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Pace and stability of embryonic development affect telomere dynamics: an experimental study in a precocial bird model

Antoine Stier, Neil B. Metcalfe and Pat Monaghan

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Original submission:	21 October 2019
1st revised submission:	11 June 2020
2nd revised submission:	24 July 2020
Final acceptance:	3 August 2020

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

Review History

RSPB-2019-2456.R0 (Original submission)

Review form: Reviewer 1

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?
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.
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
In this experimental study, the authors tested how incubation temperature and the stability of incubation conditions influence telomere length and dynamics before and after hatching in a bird and explored two possible mechanisms underlying the long-lasting effects of the prenatal conditions on telomere dynamics. Although it is well expected from previous evidence that the detrimental effects of fast growth or stress during early life on telomere dynamics can be extended to the prenatal period, it has rarely been tested in an experimental study. This paper shows that incubation conditions affect the pace of embryonic growth and the level of stress, thereby influencing telomere dynamics during the embryonic and post-natal periods in an expected way. The experiment was carefully designed and well conducted, and the manuscript is generally well written. Nevertheless, I have some suggestions to improve the paper as detailed below.

Pace and stability of prenatal growth: By experimentally increasing and decreasing incubation temperatures, embryonic growth rates were successfully accelerated and slowed down in the High and Low groups, respectively. However, there's no evidence that the manipulation of incubation temperature produced "unstable" prenatal growth in the Unstable group. What I learn from the results is that the unstable incubation conditions slowed down embryonic growth rates possibly by increasing the level of stress (as evidenced by plasma corticosterone level). This should be clarified throughout the manuscript, including the title, abstract, introduction and discussion. Perhaps "pace and stability of prenatal growth" can be clarified as "pace of growth and environmental stress during the embryonic period" or "incubation temperature and stability".

The mechanisms form an important part in this paper, but the authors do not provide sufficient information (lines 110-114) about why oxidative stress and stress hormones are important candidate mechanisms underlying the relationship between prenatal conditions and telomere dynamics in the introduction. Please extend the provided explanation.

It is not really clear why the effects of embryonic growth rate and the stability of incubation conditions on telomere length appeared in hatchlings (at day 1) but not in late embryos (at stED13), although the authors discussed that it may be due to telomerase activity (lines 417-419). I wonder if you could also analyse telomere length in the subsample euthanized at ED13. At ED13, the embryos of the H, M, L and U groups were at different developmental stages (H > M > L, U). If the telomere length at ED13 also did not differ between the experimental groups (thus showing

the similar levels as at stED13), this may suggest that constant telomere length is maintained by telomerase activity until this developmental stage, and thus telomere length is unaffected by prenatal conditions at least until the late embryonic stage. I think that it is worth a try.

Growth and survival: It is unclear whether all hatched individuals were included in the analyses of growth. At hatching sample sizes of the H, M, L and U groups were 27, 25, 26 and 25 (Fig 2D, time to hatching), but these number are reduced to 15, 15, 16 and 15 in the analysis of growth (Fig S2). Were individuals died before D90 excluded in the growth analysis to perform a longitudinal analysis? If it is the case, you should also present the analysis including all individuals or at least indicate whether the results change. It is necessary to show that indeed body mass at hatching (D1) did not differ among the experimental groups. Did the experimental treatment influence survival to D90?

Minor suggestions:
Line 104: Is the stability of incubation temperature variable, for example due to individual incubation behaviour or environmental conditions? This information may be helpful to understand why it is important (or ecologically relevant) to study the effects of the stability of prenatal conditions in addition to the effects of constantly better or worse conditions for embryonic development.
Lines 132-135: Here, more information is necessary. For example, in total how many eggs were obtained and used in the study? In which year and months were the eggs collected? What was the range of the laying dates? Were the individual eggs incubated immediately after the delivery to the laboratory? Or were they stored until the simultaneous onset of incubation (then for how long)?
Line 163: What percentage of the hatching events did occur when not observed? Did it vary among different experimental groups?
Line 168: How many replicates of enclosures were used?
Line 228: Please give the full name of 8-OHdG in the first mention.
Lines 296-303: Did you check whether egg size influenced body mass, telomere length and RBC DNA damage at hatching?
Fig 2D: Days may be a more informative unit for y-axis than hours here because days of incubation and chick age are used throughout the manuscript.
Lines 398-399: Is there any evidence from other studies that embryo (or chick) heart rate is indeed related to metabolic rate?

Review form: Reviewer 2

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

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

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

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

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Is it accessible?
Yes

Is it clear?
Yes

Is it adequate?
Yes

Do you have any ethical concerns with this paper?
No

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This is a very well controlled and executed experimental study that aims to determine the impact that prenatal conditions have on development and post natal telomere length and dynamics. It is very precise, clear and well written. The question is timely and interesting given the need to understand both telomere dynamics and what they mean.

The evidence that effects on telomeres are causal of later life patterns is weak/absent across species. This should be highlighted more and less stress put on the idea that effects on telomere length may be causal. Explanation of the value of understanding the impact on telomere length of the factors assessed even that impact is only correlated with late life problems is worth adding. For example Line 27 – I think it would be better to say they are 'correlated' ....this emphasis on a causative effect is too strong throughout.

The use of CoV has been much criticised in terms of telomere measures. You need to use other metrics. See Nettle et al 2019.

