nature portfolio

Peer Review File

Proper micro-environment alleviates mortality in laboratory mouse breeding induced by litter overlap and older dams

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

Overview
This is a potentially interesting study. The study design demonstrates a thoughtful consideration of both social and environmental variables, which are crucial for understanding the complexities of pup survival rates. I would like to congratulate the authors for their novel work. I request that the authors clarify some doubts along the text esp. in Statistical Analysis. In addition, I would also recommend checking the Results and Discussion structure and re-write it. As the study is well designed and my main concerns can be easily addressed, I truly believe that the authors can make this manuscript suitable to be accepted for publication in Communications Biology. For more details, please see my comments below.
Abstract
1. I would suggest including a brief statement about the hypothesis to provide readers with a clear understanding of the study's objectives and the problems being addressed.
Introduction
2. Page 4, Line 60 to 64: I would suggest re-writing the sentence specifying what the social factors are and what the environmental factors are. Here since only micro-environmental factors are mentioned, it would be appropriate to use the word micro-environment instead of environment.
3. In some places, the term environmental factor is used interchangeably throughout the manuscript for both macro and micro-environment. To make it clear to the reader, Please use the term "cage macro-environment" for variable Season, weekdays, months, breeding room and "cage micro-environment" for variables cage temperature, vibration, motion events, and light intensity.
4. Page 4, Line 64 to 67: The sentence is unclear. I would suggest re-writing it as "In our aforementioned experimental study, we found risks for entire litter loss and individual pup loss for overlapped litters increased by 2.3 and 1.8 times, respectively, compared to non-overlapped litters in trio-cages (with two adult males and one male)
5. Page 4, Line 80 to 82 : Here, mention the social and environmental factors (macro and micro) included in the study.
Results
6. Page 12, Line 217 to 219: 98.3%+66.3%+29.1%_8.1% =201.8%. It seems there might be an error. The percentage provided should add up to 100% if it represents the complete distribution of data.
7. Page 14, Line 252 to 253: "The Decision Tree obtained was able to describe pup death based on 10 social and environmental attributes (factors)…". Here I would suggest mentioning the social and environmental factors that were included in the decision tree.
8. Page 15, Line 276 to 277: Sentence is incomplete/unclear.
9. Overall, I would recommend providing model details (with ANOVA table) as supplementary information for all three models run. Were there any significant interaction effects? Were all possible two-way and three-way interactions for both social factors and environmental factors (Macro and micro) considered in the Model? If not, I would suggest re-running the models including them.
10. I would request the authors to please specify whether the nest score variable was categorized under social factors or cage micro-environmental factors in this study or was it considered a separate factor altogether (since nest score can be influenced by both social and environmental factors)? Additionally, I would like to suggest that results pertaining to pup mortality related to nest score be presented under a separate subheading labeled "Nest Score" for clarity.
11. Also, I would also recommend combining the results for social factors currently mentioned under the same subheads given under Litter characteristics and Probability of pup mortality. I would suggest re-writing the results section and focus mainly on how pup mortality is affected by social and environmental (macro and micro factors (which is the aim of the study) and all additional information pertaining to the characteristics of litter and cage microenvironment can be moved to the supplementary information section, with proper citations given in the results section.
Discussion

12. Page 17, Line 299 to 305; Page 19, Line 342 to 343 and Page 26, Line 496 to 106: I would suggest moving these under a separate subhead “Nest score”, where results for pup mortality based on nest score could be discussed.
13. Page 18, Line 311 to 312 – Already mentioned in Introduction (See Page 4, Line 64 to 67).
Statistical Analysis
14. Page 36, Line 704 to 706 : Sentence is incomplete.
15. As mentioned earlier in the Results section, could the authors kindly clarify whether the “nest score” variable was included as part of the social factors or the cage micro-environmental factors considered in your study? Why is it not mentioned in the model? In case of a misunderstanding from my side, I apologize and urge the authors to clarify. Given the significance of nest characteristics in influencing litter survival, I believe clarifying this aspect would greatly enhance the interpretation of your findings.
16. As suggested earlier in the results section, I would suggest running the models with all possible two-way and three-way interactions for social and environmental (macro and micro) factors.

References
17. Please check the whole list. Please ensure all references are formatted according to the Communications Biology’s standard referencing style. Please see Submission guidelines | Communications Biology (nature.com).
Other considerations
18. Word limit for the main text is ~ 5000 words (not including abstract, methods, references and figure legends). It currently exceeds 7500 words. Please concise the manuscript accordingly.

