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PROCEEDINGS B

Evolution of drug-resistant and virulent small colonies in phenotypically diverse populations of the human fungal pathogen *Candida glabrata*

Sarah J. N. Duxbury, Steven Bates, Robert E. Beardmore and Ivana Gudelj

Article citation details
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Review timeline

Original submission:	14 August 2019
1st revised submission:	8 April 2020
2nd revised submission:	8 June 2020
Final acceptance:	22 June 2020

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

Review History

RSPB-2019-1902.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?
Acceptable

General interest: Is the paper of sufficient general interest?
Acceptable

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?
Yes

Is it adequate?
Yes

Do you have any ethical concerns with this paper?
No

Comments to the Author
This manuscript describes interesting findings on the eventual correlation between drug resistance, fitness and virulence in C. glabrata populations. The subject is timely and interesting.

However, a few major issues should be addressed to improve the quality and impact of the manuscript:

1 - the title should refer to C. glabrata, as only this microbe is evaluated in this manuscript.
2 - is there a formal "classical pathogen life history theory"? or "life-history traits". Please rephrase the sentences including these concepts.
3 - Introduction - why is C. glabrata an ideal system to probe to relationship between resistance and virulence? The results from this study may not necessarily be extrapolated to other systems.
4 - Introduction - The sentence "These results suggest that compensatory mutations that ameliorate a low growth rate are not needed for the resistance phenotypes to maintain virulence" is incomprehensible.
5 - Where are the results of experiment C mentioned in the Methods section?
6 - The subtitle "Stable and unstable small colony, resistant phenotypes are not attenuated in virulence" is incomprehensible.
7 - The Galleria results should be complemented with measurement of cell proliferation within the larvae, so that one may verify if low growth rate is still observable within the host.
8 - The reversibility phenotype should be compared with presence of hot spot mutations in the Fks genes.

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?
Good

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.
Yes

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Is it accessible?
Yes

Is it clear?
Yes

Is it adequate?
Yes

Do you have any ethical concerns with this paper?
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Comments to the Author
In this manuscript, Duxbury and colleagues investigate some Candida glabrata strains which had evolved in the presence of the antifungal caspofungin, examining correlations between virulence, growth rate, and resistance. They find the emergence of small colony variants which display increased resistance with the trade-off of slower growth, but contrary to expectations maintaining high levels of virulence in an animal model. Overall I found this an interesting case that contributes to the literature on correlations between pathogen life-history traits, but some work is required for it to meet the standards required for publication. In some cases these might be met by increased clarity in the text and figure legends, in other cases further analysis and interpretation may be necessary.

I have two principal concerns. The first relates to the level of replication in the study and how replicates are presented. There are several issues here:

1. The text states that the small colony variants emerged from three capsofungin concentrations (0.78, 1.37 and 2.4 μg/ml, page 7), that there were three replicate populations for each treatment for each experiment (page 4), and that 1/3 replicates had joint RCVs and SCVs. I assume from this that the 'three biological replicates' (page 18) refer to individual colonies of SCV and RCV isolated from these 3 x 3 / 3 populations, but it is far from clear, particularly as the authors often use the definite article (e.g. "the regular colony variant"). Please provide more details on the source of the lines used for subsequent analysis. Specifically, what caspofungin concentrations did the different lines come from? Was it a single colony from each evolved population that was used for growth rates etc.? How were the colonies picked? Such information could also be conveyed by extending Supplementary Figure 1.

2. The legend to Figure 1 refers to 'biological replicates' and 'technical replicates' but it is unclear exactly what this means. I again assume that each 'biological replicate' refers to the independently evolved populations from which single RCV and SCV were reisolated, if so this should be specified. Are 'technical replicates' measurements derived from different cultures, or multiple measurements taken from the same culture? Again the level of replication should be clarified.
3. It appears as though the analysis and presentation considers all 12 replicates in Figure 1 as independent, however they are not (since each 'biological' replicate is measured multiple times). This can result in pseudoreplication issues, and should be accounted for in presentation and analysis, e.g. by presenting each biological replicate independently, and including biological replicate as a random effect in the modelling.
4. The wild-type data is the same in Figure 1c and 1d; this is not a problem but it should be made clear in the legend.
5. Figure 2 is confusing — my understanding is that Experiment A yielded three populations with SCV lines, and a single colony from each was grown without caspofungin and all gave the 'stable' type, whereas those similarly treated from Experiment B gave the 'unstable' type. The fact that experiment A gave 3 replicates of one SCV type and experiment B gave 3 replicates with the other type is not statistically significant (Fisher's Exact $p = 0.1$) but raises the question as to whether there any possible reasons that might lead the experiments to give consistently different evolutionary trajectories (e.g. media formulations, opportunities for cross-contamination, etc.). It would help to mention this in the text, particularly as the current presentation suggests to readers that only one SCV was tested from each experiment (e.g. header to Figure 1a/b is "stable SCV" rather than "SCVs"). Or perhaps I am misunderstanding, and a single SCV colony was selected from each experiment and then passaged in replicate (if so, how was it chosen? And how generalisable are the results?)

