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PROCEEDINGS B

Antioxidant supplementation slows telomere shortening in free-living white stork chicks

Javier Pineda-Pampliega, Amparo Herrera-Dueñas, Ellis Mulder, José I. Aguirre, Ursula Höfle and Simon Verhulst

Article citation details
Proc. R. Soc. B **287**: 20191917.
http://dx.doi.org/10.1098/rspb.2019.1917

Review timeline

Original submission:	13 June 2019
1st revised submission:	16 August 2019
2nd revised submission:	2 December 2019
Final acceptance:	5 December 2019

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

Review History

RSPB-2019-1386.R0 (Original submission)

Review form: Reviewer 1

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?
Excellent

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
There are few studies in vivo on the potential effects of antioxidants on telomere length and clearly more studies on different species are needed to obtain a better picture of the role of antioxidants in alleviating shortening of telomeres. This study is well-designed and analysed and present interesting data on the effect of antioxidant supplementation on a long-lived species not previously studied in this respect. I have some minor comments to be addressed by authors:

Methods: Please explain better how hatching order/age of nestlings was calculated.

Line 240 jackdaws
Line 364: Title incomplete: "Ageing and reproduction: Antioxidant supplementation alleviates telomere loss in wild birds." Please check references

Review form: Reviewer 2 (Antoine Stier)

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?
Good

Is the length of the paper justified?
No

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?
Yes

Is it clear?
Yes

Is it adequate?
No

Do you have any ethical concerns with this paper?
No

Comments to the Author
See pdf attached. (See Appendix A)

Decision letter (RSPB-2019-1386.R0)

31-Jul-2019

Dear Mr Pineda-Pampliega,

I am writing to inform you that your manuscript RSPB-2019-1386 entitled "Antioxidant supplementation slows telomere shortening in free-living white stork chicks" has, in its current form, been rejected for publication in Proceedings B.

This action has been taken on the advice of referees, whose reports are given below; we are all agreed that this is a very interesting experiment on an important topic. However as you will see, one reviewer provides a very detailed assessment and concludes that in its current form the paper does not contain sufficient results for a full Proc B paper: he/she therefore suggests that either you add some additional data, or submit to a shorter-format journal. In the hope that you will be able to address this reviewer's concerns by doing the former, we are happy to consider a resubmission, provided the comments of the referees are fully addressed. However please note that this is not a provisional acceptance.

The resubmission will be treated as a new manuscript. However, we will approach the same reviewers if they are available and it is deemed appropriate to do so by the Editor. 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.

Yours sincerely,
Professor Loeske Kruuk
Editor
mailto: proceedingsb@royalsociety.org

Associate Editor
Board Member: 1
Comments to Author:
The first reviewer offers some suggestions (including for the title), and only a minor changes for the ms (i.e., please explain better how hatching order/age of nestlings was calculated).

The second reviewer, however, suggests that the ms is more suitable for a brief communication (e.g., Biol Letters) due to limited amount of results. For a full paper in Proc B, s/he argues that additional information should be provided about the effects of the treatment on growth, the relationships between oxidative stress markers and telomere length/shortening, and the relationships between growth rate and telomere length/shortening. If the authors have such results, then it seems reasonable to includes some or all of them (and it would be odd to exclude them otherwise).

Reviewer(s)' Comments to Author:
Referee: 1

Comments to the Author(s)
There are few studies in vivo on the potential effects of antioxidants on telomere length and clearly more studies on different species are needed to obtain a better picture of the role of antioxidants in alleviating shortening of telomeres. This study is well-designed and analysed and present interesting data on the effect of antioxidant supplementation on a long-lived species not previously studied in this respect. I have some minor comments to be addressed by authors:

Methods: Please explain better how hatching order/age of nestlings was calculated.

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Line 364: Title incomplete: "Ageing and reproduction: Antioxidant supplementation alleviates telomere loss in wild birds." Please check references

Referee: 2

Comments to the Author(s)
See pdf attached

Author's Response to Decision Letter for (RSPB-2019-1386.R0)

See Appendix B.

RSPB-2019-1917.R0

Review form: Reviewer 2 (Antoine Stier)

Recommendation
Accept with minor revision (please list 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?
Good

Is the length of the paper justified?
No

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.
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?
Yes

Is it clear?
Yes

Is it adequate?
No

Do you have any ethical concerns with this paper?
No

Comments to the Author
Providing a detailed response to minor comments would have been appreciated, since some have been incorporated, some have been 'fixed' by deleting parts of the manuscript without appearing as such in the track change version, and some have been ignored. I still have some points that would need to be addressed/clarified by the authors (see pdf attached). Regarding my comment on the statistical analysis, part of the author's response seems to be based on a misinterpretation of the Nettle preprint they cite.
Antoine Stier [please note that I sign all my reviews]. (See Appendix C)

Review form: Reviewer 3

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?
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.
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?
Yes

Is it clear?
Yes

Is it adequate?
Yes

Do you have any ethical concerns with this paper?
No

Comments to the Author
I have been asked to review the statistical methods so will restrict my comments to this aspect of the manuscript. I think that there are a few issues that should be addressed with regards to how the authors have analysed this data set.

From the previous round of reviews, I see there was some discussion of the use of samples from pre-treatment individuals within the treatment group being set as 'control' – this seems fine to me.

I don't see why the authors have analysed effects of treatment, sex, mass and hatch order in separate models. No reasoning is given for this; the only thing I can think of is that the authors perhaps think that some of these effects are correlated(?), but that is certainly no reason to analyse them separately. See, for example, this paper by Morrissey & Ruxton on multiple regression: http://dx.doi.org/10.3998/ptpbio.16039257.0010.003

Similarly, I found it difficult to parse why the authors have analysed the percentiles both in separate models (Table S1) and in a single model (Table S3). Looking at the paper referenced (Bauch et al 2013) made it a little clearer as to why there were separate models for percentiles, but (i) I think the reasoning needs to be explicit within this paper, and (ii) couldn't random regression models be used to test between- versus within-individual variation in telomere shortening in a single framework?

Usually I would say that the relation between telomeres and oxidative stress variables would really be better estimated using multivariate mixed models, given the authors consider sex, mass, hatch order and treatment as potentially affecting both, but I guess here it is more about looking at the general phenotypic correlation between these values? So I think this is okay (although for future reference, I do think a 4-trait model would be cool to investigate patterns of covariance among all of these traits).

Figure 3A is hard to interpret: perhaps this is because the legend is on a separate page from the figure in the manuscript file, but I feel that it should be obvious what the axes are without having to get into the legend to find out that the slopes are actually age-related. Some tweaking of this figure would be helpful.

All tables: I don't really get the 'Intercept' column. I guess this is to show how the intercept has changed depending on inclusion / exclusion of rejected terms? But as this is non-standard then a note in the methods or the table legends would be helpful for your readers.

Decision letter (RSPB-2019-1917.R0)

11-Nov-2019

Dear Mr Pineda-Pampliega:

Your manuscript has now been peer reviewed and I have assessed the reviews. My apologies for the time this has taken: in short, we received one review that indicated the need for assessment by a statistical reviewer, and doing so then took additional time.

