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Plumage redness signals mitochondrial function in the house finch

Geoffrey E. Hill, Wendy R. Hood, Zhiyuan Ge, Rhys Grinter, Chris Greening, James D. Johnson, Noel R. Park, Halie A. Taylor, Victoria A. Andreasen, Matthew J. Powers, Nicholas M. Justyn, Hailey A. Parry, Andreas N. Kavazis and Yufeng Zhang

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Review timeline

Original submission:	15 April 2019
1st revised submission:	31 July 2019
2nd revised submission:	28 August 2019
3rd revised submission:	2 September 2019
Final acceptance:	3 September 2019

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

Review History

RSPB-2019-0889.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?
Good

Is the length of the paper justified?
Yes

Should the paper be seen by a specialist statistical reviewer?
No

Do you have any concerns about statistical analyses in this paper? If so, please specify them explicitly in your report.
No

It is a condition of publication that authors make their supporting data, code and materials available - either as supplementary material or hosted in an external repository. Please rate, if applicable, the supporting data on the following criteria.

Is it accessible?
Yes

Is it clear?
Yes

Is it adequate?
Yes

Do you have any ethical concerns with this paper?
No

Comments to the Author
The red feathers of the house finch are a textbook example in signaling theory. However, it is still controversial which mechanism links ornamentation to condition. The color-defining red keto-carotenoids are produced from yellow dietary precursor carotenoids. The ketolase enzyme has been identified as CypP2J19 by genetic means. The present study tests whther keto-carotenoid production is linked to mitochondrial respiration. The study shows that keto-carotenoids accumulate in the inner mitochondrial membrane (IMM). Furthermore, the redness of feathers of wild-caught molting male finches was found to be positively correlated with the respiratory control ratio of isolated hepatic mitochondria from the animals. From these findings, the authors concluded that the redness of the feathers represents an honest signal of condition because mitochondrial functioning, similar to color ornamentation, is well correlated with survival rate and reproductive success in this bird species.
This study is well conduced and follows an interesting hypothesis. It provides evidence for an as yet not appreciated connection between carotenoid metabolism and mitochondrial functioning. However, this reviewer raises a few concerns that should be addressed prior to publication.

Concerns:
Line 28: The sentence needs to be revised. Structural modeling cannot predict that ketolation is linked to cellular respiration. Additionally, mitochondrial P450 enzymes receive electrons from reduced nicotinamide adenine dinucleotide (NADPH) via the intermediacy of two proteins—ferredoxin reductase (a flavoprotein) and ferredoxin (an iron/sulfur protein). Thus, there is only a distant link between respiration and ketolation.

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Minor concerns:
Line337: PGC1-a needs to be replaced by PGC-1alpha.
Line338: associated with mitochondrial remodeling.
Legend Fig. 2 PGC- needs to be replaced with PGC-1alpha.

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?
Excellent

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

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Is it clear?
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Is it adequate?
No

Do you have any ethical concerns with this paper?
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Comments to the Author
The authors present a study connecting variation in mitochondrial function, plumage differences based on carotenoid pigments, and a putative candidate gene connecting both. More specifically, the paper suggests a molecular model linking red plumage hue to mitochondrial function by providing evidence that red ketocarotenoids are found in the inner mitochondrial membrane (IMM) of mitochondria, and that variation in red plumage hue is associated with certain aspects of mitochondrial activity. Understanding the genetics and evolution of carotenoid pigment processing in this case is timely, given the molecular basis of processing these molecules in vertebrates is poorly characterized. Moreover, the authors highlight the fact that carotenoids have been intensely studied in birds as a possible "honest signal" of quality, although how carotenoids might be connected to variation in physiological capacity (i.e. "quality") has been unclear. The current study tests whether this may directly signal differences in mitochondrial efficiency, and and uses data from house finches to show that carotenoids are found directly in the mitochondria, and differences in carotenoid "redness" is associated with mitochondrial respiration.

Their main findings are: 1) while carotenoids have been previously shown to be found in the mitochondria of birds (unlike other vertebrates, which actively avoid sequestering carotenoids in mitochondria), this study was able to localize it to the IMM. 2) That certain aspects of mitochondrial respiration are associated with differences in hue, presenting a functional link between mitochondrial function and plumage color. 3) Using biochemical modelling of a putative ketolation enzyme (CYP2J19), the authors present additional evidence a) that this enzyme has the shape and characteristics of one that would be predicted to interact with carotenoid molecules, and b) that it may be also be directly associated with the mitochondria. I found result #2 was the most important, while #1 seemed like an incremental improvement over existing knowledge and #3 somewhat tangential to the main thrust of the paper (see below).

