Migrating birds rapidly increase constitutive immune function during stopover

Cas Eikenaar, Sven Hessler and Arne Hegemann

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Original submission: 20 November 2019
Revised submission: 20 December 2019
Final acceptance: 6 January 2020

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

Note: This manuscript was transferred from another Royal Society journal with peer review.

Review History

RSOS-192031.R0 (Original submission)

Review form: Reviewer 1 (Camila Gómez)

Is the manuscript scientifically sound in its present form?
Yes

Are the interpretations and conclusions justified by the results?
Yes

Is the language acceptable?
Yes

Do you have any ethical concerns with this paper?
No

Have you any concerns about statistical analyses in this paper?
No

Recommendation?
Accept with minor revision (please list in comments)
Comments to the Author(s)

This manuscript presents very interesting data on recovery and activation of the immune system in a migratory species on stopover. They have addressed well the previous reviewer's comments and their manuscript has improved even more as a result. I only had very minor observations that authors can address easily.

L44 – Why (novel) in parenthesis?
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L161 – Please add a short sentence saying that you could not test for age effects.
Supplementary data - Please add a column for sex and one for age in the supplementary material.

Review form: Reviewer 2 (Nicholas P. C. Horrocks)

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Yes

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Comments to the Author(s)

This study describes the ability of a long-distance migrant to rapidly increase constitutive innate immunity during a stopover with ad libitum food. The study is well written and clearly laid out. It adds to our knowledge of the role that stopovers play in the ecology and physiology of migrating birds as well as emphasising how immune function is a dynamic process that can change over quite short time periods.

I have been asked to review this manuscript for a second time (previously for Biology Letters), so I only have minor comments that relate to improving the clarity, and perhaps making use of the fact that the longer format of RSOS compared to Biology Letters allows for a more comprehensive presentation and discussion of the results. Thank you for addressing so thoroughly all the comments and suggestions that I made in my previous review of your manuscript. I hope you find these additional comments useful.
L37 - Change to “Here, we show that…”

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At L76 you say that stopover migrants might prefer to use CIF because they are keen on avoiding immune responses, but CIF is a form of immune response/defence. I think you mean that stopover migrants might not want to use induced or adaptive immune responses, because these take longer to develop, and in the case of induced innate immune defences, are energetically costly?

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L96 – Perhaps consider adding in a sentence here to make it clear how long you held birds for. Otherwise it feels a little like you dive straight into the methods. Something like “We captured migrating wheatears at stopover and held them for three days in captivity (which mimics the average stop-over time of this species at our study site), to assess whether they could boost CIF during this time. Each bird was blood-sampled…” Currently the text reads a bit like you are still in Biology Letters format, with minimal space for providing more thorough details. I think you can afford to include a few more words now. I also think it is worth to state upfront that the three days in captivity matches a typical stop-over period for this species. I realise that you do this at L187-190 (and should continue to do so – thanks for making that change from the previous version), but I think including it in brief detail early on helps reassure the reader that your study has direct biological relevance to stopover behaviour in this species.

L101 – I think it would be beneficial to finish with some predictions here, just to remind the reader what it is you are aiming to test with your study. Something along the lines of “If migratory wheatears are able to boost CIF during stop-over, then this predicts that CIF should be higher at day three than at day one, whereas if wheatears are unable to boost CIF, we would expect no difference in levels between the start and end of captivity.”

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L181-182 – This point about encountering novel pathogens seems particularly relevant to young birds that have not previously migrated. Given that your sample included mostly first year birds, do you think you would have found similar results if you had only captured adult birds?

Your sample sizes may not permit it, but I wondered if it might be informative to test for an age effect (first year vs adult birds) on change in immune parameters? Perhaps older birds are more experienced which might affect their flight performance and thus physical state when captured, which could in turn affect ability to upregulate CIF? The fact that you found no effect of food
intake or changes in fuel stores would suggest this is not the case, but it could be interesting to check, just in case.