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. As you will see, we are all agreed that this is a very interesting topic and data-set. However the reviewers still have concerns with your manuscript, in particular with regard to the statistics, and we would like to invite you to revise your manuscript to address them. The (new) statistical reviewer raises an important point that it is not appropriate to test each effect in a separate model, when you could include them all in a multiple regression.

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, or to new reviewers. I need to emphasise that we cannot guarantee eventual acceptance of your manuscript at this stage, especially given the requirement for new analyses.

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. Please note that the referee from the previous version found your response to reviewers inadequate, so please ensure this time that every point is detailed.

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:

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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.

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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), 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,
Professor Loeske Kruuk
mailto: proceedingsb@royalsociety.org

Board Member:
Comments to Author:
It would be better to reply to all of the questions and comments made by Reviewer 2, and not to delete parts of the manuscript without explaining. The reviewer would still would like some points to be clarified by the authors. I feel that the ms should be proof-read and corrected by a native English speaker. Finally, a pdf of the data that was provided is not useful to check sample sizes and analyses or for re-use by others for meta-analyses. The manuscript has potential, but it still needs some work to improve the clarity.

Reviewer(s)' Comments to Author:

Referee: 2

Comments to the Author(s).
Providing a detailed response to minor comments would have been appreciated, since some have been incorporated, some have been 'fixed' by deleting parts of the manuscript without appearing as such in the track change version, and some have been ignored. I still have some points that would need to be addressed/clarified by the authors (see pdf attached). Regarding my comment on the statistical analysis, part of the author's response seems to be based on a misinterpretation of the Nettle preprint they cite.
Antoine Stier [please note that I sign all my reviews]

Referee: 3

Comments to the Author(s).
I have been asked to review the statistical methods so will restrict my comments to this aspect of the manuscript. I think that there are a few issues that should be addressed with regards to how the authors have analysed this data set.

From the previous round of reviews, I see there was some discussion of the use of samples from pre-treatment individuals within the treatment group being set as 'control' – this seems fine to me.

I don't see why the authors have analysed effects of treatment, sex, mass and hatch order in separate models. No reasoning is given for this; the only thing I can think of is that the authors perhaps think that some of these effects are correlated(?), but that is certainly no reason to analyse them separately. See, for example, this paper by Morrissey & Ruxton on multiple regression: http://dx.doi.org/10.3998/ptpbio.16039257.0010.003

Similarly, I found it difficult to parse why the authors have analysed the percentiles both in separate models (Table S1) and in a single model (Table S3). Looking at the paper referenced (Bauch et al 2013) made it a little clearer as to why there were separate models for percentiles, but (i) I think the reasoning needs to be explicit within this paper, and (ii) couldn't random regression models be used to test between- versus within-individual variation in telomere shortening in a single framework?

Usually I would say that the relation between telomeres and oxidative stress variables would really be better estimated using multivariate mixed models, given the authors consider sex, mass, hatch order and treatment as potentially affecting both, but I guess here it is more about looking at the general phenotypic correlation between these values? So I think this is okay (although for

future reference, I do think a 4-trait model would be cool to investigate patterns of covariance among all of these traits).

Figure 3A is hard to interpret: perhaps this is because the legend is on a separate page from the figure in the manuscript file, but I feel that it should be obvious what the axes are without having to get into the legend to find out that the slopes are actually age-related. Some tweaking of this figure would be helpful.

All tables: I don't really get the 'Intercept' column. I guess this is to show how the intercept has changed depending on inclusion / exclusion of rejected terms? But as this is non-standard then a note in the methods or the table legends would be helpful for your readers.

Author's Response to Decision Letter for (RSPB-2019-1917.R0)

See Appendix D.

Decision letter (RSPB-2019-1917.R1)

05-Dec-2019

Dear Mr Pineda-Pampliega

I am pleased to inform you that your manuscript entitled "Antioxidant supplementation slows telomere shortening in free-living white stork chicks" 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

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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,
Professor Loeske Kruuk
Editor, Proceedings B
mailto: proceedingsb@royalsociety.org

Associate Editor:
Board Member
Comments to Author:
The authors have done a fine job in addressing Reviewer 2's major and minor comments, and the manuscript has been greatly improved.

Appendix A

This paper reports interesting findings about the effects of an antioxidant supplementation on telomere length and shortening in a wild bird species. This is an interesting study on a timely topic, but I feel that in the current form it might be more appropriate for Biology Letters (*i.e.* transfer is possible from ProcB to Biol Let. from what I gathered on the Royal Society website) considering the (limited) amount of important results presented. Indeed, it is an important result that antioxidant supplementation is able to limit telomere shortening in a wild bird species, but considering the absence of effect related to telomere distribution/sex/hatching asynchrony, this could be easily framed as a short communication (1 Figure / 2 Tables). Alternatively, it would be suitable for Proc B in my opinion, but would at least require providing additional information about the effects of the treatment on growth, effects of the treatment on the relationships between oxidative stress markers and telomere length/shortening, effects of the treatment on the relationships between growth rate and telomere length/shortening. I am convinced that this paper will be an important contribution in the field of telomeres in ecology and evolution. My main comments and a few minor ones are outlined below. Despite such limitations, the research question is important and merits to be addressed and published in a high-quality journal such as Biology Letters or alternatively in Proceedings of the Royal Society B. I really hope that my comments will be useful to the authors in revising their manuscript.

Antoine Stier [Please note that I sign all my reviews]

Comment to the editor:

Major comments:

1) **Figures + sample size**

My opinion is that your figures are somewhat uselessly complex compared to the somewhat limited important biological results you have to communicate. Indeed, your data is mostly showing one main result: antioxidant supplementation alleviate telomere shortening during growth. Fig 1 is not necessary in my opinion since your experimental design is not excessively complex. Then Figure 2 could be kept or placed as ESM, but figures 3/4/5 could be one single figure showing the effect of treatment on telomere length per age group. Indeed, there is no effect of sex, or no difference in the effect between different percentiles of the telomere distribution, so there is no specific need to plot it in such complex ways. What you have to show is mainly the treatment effect (novel result) and the age effect, which of course was expected but is also worth being shown considering telomere length and shortening has never been investigated in this species.

The sample size is not appropriately reported since some individuals do not have 3 measurements according to Fig 3. This should be corrected.

2) **Including d1 data in models while before treatment**

I am not comfortable with your way of analysing the data / coding the treatment as 0 (control) for supplemented group at d1. At day 1, of course you're expecting no difference since it is before the treatment starts (but it is important to verify this assumption, but could be placed in the methods). In my opinion, the point of having measurement at d1 would be to control for individual differences in OS markers / TL prior to the experiment, and therefore including d1 values as covariate in your models rather than analysing it along with post-treatment data would make more sense in my opinion. It would allow controlling for initial differences between individuals, and avoid the complex situation of considering chicks in the supplemented group as controls at day 1.

