

Cellular and immunological mechanisms influence host-adapted phenotypes in a vector-borne microparasite

Yi-Pin Lin, Danielle M. Tufts, Matthew Combs, Alan P. Dupuis II, Ashley L. Marcinkiewicz, Andrew D. Hirsbrunner, Alexander J. Diaz, Jessica L. Stout, Anna M. Blom, Klemen Strle, April D. Davis, Laura D. Kramer, Sergios-Orestis Kolokotronis and Maria A. Diuk-Wasser

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Original submission:	1 February 2021
1st revised submission:	20 September 2021
2nd revised submission:	8 January 2022
Final acceptance:	18 January 2022

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

Review History

RSPB-2021-0162.R0 (Original submission)

Review form: Reviewer 1

Recommendation

Major revision is needed (please make suggestions in comments)

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

Excellent

General interest: Is the paper of sufficient general interest?

Good

Quality of the paper: Is the overall quality of the paper suitable?

Good

Is the length of the paper justified?

Yes

Should the paper be seen by a specialist statistical reviewer?

No

Do you have any concerns about statistical analyses in this paper? If so, please specify them explicitly in your report.

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 study by Lin et al. examines the host specific infection and response to three different pathogenic strains of *Borrelia burgdorferi* to determine if there is molecular evidence of host adaptation. There are several important and valuable experimental results presented here that detail the disease progression of different strains through time in the different experimental groups. The overarching motivation for this work is to better understand the mechanisms involved in producing and maintaining such high diversity of *B. burgdorferi* genotype diversity with respect to the outer surface protein C and 16S rRNA strain typing. Two mechanisms of balancing selection have been proposed by researchers to explain genotype diversity in *B. burgdorferi*. The authors here were seeking evidence for one of these hypotheses, multiple niche polymorphism, by determining if there was evidence for host specificity. This is in contrast to the neutral processes and lack of host specificity in negative frequency mechanisms. These ideas have been debated for over a decade now and this was a novel approach to addressing these questions but the manuscript fails to be clear what hypothesis the data support and how. I would like to see more synthesis of all the different experimental results to be able to develop a cohesive picture of what the different results show us. For instance, the cell adhesion results, cytokine responses, and spirochete burden data should be interpreted collectively. There certainly does seem to be concordance in the different experiments but it would be good for the authors do the leg work for the reader.

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Additional comments:

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Format consistently: Italicize ospC throughout

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isolates in the different hosts while the third does not. All three isolates were transmitted by the different hosts to Ixodes ticks. Thus, in my view the results clearly show that both, birds and rodents, can serve as reservoir hosts for the all the investigated *Borrelia* isolates (albeit with different efficiencies for two isolates, not the third).

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differed qualitatively, for example whether the antibody responses were against different antigens. I would suggest using Western Blot analyses to further explore this interesting finding. Other issues:

- a) Line 50: The first sentence in the introduction starts „The host ranges of parasite infection is pivotal...“. I do not understand what it refers to? Does it mean host ranges of parasites? Host ranges that parasites are able to infect? Please rephrase.
 - b) Lines 70/71: the authors refer to ospC and RST types but it has also been shown that different multilocus sequence types may have preferences for different hosts. Pls include appropriate references.
 - c) Lines 77 – 88: While it may be the case that OspC has been used as marker in the USA, this is not generally the case and studies in other *Borrelia* species have not shown the pattern that is described here (e.g. Durand et al. 2017, Scientific Reports; Raberg et al. 2017, J. Evol. Biol). The references given in line 86 (23-25) do not refer to theoretical studies of OspC diversity. Pls explain or rephrase.
 - d) Line 90: „strains with variable ospC genotypes“ – does this refer to strains that are able to change their ospC?
 - e) Line 91: „We identified genotypic differences...“ – was it not phenotypic differences? The genetics of the isolates was not investigated.
 - f) In line 103, perhaps you could add whether that was investigated by PCR or microscopy.
 - g) Line 109: „... established infection selectively...“ may be true for 297 and cN40 but not for B31-5A4?
 - h) The results described in lines 133-137 are unclear. It says that spirochetes transmission from host to feeding larvae was assessed but then it further states that unfed *I. scapularis* nymphs were allowed to feed until repletion. Pls clarify what was done. See also line 142 where it says that robin-derived larvae were below the detection limitresulting inpost nymph feeding? Please correct and rephrase this and adjust the figure legend of fig 2 so it is clear to the reader what was done.
 - i) The result shown in Fig 3 panel I is interesting, in that it shows similar infection burden in the skin of robins 56 dpf. Yet, the transmission success shown in Fig 2 panel E is quite different; perhaps you could expand on this in the discussion?
 - j) Line 212: CVF -> perhaps you want to explain the abbreviation. It is done in supplementary information but it would be easier for the reader if abbreviations were explained when first used.
 - k) Lines 237 and 239: „297 induced IgG titers significantly higher than other....“ sounds a little repetitive and could perhaps be combined?
 - l) Line 260: Theory predicts? What theory are you referring to? Pls rephrase.
 - m) Line 274: what is meant with LD prevalent regions of North America?
 - n) Line 287: what is meant by „robin competence“? pls explain, rephrase.
- Figure legend Fig 2: „the mean plus three-fold standard deviation of spirochete burdens in the uninfected group“ was used. According to the results described in lanes 141/142, larvae in the uninfected control group had zero spirochete positivity, it makes me wonder what is the three-fold standard deviation of zero?

Decision letter (RSPB-2021-0162.R0)

19-Mar-2021

Dear Dr Lin:

I am writing to inform you that your manuscript RSPB-2021-0162 entitled "Host specialization in microparasites transmitted by generalist vectors: insights into the cellular and immunological mechanisms" has, in its current form, been rejected for publication in Proceedings B.

This action has been taken on the advice of referees, who have recommended that substantial revisions are necessary. With this in mind we would be happy to consider a resubmission, provided the comments of the referees are fully addressed. However please note that this is not a provisional acceptance.

The resubmission will be treated as a new manuscript. However, we will approach the same reviewers if they are available and it is deemed appropriate to do so by the Editor. Please note that resubmissions must be submitted within six months of the date of this email. In exceptional circumstances, extensions may be possible if agreed with the Editorial Office. Manuscripts submitted after this date will be automatically rejected.

Please find below the comments made by the referees, not including confidential reports to the Editor, 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.
- 4) Data - please see our policies on data sharing to ensure that you are complying (<https://royalsociety.org/journals/authors/author-guidelines/#data>).

To upload a resubmitted manuscript, log into <http://mc.manuscriptcentral.com/prsb> and enter your Author Centre, where you will find your manuscript title listed under "Manuscripts with Decisions." Under "Actions," click on "Create a Resubmission." Please be sure to indicate in your cover letter that it is a resubmission, and supply the previous reference number.

Sincerely,
 Professor Hans Heesterbeek
 mailto: proceedingsb@royalsociety.org

Associate Editor
 Board Member: 1
 Comments to Author:

Thank you for giving Proc B the opportunity to evaluate this interesting manuscript. The reviewers agree that this MS describes an interesting and well-conducted set of experiments that address an important hypothesis. However, they have also flagged concerns that must be addressed in the next iteration of the MS. Please have a look at the thoughtful and detailed commentary provided by the reviewers below and be sure to address all of their concerns. Please pay special attention to the following:

1. Reviewer 1 points out that the MS fails to clearly explain the agreement in results among the various experiments and to make an explicit statement of which hypothesis is supported by the data. This is the most important part of the MS, so in the revision please be sure that the final answer is laid out plainly for the reader.
2. Similarly, Reviewer 2 points out a lack of alignment between data and conclusions - particularly in the title, but also throughout the MS.

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

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- c) Lines 77 – 88: While it may be the case that OspC has been used as marker in the USA, this is not generally the case and studies in other *Borrelia* species have not shown the pattern that is described here (e.g. Durand et al. 2017, Scientific Reports; Raberg et al. 2017, J. Evol. Biol). The references given in line 86 (23-25) do not refer to theoretical studies of OspC diversity. Pls explain or rephrase.
- d) Line 90: „strains with variable ospC genotypes“ – does this refer to strains that are able to change their ospC?
- e) Line 91: „We identified genotypic differences...“ – was it not phenotypic differences? The genetics of the isolates was not investigated.
- f) In line 103, perhaps you could add whether that was investigated by PCR or microscopy.
- g) Line 109: „... established infection selectively...“ may be true for 297 and cN40 but not for B31-5A4?
- h) The results described in lines 133-137 are unclear. It says that spirochetes transmission from host to feeding larvae was assessed but then it further states that unfed *I. scapularis* nymphs were allowed to feed until repletion. Pls clarify what was done. See also line 142 where it says that robin-derived larvae were below the detection limitresulting inpost nymph feeding? Please correct and rephrase this and adjust the figure legend of fig 2 so it is clear to the reader what was done.
- i) The result shown in Fig 3 panel I is interesting, in that it shows similar infection burden in the skin of robins 56 dpf. Yet, the transmission success shown in Fig 2 panel E is quite different; perhaps you could expand on this in the discussion?
- j) Line 212: CVF -> perhaps you want to explain the abbreviation. It is done in supplementary information but it would be easier for the reader if abbreviations were explained when first used.
- k) Lines 237 and 239: „297 induced IgG titers significantly higher than other....“ sounds a little repetitive and could perhaps be combined?
- l) Line 260: Theory predicts? What theory are you referring to? Pls rephrase.
- m) Line 274: what is meant with LD prevalent regions of North America?
- n) Line 287: what is meant by „robin competence“? pls explain, rephrase.
- Figure legend Fig 2: „the mean plus three-fold standard deviation of spirochete burdens in the uninfected group“ was used. According to the results described in lanes 141/142, larvae in the uninfected control group had zero spirochete positivity, it makes me wonder what is the three-fold standard deviation of zero?