The second is that one of the key claims of the authors is based on not finding an association between virulence and growth rate. However, a lack of statistically significant difference is not evidence for a lack of difference. Power calculations, and/or equivalence tests are required.

Other comments/suggestions:

- Page 5 line 5 states that 'Experiment' was treated as a random effect, but it seems to me that 'population' is also a key random effect to include since you are repeatedly measuring the same population and thus measurements on different days are not independent.
- What were the relative frequencies of SCV and RCV from individual populations at the endpoint of the evolution experiment?
- Page 8 line 10 refers to Experiment 1 and Experiment 3, should this be Experiments A and B respectively?
- Page 8 line 23 states a P value (0.045) but says that this is 'similar' to the wild type, the fact that this is a marginally significant value (but small effect size?) should be acknowledged.
- Page 9 paragraph 2 has a repetition of 'in contrast', the authors may wish to consider rewording.
- Page 10 paragraph 1. Did the colonies retrieved from G. mellonella have a regular or small phenotype?
- Figure 2 uses bar-and-whisker summaries to present three datapoints. It would be clearer to plot the raw datapoints.
- Figure 4 plots the mean value for strains. It would be good to see the variance/range of the values as well.

Decision letter (RSPB-2019-1902.R0)

17-Oct-2019

Dear Professor Gudelj:

I am writing to inform you that your manuscript RSPB-2019-1902 entitled "Evolution of drug-resistant and virulent small colonies in phenotypically diverse microbial populations" 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 important revisions are necessary. With this in mind we would be happy to consider a resubmission, provided the comments of the referees are fully addressed. However please note that this is not a provisional acceptance.

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

Please find below the comments made by the referees, not including confidential reports to the Editor, which I hope you will find useful. If you do choose to resubmit your manuscript, please upload the following:

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

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

Associate Editor
Comments to Author:
This contributes to a long standing argument in the evolutionary ecology of pathogens with new experimental facts, and as such is of interest to a wider audience. The reviewers are guardedly positive, but have a number of comments that need to be addressed. Could you please address these comments in a revised manuscripts and respond to the reviewers' comments in your reply to that reviewers. If further experimentation is required, could you please carry that out where feasible and practical.

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

Comments to the Author(s)
This manuscript describes interesting findings on the eventual correlation between drug resistance, fitness and virulence in C. glabrata populations. The subject is timely and interesting.

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Referee: 2

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Author's Response to Decision Letter for (RSPB-2019-1902.R0)

See Appendix A.

RSPB-2020-0761.R0

Review form: Reviewer 1

Recommendation
Accept as is

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

General interest: Is the paper of sufficient general interest?
Good

Quality of the paper: Is the overall quality of the paper suitable?
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 issues raised by the reviewers were adequately addressed.

Review form: Reviewer 2

Recommendation
Accept with minor revision (please list in comments)

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

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.
Yes

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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 revised manuscript is greatly improved — the explanation of replication is much clearer, the additional experiments are relevant and interesting, and the revised statistical analyses are also welcome.

Unfortunately, I have to press the issue of statistical power in the interpretation of the data presented. In their response, the authors state:

> "A reduction in growth rate in our strains due to costly resistance was not associated with a reduction in virulence."