3) **Links between OS markers and TL length/shortening, lack of effect on MDA**

If going for a full paper in Proc B or another "full-format" journal, it would be important to present (likely as ESM if nothing significant, since I presume you might have already tested it) the relationship, or lack of relationship between your oxidative stress markers (absolute value / change) and telomere length/shortening. Indeed, since your main question is the *in vivo* effects of oxidative stress on TL and shortening, the main point is indeed your experimental approach, but additional support from more correlative data (*e.g.* are the chicks showing the higher increase in antioxidants the ones showing the least telomere shortening?) would be helpful to strengthen your conclusions.

You conclude that oxidative stress is a driver of telomere shortening in vivo, but you had no effect on oxidative damage *per se* (and you do not discuss this in the discussion at all, which is problematic). This could indicate that MDA in red blood cells is not the adequate marker of oxidative damage (damage on DNA: 8-OHdG would be way more relevant in my opinion), or alternatively that your treatment has effects on telomere shortening without diminishing oxidative damage (*e.g*. through redox regulation of other pathways?). Measuring 8-OHdG on the DNA extracted for telomere length assay would be the ideal way to test if the effect you observe is indeed due to oxidative stress. If not possible, this should at least be acknowledged in the discussion, especially if going for a full-format paper.

4) **Effects on antioxidant supplementation on growth / relationship between growth rate and telomere shortening according to experimental treatment group**

If going for a full paper in Proc B or another "full-format" journal, it would be important to present:

1) the results of the antioxidant on growth rate. Indeed, growth rate is suggested to be one important determinant of early-life telomere shortening (Vedder et al. 2018), so any effect of the antioxidant treatment on growth could have subsequent effect on telomere dynamics, maybe even independently of oxidative stress. Antioxidant supplementation can have effects on growth depending on context/species (see Smith et al. 2016 for a meta-analysis), which would be interesting data to present as well in your study.

2) if your treatment changed the relationship between growth rate and telomere dynamics, as it is possible that growing fast while having extra-antioxidant could alleviate the potential

oxidative/telomere loss cost of fast growth (*e.g.* Stier et al. 2015 that you're already citing Fig 3: context-dependent relationship between growth rate and telomere shortening).

5) **Strong focus on telomere distribution**

While I agree that average telomere length is not necessarily the best parameter, you are putting a strong emphasis on telomere distribution while your treatment did not have a significantly different impact depending on the percentile of telomere length you analyzed. Therefore, any info about the impact on different percentiles is of secondary importance here in my opinion, and could be moved to ESM, mentioning in the main text that the effect did not significantly differ across telomere distribution.

6) **English**

Not being a native English-speaker myself, I am probably not the best person to comment on this aspect, but the manuscript might need some edition of the English.

Other comments:

Line 24: "Telomere length and shortening ~~is~~ are…"? I guess

Line 25: "raising the question **of** what causes variation"?

Lines 33-34: Not sure it is useful in the abstract: "Within individuals, telomeres vary in length between cells and chromosomes, and telomere attrition was faster in the longer telomeres", especially since this is not directly tested and that Table S1 shows that age effect is not significant in the longest telomeres.

Line 49: citing the recent meta-analysis (Wilbourn et al. 2018) on the topic would be good here

Lines 56-57: "due to the action of the electron transport chain to generate energy » is quite vague and should be rephrased to be more specific.

Line 72: would be good to cite reference 24 here as well.

Lines 78-79: [23] still found that VitE affected the relationship between initial telomere length and the amount of shortening during growth, and this should be mentioned in my opinion

Line 85: "with high quality TL measurements » is maybe too vague, you should mention TRF and why it is important (precision + not measuring ITS) and give the reference of one paper showing that TRF >> qPCR .

Lines 96-97: this assumption is partially backed-up by [23]

Lines 112-113: any reference to support this?

Lines 126-127 + Fig 3: It seems that the age at first sampling is quite variable between birds/nests. Could this be used in the model as a covariate to try to control for any potential effect of initial age? Indeed, telomere shortening is likely faster during the peak of growth, and therefore sampling chicks of different initial age could create some extra-noise in your data.

Lines 135-137: no CV or repeatability estimate is provided for tocopherol measurement. Adding technical repeatability estimate to CV based on duplicate measurements would better represent the precision/biological relevance of your assays.

Lines 144-147: no CV or repeatability estimate is provided for MDA measurement.

Lines 169-175: no intra-gel CV is provided

Line 186 + 204: "Age in days" → this is confusing since it is probably not the age you are testing here, but the time point at which you sampled the birds (day 1 = first day of treatment ca. 20 days, not hatching). Maybe speaking about *Time effect* would be more appropriate.

Lines 202-204: "external" is confusing, please rephrase maybe saying Experimental? Additionally, you did not only supplement with tocopherol, and the effect you mention here is on plasma tocopherol, not on antioxidants in general.

Lines 206-209 + 212-215: This belongs to the discussion, not the result section in my opinion.

Lines 224-227 + 239-242: this is not supported by your data (Table S1 showing age effect being ns in the longest telomeres) and you did not test specifically if shortening was faster in the longer telomeres

Result section in general: when $p > 0.05$, please use the wording not significantly differ, because without a power analysis, you cannot exclude the null hypothesis with some level of confidence.

Line 240: "jackdays » → jackdaws

Line 250: Effects on growth are relevant to understand telomere dynamics, so presenting such data would be important.

Line 255: regarding reference [48], please have a look at the recent commentary on this topic Costantini 2019 J Exp Biol

Discussion: There is a lack of conclusion in my opinion here. It would be important to stress that effects on long-term performance (reproductive success/survival) would be important to assess the biological importance of the effect you found, because we cannot exclude that 100bp might be of little relevance for individual fitness.

References:

R. V. Wilbourn, J. P. Moatt, H. Froy, C. A. Walling, D. H. Nussey, and J. J. Boonekamp, “The relationship between telomere length and mortality risk in non-model vertebrate systems: a meta-analysis.,” *Philos. Trans. R. Soc. Lond., B, Biol. Sci.*, vol. 373, no. 1741, pp. 20160447–9, Mar. 2018.

O. Vedder, S. Verhulst, E. Zuidersma, and S. Bouwhuis, “Embryonic growth rate affects telomere attrition: an experiment in a wild bird.,” *J Exp Biol*, vol. 221, no. 15, pp. jeb.181586–17, Jun. 2018.

S. M. Smith, R. G. Nager, and D. Costantini, “Meta-analysis indicates that oxidative stress is both a constraint on and a cost of growth,” *Ecol Evol*, pp. n/a–n/a, Mar. 2016.

D. Costantini, “Understanding diversity in oxidative status and oxidative stress: the opportunities and challenges ahead.,” *J Exp Biol*, vol. 222, no. 13, pp. jeb194688–9, Jul. 2019.

Appendix B

Referee comments, authors responses and manuscript changes

Referee 1:

Comment 1: Methods: Please explain better how hatching order/age of nestlings was calculated.

Response: The explanation of how hatch order has been calculated has been rewritten to clarify (Line 80-81, 136-138).

Comment 2: line 240 jackdaws.

Response: Thank you - spelling has been corrected (Line 196).

Comment 3: Title incomplete: "Ageing and reproduction: Antioxidant supplementation alleviates telomere loss in wild birds." Please check references.

Response: Thank you - title is now complete, and we have checked all the references to ensure their complete title.