Author's Response to Decision Letter for (RSPB-2021-0162.R0)

See Appendix A.

RSPB-2021-2087.R0

Review form: Reviewer 1

Recommendation

Major revision is needed (please make suggestions in comments)

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

Excellent

General interest: Is the paper of sufficient general interest?

Good

Quality of the paper: Is the overall quality of the paper suitable?

Acceptable

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?

No

Is it adequate?

No

Do you have any ethical concerns with this paper?

No

Comments to the Author

I appreciate the authors' extensive responses and edits to the last round of reviews. The experiments conducted and the results are of great scientific interest. However, I think the framing and interpretation of results could still use some clarification and careful review of theory. The Introduction has been edited but has gotten more difficult to understand in some respects. The introduction could use further clarification of the theoretical benefits and drivers of specialist vs. generalist pathogens. The third paragraph in the intro discusses the *B. burgdorferi sensu lato* complex but all the strains examined are *B. burgdorferi sensu stricto*. In reference to line 77, I don't think Bbsl is generally characterized as being "exclusively associated with one or a few host taxa". Most are quite generalist, certainly *B.b. sensu stricto* (Bbss) and other Lyme disease causing species are more general than not. I am also a little confused by the introduction and the background information that suggests that genospecies of Bbsl are adapted to different niches as defined by multiple niche polymorphism (MNP). MNP is generally used to describe the maintenance of variability of genotypes within a species, in this case Bbss.

The discussion section should clearly circle back to the three primary research questions that were presented at the end of the introduction. Namely:

"1) Do strains vary in their fitness across hosts, and are 'specialist' strains fitter than 'generalist' strains in the hosts they are adapted to?

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3) Do generalist strains have more flexible capacity of cellular and immunological mechanisms to increase fitness when interacting with multiple hosts, compared to a particular host-adapted strain?"

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The issue of genotype diversity within *B. burgdorferi sensu stricto* is a different matter than speciation. The maintenance of different genotypes within a species is the main theoretical thrust of MNP and other balancing selection hypotheses such as negative frequency distribution. A more careful interpretation of these theories would help hone the application of these interesting experimental results with evolutionary theory. Qiu and Martin 2014 may be helpful in this regard.

Abstract: The second sentence suggests that all microparasites are transmitted by generalist vectors, please reword. Not sure that “propagules” is the correct word here. This sentence also does not capture the theoretical underpinnings of a generalist vs. specialist pathogen which should extend to a pathogen’s adaptive potential versus host immune evasion.

Other comments:

Line 55: Can you streamline and just say host instead of habitat (host)

Line 55: Clarify what “narrower niches” means. Do you mean host specialization? Further, what does fitness components refer to here? Do you mean transmission efficiency or some other aspect of fitness, namely reproductive potential? Please clarify and define accordingly here and throughout the manuscript (e.g. line 320, etc).

Line 59 - Add an example of fitness of multi-host parasites varying across host species (because you say this is a common occurrence)

Line 59 - What are the fitness components?

Line 62 - Explain why vector-borne microparasites are ideal for this

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Line 117-118: what tick infection routes do you mean? How are they variable?

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Line 194 - 195 - Shouldn't this be the case since they were uninfected?

Line 304 - Can you switch it and make mice come first to keep it more consistent with the order of strains used throughout the paper? -- for the next several lines, as well

Line 343 - So, are you saying generalists may specialize? Or could the opposite also happen?

Line 251-252 - Does this need a source?

Line 313-315: “...WERE selectively adapted TO wfm and robin...”

Line: 315 is “laboratorial” should be “laboratory” ?

Line 327 and elsewhere: Should not start a sentence with a numbered list “2) robin...”

Could have more clarity in result, perhaps use more subheadings denoting mice or robin results?

Figure 2 can you align similar tissues types where relevant. For instance, align heart figures in the 3rd and 4th rows. It would also help clarify the figures if the Bb strains were labeled with picture or words indicating host adaptation (e.g. mouse or robin).

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?

Good

Is the length of the paper justified?

Yes

Should the paper be seen by a specialist statistical reviewer?

Yes

Do you have any concerns about statistical analyses in this paper? If so, please specify them explicitly in your report.

No

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Is it accessible?

Yes

Is it clear?

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Is it adequate?

Yes

Do you have any ethical concerns with this paper?

No

Comments to the Author

The revised version of the manuscript has improved in clarity. However, there are still some issues that need to be solved. In particular the following points should be addressed:

1/ My first concern relates to the antibody responses. As I said before, the quantitative antibody response may be interesting but there are several limitations with it: 1) you do not know to which antigens the response is to and nothing about cross-reactivity of antibodies. 2) the lysates used in ELISA are from in vitro cultures borreliae, thus you do not know whether relevant antigens (e.g. ospC, vlsE and others) are expressed. It is true as you say in your response to my previous comment that gene expression levels in vitro differ from those in the vertebrate host and this argument also applies to the ELISA results presented in the manuscript. It is not explicitly stated but I assume that the lysate mix used in ELISA was from cultured Borrelia. It would be much more interesting to support your data by a qualitative antibody analysis and I would strongly suggest to do Western blot analysis not only with lysates of the respective isolates but also with commercially available recombinant antigens (e.g. Mikrogen, Viramed and others). This will help

clarifying qualitative differences in antibody responses against the different isolates (see Baum et al. 2012, mBio 3(6):e00434-12 in this context). I do understand that this is additional work but without this – in my view – the results of the ELISA assay are somewhat trivial. After all the effort that has gone into this study, it would be a shame to not round it up.

Besides, it is not mentioned what controls were used (e.g. sera of non-infected animals) and how cut-off values were determined for the ELISA assay (Supplemental Material lines 234-246).

2/ My second concern relates to comment 14 in the previous review. It is my understanding from materials and methods that the spirochete burden in ticks and tissues was assessed by means of qPCR. Ct values of these qPCR were compared to a standard curve of known 16S rRNA gene copies (presumable from cultured material?). On page 9, lines 195-196 it is stated that the spirochete burden in the uninfected group were below the detection limit. In lines 196-199 it is outlined that the mean plus three-fold standard deviation of spirochete burden of the uninfected group was used for determining the number of xenodiagnostic-positive larvae and the percentage of xenodiagnostic-positive larvae. If you do qPCR, your negative control (=uninfected group) should be above a given cycle threshold (often 40) and receive „no Ct“. When qPCR is used to determine the number of *Borrelia*-infected ticks post-feeding, the same applies. It is unclear to me what the purpose of this procedure is?

I am not clear about this because in lines 14-17 of SM it says that „birds with positive ticks attached were removed“ and robins used in this study „were considered non-infectious“.

3/ In Supplemental materials

Lines 33-38 and lines 255-258: was a live-dead stain used to assess the number of spirochetes? A bacterial counting chamber? How many fields of view?

lines 248-259: the borreliacidal assays are described. Why was only serum from 7 dpi was used for this?

4/ please check all references in your manuscript carefully; I have outlined below some examples where the reference did not support the statement that was made.

5/ discussion – In my view there is a lack of synthesis in the discussion. It is in part a summary of the results. One could use the discussion to explain the choice of molecules analysed, e.g. IFN and TNF – why were those chosen, what is their role in immunological processes etc.. In addition, limitations of the study, e.g. that three isolates were used, that in vitro cultured specimen were used for certain experiments etc..

Other issues:

Title: I would suggest changing the title to „Host adaptive processes in generalist microparasites:...”

Line 28: ...Transmitted by generalists vectors, – not all parasites are transmitted by generalist vectors. Pls rephrase. E.g. when transmitted by

Line 30-31: The sentence starting „The Lyme disease.. pathogen..“ requires rephrasing.

Line 54: Selection may drive specialism....?

Line 56: either „generalists“ or „generalism“

Line 63: ..host-specialism would be favored... by what?

Line 67: ...they are not adapted to.

Line 78: Adaptation of Bbsl to different host niches is not multiple niche polymorphism (MNP) because MNP relates to populations, i.e. it acts within species, see Brisson 2018, Negative Frequency-Dependent Selection Is Frequently Confounding, *Frontiers in Ecology and Evolution*.

Line 81-83: Bbss is considered a host generalist because it can use different taxa as reservoir hosts (i.e. it is transmissible to ticks from these hosts, that means they are able to maintain natural transmission cycles; see Kahl et al. 2002 in *Lyme borreliosis: Biology, Epidemiology and Control for definitions*). Thus, even if the fitness in various hosts differs or the efficiency with which Bbss is transmitted, they are still reservoir hosts. This relates also to the next sentence and the cited references. In these laboratory studies it was shown that the duration of transmissibility varied between isolates (fitness variation) but that is not to say that the host cannot act as reservoir.

Line 85: Reference 13 does not report on natural populations of Bbss. Pls use appropriate references, e.g. Hanincova et al. 2006 *Epidemic Spread of Lyme Borreliosis, Northeastern United States, EID* or others.

Line 89-93: The references given for OspC diversity of Bb genotypes in North America do not fit because both references, 18 and 19, report on *Borrelia* sp. from Europe. In fact, one of the references (19) suggest that there are no specific host-OspC interactions (arguing against multiple niche polymorphism) and that other drivers likely maintain ospC diversity. The long-term study conducted by ref 18 showed that in natural populations of *B. afzelii* and *B. garinii* there was no fluctuation of ospC-types over time but dominant ospC-types remained dominant over the whole time period. So both these studies, given as ref. 18 and 19, do neither support multiple niche polymorphism nor negative frequency-dependent selection. Pls rectify the statements/references. Lines 94-95: the references given here, 1, 24 and 25, have not examined OspC diversity but explore in general host-parasite systems and the emergence of human pathogens. Pls put references in appropriate context.