While I found that the paper was generally well-written, with a good flow, my main concern—particularly for the broad readership of Proc B—is how technical some of the details of the biochemical methods and modelling are. This is especially true when trying to explain how the importance of different complexes of mitochondrial function and respiration may play a role in carotenoid ketolation (see comment below for lines 354 – 361). In addition, it really was not clear until the discussion the central hypotheses motivating the in silico biochemical modelling of CYP2J19. I think the discussion could be improved by clarifying these specific aspects of mitochondrial biochemistry, and length could be cut down by moving some of the technical parts of the methods into a supplement.

Other major points:
- It was not clear, based on the description, whether the birds were still molting? Therefore, have they deposited all of the carotenoids for their prebasic molt? I don't think this would necessarily alter the conclusions of the study, but it does complicate things a bit if there is unresolved temporal variation.
- I generally thought it would be more common (and much more objective) to use a

spectrophotometer to measure hue? Was there a strong reason why the manual inspection and quantification with Photoshop was desirable in this case? Also, while it is unlikely to alter the main conclusions, many authors of avian coloration impute their color data with TetraColorSpace to account for the differences in the avian visual spectrum.
- I think it would be useful to include some example pictures from birds sampled from different parts of the hue spectrum, so that readers have an idea the range that is being assayed. This also be useful if other future studies want to use similar methods for measuring color.
- More generally, I found the modelling of CYP2J19 not particularly well integrated with the rest of the study. For example, it is not altogether clear the supposed 'direct link' of this protein to the mitochondria (lines 384-385). Obviously it is non-trivial to provide this kind of molecular evidence, but that seems to me the most direct evidence of this molecules localization to the mitochondria. Moreover, why in the case the strict focus on CYP2J19, as opposed to other recently described carotenoid processing enzymes (e.g. scavenger receptors or BCOs?). While I am not convinced that this modelling piece is the most appropriate complement to the rest of the paper, I will leave it to the editor to decide whether it is desirable to keep this finding within the main text of the manuscript.

More specific points:

Line 29 – Should be "as a signal" instead of "signal".

Line 30 – Not sure if "inexorably" is the right word here.

Line 87- Has it been verified that putting a bird in a paper bag is not stressful? Holding birds in bags is used as a standard stress inducer in a number of stress studies. While I doubt it will affect the results—presumably birds were treated similarly—I don't think it is necessarily appropriate to suggest that these were not stressful conditions. I would suggest that either additional evidence is used to support this statement, or the statement is tempered. I also think including some verification that birds held for shortest/longest time did not show the most extreme values of mitochondrial performance.

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Line 154-159 – This is a very interesting point. It could also be useful to say from the beginning of methods that microsome = ER, I think it would make these technical methods easier to follow (e.g. what parts of the cell is being separated during each step).

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Figure 3 – What are the units used for hue?

Table S1 – In my version, the table label is incomplete.

Decision letter (RSPB-2019-0889.R0)

28-May-2019

Dear Dr Hill:

I am writing to inform you that your manuscript RSPB-2019-0889 entitled "Plumage redness signals mitochondrial function in the House Finch" has, in its current form, been rejected for publication in Proceedings B.

This action has been taken on the advice of referees, who have, although with positive attitude overall, 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, which I hope you will find useful. If you do choose to resubmit your manuscript, please upload the following:

1) A 'response to referees' document including details of how you have responded to the comments, and the adjustments you have made.
2) A clean copy of the manuscript and one with 'tracked changes' indicating your 'response to referees' comments document.
3) Line numbers in your main document.