Referee 2:

Major comments

1) **Figures and sample size:**

Comment: My opinion is that your figures are somewhat uselessly complex compared to the somewhat limited important biological results you have to communicate. Indeed, your data is mostly showing one main result: antioxidant supplementation alleviate telomere shortening during growth. Fig 1 is not necessary in my opinion since your experimental design is not excessively complex. Then Figure 2 could be kept or placed as ESM, but figures 3/4/5 could be one single figure showing the effect of treatment on telomere length per age group. Indeed, there is no effect of sex, or no difference in the effect between different percentiles of the telomere distribution, so there is no specific need to plot it in such complex ways. What you have to show is mainly the treatment effect (novel result) and the age effect, which of course was expected but is also worth being shown considering telomere length and shortening has never been investigated in this species.
The sample size is not appropriately reported since some individuals do not have 3 measurements according to Fig 3. This should be corrected.

Response: Following the suggestions of the reviewer, we moved figure 1 to the ESM (now Figure S1). We retained figure 2 (now Figure 1) because we consider that this figure is important to show that the experiment worked. Figure 4 and 5 were join in a single figure as the reviewer suggest (now Figure 3). We prefer to keep Figure 3 (now Figure 2) independent because figures 4 and 5 are about percentiles and having them in one figure makes sense, while figure 3 shows the effect of age on average TL. Also, we want to keep Figure 3 also because it illustrates the high quality of the measurements, shown in the strong effect of individual identity on TL (we now draw attention to this in the text, line 176). Sample size is now appropriately reported (lines 79 and 80).

2) Including d1 data in models while before treatment

Comment: I am not comfortable with your way of analysing the data / coding the treatment as 0 (control) for supplemented group at d1. At day 1, of course you're expecting no difference since it is before the treatment starts (but it is important to verify this assumption, but could be placed in the methods). In my opinion, the point of having measurement at d1 would be to control for individual differences in OS markers / TL prior to the experiment, and therefore including d1 values as covariate in your models rather than analysing it along with post-treatment data would make more sense in my opinion. It would allow controlling for initial differences between individuals and avoid the complex situation of considering chicks in the supplemented group as controls at day 1.

Response: The reviewer writes that he is 'uncomfortable' with our statistical approach, where we code the first telomere measurement of treated individuals as controls (because they were not treated prior to the first telomere measurement), while coding later measurements of those same individuals as 'treated'. Instead, the reviewer proposes to incorporate the first measurement as covariate.

With respect to the latter proposal: this is indeed the approach that we ourselves applied in the past, but we have in the meantime learned that this approach yields biased estimates when the means of the experimental groups are not exactly the same at the first time point, which condition will rarely be the fulfilled. See paper 169 on https://www.danielnettle.org.uk/publications/.

Two points with respect to our coding: firstly, in response to the reviewer's comments we simulated data and analysed them using our coding approach, as well as the conventional approach of testing the interaction between time and treatment. These approaches yield almost the same result when there is only one follow-up measurement. Our coding approach however has a clear advantage over the 'interaction approach' when the control time point is followed by more than 1 follow-up measurement (as in our study, and as confirmed by simulations):

When there is more than one follow-up measurement, time can either be coded as a covariate, or as a factor.

When time is coded as covariate, and treatment * time is used to test for a treatment effect, this model assumes that the treatment effect becomes proportionally stronger as time goes on, and hence this model will not yield an optimal fit when the treatment effect does not become proportionally stronger with time. Moreover, the test of whether there is a treatment effect at all is confounded with a test of whether the treatment effect varies with time. In our approach, these tests are coded separately, with treatment as main effect and, secondly, in interaction with time.

Alternatively, time is coded as a factor, and the treatment effect is tested through the interaction between treatment and time. This approach does not assume that the treatment effect increases with time but has as disadvantage that the test of treatment consumes more d.f., and hence has lower power.

3) **Links between OS markers and TL length/shortening, lack of effect on MDA**

Comment: If going for a full paper in Proc B or another "full-format" journal, it would be important to present (likely as ESM if nothing significant, since I presume you might have already tested it) the relationship, or lack of relationship between your oxidative stress markers (absolute value / change) and telomere length/shortening. Indeed, since your main question is the *in vivo* effects of oxidative stress on TL and shortening, the main point is indeed your experimental approach, but additional support from more correlative data (*e.g.* are the chicks showing the higher increase in antioxidants the ones showing the least telomere shortening?) would be helpful to strengthen your conclusions. You conclude that oxidative stress is a driver of telomere shortening in vivo, but you had no effect on oxidative damage *per se* (and you do not discuss this in the discussion at all, which is problematic). This could indicate that MDA in red blood cells is not the adequate marker of oxidative damage (damage on DNA: 8-OHdG would be way more relevant in my opinion), or alternatively that your treatment has effects on telomere shortening without diminishing oxidative damage (*e.g.* through redox regulation of other pathways?). Measuring 8-OHdG on the DNA extracted for telomere length assay would be the ideal way to test if the effect you observe is indeed due to oxidative stress. If not possible, this should at least be acknowledged in the discussion, especially if going for a full-format paper.

Response: Following the recommendation of the referee we have added the suggested new results to the manuscript on the treatment effect on growth (see comment 4) and on the relation of the oxidative stress variables with TL (Table S4; Lines 151-153, 189-190, 213-215). As suggested, we added the suggestion that the measurement of 8-OHdG may be a better indicator of oxidative stress DNA damage than MDA (Lines 215-219), although it is worth adding that 8-OHdG is a DNA-repair product and to what extent it is informative with respect to net DNA-damage is not that clear given that virtually all DNA-damage is thought to be almost instantaneously repaired.

4) **Effects on antioxidant supplementation on growth / relationship between growth rate and telomere shortening according to experimental treatment group.**

Comment: If going for a full paper in Proc B or another "full-format" journal, it would be important to present:

1) the results of the antioxidant on growth rate. Indeed, growth rate is suggested to be one important determinant of early-life telomere shortening (Vedder et al. 2018), so any effect of the antioxidant treatment on growth could have subsequent effect on telomere dynamics, maybe even independently of oxidative stress. Antioxidant supplementation can have effects on growth depending on context/species (see Smith et al. 2016 for a metaanalysis), which would be interesting data to present as well in your study.

2) if your treatment changed the relationship between growth rate and telomere dynamics, as it is possible that growing fast while having extra-antioxidant could alleviate the potential oxidative/telomere loss cost of fast growth (*e.g.* Stier et al. 2015 that you're already citing

Response: We totally agree with the recommendation and have added two new tables that address these points: Table S2 shows the effect of the treatment on mass and size, and table S5 shows the relation between mass and telomeres, comment these results in the text (Lines 33,68, 92, 145-146,170-171, 200).

5) **Strong focus on telomere distribution**

Comment: While I agree that average telomere length is not necessarily the best parameter, you are putting a strong emphasis on telomere distribution while your treatment did not have a significantly different impact depending on the percentile of telomere length you analysed. Therefore, any info about the impact on different percentiles is of secondary importance here in my opinion, and could be moved to ESM, mentioning in the main text that the effect did not significantly differ across telomere distribution.