Figure 1 - This figure really clearly illustrates your results. One striking thing is the result for microbial killing capacity. You can almost completely split the data here into two groups. Those birds that had 100% killing ability at day 1 maintained it at day 3. Perhaps these individuals can be considered as ‘high quality’ in some way? For most other individuals, killing capacity at day 1 was very low – less than 20%. Yet at day 3 it had shot up to greater than 80%. This seems like a dramatic change, and worthy of more discussion, especially when contrasted with the results for lysis and agglutination, where there was much more variation. Is there something specific about microbial killing capacity that makes it more important to up-regulate than lysis or agglutination? Or is this a cheaper form of CIF, so can be more easily up-regulated? Given that you are now not word count-limited, you might consider elaborating on your results a bit more in the discussion.

Supplementary data – Please include sex and age data in your Excel file.

Decision letter (RSOS-192031.R0)

17-Dec-2019

Dear Dr Eikenaar

On behalf of the Editors, I am pleased to inform you that your Manuscript RSOS-192031 entitled "Migrating birds rapidly increase constitutive immune function during stopover" has been accepted for publication in Royal Society Open Science subject to minor revision in accordance with the referee suggestions. Please find the referees' comments at the end of this email.

The reviewers and handling editors have recommended publication, but also suggest some minor revisions to your manuscript. Therefore, I invite you to respond to the comments and revise your manuscript.

• Ethics statement
  If your study uses humans or animals please include details of the ethical approval received, including the name of the committee that granted approval. For human studies please also detail whether informed consent was obtained. For field studies on animals please include details of all permissions, licences and/or approvals granted to carry out the fieldwork.

• Data accessibility
  It is a condition of publication that all supporting data are made available either as supplementary information or preferably in a suitable permanent repository. The data accessibility section should state where the article's supporting data can be accessed. This section should also include details, where possible of where to access other relevant research materials such as statistical tools, protocols, software etc can be accessed. If the data has been deposited in an external repository this section should list the database, accession number and link to the DOI for all data from the article that has been made publicly available. Data sets that have been deposited in an external repository and have a DOI should also be appropriately cited in the manuscript and included in the reference list.

If you wish to submit your supporting data or code to Dryad (http://datadryad.org/), or modify your current submission to dryad, please use the following link: http://datadryad.org/submit?journalID=RSOS&manu=RSOS-192031
• Competing interests
Please declare any financial or non-financial competing interests, or state that you have no competing interests.

• Authors' contributions
All submissions, other than those with a single author, must include an Authors’ Contributions section which individually lists the specific contribution of each author. The list of Authors should meet all of the following criteria; 1) substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data; 2) drafting the article or revising it critically for important intellectual content; and 3) final approval of the version to be published.

All contributors who do not meet all of these criteria should be included in the acknowledgements.

We suggest the following format:
AB carried out the molecular lab work, participated in data analysis, carried out sequence alignments, participated in the design of the study and drafted the manuscript; CD carried out the statistical analyses; EF collected field data; GH conceived of the study, designed the study, coordinated the study and helped draft the manuscript. All authors gave final approval for publication.

• Acknowledgements
Please acknowledge anyone who contributed to the study but did not meet the authorship criteria.

• Funding statement
Please list the source of funding for each author.

Please ensure you have prepared your revision in accordance with the guidance at https://royalsociety.org/journals/authors/author-guidelines/ -- please note that we cannot publish your manuscript without the end statements. We have included a screenshot example of the end statements for reference. If you feel that a given heading is not relevant to your paper, please nevertheless include the heading and explicitly state that it is not relevant to your work.

Because the schedule for publication is very tight, it is a condition of publication that you submit the revised version of your manuscript before 26-Dec-2019. Please note that the revision deadline will expire at 00.00am on this date. If you do not think you will be able to meet this date please let me know immediately.