Reviewer #2 (Remarks to the Author):

Review for:
Laboratory mouse mortality: younger dams without older litters in the cage increases new-born survival
Gabriela Munhoz Morello1,2*, Sara Capas-Peneda1,2, Sophie Brajon1,2, Sofia Lamas1,2, Igor M Lopes3, Colin Gilbert4, I. Anna S. Olsson1,2

The authors have identified a problem of pup mortality in laboratory mice that affects research and sustainability. In the present study, the authors suggest litter overlapping, dam age, temperature and other factors may be driving mortality rates and they make straightforward suggestions to mitigate these factors.

This reviewer is overall impressed by the statistical and methodological rigor the authors have taken in drafting their manuscript. The coverage and depth of their introduction is noteworthy and highly informative. One potential major issue of a conceptual nature and a few minor issues are described below for the authors’ consideration.

Minor issue: Change title to reflect the main point of the paper. As is, the subtitle is a bit convoluted and it is difficult to unravel what it means. This reviewer suggests something broader and more descriptive. For example:
Laboratory mouse mortality: litter overlap, dam age, and other considerations

Major issue: The manuscript suffers considerably from confounding cohabitation factors. The authors describe an important problem in animal care and husbandry occurring during pre-weaning, but in their study, they introduce potential effects of sire and alt-dam cage-mates. The authors show that overlapping litters is an important driver of pup mortality, but this result only seems relevant to animal care facilities that permit such co-habitation in their protocols. Is such a practice widespread in the UK (perhaps in response to space limitations and adherence to the

Three R's Principle)? This reviewer, for example, has only ever worked on dams that were single-housed before giving birth, if only to maintain strict pedigrees. Sires are removed shortly after successful breeding is confirmed. Can the authors comment on how common co-habitability is? In other words, how relevant is the current study in interpreting the 7.5% pre-weaning mortality mentioned in the introduction (line 38)?

Minor issue: Figure 1: Is there a reason that Fig1 a, b, and c show the overlapping and non-overlapping bars in three different formats? (i.e., stacked in a, touching pairs in b, and a gap between pairs in c.)

Minor issue: Line 601 (Animals and Housing) The detail about co-habitation seems essential for understanding the entire study. If Materials and Methods are to remain last in this manuscript, it would serve the authors' immensely to repeat this detail above in the abstract and/or introduction to provide better context of the cage environment and the meaning of overlapping litters.

Minor issue: Line 694 (Analysis…) The authors describe building models by adding one independent variable at a time and keeping variables with $p < 0.05$. Are they referring to only main effects or interaction terms as well when considering significance? (This reviewer recommends specifying this in the text.)

Dear all –

We thank you very much for your careful reading and positive appraisal of the manuscript. We have endeavoured to address your comments as set out below.

Best regards,

Gabriela Morello and co-authors

Reviewer #1

Abstract

1. I would suggest including a brief statement about the hypothesis to provide readers with a clear understanding of the study's objectives and the problems being addressed.
Hypothesis was added in Lines 18-20 of the Abstract: "We hypothesized that pre-weaning mortality in laboratory mouse breeding is associated with cage social and macro/micro-environment conditions.".

Introduction

2. Page 4, Line 60 to 64: I would suggest re-writing the sentence specifying what the social factors are and what the environmental factors are. Here since only micro-environmental factors are mentioned, it would be appropriate to use the word micro-environment instead of environment.
We specified which factors of which category (social, macro-, micro-environment) were studied, starting in Lines 77-81 and wherever necessary throughout the text. Lines 77-81: "The present study aimed to evaluate pup survival as a function of cage social factors (Dam Age, Litter Overlap, Age and Size of the Older Litter in the Cage when the focal litter is born, and Number of Pups Born in the Focal Litter), cage micro-environment (Cage Temperature, Light Intensity, Vibration, frequency and duration of human Motion Events near the cage, and Nest Score), and cage macro-environment (Season and Weekday of Birth of the Focal Litter)."

3. In some places, the term environmental factor is used interchangeably throughout the manuscript for both macro and micro-environment. To make it clear to the reader, Please use the term "cage macro-environment" for variable Season, weekdays, months, breeding room and "cage micro-environment" for variables cage temperature, vibration, motion events, and light intensity.
The distinction between macro and micro environment has been clarified as advised throughout the entire text.