Response: In our opinion, so little is still known about effect of age and other factors (experimental and non-experimental) on different parts of the telomere distribution that this is a useful addition to the manuscript. Moreover, we are a little wary of referring results to the ESM on the basis of the statistical significance alone, given that information presented in the ESM is considerably less likely to be taken up by readers.

6) **English**

Comment: Not being a native English-speaker myself, I am probably not the best person to comment on this aspect, but the manuscript might need some edition of the English.

Response: The entire document has been checked.

Appendix C

Referee comments, authors responses and manuscript changes

Providing a detailed response to minor comments would have been appreciated, since some have been incorporated, some have been 'fixed' by deleting parts of the manuscript without appearing as such in the track change version, and some have been ignored. I still have some points that would need to be addressed/clarified by the authors (see below). Regarding my comment on the statistical analysis, part of the author's response seems to be based on a misinterpretation of the Nettle preprint they cite.

Antoine Stier [please note that I sign all my reviews]

Referee 1:

Comment 1: Methods: Please explain better how hatching order/age of nestlings was calculated.

Response: The explanation of how hatch order has been calculated has been rewritten to clarify (Line 80-81, 136-138).

This is not clarifying how hatching order was determined → visiting the nest every day to record hatching order? Or assigning asynchrony based on size differences at the first visit? If it is the latter, is it a valid approach in this species, because if the asynchrony is moderate, it is not impossible for a late-hatched chick to be larger that a first-hatched one if the first visit only occurs a few days later

Comment 2: line 240 jackdaws.

Response: Thank you - spelling has been corrected (Line 196).

Comment 3: Title incomplete: "Ageing and reproduction: Antioxidant supplementation alleviates telomere loss in wild birds." Please check references.

Response: Thank you - title is now complete, and we have checked all the references to ensure their complete title.

Referee 2:

Major comments

1) **Figures and sample size:**

Comment: My opinion is that your figures are somewhat uselessly complex compared to the somewhat limited important biological results you have to communicate. Indeed, your data is mostly showing one main result: antioxidant supplementation alleviate telomere shortening during growth. Fig 1 is not necessary in my opinion since your experimental design is not excessively complex. Then Figure 2 could be kept or placed as ESM, but figures 3/4/5 could be one single figure showing the effect of treatment on telomere length per age group. Indeed, there is no effect of sex, or no difference in the effect between different percentiles of the telomere distribution, so there is no specific need to plot it in such complex ways. What you have to show is mainly the treatment

effect (novel result) and the age effect, which of course was expected but is also worth being shown considering telomere length and shortening has never been investigated in this species.
The sample size is not appropriately reported since some individuals do not have 3 measurements according to Fig 3. This should be corrected.

Response: Following the suggestions of the reviewer, we moved figure 1 to the ESM (now Figure S1). We retained figure 2 (now Figure 1) because we consider that this figure is important to show that the experiment worked. Figure 4 and 5 were join in a single figure as the reviewer suggest (now Figure 3). We prefer to keep Figure 3 (now Figure 2) independent because figures 4 and 5 are about percentiles and having them in one figure makes sense, while figure 3 shows the effect of age on average TL. Also, we want to keep Figure 3 also because it illustrates the high quality of the measurements, shown in the strong effect of individual identity on TL (we now draw attention to this in the text, line 176). Sample size is now appropriately reported (lines 79 and 80).

The new figure 2 is indeed providing interesting info about within-individual consistency in TL, but since there is no sex difference and the focus of the paper is about antioxidant supplementation, it would be more logical in my opinion to replace the grouping factor sex by treatment here.

The new figure 3A might benefit of some clarification on the x-axis, since it is not evident at the first glance that it represents age for each percentile.

2) **Including d1 data in models while before treatment**

Comment: I am not comfortable with your way of analysing the data / coding the treatment as 0 (control) for supplemented group at d1. At day 1, of course you're expecting no difference since it is before the treatment starts (but it is important to verify this assumption, but could be placed in the methods). In my opinion, the point of having measurement at d1 would be to control for individual differences in OS markers / TL prior to the experiment, and therefore including d1 values as covariate in your models rather than analysing it along with post-treatment data would make more sense in my opinion. It would allow controlling for initial differences between individuals and avoid the complex situation of considering chicks in the supplemented group as controls at day 1.

Response: The reviewer writes that he is 'uncomfortable' with our statistical approach, where we code the first telomere measurement of treated individuals as controls (because they were not treated prior to the first telomere measurement), while coding later measurements of those same individuals as 'treated'. Instead, the reviewer proposes to incorporate the first measurement as covariate.

With respect to the latter proposal: this is indeed the approach that we ourselves applied in the past, but we have in the meantime learned that this approach yields biased estimates when the means of the experimental groups are not exactly the same at the first time point, which condition will rarely be the fulfilled. See paper 169 on https://www.danielnettle.org.uk/publications/.

While I understand your point, I do not think it is justified in your case here. First the Nettle preprint you are referring to is highlighting that we should not correct for pre-treatment TL when analysing telomere rate of change (ΔTL), not when analysing telomere length *per se* to control for differences in pre-treatment TL (what I suggested here). Secondly, Nettle points out that such approach (correcting ΔTL by initial TL) is biased when initial TL differs between groups (in the case of smokers for instance), but this should not be the case in theory in your study since individuals were randomly assigned to the treatment.

Two points with respect to our coding: firstly, in response to the reviewer's comments we simulated data and analysed them using our coding approach, as well as the conventional approach of testing the interaction between time and treatment. These approaches yield almost the same result when there is only one follow-up measurement. Our coding approach however has a clear advantage over the 'interaction approach' when the control time point is followed by more than 1 follow-up measurement (as in our study, and as confirmed by simulations):

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When time is coded as covariate, and treatment * time is used to test for a treatment effect, this model assumes that the treatment effect becomes proportionally stronger as time goes on, and hence this model will not yield an optimal fit when the treatment effect does not become proportionally stronger with time. Moreover, the test of whether there is a treatment effect at all is confounded with a test of whether the treatment effect varies with time. In our approach, these tests are coded separately, with treatment as main effect and, secondly, in interaction with time.

Alternatively, time is coded as a factor, and the treatment effect is tested through the interaction between treatment and time. This approach does not assume that the treatment effect increases with time but has as disadvantage that the test of treatment consumes more d.f., and hence has lower power.

These seem like reasonable explanations, but I am probably not skilled enough in statistics to ensure that this somewhat unusual approach is fully valid.

Please mention explicitly that age was used as a covariate, and that individuals differ in age at sampling.

3) Links between OS markers and TL length/shortening, lack of effect on MDA

Comment: If going for a full paper in Proc B or another "full-format" journal, it would be important to present (likely as ESM if nothing significant, since I presume you might have already tested it) the relationship, or lack of relationship between your oxidative stress markers (absolute value / change) and telomere length/shortening. Indeed, since your main question is the *in vivo* effects of oxidative stress on TL and shortening, the main point is indeed your experimental approach, but additional support from more correlative data (*e.g.* are the chicks showing the higher increase in antioxidants the ones showing the least telomere shortening?) would be helpful to strengthen your conclusions.

