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Corresponding Author:	Matthew Z Anderson Ohio State University Columbua, Ohio UNITED STATES
Corresponding Author's Institution:	Ohio State University
Corresponding Author E-Mail:	anderson.3196@osu.edu
Order of Authors:	Matthew J Dunn
	Andrew L Woodruff
	Matthew Z Anderson
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TITLE:

The *Galleria mellonella* Waxworm Infection Model for Disseminated Candidiasis

AUTHORS & AFFILIATIONS:

Matthew J Dunn[1], Andrew L Woodruff[1], Matthew Z Anderson[1,2]

[1]*Department of Microbiology, The Ohio State University, Columbus, OH, USA*
[2]*Department of Microbial Infection and Immunity, The Ohio State University, Columbus, OH, USA*

Matthew J Dunn: dunn.658@osu.edu
Andrew L Woodruff: woodruff.207@osu.edu

Corresponding Author:
Matthew Z Anderson
anderson.3196@osu.edu

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SUMMARY:

Galleria mellonella serves as an invertebrate model for disseminated candidiasis. Here, we detail the infection protocol and provide supporting data for the model's effectiveness.

ABSTRACT:

Candida species are common fungal commensals of humans colonizing the skin, mucosal surfaces, and gastrointestinal tract. Under certain conditions, *Candida* can overgrow their natural niches resulting in debilitating mucosal infections as well as life-threatening systemic infections, which are a major focus of investigation due to their associated high mortality rates. Animal models of disseminated infection exist for studying disease progression and dissecting the characteristics of *Candida* pathogenicity. Of these, the *Galleria mellonella* waxworm infection model provides a cost-effective experimental tool for high-throughput investigations of systemic virulence. Many other bacterial and eukaryotic infectious agents have been effectively studied in *G. mellonella* to understand pathogenicity, making it a widely accepted model system. Yet, variation in the method used to infect *G. mellonella* can alter phenotypic outcomes and complicate interpretation of the results. Here, we outline the benefits and drawbacks of the waxworm model to study systemic *Candida* pathogenesis and detail an approach to improve reproducibility. Our results highlight the range of mortality kinetics in *G. mellonella* and describe the variables which can modulate these kinetics. Ultimately, this method stands as an ethical, rapid, and cost-effective approach to study virulence in a model of disseminated candidiasis.

INTRODUCTION:

Candida species are common human commensals that are capable of emerging as opportunistic pathogens in severely immunocompromised and dysbiotic patients. Although many *Candida* species can cause disease, *C. albicans* is the most prevalent cause of disseminated candidiasis[1,2]. Systemic disease results from *C. albicans* accessing the bloodstream through either direct penetration of previously restrictive host barriers or introduction at surgical sites and other breaches of the body[3]. *Candida* species utilize a range of pathogenic processes to cause systemic disease within the host including filamentation, biofilm formation, immune cell evasion and escape, and iron scavenging[4]. *In vitro* approaches exist to investigate individual pathogenic mechanisms, but animal models continue to provide the best option to investigate the entirety of disease outcome[5,6]. Previous research has detailed many instances of promising *in vitro* investigations of virulence failing to reproduce *in vivo*[7,8]. Thus, animal models are still required to assess virulence *in vivo.* Most disease models rely on mice to serve as a surrogate for human infections despite *C. albicans* inability to naturally colonize murine systems as a commensal[9]. Invertebrate models of disseminated candidiasis include the nematode *Caenorhabditis elegans*, the fruit fly *Drosophila melanogaster*, and the waxworm *Galleria mellonella*, although concerns about fundamental differences in basic physiology, host body temperatures, and routes of exposure have hampered their broad acceptance[10,11].

Most recently, the *G. mellonella* waxworm infection model has been adopted to model pathogenicity of a wide range of bacterial and fungal pathogens[12-14]. Advantages of this model include its relatively low cost, increased throughput, ease of use, and reduced ethical concerns regarding animal beneficence compared to murine models. For researchers, this translates into increased ability to test multiple variables, stronger confidence intervals, more rapid experimentation, and bypass of animal protocols. *G. mellonella* has served as a platform to rapidly assess *C. albicans* virulence following perturbation of genes required for biofilm formation, filamentation, and gene regulation across clinical isolates[11,15,16]. Recent studies have incorporated investigation of antifungal efficacy using *G. mellonella* to assess the pharmacokinetics of drug activity and resistance under *in vivo* settings, which are otherwise challenging and time consuming[17,18]. Yet, studies of *C. albicans* virulence in *G. mellonella* have been complicated by reportedly high levels of variation within experiments and inconsistent protocols between research groups that produce differing virulence phenotypes between mice and waxworms[11,13,19-21]. Here, we outline a *G. mellonella* protocol to standardize *C. albicans* infections, increase reproducibility in virulence experiments, and demonstrate consistency with previously described studies of virulence in murine models.