4. Page 4, Line 64 to 67: The sentence is unclear. I would suggest re-writing it as "In our aforementioned experimental study, we found risks for entire litter loss and individual pup loss for overlapped litters increased by 2.3 and 1.8 times, respectively, compared to non-overlapped litters in trio-cages (with two adult males and one male)
Suggestion accepted, thank you. Lines 69-71 state: "In an experimental study with 109 litters (55 in trio-cages), we found risks for entire litter loss and individual pup loss increased by multiples of 2.3 and 1.8, respectively, compared to non-overlapped litters[8]."

5. Page 4, Line 80 to 82 : Here, mention the social and environmental factors (macro and micro) included in the study.
I mentioned all social, macro-, and micro-environment as advised, following the structure presented in reply #2. In the methodology section, I state the additional macro-environment and social factors that were initially considered, but dropped due to multicollinearity issues.

Results

6. Page 12, Line 217 to 219: 98.3%+66.3%+29.1%_8.1% =201.8%. It seems there might be an error. The percentage provided should add up to 100% if it represents the complete distribution of data.
This is actually not an error, but I can see why this could be confusing to read, thank you for pointing this out. Perhaps the following alteration reads slightly better: "Cage Temperature fluctuations of at least 2.0°C, ≥1.5°C, ≥1.0°C, and ≥0.5°C were experienced by 8.1%, 29.1%, 66.3%, and 98.3% litters, respectively". The idea here is to show that pups experienced substantial temperature variation: a few experienced a high temperature variation of 2.0°C or more, but a lot of them experienced 0.5°C or more (thus the latter group includes pups that experienced 0.5°C all the way to 2.0°C and above). This information, however, was moved to the Supplementary Material.

7. Page 14, Line 252 to 253: "The Decision Tree obtained was able to describe pup death based on 10 social and environmental attributes (factors)…". Here I would suggest mentioning the social and environmental factors that were included in the decision tree.
This sentence was eliminated entirely, as I re-structured the Results section, as advised. Nevertheless, I added the description of factors wherever it was appropriate throughout the text and in the Tree´s legend.

8. Page 15, Line 276 to 277: Sentence is incomplete/unclear.
The entire paragraph was re-worded to (Lines 145-148): "On the right side of the Decision Tree, approximately 59.1% of the 716 pups of younger dams in overlapped litters died. When five older pups or more (≥ 7 d-old) were present in the cage, 66.4% of 581 new-born pups died (Fig. 3, Node 9). Overall, the left upper side of the Decision Tree (green portion of Fig. 3) depicts a path of pup survival."

9. Overall, I would recommend providing model details (with ANOVA table) as supplementary information for all three models run. Were there any significant interaction effects? Were all possible two-way and three-way interactions for both social factors and environmental factors (Macro and micro) considered in the Model? If not, I would suggest re-running the models including them.
I apologize for not having that information as clear as it should be. I re-structured the Results section, as advised, but left the sub-section "Pre-Weaning Mortality". In this sub-section, I mentioned the overall results from the statistical models, as stated in Lines 105-111: "Results considering the whole dataset (Suppl. Table 1) revealed that Pup Probability of Death was affected by Litter Overlap while interacting with Size of Focal Litter ($t_{(1, 2893)} = -2.34$, $P = 0.019$), Size of Focal Litter in a quadradic fashion ($t_{(1, 2893)} = -4.21$, $P = 0.068$), and Dam Age ($t_{(1, 2893)} = 4.64$, $P < 0.001$). When analysing only the overlapped litters, Pup Probability of Death was affected by Number of Older Pups ($t_{(1, 2893)} = 4.54$, $P < 0.001$), Age of Older Pups in a quadratic

fashion ($t_{(1, 2893)} = -3.33$, $P < 0.001$), Size of Focal Litter in a quadratic Fashion ($t_{(1, 2893)} = 2.16$, $P = 0.031$), and Dam Age ($t_{(1, 2893)} = 2.87$, $P = 0.004$, Suppl. Table 2)."

As referred in this paragraph, I provided all the statistical information (solutions for fixed effects) as Supplementary Tables (ANOVA), as requested.

We did consider all possible interactions and even higher-order terms, as stated in lines 525-529: "The macro-environmental factors Weekday, Season, and Breeding Room were then tested one at a time as confounders, followed by possible two- and three-way interactions and higher order terms, eliminated from the models when not significant ($P \leq 0.050$). Least-square means were compared for Litter Overlap (Model 1) considering 95% confidence interval, and probability predictions for pup death were obtained from Model 2."