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Sincerely,

Proceedings B
mailto: proceedingsb@royalsociety.org

Associate Editor
Board Member: 1
Comments to Author:
This study addresses a question that is significant, of broad interest: whether there is a functional link between feather coloration, carotenoid oxidation, and mitochondrial respiration. If true, this hypothesis can explain the honesty of red colour signals in this species and others that use carotenoid oxidation to produce red colour ornaments. The paper provides several lines of evidence to support this hypothesis and I agree with both reviewers that the combined evidence is generally compelling. However, the evidence that carotenoid metabolism is functionally linked to mitochondrial respiration is somewhat circumstantial. For example, reviewer 1 points out that structural modeling cannot predict that ketolation is linked to cellular respiration, and co-localistation doesn't conclusively show a functional link. I therefore suggest that you provide a more cautious and nuanced interpretation, and address the detailed reviewer comments regarding interpretation of the data. The structural modelling is valuable but could be better integrated, for example by clearly articulating the hypotheses motivating and/or additional insight provided by structural modelling. Some aspects of the methods require additional clarification and justification. Once these points are addressed, I think this paper will make a very valuable contribution that has the potential to really shift our understanding of how the honesty of carotenoid-based ornaments is enforced.

Reviewer(s)' Comments to Author:

Referee: 1

Comments to the Author(s)
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Author's Response to Decision Letter for (RSPB-2019-0889.R0)

See Appendix A.

RSPB-2019-1354.R0

Review form: Reviewer 2

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?
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.
No

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Is it adequate?
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Do you have any ethical concerns with this paper?
No

Comments to the Author
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Decision letter (RSPB-2019-1354.R0)

21-Aug-2019

Dear Dr Hill

I am pleased to inform you that your Review manuscript RSPB-2019-1354 entitled "Plumage redness signals mitochondrial function in the House Finch" has been accepted for publication in Proceedings B.

The referee and the Associate Editor do not recommend any further changes. Therefore, please proof-read your manuscript carefully and upload your final files for publication. 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 me know immediately.

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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. Please note that PowerPoint files are not accepted.

3) Electronic supplementary material: this should be contained in a separate file from the main text and the file name should contain the author's name and journal name, e.g authorname_procb_ESM_figures.pdf
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 see: https://royalsociety.org/journals/authors/author-guidelines/

4) Data-Sharing and data citation
It is a condition of publication that data supporting your paper are made available. Data should be made available either in the electronic supplementary material or through an appropriate repository. Details of how to access data should be included in your paper. Please see https://royalsociety.org/journals/ethics-policies/data-sharing-mining/ for more details.

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

Sincerely,

Professor Hans Heesterbeek
mailto:proceedingsb@royalsociety.org

Associate Editor
Comments to Author:
The revised manuscript addresses the reviewer concerns well and will make a very nice (potentially transformative) contribution to the field. Ironically, being more careful in the wording regarding what we know about CYP2J19 has underscored the value of the structural modelling, which is now better integrated. I hope this paper stimulates research into the functional link between red ornamental colours and mitochondrial performance - it would be fascinating to know whether this link extends to other taxa.

Reviewer(s)' Comments to Author:

Referee: 2

Comments to the Author(s).
In general, authors revised the manuscript in response to comments, and am okay with recommending acceptance. One of my original concerns was whether the structural modelling of CYP2J19 fit well in the paper. The way authors revised the wording to be more cautious about what the modelling demonstrates—and acknowledging more work would need to be done to show that the enzyme is indeed found in the IMM—makes the connection between the biochemistry and modelling clearer.

Decision letter (RSPB-2019-1354.R1)

30-Aug-2019

Dear Dr Hill

I am pleased to inform you that your manuscript RSPB-2019-1354.R1 entitled "Plumage redness signals mitochondrial function in the house finch" has been accepted for publication in Proceedings B, subject to some final revision.

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

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

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

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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.

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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.

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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,

Professor Hans Heesterbeek
Editor, Proceedings B
mailto:proceedingsb@royalsociety.org

Associate Editor:
Board Member
Comments to Author:
The manuscript has been shortened so I read it thoroughly again and picked up a couple of things that I really should have picked up in the previous version. I apologise for this. Please add the sample size for the number of birds for which you measured plumage colour (i.e. in the multiple regression) - in both sections b and g of the methods. Although it says in section c of the methods that 91 birds were used in the study, looking at figure 4 and the model degrees of freedom, only a subset of these had colour data. Also, only the best fit model (i.e. following backwards model selection) should be interpreted (given the sample size and number of predictors, the full model is overspecified). This is a simple matter of deleting a few phrases, which I have marked up in the attached pdf, so the changes are very minor. Thank-you for your understanding regarding these final changes.

Decision letter (RSPB-2019-1354.R2)

03-Sep-2019

Dear Dr Hill

I am pleased to inform you that your manuscript entitled "Plumage redness signals mitochondrial function in the house finch" 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.