Previous studies demonstrated that the *C. albicans* mating type-like (*MTL*) locus on chromosome 5 regulates cell identity and mating competence similar to *Saccharomyces cerevisiae* and other Ascomycete fungi[22]. The majority of *C. albicans* isolates are heterozygous at the *MTL* locus, encoding one of each of the *MTL***a** and *MTL*α alleles (*MTL***a**/α), and are consequently sterile[15,23,24]. Loss of one of the *MTL* alleles through loss of heterozygosity (LOH) or mutation leads to homozygous *MTL***a** or *MTL*α strains that can undergo a phenotypic switch from the sterile 'white' state to the mating competent 'opaque' state[25]. Previous work has highlighted that loss of *MTL* heterozygosity also reduces virulence in murine models of systemic infection across different strain backgrounds[26]. Here, we detail the *G. mellonella* model for

disseminated candidiasis using a genetically similar experimental set to depict the contribution of *MTL* heterozygosity to virulence in *G. mellonella*. We show that *MTL* configuration influenced *C. albicans* pathogenicity, where *MTL*α strains were less virulent with respect to both *MTL***a**/α and *MTL***a** cells, similar to findings within murine infection model[26].

PROTOCOL:

All methods described rely on use of invertebrate hosts and do not require Institutional Animal Care and Use Committee (IACUC) approval.

1. *Galleria mellonella* Waxworm Larvae

1.1. Order larvae from wholesalers and suppliers that do not introduce hormones, antibiotics, or other treatments to the larvae and which are able to ship and deliver live specimens.

1.1.1. Be sure to purchase all larvae from the same supplier during the course of experimentation. Be careful when ordering larvae during summer months as temperatures exceeding 30 °C decrease larvae viability.

1.1.2. Monitor temperatures across the expected shipping route and plan shipping and delivery accordingly or choose expedited shipping to minimize their exposure to environmental conditions.

1.2. When larvae arrive, check each container to ensure viable, healthy larvae are present. Healthy larvae will be completely submerged beneath the bedding, moving, and possess a light yellow/tan coloration with few larvae containing black marks or discolorations along the body.

1.3. Store larvae in their container in a ventilated space at ambient temperature (25 °C). Depending on the initial condition of the larvae, they may be used for up to two weeks.

2. Culture Preparation for *Galleria mellonella* Infection

Note: Proper lab attire should be worn throughout this portion of the protocol including gloves, lab coat, and safety glasses.

2.1. Make yeast peptone dextrose (YPD) liquid and agar plates. Prepare 1x phosphate buffered saline (PBS) and 70% ethanol solutions.

2.2. Grow *C. albicans* strains to be injected overnight in 3 mL of fresh YPD in standard culture tubes on a rotating drum at medium speed and 30 °C.

2.3. Spin cells down at 2,500 x g for 5 min in a tabletop centrifuge. Pour off supernatant, being careful not to disturb the cell pellet.

2.3.1. Resuspend cells fully in 5 mL of 1x PBS by repeated pipetting or vortexing. Repeat the centrifugation and resuspension of cells in PBS twice more to ensure removal of all culture media.

2.4. After a final wash, resuspend cells in 1 mL of PBS and transfer them to a microcentrifuge tube.

2.5. Conduct a set of serial dilutions in PBS to generate a 1:100, 1:1,000, and 1:100,000 dilution series.

Note: Alternative dilutions may be necessary to reach desired cell counts.

2.5.1. Using a hemocytometer, count cells from either the 1:100 or the 1:1,000 dilution. Most often, the 1:1,000 dilution provides the appropriate cell counts for *C. albicans* cultures.

2.5.2. Calculate total concentration of cells within the initial culture using the grid math of the hemocytometer, aiming for between 30-300 cells to be counted in the central 5x5 grid. An automated cell counter may be used as an alternative; however, use of optical density to determine cell counts is discouraged as cell morphology (size, shape, *etc.*) can significantly affect its accuracy.

2.6. Using the measured cell counts, make a new dilution of $2.5x10^7$ cells/mL in 1x PBS in a microcentrifuge tube. This dilution will serve as the basis for each inoculation of *G. mellonella* with *C. albicans*. Each injection will require 10 µL of this inoculum for an infectious dose of 250,000 *C. albicans* cells.