You conclude that oxidative stress is a driver of telomere shortening in vivo, but you had no effect on oxidative damage *per se* (and you do not discuss this in the discussion at all, which is problematic). This could indicate that MDA in red blood cells is not the adequate marker of oxidative damage (damage on DNA: 8-OHdG would be way more relevant in my opinion), or alternatively that your treatment has effects on telomere shortening without diminishing oxidative damage (*e.g.* through redox regulation of other pathways?). Measuring 8-OHdG on the DNA extracted for telomere length assay would be the ideal way to test if the effect you observe is indeed due to oxidative stress. If not possible, this should at least be acknowledged in the discussion, especially if going for a full-format paper.

Response: Following the recommendation of the referee we have added the suggested new results to the manuscript on the treatment effect on growth (see comment 4) and on the relation of the oxidative stress variables with TL (Table S4; Lines 151-153, 189-190, 213-215). As suggested, we added the suggestion that the measurement of 8-OHdG may be a better indicator of oxidative stress DNA damage than MDA (Lines 215-219), although it is worth adding that 8-OHdG is a DNA-repair product and to what extent it is informative with respect to net DNA-damage is not that clear given that virtually all DNA-damage is thought to be almost instantaneously repaired.

There are significant amounts of 8-OHdG being integrated in genomic DNA (~0.05-0.10pg/ng of DNA in avian RBCs in my experience), suggesting that repair is not so instantaneous. Measuring 8-OHdG in urine or plasma indeed reflects 'repaired' 8-OHdG, but measuring cellular 8-OHdG on DNA extracted from blood cells is supposed to reflect unrepaired 8-OHdG to the best of my knowledge.

You still do not discuss the possibility that your antioxidant treatment had effects on telomere shortening unrelated to oxidative damage prevention (what the absence of effect on MDA could suggest), which might be linked to other phenomenon such as changes in redox signalling.

4) **Effects on antioxidant supplementation on growth / relationship between growth rate and telomere shortening according to experimental treatment group.**

Comment: If going for a full paper in Proc B or another "full-format" journal, it would be important to present:

1) the results of the antioxidant on growth rate. Indeed, growth rate is suggested to be one important determinant of early-life telomere shortening (Vedder et al. 2018), so any effect of the antioxidant treatment on growth could have subsequent effect on telomere dynamics, maybe even independently of oxidative stress. Antioxidant supplementation can have effects on growth depending on context/species (see Smith et al. 2016 for a metaanalysis), which would be interesting data to present as well in your study.

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Response: We totally agree with the recommendation and have added two new tables that address these points: Table S2 shows the effect of the treatment on mass and size, and table S5 shows the relation between mass and telomeres, comment these results in the text (Lines 33,68, 92, 145-146,170-171, 200).

I appreciate the addition of such details as ESM.

5) **Strong focus on telomere distribution**

Comment: While I agree that average telomere length is not necessarily the best parameter, you are putting a strong emphasis on telomere distribution while your treatment did not have a significantly different impact depending on the percentile of telomere length you analysed. Therefore, any info about the impact on different percentiles is of secondary importance here in my opinion, and could be moved to ESM, mentioning in the main text that the effect did not significantly differ across telomere distribution.

Response: In our opinion, so little is still known about effect of age and other factors (experimental and non-experimental) on different parts of the telomere distribution that this is a useful addition to the manuscript. Moreover, we are a little wary of referring results to the ESM on the basis of the statistical significance alone, given that information presented in the ESM is considerably less likely to be taken up by readers.

I agree that ESM is less likely to be taken up by readers, but the current focus on telomere distribution here is not conveying additional information related to your experiment, so it is somewhat diluting your interesting result on the effect of antioxidant supplementation in my opinion.

6) **English**

Comment: Not being a native English-speaker myself, I am probably not the best person to comment on this aspect, but the manuscript might need some edition of the English.

Response: The entire document has been checked.

A new check might be needed, for instance:

Line 35: the % after 31 seems to have disappear

Line 139: we create**d**

Line 179: there **were**

Line 198: between TL **and** any..

Appendix D

Referee comments, authors responses and manuscript changes

Board Member:
Comments to Author:
It would be better to reply to all of the questions and comments made by Reviewer 2, and not to delete parts of the manuscript without explaining. The reviewer would still would like some points to be clarified by the authors. I feel that the ms should be proof-read and corrected by a native English speaker. Finally, a pdf of the data that was provided is not useful to check sample sizes and analyses or for re-use by others for meta-analyses. The manuscript has potential, but it still needs some work to improve the clarity.

Thank you very much for your advice. We apologize for the insufficient replies in the last revision that were unintended and were partly due to the aim of reducing the length of the manuscript.

The comments and answers of the first revision are in black, the new comments of the referees are in red, and the new answers to these comments are in blue. Line numbers corresponds to "Manuscript_tracked_changes" file.

Referee 2 (Antoine Stier)

Comment: Providing a detailed response to minor comments would have been appreciated, since some have been incorporated, some have been 'fixed' by deleting parts of the manuscript without appearing as such in the track change version, and some have been ignored. I still have some points that would need to be addressed/clarified by the authors (see below). Regarding my comment on the statistical analysis, part of the author's response seems to be based on a misinterpretation of the Nettle preprint they cite.

Antoine Stier [please note that I sign all my reviews]

Thank you for your comments that have helped to greatly improve the manuscript. We are sorry that the response to the prior minor comments was not adequate. In some cases, we decided to delete parts since the paper was very long, and since the referee was not convinced by some of the contents, we thought it was better to eliminate that part and thus shorten the manuscript. However, it was a mistake not to add this in the tracked changes. Likewise, we are sorry if any of the comments were not answered properly.

From the enclosed PDF by Stier:

Comment 1: Methods: Please explain better how hatching order/age of nestlings was calculated.

Response: The explanation of how hatch order has been calculated has been rewritten to clarify (Line 80-81, 136-138).

This is not clarifying how hatching order was determined a→ visiting the nest every day to record hatching order? Or assigning asynchrony based on size differences at the first

visit? If it is the latter, is it a valid approach in this species, because if the asynchrony is moderate, it is not impossible for a late-hatched chick to be larger that a first-hatched one if the first visit only occurs a few days later

Thank You for this comment. In fact, we did assign hatch order by nestling size and this has now been clarified in the text (Line 80-84). In white stork parents begin incubation with the first or second egg and laying occurs at intervals of two days. Furthermore, egg mass also tends to decrease with laying order, and this effect, combined with hatching asynchrony, results in a marked size hierarchy among nestmates.

Referee 2:

Major comments

1) Figures and sample size:

Comment: My opinion is that your figures are somewhat uselessly complex compared to the somewhat limited important biological results you have to communicate. Indeed, your data is mostly showing one main result: antioxidant supplementation alleviate telomere shortening during growth. Fig 1 is not necessary in my opinion since your experimental design is not excessively complex. Then Figure 2 could be kept or placed as ESM, but figures 3/4/5 could be one single figure showing the effect of treatment on telomere length per age group. Indeed, there is no effect of sex, or no difference in the effect between different percentiles of the telomere distribution, so there is no specific need to plot it in such complex ways. What you have to show is mainly the treatment effect (novel result) and the age effect, which of course was expected but is also worth being shown considering telomere length and shortening has never been investigated in this species.
The sample size is not appropriately reported since some individuals do not have 3 measurements according to Fig 3. This should be corrected.

