measure behaviour.

P7: uncertain why only 9 (from supplementary materials) birds were measured for oxalic acid levels. There should be blood samples from all birds?

P8: How was disturbance minimized during sacrifice throughout one day?

P8 and in general: was behaviour measured during the day? It would be good to see recovery sleep. Figure 1D would be good to extend to day hours.

P8: why were the other genes only measured only once during day and night? How was 4 h into each state assigned?

Figure 1: A and B: I would have preferred an actogram instead of a sleepogram (more standard in chronobiology). Was sleep measured during the daylight hours? It does not seem like it from the methodology. Therefore, the daylight hours should be removed if there is no information (rather than just blank, which would indicate that there was no sleep during daylight hours). Figure 1 legend should have C labelled similarly to the other letters. Am I correct that LD had more sleep bouts but also longer duration? If this is true, then from first paragraph in discussion, it's not just the sleep onset and offset but also bout length that is affected. Essentially, were LD birds just sleeping longer but also had longer bouts? From the figure, It doesn't seem like dimLAN birds had less sleep bouts, their sleep bouts seemed more than the controls from sleepogram.

Figure 2: It seems that there is a lot of variation in oxalate levels in dLAN. Some birds did not seem to decrease compared to controls. Was oxalate measured before treatment during LD? It would be useful to check decrease within individuals than between the two treatments. Why is there so much more variation in oxalate levels for dLAN than control birds?

P10: consider within individual LMMs that would account for within individual differences from LD to dLAN. Here multiple T-tests were performed on 6 time points and could be subject to error whereas one model would take care of the analyses.

P10: line about sleep and awake latencies: reword such that it is clear what is increasing and what is decreasing.

P13 first sentence: is this a question?

P12-13: no discussion of bmal

P13: the discussion on the cytokines could benefit from a discussion at multiple time points

P14: suggest removing last part about humans to avoid over-speculation

Review form: Reviewer 2

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?

N/A

Is it clear?

N/A

Is it adequate?

N/A

Do you have any ethical concerns with this paper?

No

Comments to the Author

RSPB-2019-2952

Sleep in unnatural times: Illuminated night negatively affects sleep and associated hypothalamic gene expression in diurnal zebra finches

By Batra et al.

The authors analyze changes in sleep and changes in gene transcription involved in circadian and sleep regulation in the zebra finch under influence of dim light at night. This is a very hot topic and probably one of the first studies analyzing this in this detail in a day active animal.

I only have minor comments.

Minor comments

In general it seems that sometimes in the sentences the articles of some words are missing. This needs to be checked throughout the manuscript. For example

Page 3, 2nd paragraph line 3: "[The] daily sleep-wake pattern is..."

Page 6, 1st paragraph, line 13 "... 1 lux) for [the] next three weeks."

Page 5, 2nd paragraph, 1st line "We monitored [the] sleep state of [the] bird[s] by [their] posture in [the]cage using [a] night vision camera and..."

Page 10 2nd paragraph, line 7 "... acrophase of [the] daily rhythm in ..."

Page 12 2nd paragraph, 1st line "... sleep effects involve [the] endogenous circadian ..."

Page 3, 1st paragraph line 4. Next to evidence that light at night influences metabolism in rodents, it was also shown that it can influence sleep in rodents (see for instance Stenvers et al Sci Rep 2016).

Page 4 last line "... 12 h light coupled [to] 12 h in absolute darkness..."

Page 8 2nd paragraph. It is unclear which animals gave blood for the determination of the oxalic acid levels. From the data I understand these are 9 animals per group, but that means they do not (only) come from the group where the behavior was recorded, but they also do not cover the

group that was sampled later in 4h intervals, since the latter group was much larger. And at which time point were these samples taken?

Page 11, line 19 and 20, there are two sentences starting with "however". Particularly the second sentence I am wondering why it starts with this word.

Page 12 1st paragraph, line 11. "... associated to [an] attenuated nocturnal melatonin..."

Page 12 2nd paragraph. I think the authors are aware of the problem of the chicken and the egg: The question whether sleep deprivation causes changes in circadian mRNA oscillations or whether the changes in circadian mRNA oscillations are causing the increase in waking, but this is not well fleshed out in this paragraph. Only at the end it becomes clear, so maybe it is better to also introduce this at the beginning of the paragraph.