To revise your manuscript, log into https://mc.manuscriptcentral.com/rsos and enter your Author Centre, where you will find your manuscript title listed under "Manuscripts with Decisions". Under "Actions," click on "Create 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 referees and upload a file "Response to Referees" in "Section 6 - File Upload". You can use this to document any changes you make to the original manuscript. In order to expedite the processing of the revised manuscript, please be as specific as possible in your response to the referees. We strongly recommend uploading two versions of your revised manuscript:

1) Identifying all the changes that have been made (for instance, in coloured highlight, in bold text, or tracked changes);
2) A 'clean' version of the new manuscript that incorporates the changes made, but does not highlight them.
When uploading your revised files please make sure that you have:

1) A text file of the manuscript (tex, txt, rtf, docx or doc), references, tables (including captions) and figure captions. Do not upload a PDF as your "Main Document";
2) A separate electronic file of each figure (EPS or print-quality PDF preferred (either format should be produced directly from original creation package), or original software format);
3) Included a 100 word media summary of your paper when requested at submission. Please ensure you have entered correct contact details (email, institution and telephone) in your user account;
4) Included the raw data to support the claims made in your paper. You can either include your data as electronic supplementary material or upload to a repository and include the relevant doi within your manuscript. Make sure it is clear in your data accessibility statement how the data can be accessed;
5) All supplementary materials accompanying an accepted article will be treated as in their final form. Note that the Royal Society will neither edit nor typeset supplementary material and it will be hosted as provided. Please ensure that the supplementary material includes the paper details where possible (authors, article title, journal name).

Supplementary files will be published alongside the paper on the journal website and posted on the online figshare repository (https://rs.figshare.com/). The heading and legend provided for each supplementary file during the submission process will be used to create the figshare page, so please ensure these are accurate and informative so that your files can be found in searches. 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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If your manuscript is newly submitted and subsequently accepted for publication, you will be asked to pay the article processing charge, unless you request a waiver and this is approved by Royal Society Publishing. You can find out more about the charges at https://royalsocietypublishing.org/rsos/charges. Should you have any queries, please contact openscience@royalsociety.org.

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

Kind regards,
Andrew Dunn
Royal Society Open Science Editorial Office
Royal Society Open Science
openscience@royalsociety.org

on behalf of Prof Kevin Padian (Subject Editor)
openscience@royalsociety.org

Associate Editor Comments to Author:
The reviewers are largely pleased with the changes you have implemented following prior review at one of our sister journals; however, a few recommendations have been made in this version that we'd like you to take into consideration.
Reviewer comments to Author:
Reviewer: 1

Comments to the Author(s)
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Reviewer: 2

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I have been asked to review this manuscript for a second time (previously for Biology Letters), so I only have minor comments that relate to improving the clarity, and perhaps making use of the fact that the longer format of RSOS compared to Biology Letters allows for a more comprehensive presentation and discussion of the results. Thank you for addressing so thoroughly all the comments and suggestions that I made in my previous review of your manuscript. I hope you find these additional comments useful.

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At L76 you say that stopover migrants might prefer to use CIF because they are keen on avoiding immune responses, but CIF is a form of immune response/defence. I think you mean that stopover migrants might not want to use induced or adaptive immune responses, because these take longer to develop, and in the case of induced innate immune defences, are energetically costly?

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Consider changing to “…can negatively affect immune defences.”

Perhaps consider adding in a sentence here to make it clear how long you held birds for. Otherwise it feels a little like you dive straight into the methods. Something like “We captured migrating wheatears at stopover and held them for three days in captivity (which mimics the average stop-over time of this species at our study site), to assess whether they could boost CIF during this time. Each bird was blood-sampled…” Currently the text reads a bit like you are still in Biology Letters format, with minimal space for providing more thorough details. I think you can afford to include a few more words now. I also think it is worth to state upfront that the three days in captivity matches a typical stop-over period for this species. I realise that you do this at L187-190 (and should continue to do so – thanks for making that change from the previous version), but I think including it in brief detail early on helps reassure the reader that your study has direct biological relevance to stopover behaviour in this species.

I think it would be beneficial to finish with some predictions here, just to remind the reader what it is you are aiming to test with your study. Something along the lines of “If migratory wheatears are able to boost CIF during stop-over, then this predicts that CIF should be higher at day three than at day one, whereas if wheatears are unable to boost CIF, we would expect no difference in levels between the start and end of captivity.”

Change to “After capture, birds…”

Thanks for now including this clear explanation.

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Your sample sizes may not permit it, but I wondered if it might be informative to test for an age effect (first year vs adult birds) on change in immune parameters? Perhaps older birds are more experienced which might affect their flight performance and thus physical state when captured, which could in turn affect ability to upregulate CIF? The fact that you found no effect of food intake or changes in fuel stores would suggest this is not the case, but it could be interesting to check, just in case.

