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Dietary antioxidants attenuate the endocrine stress response during long-duration flight of a migratory bird

Stefania Casagrande, Kristen DeMoranville, Lisa Trost, Barbara Pierce, Amadeusz Bryła, Maciej Dzialo, Edyta T. Sadowska, Ulf Bauchinger and Scott R. McWilliams

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Review timeline
Original submission: 3 April 2020
Revised submission: 21 May 2020
Final acceptance: 21 May 2020

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

Review History

RSPB-2020-0744.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?
Yes

Is it clear?
Yes

Is it adequate?
Yes

Do you have any ethical concerns with this paper?
No

Comments to the Author
This is an interesting study on the impacts of antioxidants on corticosterone responses during extended flight in a wind tunnel. The results are exciting and address a major outstanding question in the field. Many studies have attempted to tease apart the role of corticosterone during migratory flight, and several investigators have posited that oxidative stress is a major component of migratory metabolism. This manuscript does an excellent job of teasing apart the relative roles of antioxidants and corticosterone in regulating metabolism during flight. I only had a few comments.

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1) Figures 1 and 2: For Fig. 1, the units on the y-axis don't make sense. Table S1 indicates flight means as about 39 and 62 ng/ml for anthocyanin and controls, whereas the figure indicates about 0.3 and 1.0 for those values. Does the figure plot z-scores instead? If so, I recommend graphing ng/ml. Even if the stats were run on z-scores, the graphing of ng/ml would make the data more comparable to other published studies. I think this is what was intended in the legend of Figs. 2 and 3, but the y-axis units again seem to be z-scores instead of what the legend claims.

Minor comments:

1) Line 131: The "<3 min" I believe refers to the time to take a blood sample, but the way it is written seems to refer to the timing of the longest flight. Please adjust to make this clearer.

2) Line 136: the word "deprivation" often implies starvation, which is not what was done here. I might suggest "restriction" instead.

3) In the supplemental data file, I believe there is a typographical error. In column F, the title is Cort (ng/ml). Could this be pg/ml instead? I don't believe that the first bird had a value off 73004 ng/ml, especially since 73.004 ng/ml would better match the data that are reported in table S1.

Review form: Reviewer 2 (Hubert Schwabl)

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

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duration flight.
The study is well designed and conducted, the data analyses proper, the conclusions justified, and the paper well written. I comment the authors for their carefully avoiding to couch their results in Cort being a stress hormone and migration and duration flight representing stress or causing a stress response. Rather, the authors emphasize the role of GC in energy metabolism with acute increases in Cort serving adaptive metabolic functions. Only in their conclusion of the role of complementing effects of AO they refer to deleterious effects of chronically elevated Cort. Nevertheless, I have some comments and suggestions that might further improve the paper.

Comments:

Line 30 to 36: the two sentences are quite long, suggest to break up.

Line 51: I wonder if the term "upregulation" can be avoided. To me, it implies a preparatory modification of the function of the entire HPA axis (i.e. change in CORT set point). Rather, I see the Cort response observed here as an "increase" in Cort secretion in response to energy demand during flight. Moreover, the mentioned long-term costs of "upregulated" GC mentioned here imply chronic stress with chronically elevated GCs, which is not what the study is interested in and shows. (Migratory flight might not cause stress …)

Line 57: consider changing fine-tuned "upregulation".

Line 120: I wonder if results would be the same if wind-tunnel flights would have been performed at other times than Sept – Dec and Feb – April, the time periods when starlings can be assumed to be in a physiological state of migration (with preparatory physiological adjustment to high energy demand and endurance flight).

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Line 171: The Cort statistics model includes assay (plate number) as a random factor. Inter-plate cv is reported as 8.73%, but were samples randomized across plates?

Line 190: "birds fed with anthocynanins had lower levels of Cort than control birds after flight". Can you say that without reporting a post-hoc p-value?