Page 13 2nd paragraph, line 4. I think at "in turn..." a new sentence should start.

Page 13, last line. Please do not end the sentence and paragraph with etc., but be more specific.

Decision letter (RSPB-2019-2952.R0)

03-Feb-2020

Dear Dr Kumar:

Your manuscript has now been peer reviewed and the reviews have been assessed by an Associate Editor. As you will see, the reviewers and the AE have raised some concerns with your manuscript and we would like to invite you to revise your manuscript to address them. In particular, your reviewers expressed very different views in some aspects of your study. Both the AE and I conclude that the concerns raised by reviewer 1 do not preclude publication, however please be sure to address the other comments in detail. The reviewers' comments (not including confidential comments to the Editor) and the comments from the Associate Editor are included at the end of this email for your reference.

We do not allow multiple rounds of revision so we urge you to make every effort to fully address all of the comments at this stage. If deemed necessary by the Associate Editor, your manuscript will be sent back to one or more of the original reviewers for assessment. If the original reviewers are not available we may invite new reviewers. Please note that we cannot guarantee eventual acceptance of your manuscript at this stage.

To submit your revision please 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 Revision". Your manuscript number has been appended to denote a revision.

When submitting your revision please upload a file under "Response to Referees" - in the "File Upload" section. This should document, point by point, how you have responded to the reviewers' and Editors' comments, and the adjustments you have made to the 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.

Your main manuscript should be submitted as a text file (doc, txt, rtf or tex), not a PDF. Your figures should be submitted as separate files and not included within the main manuscript file.

When revising your manuscript you should also ensure that it adheres to our editorial policies (<https://royalsociety.org/journals/ethics-policies/>). You should pay particular attention to the following:

Research ethics:

If your study contains research on humans please ensure that you detail in the methods section whether you obtained ethical approval from your local research ethics committee and gained informed consent to participate from each of the participants.

Use of animals and field studies:

If your study uses animals please include details in the methods section of any approval and licences given to carry out the study and include full details of how animal welfare standards were ensured. Field studies should be conducted in accordance with local legislation; please include details of the appropriate permission and licences that you obtained to carry out the field work.

Data accessibility and data citation:

It is a condition of publication that you make available the data and research materials supporting the results in the article. Datasets should be deposited in an appropriate publicly available repository and details of the associated accession number, link or DOI to the datasets must be included in the Data Accessibility section of the article (<https://royalsociety.org/journals/ethics-policies/data-sharing-mining/>). Reference(s) to datasets should also be included in the reference list of the article with DOIs (where available).

In order to ensure effective and robust dissemination and appropriate credit to authors the dataset(s) used should also be fully cited and listed in the references.

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=\(Document not available\)](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.

For more information please see our open data policy <http://royalsocietypublishing.org/data-sharing>.

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. Please try to submit all supplementary material as a single file.

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].

Please submit a copy of your revised paper within three weeks. If we do not hear from you within this time your manuscript will be rejected. If you are unable to meet this deadline please let us know as soon as possible, as we may be able to grant a short extension.

Thank you for submitting your manuscript to Proceedings B; we look forward to receiving your revision. If you have any questions at all, please do not hesitate to get in touch.

Best wishes,
Dr Sarah Brosnan
Editor, Proceedings B
mailto: proceedingsb@royalsociety.org

Associate Editor
Comments to Author:

Both referees agree in that this is a highly timely and also largely novel study (but see additional recent references to be considered that were pointed out by referee 1). The experts disagreed in their evaluation, however. Referee 2 has only minor issues and is generally highly in favour of the study, whereas referee 1 would have preferred higher/more equal sample sizes in some analyses, a consideration of more clock genes, and the measurement of more time points in the analysis of cytokines. While I agree that the differences in samples sizes are not very elegant, I find that the results of the analyses with reduced samples sizes are still very clear, so I do not consider this a major issue. I agree with referee 2 that – within the limitations outlined by referee 1 - this is still a very detailed study in field where research is still scarce but urgently needed. Both referees made a long list of invaluable feedback for improvement of clarity of content, interpretation and language to be considered by the authors.