However, as I said before, an inability to statistically detect an association is not necessarily support for the lack of an association. For example, Figure 4 does not appear to show a trade-off (negative correlation) between virulence and costly resistance. This observation is supported by a statistical analysis (Spearman's Rank Correlation test) that gives p = 0.2667. They therefore do not have evidence to reject the null hypothesis. But, there are only seven datapoints being analysed. A power calculation suggests that even if there was a strong association between these variables (r = 0.7), the probability of detecting this association (and rejecting the null) would be about 50%.

```r
library(pwr)
pwr.r.test(n=7, r=0.7, sig.level=0.05)
## 0.45
```

Similar arguments could be made about the data presented in Figure 3 (I have not done the power calculations here). In short, I am concerned about Type 2 error. Positive support for the hypothesis of 'no difference' could be obtained by equivalence tests, but I suspect the low number of samples will be an issue.

I am not necessarily disputing the conclusions of the authors — just presenting an alternative hypothesis that has not been conclusively ruled out. The issue of power and/or the potential for an undetected association should be better discussed as a caveat to the conclusions. Ideally,

further experiments with larger sample sizes would provide more insight, but I suspect that is not an option here. I would note that despite this, I think that these data are very interesting and make a valuable contribution to the literature.

Decision letter (RSPB-2020-0761.R0)

18-May-2020

Dear Professor Gudelj:

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 have raised some issues with your manuscript and 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. 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), 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
Comments to Author:
Thank you for your revision, which has improved the paper substantially. Both reviewers are happy bar for one point. Reviewer 2 raises the issue that the argument is centred around not rejecting a null hypothesis and taking this as support for your conclusion. The reviewer also raises issues about the power of your analysis. This issue appears to be inherent in the statistical analysis that you carry out (to arrive at this conclusion the null hypothesis used is not ideal). I agree with the reviewer that the data and results are important and do not want this to stand in the way of dissemination of this work. It is, however, a point that needs to be addressed. At a minimum, could you discuss this issue as suggested by the reviewer? If possible, consider

modifying or adding a statistical analysis that is better suited to answer the question that the papers sets out to ask.

Reviewer(s)' Comments to Author:

Referee: 1

Comments to the Author(s).
The issues raised by the reviewers were adequately addressed.

Referee: 2

Comments to the Author(s).
The revised manuscript is greatly improved — the explanation of replication is much clearer, the additional experiments are relevant and interesting, and the revised statistical analyses are also welcome.

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> "A reduction in growth rate in our strains due to costly resistance was not associated with a reduction in virulence."

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I am not necessarily disputing the conclusions of the authors — just presenting an alternative hypothesis that has not been conclusively ruled out. The issue of power and/or the potential for an undetected association should be better discussed as a caveat to the conclusions. Ideally, further experiments with larger sample sizes would provide more insight, but I suspect that is not an option here. I would note that despite this, I think that these data are very interesting and make a valuable contribution to the literature.

Author's Response to Decision Letter for (RSPB-2020-0761.R0)

See Appendix B.

Decision letter (RSPB-2020-0761.R1)

22-Jun-2020

Dear Professor Gudelj

I am pleased to inform you that your manuscript entitled "Evolution of drug-resistant and virulent small colonies in phenotypically diverse populations of the human fungal pathogen Candida glabrata" 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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You are invited to opt for Open Access, making your freely available to all as soon as it is ready for publication under a CCBY licence. Our article processing charge for Open Access is £1700. Corresponding authors from member institutions (http://royalsocietypublishing.org/site/librarians/allmembers.xhtml) receive a 25% discount to these charges. For more information please visit http://royalsocietypublishing.org/open-access.

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

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

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

Associate Editor:
Board Member
Comments to Author:

Thank you for addressing the issues raised and including extra data and further tests. it has improved the paper.

Appendix A

Associate Editor
Comments to Author:
This contributes to a long standing argument in the evolutionary ecology of pathogens with new experimental facts, and as such is of interest to a wider audience. The reviewers are guardedly positive, but have a number of comments that need to be addressed. Could you please address these comments in a revised manuscripts and respond to the reviewers' comments in your reply to that reviewers. If further experimentation is required, could you please carry that out where feasible and practical.

We would like to thank the reviewers and the editor for their time and thoughtful comments. We have fully addressed all referees comments. In addition we have added two new experimental data sets: (a) we tested for mutations in the hotspot regions of the *FKS* genes and other gene targets associated with caspofungin resistance evolution (as suggested by Reviewer 1); (b) We also performed competition experiments in caspofungin to infer final frequencies of the two types (in response to Referee 2). Finally, for clarity we expanded Supplementary Figure 1 to include a detailed schematic of all experimental methods and have included additional information regarding Methods and Results in the Electronic Supplementary Material.