Response: Following the suggestions of the reviewer, we moved figure 1 to the ESM (now Figure S1). We retained figure 2 (now Figure 1) because we consider that this figure is important to show that the experiment worked. Figure 4 and 5 were join in a single figure as the reviewer suggest (now Figure 3). We prefer to keep Figure 3 (now Figure 2) independent because figures 4 and 5 are about percentiles and having them in one figure makes sense, while figure 3 shows the effect of age on average TL. Also, we want to keep Figure 3 also because it illustrates the high quality of the measurements, shown in the strong effect of individual identity on TL (we now draw attention to this in the text, line 176). Sample size is now appropriately reported (lines 79 and 80).

The new figure 2 is indeed providing interesting info about within-individual consistency in TL, but since there is no sex difference and the focus of the paper is about antioxidant supplementation, it would be more logical in my opinion to replace the grouping factor sex by treatment here.
The new figure 3A might benefit of some clarification on the x-axis, since it is not evident at the first glance that it represents age for each percentile.

Thank You for these comments. As you suggest, we have now used "Treatment" as grouping factor for Figure 2. In the Figure 3A we have added on the x-axis more information to clarify we are representing the association with age for each percentile.

2) Including d1 data in models while before treatment.

Comment: I am not comfortable with your way of analysing the data / coding the treatment as 0 (control) for supplemented group at d1. At day 1, of course you're expecting no difference since it is before the treatment starts (but it is important to verify this assumption but, could be placed in the methods). In my opinion, the point of having measurement at d1 would be to control for individual differences in OS markers / TL prior to the experiment, and therefore including d1 values as covariate in your models rather than analysing it along with post-treatment data would make more sense in my opinion. It would allow controlling for initial differences between individuals and avoid the complex situation of considering chicks in the supplemented group as controls at day 1.

Response: The reviewer writes that he is 'uncomfortable' with our statistical approach, where we code the first telomere measurement of treated individuals as controls (because they were not treated prior to the first telomere measurement), while coding later measurements of those same individuals as 'treated'. Instead, the reviewer proposes to incorporate the first measurement as covariate.
With respect to the latter proposal: this is indeed the approach that we ourselves applied in the past, but we have in the meantime learned that this approach yields biased estimates when the means of the experimental groups are not exactly the same at the first time point, which condition will rarely be the fulfilled. See paper 169 on https://www.danielnettle.org.uk/publications/.

While I understand your point, I do not think it is justified in your case here. First the Nettle preprint you are referring to is highlighting that we should not correct for pre-treatment TL when analysing telomere rate of change (ΔTL), not when analysing telomere length *per se* to control for differences in pre-treatment TL (what I suggested here). Secondly, Nettle points out that such approach (correcting ΔTL by initial TL) is biased when initial TL differs between groups (in the case of smokers for instance), but this should not be the case in theory in your study since individuals were randomly assigned to the treatment.

Again, thank you for your comment, but we do not agree. With respect to your first point, it is worth noting that Bateson et al (including Nettle, now published: https://royalsocietypublishing.org/doi/full/10.1098/rsos.190937) also investigated bias when analysing the data as suggested by the reviewer, i.e. using the first measurement as covariate and the follow-measurement(s) as dependent variable, and show that this also yields a bias when examining the effect of a factor on telomere attrition (see model 3 as described in table 1 in their paper). With respect to the second point: even small differences between groups in baseline values cause bias, regardless of whether that difference is significant. The prudent approach therefore in our view is one in which it is not necessary to make an assumption about the absence of a difference between groups

in baseline value, in particular when there are no obvious costs to the alternative approach that avoids this assumption.
We further note that referee 3, who was asked to look at our statistical approach specifically stated "this seems fine to me".

Two points with respect to our coding: firstly, in response to the reviewer's comments we simulated data and analysed them using our coding approach, as well as the conventional approach of testing the interaction between time and treatment. These approaches yield almost the same result when there is only one follow-up measurement. Our coding approach however has a clear advantage over the 'interaction approach' when the control time point is followed by more than 1 follow-up measurement (as in our study, and as confirmed by simulations):
When there is more than one follow-up measurement, time can either be coded as a covariate, or as a factor.
When time is coded as covariate, and treatment * time is used to test for a treatment effect, this model assumes that the treatment effect becomes proportionally stronger as time goes on, and hence this model will not yield an optimal fit when the treatment effect does not become proportionally stronger with time. Moreover, the test of whether there is a treatment effect at all is confounded with a test of whether the treatment effect varies with time. In our approach, these tests are coded separately, with treatment as main effect and, secondly, in interaction with time.
Alternatively, time is coded as a factor, and the treatment effect is tested through the interaction between treatment and time. This approach does not assume that the treatment effect increases with time but has as disadvantage that the test of treatment consumes more d.f., and hence has lower power.

These seem like reasonable explanations, but I am probably not skilled enough in statistics to ensure that this somewhat unusual approach is fully valid. Please mention explicitly that age was used as a covariate, and that individuals differ in age at sampling.

Thank you for the comment. We have now included a clarification that states that age is used as covariate (line 144-145, 155). The indication that individuals differ in age at sampling was also included in line 92.

3) Links between OS markers and TL length/shortening, lack of effect on MDA.

Comment: If going for a full paper in Proc B or another "full-format" journal, it would be important to present (likely as ESM if nothing significant, since I presume you might have already tested it) the relationship, or lack of relationship between your oxidative stress markers (absolute value / change) and telomere length/shortening. Indeed, since your main question is the *in vivo* effects of oxidative stress on TL and shortening, the main point is indeed your experimental approach, but additional support from more correlative data (*e.g.* are the chicks showing the higher increase in antioxidants the ones showing the least telomere shortening?) would be helpful to strengthen your conclusions. You conclude that oxidative stress is a driver of telomere shortening in vivo, but you had no effect on oxidative damage *per se* (and you do not discuss this in the discussion at all, which is problematic). This could indicate that MDA in red blood cells is not the adequate marker of oxidative damage (damage on DNA: 8-OHdG would be way more relevant in

my opinion), or alternatively that your treatment has effects on telomere shortening without diminishing oxidative damage (*e.g.* through redox regulation of other pathways?). Measuring 8-OHdG on the DNA extracted for telomere length assay would be the ideal way to test if the effect you observe is indeed due to oxidative stress. If not possible, this should at least be acknowledged in the discussion, especially if going for a full-format paper.

Response: Following the recommendation of the referee we have added the suggested new results to the manuscript on the treatment effect on growth (see comment 4) and on the relation of the oxidative stress variables with TL (Table S4; Lines 151-153, 189-190, 213-215). As suggested, we added the suggestion that the measurement of 8-OHdG may be a better indicator of oxidative stress DNA damage than MDA (Lines 215-219), although it is worth adding that 8-OHdG is a DNA-repair product and to what extent it is informative with respect to net DNA-damage is not that clear given that virtually all DNA-damage is thought to be almost instantaneously repaired.