Reviewer(s)' Comments to Author:

Referee: 1

Comments to the Author(s)

Title: Sleep in unnatural times: Illuminated night negatively affects sleep and associated hypothalamic gene expressions in diurnal zebra finches

Note: there were no line numbers in the text so my comments generally refer to the page numbers and rough paragraphs.

The authors exposed female zebra finches to dim light at night and checked *bmal1* and *per2* pacemakers in the hypothalamus at 6 time points, oxalate and sleep behaviour of a subset of the birds (9 for oxalate, 6 for sleep) at one time point and only over 2 days (not all 3 weeks), and sleep regulatory pathways at day and night. While the questions are interesting, the various (non-agreeing) sample sizes used during the experiment is potentially problematic. There are no reports of daytime activity so diurnal differences in *achm3* levels are difficult to interpret. Lastly, the authors only picked two pacemaker genes for study. While there is significant change of *per2* in LD and dLAN, for *bmal1* there is no difference. Without testing more genes such as *Cry* in the repression loop or *Clk* in the activation loop, it is difficult to draw any conclusion whether this effect is specific or not.

Abstract: please add how long birds were exposed and state in the abstract this was just performed on females

Introduction:

P4: There is some evidence from Dominoni group recent publication and following citation that oxalate may not be representative for birds:

Raap, T., Pinxten, R., & Eens, M. (2018). Artificial light at night causes an unexpected increase in oxalate in developing male songbirds. *Conservation Physiology*, 6(1). doi: 10.1093/conphys/coy005

P4-on: For all cytokine work, authors should consider this recent publication: Mishra, I., Knerr, R. M., Stewart, A. A., Payette, W. I., Richter, M. M., & Ashley, N. T. (2019). Light at night disrupts diel patterns of cytokine gene expression and endocrine profiles in zebra finch (*Taeniopygia guttata*). *Scientific Reports*, 9(1), 15833. doi: 10.1038/s41598-019-51791-9

P4-on: Authors should consider clock gene recent work on dim light and urban/rural areas: Renthlei, Z., & Trivedi, A. K. (2019). Effect of urban environment on pineal machinery and clock

genes expression of tree sparrow (*Passer montanus*). *Environmental Pollution*, 255, 113278.
<https://doi.org/10.1016/j.envpol.2019.113278>

Methods:

P6: It would be interesting to have measured males especially for sleep reducing reasons

P6: state what types of light were used, spectra and radiance

Why did the authors pick only *per2* and *bmal1* and not other circadian genes? It's not clear from the discussion or introduction.

P6: only 6 birds were videographed. There is ample evidence from Niels Rattenborg's group that there is large intra- and inter-individual variation in sleep behaviours. There were 72 birds total but effective ample size for behaviours is 6 per group. Authors mention video limitations but videos could be cycled during last week of the experiment, assuming authors had 12 cameras, one week would have been enough to measure behaviour.

P7: uncertain why only 9 (from supplementary materials) birds were measured for oxalic acid levels. There should be blood samples from all birds?

P8: How was disturbance minimized during sacrifice throughout one day?

P8 and in general: was behaviour measured during the day? It would be good to see recovery sleep. Figure 1D would be good to extend to day hours.

P8: why were the other genes only measured only once during day and night? How was 4 h into each state assigned?

Figure 1: A and B: I would have preferred an actogram instead of a sleepogram (more standard in chronobiology). Was sleep measured during the daylight hours? It does not seem like it from the methodology. Therefore, the daylight hours should be removed if there is no information (rather than just blank, which would indicate that there was no sleep during daylight hours). Figure 1 legend should have C labelled similarly to the other letters. Am I correct that LD had more sleep bouts but also longer duration? If this is true, then from first paragraph in discussion, it's not just the sleep onset and offset but also bout length that is affected. Essentially, were LD birds just sleeping longer but also had longer bouts? From the figure, It doesn't seem like dimLAN birds had less sleep bouts, their sleep bouts seemed more than the controls from sleepogram.

Figure 2: It seems that there is a lot of variation in oxalate levels in dLAN. Some birds did not seem to decrease compared to controls. Was oxalate measured before treatment during LD? It would be useful to check decrease within individuals than between the two treatments. Why is there so much more variation in oxalate levels for dLAN than control birds?