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Line 253 ff: Here you get into Cort and "stress physiology". I suggest to not say that your study "allowed us to specifically narrow the perspective on the effect of flight on stress physiology…" You did not study stress physiology here, you looked at Cort levels. These Cort levels are rather high (mean 39.33 and 62.18 ng/ml), comparable to levels that can be induced by a potent stressor (such as that used by the capture and handling protocol that is commonly applied to evaluate the

scope of the Cort stress response). This is an important result that prompts me to suggest showing the raw values in a main figure. I also suggest to compare these levels to those reported for other passerines or even the starling, if available. The reported levels at rest, contrast, are comparable to levels usually reported for levels without stress. As often in ecological research absolute levels of molecules are not reported as authors are interested in relationships with other factors and do not care about absolute concentrations. In the endocrine stress response field it is, however, important to see absolute levels as they allow the reader to evaluate the strength of the HPA activity in different conditions. In this context, it would also be interesting to see a comparison to the change in starling Cort levels with time of day and other natural factors such as food availability, weather, season. But, I am not sure if such information is available for the starling. Nevertheless, the discussion of the results should keep these considerations in mind.

Line 258/259: Whom should we believe? The authors who conclude that Cort levels did not change after flight or your interpretation of their results. I suggest going with the statistics in the paper.

Fig. 1 and Figure 2: Are these the combined data for Fall and Spring?

Table 1 and 2 legends: It is not clear to me why in the comparison of Cort during Fall and Spring, Spring is referred to as reference group.

Decision letter (RSPB-2020-0744.R0)

18-May-2020

Dear Dr Casagrande

I am pleased to inform you that your manuscript RSPB-2020-0744 entitled "Dietary antioxidants attenuate the endocrine stress response during long-duration flight of a migratory bird" has been accepted for publication in Proceedings B. Congratulations!!

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

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

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

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

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

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

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

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

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

In order to ensure effective and robust dissemination and appropriate credit to authors the dataset(s) used should be fully cited. To ensure archived data are available to readers, authors should include a 'data accessibility' section immediately after the acknowledgements section. This should list the database and accession number for all data from the article that has been made publicly available, for instance:
- DNA sequences: Genbank accessions F234391-F234402
- Phylogenetic data: TreeBASE accession number S9123
- Final DNA sequence assembly uploaded as online supplemental material
- Climate data and MaxEnt input files: Dryad doi:10.5521/dryad.12311

NB. From April 1 2013, peer reviewed articles based on research funded wholly or partly by RCUK must include, if applicable, a statement on how the underlying research materials – such as data, samples or models – can be accessed. This statement should be included in the data accessibility section.

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

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

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

Sincerely,
Dr John Hutchinson, Editor
mailto: proceedingsb@royalsociety.org

Associate Editor
Comments to Author:
We have now obtained two expert reviews of your manuscript, and I am pleased to say that both reviewers found your manuscript interesting and thought that it provided novel information on the interplay of migration physiology and the glucocorticoid axis. Based upon my own reading of the I concur with their assessment.

Although the reviews were generally positive, both reviewers provide useful commentary that will improve the presentation of the manuscript. In particular, reviewer 1 identifies some issues associated with data presentation that, if followed, would not only make the data more accessible to readers but would also make it more useful for comparison across studies. They also note suggest reviewing the supplemental data file and table S1 as there may be a labeling error in the data file. Reviewer 2 provides a more extensive list of suggested revisions that are aimed at clarifying some of the language including the use of the term "upregulation". I agree that the manuscript would benefit from either changing this terminology, or clearly defining how you are using the term to avoid unnecessary confusion. They also provide a detailed suggestion on the use of "stress physiology", and whether it is appropriate for the current study, along with some suggested comparisons that, if available for starlings, would help put the current study into a broader context.

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Author's Response to Decision Letter for (RSPB-2020-0744.R0)

See Appendix A.

Decision letter (RSPB-2020-0744.R1)

21-May-2020

Dear Dr Casagrande

I am pleased to inform you that your manuscript entitled "Dietary antioxidants attenuate the endocrine stress response during long-duration flight of a migratory bird" 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.

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

Sincerely,
Editor, Proceedings B
mailto: proceedingsb@royalsociety.org

Appendix A

Associate Editor
Comments to Author:
We have now obtained two expert reviews of your manuscript, and I am pleased to say that both reviewers found your manuscript interesting and thought that it provided novel information on the interplay of migration physiology and the glucocorticoid axis. Based upon my own reading of the I concur with their assessment.

