**We thank the reviewers for their thoughtful comments. We have taken the time and effort to insure we address each comment and suggestion made. In making edits, use of track changes produced an unsightly mess. Instead, we highlight all lines containing major edits in our responses and hope that this is sufficient to highlight the changes. Our responses to specific reviewer comments are included below.**

**Thank you**

**Matt Anderson**

**Editorial comments:**  
Changes to be made by the Author(s):  
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

2. Please provide an email address for each author.

Email addresses for each author have been included on Page 1.

3. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

All instances of personal pronouns in the protocol have been altered.

4. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible.

All four instances of “would”, “should”, “Could”, and “can” have been replaced with imperative tense directives.

5. 3.2: How large is the petri dish?

This detail has been added in line 161. They are 15 mm petri dishes.

6. 3.4: Please break up into two steps.

This step has been broken into steps 3.4 and 3.5, lines 170-179.

7. Figure 1: Please use SI abbreviations for volume units (mL, µL) and include a space between numbers and their units (10 µL).

This change has been made per the editor’s recommendation.

8. Figure 2A: PBS-1 and PBS-2 are difficult to distinguish from the figure. Please revise to be clear.

The individual PBS control lines in Figure 2A have been recolored to provide better contrast using a red/blue color scheme.

9. Table of Materials: Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc. Please include a space between all numerical values and their corresponding units: 15 mL, 37 °C, 60 s; etc.

The requested format changes (spacing and unit changes) have been made throughout the manuscript and Table of Materials.

10. Discussion: Please discussion any limitation of the technique.

We have expanded on the limitations of the technique by adding lines 286-291 and 311-326. Previously, limitations were discussed in lines 297-310.

11. References: Please do not abbreviate journal titles.

This has been edited for the editor’s recommendation. The ‘JoVE’ Endnote citation format has been imported and implemented with the ‘full journal name’ selected. We have selected options for “Full journal title”. This should provide the requested format.  
  
**Reviewers' comments:**  
  
Reviewer #1:  
  
Manuscript Summary:  
The paper by Dunn, Woodruff, and Anderson described the protocol used to assess the inoculum and age effect on systemic infection with yeasts.  
The authors aimed at outlining benefits and drawbacks of the model host Galleria mellonella to study systemic Candida pathogenesis and at detailing an approach to improve reproducibility. The same model has already been published in JOVE in 2012 (Ramarao et al., J. Vis. Exp. (70), e4392) and in 2013 (Harding et al., J. Vis. Exp. (81), e50964), and other papers deeply described its usefulness for systemic candidiasis studies (including inoculum size, different hemocyte recruitment, histology, strains varying in virulence potential…)( Fuchs et al., Microbes Infect. 2010; Mesa-Arango et al., Med Mycol. 2013; Sheehan and Kavanagh, Virulence 2018; Rajendran et al. Eukaryot Cell. 2015; Amorim-Vaz et al., Front Microbiol. 2015).  
  
Despite the lack of novelty, experiments on C. albicans strains encoding either heterozygous (a/α) or homozygous (a/- or α/-) MTL loci are interesting.  
  
Major Concerns:  
- I suggest to avoid the term "worms" as G. mellonella is a Lepidoptera belonging to the Pyralidae family. The term "larvae" is more appropriate.

The authors appreciate the clarification and all terminology has been updated.

- The authors suggest the readers to buy larvae from "any bait shop"; however, many breeders add hormones and/or antibiotics to larvae food constituting a bias for the experimental use.

We have removed this phrase and included language to highlight the importance of vendor consistency across experiment sets. See lines 96-97.

- larvae that begin to pupate into moths are usually considered "censored" in the statistical analysis. In figure 2 (panel C), mainly at day 5, most of the larvae turned into pupae. This could suggest that the used larval batch was quite old and could represent a bias in survival experiments.

We acknowledge that pupae formation represents a transition from the waxworm to moth forms of *G. mellonella* and could affect how virulence progresses as this has not been addressed in the literature to our knowledge. All experiments used larvae the day following their receipt in the lab and is unlikely to be due to unnecessary aging in the lab. It is difficult for us to know the age of the worms prior to shipping but could represent a confounding variable.