Line 117:growth rates in vitro ...

Line 188: ...is variable for tick infection route... - what is meant here? That it varies from tick to tick or that tick infection differs from needle inoculation?

Line 123: ...these strains in mouse blood,...

Lines 134-135: 297 bound to white-footed mouse fibroblasts at higher levels than cN40 - what about B31? Did it also bind at significantly higher levels than cN40? If not, what does it mean?

Lines 140-146: You do not refer to Fig 1E and 1H, maybe you want to briefly mention the controls? In addition, I believe there is a mistake in the figure: robin endothelial cells incubated with 297 showed significant greater expression of IFN and TNF than B31 and cN40 incubated cells. There is also a mistake mouse cells or the figure labelling is wrong. Pls check and correct as appropriate.

Line 165: it is about the presence in blood, isn't it? Perhaps you can make that point.

Lines 167-168: ...consistent with early and transient blood passage?

Line 213: ... set up the threshold... see my comment above.

Line 220: ... uninfected mice only at 7 dpi...

Line 238:after incubation ... or ...after being incubated...

Line 240: OmCI - please define what it is

Line 243: ...after being treated...

Line 272:other strain-infected...

Lines 286 and 288: ...eliminated 50 % of the respective..? Do I get it correctly that only the isolate against which the antibodies were induced was lysed?

Line 295: what are ..barriers for fitness components..?

Line 307: "At 56 dpf, cN40 infection yielded the greatest number of robin tissues with detectable bacterial burdens..." - from your time line it seems that at day 56, xenodiagnosis was done but at day 64 tissues were examined. Pls correct as appropriate

Line 310 and comment 15: what is meant by skin-specific phenotypes that do not differentiate the strains? The reference that is given in response to comment 15 (Sertour et al.) describes that *Borrelia* isolates/species differ in their tissue tropism. I do not see how that would explain the difference of burden of 297 in skin compared to other tissues of robins.

Line 314: better - ..showed a higher degree of adaptation to? If you keep your wording, please correct ..adapted to...

Line 320: ...overall fitness in the respective reservoir

Line 323: ...in the respective hosts...

Line 332: ...of the tested strains...

Line 333: ...shaping der association of Bbss and reservoir hosts..? MNP relates to populations, not species.

Lines 341-343: please include reference for genetic variation in core genome elements: Gatzmann et al. 2015 NGS population genetics analyses reveal divergent evolution of a Lyme Borreliosis agent in Europe and Asia Ticks and Tick-borne diseases 6; Becker et al. 2020 High conservation combined with high plasticity: genomics and evolution of *Borrelia bavariensis*. BMC Genomics 21, 702 (2020). <https://doi.org/10.1186/s12864-020-07054-3>.

Line 349: please include Margos et al. 2019 as a reference.

Figure 3: please add the respective hosts to the upper and lower panel

Supplementary methods line 44: you refer to a shuttle plasmids - what is this for?

Supplementary table S1: in the introduction you now mention multilocus sequence typing, why not include the MLST sequence types in the table?

Supplementary table S3: there is an "a" to many in the first line of data

Decision letter (RSPB-2021-2087.R0)

28-Oct-2021

Dear Dr Lin:

Your manuscript has now been peer reviewed and the reviews have been assessed by an Associate Editor. The reviewers' comments (not including confidential comments to the Editor) and the comments from the Associate Editor are included at the end of this email for your reference. As you will see, the reviewers and the Associate Editor are not yet sufficiently convinced and have raised several concerns with your manuscript. We would like to invite you to revise your manuscript to address them.

We do not allow multiple rounds of revision so we urge you to make every effort to fully address all of the comments at this stage. If deemed necessary by the Associate Editor, your manuscript will be sent back to one or more of the original reviewers for assessment. If the original reviewers are not available we may invite new reviewers. Please note that we cannot guarantee eventual acceptance of your manuscript at this stage.

To submit your revision please log into <http://mc.manuscriptcentral.com/prsb> and enter your Author Centre, where you will find your manuscript title listed under "Manuscripts with Decisions." Under "Actions", click on "Create a Revision". Your manuscript number has been appended to denote a revision.

When submitting your revision please upload a file under "Response to Referees" in the "File Upload" section. This should document, point by point, how you have responded to the reviewers' and Editors' comments, and the adjustments you have made to the manuscript. We require a copy of the manuscript with revisions made since the previous version marked as 'tracked changes' to be included in the 'response to referees' document.

Your main manuscript should be submitted as a text file (doc, txt, rtf or tex), not a PDF. Your figures should be submitted as separate files and not included within the main manuscript file.

When revising your manuscript you should also ensure that it adheres to our editorial policies (<https://royalsociety.org/journals/ethics-policies/>). You should pay particular attention to the following:

Research ethics:

If your study contains research on humans please ensure that you detail in the methods section whether you obtained ethical approval from your local research ethics committee and gained informed consent to participate from each of the participants.

Use of animals and field studies:

If your study uses animals please include details in the methods section of any approval and licences given to carry out the study and include full details of how animal welfare standards were ensured. Field studies should be conducted in accordance with local legislation; please include details of the appropriate permission and licences that you obtained to carry out the field work.

Data accessibility and data citation:

It is a condition of publication that you make available the data and research materials supporting the results in the article (<https://royalsociety.org/journals/authors/author-guidelines/#data>). Datasets should be deposited in an appropriate publicly available repository and details of the associated accession number, link or DOI to the datasets must be included in the Data Accessibility section of the article (<https://royalsociety.org/journals/ethics-policies/data-sharing-mining/>). Reference(s) to datasets should also be included in the reference list of the article with DOIs (where available).

In order to ensure effective and robust dissemination and appropriate credit to authors the dataset(s) used should also be fully cited and listed in the references.

If you wish to submit your data to Dryad (<http://datadryad.org/>) and have not already done so you can submit your data via this link [http://datadryad.org/submit?journalID=RSPB&manu=\(Document not available\)](http://datadryad.org/submit?journalID=RSPB&manu=(Document%20not%20available)), which will take you to your unique entry in the Dryad repository.

If you have already submitted your data to dryad you can make any necessary revisions to your dataset by following the above link.

For more information please see our open data policy <http://royalsocietypublishing.org/data-sharing>.

Electronic supplementary material:

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

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

Please submit a copy of your revised paper within three weeks. If we do not hear from you within this time your manuscript will be rejected. If you are unable to meet this deadline please let us know as soon as possible, as we may be able to grant a short extension.

Thank you for submitting your manuscript to Proceedings B; we look forward to receiving your revision. If you have any questions at all, please do not hesitate to get in touch.

Best wishes,

Professor Hans Heesterbeek

<mailto:proceedingsb@royalsociety.org>

Associate Editor Board Member

Comments to Author:

Many thanks for your extensive revision of this MS in response to the previous round of suggestions by the two peer reviewers. However, concerns remain about several aspects of this paper, and the review team needs to see another round of major revisions before this MS can be further considered for publication in Proc B. Detailed critiques from the reviewers are below, but please pay special attention to the following:

1. ANTIBODY RESPONSES - Reviewer 2 suggests some additional lab work that would make the results of the ELISA assay much more impactful. Please consider adding these data to the MS.
2. EXPLANATION OF METHODS - Reviewer 2 also raises several concerns about the inclusion of controls and other aspects of the methodological approach, most areas where the methods need to be explained more clearly. Please see Reviewer 2's notes below for a detailed list of issues that should be addressed.
3. DISCUSSION - Both reviewers noted that the Discussion fails to synthesize the results of the experiments and to return to the central questions outlined in the Introduction.
4. INTRODUCTION - Reviewer 1 suggests that the Introduction should be enriched with greater theoretical grounding on the tradeoffs of specialization.

Reviewer(s)' Comments to Author:

Referee: 1

Comments to the Author(s).

I appreciate the authors' extensive responses and edits to the last round of reviews. The experiments conducted and the results are of great scientific interest. However, I think the framing and interpretation of results could still use some clarification and careful review of theory. The Introduction has been edited but has gotten more difficult to understand in some respects. The introduction could use further clarification of the theoretical benefits and drivers of specialist vs. generalist pathogens. The third paragraph in the intro discusses the *B. burgdorferi* sensu lato complex but all the strains examined are *B. burgdorferi* sensu stricto. In reference to line 77, I don't think Bbsl is generally characterized as being "exclusively associated with one or a few host taxa". Most are quite generalist, certainly *B.b. sensu stricto* (Bbss) and other Lyme disease causing species are more general than not. I am also a little confused by the introduction and the background information that suggests that genospecies of Bbsl are adapted to different niches as defined by multiple niche polymorphism (MNP). MNP is generally used to describe the maintenance of variability of genotypes within a species, in this case Bbss.

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Referee: 2

Comments to the Author(s).

The revised version of the manuscript has improved in clarity. However, there are still some issues that need to be solved. In particular the following points should be addressed:

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commercially available recombinant antigens (e.g. Mikrogen, Viramed and others). This will help clarifying qualitative differences in antibody responses against the different isolates (see Baum et al. 2012, mBio 3(6):e00434-12 in this context). I do understand that this is additional work but without this – in my view – the results of the ELISA assay are somewhat trivial. After all the effort that has gone into this study, it would be a shame to not round it up.