P10: consider within individual LMMs that would account for within individual differences from LD to dLAN. Here multiple T-tests were performed on 6 time points and could be subject to error whereas one model would take care of the analyses.

P10: line about sleep and awake latencies: reword such that it is clear what is increasing and what is decreasing.

P13 first sentence: is this a question?

P12-13: no discussion of *bmal*

P13: the discussion on the cytokines could benefit from a discussion at multiple time points

P14: suggest removing last part about humans to avoid over-speculation

Referee: 2

Comments to the Author(s)

RSPB-2019-2952

Sleep in unnatural times: Illuminated night negatively affects sleep and associated hypothalamic gene expression in diurnal zebra finches

By Batra et al.

The authors analyze changes in sleep and changes in gene transcription involved in circadian and sleep regulation in the zebra finch under influence of dim light at night. This is a very hot topic and probably one of the first studies analyzing this in this detail in a day active animal.

I only have minor comments.
Minor comments

In general it seems that sometimes in the sentences the articles of some words are missing. This needs to be checked throughout the manuscript. For example

Page 3, 2nd paragraph line 3: “[The] daily sleep-wake pattern is...”

Page 6, 1st paragraph, line 13 “... 1 lux) for [the] next three weeks.”

Page 5, 2nd paragraph, 1st line “We monitored [the] sleep state of [the] bird[s] by [their] posture in [the]cage using [a] night vision camera and...”

Page 10 2nd paragraph, line 7 “... acrophase of [the] daily rhythm in ...”

Page 12 2nd paragraph, 1st line “... sleep effects involve [the] endogenous circadian ...”

Page 3, 1st paragraph line 4. Next to evidence that light at night influences metabolism in rodents, it was also shown that it can influence sleep in rodents (see for instance Stenvers et al Sci Rep 2016).

Page 4 last line “... 12 h light coupled [to] 12 h in absolute darkness...”

Page 8 2nd paragraph. It is unclear which animals gave blood for the determination of the oxalic acid levels. From the data I understand these are 9 animals per group, but that means they do not (only) come from the group where the behavior was recorded, but they also do not cover the group that was sampled later in 4h intervals, since the latter group was much larger. And at which time point were these samples taken?

Page 11, line 19 and 20, there are two sentences starting with “however”. Particularly the second sentence I am wondering why it starts with this word.

Page 12 1st paragraph, line 11. “... associated to [an] attenuated nocturnal melatonin...”

Page 12 2nd paragraph. I think the authors are aware of the problem of the chicken and the egg: The question whether sleep deprivation causes changes in circadian mRNA oscillations or whether the changes in circadian mRNA oscillations are causing the increase in waking, but this is not well fleshed out in this paragraph. Only at the end it becomes clear, so maybe it is better to also introduce this at the beginning of the paragraph.

Page 13 2nd paragraph, line 4. I think at “in turn...” a new sentence should start.

Page 13, last line. Please do not end the sentence and paragraph with etc., but be more specific.

Author's Response to Decision Letter for (RSPB-2019-2952.R0)

See Appendix A.

Decision letter (RSPB-2019-2952.R1)

12-May-2020

Dear Dr Kumar

I am pleased to inform you that your manuscript RSPB-2019-2952.R1 entitled "Sleep in unnatural times: Illuminated night negatively affects sleep and associated hypothalamic gene expressions in diurnal zebra finches" has been accepted for publication in Proceedings B pending some minor revisions.

The AE has made some excellent suggestions to further strengthen your manuscript (please see below and attached). Therefore, I invite you to respond to the 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(s) 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=\(Document not available\)](http://datadryad.org/submit?journalID=RSPB&manu=(Document%20not%20available)) 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,
Dr Sarah Brosnan
Editor, Proceedings B
<mailto:proceedingsb@royalsociety.org>

Associate Editor:
Board Member
Comments to Author:

I have carefully studied the revisions made on this manuscript and I am satisfied with how the authors dealt with the comments. In particular they added a lot of additional data which had been requested by referee 1. I am attaching marked files of

- the main text
- Table S3, and
- Supplementary methods

containing my corrections of typos and language. The main text files also contains comments in places where the meaning was not clear to me. Moreover table 3 needs to be structured in two parts, as highlighted in my comment. All edits were done in the track changes mode of Word.