10. I would request the authors to please specify whether the nest score variable was categorized under social factors or cage micro-environmental factors in this study or was it considered a separate factor altogether (since nest score can be influenced by both social and environmental factors)? Additionally, I would like to suggest that results pertaining to pup mortality related to nest score be presented under a separate subheading labeled "Nest Score" for clarity.

Nest Score has been classified as a micro-environmental factor for the pups, although we agree that this could also fit the social-factor category. A third-level sub-heading "Nest Score" was added both in the Results and Discussion sections, as advised.

11. Also, I would also recommend combining the results for social factors currently mentioned under the same subheads given under Litter characteristics and Probability of pup mortality. I would suggest re-writing the results section and focus mainly on how pup mortality is affected by social and environmental (macro and micro factors (which is the aim of the study) and all additional information pertaining to the characteristics of litter and cage microenvironment can be moved to the supplementary information section, with proper citations given in the results section.

The majority of information stated under Litter Characteristics was moved to the Supplementary Material and cited wherever appropriate. Both the results from the statistical models and from the Decision Tree were combined and are presented separately per each of the factors studied, as advised, thank you for this suggestion.

Discussion

12. Page 17, Line 299 to 305; Page 19, Line 342 to 343 and Page 26, Line 496 to 106: I would suggest moving these under a separate subhead "Nest score", where results for pup mortality based on nest score could be discussed.

All suggested sentences were moved to the sub-section Nest Score.

13. Page 18, Line 311 to 312 – Already mentioned in Introduction (See Page 4, Line 64 to 67).

This statement was replaced to (Lines 231-232): "This finding is consistent with our previous work, recorded in both experimental (n=109)[8] and observational (n>34k) litters[10]."

Statistical Analysis

14. Page 36, Line 704 to 706 : Sentence is incomplete.
This entire paragraph was re-written to (Lines 531-535): “Cage micro-environment (Nest Score, Cage Temperature, Light Intensity, Cage Vibration, and Motion Events) was monitored for Day 0 of each studied litter, the most critical period for pup mortality, in addition to the aforementioned macro-environmental and social data. A Data Mining approach was used to evaluate the effects of all measured conditions of cage micro-environment and social factors. ”

15. As mentioned earlier in the Results section, could the authors kindly clarify whether the “nest score” variable was included as part of the social factors or the cage micro-environmental factors considered in your study? Why is it not mentioned in the model? In case of a misunderstanding from my side, I apologize and urge the authors to clarify. Given the significance of nest characteristics in influencing litter survival, I believe clarifying this aspect would greatly enhance the interpretation of your findings.
Yes, we absolutely needed to clarify this, thank you for pointing this out, we appreciate it. Nest score, as I replied above, was considered a micro-environment factor, thus monitored for the subset of 172 litters. This is why it does not show up in the statistical models (used to explain pup-probability of death by using the entire dataset and the dataset with only overlapped litters, please see Lines 490-493), but it does show in the Decision Tree, which was the classification method that we used to describe mortality in the micro-environment subset. Nest Score has now been referred throughout the text as a micro-environment factor, and added as one of the factors that entered the Decision Tree modelling, as stated in Line 531).

16. As suggested earlier in the results section, I would suggest running the models with all possible two-way and three-way interactions for social and environmental (macro and micro) factors.
Interactions and higher-order terms had been added in the statistical models before the initial submission, as now clarified in the text (Line 526). The interactions among micro-environment attributes are visually seen throughout the Decision Tree, as the conditions of nodes towards the bottom are predicted on the conditions of the upper Decision Nodes.

References

17. Please check the whole list. Please ensure all references are formatted according to the Communications Biology’s standard referencing style. Please see Submission guidelines | Communications Biology (nature.com). Other considerations
References were reviewed.

18. Word limit for the main text is ~ 5000 words (not including abstract, methods, references and figure legends). It currently exceeds 7500 words. Please concise the manuscript accordingly.
We have reduced the manuscript to 4976 words.