Besides, it is not mentioned what controls were used (e.g. sera of non-infected animals) and how cut-off values were determined for the ELISA assay (Supplemental Material lines 234-246).

2/ My second concern relates to comment 14 in the previous review. It is my understanding from materials and methods that the spirochete burden in ticks and tissues was assessed by means of qPCR. Ct values of these qPCR were compared to a standard curve of known 16S rRNA gene copies (presumable from cultured material?). On page 9, lines 195-196 it is stated that the spirochete burden in the uninfected group were below the detection limit. In lines 196-199 it is outlined that the mean plus three-fold standard deviation of spirochete burden of the uninfected group was used for determining the number of xenodiagnostic-positive larvae and the percentage of xenodiagnostic-positive larvae. If you do qPCR, your negative control (=uninfected group) should be above a given cycle threshold (often 40) and receive „no Ct“. When qPCR is used to determine the number of *Borrelia*-infected ticks post-feeding, the same applies. It is unclear to me what the purpose of this procedure is?

I am not clear about this because in lines 14-17 of SM it says that „birds with positive ticks attached were removed“ and robins used in this study „were considered non-infectious“.

3/ In Supplemental materials

Lines 33-38 and lines 255-258: was a live-dead stain used to assess the number of spirochetes? A bacterial counting chamber? How many fields of view?

lines 248-259: the borreliacidal assays are described. Why was only serum from 7 dpi was used for this?

4/ please check all references in your manuscript carefully; I have outlined below some examples where the reference did not support the statement that was made.

5/ discussion – In my view there is a lack of synthesis in the discussion. It is in part a summary of the results. One could use the discussion to explain the choice of molecules analysed, e.g. IFN and TNF – why were those chosen, what is their role in immunological processes etc.. In addition, limitations of the study, e.g. that three isolates were used, that in vitro cultured specimen were used for certain experiments etc..

Other issues:

Title: I would suggest changing the title to „Host adaptive processes in generalist microparasites:...”

Line 28: ...Transmitted by generalists vectors, – not all parasites are transmitted by generalist vectors. Pls rephrase. E.g. when transmitted by

Line 30-31: The sentence starting „The Lyme disease.. pathogen..“ requires rephrasing.

Line 54: Selection may drive specialism....?

Line 56: either „generalists“ or „generalism“

Line 63: ..host-specialism would be favored... by what?

Line 67: ...they are not adapted to.

Line 78: Adaptation of Bbsl to different host niches is not multiple niche polymorphism (MNP) because MNP relates to populations, i.e. it acts within species, see Brisson 2018, Negative Frequency-Dependent Selection Is Frequently Confounding, *Frontiers in Ecology and Evolution*.

Line 81-83: Bbss is considered a host generalist because it can use different taxa as reservoir hosts (i.e. it is transmissible to ticks from these hosts, that means they are able to maintain natural transmission cycles; see Kahl et al. 2002 in *Lyme borreliosis: Biology, Epidemiology and Control for definitions*). Thus, even if the fitness in various hosts differs or the efficiency with which Bbss is transmitted, they are still reservoir hosts. This relates also to the next sentence and the cited references. In these laboratory studies it was shown that the duration of transmissibility varied between isolates (fitness variation) but that is not to say that the host cannot act as reservoir.

Line 85: Reference 13 does not report on natural populations of Bbss. Pls use appropriate references, e.g. Hanincova et al. 2006 *Epidemic Spread of Lyme Borreliosis, Northeastern United States, EID* or others.

Line 89-93: The references given for OspC diversity of Bb genotypes in North America do not fit because both references, 18 and 19, report on *Borrelia* sp. from Europe. In fact, one of the references (19) suggest that there are no specific host-OspC interactions (arguing against multiple niche polymorphism) and that other drivers likely maintain ospC diversity. The long-term study conducted by ref 18 showed that in natural populations of *B. afzelii* and *B. garinii* there was no fluctuation of ospC-types over time but dominant ospC-types remained dominant over the whole time period. So both these studies, given as ref. 18 and 19, do neither support multiple niche polymorphism nor negative frequency-dependent selection. Pls rectify the statements/references. Lines 94-95: the references given here, 1, 24 and 25, have not examined OspC diversity but explore in general host-parasite systems and the emergence of human pathogens. Pls put references in appropriate context.

Line 117:growth rates in vitro ...

Line 188: ...is variable for tick infection route... - what is meant here? That it varies from tick to tick or that tick infection differs from needle inoculation?

Line 123: ...these strains in mouse blood,...

Lines 134-135: 297 bound to white-footed mouse fibroblasts at higher levels than cN40 - what about B31? Did it also bind at significantly higher levels than cN40? If not, what does it mean?

Lines 140-146: You do not refer to Fig 1E and 1H, maybe you want to briefly mention the controls? In addition, I believe there is a mistake in the figure: robin endothelial cells incubated with 297 showed significant greater expression of IFN and TNF than B31 and cN40 incubated cells. There is also a mistake mouse cells or the figure labelling is wrong. Pls check and correct as appropriate.

Line 165: it is about the presence in blood, isn't it? Perhaps you can make that point.

Lines 167-168: ...consistent with early and transient blood passage?

Line 213: ... set up the threshold... see my comment above.

Line 220: ... uninfected mice only at 7 dpi...

Line 238:after incubation ... or ...after being incubated...

Line 240: OmCI - please define what it is

Line 243: ...after being treated...

Line 272:other strain-infected...

Lines 286 and 288: ...eliminated 50 % of the respective..? Do I get it correctly that only the isolate against which the antibodies were induced was lysed?

Line 295: what are ..barriers for fitness components..?

Line 307: "At 56 dpf, cN40 infection yielded the greatest number of robin tissues with detectable bacterial burdens..." - from your time line it seems that at day 56, xenodiagnosis was done but at day 64 tissues were examined. Pls correct as appropriate

Line 310 and comment 15: what is meant by skin-specific phenotypes that do not differentiate the strains? The reference that is given in response to comment 15 (Sertour et al.) describes that *Borrelia* isolates/species differ in their tissue tropism. I do not see how that would explain the difference of burden of 297 in skin compared to other tissues of robins.

Line 314: better - ..showed a higher degree of adaptation to? If you keep your wording, please correct ..adapted to...

Line 320: ...overall fitness in the respective reservoir

Line 323: ...in the respective hosts...

Line 332: ...of the tested strains...

Line 333: ...shaping der association of Bbss and reservoir hosts..? MNP relates to populations, not species.

Lines 341-343: please include reference for genetic variation in core genome elements: Gatzmann et al. 2015 NGS population genetics analyses reveal divergent evolution of a Lyme Borreliosis agent in Europe and Asia *Ticks and Tick-borne diseases* 6; Becker et al. 2020 High conservation combined with high plasticity: genomics and evolution of *Borrelia bavariensis*. *BMC Genomics* 21, 702 (2020). <https://doi.org/10.1186/s12864-020-07054-3>.

Line 349: please include Margos et al. 2019 as a reference.

Figure 3: please add the respective hosts to the upper and lower panel

Supplementary methods line 44: you refer to a shuttle plasmids - what is this for?

Supplementary table S1: in the introduction you now mention multilocus sequence typing, why not include the MLST sequence types in the table?

Supplementary table S3: there is an "a" to many in the first line of data

Author's Response to Decision Letter for (RSPB-2021-2087.R0)

See Appendix B.

Decision letter (RSPB-2021-2087.R1)

18-Jan-2022

Dear Dr Lin

I am pleased to inform you that your manuscript entitled "Cellular and immunological mechanisms influence host-adapted phenotypes in a vector-borne microparasite" 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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Please remember to make any data sets live prior to publication, and update any links as needed when you receive a proof to check. It is good practice to also add data sets to your reference list.

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

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

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

Associate Editor:
Board Member

Comments to Author:

Thank you for your careful attention to the reviewers' suggestions. I am pleased to accept this interesting manuscript for publication in Proc B.

Appendix A



Dr Antoine Barreaux
Research Associate

School of Biological Sciences, Life Sciences Building
email:antoine.barreaux@bristol.ac.uk

08/12/21

Dear Prof Kruuk

We are resubmitting our research manuscript entitled "*Incorporating effects of age on energy dynamics predicts non-linear maternal allocation patterns in iteroparous animals*" for consideration for publication in *Proceedings of the Royal Society B*. We thank you for considering our manuscript and for the opportunity to resubmit.

We are grateful to the associate editor and reviewers for the positive reviews and helpful comments that we feel have improved the manuscript. We have significantly changed sections of this manuscript in light of each reviewer's comments and have incorporated all the stylistic and grammatical corrections suggested. Our detailed responses are provided below.

We describe original work that is not being considered for publication in any other journal. We declare having no competing interests. All the authors gave final approval for publication and submission to *Proceedings B* of the final manuscript.

We appreciate your time and are looking forward to your response.

Best regards,

Antoine Barreaux, on behalf of all authors

Associate Editor

Board Member: 1

Comments to Author:

I enjoyed reading this paper which tackles an important question of optimal allocation to reproduction in iteroparous animals. I was particularly intrigued by the lack age-dependent extrinsic mortality effects and the evidence pointing to reproductive restraint. I think this paper can make an important contribution to the field. The paper has been reviewed by three experts that provided a number of queries and suggestions for improvement. I encourage the authors to address all of the points raised by the reviewers in detail.

RESPONSE: We thank the associate editor for their time, positive comments, and consideration for our manuscript. We have made sure to answer all the helpful queries and suggestions made by the three referees, and to address all the points raised. Please find our detailed answer below.