I would like to ask the authors to carefully attend to these edits and questions to get the paper in shape for publication.

Author's Response to Decision Letter for (RSPB-2019-2952.R1)

See Appendix B.

Decision letter (RSPB-2019-2952.R2)

12-May-2020

Dear Dr Kumar

I am pleased to inform you that your manuscript entitled "Sleep in unnatural times: Illuminated night negatively affects sleep and associated hypothalamic gene expressions in diurnal zebra finches" 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 9 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.

You are allowed to post any version of your manuscript on a personal website, repository or preprint server. However, the work remains under media embargo and you should not discuss it with the press until the date of publication. Please visit <https://royalsociety.org/journals/ethics-policies/media-embargo> for more information.

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,

Editor, Proceedings B
<mailto:proceedingsb@royalsociety.org>

Appendix A

Response to Editor's and Reviewers' comments

Associate Editor

Comments to Author:

Both referees agree in that this is a highly timely and also largely novel study (but see additional recent references to be considered that were pointed out by referee 1). The experts disagreed in their evaluation, however. Referee 2 has only minor issues and is generally highly in favour of the study, whereas referee 1 would have preferred higher/more equal sample sizes in some analyses, a consideration of more clock genes, and the measurement of more time points in the analysis of cytokines. While I agree that the differences in sample sizes are not very elegant, I find that the results of the analyses with reduced samples sizes are still very clear, so I do not consider this a major issue. I agree with referee 2 that – within the limitations outlined by referee 1 - this is still a very detailed study in field where research is still scarce but urgently needed. Both referees made a long list of invaluable feedback for improvement of clarity of content, interpretation and language to be considered by the authors.

Thank you so very much for your encouragement, and very valuable comments.

Reviewer(s)' Comments to Author:

Referee: 1

Comments to the Author(s)

Title: Sleep in unnatural times: Illuminated night negatively affects sleep and associated hypothalamic gene expressions in diurnal zebra finches

Note: there were no line numbers in the text so my comments generally refer to the page numbers and rough paragraphs.

Comment #1. The authors exposed female zebra finches to dim light at night and checked *bmal1* and *per2* pacemakers in the hypothalamus at 6 time points, oxalate and sleep behaviour of a subset of the birds (9 for oxalate, 6 for sleep) at one time point and only over 2 days (not all 3 weeks), and sleep regulatory pathways at day and night. While the questions are interesting, the various (non-agreeing) sample sizes used during the experiment is potentially problematic.

Response #1. Thank you. We had to use a varying sample size because of the limitations at our end. For example, we recorded sleep behaviour of six birds because of the technical limitation for videographing, but we measured plasma oxalate levels of nine birds to have a larger sample size. This was also to match the sample size of the other blood variable, melatonin that we had measured and included in a previous publication (Batra et al., 2019). We believe that $n = 6$ was adequate to show the effect, since both treatment and control groups had similar sample size. This is now mentioned in methods section at page 8, lines 152-158.

Comment #2. There are no reports of daytime activity so diurnal differences in *achm3* levels are difficult to interpret.

Response #2. Thank you. Representative actogram and activity profile for 24-h period is now in the figure 1. There was no difference in the total 24-h activity levels between the control and dLAN group, but the nighttime activity was significantly increased under dLAN (12L: 12dLAN), compared to control (12L: 12D) group.

Comment #3. Lastly, the authors only picked two pacemaker genes for study. While there is significant change of *per2* in LD and dLAN, for *bmal1* there is no difference. Without testing more genes such as *Cry* in the repression loop or *Clk* in the activation loop, it is difficult to draw any conclusion whether this effect is specific or not.

Response #3. Thank you. We have included four more genes of the circadian transcriptional-translational (TTFL) loop, as suggested.

Comment #4. Abstract: please add how long birds were exposed and state in the abstract this was just performed on females

Response #4. Thank you. Incorporated in the abstract in lines 20-21.