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Electronic supplementary material:
All supplementary materials accompanying an accepted article will be treated as in their final form. They will be published alongside the paper on the journal website and posted on the online figshare repository. Files on figshare will be made available approximately one week before the accompanying article so that the supplementary material can be attributed a unique DOI.

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

Sincerely,

Editor, Proceedings B
mailto: proceedingsb@royalsociety.org

Appendix A

Associate Editor
Board Member: 1
Comments to Author:
This study addresses a question that is significant, of broad interest: whether there is a functional link between feather coloration, carotenoid oxidation, and mitochondrial respiration. If true, this hypothesis can explain the honesty of red colour signals in this species and others that use carotenoid oxidation to produce red colour ornaments. The paper provides several lines of evidence to support this hypothesis and I agree with both reviewers that the combined evidence is generally compelling. However, the evidence that carotenoid metabolism is functionally linked to mitochondrial respiration is somewhat circumstantial. For example, reviewer 1 points out that structural modeling cannot predict that ketolation is linked to cellular respiration, and co-localistation doesn't conclusively show a functional link. I therefore suggest that you provide a more cautious and nuanced interpretation, and address the detailed reviewer comments regarding interpretation of the data. The structural modelling is valuable but could be better integrated, for example by clearly articulating the hypotheses motivating and/or additional insight provided by structural modelling. Some aspects of the methods require additional clarification and justification. Once these points are addressed, I think this paper will make a very valuable contribution that has the potential to really shift our understanding of how the honesty of carotenoid-based ornaments is enforced.

In revising the paper, we have changed wording to more cautiously express what our data actually say and what they may imply about connections between OXPHOS and production of red pigments. In making these changes, we feel that we have shown the value of the biochemical model and better integrated it into the paper. Our specific changes are outlined below.

Reviewer(s)' Comments to Author:

Referee: 1

Comments to the Author(s)
The red feathers of the house finch are a textbook example in signaling theory. However, it is still controversial which mechanism links ornamentation to condition. The color-defining red keto-carotenoids are produced from yellow dietary precursor carotenoids. The ketolase enzyme has been identified as CypP2J19 by genetic means. The present study tests whther keto-carotenoid production is linked to mitochondrial respiration. The study shows that keto-carotenoids accumulate in the inner mitochondrial membrane (IMM). Furthermore, the redness of feathers of wild-caught molting male finches was found to be positively correlated with the respiratory control ratio of isolated hepatic mitochondria from the animals. From these findings, the authors concluded that the redness of the feathers represents an honest signal of condition because mitochondrial functioning, similar to color ornamentation, is well correlated with survival rate and reproductive success in this bird species.

This study is well conduced and follows an interesting hypothesis. It provides evidence for an as yet not appreciated connection between carotenoid metabolism and mitochondrial functioning. However, this reviewer raises a few concerns that should be addressed prior to publication.

Concerns:
Line 28: The sentence needs to be revised. Structural modeling cannot predict that ketolation is linked to cellular respiration.

Agreed. We revised the sentence and now we state that the proposed mechanisms to link ketolation to mitochondrial function are hypotheses at this point and the data at hand do not specifically support the particular mechanism in the model.

Additionally, mitochondrial P450 enzymes receive electrons from reduced nicotinamide adenine dinucleotide (NADPH) via the intermediacy of two proteins—ferredoxin reductase (a flavoprotein) and ferredoxin (an iron/sulfur protein). Thus, there is only a distant link between respiration and ketolation.

The prevailing dogma, based on characterized mitochondrial P450 enzymes, is that they receive electrons from NADPH via intermediates (ferredoxin reductase/ ferredoxin). However, it is too early in our opinion to speculate about the specifics of the electron donor for CYP2J19, as it is not closely related to known mitochondrial P450 enzymes.
However, the fact that electrons likely reach CYP2J19 from NAD(P)H through intermediates does not in our opinion effect our hypothesis that the carotenoid conversion of this enzyme maybe serve as a link between mitochondrial performance and signaling. A lack of NAD(P)H due to poorly performing mitochondria would still reduce the flow of electrons to CYP2J19 and thus the rate of carotenoid conversion, even if this occurs through intermediates.