Referee: 1

Many organisms show a decline in reproductive success with age. This pattern, referred to as reproductive senescence, is often seen as a hump-shaped relationship between age and a fitness component (e.g., offspring size, clutch size, etc.). The evolutionary theory of aging proposes that a decline in reproductive success with age is the result of weak selection on fitness components expressed late in life. This can result in the accumulation of late-acting deleterious alleles (or alleles with antagonistic effects on early and late life fitness). Although the evolutionary theory of aging is well supported, less is known about the specific impact of ecological and energetic factors in determining the relationship between age and reproductive fitness (although extrinsic mortality is expected to be important).

In this manuscript, the authors use a dynamic state variable model to predict optimal maternal allocation patterns as a function of age, when there is stochasticity in energy dynamics and age dependent changes in factors such as feeding success and external mortality rate. The model is parameterized with data from tsetse. Using the results of their models, the authors determine what combination of factors successfully recapitulate the hump-shaped relationship between age and maternal allocation in tsetse.

The manuscript is well written and addresses a topic that is certainly of interest to the readers of Proc. R. Soc. B. I am not a theoretician, so I am not able to comment on the modeling approach. However, I have some more general comments and questions regarding the author's approach and conclusions.

RESPONSE: We thank the referee for their interest in our manuscript. We appreciate the helpful general comments and suggestions on our approach and conclusions.

1. A central theme in the evolution of senescence is that selection acting late in life is weak compared to selection acting early in life. I really struggled to see how variation in the strength of selection is incorporated in to their modeling approach (is this just through extrinsic mortality?). This was especially confusing for me because the dynamic programming approach finds the optimal strategy by beginning with the oldest age class and working backward. This seems biologically implausible, but I'm sure that I am missing something here?

RESPONSE: While a decline in the strength of selection in later ages is indeed a central theme in evolutionary explanations for actuarial senescence, our intention was to identify if and when observed patterns of reproductive senescence arose through optimal maternal allocation. In our model, there is no forced decline in selective pressure later in life because all offspring are equally valuable at all ages (offspring quality depends on maternal allocation itself) and there are no mutations. Mothers may vary allocation of resources to offspring with age, which will then result in offspring of different quality and may lead to reproductive senescence if offspring quality decreases with maternal age. In the baseline model, the input parameters are age independent. We compare outputs of this model to other scenarios which include age-dependence in energy dynamics and environmentally-driven mortality ("extrinsic mortality") to study how these evolutionary constraints affect optimal allocation and whether they lead to reproductive senescence.

We have added the following clarification about how our model fits in the context of declining selection as an explanation for senescence, at 235-238:

"Our aim is to identify whether observed senescence can arise from optimal maternal allocation. As such, we do not impose a decline in selection in later life as all offspring are equally valuable at all ages (for a given maternal allocation), and there are no mutations."

To address the point about dynamic programming, we note that the approach of dynamic programming is not intended to mimic any biological process, but to find an optimal strategy. Decisions (e.g. amount of resources to allocate to an offspring) are assumed to depend on the animal's current state, regardless of how this state was reached. However, the current decision of an animal affects its future state and so future decisions. When finding the optimal current decision, it is therefore necessary to know what the future states (and decisions) will be, so we have to work backwards. This is a standard approach in dynamic programming in general introduced by Bellman in the 1950s [1] and widely applied to behavioural ecology in the 1980s and 1990s [2,3]. We do not claim that animals themselves follow such complex calculations, but follow evolved rules of thumb that generate behaviour similar to the strategy [4].

2. The authors parameterize the model using data from a laboratory population of tsetse. They then determine the set of environmental conditions that generate a hump-shaped relationship between age and maternal allocation that is most similar to the relationship observed in the lab. This approach seems to assume that the tsetse population has adapted to the lab conditions. Is there evidence for this? It's not clear from the methods whether there is evidence that the population is lab adapted or even how many generations the population has been maintained in the lab. The approach in the paper only works if the population is lab adapted.

RESPONSE: We agree with the referee that there are potential limitations when relying too heavily on a single study from a long-standing laboratory population, and we cannot rule out effects of inbreeding or inadvertent selection. We do not assume, however, that the population has adapted to the lab, rather we ask if this laboratory population might follow the evolved strategy of field flies. This is because our assumptions are based on field conditions, including uncertain blood supply and environmentally-driven mortality. Ideally, we would fit our model to within-individual allocation data from field flies but owing to current sampling limitations, such data are not available.

We clarified this caveat in the discussion at lines 376-385:

“A caveat is that the only available data on within-individual patterns of allocation with age in tsetse is from a laboratory study with a population of flies that has been in the laboratory many generations [24], and we cannot conclude how well our model would explain patterns in the wild. In cross-sectional studies, there is a slight increase in allocation with age, as observed at earlier ages both in the laboratory [24] and in our model, but no later-life decline [51,80]. The lack of reproductive senescence in the wild could be linked to shorter lifespans, with wild flies being more susceptible to death from starvation and predation [44,81]. A limitation of the field data is that individual tsetse cannot be tracked across their lifespan and pregnant females are only caught during particular seasons of the year. As such, we may not be able to observe reproductive senescence in the wild, even if it occurred, due to the cross-sectional data currently available [51,80].”

3. The authors main conclusion is that when there is age-dependency in energy dynamics, the optimal relationship between age and maternal allocation is hump-shaped. It was never really clear to me whether the authors are proposing this as an alternative to reproductive senescence driven by relaxed selection on maternal investment late in life. I guess this gets back to my first point of confusion (related to point 1 above).

RESPONSE: Yes, in our study we ask whether reproductive senescence can be predicted without invoking a mutation-selection balance. As explained above, there is no forced changing selective pressure later in life in our model because all offspring are equally valuable at all ages and there are no mutations. We here predict reproductive senescence with our model from an optimal allocation perspective. This concurs with the findings of McNamara et al. [5] showing how reproductive senescence can be condition-dependent, with later-life reproductive restraint being due to damage accumulation. Here, we show that an evolutionary explanation for reproductive senescence could be age-dependent changes in resource availability and energetic costs (energy dynamics) and not variation in environmentally-driven mortality – at least in a tsetse-like organism.

We clarified lines 369-374:

“Relative allocation decreases with age and older females allocate less reserves to reproduction in comparison to younger females, regardless of their own reserves. This concurs with predictions of adaptive later-life reproductive restraint as a functional explanation for reproductive senescence [20], whereby maternal allocation decreases with age to reduce risks of increased mortality associated with accrued damage due to reproduction [20] or starvation with declining energy dynamics.”

We also added lines 387-389:

“In summary, we provide a mechanistic explanation behind the pattern of increase-then-decrease in optimal allocation which is driven by evolutionary constraints with age-dependent effects on energy dynamics, confirming the possibility of later-life reproductive restraint.”

4. Line 280-281: These sentences were slightly confusing. The first sentence tells us the fraction of scenarios that generated hump-shaped allocation patterns (749/1403). The next sentence considers the “models with a negative quadratic term” that had an $R^2 > 0.7$ (35/1403). Shouldn't this fraction be 35/749? The first sentence implies that 749 of the scenarios had a negative quadratic term, so that should be the denominator in the second proportion.

RESPONSE: This is a good point. To avoid confusion about percentage values, we now report the absolute number in the second statement.

Lines 277-279:

"We predicted a non-linear (hump-shaped) pattern of allocation in 53% (749/1403) of the scenarios evaluated. 35 of these quadratic downward scenarios fit the simulated data with a conditional pseudo R^2 value above 0.7 (Table 3, Figure 2)."

5. Line 351: "have" should be "has"

RESPONSE: Corrected.

6. Line 384-387: I agree that using this same approach to predict patterns of reproductive senescence in other species would be very illuminating! In my opinion, the manuscript would be of interest to a much broader audience if it included such a comparison.

RESPONSE: We agree that it could be interesting and insightful to apply our model to data from a range of species, yet this is beyond the scope of our current study. Here, we predict reproductive senescence from an optimal allocation perspective. We believe our model set-up to be already quite general as it allows the optimal strategy for iteroparous animals to be anywhere on the continuum from extreme capital to extreme income breeding (see also our reply to Referee 2). We are indeed concerned with predicting where animals will lie on the continuum given their ecology (see [6]). Besides, given the range of parameters we explored, we were able to investigate a range of age-dependent allocation patterns (see Fig. 2 for those with a quadratic downward fit) without too many constraints. Finally, we note that, while we fit our model to tsetse data, it remains a general approach as the age-dependence in energy-related traits underlying the hump-shaped pattern are observed across numerous animal systems. For example, age-dependence in energy intake or feeding success is likely to be commonly observed given that foraging efficiency improves with age in many taxa. We appreciate that interesting contrasts may be raised by tailoring our model to the biology of other iteroparous systems across the phylogenetic tree, particularly those with detailed field data on age-specific reproductive allocation (e.g. long-term studies of individually marked vertebrates), and we hope our model will inspire such future work.

We added lines 120-123:

"The model set-up allows the optimal strategy to be anywhere on the continuum from extreme capital (females build up stored reserves which are used for reproduction) to extreme income breeding (females do not store reserves across breeding events and use only those acquired during feeding), given their ecology [50]."

We clarified at lines 329-337:

"These scenarios confirm the impact on allocation of gains in experience in breeding and in acquiring food [7,15,23] and increasing energetic costs across the lifespan because of damage accumulation [7,15,19,23]. The evidence for age-dependence in such traits is wide-ranging across systems from a decrease in energetic costs with an improved lactation ability (e.g. seals [25]), an improved energy transfer efficiency (rats [77]), or reduced metabolic requirements post maturation (tsetse [60,61,69]), to an increase in energy intake with an improved mobility post maturation (tsetse [60,61,70]), or a decrease in energy intake later in life because of gut deterioration (Drosophila [78]) or other physiological deteriorations. Such evidence could explain why these non-linear patterns of maternal allocation are found across diverse taxonomic groups."