Below are detailed point-by-point responses.

Reviewer(s)' Comments to Author:

Referee: 1

Comments to the Author(s)
This manuscript describes interesting findings on the eventual correlation between drug resistance, fitness and virulence in C. glabrata populations. The subject is timely and interesting.

Thank you.

However, a few major issues should be addressed to improve the quality and impact of the manuscript:

1 - the title should refer to C. glabrata, as only this microbe is evaluated in this manuscript.

We have changed the title as suggested

2 - is there a formal "classical pathogen life history theory"? or "life-history traits". Please rephrase the sentences including these concepts.

To improve clarity, we have now rephrased the sentences that include the above concepts (lines 20-21, 58-60, 75-76, 402-403, 411-412).

3 - Introduction - why is C. glabrata an ideal system to probe to relationship between resistance and virulence? The results from this study may not necessarily be extrapolated to other systems.

The choice of *C. glabrata* as a model system has now been further explained in the Introduction section (paragraphs 4 and 5, lines 54-67). To test our hypothesis it is crucial to choose an organism where (a) resistance rapidly evolves to commonly used drugs, (b) resistance can impart a growth fitness cost and (c) our previous work provides evidence of potential lack of positive relationship between growth rate and virulence [23, 24]. We also needed a simple and effective model host for studying virulence and the impact of fitness costs. *C.glabrata* and its host *Galleria mellonella* fulfil all the above criteria. Importantly, *C. glabrata* is closely associated with the well-established model yeast *Saccharomyces cerevisiae* and as such molecular, genetic and evolutionary techniques can readily be transferred from *S. cerevisiae* to *C. glabrata* making the latter an increasingly popular infection model organism.

4 - Introduction - The sentence "These results suggest that compensatory mutations that ameliorate a low growth rate are not needed for the resistance phenotypes to maintain virulence" is incomprehensible.

We have edited this sentence on lines 74-76 to clarify, namely " These results suggest that virulence could be maintained in the presence of costly resistance that results in a reduced growth rate."

5 - Where are the results of experiment C mentioned in the Methods section?

We have clarified the Methods section throughout linking particular experiments with the figures within which the data is shown. We further clarify that the results of Experiment C are contained in Supplementary Figure 2 and this figure has been re-plotted to highlight data points that originate from each of Experiments A, B and C.

We state that colony size variation was not observed in any of the nine revived populations from Experiment C (lines 109-112 of Methods and lines 228-229 of Results).

6 - The subtitle "Stable and unstable small colony, resistant phenotypes are not attenuated in virulence" is incomprehensible.

We have re-written the subtitle which now reads “Independently-evolved, drug-resistant SCVs are not attenuated in virulence”.

7 - The Galleria results should be complemented with measurement of cell proliferation within the larvae, so that one may verify if low growth rate is still observable within the host.

Thank you for your suggestion. However, we argue that these experiments would not alter the main message of the paper. The key point is that the resistance costs are only ever measured in terms of reduced growth rate *in vitro* [as indicated in references 5,7,9 of the main paper]. Such measurements are subsequently used to infer a reduction in virulence. Our study provides experimental evidence to the contrary. Therefore, it is imperative that we follow the standard procedures of measuring cost of resistance.

8 - The reversibility phenotype should be compared with presence of hot spot mutations in the Fks genes.

We now include the new result as suggested. In particular, we tested for mutations in the hotspot regions of the *FKS* genes and other gene targets associated with caspofungin resistance evolution described in [11]. No nucleotide changes were detected in any of the strains for all the targets tested. The experimental procedure has been described in the Methods section “Characterisation of genomic targets” (lines 161-169) and the results reported in the Results section (lines 297-303).

Referee: 2

Comments to the Author(s)
In this manuscript, Duxbury and colleagues investigate some Candida glabrata strains which had evolved in the presence of the antifungal caspofungin, examining correlations between virulence, growth rate, and resistance. They find the emergence of small colony variants which display increased resistance with the trade-off of slower growth, but contrary to expectations maintaining high levels of virulence in an animal model. Overall I found this an interesting case that contributes to the literature

on correlations between pathogen life-history traits, but some work is required for it to meet the standards required for publication. In some cases these might be met by increased clarity in the text and figure legends, in other cases further analysis and interpretation may be necessary.