2.7. Confirm accuracy of the infectious dose by plating the correct volume of the 1:100,000 dilution for 100 colony forming units (CFUs) on YPD agar for each culture to be used in infection.

2.8. Incubate cell count plates from step 2.7 for 48 hours (h) at 30 °C and count the number of colonies per plate. Between 80 and 120 colonies constitutes an acceptable range for accuracy in the inoculum.

3. **Infection of *Galleria mellonella* Larvae with *Candida* Cultures**

Note: Proper lab attire should be worn throughout this portion of the protocol including gloves, lab coat, and safety glasses.

3.1. Add 1 mL of 100% ethanol and 1 mL of 1x PBS to two separate microcentrifuge tubes. The ethanol and PBS will be used to wash the injection needle between infections.

3.2. Spread a small amount of waxworm bedding in an empty, 100 x 15 mm sterile Petri dish, which will house the larvae following inoculation for each independent strain. Addition of the bedding limits adherence of dead *G. mellonella* larvae to the Petri dish surface.

3.3. Sterilize a 26 G, 10 µL syringe by taking up and expunging 10 µL of 70% ethanol three times followed by three washes with 10 µL of 1x PBS. Vortex the inoculation dilution of *C. albicans* and take up 10 µL of culture. Ensure that there are no air bubbles within the syringe as injection of air promotes death and will complicate downstream analysis.

3.4. Open a container holding the *G. mellonella* larvae and carefully turn bedding over with a finger to uncover larvae. Select larvae which are in good health as identified by active movement, a light yellow/tan color, and a lack of black pigmentation on the larvae body. These larvae should be of similar size across the full experimental set.

3.5. Pick up a single larvae and hold it gently between the index/middle finger and thumb similar to holding a pencil. Roll the larvae onto its back so the legs are facing up. Hold the full length of the body between the fingers and thumb so that the larvae is unable to curl or pull away from the injection.

NOTE: If desired, sterilize the site of injection using a cotton swab soaked in 100% ethanol.

3.6. Rotate the syringe so the needle's bevel faces up and slowly insert the needle tip including the length of the bevel into the body at the junction with the rearmost left leg. Ensure that the needle penetrates the body and does not simply push the body of the larvae inwards. Do not use force in penetrating the body as the needle may pierce completely through the larvae quickly. Gently lifting with the needle will confirm appropriate penetration. Inject the full 10 µL *Candida* inoculum and extract the needle.

3.7. Repeat step 3.3-3.5 for each larvae. To prevent cell settling, vortex the *Candida* cultures for inoculation after every third injection. As a control, inject a set of *G. mellonella* with 10 µL of 1X PBS.

3.8. Co-house 10 larvae injected from the same *Candida* culture in each 100 x 15 mm Petri dish following inoculation. Incubate larvae at 37 °C for eight days, checking larvae every 24 h to monitor death.

3.8.1. Assess mortality initially by darkened pigmentation, the formation of black patches or bodies, and a lack of movement. To confirm mortality, use tweezers to gently roll moribund larvae onto their back and lightly poke the larvae's underside with the tweezers. Non-responsiveness as observed by a lack of visible body or leg movement is scored as death and the larvae are removed from the Petri dish.

3.9. Record larvae mortality over the time course. Larvae that begin to pupate into moths can be included in the analysis but should be removed if they begin to molt. Pupating larvae can be

censored from end point analysis as the virulence differences between larvae and pupa are unclear.

3.10. Assess statistical significance using Mantel-Cox statistical test.

REPRESENTATIVE RESULTS:

Here, we demonstrate a reproducible method for the use of *G. mellonella* waxworms to investigate a disseminated candidiasis model of infection using *C. albicans*. The appropriate storage, maintenance, and selection of larvae for infection are critical component of insuring reproducibility in *G. mellonella* mortality (**Figure 1A**). Healthy larvae that are active, have a light yellow/tan color, and lack black patches on the body should be used for this system and are typically viable for infection up to two weeks following arrival. To minimize any potentially confounding effects of *G. mellonella* physiology, larvae were selected of similar size and weight. A well-mixed inoculum and consistent injection at the same anatomical site of the larvae body also stand as important variables in promoting reproducibility between experiments. Images showcase maintenance of infected larvae through the duration of experimentation (**Figure 1B**). To assess the impact of larvae age on virulence, separate PBS control infections were performed with larvae either zero or ten days after arriving. No significant difference in the kinetics of *G. mellonella* death existed between these groups (**Figure 2A**). Characteristic signs of illness arose in larvae from both groups that succumbed to the infection; the originally yellow/tan coloration of the larvae became discolored and gave way to black patches before total loss of motility and, eventually, death (**Figure 2B**). Infection with *C. albicans* speeds up this process of discoloration and morbidity but does not significantly alter the cascade of phenotypic markers leading to death compared to uninfected larvae or PBS injected larvae (**Figure 2C**).