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Supplementary data – Please include sex and age data in your Excel file.
Author's Response to Decision Letter for (RSOS-192031.R0)

See Appendix A.

Decision letter (RSOS-192031.R1)

06-Jan-2020

Dear Dr Eikenaar,

It is a pleasure to accept your manuscript entitled "Migrating birds rapidly increase constitutive immune function during stopover" in its current form for publication in Royal Society Open Science. The comments of the reviewer(s) who reviewed your manuscript are included at the foot of this letter.

Please ensure that you send to the editorial office an editable version of your accepted manuscript, and individual files for each figure and table included in your manuscript. You can send these in a zip folder if more convenient. Failure to provide these files may delay the processing of your proof. You may disregard this request if you have already provided these files to the editorial office.

You can expect to receive a proof of your article in the near future. Please contact the editorial office (openscience_proofs@royalsociety.org) and the production office (openscience@royalsociety.org) to let us know if you are likely to be away from e-mail contact -- if you are going to be away, please nominate a co-author (if available) to manage the proofing process, and ensure they are copied into your email to the journal.

Due to rapid publication and an extremely tight schedule, if comments are not received, your paper may experience a delay in publication. Royal Society Open Science operates under a continuous publication model. Your article will be published straight into the next open issue and this will be the final version of the paper. As such, it can be cited immediately by other researchers. As the issue version of your paper will be the only version to be published I would advise you to check your proofs thoroughly as changes cannot be made once the paper is published.

Please see the Royal Society Publishing guidance on how you may share your accepted author manuscript at https://royalsociety.org/journals/ethics-policies/media-embargo/.

Thank you for your fine contribution. On behalf of the Editors of Royal Society Open Science, we look forward to your continued contributions to the Journal.

Kind regards,
Royal Society Open Science Editorial Office
Royal Society Open Science
openscience@royalsociety.org

on behalf of Mr Andrew Dunn (Associate Editor) and Kevin Padian (Subject Editor)
openscience@royalsociety.org
Associate Editor Comments to Author (Mr Andrew Dunn):
Associate Editor: 1
Comments to the Author:
(There are no comments.)

Reviewer comments to Author:

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https://www.facebook.com/RoyalSocietyPublishing.FanPage/
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Dear Editor,

Thank you very much for your positive letter. We were glad to see that the reviewers were content with the changes we made in response to their comments, and for their last few minor comments, all of which we have incorporated in the manuscript.

For clarity we have pasted the comments in bold and our responses in normal format immediately below each comment. Where necessary, page and line numbers in the responses are given to indicate the position of text in the revised manuscript.

Kind regards on behalf of all authors,
Cas Eikenaar

Reviewer: 1

Comments to the Author(s)
This manuscript presents very interesting data on recovery and activation of the immune system in a migratory species on stopover. They have addressed well the previous reviewer’s comments and their manuscript has improved even more as a result. I only had very minor observations that authors can address easily.

Thank you for your supportive words.

L44 – Why (novel) in parenthesis?
We agree that the parentheses are not necessary and removed these.

L70, 77, 85, 177 – Perhaps these lines should be the start of new paragraphs? It might be that something happened when formatting the proofs, but both the introduction and the discussion seem to be one single huge paragraph. Please check and make sure that the text is appropriately formatted with paragraphs.

We agree that for readability, it is better to start a new paragraph at line 70, however, we do not think new paragraphs are appropriate at the other indicated positions (there is no switch in topic there and a new paragraph we believe would break the flow).

L76 – Please check whether the expression ‘birds at stopover’ should be changed to ‘birds on stopover’. This is repeated in various sections throughout the manuscript and I believe that most literature uses ‘on stopover’. I may be wrong.

We are not sure whether one of the two is preferable, but the first author (CE) has always used “at stopover” in his previous papers. Therefore we would like to keep this wording.

L161 – Please add a short sentence saying that you could not test for age effects.
Supplementary data - Please add a column for sex and one for age in the supplementary material.

We have done so: “The limited number of adult birds in our sample precluded testing for an age effect on the changes in CIF.” line 169-170

Sex and age have been added to the supplementary material, thank you for pointing this out.