Introduction:

Comment #5. P4: There is some evidence from Dominoni group recent publication and following citation that oxalate may not be representative for birds:

Raap, T., Pinxten, R., & Eens, M. (2018). Artificial light at night causes an unexpected increase in oxalate in developing male songbirds. *Conservation Physiology*, 6(1). doi: 10.1093/conphys/coy005

Response #5. Thank you. There are conflicting reports regarding the relationship of plasma oxalate levels with sleep loss in birds. In adult great tits exposed to white light at night, the loss of night time activity was correlated with decreased oxalate levels (Ouyang et al., 2017). However, there were increased oxalate levels in developing male great tits under illuminated night environment (Raap et al., 2018). Could it be that the relationship of sleep loss with plasma oxalate levels differed between developing and adult birds? We have included both references in the Introduction in lines 57-60.

Comment #6. P4-on: For all cytokine work, authors should consider this recent publication: Mishra, I., Knerr, R. M., Stewart, A. A., Payette, W. I., Richter, M. M., & Ashley, N. T. (2019). Light at night disrupts diel patterns of cytokine gene expression and endocrine profiles in zebra finch (*Taeniopygia guttata*). *Scientific Reports*, 9(1), 15833. doi: 10.1038/s41598-019-51791-9

Response #6. Thank you. We have considered and included this reference in the revised version in lines 62-65.

Comment #7. P4-on: Authors should consider clock gene recent work on dim light and urban/rural areas: Renthlei, Z., & Trivedi, A. K. (2019). Effect of urban environment on pineal machinery and clock genes expression of tree sparrow (*Passer montanus*). *Environmental Pollution*, 255, 113278. <https://doi.org/10.1016/j.envpol.2019.113278>

Response #7. Thank you. We have considered and included this reference in the revised version at page 15, lines 307-309.

Methods:

Comment #8. P6: It would be interesting to have measured males especially for sleep reducing reasons.

Response #8. Thank you. To avoid any potential sex-difference effects, we used only one sex, and specifically chose females since they could show a greater response to the altered light environment. Raap et al. (2015) reported a sex-dependent effect on sleep proportion, with female great tits spending a greater proportion of night awake, as compared to males. Few other studies have also investigated the effect of dim light at night on sleep behaviour in both males and females (Ouyang et al., 2017; Sun et al., 2017). A future study might could

include both male and female birds, as well as investigate dLAN effects both during early and late life in zebra finches. We have mentioned this in methods section at page 6, lines 102-106.

Comment #9. P6: state what types of light were used, spectra and radiance

Response #9. Thank you. We used Philips (India, 220V-240V) white light with a radiance of 220 lumens. This is now mentioned in the methods section at page 6, lines 116-122.

Comment #10. Why did the authors pick only *per2* and *bmal1* and not other circadian genes? It's not clear from the discussion or introduction.

Response #10. Thank you. We have now included four more clock genes of the circadian TTFL (see also response #3).

Comment #11. P6: only 6 birds were videographed. There is ample evidence from Niels Rattenborg's group that there is large intra- and inter-individual variation in sleep behaviours. There were 72 birds total but effective sample size for behaviours is 6 per group. Authors mention video limitations but videos could be cycled during last week of the experiment, assuming authors had 12 cameras, one week would have been enough to measure behaviour.

Response #11. Thank you. We take your point. Here, we videographed 6 of 36 birds in each group due to the technical limitation at our end, as we did not want to spread video recordings over several days to avoid any possible exposure duration (of experiment) effect. Although a larger sample size would always be better, we believe $n = 6$ chosen randomly would represent the response pattern of 36 birds. We had similar sample size in both conditions, and statistical analysis showed the treatment effect. We have mentioned this in methods section at page 8, lines 154-158.

Comment #12. P7: uncertain why only 9 (from supplementary materials) birds were measured for oxalic acid levels. There should be blood samples from all birds?

Response #12. Thank you. $n = 9$ was used for oxalate assay from daytime samples, and same sample size was used for the measurement of melatonin (Batra et al., 2019). The sample size was also close to what we included for the cytokine gene expression assays. We have mentioned this in methods section at page 8, lines 167-168.

Comment #13. P8: How was disturbance minimized during sacrifice throughout one day?

Response #13. Thank you. Birds were singly housed in the light-proof and sound-proof photoperiodic boxes. We sacrificed one bird at a time (when other birds were inside their boxes), and this minimized disturbance to its neighbor. This was evidenced also by activity recordings of other birds in the group. This is now mentioned in the methods section at page 6, lines 107-110.