Line 293: Carotenoid accumulation at the IMM has been previously reported in mammals (see, Ref. 38,39). These studies showed that carotenoid metabolizing enzyme BCO2 localizes to the IMM. Interestingly, the accumulation of carotenoids was linked to adverse health effects in the previous study. It would be worthwhile to discuss these studies in light of the present findings.

We agree with the reviewer that this is interesting and potentially of health relevance and we have revised the text accordingly.

Line 358: Control of energy homeostasis is complex. The liver is a metabolic buffer, ensuring that circulating glucose and TG are neither too high nor too low. It also is critical for the interconversion of metabolites. Muscles are end organs that consume energy. One cannot compare hepatic and muscle mitochondrial physiology.

This is a good point. We have removed this paragraph.

Line 363 f. Does CYP2J19 localize to the inner membrane of the mitochondria? A demonstration that CYP2J19 resides at IMM would corroborate the conclusions of the paper. Is there any evidence from other studies that mitochondrial P450 enzyme activity is affected by mitochondrial condition?

We do not know whether or not CYP2J19 localizes in the IMM, but it is clearly a critical piece of information. This question is challenging to address and we need funding to do this. Our approach is to proceed one step at a time. Here, we show a link between mitochondrial function and redness. Hopefully this gives us the foundation to compete for grant money to move to the next step, which will include a detailed study of the site of ketolation.

Line 396 ff. Can the authors exclude that the expression level and/or genetic variability modulate the activity of the CYP2J19 enzyme? These parameters would also explain the observed color differences.

We do not see variation in gene expression levels or genetic variability as alternatives to the general hypothesis that redness signals mitochondrial function. We think that is likely that gene expression of enzymes like SRB1 and BC02 modulate the activity of CYP2J19, in response to the functionality of the mitochondrion. It would be surprising if there were not such feedback networks. With the publication of this paper, we are hopeful that scientists interested in carotenoid signaling will agree that a functional genomics study would complement the current important but correlative study.

Minor concerns:
Line337: PGC1-a needs to be replaced by PGC-1alpha.

Corrected.

Line338: associated with mitochondrial remodeling.

Corrected.

Legend Fig. 2 PGC-□ needs to be replaced with PGC-1alpha.

We believe this is a conversion issue of the journal's system.

Referee: 2

Comments to the Author(s)
The authors present a study connecting variation in mitochondrial function, plumage differences based on carotenoid pigments, and a putative candidate gene connecting both. More specifically, the paper suggests a molecular model linking red plumage hue to mitochondrial function by providing evidence that red ketocarotenoids are found in the inner

mitochondrial membrane (IMM) of mitochondria, and that variation in red plumage hue is associated with certain aspects of mitochondrial activity. Understanding the genetics and evolution of carotenoid pigment processing in this case is timely, given the molecular basis of processing these molecules in vertebrates is poorly characterized. Moreover, the authors highlight the fact that carotenoids have been intensely studied in birds as a possible "honest signal" of quality, although how carotenoids might be connected to variation in physiological capacity (i.e. "quality") has been unclear. The current study tests whether this may directly signal differences in mitochondrial efficiency, and and uses data from house finches to show that carotenoids are found directly in the mitochondria, and differences in carotenoid "redness" is associated with mitochondrial respiration.

Their main findings are: 1) while carotenoids have been previously shown to be found in the mitochondria of birds (unlike other vertebrates, which actively avoid sequestering carotenoids in mitochondria), this study was able to localize it to the IMM. 2) That certain aspects of mitochondrial respiration are associated with differences in hue, presenting a functional link between mitochondrial function and plumage color. 3) Using biochemical modelling of a putative ketolation enzyme (CYP2J19), the authors present additional evidence a) that this enzyme has the shape and characteristics of one that would be predicted to interact with carotenoid molecules, and b) that it may be also be directly associated with the mitochondria. I found result #2 was the most important, while #1 seemed like an incremental improvement over existing knowledge and #3 somewhat tangential to the main thrust of the paper (see below).

While I found that the paper was generally well-written, with a good flow, my main concern—particularly for the broad readership of Proc B—is how technical some of the details of the biochemical methods and modelling are. This is especially true when trying to explain how the importance of different complexes of mitochondrial function and respiration may play a role in carotenoid ketolation (see comment below for lines 354 – 361). In addition, it really was not clear until the discussion the central hypotheses motivating the in silico biochemical modelling of CYP2J19. I think the discussion could be improved by clarifying these specific aspects of mitochondrial biochemistry, and length could be cut down by moving some of the technical parts of the methods into a supplement.