Previous studies have included pupae in their recordings of survival as we have included here PMID 28898264. Censoring pupating larvae could alter the description of the data towards the end of the experiment but would not alter the results presented here as death generally occurred prior to the day 5 time point where pupation was seen as its earliest. From our experience we have not seen observable differences in death between larvae and pupating larvae. Pupa have succumbed to the infection and biological replicates in which no larvae or a portion of larvae have pupated were statistically indistinguishable. We had included a sentence on pupating larvae in 3.9 of the Methods previously and have extended that line to more comprehensively describe the caveats of including pupa (lines 201-203).

- PBS-injected larvae usually show no mortality. In figure 2 (panel A), we can observe about 25% mortality at day 5. Could authors comment on this data? The same observation can be done about figure 3: the mortality of PBS-injected larvae is too high and authors should consider to repeat the all experiment.

The reviewer is correct in noting experiments in which PBS-injected worms die during the course of the experiment. This is in contrast to a number of studies, especially of bacterial pathogens, in which death is not observed during the course of the experiment. It is worth noting bacterial infection studies typically conclude within three days of infection for many of the organisms (*Psuedomonas, Salmonella*, *Legionella*, etc.,), often before we see significant drop off in PBS-injected worm survival.

In contrast, the level of mortality associated with PBS injection is not outside that seen commonly in *G. mellonella* infections for *C. albicans*. We would like to note the following publications all have similar or increased mortality following injection with PBS: PMID 26549450, 25504520, 21178491, 27458452, and, 20223293. Similar mortality is observed in other studies not centered on *C. albicans* including 24743168, 29458721, 17400503, and 19572230 that extend out to 5-8 days as is detailed here for *C. albicans*. Within this study, there are experiments lacking any worm mortality (Figure 2B), demonstrating that there is some variability in mortality due to PBS injection as depicted in Figure 2A. Thus, although there is death in the control animals, it is not beyond the range observed in similar studies with *G. mellonella* in *C. albicans.*

Minor Concerns:  
- line 52: Cenaerahditis elegans needs to be correct in Caenorhabditis elegans

This change has been made per the reviewer’s suggestion.

- line 56: Although a recent study promoted the use of Galleria mellonella hemocytes for a phagocytic assay for Leishmania braziliensis, I believe that, when using the term eukaryotic pathogens, the authors refer to fungal pathogens. I suggest to amend "eukaryotic" in "fungal".

This change has been made per the reviewer’s suggestion. We would like to note that the use of *G. mellonella* to study parasites is increasing. Of particular note is its use investigating oomycete virulence as seen in PMID 29458721 and 29904064.

- line 117: when spinning down yeast cells for inoculum preparation, authors suggested 2,500 x g for 5 min. I believe it is a too high speed to recover fully vital cells.

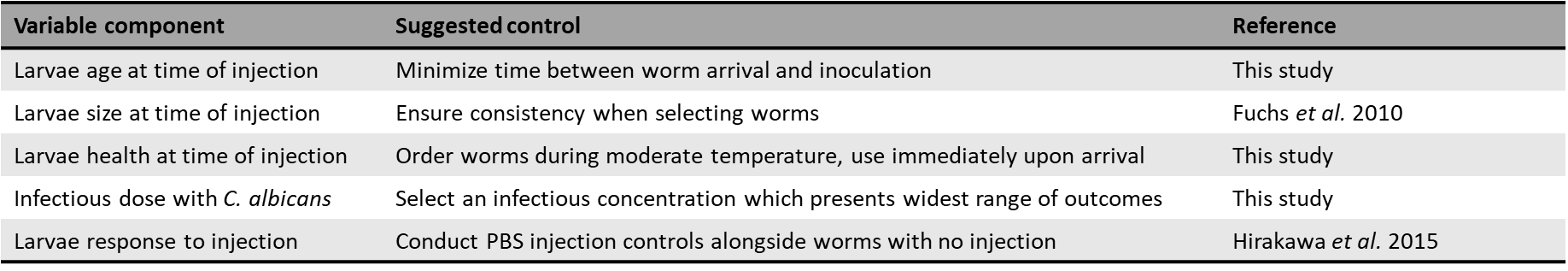
The speed mentioned here does not impact viability and has been used extensively in *Candida* biology by the author and other groups (PMID 3900217, 27711197, etc.).

