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Nonlinear disease tolerance curves reveal distinct components of host responses to viral infection

Vanika Gupta and Pedro F. Vale

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Original submission: 11 April 2017
Revised submission: 30 May 2017
Final acceptance: 31 May 2017

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

Review History

RSOS-170342.R0 (Original submission)

Review form: Reviewer 1

Is the manuscript scientifically sound in its present form?
Yes

Are the interpretations and conclusions justified by the results?
Yes

Is the language acceptable?
Yes

Is it clear how to access all supporting data?
Yes

Do you have any ethical concerns with this paper?
No

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

Recommendation?
Accept with minor revision (please list in comments)

Comments to the Author(s)
Infection tolerance is likely a critical contributor to the evolution of immune systems and host-microbe interaction dynamics, but it can be a tricky thing to measure, and analytical tools for estimating variation in tolerance are still in their infancy. In this manuscript, the contribution of a JAK-STAT pathway epigenetic regulator to fly tolerance of DCV is analyzed using both linear and non-linear statistics on the relationship between viral dose and host survival. The results highlight that while linear methods might give a better resolution on the coarse relationship between increasing titer and the decline in host health, non-linear models give better leverage for defining subtler but important parameters associated with variation in tolerance. For example, while linear analyses suggest that G9a affects tolerance only in female flies, the non-linear analysis reveals that the gene affects tolerance sensitivity in both sexes, albeit with different effect sizes.

This manuscript is polished and well-written. The experiments are well-designed and the statistics are appropriate for the questions and type of data. The discussion is appropriately written in the context of the results. I have a few minor comments to increase the clarity of the reasoning behind some of the decisions made in the experimental design of this work.

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While the authors cite that they use initial dose rather than viral load at 5 dpi in order to reduce error and conform to the restrictions of the statistical method of analysis (eg. Line 137), I am keen to know whether using 5dpi viral load instead changes any of the major conclusions. For example, a new paper on resistance and tolerance to infection in flies (Howick and Lazzaro 2017, Mol. Ecol, DOI 10.1111/mec.14017) suggests that the growth dynamics of microbes after the initial dose are not always easy to extrapolate among genotypes, thus rendering initial dose something of a dicey thing to use for estimating tolerance. While there does seem to be a reasonable relationship between viral dose and viral load in this manuscript (and after it concerns a pair of genotypes rather than dozens), I think it might be helpful to highlight that initial dose might not always be the best idea, for those who wish to build off of this work. It might also be useful to highlight that the distribution of viral loads may not be normal (perhaps another reason why you chose to use initial dose, particularly given sample sizes?).

Section beginning on Line 169 – given the context of the dependence of results on dose, I think some justification for using the highest dose to measure gene expression is needed. I can imagine both pros and cons to this approach, and think it might be useful for some extra discussion to highlight how the chosen dose might affect the conclusions from these results. On a minor note, this section could use a line or two of explanation for the choice of these targets, considering the full justification doesn't come until later, in the methods section.

Line 192-193 – both here and in the introduction, you refer to the "separate" measurement of host survival and pathogen burdens. What do you mean by this? That they are measured in separate experiments? I assume that you are not referring to the restrictions imposed by destructive sampling, as they are present in this experiment as well.

Review form: Reviewer 2

Is the manuscript scientifically sound in its present form?
Yes

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No

Have you any concerns about statistical analyses in this paper?
Yes

Recommendation?
Accept with minor revision (please list in comments)

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L 154-155: 'A comparison of the overall fit of the curves showed that G9a+/+ and G9a-/- genotypes have distinct tolerance profiles during DCV infection': Do the slopes of the curves differ for the two genotypes?

L 185-190: A stylistic suggestion: Perhaps include the main finding from this study at the end of the first paragraph? At the moment the reader has to wait until the end of the second page of the discussion.

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L 283-287: A few comments about this paragraph:

1. The description of the generation of the fly mutants could be explained in a little bit more detail, especially so that people unfamiliar with these methods could understand more clearly how these mutants were generated. If I have understood correctly, the G9a-/- flies were generated by imprecise/imperfect excision of the P-element, thereby part of the 5' UTR region was removed along with the P-element. The control on the other hand was generated by precise excision whereby only the P-element was removed. I thought that 'restored the functional phenotype of G9a' was a bit confusing in this context.
2. The authors refer to reference 16 for the generation of the mutants however, in the methods of reference 16 they write that the mutants were generated previously and cite another article (Kramer et al 2011; PloS Biol 9: e1000569) so perhaps the reference should be changed at line 286?
3. Were the fly lines back-crossed into a wild-type background or do they have e.g. y1 or w* on the x-chromosome? Please give the full genotype information for each chromosome/mutant line.