I have two principal concerns. The first relates to the level of replication in the study and how replicates are presented. There are several issues here:

1. The text states that the small colony variants emerged from three capsofungin concentrations (0.78, 1.37 and 2.4 µg/ml, page 7), that there were three replicate populations for each treatment for each experiment (page 4), and that 1/3 replicates had joint RCVs and SCVs. I assume from this that the 'three biological replicates' (page 18) refer to individual colonies of SCV and RCV isolated from these 3 x 3 / 3 populations, but it is far from clear, particularly as the authors often use the definite article (e.g. "the regular colony variant"). Please provide more details on the source of the lines used for subsequent analysis. Specifically, what caspofungin concentrations did the different lines come from? Was it a single colony from each evolved population that was used for growth rates etc.? How were the colonies picked? Such information could also be conveyed by extending Supplementary Figure 1.

We apologise for the lack of clarity, we have fully revised the Methods section (lines 106-117) and have extended Supplementary Figure 1.

Namely:

1. Experiment A: triplicate populations were evolved in eight different caspofungin concentrations and drug-free control in a 96-well plate over 14 days
2. The same setup as in 1. was repeated an additional two times for Experiment B and C.
3. Frozen day 14 populations evolved at the post-IC50 concentrations of 0.78, 1.37 and 2.40 µg/ml were revived from Experiments A-C, by streaking on agar.
4. Colony size variation was only found at 0.78 µg/ml caspofungin, in a **single** (1 out of 3) population from Experiment A and a **single** (1 out of 3) population in Experiment B.
5. We randomly selected a single colony of both the SCV and the RCV from each of the two populations (one from Experiment A and one from Experient B) described in 4. for all phenotypic assays.
6. Figure 1 shows growth fitness and drug susceptibility data for a single SCV and single RCV co-isolated from the single (1 out of 3) population passaged at

0.78 µg/ml in Experiment A (which showed colony size variation). Growth fitness data in Figure 1a and b were measured in 4 replicate wells on a 96-well plate and repeated on an additional two days (N=4 X 3 =12). Drug susceptibility data in Figure 1c and d were measured in 3 replicate wells on a 96-well plate and repeated on an additional two days (N=3 x 3 = 9).

7. Supplementary Figure 4 shows growth fitness and drug susceptibility data for a single SCV and single RCV co-isolated from the single (1 out of 3) population passaged at 0.78 µg/ml in Experiment B (which showed colony size variation). This data is compared to that of the 2001WT strain, using the same data points as in Figure 1. Growth fitness data for the SCV and RCV in Supplementary Figure 4a and b were measured in 4 replicate wells on a 96-well plate (N=4). Drug susceptibility data in Figure 4c and d were measured in 3 replicate wells on a 96-well plate (N=3).

2. The legend to Figure 1 refers to 'biological replicates' and 'technical replicates' but it is unclear exactly what this means. I again assume that each 'biological replicate' refers to the independently evolved populations from which single RCV and SCV were reisolated, if so this should be specified. Are 'technical replicates' measurements derived from different cultures, or multiple measurements taken from the same culture? Again the level of replication should be clarified.

We apologise for the lack of clarity and misunderstanding about biological and technical replicates. In our original version of the manuscript we referred to populations growing in replicate wells on a 96-well plate as “technical replicates” while replications of the same experiment conducted on different days was referred to as “biological replicates”.

For clarity, we now refer to “replicates” throughout the manuscript, specifying whether these are replicate wells on a 96-well plate originating from the same overnight culture or whether they come from a 96-well plate run on a different day and originating from a separate overnight culture. For example, Figure 1 legend now contains an explanation

“N = 12 measurements per colony variant (measurement in four individual wells of a microtiter plate, repeated separately on three days). Different symbol shapes (separated by horizontal noise on the x-axis) represent measurements from separate days. Black points and error bars show overall means and standard errors.”

3. It appears as though the analysis and presentation considers all 12

replicates in Figure 1 as independent, however they are not (since each 'biological' replicate is measured multiple times). This can result in pseudoreplication issues, and should be accounted for in presentation and analysis, e.g. by presenting each biological replicate independently, and including biological replicate as a random effect in the modelling.