Although the reviews were generally positive, both reviewers provide useful commentary that will improve the presentation of the manuscript. In particular, reviewer 1 identifies some issues associated with data presentation that, if followed, would not only make the data more accessible to readers but would also make it more useful for comparison across studies. They also note suggest reviewing the supplemental data file and table S1 as there may be a labeling error in the data file. Reviewer 2 provides a more extensive list of suggested revisions that are aimed at clarifying some of the language including the use of the term “upregulation”. I agree that the manuscript would benefit from either changing this terminology, or clearly defining how you are using the term to avoid unnecessary confusion. They also provide a detailed suggestion on the use of “stress physiology”, and whether it is appropriate for the current study, along with some suggested comparisons that, if available for starlings, would help put the current study into a broader context.
AU: We thank the Associated Editor for the positive comments to our study. We have addressed all the points raised by the reviewers and by you (please see below our reply in bold; line number refers to the file with tracked changes). We would only like to point out that we changed the labelling of the data set that now are available on Dryad repository, while the ones of Table S1 were correct.

Reviewer(s)' Comments to Author:

Referee: 1

Comments to the Author(s)
This is an interesting study on the impacts of antioxidants on corticosterone responses during extended flight in a wind tunnel. The results are exciting and address a major outstanding question in the field. Many studies have attempted to tease apart the role of corticosterone during migratory flight, and several investigators have posited that oxidative stress is a major component of migratory metabolism. This manuscript does an excellent job of teasing apart the relative roles of antioxidants and corticosterone in regulating metabolism during flight. I only had a few comments.
AU: we are very grateful to the reviewer for this very positive comment.

Major comment:

1) Figures 1 and 2: For Fig. 1, the units on the y-axis don’t make sense. Table S1 indicates flight means as about 39 and 62 ng/ml for anthocyanin and controls, whereas the figure indicates about 0.3 and 1.0 for those values. Does the figure plot z-scores instead? If so, I recommend graphing ng/ml. Even if the stats were run on z-scores, the graphing of ng/ml would make the data more comparable to other published

studies. I think this is what was intended in the legend of Figs. 2 and 3, but the y-axis units again seem to be z-scores instead of what the legend claims.

AU: we thank the reviewer for bringing up this point because the specification about z-score transformed data was actually missing from the legend of Figure 1, where we recalled only that raw data were available in Table S1. We apologize for this mistake. We have addressed the issue by reporting in this legend for Fig 1: "All variables were z-scored but units of measures were reported for clarity." We would like to keep the present form of the Figure 1 because Figure 2 has to be represented by z-score values of correlated variables that require the standardization of covariates' scale. Thus, we think that representing the data in the same way in all figures is more correct and clearer. In any case, raw data are available in Table S1.

Minor comments:

1) Line 131: The "<3 min" I believe refers to the time to take a blood sample, but the way it is written seems to refer to the timing of the longest flight. Please adjust to make this clearer.

AU: we moved "<3 min" after "Immediately", line 132

2) Line 136: the word "deprivation" often implies starvation, which is not what was done here. I might suggest "restriction" instead.

AU: changed accordingly (line 140)

3) In the supplemental data file, I believe there is a typographical error. In column F, the title is Cort (ng/ml). Could this be pg/ml instead? I don't believe that the first bird had a value off 73004 ng/ml, especially since 73.004 ng/ml would better match the data that are reported in table S1.

AU: we thank very much the reviewer for this observation. We have changed the units of the data set to pg/mL.

Referee: 2

Comments to the Author(s)

This research paper reports "baseline" plasma concentrations of the glucocorticoid corticosterone (Cort) in birds after flight in a wind tunnel. It tested the hypothesis that Cort functions to support metabolic demand by flight and that dietary antioxidants (anthocyanins) complement Cort function, thereby possibly avoiding potential detrimental effects of high Cort levels. To this end, the experiment compared Cort levels between two groups of European starlings immediately after sustained flight (up to 2 hours) in a wind tunnel and after rest. The experimental group was fed a diet supplemented with anthocyanin, the control group was fed an iso-caloric diet without anthocyanin. The study was performed in two seasons, fall and spring (related to the two migratory periods of starlings). Results report 1) higher Cort levels immediately after flight that at rest in both groups and 2) lower Cort levels in anthocyanin supplemented birds after flight; 3) no difference between spring and fall in the Cort response; 4) positive relationship of Cort with flight duration (up to 2 hours) in controls, negative relationship in AO-fed birds;

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AU: we thank the reviewer for this positive overview. For clarity, we would like just to point out that our birds flew up to 6 hours, not 2.