L 307-310: I suggest to present the full RT and qPCR methods in this section rather than in the later section (line 329 onwards). Please also include qPCR conditions and primer efficiencies.

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Fig. 3 legend: What does 'ultimate severity' mean? Asterisks are mentioned in the legend but are not in the figure itself. Include in the legend what the vertical dotted lines in c and d illustrate. Also, probably a silly question, if sensitivity is one value estimated from the logistic curve, where do the replicate values come from that are used in the ANOVA?

Minor points

L 120-122: Sentence starting 'This…'. This interpretation would fit better in the discussion.

L 128-130: Sentence starting ' These results…'. Did reference 16 also examine the same time period after infection as in this study? This interpretive sentence would fit better in the discussion.

L 165-167: This sentence might also fit better in the discussion.

L 238: '…in mediating host sensitivity to increases in DCV…'. Typo? This doesn't quite make sense.

L 343: suggest: '…be affected by a…'

L 361: Presumably the response variable 'fly health (DPI)' means fly survival. If so, I suggest to write this more explicitly.

Figs 2-4: Sometimes the figure legends contain the 'a' in G9a and sometimes they do not.

Fig. 4 legend: There is no reference to the asterisks that are found in the figure.

Decision letter (RSOS-170342)

09-May-2017

Dear Dr Vale

On behalf of the Editors, I am pleased to inform you that your Manuscript RSOS-170342 entitled "Non-linear disease tolerance curves reveal distinct components of host responses to viral infection" has been accepted for publication in Royal Society Open Science subject to minor revision in accordance with the referee suggestions. Please find the referees' comments at the end of this email.

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

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

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

If you wish to submit your supporting data or code to Dryad (http://datadryad.org/), or modify your current submission to dryad, please use the following link:
http://datadryad.org/submit?journalID=RSOS&manu=RSOS-170342

• Competing interests
Please declare any financial or non-financial competing interests, or state that you have no competing interests.
• Authors' contributions
All submissions, other than those with a single author, must include an Authors' Contributions section which individually lists the specific contribution of each author. The list of Authors should meet all of the following criteria; 1) substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data; 2) drafting the article or revising it critically for important intellectual content; and 3) final approval of the version to be published.

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

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

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

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

Please note that we cannot publish your manuscript without these end statements included. We have included a screenshot example of the end statements for reference. If you feel that a given heading is not relevant to your paper, please nevertheless include the heading and explicitly state that it is not relevant to your work.

Because the schedule for publication is very tight, it is a condition of publication that you submit the revised version of your manuscript within 7 days (i.e. by the 18-May-2017). If you do not think you will be able to meet this date please let me know immediately.

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

When submitting your revised manuscript, you will be able to respond to the comments made by the referees and upload a file "Response to Referees" in "Section 6 - File Upload". You can use this to document any changes you make to the original manuscript. In order to expedite the processing of the revised manuscript, please be as specific as possible in your response to the referees.

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

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2) A separate electronic file of each figure (EPS or print-quality PDF preferred (either format should be produced directly from original creation package), or original software format)
3) Included a 100 word media summary of your paper when requested at submission. Please ensure you have entered correct contact details (email, institution and telephone) in your user account
4) Included the raw data to support the claims made in your paper. You can either include your data as electronic supplementary material or upload to a repository and include the relevant doi within your manuscript
5) All supplementary materials accompanying an accepted article will be treated as in their final form. Note that the Royal Society will neither edit nor typeset supplementary material and it will be hosted as provided. Please ensure that the supplementary material includes the paper details where possible (authors, article title, journal name).

Supplementary files will be published alongside the paper on the journal website and posted on the online figshare repository (https://figshare.com). The heading and legend provided for each supplementary file during the submission process will be used to create the figshare page, so please ensure these are accurate and informative so that your files can be found in searches. Files

on figshare will be made available approximately one week before the accompanying article so that the supplementary material can be attributed a unique DOI.

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

Kind regards,
Alice Power
Editorial Coordinator
Royal Society Open Science
openscience@royalsociety.org

on behalf of Kevin Padian
Subject Editor, Royal Society Open Science
openscience@royalsociety.org

Associate Editor Comments to Author:

Comments to the Author:
This paper was recommended by both reviewers who recognised the importance and novelty of the results. However, both made useful comments that could help the authors improve the interpretations and paper quality.

Reviewer comments to Author:
Reviewer: 1

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Author's Response to Decision Letter for (RSOS-170342)

See Appendix A.