Other major points:
- It was not clear, based on the description, whether the birds were still molting? Therefore, have they deposited all of the carotenoids for their prebasic molt? I don't think this would necessarily alter the conclusions of the study, but it does complicate things a bit if there is unresolved temporal variation.

In our original version, we stated that all birds included in this study were molting males. The fact that the reviewer missed our statement indicates that we did not make the point clearly enough. So, we have added this statement to the methods:

"It was crucial to use birds in the process of molt and hence actively engaged in the production of red feather pigments, given this allowed us to match the current physiological state of birds to ornamentation that was actively being produced."

- I generally thought it would be more common (and much more objective) to use a spectrophotometer to measure hue? Was there a strong reason why the manual inspection and quantification with Photoshop was desirable in this case? Also, while it is unlikely to alter the main conclusions, many authors of avian coloration impute their color data with TetraColorSpace to account for the differences in the avian visual spectrum.

It is well established that all diurnal birds have very good perception of colors in the yellow/orange/red portion of the spectrum, so there was no point to using visual models in this study. The point about measuring color from photos rather than with a spectrometer is important. Over the last 20 years, Hill and his students have published over 50 papers using every technique for measuring coloration of feathers, writing methods papers on color measurement, and editing the most widely cited synthesis paper on color measurement. For this study, we chose to use digital photos because it was the tool that was most appropriate for the job. Some of the molting males used in this study had grown only scattered colored feathers. The human eye could see the color of growing feathers and digital camera images captured such coloration, but there was no colored region large enough to allow for accurate measurement with a spectrometer. In our experience it takes at least a dozen colored feathers in patch to produce a colored area large enough for accurate spectrometer readings. With digital photos we were able to get accurate color measurements from all birds. The validity, repeatability, and accuracy of digital images for color quantification has been documented in recent papers, which we now cite.

McKay, B.D., 2013. The use of digital photography in systematics. *Biological Journal of the Linnean Society*, *110*(1), pp.1-13.

Hill GE, Hood WR, Huggins K. 2009 A multifactorial test of the effects of carotenoid access, food intake and parasite load on the production of ornamental feathers and bill coloration in American goldfinches. J. Exp. Biol. 212. (doi:10.1242/jeb.026963)

- I think it would be useful to include some example pictures from birds sampled from different parts of the hue spectrum, so that readers have an idea the range that is being assayed. This also be useful if other future studies want to use similar methods for measuring color.

We think this is an excellent suggestion. We have added a new supplementary figure of two molting male house finches showing the range of color variation in this study.

More generally, I found the modelling of CYP2J19 not particularly well integrated with the rest of the study. For example, it is not altogether clear the supposed 'direct link' of this protein to the mitochondria (lines 384-385). Obviously it is non-trivial to provide this kind of molecular

evidence, but that seems to me the most direct evidence of this molecules localization to the mitochondria. Moreover, why in the case the strict focus on CYP2J19, as opposed to other recently described carotenoid processing enzymes (e.g. scavenger receptors or BCOs?). While I am not convinced that this modelling piece is the most appropriate complement to the rest of the paper, I will leave it to the editor to decide whether it is desirable to keep this finding within the main text of the manuscript.

The purpose of the structural model is to establish the plausibility of a link between mitochondrial function and red pigmentation. We now state this explicitly. We would make a case to keep the modeling because it is additional support for the study's findings and some readers need to see a plausible mechanism before they will begin to think about the empirical observation. The functional model is a hypothesis and future research will surely reveal the actual mechanisms that link redness to mitochondrial function.

We are beyond a list of candidate enzymes for carotenoid ketolation in birds. CYP2J19 has been shown to be an enzyme that birds and turtles use to convert yellow carotenoids to red carotenoids for both integumentary coloration and for oil droplets in the retina—hence we focused on CYP2J19 instead of other enzymes. We now cite the papers that establish CYP2J19 as the enzyme used for ketolation by birds and turtles.

More specific points:

Line 29 – Should be "as a signal" instead of "signal".

Change made.

Line 30 – Not sure if "inexorably" is the right word here.