Comment #14. P8 and in general: was behaviour measured during the day? It would be good to see recovery sleep. Figure 1D would be good to extend to day hours.

Response #14. Thank you. Yes, we had measured the behaviour both during the day and night. Birds did not show a sleep bout during the day; hence the data was not presented earlier. However, we have now added data for the daytime also to show the day-night sleep pattern (Figure 1).

Comment #15. P8: why were the other genes only measured only once during day and night? How was 4 h into each state assigned?

Response #15. Thank you. We did not think that daily change in expressions of genes related to sleep was relevant to the question that we addressed in this study; therefore, we chose hour 4 after the light on and light off in both control and dLAN birds. However, we have now extended this, and have measured mRNA expression for all the six timepoints, and presented data in the figure 4.

Comment #16. Figure 1: A and B: I would have preferred an actogram instead of a sleepogram (more standard in chronobiology). Was sleep measured during the daylight hours? It does not seem like it from the methodology. Therefore, the daylight hours should be removed if there is no information (rather than just blank, which would indicate that there was no sleep during daylight hours).

Response #16. Thank you. We have added actograms in the figure 1. We measured sleep behaviour also during the day; the blank portion in a sleepogram indicates no sleep bout during the daylight hours. We have mentioned now this clearly in the methods section. Having a sleepogram is useful, since birds at night might not be active, but awake; an actogram therefore may not adequately represent the sleep behaviour.

Comment #17. Figure 1 legend should have C labelled similarly to the other letters.

Response #17. Thank you. Corrected.

Comment #18. Am I correct that LD had more sleep bouts but also longer duration? If this is true, then from first paragraph in discussion, it's not just the sleep onset and offset but also bout length that is affected. Essentially, were LD birds just sleeping longer but also had longer bouts? From the figure, It doesn't seem like dimLAN birds had less sleep bouts, their sleep bouts seemed more than the controls from sleepogram.

Response #18. Thank you. Yes, there were more sleep bouts and also of the longer duration in LD, as compared to dLAN. To make this clear, we have reworded lines 281-286 in the first paragraph. Furthermore, sleep bouts are quite close in LD, but sparse in dLAN; hence, it is unclear in the sleepogram that there were more bouts in LD. For this reason, we have also plotted sleep bouts in a separate graph.

Comment #19. Figure 2: It seems that there is a lot of variation in oxalate levels in dLAN. Some birds did not seem to decrease compared to controls. Was oxalate measured before treatment during LD? It would be useful to check decrease within individuals than between the two treatments. Why is there so much more variation in oxalate levels for dLAN than control birds?

Response #19. Thank you. There was indeed larger variation in oxalate levels in dLAN, but we would usually expect such a variation in the experimental group as compared to controls. However, despite a higher individual variation, there was a significant difference in mean oxalate levels between LD and dLAN birds. We had not measured oxalate before the experiment began. We have mentioned this at page 14, lines 290-297.

Comment #20. P10: consider within individual LMMs that would account for within individual differences from LD to dLAN. Here multiple T-tests were performed on 6 time points and could be subject to error whereas one model would take care of the analyses.

Response #20. Thank you. We have now performed LMMs: GLM for gene expression data (since different birds contributed to different points of the day) and GLMM for activity and sleep behaviour, since the data are repeated measures.

Comment #21. P10: line about sleep and awake latencies: reword such that it is clear what is increasing and what is decreasing.

Response #21. Thank you. We have reworded the sentence at lines 227-229.

Comment #22. P13 first sentence: is this a question?

Response #22. Thank you. We have reworded this sentence.

Comment #23. P12-13: no discussion of bmal

Response #23. Thank you. We have now added discussion of bmal1 in lines 305-313.

Comment #24. P13: the discussion on the cytokines could benefit from a discussion at multiple time points

Response #24. Thank you. We have now added this in the discussion section.

Comment #25. P14: suggest removing last part about humans to avoid over-speculation

Response #25. Thank you. We have removed this in the discussion.

Referee: 2

Comments to the Author(s)

RSPB-2019-2952

Sleep in unnatural times: Illuminated night negatively affects sleep and associated hypothalamic gene expression in diurnal zebra finches

By Batra et al.