Reviewer #2

1. Minor issue: Change title to reflect the main point of the paper. As is, the subtitle is a bit convoluted and it is difficult to unravel what it means. This reviewer suggests something broader and more descriptive. For example:
Laboratory mouse mortality: litter overlap, dam age, and other considerations
Thank you for your suggestion. The title was changed to “Mouse breeding: increased pup mortality with litter overlap and older dams, alleviated by proper micro-environment”, in an attempt to be more informative, less convoluted, and following the journal’s Style and Formatting Guide in that “The title should summarize the key findings in a single statement of 15 words or fewer”.

2. Major issue: The manuscript suffers considerably from confounding cohabitation factors. The authors describe an important problem in animal care and husbandry occurring during pre-weaning, but in their study, they introduce potential effects of sire and alt-dam cage-mates. The authors show that overlapping litters is an important driver of pup mortality, but this result only seems relevant to animal care facilities that permit such co-habitation in their protocols. Is such a practice widespread in the UK (perhaps in response to space limitations and adherence to the Three R’s Principle)? This reviewer, for example, has only ever worked on dams that were single-housed before giving birth, if only to maintain strict pedigrees. Sires are removed shortly after successful breeding is confirmed. Can the authors comment on how common co-habitability is? In other words, how relevant is the current study in interpreting the 7.5% pre-weaning mortality mentioned in the introduction (line 38)?
Thank you for your feedback, I appreciate you sharing your experience.

Regarding the issue of Dam and Sire age being confounded, we added to the discussion the following statement (Lines 226-228):” Furthermore, interpretation of results related to Dam Age should be done with caution, for being confounded with Sire Age in this study. It is known that the presence of the sire can facilitate pup survival23, but not much has been done to evaluate the effect of Sire Age on paternal care.”

Regarding the incidence of Litter Overlap in “research”, I now see that I have not conveyed exactly the message I was originally planning about the overlap problem. This study addresses Litter Overlap as a problem in laboratory **mouse breeding** as well as in cages with mice directly acquired for/used in research. In fact, I believe ethical committees should question the occurrence of overlapping litters in the context of research. However, in 100% of all breeding facilities that my peers and I have visited or collaborated with in three different countries (including the Wellcome Sanger Institute, Crick Institute, Babraham Institute, and Charles River, UK), where mice are mated to produce the mice that we use in research, housing configurations included breeding continuously in pairs or in cages with multiple females (mostly trios), with Litter Overlap often occurring. For pairs it is less typical but does happen, particularly if females become pregnant again by being mated to the post-partum oestrus, and the weaning of their previous litter is delayed. Breeding males are usually maintained in the cage with the female(s) to allow for uninterrupted breeding. I can share some of the results from our previous studies that addressed Litter Overlap in breeding facilities since 2017:

- In a retrospective study with 219975 pups from 34949 C57BL/6 litters from trio- (2F+1M) and pair- (1F+1M) housed breeding mice (data from 2010-2019), Litter

Overlap happened in 50% and 57% of the litters in two model breeding facilities in the UK, the Wellcome Sanger and the Babraham Institue (Please see Morello et al., 2020. PloS one 15.8 (2020): e0236290).

- In an experimental study in the Babraham Institute, with 54 C57BL/6 single housed breeding female mice and 55 trio-housed breeding mice, Litter Overlap happened in 36% of the litters from trio-housed mice (Please, see Brajon et al. 2019. Applied animal behaviour science, 218, 104827).

- In recently unpublished data collected in our AAALAC-accredited Breeding facility (i3S, Portugal) for the years of 2022 and 2023 (of 2986 pups from 529 C57BL/6 litters), Litter Overlap occurred in 42% of the litters from trio- and pair-housed breeding mice (60% and 27% of the 238 trio- and 291 pair-housed litters, respectively).

- Additionally, we interact with several breeding facilities across Europe and a few in the US, and Litter Overlap is something that is often seen by animal care-takers in most of our collaborating facilities.

Regarding the comment on the 7.5% mortality rate, as we state in Lines 44-47 and 241-245, we have reasons to suspect that the actual pre-weaning mortality is substantially higher than the 7.5% given by the main B6 supplier. In our previous mortality studies, we compared the mortality rate that we obtained by counting pups daily with historical records from the same breeding mice (whose pups were counted once a week by animal care-takers, not necessarily within 48h post-partum). We learned that actual mortality was most often 10%-30% higher than the historical records of the same breeding mice (historical and counted litters were staggered). Later, we compared the same groups for number of pups weaned/litter and found no difference. This was an important comparison to do, so that we knew that our pup-counting protocol was safe and not increasing mortality. We concluded that counting pups daily led to a more accurate picture of the actual number of pups born in a litter, thus a more accurate mouse pre-weaning mortality estimate. We believe, therefore, that the mortality figures are more coherent with 15%-30% in the context of breeding.