We have re-run the analysis of the data in Figure 1a and b using linear mixed effects models as suggested and have also re-run the same analysis for the data in Supplementary Figure 4a and b. The outcomes are now presented in the manuscript (lines 234-243) and the Methods section updated accordingly (lines 122-126). Please note that the outcomes and conclusions remain the same.

4. The wild-type data is the same in Figure 1c and 1d; this is not a problem but it should be made clear in the legend.

We have clarified this in the Figure legend.

5. Figure 2 is confusing — my understanding is that Experiment A yielded three populations with SCV lines, and a single colony from each was grown without caspofungin and all gave the 'stable' type, whereas those similarly treated from Experiment B gave the 'unstable' type. The fact that experiment A gave 3 replicates of one SCV type and experiment B gave 3 replicates with the other type is not statistically significant (Fisher's Exact p = 0.1) but raises the question as to whether there any possible reasons that might lead the experiments to give consistently different evolutionary trajectories (e.g. media formulations, opportunities for cross-contamination, etc.). It would help to mention this in the text, particularly as the current presentation suggests to readers that only one SCV was tested from each experiment (e.g. header to Figure 1a/b is "stable SCV" rather than "SCVs"). Or perhaps I am misunderstanding, and a single SCV colony was selected from each experiment and then passaged in replicate (if so, how was it chosen? And how generalisable are the results?)

We apologise for not making the methodology clearer, it is the latter. We have fully revised the Methods section (lines 145-159) and have extended Supplementary Figure 1 to provide a detailed schematic of the experimental design.

In summary:

1. 1 out of 3 populations in Experiment A yielded SCV colonies
2. 1 out of 3 populations in Experiment B yielded SCV colonies

3. 0 out of 3 populations in Experiment C yielded SCV colonies
4. The same randomly-selected single SCV colony that was used for phenotypic assays from each of Experiment A (Figure 1) and B (Supplementary Figure 4) was used to test stability. Each SCV was subsequently passaged in triplicate populations (wells on a single 96-well plate) founded from a single culture of the respective SCV.
5. A single growth rate and yield measurement was taken for each passaged replicate population during the final transfer (day 14).

We do not make generalisability claims in our paper. Instead we provide examples of two different reversibility properties that our SCVs of *C. glabrata* evolved in the presence of caspofungin can have. We passaged (in triplicate) a randomly-selected SCV colony from each of Experiment A and B that was isolated from the distinct sub-population in each Experiment. The SCV from Experiment A consistently demonstrated irreversibility across the three passaged replicate populations, whilst the SCV from Experiment B was consistently reversed across its three replicate populations

We passaged the three replicate populations founded from the SCV from each of Experiment A and B concurrently, using the same drug-free media, over the same 14-day period with all populations in the same microtiter plate. Therefore, the differences seen would not have been caused by different culture conditions.

The second is that one of the key claims of the authors is based on not finding an association between virulence and growth rate. However, a lack of statistically significant difference is not evidence for a lack of difference. Power calculations, and/or equivalence tests are required.

This is a misunderstanding. Our key claim is not based on "not finding an association between virulence and growth rate". Moreover, we do not conclude that a lack of statistically significant difference is evidence for a lack of difference.

The existing empirical evidence suggest that resistant pathogens that suffer a reduction in growth rate as a result of costly resistance, also suffer a reduction in virulence. Here we provide the first experimental evidence that virulence could be maintained following the evolution of costly resistance. A reduction in growth rate in our strains due to costly resistance was not associated with a reduction in virulence. This is our key novel result.

In addition, we pooled virulence data for different isolates and tested for association between growth rate and virulence. We found no statistically significant correlation (Fig 4) in our data. Given that previous studies on different systems found either positive or negative correlations (please see Discussion section third paragraph from the bottom (lines 402-409)) we argue that our data further highlights the complexity of the growth rate-virulence relationship. Thus, we conclude that " we need to develop an in-depth, mechanistic understanding of the relationship between growth rate and virulence" (second to last and the last paragraph of Discussion (lines 411-421)).