Decision letter (RSOS-170342.R1)

31-May-2017

Dear Dr Vale,

I am pleased to inform you that your manuscript entitled "Non-linear disease tolerance curves reveal distinct components of host responses to viral infection" is now accepted for publication in Royal Society Open Science.

You can expect to receive a proof of your article in the near future. Please contact the editorial office (openscience_proofs@royalsociety.org and openscience@royalsociety.org) to let us know if you are likely to be away from e-mail contact. Due to rapid publication and an extremely tight schedule, if comments are not received, your paper may experience a delay in publication.

Royal Society Open Science operates under a continuous publication model (http://bit.ly/cpFAQ). Your article will be published straight into the next open issue and this will be the final version of the paper. As such, it can be cited immediately by other researchers. As the issue version of your paper will be the only version to be published I would advise you to check your proofs thoroughly as changes cannot be made once the paper is published.

In order to raise the profile of your paper once it is published, we can send through a PDF of your paper to selected colleagues. If you wish to take advantage of this, please reply to this email with the name and email addresses of up to 10 people who you feel would wish to read your article.

On behalf of the Editors of Royal Society Open Science, we look forward to your continued contributions to the Journal.

Best wishes,
Alice Power
Editorial Coordinator
Royal Society Open Science
openscience@royalsociety.org

Appendix A

Dear Editorial Board of Royal Society Open Science

We are happy to submit the revised version of our manuscript. The reviewers' comments and suggestions were very helpful in improving our manuscript. We have modified our manuscript addressing all issues raised by the reviewers. Please find below, our response to the reviewers' comments in italics.

Sincerely,

Vanika Gupta, Pedro Vale.

Comments to Author:

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written in the context of the results. I have a few minor comments to increase the clarity of the reasoning behind some of the decisions made in the experimental design of this work.

We thank the reviewer for finding merit in our work.

Comments:
While the authors cite that they use initial dose rather than viral load at 5 dpi in order to reduce error and conform to the restrictions of the statistical method of analysis (eg. Line 137), I am keen to know whether using 5dpi viral load instead changes any of the major conclusions For example, a new paper on resistance and tolerance to infection in flies (Howick and Lazzaro 2017, Mol. Ecol, DOI 10.1111/mec.14017) suggests that the growth dynamics of microbes after the initial dose are not always easy to extrapolate among genotypes, thus rendering initial dose something of a dicey thing to use for estimating tolerance.

As reviewer 2 also pointed out, we agree that it would be interesting to see the analyses with viral load instead of viral dose. However, differences in the sample sizes used to measure viral titers and survival post-infection in this experiment posits a problem on the suggested analyses. In our experiment, the number of replicates for viral titers' measurement were five whereas survival was measured using 20 replicates. To effectively use viral load in the analysis we would require survival and viral titre data for each individual fly. Indeed this is a further advantage of using viral dose as a reasonable proxy for viral titre without requiring large sample sizes to be processed.

While there does seem to be a reasonable relationship between viral dose and viral load in this manuscript (and after it concerns a pair of genotypes rather than dozens), I think it might be helpful to highlight that initial dose might not always be the best idea, for those who wish to build off of this work. It might also be useful to highlight that the distribution of viral loads may not be normal (perhaps another reason why you chose to use initial dose, particularly given sample sizes?).

Thanks for pointing this out. We agree with the reviewer that initial dose might not always be the best way to measure tolerance. We have included this caveat in the manuscript (Line 138-141). We have also incorporated the usefulness of pathogen dose if pathogen loads are not normally distributed especially when sample sizes are small. However, we would like to point out that in our case viral load residuals were indeed normally distributed.

Section beginning on Line 169 – given the context of the dependence of results on dose, I think some justification for using the highest dose to measure gene expression is needed. I can imagine both pros and cons to this approach, and think it might be useful for some extra discussion to highlight how the chosen dose might affect the conclusions from these results. On a minor note, this section could use a line or two of explanation for the choice of these targets, considering the full justification doesn't come until later, in the methods section.

We chose this dose because we detected high viral titres at 5DPI, and were therefore more likely to detect changes in immune gene expression. We have included this explanation both in the methods and the results section. Please refer to Line 175-18.

Line 192-193 – both here and in the introduction, you refer to the "separate" measurement of host survival and pathogen burdens. What do you mean by this? That they are measured in separate experiments? I assume that you are not referring to the restrictions imposed by destructive sampling, as they are present in this experiment as well.

We meant independent analyses performed on survival and pathogen burden as opposed to the restriction imposed by the destructive sampling. We have modified the text (Line 204) to make it clearer.