As we mentioned above, the males (and mate females in trios) are not removed from breeding cages before retirement. Considering the number of mice used in research in the EU and that trio- and pair- housing of mice is fairly common, and a very conservative mortality rate of 10%, this means that half a million additional mice are produced yearly just in the EU, to compensate for pre-weaning mortality and still supply the demand. Now, if we consider that C57BL/6 mice (study subjects) are one of the most used mouse strains for science, and the background to many other GM strains, accept an estimated pup mortality rate of 20%, that we are able to create strategies in the future to avoid litter overlap, and that overlap happens in 30-50% of the litters (let´s consider 40%), on average, eventually we may be able to prevent the birth and death of over 300k mice yearly just in the EU (i.e. 1.42M – 1.09M, which would be the number of dead pups if there were no overlapped litters, considering that mortality in overlapped litters is on average 1.6 higher than that of non-overlapped litters), in addition to a reduction in the use of adult breeders. These may be optimistic estimates, but even if we achieve half of this goal, that still means over 150k mice/yr, or 1.5M mice in 10 years, just in Europe.

Thus, I would argue that this study is very relevant for reducing the number of mice produced and making mouse production more sustainable, cost-effective, and welfare-friendly (by consequently reducing the number of adult breeding mice needed). Housing schemes and

weaning assiduity is something we may be able to change/adapt. Therefore, publishing these results has some urgency.

I thank you for questioning this, because my team and I failed to realize that mice users do not necessarily know how mice are commonly bred. For this reason, the following statement was added to the Introduction:

Lines 62-68: “Litter overlap happens in any housing configuration in which males and females’ co-habit to allow for uninterrupted breeding. To our knowledge, many breeding facilities house mice continuously either in pairs (one male and one female) or cages with a male and multiple females. In the latter configuration, litter overlap is a normal consequence of the set-up. For pairs, it is rare but does happen, particularly if females become pregnant again by being mated to the post-partum oestrus, and the weaning of their previous litter is delayed.”

3. Minor issue: Figure 1: Is there a reason that Fig1 a, b, and c show the overlapping and non-overlapping bars in three different formats? (i.e., stacked in a, touching pairs in b, and a gap between pairs in c.).
No specific reason. In fact, two of these figures were conveying the same information, thus Fig 1a was removed. The remaining graphs were standardized and re-organized to be coherent with the changes in the structure of the Results section, as advised by Reviewer 1.

4. Minor issue: Line 601 (Animals and Housing) The detail about co-habitation seems essential for understanding the entire study. If Materials and Methods are to remain last in this manuscript, it would serve the authors’ immensely to repeat this detail above in the abstract and/or introduction to provide better context of the cage environment and the meaning of overlapping litters.
Thank you for pointing this out. This detail was added to Line 23 of the Abstract, and to Lines 62-68 of the Introduction. Please, refer to my answer to question # 4.

5. Minor issue: Line 694 (Analysis…) The authors describe building models by adding one independent variable at a time and keeping variables with $p < 0.05$. Are they referring to only main effects or interaction terms as well when considering significance? (This reviewer recommends specifying this in the text.)
We did apply a stepwise process with bidirectional elimination considering 0.05 significance for both main effects, interaction and higher order terms. To clarify this, we rewrote Lines 525-529 to: “The macro-environmental factors Weekday, Season, and Breeding Room were then tested one at a time as confounders, followed by possible two- and three-way interactions and higher order terms, eliminated from the models when not significant ($P \leq 0.050$).”

REVIEWERS' COMMENTS:

Reviewer #1 (Remarks to the Author):

After reviewing the revised manuscript, I found that all the suggestions have been effectively addressed. I am pleased to inform that I recommend acceptance of the manuscript.

Reviewer #2 (Remarks to the Author):

This reviewer appreciates the considerable effort the authors have taken in addressing all issues. Their responses have clarified much and more. This reviewer has no further comments or recommendations for the authors.

Dear reviewers –

Thank you for taking the time to review the first and revised versions of this manuscript. We believe that the manuscript has substantially improved since its first version, thanks to your much appreciated suggestions and comments.

Best regards,

Gabriela Morello and co-authors.