We have also now further clarified that our null hypothesis is: there is no rank order relationship between the growth rate and larval survival time. Critical ρ for our sample size is ρ(N=7, α=0.05)=0.786. Given that our computed Spearman's correlation ρ=-0.5, we cannot reject the null hypothesis.

Other comments/suggestions:

- Page 5 line 5 states that 'Experiment' was treated as a random effect, but it seems to me that 'population' is also a key random effect to include since you are repeatedly measuring the same population and thus measurements on different days are not independent.

Originally, we did not include “population” as a random variable because we reasoned that the growth measurements made of the populations were not repeated measures of the same populations, due to genetic turnover during generations of serial passaging and emergence of sub-population diversity. In general, given that all populations originate from a common ancestor, true population independence is impossible to achieve.

However, as suggested we have now also adjusted the random effect included in the linear mixed effects model to reflect repeated measures of the same evolving populations over time. We now include a single nested random effect of ‘Population’ within ‘Experiment’ and have adjusted the methods section on lines 102-104 and the results section on lines 215-221 to take this into account. We have re-plotted all individual populations in Supplementary Figure 2, labelling Experiment by different symbol shapes and labelling Day by different colours. We now find significant effects of both fixed factors (day and caspofungin concentration) on relative growth, whereas in our previous analysis only the fixed effect of day was significant. This additional significant result does not alter our main conclusion, as we already reported dose response effects of caspofungin concentration on relative growth of the wild-type ancestral strain and sub-population RCV strains presented in Figures 1c and Supplementary Figure 4c.

- What were the relative frequencies of SCV and RCV from individual populations at the endpoint of the evolution experiment?

Whilst we didn't determine the relative frequency directly at the end-point of the evolutionary experiment we now include new data from which the frequencies can be inferred.

In particular we tested competitive fitness of the SCV (Small_Col in Figure 1a) against its co-isolated RCV (Reg_Col in Figure 1a) from the evolved population in Experiment A. The competitive fitness was measured over 24 hours mixed growth in the presence of caspofungin at 0.78 ug/ml. The SCV showed negative frequency-dependent fitness, with coexistence between SCV and RCV predicted above an approximately 85% frequency of SCV in the mixed culture. We have included details of the competitive fitness assay in the methods section on lines 171-184 and in the results on lines 305-314. We have added an extra figure (new Supplementary Figure 6) and legend.

- **Page 8 line 10 refers to Experiment 1 and Experiment 3, should this be Experiments A and B respectively?**

Yes, apologies for the typo. We have made the edits on lines 236-237 within the newly edited paragraph in the Results.

- **Page 8 line 23 states a P value (0.045) but says that this is 'similar' to the wild type, the fact that this is a marginally significant value (but small effect size?) should be acknowledged.**

We have now acknowledged this as suggested on lines 247-249.

- Page 9 paragraph 2 has a repetition of 'in contrast', the authors may wish to consider rewording.

We have removed the second "in contrast" on line 285.

- Page 10 paragraph 1. Did the colonies retrieved from G. mellonella have a regular or small phenotype?

This is an interesting question, however we did not harvest fungal colonies from *G. mellonella,* as the focus of these experiments was to test virulence via larval survival.

- Figure 2 uses bar-and-whisker summaries to present three datapoints. It would be clearer to plot the raw datapoints.

We have now replotted the data for Figures 1, 2 and Supplementary Figure 4 displaying the raw data points.

- Figure 4 plots the mean value for strains. It would be good to see the variance/range of the values as well.

We thank the reviewer for this suggestion, however replicate growth rate and yield data presented in Figures 1, 2 and Supplementary Figure 4 were collected independently from larval survival time data presented in Figure 3. This means that each growth value data point (replicate) is not paired with a larval survival time measurement, therefore it is not possible to present a horizontal and vertical scatter of points surrounding each mean value. We therefore have not made changes to the data plotted in Figure 4. Average growth rates and yields (Figures 1, 2 and Supplementary Figure 4) were calculated across replicate wells in a 96-well plate, whereas average larval survival times (Figure 3) were calculated across replicate larvae, each infected with an inoculum dose from a single strain overnight culture. We have included a description of the data analysis in the Methods on lines 200-208.