Overall this is an interesting experiment but the key to testing the overall hypothesis would be to determine if these development/telomere differences are then linked to differences in later life health and survival. Given the lifespan of the model species used this seems possible and it is a pity that this wasn't done.

I don't agree with the argument that this experiment shows the 'extreme importance of the prenatal stage in determining telomere length over the life course of individuals'. In the experimental individuals all other conditions after the experiment are the same for all individuals, i.e. they are exposed to no other variation. Therefore it seems obvious that the telomere length differences caused by the prenatal experiment will predominate....however large or small that actual effect may be. However in a natural system many other factors and variants may come into play and may totally swamp the effects on telomere length of the prenatal conditions. What we need to know is how relatively much the prenatal impact effects telomeres compared to the other variation individuals may normally be exposed to.

Decision letter (RSPB-2019-2456.R0)

12-Dec-2019

Dear Mr Stier,

I am writing to inform you that your manuscript RSPB-2019-2456 entitled "Ageing before birth: pace and stability of prenatal growth affect telomere dynamics" has, in its current form, been rejected for publication in Proceedings B.

This action has been taken on the advice of referees, who have recommended that substantial revisions are necessary. With this in mind we would be 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, and some comments from me. 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

Editor comments

Both the referees and I are positive about the overall interest of hte paper and the value of the design, but the referees' comments raise some important issues to address. Both have suggested additional information that it would be good to include if possible; in particular, see Ref 1's suggestion about telomerase activity, which would greatly add to the paper. Ref 2 acknowledges that their suggestions may not be feasible, but the issues do need to be acknowledged in the Discussion.

L36: 'These early life differences in telomere length persisted…' would be clearer.

L134: 'Identity of the parents was unfortunately unavailable, but since eggs were collected every

day and Japanese quail lay a maximum of one egg a day [34], it is unlikely that several eggs originated from the same female.'
I'm not sure I understand this: do you mean that all eggs were collected on the same day? Presumably if they were collected on multiple days, it's likely there were some from the same female. What was the total number of females from whom eggs were collected, as compared to the number of eggs collected? This is obviously a minor point, but it would be good to clarify the sample sizes.

The Methods are long. Can you move some of the details into Supplementary Information?

Where you give a chi-squared statistic, it needs an associated d.f. value.

It would be interesting to see the associations between your different traits: can you give the correlations at each stage?

Is it appropriate to use 'instability' as the term for the treatment that actually mimics the natural situation, of the female leaving incubation for short periods? How about 'variable', or 'natural', as opposed to the constant temperatures of the other treatments?

The key results are in Figure 3A. As a small sub-panel, it's a difficult to see the details. I suggest replacing 3A with your four panels in Fig S3 E-H (and put 3A in Supplementary instead).

Reviewer(s)' Comments to Author:

Referee: 1

Comments to the Author(s)
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Lines 398-399: Is there any evidence from other studies that embryo (or chick) heart rate is indeed related to metabolic rate?

Referee: 2

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The use of CoV has been much criticised in terms of telomere measures. You need to use other metrics. See Nettle et al 2019.

Overall this is an interesting experiment but the key to testing the overall hypothesis would be to determine if these development/telomere differences are then linked to differences in later life health and survival. Given the lifespan of the model species used this seems possible and it is a pity that this wasn't done.

I don't agree with the argument that this experiment shows the 'extreme importance of the prenatal stage in determining telomere length over the life course of individuals'. In the experimental individuals all other conditions after the experiment are the same for all individuals, i.e. they are exposed to no other variation. Therefore it seems obvious that the telomere length differences caused by the prenatal experiment will predominate....however large or small that actual effect may be. However in a natural system many other factors and variants may come into play and may totally swamp the effects on telomere length of the prenatal conditions. What we need to know is how relatively much the prenatal impact effects telomeres compared to the other variation individuals may normally be exposed to.

Author's Response to Decision Letter for (RSPB-2019-2456.R0)

See Appendix A.

RSPB-2020-1378.R0

Review form: Reviewer 1

Recommendation
Accept as is

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

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?
Yes

Is it clear?
Yes

Is it adequate?
Yes

Do you have any ethical concerns with this paper?
No

Comments to the Author
The authors considered my previous comments carefully in the revised manuscript. I am satisfied with the improvement.

Review form: Reviewer 3

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?
Yes

Is it clear?
Yes

Is it adequate?
Yes

Do you have any ethical concerns with this paper?
No

Comments to the Author
General comments:

This study has experimentally manipulated incubation conditions of Japanese quail embryos and tested for effects on pre-and post-natal telomere length, together with potential causes of telomere shortening (growth rate, oxidative damage, and prenatal glucocorticoid levels). The experiment is well designed and executed, the manuscript well written, and the results are very interesting for a diverse audience, as they are relevant for the study of pre-natal maternal effects, ageing, life-history trade-offs and telomere dynamics. In addition to the results of the experiment, the study also provides a very detailed description of the general telomere dynamics (i.e. repeatability, shortening rate with age) of this relatively short-lived bird species. I only have a few minor suggestions for improvement (see specific comments below), of which the two most important are:

1. The authors should try to correct for methodological between-gel differences in calculating the intra-individual repeatability of telomere length. Moreover, providing the intra-individual repeatability with, and without, correcting for the fixed effect of the incubation experiment on telomere length, would provide insight into what proportion of the repeatability is actually caused by the manipulated pre-natal conditions.
2. Because layer-type Japanese quail will not have been selected for lifespan, but only for early-life productivity, while in the wild the individuals with the longest lifespan in most species also have the highest lifetime reproductive success, there may be little selection for telomere maintenance in this model species. Although I do not think that this is a problem for showing 'proof of principle', I do think that a few lines in the Discussion about why effects in quail may be more pronounced than in longer-lived wild species could add to the quality of the Discussion.