We added lines 389-399:

"Our model also provides a more general framework to understand optimal reproductive allocation in iteroparous breeders. By tracking maternal allocation, maternal reserves, and relative allocation we show what strategic choices individuals make given their ecology, anywhere on the continuum from extreme capital to extreme income breeding. With our particular parameters tailored to tsetse biology, we find an income breeding strategy as we predicted given that tsetse acquire resources through feeding on protein-rich blood multiple times for each gestation cycle [51]. However, the same model could also predict a capital breeding strategy when applied to specific biology of other iteroparous breeders. Indeed, we hope that this framework inspires future models which could be fitted to long-term individual studies from wild

vertebrate populations such as red deer, bison, or terns [10, 15] and thus ascertain the generality of our findings both in field conditions and in diverse taxonomic groups.”

7. In the abstract, the authors point out that diverse scenarios can generate hump-shaped relationships between maternal allocation and age. They then conclude that this is why so many organisms display this pattern in nature. I'm not sure that their data allow them to make such a conclusion. It is true that there appear to many routes to a hump-shaped distribution. However, without a cross-species comparison we don't know whether different species take these different routes! This conclusion would be much stronger if the authors used data from another organism to parameterize the model and explore which environmental / energetic conditions generate the pattern they are trying to explain.

RESPONSE: It's true that a large parameter space doesn't necessarily equate to a large number of species. However, we have linked empirical patterns from different species more specifically to our model by discussing the wide-ranging examples which apply to the key parameters involved, i.e. species which have age-dependence in specific traits linked to energy dynamics (see response to point 6).

As explained in our response to point 6, we believe it to be beyond the scope of this current study to use data from other organisms. Here, we predict reproductive senescence from an optimal allocation perspective, showing that an evolutionary and mechanistic explanation could be age-dependent changes in energy dynamics. We believe our current model set-up to be already quite general as it allows the optimal strategy for iteroparous animals to be anywhere on the continuum from extreme capital to extreme income breeding.

8. Hargrove et al. 2011 appears twice in reference list (37 and 41) but appears to be the same paper.

RESPONSE: Corrected.

Referee: 2

Comments to the Author(s)

This original work proposes a model of optimal allocation to reproduction in iteroparous animals with an application to the case study of tsetse flies.

I really enjoyed reading that nice piece of work that can potentially offer a solid contribution to better understand life history variation. However, I found three major problems that need to be solved.

RESPONSE: Thank you for positive comments and suggestions.

First and most importantly, the proposed model targets the amount of reserves that mothers allocate and seems at first sight limited to extreme capital breeders (sensu Jonsson 1997 Oikos). Then, the model also includes feeding opportunities, indicating that it might be applied to income breeders. Lastly, the statement in line 142 clearly shows that the model is restricted to capital breeders: income breeders produce offspring without storing any reserves!

It is thus impossible to assess how the model proposed matches with the major axis of variation in resource allocation to reproduction: the capital-income breeder continuum. A thorough discussion about how the model should be influenced by the ranking of a given species on the capital-income breeder continuum is required. In particular, purely capital breeders should be insensitive to food restriction during the rearing period. They have information about their body reserves and can adjust their reproductive allocation to these reserves. On the contrary, purely income breeders have no information and strongly depend on resource availability during the rearing period. Moreover, storing, carrying, and handling body reserves is more costly than directly allocating energy assimilated to offspring. How the proposed model accounts for these opposed reproductive tactics and their associated constraints has to be made explicit. For instance, the conclusion that age-dependence in feeding success does not drive the shape of age-specific allocation is likely a consequence of the model structure and might not apply to income breeders.

RESPONSE: We appreciate that setting our model in the context of the capital-income breeding continuum is an important point. While our manuscript may not have been explicitly clear on this point, our model does indeed allow us to predict where animals will lie on the continuum given their ecology (see [6]). One of the main model outputs is the amount of reserves females store versus use up for each offspring, thus allowing us to predict the optimal strategy anywhere along the continuum from extreme capital (rely only on stored reserves) to income (rely only on resources from food intake) breeding.

What emerges in our baseline tsetse model is almost pure income breeding: females have a minimum reserve level and every period use everything they have above this. We emphasise that this is an output rather than an assumption of the model. We have previously hypothesised that tsetse are income breeders due to two lines of evidence: first, female acquire resources through feeding on protein-rich blood multiple times for each gestation cycle, and, second, mothers delay allocation of resources to offspring until late into gestation. This latter strategy may be adaptive if it benefits females to allocate resources significantly towards offspring growth only at the point during gestation when they are certain to have sufficient reserves available to produce a viable offspring, otherwise they can abort that offspring [7].

We clarified lines 146-147 that individuals have the choice to allocate resources or not and we replaced “reserves” by “resources”:

“If the optimal decision is not to allocate any resources ($M_t^ = 0$), then no offspring can be produced. If resources are allocated ($M_t^* > 0$), then a juvenile offspring is produced.”*

As explained in our response to Referee 1 point 6 above, we have clarified our text, in the introduction at lines 120-123 and in the discussion at lines 389-399, to incorporate the point about capital versus income breeding more explicitly.

Second, the authors often refers to « offspring quality » without defining what they mean by that. Is it offspring survival? Offspring mass? Moreover, the justification of using a Gompertz model to relate this « offspring quality » with fitness gain has to be provided.

RESPONSE: In our model, offspring quality is a measure of an offspring’s expected reproductive success during its lifetime given its energy reserves at the start of adulthood. It is the value of the offspring’s life defined as the immediate gain in fitness for a mother for a given offspring based on the expected fitness of the offspring upon reaching adulthood.

We clarified this at lines 174-176:

“The immediate gain in fitness f is a function of offspring quality (i.e. the expected reproductive success during the offspring’s lifetime given its energy reserves at the start of adulthood) and depends on maternal allocation M_t minus the energy needed to survive the non-feeding phase p .”

The Gompertz assumption has no effect on our model predictions, since it is replaced by the outcome of the previous iteration over several backwards iterations (i.e. several generations). We use this Gompertz assumption as it provides an initial starting assumption about the value of offspring, and choose a sensible shape given the assumed selective mortality of smaller offspring. Since we iterate to convergence of the offspring value function, the actual shape of the initial function has no effect on our predictions. We apologise that this was unclear, and we have now clarified this in the ESM lines 20-25:

“For the immediate gain in fitness f (figure S1), the Gompertz assumption has no effect on the predictions, since it is replaced by the outcome of the previous iteration over several backwards iterations (i.e. several generations). We have to start with some assumption about the value of offspring, and choose a sensible shape given the assumed selective mortality of smaller offspring. Since we iterate to convergence of the offspring value function, the actual shape of the initial function has no effect on model predictions.”

Lastly, the use of quadratic model to describe age-specific changes in reproductive allocation is misleading. A decreasing rate of increase in reproductive performance among young and prime-aged adults with no senescence is well fitted by a quadratic model. However, to model the standard three stages expected in age-specific variation in reproduction (Emlen 1970 Ecology, which would warrant to be quoted in that paper), a threshold model is required. Threshold models are indeed more appropriate than quadratic models to assess senescence patterns (see e.g. Bermann et al. 2009 PRSB for a

discussion).

RESPONSE:

We thank the referee for their suggestion, and we have quoted the Emlen [8] and Berman [9] references in our manuscript.

We clarified lines 64-66 adding the Emlen reference:

“To our knowledge, only one study predicted an increase and decrease in fecundity as a by-product of natural selection [26] and few – if any – theoretical studies predict this lifetime reproductive resources allocation pattern, from an initial increase to a later-life decline.”

We added the Berman reference at lines 49-50:

“In many systems, maternal allocation tends to decline with age, termed reproductive senescence [10,15,16].”

And at line 99:

“similar to long-lived vertebrates [10,15,16]”

However, we respectfully disagree with the referee about a threshold model (a 3 splines threshold model for example) being required here. These splines are low order polynomials that use knot/data breakpoints to determine relationships to data and are similar to our quadratic approach. We here explain the increase-then-decrease specifically, using the quadratic model fitting with a negative quadratic term to find model scenarios with the senescence part as well as the initial increase. Our quadratic model captures increase-then-decrease patterns without assuming a straight plateau, as organisms do not necessarily show such hard switches – reproductive competence and senescence can be quite gradual.

Besides, we acknowledge the willingness to fit the best model when considering specific empirical data, as is the case for the threshold model fit to southern fulmar data in the Berman study [9]. However, in our previous study on reproductive allocation in laboratory tsetse [10], we also fitted (in addition to a quadratic model) a model assuming a relationship between offspring quality and the logarithm of maternal age, as in Berman et al. [9], as well as more flexible generalized additive models. Neither approaches resulted in an improved fit to the data compared to the quadratic fit. This brings further support to quadratic models being an appropriate and similar approach to threshold models to fit the entire life-long allocation pattern in our study, from the increase to the decrease in allocation, both from a general and tsetse-specific perspective. We accept that the model fitting algorithm may differ when applying our framework to other studies in different systems (see response to Referee 1 point 6).