The infective dose also significantly impacts mortality kinetics of *G. mellonella* infections. To assess the effects of *C. albicans* inoculum size, we infected *G. mellonella* with four different clinical isolates (12C, P60002, P75010, and SC5314: **Table 1**) at three different doses. Larvae infected with SC5314 displayed mortality at all doses, consistent with increased pathogenicity of this isolate relative to most others used within the experiment (**Figure 2D**). More larvae succumbed to infection with 1.0×10^5 SC5314 or 12C cells compared to 1.0×10^4 cells. The highest dose of 1.0×10^6 cells proved excessively lethal with larvae dying following infection of all strains including the less virulent isolates, P60002 and 12C. Larvae injected with P75010 and SC5314 succumbed to the infection within 24 h, suggesting a more moderate infectious dose is appropriate to determine differences in virulence. Consequently, an infectious dose of 2.5×10^5 was used for all subsequent experiments.

To demonstrate similarity in virulence between murine and *G. mellonella* models of systemic disease, we assayed virulence of SC5314-derived *C. albicans* strains encoding either heterozygous (**a**/α or homozygous (**a**/- or α/-) *MTL* loci. Infection of *G. mellonella* with *MTL* heterozygotes from either the SC5314 or its prototrophic derivative BWP17 genetic background produced high rates of mortality compared to PBS-injected animals. Three-quarters of the control larvae survived to day 8 compared to loss of over half of all *C. albicans* injected animals

within 3 days post infection (dpi) that further increased at 8 dpi (**Figure 3A, 3B**). Infection with BWP17 dampened *C. albicans* lethality in this model (27% survival compared to 4% survival in SC5314 *MTL* heterozygotes at 8 dpi; **Figure 3C, 3D**). *G. mellonella* larvae infected with *MTL*α/- strains survived significantly longer compared to the *MTL* heterozygous parental strains (log-rank test, SC5314; p=0.0006, BWP17; p=0.0002). Interestingly, virulence of *MTL***a**/- *C. albicans* cells matched its *MTL* heterozygous counterpart in both the SC5314 and BWP17 backgrounds. Thus, *MTL* configuration does have an influence on *C. albicans* virulence with *MTL*α/- strains displaying decreased killing compared to either *MTL***a**/α or *MTL***a**/- cells.

FIGURE AND TABLE LEGENDS:

Figure 1. Overview of infection procedure. (A) A flowchart highlights the key elements of the infection procedure including ordering and storage of larvae, infection culture preparation, and injection. **(B)** Representative images show healthy storage conditions, preparation of the infection space, injection, and larvae maintenance following infection.

Figure 2. The effects of *G. mellonella* infection procedures. (A) PBS controls were infected either immediately upon arrival (PBS-D0, blue) or after 10 days (PBS-D10, red). **(B)** Representative images highlight larvae discoloration and death. **(C)** An image series of infection plates detail the effects of injection with PBS (middle) or with SC5314 (lower) compared to uninfected larvae (top) on days 1, 3, 5, and 7 following injection. **(D)** Dosage effects are shown within a collection of clinical isolates.

Figure 3. The *MTL*a allele promotes *C. albicans* killing of *G. mellonella*. *MTL* locus heterozygous (**a**/α) and homozygous (**a**/- and α/-) SC5314 **(A)** and BWP17 **(C)** derivatives are plotted for larvae mortality over eight days. Confidence intervals are plotted for each SC5314 **(B)** and BWP17 **(D)** derivative strain to highlight consistency in the kinetics of larvae mortality across experiments. The average (solid line) is plotted with standard deviations (filled in space) for three biological replicates of 10 larvae each.

Table 1. *C. albicans* strains used in this study.

Table 2. Considerations regarding *G. mellonella* infection.

DISCUSSION:

The *G. mellonella* waxworm model stands as an effective tool for the rapid and reproducible analysis of *C. albicans* virulence. This detailed protocol relies upon consistent delivery of a defined infectious dose to the same site across a batch of larvae. Infectious dose has a profound impact on *G. mellonella* mortality whereas use of larvae between their initial arrival and ten days following receipt produced similar results. Loss of the *C. albicans MTL***a** allele results in decreased virulence consistent with previous experiments in mice although disruption of the *MTL*α allele did not alter larvae death.