Specific comments:

Line 1: I do not think that the title is completely appropriate. Because of the term 'birth' my first impression would be that this is a study on humans (or on another mammal species). Moreover, the term 'ageing' is inappropriate, because there is no evidence that telomere length predicts individual rate of ageing (only that it predicts lifespan, which does not need to be related to ageing). I suggest to omit the first part, and add something like 'in a precocial bird' at the end.

Line 29: I would omit 'strongly'. Work in humans, and in some other species in which telomeres were measured with TRF, suggests that the majority of between-individual variation is due to additive genetic effects, with comparatively small permanent environmental effects.

Lines 65-66: I would omit this explanation. This is only a mechanistic/proximate explanation, and the fact that germline telomere length can be protected against telomere loss without a high risk of cancer makes this explanation not very credible from an evolutionary point of view.

Line 103: I would omit 'slightly'. If a small difference has a large effect on fitness I would not call it a slight or small effect. Since causal effects on fitness of these type of manipulations are unknown, it is best to refrain from speculation on effect size.

Line 138: I think you forgot to omit a part of the sentence.

Line 163: Although I understand the reasoning behind your different treatments, I object to calling some treatments more or less 'natural'. Incubation in an incubator is by definition not 'natural' because in the wild a female would have continuous feedback from the temperature of her eggs, and adjust her incubation intensity accordingly (but only if she has enough energy to put in the optimal amount of heat). Since an embryo is generating more heat the bigger it gets, in the wild a female would lower incubation intensity further into the incubation phase, to keep the eggs at a constant temperature. This is a process that cannot be achieved with small commercial incubators, but seems to be the norm in the poultry industry (e.g. see Lourens et al. 2005, Poultry Science).

Moreover, the type of Japanese quail that is used in laying farms has completely lost its ability to incubate its own eggs (because there is a trade-off between laying and incubation) and will have been artificially incubated for perhaps more than a 100 generations (generation time of industrial laying quail will be less than a year, as laying rate and egg hatchability steeply decline with age (see Woodard & Abplanalp 1971, Poultry Science) making it not commercially profitable to keep them alive for longer than that). Hence, in these quail there will have been generations of selection for having the highest hatchability, and early-life productivity, under the incubation temperature chosen by most breeders (which may not coincidentally be a 100 degrees Fahrenheit), and not for having the longest possible lifespan. This may even be an explanation for why the effect of incubation temperature on telomere length of Japanese quail seems to be so much larger (in terms of base pairs) than in common terns. With this type of selection regime there will be little selection to prevent telomere loss due to a trade-off with development speed, as they will never reach the age at which telomere length becomes critical (or at least eggs laid at that age will not be used to produce the next generation, as they have lower hatchability). This may make the quail an ideal model for showing the 'proof of principle' for such trade-offs, but it may not reflect the 'natural situation', regardless of what incubation profile has been used.

Lines 236-238: Interesting that they lose so many base pairs of telomere length per year. These type of details, which can only be obtained from TRF studies, make the study extra valuable.

Lines 238-240: Is this repeatability corrected for the effects of the incubation treatment? If not, I would also present a repeatability value that is corrected for variation caused by the treatment (by adding treatment as a fixed effect). It would be very interesting to know if they are also very repeatable without the extra between-individual variation in embryonic development rate caused by the experiment, as that would suggest a high heritability (or strong maternal effects that are independent of incubation procedure).

Moreover, I noticed in the ESM that repeated samples of individuals were always run on the same gel. In the future it would be better to randomize samples over gels, as now your between-individual variance may be artificially increased due to methodological between-gel differences. Perhaps you can add the gel-specific value of the standard sample as a fixed effect to see if that explains some of the between-gel variance, which would make your repeatability estimate more accurate.

Lines 240-245: Again, these extra results make this one of the most detailed telomeres studies.

Line 338: I would tone down the statement on selective mortality, because it is still possible that the unhatched embryos of the different incubation treatments differ in telomere length.

Figures: I think that Fig. S2 would be much more valuable to include in the main manuscript than Fig. 1.

Decision letter (RSPB-2020-1378.R0)

06-Jul-2020

Dear Dr Stier,

Thank you for the revised version of this manuscript, which has now been reviewed by one of the original reviewers and a new reviewer. The reviewers' comments (not including confidential comments to the Editor) are included at the end of this email. As you will see, the reviewers are very positive about the manuscript, but the new reviewer has made some useful suggestions for improvement, so we would like to invite you to revise your manuscript to address them.

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), 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.

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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. 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 you are unable to meet this deadline - especially given the current circumstances - 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.

My best wishes to you and co-authors,
Loeske Kruuk
Editor
mailto: proceedingsb@royalsociety.org

Reviewer(s)' Comments to Author:

Referee: 1

Comments to the Author(s).
The authors considered my previous comments carefully in the revised manuscript. I am satisfied with the improvement.

Referee: 3

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Line 103: I would omit 'slightly'. If a small difference has a large effect on fitness I would not call it a slight or small effect. Since causal effects on fitness of these type of manipulations are unknown, it is best to refrain from speculation on effect size.