Comments:

Line 30 to 36: the two sentences are quite long, suggest to break up.
AU: we have changed the sentences in accordance with what suggested, line 32-34: “Dietary antioxidants (e.g., anthocyanins) support metabolism by quenching excess reactive oxygen species produced during aerobic metabolism, and also by activating specific metabolic pathways. For example, similar to GCs’ function, anthocyanins promote the release of stored energy, although the extent of complementarity between GCs and dietary antioxidants is not well known.”

Line 51: I wonder if the term “upregulation” can be avoided. To me, it implies a preparatory modification of the function of the entire HPA axis (i.e. change in CORT set point). Rather, I see the Cort response observed here as an “increase” in Cort secretion in response to energy demand during flight. Moreover, the mentioned long-term costs of “upregulated” GC mentioned here imply chronic stress with chronically elevated GCs, which is not what the study is interested in and shows. (Migratory flight might not cause stress …)
AU: We changed “upregulation” with “increase of GCs secretion” as suggested (line 43, 52, 58, 215, 264) whereas we left it in line 275 because we were referring to more general conditions where anticipatory functions of GCs can occur. About the second comment, we kindly disagree with the concept that costs due to increase Cort secretion imply chronic stress. However, we feel this was a general consideration of the reviewer that doesn’t require a specific action from our side.

Line 57: consider changing fine-tuned “upregulation”.
AU: changed with “secretion” (line 58).

Line 120: I wonder if results would be the same if wind-tunnel flights would have been performed at other times than Sept – Dec and Feb – April, the time periods when starlings can be assumed to be in a physiological state of migration (with preparatory physiological adjustment to high energy demand and endurance flight).
AU: good point that could be addressed in future studies?

Lines 131/137: Can it be assumed that a delay of < 3min is safe to obtain baseline Cort levels? Did you look at the relationship of sampling delay and Cort (see for example Steenweg et al. 2015, Schwabl et al. 2016)?

AU: Following the suggestion we looked at this relationship by including exact time of bleeding in the corticosterone model but we did not find any effect of time (in seconds) of bleeding (estimate: 0.0027, $F_{(1,80.41)}$=0.55, p=0.46). Thus, we can assume that bleeding within 3 minutes is adequately quick to obtain baseline Cort levels and so the results presented are correct.

Lines 130/138: birds flew for different periods of time. Did they all fly at about the same time of day? Were birds 'at rest" and "after flight" sampled at the same time of day? Can you rule out a time of day effect on Cort levels.

AU: we have added the required information in line 129: "On Day 15, and 105 min after lights on (6:30 a.m. until 30 Oct, 5:30 a.m. until 26 March, 6:30 a.m. thereafter; daily light schedule in ESM)". Please note that time of sampling after flight and after resting were comparable as both occurred in the first part of the day. However, time of sampling after flight had a broader range than sampling after rest, and we controlled for the effect of day time on corticosterone level after flight. We now report this on line 136: "Since birds finished their endurance flight at different times of the day and since corticosterone can show circadian fluctuation, we checked and found no effect of time-of-day on corticosterone values ($F_{(1,40)}$=1.24, p=0.47)."

Line 171: The Cort statistics model includes assay (plate number) as a random factor. Inter-plate cv is reported as 8.73%, but were samples randomized across plates?

AU: we have now specified in ESM that: "Individuals were randomized across plates with repeated measures for the same individual run in the same plate".

Line 190: "birds fed with anthocynanins had lower levels of Cort than control birds after flight". Can you say that without reporting a post-hoc p-value?

AU: Here and throughout the Results we support such statements by referring to the appropriate table and figure that shows the patterns and reports the statistical analyses including p-values (in this case, table 1 and figure 1). We would prefer to report the complete set of analyses as reported in the tables rather than repeating these values in the text.

Line 200: statement "suggesting that the effect of anthocyanins in controlling corticosterone levels was not mediated by change in body condition." This should go into the discussion. Moreover, "controlling" could be replaced with "influencing", as you have not shown that AC control Cort or the HPA-axis.

AU: we replaced "controlling" as suggested (204). However, we would like to leave this sentence in the results because we discussed other relevant results in this section and because we did not address the lack of effect on body mass in the discussion. We hope that the reviewer will agree with this choice.