The outcome of *G. mellonella* infections can be significantly impacted by the experimental design. In contrast to vertebrate models of infection, small variation in the injection site, inoculum volume, and inoculum size may impact interpretations of the data. Infections with increasing numbers of *C. albicans* cells led to greater and more rapid morbidity of infected larvae. At a certain point, injecting too many *C. albicans* appears capable of overwhelming host defenses leading to rapid death of the host, potentially through toxic shock. However, SC5314 was consistently the most virulent strain at all doses, followed by 12C, and then the other strains. Thus, infection parameters have a profound impact on the absolute kinetics of infections but may still provide estimates of relative virulence. Prior work within these experiments further demonstrated the importance of thoroughly washing the *Candida* cells. Residual YPD included in the injection bolus significantly decreases larvae survival (data not shown).

Worm health is a critical variable when conducting *G. mellonella* experiments. Larvae transported using standard shipping in the middle of summer often arrive prematurely aged or stressed, covered with black patches, with decreased viability. No significant role for larvae size on morbidity has been previously observed although the experiments described here focused on assaying larvae of roughly equivalent mass. Healthy larvae and PBS control larvae should always be run alongside infection groups to account for any changes in larvae viability between biological replicates performed on separate days. *G. mellonella* infected ten days after arriving matched the kinetics of larvae infected immediately upon receipt. Thus, a single shipment of larvae can provide sufficient material and time to perform full experiments. These experiments relied upon 30 animals per strain to determine differences in virulence, which is significantly more animals than typically utilized in murine models, resulting in stronger confidence of observed results[11,27,28]. This model also reduced ethical concerns, time, and costs compared to vertebrate models of infection. Thus, the use of wax larvae may facilitate identification of smaller effects on systemic virulence than is possible in other systems.

Inherent caveats to the *G. mellonella* system exist that limit its utility in studying host-pathogen interactions. First, *G. mellonella* lack an adaptive immune system and cannot be used to investigate antigenicity or immunological memory as in higher eukaryotes[29]. The body of the larvae is composed of a single continuous cavity filled with hemolymph, which function to circulate nutrients, signaling molecules, immune cells, and antimicrobial peptides. These immune cells, hemocytes, behave similarly to neutrophils and can therefore phagocytose, generate oxidative bursts, and secrete lytic enzymes following activation through extracellular pathogen recognition receptors[30]. Yet, this function is a subset of innate immune cell processes within chordate systems that may not fully reflect all leukocyte activities. Another major caveat is the lack of a genome assembly for *G. mellonella* although the genome was recently sequenced[31]. Consequently, the extent of genotypic diversity within the species is unknown and most suppliers likely ship genetically heterogeneous populations that may affect mortality across experiments. Furthermore, molecular techniques do not exist for the organism and complicate any attempts to dissect host contributions to pathogenesis. Yet, consistent virulence results following infection with *C. albicans* across experiments, vendors, time, and labs supports their utility in fundamental questions of pathogenicity.

Loss of *MTL***a** reduced virulence of *C. albicans* relative to *MTL* heterozygotes and *MTL*α homozygote backgrounds. This trend is consistent with prior work performed using mouse models of systemic virulence[26]. In contrast to murine models, loss of *MTL*α did not alter virulence relative to the *MTL***a**/α parental strain. The discrepancy between these results is unclear but may include components of adaptive immunity that are missing in invertebrate organisms. Alternatively, increased virulence associated with *MTL* heterozygosity may be attributable only to the *MTL*α allele in SC5314, potentiating the need for assessment across multiple strain backgrounds. Therefore, we suggest a differential role for virulence between the two *MTL* alleles.

Overall, the *G. mellonella* model serves as a rapid and reproducible model for assessing systemic candidiasis. Other readouts of virulence can be assayed with this model including fungal prevalence by plating larvae homogenate for colony forming units (CFUs)[11] or bioluminescence, which also provides fungal localization[32]. In the future, this model can be expanded to assess virulence characteristics of other *Candida* species such as elevated drug resistance in the newly emergent *Candida auris* species[33]. Continued development of tools to visualize lymphocyte within *G. mellonella* interacting with infecting *Candida*, inbred waxworm lineages to reduce variability in infections outcomes, and visualization of the infective process *via* reporter *Candida* lines[34] will further refine this model of disease and allow deeper investigations of *in vivo* host-pathogen interactions.