Two synonyms for inexorable are: "unyielding; unalterable". That is precisely the type of connection to which we are referring. Irrespective of whether mitochondrial function controls carotenoid ketolation through direct or indirect mechanisms, this relationship is uncheatable.

Line 87- Has it been verified that putting a bird in a paper bag is not stressful? Holding birds in bags is used as a standard stress inducer in a number of stress studies. While I doubt it will affect the results—presumably birds were treated similarly—I don't think it is necessarily appropriate to suggest that these were not stressful conditions. I would suggest that either additional evidence is used to support this statement, or the statement is tempered. I also think including some verification that birds held for shortest/longest time did not show the most extreme values of mitochondrial performance.

Hill actually titled his first book "A red bird in a brown bag" because of the significance of brown paper bags in this research program. Paper bags have flat bottoms and actually form a small chamber in which House Finches can stand comfortably. Birds cannot see danger so they tend

to settle down rapidly and will eat in bags. The bags used in stress tests are cloth bags which hold birds in awkward positions, often upside down. In such cloth bags, birds struggle persistently. We cite the book "A red bird in a brown bag" for statements supporting the idea that brown bags provide a relatively low stress environment.

Line 109 – "Tissue collection" methods – how were the birds pooled together? Randomly? By plumage color?

It was challenging for us to capture seven molting males on a particular morning. All captured males that met our criteria were added to the pool, and we stopped trapping when a complete pool was formed. Thus, the collection wasn't technically random—maybe best described as haphazard. We have now added these details to the methods.

Line 148 - Not clear here how this is a "conservative method"; maybe be more explicit about this point?

The word conservative was removed.

Line 153 – IMM and OMM should be put in parenthesis the first time the full names are used (line 149-150).

Corrected.

Line 154-159 – This is a very interesting point. It could also be useful to say from the beginning of methods that microsome = ER, I think it would make these technical methods easier to follow (e.g. what parts of the cell is being separated during each step).

We added the sentence: "The microsome fraction was comprised primarily of fragments of endoplasmic reticulum (ER)."

Line 178 – Add "respiratory control ratio" when using RCR for the first time.

Corrected.

Line 280 - Cite and outline previous results from Mayne and Parker 1986?

Mayne and Parker is a study of location of beta-carotene in subcellular fractions of chicken. We did not find an appropriate place to cite this paper.

Line 314,333 – Should be Table S1, not Table 1

The Table will be in the main document.

Line 338 - Should be "associated *with* mitochondrial…"

Corrected.

Line 343 – “is has” should be “has”

Corrected.

Line 354 – 361 – Is glucose the primary substrate for complex I, while fatty acids are for complex 2, and is this why you are suggesting this relationship between complexes and possible migration vs. resident birds? If so, saying that explicitly would enhance this paragraph. It can be difficult to keep track of these details.

We removed this paragraph.

Line 384-385 - Is this really a ‘direct link’ to mitochondrial performance? Also, how does the structural modelling specifically suggest it is anchored to the IMM?

The structural modelling doesn’t show that CYP2J19 is specifically anchored to the IMM, simply that it is likely to be associated with a cellular membrane. This statement was poorly phrased and has been removed from the current version of the manuscript.

Line 399 - “Consistent with plumage as a indicator…”

Corrected.

Figure 3 – What are the units used for hue?

Hue is a unitless measure. It is a position on a 360 hue range.

Table S1 – In my version, the table label is incomplete.
Table has now been fixed.

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Abstract: Carotenoid coloration is widely recognized as a signal of individual condition in various animals, but despite decades of study, the mechanisms that link carotenoid coloration to condition remain unresolved. Most birds with red feathers convert yellow dietary carotenoids to red carotenoids in an oxidation process requiring the cytochrome P450 enzyme CYP2J19. Here, we tested the hypothesis that the process of carotenoid oxidation and feather pigmentation is functionally linked to mitochondrial performance. Consistent with this hypothesis, we observed high levels of red ketolated carotenoids associated with the hepatic mitochondria of molting wild house finches (Haemorhous mexicanus), and upon fractionation, we found the highest concentration of ketolated carotenoids in the inner mitochondrial membrane. We further found that the redness of growing feathers was positively related to the performance of liver mitochondria. Structural modeling of CYP2J19 further supports the hypothesis that ketolation is functionally linked to cellular respiration. These observations suggest that feather coloration serves a signal of core functionality through inexorably links to cellular respiration in the mitochondria.
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