Line 216: see my comment above about the use of "upregulation". Also line 219.

AU: we changed this word thorough the text (e.g. 43, 214, 264) while we left it in line 275 because we

were referring to more general conditions where anticipatory functions of GCs can occur.

Line 228/229: It is incorrect to say "that AC promotes the catabolism of fat by inhibiting lipogenesis". These are different metabolic processes involving different pathways and enzymes.
AU: we changed the sentence that now is (line 232): "Activation of AMPK by anthocyanins promotes the catabolism of fat and inhibits lipogenesis".

Line 253 ff: Here you get into Cort and "stress physiology". I suggest to not say that your study "allowed us to specifically narrow the perspective on the effect of flight on stress physiology…" You did not study stress physiology here, you looked at Cort levels.
AU: we changed "stress physiology" with "circulating corticosterone". We have already replied above why we would like to keep the figure with z-scored values.
These Cort levels are rather high (mean 39.33 and 62.18 ng/ml), comparable to levels that can be induced by a potent stressor (such as that used by the capture and handling protocol that is commonly applied to evaluate the scope of the Cort stress response). This is an important result that prompts me to suggest showing the raw values in a main figure. I also suggest to compare these levels to those reported for other passerines or even the starling, if available. The reported levels at rest, contrast, are comparable to levels usually reported for levels without stress. As often in ecological research absolute levels of molecules are not reported as authors are interested in relationships with other factors and do not care about absolute concentrations. In the endocrine stress response field it is, however, important to see absolute levels as they allow the reader to evaluate the strength of the HPA activity in different conditions. In this context, it would also be interesting to see a comparison to the change in starling Cort levels with time of day and other natural factors such as food availability, weather, season. But, I am not sure if such information is available for the starling. Nevertheless, the discussion of the results should keep these considerations in mind.
AU: We see the point of the reviewer, and we happen to agree that reporting of these absolute Cort levels are important - this is why we reported in the supplemental table the levels of corticosterone for each group and time point. Following previous comments of the reviewer, we reported the lack of significant effect of time-of-day on corticosterone. We would not like to add comparison of hormone levels with other species or studies because (a) we did not find any detrimental effect on bird condition (i.e. body mass, fat and muscle score), and (b) such broad comparisons are beyond the scope of this experimental study. Given the reviewer's suggestion, we have added to the Discussion the following (line 270): "The levels of corticosterone observed in this study after the endurance flight were relatively high (62.18 ng*mL-1, Table S1), but lower than the stress-induced levels observed in wild females of the same species (e.g. 80.05 ng*mL-1 ng ; [78]). Further studies are needed to determine how the Cort levels reported here for starlings performing endurance flights in a windtunnel are comparable to those of free-ranging birds."
Line 258/259: Whom should we believe? The authors who conclude that Cort levels did not change after flight or your interpretation of their results. I suggest going with the statistics in the paper.
AU: Based on their statistical analyses, it could be said that the model was overfitted/lacking the statistical power to reject the null hypothesis tested in a model assessing the effect of flight on corticosterone level. Specifically, the statistical model was run on 8 birds (but data were unbalanced as

not all data were available for all birds) with at least 5 factors in the model (4-levels of treatment, 2-levels of hormonal trait, body condition, interaction terms and one random factor;) and they reported a p-value=0.078 for the effect of flight. Based on the non-overlapping error bars reported in the figure, baseline corticosterone was significantly greater after 2-hours than pre- and post-flight. For this reason, we would like to keep our interpretation. Please note that the sentence has been revised (line 263): "Similar to our findings, red knots *Calidris canatus* showed higher levels of baseline corticosterone after 2 hours of flight although this increase was not statistically significant [66] perhaps because of lack of statistical power to reject the null hypothesis".

Fig. 1 and Figure 2: Are these the combined data for Fall and Spring?
AU: Yes, they are. We have now specified that data of Fall and Spring were combined.

Table 1 and 2 legends: It is not clear to me why in the comparison of Cort during Fall and Spring, Spring is referred to as reference group.
AU: The choice was arbitrary (made as default by the software that considers the last term in alphabetic order as the reference group) and does not change the result. It would not be necessary to mention reference groups as these are all 2-levels factors and reference group are unmistakable, but for clarity we think it was better to keep it to explain the output of the tables and the meaning of letters within brackets.