Reviewer: 2

Comments to the Author(s)
This study describes the ability of a long-distance migrant to rapidly increase constitutive innate immunity during a stopover with ad libitum food. The study is well written and clearly laid out. It adds to our knowledge of the role that stopovers play in the ecology and physiology

Appendix A

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For clarity we have pasted the comments in bold and our responses in normal format immediately below each comment. Where necessary, page and line numbers in the responses are given to indicate the position of text in the revised manuscript.

Kind regards on behalf of all authors,
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of migrating birds as well as emphasising how immune function is a dynamic process that can change over quite short time periods.

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Thank you for your positive words and useful comments.

L37 – Change to “Here, we show that…”

Done.

L74-L77 – I was confused by the terminology used here. Constitutive immunity often is inflammatory, so this doesn’t quite seem to make sense. Perhaps, within the innate immune system you are making the distinction between constitutive innate immunity and induced innate defences (see Lee 2006 Linking immune defenses and life history at the levels of the individual and the species. Integ. Comp. Biol. 46: 1000-1015)? If so, this needs to be made clear.

We agree that an inflammatory immune response is part of the innate immune system. However, with constitutive immune function, we refer to the baseline levels of innate immune function in the absence of an infection. This standing line of innate immune function does not include an inflammatory immune response when a pathogen starts replicating in the body, because an immune response is differently regulated and has different costs and benefits compared to baseline (constitutive) immune function. In particular the immune response includes typical sickness responses such as fever, increased body temperature, lethargy and anorexia, while those are not part of constitutive immune function. Hence it is important to separate those two. We have clarified this in the revision:

“An animal’s immune system includes both constitutive (baseline) defences, the always present immediate first lines of defence, and induced immune responses [5,6]. The main benefit of an improved constitutive immune function is probably increased likelihood of clearing pathogens before they start replicating and establishing themselves inside a host, while an innate immune response is mounted when a pathogen starts replicating and establishing itself in the body. Baseline immune function and immune responses are differently regulated and baseline immune function often does not correlate with the strength of an immune response [7,8]. While immune responses are important to ensure survival, immune responses are energetically costly [9,10], always include self-damage, and often include “opportunity costs”, i.e. lost opportunities in life-history, resulting in fitness costs [10-13]. Hence, a stronger constitutive (baseline) immune function (CIF) should be favoured in order to avoid mounting an actual immune response.” lines 71-81.

At L76 you say that stopover migrants might prefer to use CIF because they are keen on avoiding immune responses, but CIF is a form of immune response/defence. I think you mean that stopover migrants might not want to use induced or adaptive immune responses, because these take longer to develop, and in the case of induced innate immune defences, are energetically costly?

CIF indeed is a form of immune defense, but it does not include an immune response. The birds can thus use CIF to prevent having to mount an immune response. Also see our response above.

L78 – The context of racing with pigeons might be a bit lost to the reader here, unless they are familiar with the paper. Consider changing to something like “…although intensive flight during races does not affect CIF in non-migratory racing pigeons…”

We agree and thank you for this suggestion, which we have followed.

L92 – Consider changing to “…can negatively affect immune defences.”

A good idea, the word ‘negatively’ has been inserted
L96 – Perhaps consider adding in a sentence here to make it clear how long you held birds for. Otherwise it feels a little like you dive straight into the methods. Something like “We captured migrating wheatears at stopover and held them for three days in captivity (which mimics the average stop-over time of this species at our study site), to assess whether they could boost CIF during this time. Each bird was blood-sampled...” Currently the text reads a bit like you are still in Biology Letters format, with minimal space for providing more thorough details. I think you can afford to include a few more words now. I also think it is worth to state upfront that the three days in captivity matches a typical stop-over period for this species. I realise that you do this at L187-190 (and should continue to do so – thanks for making that change from the previous version), but I think including it in brief detail early on helps reassure the reader that your study has direct biological relevance to stopover behaviour in this species.