Line 138: I think you forgot to omit a part of the sentence.

Line 163: Although I understand the reasoning behind your different treatments, I object to calling some treatments more or less 'natural'. Incubation in an incubator is by definition not 'natural' because in the wild a female would have continuous feedback from the temperature of her eggs, and adjust her incubation intensity accordingly (but only if she has enough energy to put in the optimal amount of heat). Since an embryo is generating more heat the bigger it gets, in the wild a female would lower incubation intensity further into the incubation phase, to keep the eggs at a constant temperature. This is a process that cannot be achieved with small commercial incubators, but seems to be the norm in the poultry industry (e.g. see Lourens et al. 2005, Poultry Science).

Moreover, the type of Japanese quail that is used in laying farms has completely lost its ability to incubate its own eggs (because there is a trade-off between laying and incubation) and will have been artificially incubated for perhaps more than a 100 generations (generation time of industrial laying quail will be less than a year, as laying rate and egg hatchability steeply decline with age

(see Woodard & Abplanalp 1971, Poultry Science) making it not commercially profitable to keep them alive for longer than that). Hence, in these quail there will have been generations of selection for having the highest hatchability, and early-life productivity, under the incubation temperature chosen by most breeders (which may not coincidentally be a 100 degrees Fahrenheit), and not for having the longest possible lifespan. This may even be an explanation for why the effect of incubation temperature on telomere length of Japanese quail seems to be so much larger (in terms of base pairs) than in common terns. With this type of selection regime there will be little selection to prevent telomere loss due to a trade-off with development speed, as they will never reach the age at which telomere length becomes critical (or at least eggs laid at that age will not be used to produce the next generation, as they have lower hatchability). This may make the quail an ideal model for showing the 'proof of principle' for such trade-offs, but it may not reflect the 'natural situation', regardless of what incubation profile has been used.

Lines 236-238: Interesting that they lose so many base pairs of telomere length per year. These type of details, which can only be obtained from TRF studies, make the study extra valuable.

Lines 238-240: Is this repeatability corrected for the effects of the incubation treatment? If not, I would also present a repeatability value that is corrected for variation caused by the treatment (by adding treatment as a fixed effect). It would be very interesting to know if they are also very repeatable without the extra between-individual variation in embryonic development rate caused by the experiment, as that would suggest a high heritability (or strong maternal effects that are independent of incubation procedure).

Moreover, I noticed in the ESM that repeated samples of individuals were always run on the same gel. In the future it would be better to randomize samples over gels, as now your between-individual variance may be artificially increased due to methodological between-gel differences. Perhaps you can add the gel-specific value of the standard sample as a fixed effect to see if that explains some of the between-gel variance, which would make your repeatability estimate more accurate.

Lines 240-245: Again, these extra results make this one of the most detailed telomeres studies.

Line 338: I would tone down the statement on selective mortality, because it is still possible that the unhatched embryos of the different incubation treatments differ in telomere length.

Figures: I think that Fig. S2 would be much more valuable to include in the main manuscript than Fig. 1.

Author's Response to Decision Letter for (RSPB-2020-1378.R0)

See Appendix B.

RSPB-2020-1378.R1 (Revision)

Review form: Reviewer 1

Recommendation
Accept as is

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?
Yes

Is it clear?
Yes

Is it adequate?
Yes

Do you have any ethical concerns with this paper?
No

Comments to the Author
I am happy with the changes, and think that the study makes an excellent contribution to the research field.

Decision letter (RSPB-2020-1378.R1)

03-Aug-2020

Dear Dr Stier

I am very pleased to tell you that your manuscript entitled "Pace and stability of embryonic development affect telomere dynamics: an experimental study in a precocial bird model" has been accepted for publication in Proceedings B. Thank you for your careful attention to the revisions throughout the review process, and congratulations on an excellent paper!

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 submitting this paper to Proceedings B. On behalf of all the Editors of the Proceedings B, we look forward to your continued contributions to the Journal.

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

Appendix A

12-Dec-2019

Dear Mr Stier,

I am writing to inform you that your manuscript RSPB-2019-2456 entitled "Ageing before birth: pace and stability of prenatal growth affect telomere dynamics" has, in its current form, been rejected for publication in Proceedings B.

This action has been taken on the advice of referees, who have recommended that substantial revisions are necessary. With this in mind we would be 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, and some comments from me. 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

Editor comments

Both the referees and I are positive about the overall interest of hte paper and the value of the design, but the referees' comments raise some important issues to address. Both have suggested additional information that it would be good to include if possible; in particular, see Ref 1's suggestion about telomerase activity, which would greatly add to the paper. Ref 2 acknowledges that their suggestions may not be feasible, but the issues do need to be acknowledged in the Discussion.

We wish to thank both the editor and the reviewers for their positive and constructive feedback. We provide a point by point response below and a track-changed version of the manuscript.

L36: 'These early life differences in telomere length persisted…' would be clearer.

corrected

L134: 'Identity of the parents was unfortunately unavailable, but since eggs were collected every day and Japanese quail lay a maximum of one egg a day [34], it is unlikely that several eggs originated from the same female.'

I'm not sure I understand this: do you mean that all eggs were collected on the same day? Presumably if they were collected on multiple days, it's likely there were some from the same female. What was the total number of females from whom eggs were collected, as compared to the number of eggs collected? This is obviously a minor point, but it would be good to clarify the sample sizes.