Appendix B

Associate Editor
Comments to Author:
Thank you for your revision, which has improved the paper substantially. Both reviewers are happy bar for one point. Reviewer 2 raises the issue that the argument is centred around not rejecting a null hypothesis and taking this as support for your conclusion. The reviewer also raises issues about the power of your analysis. This issue appears to be inherent in the statistical analysis that you carry out (to arrive at this conclusion the null hypothesis used is not ideal). I agree with the reviewer that the data and results are important and do not want this to stand in the way of dissemination of this work. It is, however, a point that needs to be addressed. At a minimum, could you discuss this issue as suggested by the reviewer? If possible, consider modifying or adding a statistical analysis that is better suited to answer the question that the papers sets out to ask.

We thank the editor and the reviewers for their time and a positive feedback. We have addressed the final query from Reviewer 2 by:

1. including data from **two additional replicate infections studies (3 replicate studies in total)** in the new Supplementary Figures 7-11.
2. performing bootstrapping for both linear and Deming regressions and also Pearson and Spearman correlations.

None of these 4 methods detected a correlation between larval survival times and relative growth rates (or growth yields) **for any of the 3 replicate infection studies**, and thus we could not reject the hypothesis of a no correlation from any of these tests. However, we now also acknowledge in the manuscript that further *G. melonella* infection studies involving larger sample sizes would be needed to establish an absence of correlation.

Our point-by point responses are listed below

Reviewer(s)' Comments to Author:

Referee: 1

Comments to the Author(s).
The issues raised by the reviewers were adequately addressed.

Thank you for your feedback.

Referee: 2

Comments to the Author(s).
The revised manuscript is greatly improved — the explanation of replication is much clearer, the additional experiments are relevant and interesting, and the revised statistical analyses are also welcome.

Thank you for your feedback.

Unfortunately, I have to press the issue of statistical power in the interpretation of the data presented. In their response, the authors state:

> "A reduction in growth rate in our strains due to costly resistance was not associated with a reduction in virulence."

However, as I said before, an inability to statistically detect an association is not necessarily support for the lack of an association. For example, Figure 4 does not appear to show a trade-off (negative correlation) between virulence and costly resistance. This observation is supported by a statistical analysis (Spearman's Rank Correlation test) that gives p = 0.2667. They therefore do not have evidence to reject the null hypothesis. But, there are only seven datapoints being analysed. A power calculation suggests that even if there was a strong association between these variables (r = 0.7), the probability of detecting this association (and rejecting the null) would be about 50%.

```r
library(pwr)
pwr.r.test(n=7, r=0.7, sig.level=0.05)
## 0.45
```

Similar arguments could be made about the data presented in Figure 3 (I have not done the power calculations here). In short, I am concerned about Type 2 error. Positive support for the hypothesis of 'no difference' could be obtained by equivalence tests, but I suspect the low number of samples will be an issue.

I am not necessarily disputing the conclusions of the authors — just presenting an alternative hypothesis that has not been conclusively ruled out. The issue of power and/or the potential for an undetected association should be better discussed as a

caveat to the conclusions. Ideally, further experiments with larger sample sizes would provide more insight, but I suspect that is not an option here. I would note that despite this, I think that these data are very interesting and make a valuable contribution to the literature.

With respect to Figure 3, we now clarify that we conducted three independent replicate survival studies performed on separate days, each using N=20 larvae per *C. glabrata* strain (i.e. 20 x 3 = 60 larvae in total per *C. glabrata* strain). As commonly done for *G. melonella* infection studies, data from one of the replicate study is presented in Figure 3 and we now include the data for the other two replicate studies in new Supplementary Figures 7 and 8. For all three replicate infection studies we observe no marked alterations in virulence of small colony mutants.

With respect to Figure 4, we now add corresponding data for the second (new Supplementary Figure 10) and the third (new Supplementary Figure 11) replicate infection study. Bootstrapping was performed on the data in Figure 4, Supplementary Figure 10a and Supplementary Figure 11a, for both linear and Deming regressions and also Pearson and Spearman correlations. The resulting statistics are reported in Supplementary Figures 9, Supplementary Figures 10b,c and 11b,c. None of our 4 tests detected a correlation between larval survival times and relative growth rates (or growth yields) in all 3 replicate infection studies and thus we could not reject the hypothesis of no correlation. However, we now also acknowledge that further *G. melonella* infection studies involving larger sample sizes would be needed to establish an absence of correlation (lines 362-364).