Detailed comments:

l. 28: « mortality risk », not « extrinsic mortality »

l. 32: « mortality risk », not « extrinsic mortality »

l. 44: « allocation », not « investment »

l. 55: Remove « extrinsic »

l. 58: Remove « intrinsic »

l. 94: Remove « extrinsic »

l. 96: « case study » instead of « an exemplar »

l. 98: « deer », not « deers »

l. 131; What ny refers to is missing

l. 150: Remove « which is a form of intrinsic mortality »

l. 151: Replace « extrinsic » with « environmentally driven »

l. 151-152: Replace « from external factors (predation, inclement weather). » with « from predation or inclement weather. »

l. 161: Remove « extrinsic »

l. 198: Remove « extrinsic »

l. 214: Remove « extrinsic »

l. 220-221: The legend of Table 1 should be placed above the table.

l. 227: Remove « extrinsic »

l. 255-256: The legend of Table 2 should be placed above the table.

l. 256: Remove « extrinsic »

Figure 1: Display the data in addition to models in the panels

I. 291-296: The legend of Table 3 should be placed above the table.

I. 296: Remove « extrinsic »

I. 324: Remove « extrinsic »

I. 356: Remove « extrinsic »

I. 357: Remove « extrinsic »

RESPONSE: Thank you – we have made all these minor changes. We have removed “extrinsic” and intrinsic where needed and have replaced “extrinsic mortality” by “environmentally-driven mortality” to clarify (we could not just say ‘mortality’ as this is not the only source of mortality, given that individuals can starve to death)

For “ny”, we have added an explanation at lines 136-137:

“ny is the total energy intake (y units of energy per successful feeding attempt, n times per period)”

We are not sure what the referee meant by displaying the data for Figure 1 in addition to models in the panels. The simulated data is already present on the panels as we show the average maternal allocation, or average relative allocation, or average maternal reserves of 1000 mothers for 12 reproductive cycles (x-axis). An example of the raw data for the simulation of the 1000 mothers can be seen in the ESM “Figure S9. Individual forward simulation allocation data for 1000 mothers for the selected hump-shaped scenario”. However, we do not think it would help the readers understand the figure if we added these 1000 lines of raw simulated data. If the referee is instead mentioning the laboratory data from Lord et al. [10], then the quadratic fit of the tsetse laboratory data is the dashed black line visible in Figure.2. However, we do not have any empirical data for maternal reserves or relative allocation over time and we would only be able to add the empirical data for maternal allocation in panel a of figure.1. We feel such partial presentation of data would confuse the reader and decided not to modify the figure.

I. 362: I do not understand and what a balance between intrinsic and extrinsic mortality means. In nature, both types of mortality strongly interplay, leaving no chance to tease them apart.

RESPONSE: Point taken, this was indeed unclear. We have modified lines 351-354:

“We also do not consider damage accumulation depending on maintenance, which would increase the risk of damage-associated mortality with age, and potentially shift the allocation trade-off towards increased reproduction. “

I. 369-373: This is clearly an overstatement pushing too far the adaptive interpretation. Reproductive restraint has been involved for young, not old individuals (see seminal papers by Curio). From a more parsimonious viewpoint, a decreased allocation with increasing age reflects senescence.

RESPONSE: We agree with the reviewer that we here predict reproductive senescence and with our model we are looking for an evolutionary explanation for senescence from a functional perspective. We are tracking maternal reserves and relative maternal allocation, which enables us to show that individuals make the strategic choice when getting older to allocate less to reproduction despite having as much reserves as younger females. We do not agree that this pushes the adaptive interpretation too far, rather we argue that this pattern of optimal allocation is driven by evolutionary constraints with age-dependent effects on energy dynamics. This concurs with the findings of McNamara et al. [5] showing how reproductive senescence can be condition-dependent. In their model, later-life reproductive restraint is driven by reproduction damage and an increase in associated mortality risk. In our model, such later-life ‘restraint’ is driven by age-dependent energy acquisition and energetic costs, which increases the risk of condition dependent-death (starvation) when investing as much as younger mothers.

We clarified this at lines 369-374:

“Relative allocation decreases with age and older females allocate less reserves to reproduction in comparison to younger females, regardless of their own reserves. This concurs with predictions of adaptive later-life reproductive restraint as a functional explanation for reproductive senescence [20], whereby maternal allocation decreases with age to reduce risks of increased mortality associated with accrued damage due to reproduction [20] or starvation with declining energy dynamics.”

I. 382-384: Alternatively, the lack of reproductive senescence in the wild might be due to a shorter lifespan in the wild.

RESPONSE: Fair point. We have clarified lines 380-382:

“The lack of reproductive senescence in the wild could be linked to shorter lifespans, with wild flies being more susceptible to death from starvation and predation [44,81].”

J. M. Gaillard

Referee: 3

Comments to the Author(s)

In this manuscript the authors describe an optimality model that is used to investigate the conditions that favour hump shaped patterns in maternal investment in offspring throughout the life of the mother.

Overall, I think the model results are interesting and the manuscript is well written.

I therefore have only a few comments and suggestions for the authors. Some of these are just minor details, but changes would still help clarify the m.s.

RESPONSE: We thank the referee for their positive feedback and helpful comments and suggestions which helped us clarify the manuscript.

My only slightly larger/more general concern is about the way you write about results from statistical tests on simulation results. While these results represent the expected population level average maternal investment with respect to age, they do not necessarily represent very well the individual choices or strategies. I have no doubt the authors agree with me on this, but I think it is necessary to clarify this in the text, especially in the discussion.

RESPONSE: This is a good point, and we agree that it is important to consider variation at the individual level. Stable individual differences in state-dependent adaptive behaviour have been shown to occur in another theoretical study in ecological contexts of intermediate favourability [11] which could be similar to what tsetse experience with rich food (vertebrate blood) and high risk (host swatting defences and predation). Our results represent the expected population-level average maternal allocation with respect to age, and it is worth noting that there is no variation within populations in the strategies: for a given parameter set, there is a single optimal strategy, and individual-level variation *in realised behaviour* emerges from stochastic events in the simulations. The individual trajectories are fairly similar as can be seen for example in the Fig.9 of the ESM. There are no strong divergences of behaviour between individuals, and we show the variability in maternal allocation with the error bars.

We now reflect on this more in the discussion at lines 356-364:

“Our results represent the expected population level average maternal allocation with respect to age, which may not necessarily capture the individual strategies. Stable individual differences in state-dependent adaptive behaviour have been shown to occur in another theoretical study in ecological contexts of intermediate favourability [79] which could be similar to what tsetse experience with rich food (vertebrate blood) and high risk (host swatting defences and predation). Although there is no variation within populations in the strategies in our model, for a given parameter set there is a single optimal strategy, individual-level variation in realised behaviour emerges from stochastic events in the simulations. However, there were no strong divergences of behaviour between individuals with individual trajectories being fairly similar (see ESM Fig.9).”

I. 91-92: do you actually investigate stochasticity in energetic costs and feeding success? The way I read your Table 2, it seems that there is no systematic investigation of differences in stochasticity? Is this specified somewhere else?

RESPONSE: We did introduce stochasticity but did not systematically investigate differences in stochasticity and we have now clarified at lines 92-95:

"We introduce stochasticity in energetic costs– in terms of the amount of energy required to forage successfully and individual differences in metabolism – and in feeding success. We systematically assess how allocation is influenced by age-dependence in energetic costs, feeding success, energy intake per successful feeding attempt, and environmentally-driven mortality."

I.103-104 & I. 138: Although this is at least partially explained later in the ms, I think it would be valuable with a brief explanation of why or how there can be stochasticity in costs of flight and blood digestion (these could easily be thought of as relatively constant for a single individual)

RESPONSE: Each feeding attempt and consequently digestion will be quite different each time for each tsetse, because of the uncertainty in finding a host, host defences, predation risk, bloodmeal volume and so forth.

We have now explained this at lines 92-93:

"We introduce stochasticity in energetic costs– in terms of the amount of energy required to forage successfully and individual differences in metabolism – and in feeding success."

And lines 102-105:

"Tsetse have access to a rich food supply, vertebrate blood [43], but this can be highly uncertain, requiring finding a host and avoiding its defences (e.g. swatting) [37,44], which introduces stochasticity in the flight duration and distance as well as the bloodmeal volume and hence in the energetic costs of flight and blood digestion [37,39,44]."

I. 155: "fitness h", is it not better to write expected fitness here?

RESPONSE: Yes, we have added 'expected' here.

I. 186-187: It was unclear to me at first that this was the starting reserves at emergence, please rewrite to clarify.

RESPONSE: We have merged the sentences and clarified lines 190-192:

"We simulated the life histories of 1000 mothers (ESM, code modified from [53]) following the optimisation strategy and the reserves at the start of adulthood R_1 , the distribution of which was determined using an iterative procedure as described in [54] (ESM)."

I. 267-268: are you not repeating yourself here?

RESPONSE: We have now updated the text lines 270-271:

"Exploring first the baseline case of the model, the optimal allocation decision is dependent on maternal reserves but independent of age (Figure 1.a, grey line)."

I. 342-343 could you link these empirical patterns more strongly to your model? Maybe through specifying which parameters of your model might be involved? It is unclear to me how relevant these empirical results are to your model results.

RESPONSE: We specified which parameters correspond to which traits lines 331-336:

"The evidence for age-dependence in such traits is wide-ranging across systems from a decrease in energetic costs with an improved lactation ability (e.g. seals [25]), an improved energy transfer efficiency (rats [77]), or reduced metabolic requirements post maturation (tsetse [60,61,69]), to an increase in energy intake with an improved mobility post maturation (tsetse [60,61,70]), or a decrease in energy intake later in life because of gut deterioration (Drosophila [78]) or other physiological deteriorations."