We do not have precise information from the breeder on the number of laying females except that eggs are collected every day; in the case of our experimental eggs, these were indeed all collected on a single day. Consequently, there is virtually no risk that two eggs came from the same female. This has now been clarified on lines 137-142.

The Methods are long. Can you move some of the details into Supplementary Information?

We have now moved the finer technical details of measurements of heart rate, corticosterone, DNA damage and TRF to the ESM.

Where you give a chi-squared statistic, it needs an associated d.f. value.

d.f. have now been added (lines 418 to 467)

It would be interesting to see the associations between your different traits: can you give the correlations at each stage?

We now provide correlations at each stage between variables as a figure S6 in the ESM (ESM S7), and present them briefly in the results (lines 479-484) and discussion (lines 520-524) sections.

Is it appropriate to use 'instability' as the term for the treatment that actually mimics the natural situation, of the female leaving incubation for short periods? How about 'variable', or 'natural', as opposed to the constant temperatures of the other treatments?

We feel that 'instability' is appropriate even if it is indeed mimicking more the natural scenario than a constant incubation temperature. Indeed, incubation recesses are frequent in many bird species, but will be minimized under good environmental conditions (*e.g.* food supplementation, Lothery et al. 2014), suggesting that they are a more linked to environmental constraints than to an intrinsic biological pattern (this is now mentioned lines 171-176). The degree of instability created is a consequence of the outcome of a trade-off between the cost to the parent (from not feeding/drinking) and cost to the embryo. We investigate the latter here. Additionally, any incubation recess slows down embryonic metabolism/growth (see lines 160-165 and new Fig S1), therefore creating a form of instability in embryonic development.

The key results are in Figure 3A. As a small sub-panel, it's a difficult to see the details. I suggest replacing 3A with your four panels in Fig S3 E-H (and put 3A in Supplementary instead).

In our opinion, replacing Fig 3A with S3 E-H would not enable the reader to compare directly experimental groups at different stages (*i.e.* Fig S3A-D). Ideally, having 3A + full S3 in the manuscript would be good, but it is unfortunately impossible considering the space limits for Proc B (we were already at the limit of 10 pages, including 4 paid pages). Therefore, we have decided to present Fig 3A as a standalone figure (instead of a panel, now Fig 3), so allowing it to be bigger, enabling the reader to better see the necessary information on TL.

Reviewer(s)' Comments to Author:

Referee: 1

Comments to the Author(s)
In this experimental study, the authors tested how incubation temperature and the stability of incubation conditions influence telomere length and dynamics before and after hatching in a bird and explored two possible mechanisms underlying the long-lasting effects of the prenatal conditions on telomere dynamics. Although it is well expected from previous evidence that the detrimental effects of fast growth or stress during early life on telomere dynamics can be extended to the prenatal period, it has rarely been tested in an experimental study. This paper shows that incubation conditions affect the pace of embryonic growth and the level of stress, thereby influencing telomere dynamics during the embryonic and post-natal periods in an expected way. The experiment was carefully designed and well conducted, and the manuscript is generally well written. Nevertheless, I have some suggestions to improve the paper as detailed below.

Pace and stability of prenatal growth: By experimentally increasing and decreasing incubation temperatures, embryonic growth rates were successfully accelerated and slowed down in the High and Low groups, respectively. However, there's no evidence that the manipulation of incubation temperature produced "unstable" prenatal growth in the Unstable group. What I learn from the results is that the unstable incubation conditions slowed down embryonic growth rates possibly by increasing the level of stress (as evidenced by plasma corticosterone level). This should

be clarified throughout the manuscript, including the title, abstract, introduction and discussion. Perhaps "pace and stability of prenatal growth" can be clarified as "pace of growth and environmental stress during the embryonic period" or "incubation temperature and stability".
We are sorry we were not clear enough in explaining why this temperature treatment creates developmental instability. As explained above, incubation recess periods are creating instability by slowing down metabolism/embryo growth during each incubation recess. Under a constant incubation temperature, embryo growth will be constant, while under our unstable treatment, embryo development/metabolism will fluctuate, slowing down during recesses and accelerating when the temperature increases again, which is in our opinion a clear form of instability. We agree with the reviewer that our CORT results suggest that this instability acts as an environmental stressor. We now provide further evidence that incubation recesses create instability by showing (in Fig S1, also explained lines 160-165) the effect of a 15min incubation recess on embryo heart rate to better justify our choice to use the term 'instability'. We have changed the title and the abstract to refer to the pace and stability of embryonic development instead of prenatal growth to be clearer.

The mechanisms form an important part in this paper, but the authors do not provide sufficient information (lines 110-114) about why oxidative stress and stress hormones are important candidate mechanisms underlying the relationship between prenatal conditions and telomere dynamics in the introduction. Please extend the provided explanation.
We now provide more context and information on mechanisms linking prenatal conditions, oxidative stress, corticosterone and telomere dynamics (lines 114-120)