There are significant amounts of 8-OHdG being integrated in genomic DNA (~0.05 0.10pg/ng of DNA in avian RBCs in my experience), suggesting that repair is not so instantaneous. Measuring 8-OHdG in urine or plasma indeed reflects 'repaired' 8 OHdG but measuring cellular 8-OHdG on DNA extracted from blood cells is supposed to reflect unrepaired 8-OHdG to the best of my knowledge.
You still do not discuss the possibility that your antioxidant treatment had effects on telomere shortening unrelated to oxidative damage prevention (what the absence of effect on MDA could suggest), which might be linked to other phenomenon such as changes in redox signalling.

Thank you. We completely agree with your final point and want to apologize because in the prior revision we only added information about the 8-OHdG measurement but missed including an additional part discussing other possibly ways in which our treatment may have affected telomere shortening. Now, as the referee suggested, we have included a comment in which we discuss the possibility that our treatment may modify the cellular redox environment, which could affect cell cycle and consecutively the dynamics of telomeres (lines 236-245).

4) Effects on antioxidant supplementation on growth / relationship between growth rate and telomere shortening according to experimental treatment group.

Comment: If going for a full paper in Proc B or another "full-format" journal, it would be important to present:
1) the results of the antioxidant on growth rate. Indeed, growth rate is suggested to be one important determinant of early-life telomere shortening (Vedder et al. 2018), so any effect of the antioxidant treatment on growth could have subsequent effect on telomere dynamics, maybe even independently of oxidative stress. Antioxidant supplementation can have effects on growth depending on context/species (see Smith et al. 2016 for a metaanalysis), which would be interesting data to present as well in your study.

2) if your treatment changed the relationship between growth rate and telomere dynamics, as it is possible that growing fast while having extra-antioxidant could alleviate the

potential oxidative/telomere loss cost of fast growth (*e.g.* Stier et al. 2015 that you're already citing.

Response: We totally agree with the recommendation and have added two new tables that address these points: Table S2 shows the effect of the treatment on mass and size, and table S5 shows the relation between mass and telomeres, comment these results in the text (Lines 33,68, 92, 145-146,170-171, 200).

I appreciate the addition of such details as ESM.

Thank you for the comment.

5) Strong focus on telomere distribution

Comment: While I agree that average telomere length is not necessarily the best parameter, you are putting a strong emphasis on telomere distribution while your treatment did not have a significantly different impact depending on the percentile of telomere length you analysed. Therefore, any info about the impact on different percentiles is of secondary importance here in my opinion, and could be moved to ESM, mentioning in the main text that the effect did not significantly differ across telomere distribution.

Response: In our opinion, so little is still known about effect of age and other factors (experimental and non-experimental) on different parts of the telomere distribution that this is a useful addition to the manuscript. Moreover, we are a little wary of referring results to the ESM on the basis of the statistical significance alone, given that information presented in the ESM is considerably less likely to be taken up by readers.

I agree that ESM is less likely to be taken up by readers, but the current focus on telomere distribution here is not conveying additional information related to your experiment, so it is somewhat diluting your interesting result on the effect of antioxidant supplementation in my opinion.

Thank you for your comment. Although you are probably right, we still feel we need to keep this part in order to have the complete picture. Thus, we have maintained this section as it was. We hope you will find this acceptable.

6) English

Comment: Not being a native English-speaker myself, I am probably not the best person to comment on this aspect, but the manuscript might need some edition of the English.

Response: The entire document has been checked.

A new check might be needed, for instance:
Line 35: the % after 31 seems to have disappear
Line 139: we create**d**
Line 179: there **were**

Line 198: between TL **and** any..

Thank You very much for the corrections. We have included the suggested changes and checked the document again, with the help of an experienced English speaker in order to weed out additional errors.

Referee 3:

Comment: From the previous round of reviews, I see there was some discussion of the use of samples from pre-treatment individuals within the treatment group being set as 'control' – this seems fine to me.

Response: we are happy to see that you agree with our approach.

Comment: I don't see why the authors have analysed effects of treatment, sex, mass and hatch order in separate models. No reasoning is given for this; the only thing I can think of is that the authors perhaps think that some of these effects are correlated(?), but that is certainly no reason to analyse them separately. See, for example, this paper by Morrissey & Ruxton on multiple regression: http://dx.doi.org/10.3998/ptpbio.16039257.0010.003

Response: Thank you very much for your comment. The concern about correlation was the reason why we had analysed them separately but now we followed your suggestion and included all variables in a single model (Table 2, lines 142-153).

Comment: Similarly, I found it difficult to parse why the authors have analysed the percentiles both in separate models (Table S1) and in a single model (Table S3). Looking at the paper referenced (Bauch et al 2013) made it a little clearer as to why there were separate models for percentiles, but (i) I think the reasoning needs to be explicit within this paper, and (ii) couldn't random regression models be used to test between- versus within-individual variation in telomere shortening in a single framework?

Response: Thank you for this point. With view to the first part of your concerns, we have now explained in the paper that we have decided to additionally analyse the data for every 10^{th} percentiles separately, not only to compare between and within individual differences, but also to be able to disentangle if the effect of the treatment and the rest of parameters affect in a different way depending on telomere length, as has been described for attrition in other studies cited (lines 188-196). Regarding the second point: It is not clear to us from the comment what model formula the reviewer has in mind exactly, and neither is it clarified what the suggested approach would yield in terms of additional information over and above the results of the present analyses. We therefore choose to not burden the manuscript with more complex analyses, also because the other reviewer already considers our analysis of the treatment effects on the different percentiles as somewhat diverging from the focus of our paper.

Comment: Usually I would say that the relation between telomeres and oxidative stress variables would really be better estimated using multivariate mixed models, given the

authors consider sex, mass, hatch order and treatment as potentially affecting both, but I guess here it is more about looking at the general phenotypic correlation between these values? So, I think this is okay (although for future reference, I do think a 4-trait model would be cool to investigate patterns of covariance among all of these traits).

Response: Thank You for the point raised. In fact, we have decided to keep the oxidative stress variables in a different model with respect to sex, mass, hatch order and treatment because we are more interested in the phenotypic correlation of oxidative stress variables with telomeres. However, we are thankful for Your valuable recommendation, and will keep it in mind for future studies.

Comment: Figure 3A is hard to interpret: perhaps this is because the legend is on a separate page from the figure in the manuscript file, but I feel that it should be obvious what the axes are without having to get into the legend to find out that the slopes are age-related. Some tweaking of this figure would be helpful.

Response: We totally agree with both referees regarding this figure, and for this reason we have added new info to the X-axis of Figure 3A to clarify that in each percentile this axis indicates age.

Comment: All tables: I don't really get the 'Intercept' column. I guess this is to show how the intercept has changed depending on inclusion / exclusion of rejected terms? But as this is non-standard then a note in the methods or the table legends would be helpful for your readers.

Response: Thank You for this comment. We have added an explanation about why we kept the intercept in all tables in the methods section (lines 162-163).