I.389-396. While I do not strongly disagree with this paragraph in any way, I am not quite convinced it is a good ending as the potential implications mentioned here are quite far from the actual model results and new insight on these matters have yet come from the model.

RESPONSE: We agree with the referee, and we have now removed this paragraph and instead conclude lines 387-399 with the main finding of our model (in explaining increase-then-decrease optimal allocation through age-dependent energy dynamics), and a comment on the generalisability of our framework to other iteroparous breeders (see responses above, in particular to Referee 1 points 3 and 6).

ESM: Would it be possible to use the same name for the time axis in all the figures? Is there a difference between larviposition interval and number of reproductive cycles?

RESPONSE: We thank the referee for pointing it out and we have now used the same name for the time axis in all the figures: “Time (number of reproductive cycles)”.

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Appendix B



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18/01/22

Dear Prof Kruuk

We are resubmitting our research manuscript entitled "*Incorporating effects of age on energy dynamics predicts non-linear maternal allocation patterns in iteroparous animals*". We are thankful that our manuscript has been accepted for publication in *Proceedings of the Royal Society B*.

We are grateful to the associate editor and reviewers for the minor revisions suggested and their recommendation for publication. We have made the suggested changes in light of each reviewer's comments. Our detailed responses are provided below.

We describe original work that is not being considered for publication in any other journal. We declare having no competing interests. All the authors gave final approval for publication and submission to *Proceedings B* of the final manuscript.

We appreciate your time and are looking forward to your response.

Best regards,

Antoine Barreaux, on behalf of all authors

Associate Editor

Board Member: 1

Comments to Author:

The authors have done an excellent job in responding to reviewers' comments. I do encourage the authors to address the remaining issues raised by the reviewers. In particular, J.M. Gaillard explains in detail why he is concerned about the use of quadratic models in this case. I think the authors will have little problem in addressing these points and I think this paper will advance the field.

RESPONSE: We thank the associate editor for their time, their interest in our research and their support throughout the submission process. We have made sure to answer all the remaining helpful queries made by the two referees. Please find our detailed answer below.

Referee: 1

Comments to Author:

Dear Editor,

I reviewed a previous version of this manuscript and the authors have done an excellent job responding to my previous comments of the other reviewers. Nevertheless, I have a few comments regarding the revised manuscript. The first two issues are relatively minor. The third is more substantial.

RESPONSE: We thank the referee for their interest in our manuscript, their appreciation of our previous answers, and the last few comments on our approach and conclusions.

1. Line 22, line 388 (and elsewhere): There are two major explanations for the evolution of senescence: optimality and mutation pressure (e.g., Partridge and Barton 1993). The optimality explanation posits that ageing could evolve as part of an optimal life history in which there is an antagonism between performance early in life and performance late in life. In contrast, mutation pressure could lead to ageing

because selection against late-acting deleterious mutations will be weak. These two explanations cannot be distinguished based upon the relationship between parental allocation and parental age. Thus, I do not think it is correct to say that “optimal” allocation is hump shaped across ages in diverse taxa. The hump-shaped pattern may be widespread, but in most cases we do not know whether the shape of this curve can be explained by the optimality hypothesis or the mutation pressure hypothesis.

RESPONSE:

This is a fair point, in terms of distinguishing the hump-shaped pattern across empirical studies.

We have clarified lines 24 and 425 by removing the word “optimal”. That said, our model is focused on the optimality explanation, as we assume no mutations and as suggested by referee 2, we have clarified that assumption lines 246-248:

“As such, we do not impose a decline in selection in later life as all offspring are potentially equally valuable at all ages (for for the same maternal allocation), and we assume there are no mutations.”

And we have also clarified the start of the discussion lines 338-343:

“Our model predicts optimal maternal allocation of resources is non-linear with age, when there is age-dependence in key drivers of energy dynamics. Such a non-linear relationship between parental allocation and age has been found in many species. Our model assumes no mutations and hence provides further theoretical insight into the drivers of age-dependent allocation in terms of optimal life-history allocation, although we acknowledge that similar patterns can also arise from changes in mutation pressure which are not considered in our model.”

2. Line 51: genes should be alleles.

RESPONSE: Corrected.

3. Line 80-87 and 236-238: I have a hard time reconciling these two sections. In the first passage, the authors acknowledge that offspring quality may decline with parental age, and as a consequence later-born offspring might have lower reproductive value to parents than earlier-born offspring. The authors suggest that this pattern is not accounted for in current models of senescence, which focus on fecundity and not offspring quality. However, in the second passage the authors state that they are assuming that the reproductive value of earlier-born and later-born offspring is the same. I understand that the goal is to examine whether maternal allocation strategies can generate hump-shaped allocation curves without invoking a decline in the strength of selection with age. However, it is not clear whether / how the model predictions will change if the assumption that all offspring contribute equally to parental fitness is relaxed.

RESPONSE:

We appreciate that the phrasing of these two sections may have caused confusion. To clarify, we assume here that the reproductive value of earlier-born and later-born offspring is *potentially* the same for a given level of maternal allocation as we do not want to force offspring quality to decrease with age by invoking a decline in the strength of selection with age. However, mothers may vary allocation of resources to offspring with age, which will then result in offspring of different quality and may lead to reproductive senescence if offspring quality decreases with maternal age. So, offspring quality may indeed vary with age depending on the optimal strategy.

We agree that it could be interesting to relax the assumption that all offspring potentially contribute equally to parental fitness whatever the parental age, yet this is beyond the scope of our current study. Making assumptions about the offspring as well as parents would make the model considerably more complex and would require careful thought about how these would impact model predictions. We hope our model will inspire such future work exploring the effects of varying assumptions about the offspring.

We clarified lines 246-250 by adding that all offspring are potentially equally valuable, but that offspring quality may change if maternal allocation changes with age:

“As such, we do not impose a decline in selection in later life as all offspring are potentially equally valuable at all ages (for for the same maternal allocation), and we assume there are no mutations. However, mothers may vary allocation of resources to offspring with age, which will then result in

offspring of different quality and may lead to reproductive senescence if offspring quality decreases with maternal age.”

We added lines 371-374 in the discussion:

“Imposing a declining selection with age by relaxing the hypothesis that all offspring are equal may potentially nuance our predictions about non-linear parental allocation. We hope our model will inspire future work on age-dependent allocation under varying assumptions about offspring quality.”

Referee: 2

Comments to the Author(s)

I warmly thank the authors for having provided both detailed responses to comments and a carefully-revised manuscript. I was really convinced by the changes performed (in particular the link between the authors' model and the capital-income breeder continuum). However, I still have two concerns about this paper, which should be easy to solve.

RESPONSE: Thank you for warm thanks and positive and helpful comments and suggestions.

First, the two assumptions the authors clearly stated in their responses to the first reviewer's comments should be presented and discussed more thoroughly in the paper itself. The « as » in l.322 in the version with tracked changes should be replaced with « as we assume ». Indeed, there appear to be quite strong assumptions because in the real world (1) the timing of reproduction throughout the lifetime matters a lot in terms of fitness but in case the population is stationary, and (2) mutations should occur soon or later.

RESPONSE: We appreciate these are key assumptions and we have clarified these, and their implications, in the revised version. As specified above in our answer to referee 1 we have added more details about our assumptions about the absence of mutations and we emphasize that the value of all offspring is *potentially* equal across parental age.

We have added that we assume there are no mutations in our model lines 246-248 and we have clarified the start of the discussion lines 338-343 how important it is when coming back to the general theory of ageing and the distinction between optimality and mutation pressure explanations. We clarified lines 246-250 that all offspring are potentially equally valuable, but that offspring quality may change if maternal allocation changes with age. We also discussed relaxing that assumption lines 371-374.

Second, I still disagree with the use of quadratic models to infer an initial increase of reproductive performance until a peak or plateau followed by a decrease of reproductive performance. To illustrate my concern, consider the following age-specific trajectory of performance (rp):

```
age<-c(1,2,3,4,5,6,7,8,9,10,11)
```

```
rp<-c(0.01,0.05,0.20,0.60,1.2,1.5,1.6,1.7,1.75,1.75,1.75)
```

This rp trajectory is characterized by a continuous increase of reproduction with age until a plateau is reached. There is no evidence of any reproductive senescence from this trajectory.

Now fit a quadratic model:

```
mod<-lm(rp ~ age + I(age^2))
```

and look at the estimates:

Coefficients:

	Estimate	Std. Error	t value	Pr(> t)
(Intercept)	-0.708182	0.206638	-3.427	0.008992 **
age	0.469056	0.079144	5.927	0.000351 ***
I(age^2)	-0.021853	0.006424	-3.402	0.009333 **

The outcome is a statistically significant negative quadratic coefficient in absence of reproductive senescence. This clearly demonstrates that a negative quadratic term cannot be interpreted in terms of decreasing performance in late life. Although additional models ran by the authors convinced me that

their findings are robust, I would ask the authors to add an explicit statement about the caution required when interpreting quadratic parameters in terms of senescence to avoid the pitfall mentioned just above.

J.M. Gaillard

RESPONSE: Thank you for providing this effective example. We agree that caution is required when interpreting quadratic parameters in terms of senescence to infer an initial increase of reproductive performance until a peak or plateau followed by a decrease of reproductive performance.

We added the following statement in the methods lines 266-273:

“It is worth noting that caution is required when interpreting quadratic parameters in terms of senescence to infer an initial increase of reproductive performance until a peak or plateau followed by a decrease of reproductive performance. This is because the presence of a statistically significant negative quadratic coefficient does not necessarily indicate a hump-shaped curve but can also represent a case of diminishing returns where allocation plateaus in later-life but does not decline (hence, no reproductive senescence).”