It is not really clear why the effects of embryonic growth rate and the stability of incubation conditions on telomere length appeared in hatchlings (at day 1) but not in late embryos (at stED13), although the authors discussed that it may be due to telomerase activity (lines 417-419). I wonder if you could also analyse telomere length in the subsample euthanized at ED13. At ED13, the embryos of the H, M, L and U groups were at different developmental stages (H > M > L, U). If the telomere length at ED13 also did not differ between the experimental groups (thus showing the similar levels as at stED13), this may suggest that constant telomere length is maintained by telomerase activity until this developmental stage, and thus telomere length is unaffected by prenatal conditions at least until the late embryonic stage. I think that it is worth a try.
We wish to thank the reviewer for this excellent suggestion. We have now added data on telomere length in a sample of ED13 embryos (lines 441-444). Although the sample size is small (N = 3-4 per group, total N = 15)**, there is no evidence for an effect of incubation temperature/stability on these ED13 embryo telomere lengths. We are therefore proposing that most between-individual differences in telomere length appears during the last 25% of embryogenesis (>stED13), as also suggested by the positive correlation between StED13 body mass and telomere length, which became negative at day 1 post-hatching (new Fig S6, lines 479-484). This is now discussed on lines 520-524.**

Growth and survival: It is unclear whether all hatched individuals were included in the analyses of growth. At hatching sample sizes of the H, M, L and U groups were 27, 25, 26 and 25 (Fig 2D, time to hatching), but these number are reduced to 15, 15, 16 and 15 in the analysis of growth

(Fig S2). Were individuals died before D90 excluded in the growth analysis to perform a longitudinal analysis? If it is the case, you should also present the analysis including all individuals or at least indicate whether the results change. It is necessary to show that indeed body mass at hatching (D1) did not differ among the experimental groups. Did the experimental treatment influence survival to D90?

We already mentioned in the methods that "We collected blood at each of D1, D20 and at D60 for telomere length measurement for a subsample of 61 of the 103 birds that hatched (+ 8 birds at D360) **". We decided to only include body mass data for these individuals, as the main aim was to explain potential postnatal telomere dynamics by group-specific differences in growth rate. We found no evidence for effects of incubation temperature on postnatal growth, either using the full dataset or the dataset reduced to those individuals for which we have telomere data (the extra analysis is now mentioned on lines 271-272). The natural mortality before d90 was very low (4 individuals), so was extremely unlikely to bias our results.**

Minor suggestions:

Line 104: Is the stability of incubation temperature variable, for example due to individual incubation behaviour or environmental conditions? This information may be helpful to understand why it is important (or ecologically relevant) to study the effects of the stability of prenatal conditions in addition to the effects of constantly better or worse conditions for embryonic development.

The consistency of incubation temperature is indeed influenced by environmental conditions, as for instance revealed by the increased consistency found in response to experimental food supplementation (e.g. Lothery et al. 2014 Plos One). We now mention this in the methods on lines 163-165

Lines 132-135: Here, more information is necessary. For example, in total how many eggs were obtained and used in the study? In which year and months were the eggs collected? What was the range of the laying dates? Were the individual eggs incubated immediately after the delivery to the laboratory? Or were they stored until the simultaneous onset of incubation (then for how long)?

We have now added these details on lines 140-142.

Line 163: What percentage of the hatching events did occur when not observed? Did it vary among different experimental groups?

Approximately 30% of hatching occurred at night, without obvious difference between experimental groups (H = 27%, M = 32%, L = 30%, U = 28%).

Line 168: How many replicates of enclosures were used?

Due to logistical constraints, only one enclosure was used per treatment group

Line 228: Please give the full name of 8-OHdG in the first mention.

Done

Lines 296-303: Did you check whether egg size influenced body mass, telomere length and RBC

DNA damage at hatching?
Effects of egg size on body mass, telomere length and RBC DNA damage are in our opinion out of the scope of the current manuscript (which is already at the space limit of the journal and contains many variables and analyses).

Fig 2D: Days may be a more informative unit for y-axis than hours here because days of incubation and chick age are used throughout the manuscript.
Done

Lines 398-399: Is there any evidence from other studies that embryo (or chick) heart rate is indeed related to metabolic rate?
Heart rate is indeed highly correlated with metabolic rate in birds after hatching (Butler et al. 2004), but surprisingly no study to the best of our knowledge has correlated embryo heart rate and metabolic rate in birds. However, a very strong correlation has been found in reptile embryos (R^2 = 0.89; Du et al. 2010 Plos One).

Referee: 2

Comments to the Author(s)
This is a very well controlled and executed experimental study that aims to determine the impact that prenatal conditions have on development and post natal telomere length and dynamics. It is very precise, clear and well written. The question is timely and interesting given the need to understand both telomere dynamics and what they mean.

The evidence that effects on telomeres are causal of later life patterns is weak/absent across species. This should be highlighted more and less stress put on the idea that effects on telomere length may be causal. Explanation of the value of understanding the impact on telomere length of the factors assessed even that impact is only correlated with late life problems is worth adding. For example Line 27 – I think it would be better to say they are 'correlated' ....this emphasis on a causative effect is too strong throughout.
We do not feel that the original version was emphasizing causality (*e.g.* "Telomere length and/or shortening rate, especially in early life, have been shown to predict subsequent survival/lifespan in a range of species [19-23], thereby leading to the idea that telomere length could act as a biomarker of individual 'biological state'"; "short telomeres have been associated with reduced chances of survival [19,21] and increased risks of ageing-related diseases")**, but we now provide a sentence in the introduction about the causality *vs.* correlative nature of this link, and have added a recent reference providing support for a causal link (lines 71-74)**

The use of CoV has been much criticised in terms of telomere measures. You need to use other metrics. See Nettle et al 2019.
The Nettle et al. (2019) paper (titled 'Consequences of measurement error in qPCR telomere data: A simulation study') addresses issues with the qPCR approach, whereas we used the (more accurate) TRF method. Samples were analysed only once (as is mostly the case with in-gel TRF studies) considering the labour-intensive nature of this technique and our large sample

size (n = 228), and only the inter-gel standards were analysed in duplicate. Therefore we cannot calculate repeatabilities and can only provide the CV (%) here, but AS has carried out a repeatability analysis using the same methodology in another bird species (the Collared flycatcher, Stier et al. submitted to Biology Letters) and confirmed the very high repeatability of our TRF method (*R* = 0.995, N = 18, p < 0.001).