The authors analyze changes in sleep and changes in gene transcription involved in circadian and sleep regulation in the zebra finch under influence of dim light at night. This is a very hot topic and probably one of the first studies analyzing this in this detail in a day active animal.

Thank you. We appreciate your positive comments.

I only have minor comments.

Minor comments

Comment #26. In general it seems that sometimes in the sentences the articles of some words are missing. This needs to be checked throughout the manuscript. For example

Page 3, 2nd paragraph line 3: “[The] daily sleep-wake pattern is....”

Response #26. Thank you. We have checked and corrected throughout the manuscript.

Comment #27. Page 6, 1st paragraph, line 13 “... 1 lux) for [the] next three weeks.”

Response #27. Thank you. We have corrected this at line 111.

Comment #28. Page 5, 2nd paragraph, 1st line “We monitored [the] sleep state of [the] bird[s] by [their] posture in [the]cage using [a] night vision camera and...”

Response #28. Thank you. We have corrected this at line 139.

Comment #29. Page 10 2nd paragraph, line 7 “... acrophase of [the] daily rhythm in ...”

Response #29. Thank you. We have corrected this at line 205.

Comment #30. Page 12 2nd paragraph, 1st line "... sleep effects involve [the] endogenous circadian ..."

Response #30. Thank you. We have corrected this at line 304.

Comment #31. Page 3, 1st paragraph line 4. Next to evidence that light at night influences metabolism in rodents, it was also shown that it can influence sleep in rodents (see for instance Stenvers et al Sci Rep 2016).

Response #31. Thank you. We have now included the said study in introduction at line 36.

Comment #32. Page 4 last line "... 12 h light coupled [to] 12 h in absolute darkness..."

Response #32. Thank you. We have now corrected this at line 89.

Comment #33. Page 8 2nd paragraph. It is unclear which animals gave blood for the determination of the oxalic acid levels. From the data I understand these are 9 animals per group, but that means they do not (only) come from the group where the behavior was recorded, but they also do not cover the group that was sampled later in 4h intervals, since the latter group was much larger. And at which time point were these samples taken?

Response #33. Thank you. We used samples from 9 birds for the oxalate assay including six birds for which we had recorded the behaviour. These plasma samples were collected during the middle of the day. We have mentioned this at page 8, lines 167-168.

Comment #34. Page 11, line 19 and 20, there are two sentences starting with "however". Particularly the second sentence I am wondering why it starts with this word.

Response #34. Thank you. We have now corrected this at lines 251-255.

Comment #35. Page 12 1st paragraph, line 11. "... associated to [an] attenuated nocturnal melatonin..."

Response #35. Thank you. We have now corrected this at line 298.

Comment #36. Page 12 2nd paragraph. I think the authors are aware of the problem of the chicken and the egg: The question whether sleep deprivation causes changes in circadian mRNA oscillations or whether the changes in circadian mRNA oscillations are causing the increase in waking, but this is not well fleshed out in this paragraph. Only at the end it becomes clear, so maybe it is better to also introduce this at the beginning of the paragraph.

Response #36. Thank you. We have now reworded this paragraph to better state what we intended to say in lines 313-318.

Comment #37. Page 13 2nd paragraph, line 4. I think at "in turn..." a new sentence should start.

Response #37. Thank you. We have now corrected this at line 322.

Comment #38. Page 13, last line. Please do not end the sentence and paragraph with etc., but be more specific.

Response #38. Thank you. We have now corrected this.

Appendix B

Response to Editor's comments

Associate Editor:

Board Member

Comments to Author:

I have carefully studied the revisions made on this manuscript and I am satisfied with how the authors dealt with the comments. In particular they added a lot of additional data which had been requested by referee 1. I am attaching marked files of

- the main text

- Table S3, and

- Supplementary methods

containing my corrections of typos and language. The main text files also contains comments in places where the meaning was not clear to me. Moreover table 3 needs to be structured in two parts, as highlighted in my comment. All edits were done in the track changes mode of Word.

I would like to ask the authors to carefully attend to these edits and questions to get the paper in shape for publication.

Response: Thank you so very much. We have considered all the comments and edits, and have included them in the text, as appropriate.