Reviewer: 2

Comments to the Author(s)

L 56-57: Regarding 'there is no reason to expect the relationship between host health and pathogen burdens be linear'. This problem has been discussed in literature older than ref 15, (e.g. Tiffin & Inouye 2000) and quadratic terms were suggested and have been tested (e.g. Råberg et al 2007; Råberg et al 2014). I realise that quadratic terms are not as sensitive / do not go as far as 'tolerance curves', but perhaps it is worth including to demonstrate the evolution of this problem?

Thank you for pointing this out. We have included the suggested changes in the manuscript. Please refer to Line 57-61.

L 124 paragraph: I wondered if the authors had tested for non-linear relationships between viral dose and viral load. It's not so easy to see all the data points in the figure because some are overlapping, but the relationship for male and female G9a-/- does not look particularly linear in figures 3c and d. Viral dose is subsequently used on the x-axis for the tolerance curves, because of the positive relationship between dose and load (L 134-136). However, if the relationship between dose and load is not linear, using dose as a proxy for load would have consequences for the tolerance analyses. Would it make sense to check this by running additional tolerance analyses using viral load instead of viral dose, e.g. as was done by Lefèvre et al 2011 278: 751-759?

We agree with the reviewer that a non-linear relationship between dose and load could have consequences for our tolerance analyses. We also realize the usefulness of viral load for the tolerance analyses to circumvent this problem. We have addressed this issue in response to first reviewer's comments. We had different number of replicates for viral load and survival assay. Since we did not have comparable replicate numbers, using load would not be ideal in this case.

L 144-147: 'In males, however, no significant difference in slopes was detected between G9a+/+

and G9a-/-, even though the survival of G9a-/- males was lower than the survival of G9a+/+ males at almost all viral doses.' I was not sure I followed the logic of this sentence. Lower survival does not necessarily translate into differences in slopes; if G9a-/- shows a similar percent reduction in survival relative to G9a+/+ across doses then I would expect the tolerance curves of the two male genotypes to be parallel to each other (as was the case).

Thank you, we indeed wanted to convey that lower survival need not translate into lower tolerance when rate of health decline is similar. We have modified Line 149 to make it clear.

L 154-155: 'A comparison of the overall fit of the curves showed that G9a+/+ and G9a-/- genotypes have distinct tolerance profiles during DCV infection': Do the slopes of the curves differ for the two genotypes?

This comparison particularly refers to the overall curve which include all the components. For all the non-linear analyses, we fixed the slope of the curve to -1 and therefore, slope could not be compared. We fixed slopes because we did not have enough data points spanning the slope that we could use the data to accurately measure the slope.

L 185-190: A stylistic suggestion: Perhaps include the main finding from this study at the end of the first paragraph? At the moment the reader has to wait until the end of the second page of the discussion.

As per reviewer's suggestion, we have included main findings of the study after first paragraph of discussion. Please refer to Line 197-202 to see the incorporated change.

L 273 and abstract. I received slightly mixed messages from the abstract compared to the concluding paragraph of the discussion. The abstract suggests that linear models may be 'inadequate' (L 22) and that non-linear logistic models are 'better' (L 32). But the concluding

paragraph says ‘We suggest that a combination of linear and non-linear models is ideal…’

We have fixed this discrepancy between our abstract and conclusion. We have corrected the abstract suggesting that both linear and non-linear estimates of tolerance are useful.

L 283-287: A few comments about this paragraph:
1. The description of the generation of the fly mutants could be explained in a little bit more detail, especially so that people unfamiliar with these methods could understand more clearly how these mutants were generated. If I have understood correctly, the G9a-/- flies were generated by imprecise/imperfect excision of the P-element, thereby part of the 5’ UTR region was removed along with the P-element. The control on the other hand was generated by precise excision whereby only the P-element was removed. I thought that ‘restored the functional phenotype of G9a’ was a bit confusing in this context.

We thank the reviewer for pointing this out. We have changed the text Line 298 in the Methods section to make it more precise.

2. The authors refer to reference 16 for the generation of the mutants however, in the methods of reference 16 they write that the mutants were generated previously and cite another article (Kramer et al 2011; PloS Biol 9: e1000569) so perhaps the reference should be changed at line 286?

As suggested, we have changed the reference in the text.

3. Were the fly lines back-crossed into a wild-type background or do they have e.g. y1 or w* on the x-chromosome? Please give the full genotype information for each chromosome/mutant line.

As described in the methods L295, both lines were derived from the same background using P-element excision, so backcrossing was not necessary as they are identical except for the dysfunctional g9a locus.