Overall this is an interesting experiment but the key to testing the overall hypothesis would be to determine if these development/telomere differences are then linked to differences in later life health and survival. Given the lifespan of the model species used this seems possible and it is a pity that this wasn't done.

We fully agree it is a pity that we do not have this information, but this study was funded by a 2 year Marie-Curie fellowship to AS, which unfortunately was not long enough to enable survival/lifespan to be quantified, even in a short-lived species like the Japanese quail. With this paper, we aim to stimulate future studies looking at these long-term effects, as indicated in the last sentence of the conclusion.

I don't agree with the argument that this experiment shows the 'extreme importance of the prenatal stage in determining telomere length over the life course of individuals'. In the experimental individuals all other conditions after the experiment are the same for all individuals, i.e. they are exposed to no other variation. Therefore it seems obvious that the telomere length differences caused by the prenatal experiment will predominate....however large or small that actual effect may be. However in a natural system many other factors and variants may come into play and may totally swamp the effects on telomere length of the prenatal conditions. What we need to know is how relatively much the prenatal impact effects telomeres compared to the other variation individuals may normally be exposed to.

While we partly agree with the reviewer's point (and have removed "extreme" from the sentence), the fact that in our case postnatal environmental conditions were similar for all individuals does not preclude the existence of marked inter-individual differences in telomere dynamics (*e.g.* Heidinger 2012 PNAS).

Additionally, humans experience various environmental conditions postnatally, but yet show a very high consistency in telomere "ranking" between individuals through time (e.g. Benetos et al. 2019 FASEB), and the same is true in wild animals when measured with an appropriate methodology (Kärkkäinen & Stier meta-analysis in prep.; Bichet et al. 2020 Mol Ecol). This suggests that most inter-individual difference in telomere length is already set at birth. However, we have added a caveat to the discussion (lines 552-554), that "While postnatal telomere shortening rate might be more variable between individuals living in the wild, there is evidence that the rank order of telomere lengths across individuals is retained over time even in natural conditions (Bichet et al. 2020)".

Appendix B

Reviewer(s)' Comments to Author & responses (track-change document below):

Referee: 1

Comments to the Author(s).
The authors considered my previous comments carefully in the revised manuscript. I am satisfied with the improvement.
We are grateful to reviewer 1 for his/her previous comments and happy to see her/him satisfied with the changes we made to the manuscript.

Referee: 3

Comments to the Author(s).
General comments:

This study has experimentally manipulated incubation conditions of Japanese quail embryos and tested for effects on pre-and post-natal telomere length, together with potential causes of telomere shortening (growth rate, oxidative damage, and prenatal glucocorticoid levels). The experiment is well designed and executed, the manuscript well written, and the results are very interesting for a diverse audience, as they are relevant for the study of pre-natal maternal effects, ageing, life-history trade-offs and telomere dynamics. In addition to the results of the experiment, the study also provides a very detailed description of the general telomere dynamics (i.e. repeatability, shortening rate with age) of this relatively short-lived bird species. I only have a few minor suggestions for improvement (see specific comments below), of which the two most important are:

1. The authors should try to correct for methodological between-gel differences in calculating the intra-individual repeatability of telomere length. Moreover, providing the intra-individual repeatability with, and without, correcting for the fixed effect of the incubation experiment on telomere length, would provide insight into what proportion of the repeatability is actually caused by the manipulated pre-natal conditions.
As detailed below, we tried to correct for methodological between-gel differences in calculating the intra-individual repeatability of telomere length by including either the specific value of the standard sample or the gel identity, but such inclusions had no impact on our results. We now also provide an extra analysis where the incubation treatment is included as a fixed factor, but this only reduced the within-individual repeatability very slightly (0.93 to 0.91). This suggests that the high repeatability is not simply a consequence of the treatment effects.

2. Because layer-type Japanese quail will not have been selected for lifespan, but only for early-life productivity, while in the wild the individuals with the longest lifespan in most species also have the highest lifetime reproductive success, there may be little selection for telomere maintenance in this model species. Although I do not think that this is a problem for showing 'proof of principle', I do think

that a few lines in the Discussion about why effects in quail may be more pronounced than in longer-lived wild species could add to the quality of the Discussion.
We fully agree with the reviewer's point and now discuss this issue on lines 419-424.