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DISCLOSURES:
The authors have nothing to disclose.

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Figure 1

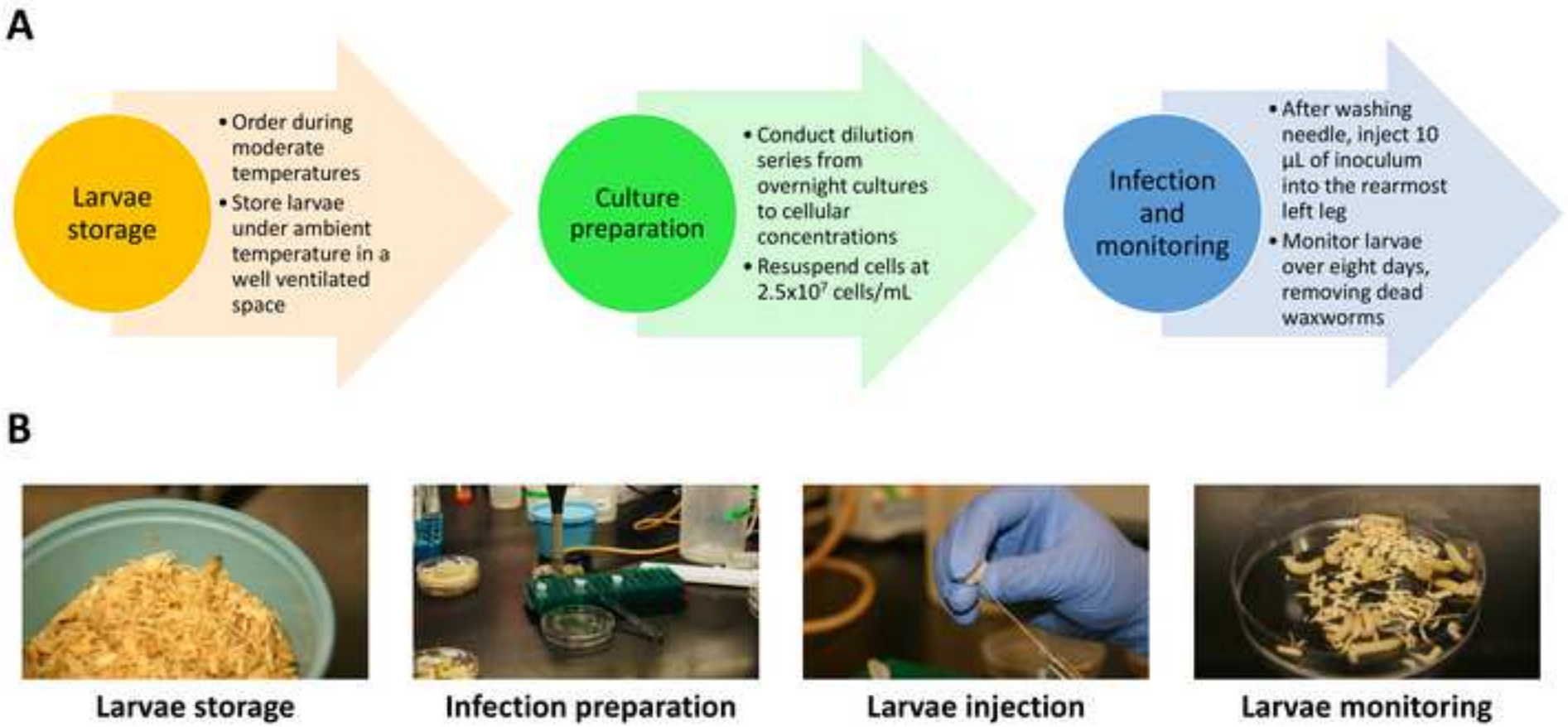

Figure 2

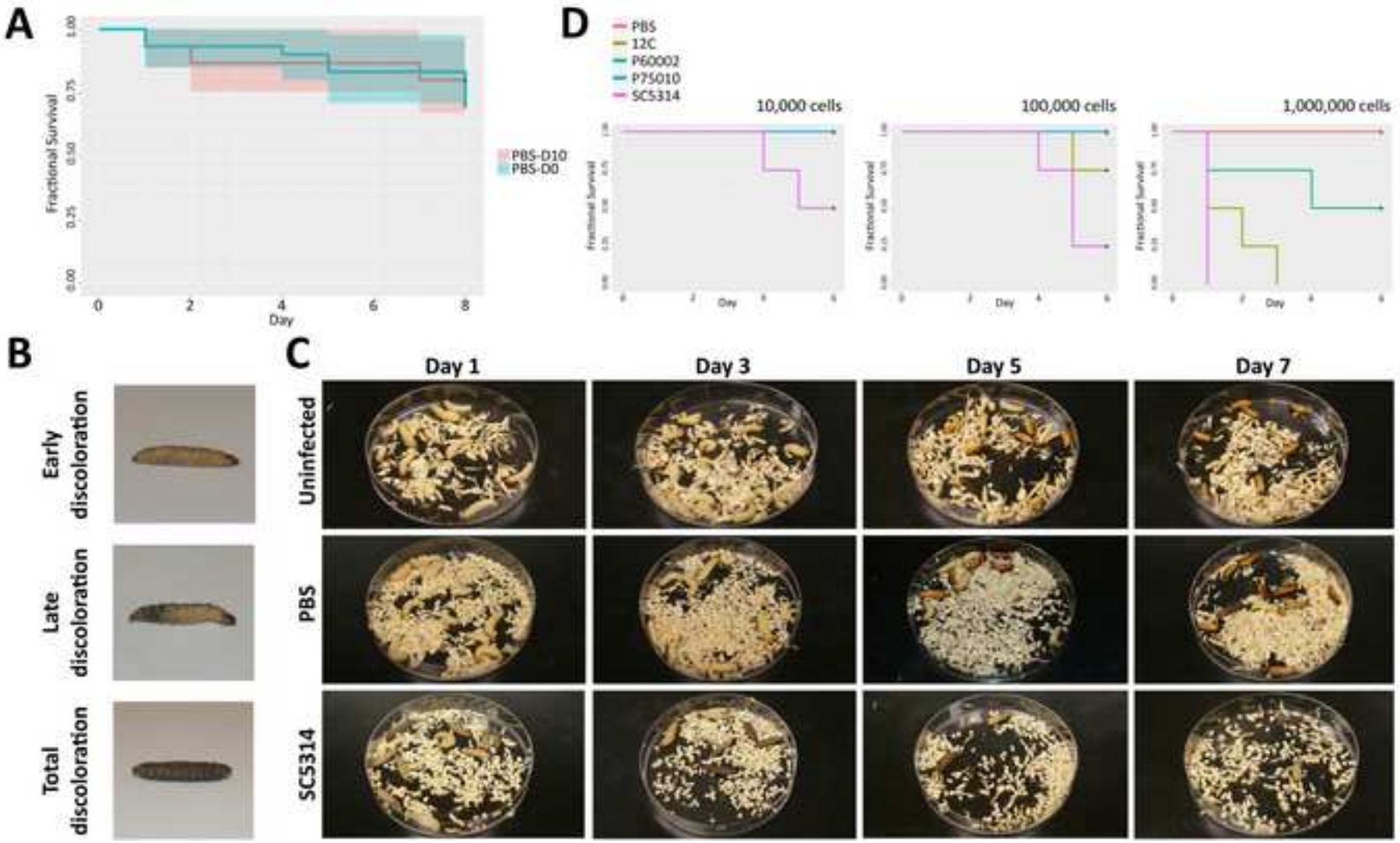

Figure 3

Click here to access/download;Figure;090618_jove_figure_Figure3.tif

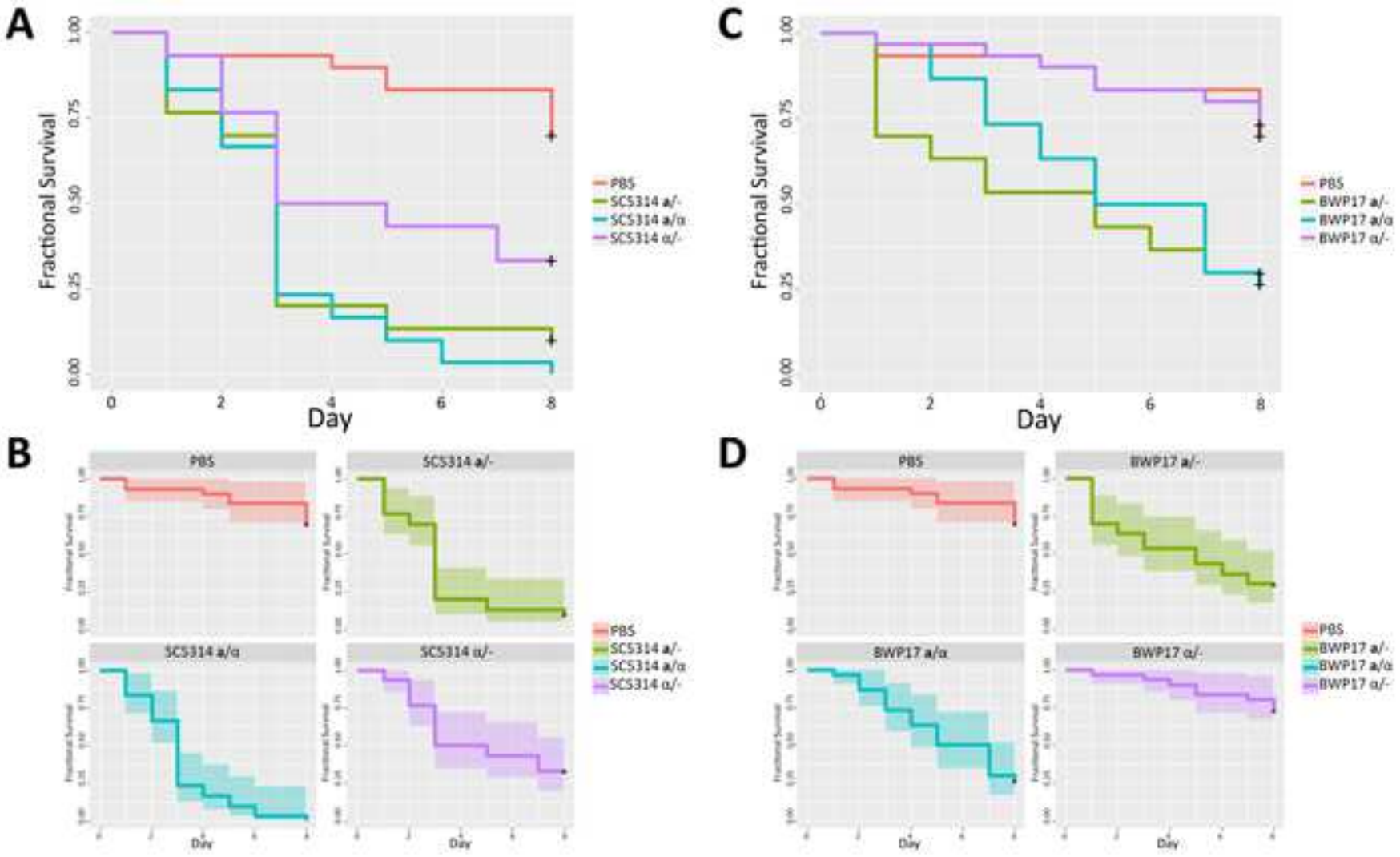

Table 1. C. albicans strains used i

Candida strain	Parental strain
MAY1	SC5314
MAY6	P60002
MAY11	P75010
MAY15	12C
MAY61	SC5314
MAY71	SC5314