Reviewer #2:  
  
Manuscript Summary:  
The manuscript provides experimental approaches to test fungal virulence with Galleria mellonella. The authors highlighted the advantages to use this simple model. They use C. albicans strains and several mutants to address potential effects on virulence. The manuscript is easy to follow and methods are precisely described.  
  
Major Concerns:  
a. It is known that Galleria infection experiments suffer from extended variability issues. In their experiments however, this variability issue seems not a major issue. It is surprising that authors did not calibrate the larvae according to their weight, which is one of the parameters influencing outcome. The authors should therefore more carefully list the parameters associated with variations in a Table format for example. I think the authors should be more precise.

We would like to thank Reviewer #2 for mentioning the consistency of the methodology described here. To specifically include the controlled variables in this experiment, Table 2 has been generated and included in the main text as well as attached below.

Of note, we have been unable to find a correlation between worm size and lethality of a consistent infection dose similar to the one used here. That work was performed but not reported as directly applicable to the study described in PMID 25504520.

**Table 2. Considerations when using this method.**



b. It is also surprising that larvae are not locally disinfected at the site of inoculation before injection.

Before this notation, the authors were unaware that such a protocol existed. Local sterilization of the worm prior to injection has not been performed in any *G. mellonella* study using *C. albicans*. We are also unable to find widespread use of this approach in other fungal pathogens using waxworms. We are unsure as to how this would alter the infection outcome nor can we find direct comparison demonstrating increased efficacy of sterilization. To allow comparison with the body of *C. albicans* literature, the protocol will be retained without the added sterilization but a note has been added to the Methods (3.5).

c. The authors should also mention other possible readouts of infections, eg CFU counts, bioluminescence, etc.

This addition has been made per the reviewer’s suggestion (line 336-338).  
  
Minor Concerns:  
a. The authors ordered larvae from a supplier that is not mentioned.

The source of the larvae (Snackworms.com) was listed in the Table of Supplies. We have left it out of the manuscript text as we do not want to push a particular supplier on the reader.

Reviewer #3:  
  
Manuscript Summary:  
This paper clearly describes how to work with G. mellonella and C. albicans and can be very useful to the community.  
  
Major Concerns:  
In the abstract and the rest of the paper precise that the model is set-up for C. albicans and that it is not necessarely identical for other species. Infection with glabrata are not at all successful in our hand (personal communication).  
Could you precise why 3 washes in PBS are so important. Did you check if we can onserve a difference with less whashes?

We have not assessed differences on virulence by the number of PBS washes prior to infection. This step is intended to remove any YPD or other culture media that can carry over from growing the inoculum. It is possible that two washes are sufficient as some studies have preferred to use (PMID 20223293). The primary goal here is to remove that media which is known to affect the outcome of infection in model hosts due to immunogenicity of media components. We have chosen to perform three washes per the protocol here.

In the same idea, did you check the efficacy of washing the needle between each larva ? In our hand we did not see any difference in term of mortality (personal communication). Other protocols were already published please cite them. For example : Fuchs et al., Virulence 1:6, 475-482; November/December 2010; KAVANAGH et al. fungal b i o l o gy r e v i ews 2 4 ( 2 0 1 0 ) 7 9 e8 3.

We have added these references as they are important to this work (line 69).

In the discussion you speak about "vizualisation of the infection via reporter Candida", A study was already performed in this sense : Delarze et al, Virulence. 2015;6(7):684-93.

We thank the reviewer for pointing out this reference. We have included it (line 338) and highlighted the ability to track the infective process as the next step in use of such a reporter line.

Minor Concerns:  
line 80, I guess you wanted to say "to depict the contribution of MTL heterozygosity to virulence in G. mellonella".

This has been edited per the reviewer’s suggestion.

Figure 2C : pictures are too small and G. mellonella are hardly visible.

The reviewer is correct in noting that images could be increased in magnification for clarity. As such, we have increased magnification of each petri dish of larvae across the time course. We have chosen not to include blown up insets as individual animals are pictured in Figure 2B.