Specific comments:

Line 1: I do not think that the title is completely appropriate. Because of the term 'birth' my first impression would be that this is a study on humans (or on another mammal species). Moreover, the term 'ageing' is inappropriate, because there is no evidence that telomere length predicts individual rate of ageing (only that it predicts lifespan, which does not need to be related to ageing). I suggest to omit the first part, and add something like 'in a precocial bird' at the end.
We have changed the title according to the reviewer comment: "Pace and stability of embryonic development affect telomere dynamics: an experimental study in a precocial bird model"

Line 29: I would omit 'strongly'. Work in humans, and in some other species in which telomeres were measured with TRF, suggests that the majority of between-individual variation is due to additive genetic effects, with comparatively small permanent environmental effects.
We removed "strongly" as suggested by the reviewer

Lines 65-66: I would omit this explanation. This is only a mechanistic/proximate explanation, and the fact that germline telomere length can be protected against telomere loss without a high risk of cancer makes this explanation not very credible from an evolutionary point of view.
We have now removed this mechanistic explanation

Line 103: I would omit 'slightly'. If a small difference has a large effect on fitness I would not call it a slight or small effect. Since causal effects on fitness of these type of manipulations are unknown, it is best to refrain from speculation on effect size.
We fully agree and have now removed 'slightly' from the text

Line 138: I think you forgot to omit a part of the sentence.
We double-checked the sentence starting line 138 and did not notice what would have been omitted. However, we have rephrased the sentence to be clearer.

Line 163: Although I understand the reasoning behind your different treatments, I object to calling some treatments more or less 'natural'. Incubation in an incubator is by definition not 'natural' because in the wild a female would have continuous feedback from the temperature of her eggs, and adjust her incubation intensity accordingly (but only if she has enough energy to put in the optimal amount of heat). Since an embryo is generating more heat the bigger it gets, in the wild a female would lower incubation intensity further into the incubation phase, to keep the eggs at a constant

temperature. This is a process that cannot be achieved with small commercial incubators, but seems to be the norm in the poultry industry (e.g. see Lourens et al. 2005, Poultry Science).
Although we agree that artificial incubators will never fully re-create the natural scenario, incubation recesses occur under the natural scenario, but not in typical studies using artificial incubators, which is why we referred to it as "more natural". Nonetheless, we have now removed the mention of a natural scenario to avoid giving the wrong impression.

Moreover, the type of Japanese quail that is used in laying farms has completely lost its ability to incubate its own eggs (because there is a trade-off between laying and incubation) and will have been artificially incubated for perhaps more than a 100 generations (generation time of industrial laying quail will be less than a year, as laying rate and egg hatchability steeply decline with age (see Woodard & Abplanalp 1971, Poultry Science) making it not commercially profitable to keep them alive for longer than that). Hence, in these quail there will have been generations of selection for having the highest hatchability, and early-life productivity, under the incubation temperature chosen by most breeders (which may not coincidentally be a 100 degrees Fahrenheit), and not for having the longest possible lifespan. This may even be an explanation for why the effect of incubation temperature on telomere length of Japanese quail seems to be so much larger (in terms of base pairs) than in common terns. With this type of selection regime there will be little selection to prevent telomere loss due to a trade-off with development speed, as they will never reach the age at which telomere length becomes critical (or at least eggs laid at that age will not be used to produce the next generation, as they have lower hatchability). This may make the quail an ideal model for showing the 'proof of principle' for such trade-offs, but it may not reflect the 'natural situation', regardless of what incubation profile has been used.
See our answer to main comment 2 and associated changes on lines 419-424

Lines 236-238: Interesting that they lose so many base pairs of telomere length per year. These type of details, which can only be obtained from TRF studies, make the study extra valuable.
Lines 240-245: Again, these extra results make this one of the most detailed telomeres studies.
I think that Fig. S2 would be much more valuable to include in the main manuscript than Fig. 1.
We have now moved former Fig 1 to the ESM and placed the former Fig. S2 in the main text as we fully agree that such information makes the study extra valuable. The fact that Japanese quails lose so many base pairs per year fits well with the fact that short-lived bird species exhibit faster telomere shortening than long-lived ones, but could also be influenced by the history of artificial selection mentioned by the reviewer.

Lines 238-240: Is this repeatability corrected for the effects of the incubation treatment? If not, I would also present a repeatability value that is corrected for variation caused by the treatment (by adding treatment as a fixed effect). It would be very interesting to know if they are also very repeatable without the extra between-individual variation in embryonic development rate caused by the experiment, as that would suggest a high heritability (or strong maternal effects that are independent of incubation procedure).

The original repeatability analysis was not including the incubation treatment as a fixed factor. We have now added this extra-analysis on lines 261-263. Including the incubation treatment reduced only very slightly (0.93 -> 0.91) the within-individual repeatability, suggesting indeed high heritability or strong maternal effects.

Moreover, I noticed in the ESM that repeated samples of individuals were always run on the same gel. In the future it would be better to randomize samples over gels, as now your between-individual variance may be artificially increased due to methodological between-gel differences. Perhaps you can add the gel-specific value of the standard sample as a fixed effect to see if that explains some of the between-gel variance, which would make your repeatability estimate more accurate.

Samples from the same individual were always run on the same gel to maximize the statistical power to detect intra-individual changes in telomere length. This strategy has however the drawback of increasing between-individual variance as pointed out by the reviewer. This is however unlikely to affect our main results as our experimental groups were equally divided among different gels.

We included either the gel-specific value of the standard sample or the gel identity in the repeatability analysis, and this does not affect the repeatability estimate (0.93 without inclusion, 0.93 with inclusion of the specific value of the standard sample, 0.93 with inclusion of the gel identity). We now specify on lines 260-261 that the within-individual repeatability is corrected for both age and between-gel effects.

Line 338: I would tone down the statement on selective mortality, because it is still possible that the unhatched embryos of the different incubation treatments differ in telomere length.

We now mention it as "unlikely to be driven".