The specific full background genotype is described in detail in Kramer, et al (2011). Epigenetic regulation of learning and memory by Drosophila EHMT/G9a. PLoS Biol, 9(1), e1000569,

where they are referred to as EHMT lines:

"For the creation of EHMT deletions y^1,$EHMT^{KG01242}$ females were crossed to w; ry^{506},Sb^1,P[Δ2-3]99B/TM6B,Tb^1 males in order to induce mobilization of the KG01242 p-element insertion, which has a y^+ marker. Male progeny with the genotype y^1,$EHMT^{KG01242}$/Y; ry^{506},Sb^1,P[Δ2-3]99B/+ were crossed to C(1)DX, y^1, f^1/Y females and male progeny with an excision of KG01242 were isolated based on the absence of the y^+ marker."

L 307-310: I suggest to present the full RT and qPCR methods in this section rather than in the later section (line 329 onwards). Please also include qPCR conditions and primer efficiencies.

We have included the reaction conditions for both RT and qPCR methods in the methods section.

L 314: If known I would suggest to include the approximate dose that these viral concentrations deliver to the flies.

On this occasion we did not measure viral titers immediately following septic injury.

L 378: The upper limit of the 4-parameter logistic model was constrained at 25 days. However, in the linear model the reaction norm for G9a+/+ passes through the y-axis at above 25 days (fig 3A and B), which does not reflect health in the absence of infection. Some authors (e.g. Graham et al 2011 Func Ecol 25 5-17) have additionally incorporated health in the absence of infection,

i.e. examined the cost of infection as the y-variable, which gets around this problem. Might it be useful or interesting to include this here as well?

We agree with the reviewer that it would be useful to include health in the absence of infection. However, the way our study was conducted limits us to use the measure of health under uninfected condition in our analyses. In this experiment, we observed our flies for only 25 days post-infection. Generally, wild-type flies under uninfected conditions survive much longer (~40-45 days). Since, this experiment was stopped earlier, our estimation of health under uninfected condition is skewed. Consequently, our estimates of vigor would be biased. For this reason, we neither compared vigor from the non-linear model nor used the intercept estimates from the linear model.

L 381: Why was the slope fixed to -1?

To accurately estimate the slope from a non-linear sigmoid curve requires more data recorded within the range spanning the slope (see H. J. Motulsky, GraphPad Curve Fitting Guide. (2016)). Our study did not have many data points (three points) and therefore, it was preferable to fix the slope at -1 to obtain more accurate fit from the available data. Furthermore, the physiological significance for disease tolerance of the slope obtained from a non-linear curve relatively less understood while the other parameters (EC50, Vigor and Severity) are well-defined See Louie, A., Song, K. H., Hotson, A., Tate, A. T., & Schneider, D. S. (2016). How many parameters does it take to describe disease tolerance?. PLoS Biol, 14(4), e1002435.

Fig. 3 legend: What does 'ultimate severity' mean? Asterisks are mentioned in the legend but are not in the figure itself. Include in the legend what the vertical dotted lines in c and d illustrate. Also, probably a silly question, if sensitivity is one value estimated from the logistic curve, where do the replicate values come from that are used in the ANOVA?

Thank you for pointing out the mistake. We have corrected the text in the legend to match with the figure description. We have also included the asterisks in the figure to highlight groups with significant differences. The sensitivity or the other parameters are estimated from 20 replicate values (sample size) that are simulated while fitting curves. Consequently, there would be an error around these estimates and that provides the standard error estimate of severity or sensitivity.

Minor points

L 120-122: Sentence starting 'This…'. This interpretation would fit better in the discussion.

We have moved this to the discussion section.

L 128-130: Sentence starting ' These results…'. Did reference 16 also examine the same time period after infection as in this study? This interpretive sentence would fit better in the discussion.

Thanks for the suggestion, we have included this in the discussion now.

L 165-167: This sentence might also fit better in the discussion.

We have incorporated this in discussion.

L 238: '…in mediating host sensitivity to increases in DCV…'. Typo? This doesn't quite make sense.

Thank you for pointing out. We have corrected this in the manuscript.

L 343: suggest: '…be affected by a…'

We have incorporated the suggestion.

L 361: Presumably the response variable 'fly health (DPI)' means fly survival. If so, I suggest to write this more explicitly.

We have included the suggestion.

Figs 2-4: Sometimes the figure legends contain the ‘a’ in G9a and sometimes they do not.

We have corrected figures and they are now consistent. Thank you for pointing out.

Fig. 4 legend: There is no reference to the asterisks that are found in the figure.

Thank you for pointing it out. We have included the reference to asterisks in the text.