This is a good point and we agree that it helps the reader to already in the Introduction better understand the design of our study. Thank you! We have added the following sentence: "We captured wheatears at stopover and held them in captivity for three days (matching stopover durations of this species [25,26]), and assessed whether they can boost CIF during this time." Lines 102-104

L101 – I think it would be beneficial to finish with some predictions here, just to remind the reader what it is you are aiming to test with your study. Something along the lines of “If migratory wheatears are able to boost CIF during stop-over, then this predicts that CIF should be higher at day three than at day one, whereas if wheatears are unable to boost CIF, we would expect no difference in levels between the start and end of captivity.”

We normally are quite keen on predictions at the end of the Introduction, but in this instance we really did not know what to expect when we started the study and we do not want to make predictions afterwards as this is unethical. Also, with the addition of the sentence containing “…and assessed whether they can boost CIF during this time” (see comment and response above), we think it is clear what we are testing.

L106 – Change to “After capture, birds…”

Done.

L124-126 – Thanks for now including this clear explanation.

You are welcome! It helped improving the manuscript.

L169 – Change to “…to substantiate this hypothesis have, until now, been lacking.”

Done.

L171-172 – Again, I would encourage greater clarity in terminology here. What do you mean by “better CIF and immune responses”? CIF is a type of immune response.

No, CIF is not a type of immune response, please also see our responses to the very similar comments above. Do note that we have changed the ‘better CIF’ to ‘stronger CIF’, as that is more appropriate.

L181-182 – This point about encountering novel pathogens seems particularly relevant to young birds that have not previously migrated. Given that your sample included mostly first year birds, do you think you would have found similar results if you had only captured adult birds?

Yes, we do think so as passerine migrants are not stopover-site faithful, and thus may encounter novel pathogens during migrations as adults too.

Your sample sizes may not permit it, but I wondered if it might be informative to test for an age effect (first year vs adult birds) on change in immune parameters? Perhaps older birds are more experienced which might affect their flight performance and thus physical state when captured, which could in turn affect ability to upregulate CIF? The fact that you found no effect of food intake or changes in fuel stores would suggest this is not the case, but it could be
interesting to check, just in case.

Indeed, with only 8 adult birds, our sample size does not allow for a proper test of an age effect. We now point this out in the revised manuscript: “The limited number of adult birds in our sample precluded testing for an age effect on the changes in CIF.” line 169-170

(FYI, when eye-balling the data, there is no hint whatsoever for a potential age effect.)

**Figure 1** – This figure really clearly illustrates your results. One striking thing is the result for microbial killing capacity. You can almost completely split the data here into two groups. Those birds that had 100% killing ability at day 1 maintained it at day 3. Perhaps these individuals can be considered as ‘high quality’ in some way? For most other individuals, killing capacity at day 1 was very low – less than 20%. Yet at day 3 it had shot up to greater than 80%. This seems like a dramatic change, and worthy of more discussion, especially when contrasted with the results for lysis and agglutination, where there was much more variation. Is there something specific about microbial killing capacity that makes it more important to up-regulate than lysis or agglutination? Or is this a cheaper form of CIF, so can be more easily up-regulated? Given that you are now not word count-limited, you might consider elaborating on your results a bit more in the discussion.

We agree that this is an interesting pattern. The reason for a stronger increase in BKA compared to lysis and agglutination could be that the bacteria killing capacity is a broader, more integrative measure compared to the other two parameters. With other words, while complement activity (lysis titer) and natural antibody titers (agglutination titer) reflect single parameters, BKA captures several sub-parameters of innate immune function simultaneously and the data suggest that some parameters can be upregulated faster than complement activity and natural antibodies titers respectively. This could include for example certain proteins or white blood cell types. We have included a short discussion in the revision:

“Interestingly, for many individuals the increase in BKA appears stronger than the increase in lysis. This is probably because the bacteria killing capacity is a broad, integrative measure of innate immune function [32,53,54]. In contrast, complement activity (lysis titer) and natural antibody titers (agglutination titer) reflect more tailored parameters [34, and references therein]. Our data suggest that some parameters (captured in the BKA) might be upregulated to a stronger degree in short time frames than complement activity and natural antibodies titers respectively.” lines 194-200

**Supplementary data** – Please include sex and age data in your Excel file.

Sex and age have been added to the supplementary material, thank you for pointing this out.