MAY313	BWP17
MAY314	BWP17
MAY315	BWP17

n this study.

Genotype	MTL
Candida albicans clinical isolate	**a**/α
Candida albicans clinical isolate	**a**/**a**
Candida albicans clinical isolate	**a**/α
Candida albicans clinical isolate	**a**/**a**
MTL **a**::*SAT1*	α/-
MTL α::*SAT1*^{S} *tac1* ::*TAC1-GFP-HYGR*	**a**/-
ura3 Δ::λimm434/*ura3* Δ::λimm434 *his1* ::hisG/*his1* ::hisG *arg4* ::hisG/*arg4* ::RS-*arg4 -ura3* -BN *sir2* ::*his1 /sir2* ::*dpl200*	**a**/α
ura3 Δ::λimm434/*ura3* Δ::λimm434 *his1* ::hisG/*his1* ::hisG *arg4* ::hisG/*arg4* ::RS-*ARG4 -URA3* -BN *sir2* ::*HIS1 /sir2* ::*dpl200*	α/-
ura3 Δ::λimm434/*ura3* Δ::λimm434 *his1* ::hisG/*his1* ::hisG *arg4* ::hisG/*arg4* ::RS-*ARG4 -URA3* -BN *sir2* ::*HIS1 /sir2* ::*dpl200*	**a**/-

Reference
Wu *et al.* 2007
Wu *et al.* 2007
Wu *et al.* 2007
Wu *et al.* 2007
This study
This study
This study
This study
This study

Table 2. Considerations when u

Variable component
Larvae age at time of injection
Larvae size at time of injection
Larvae health at time of injection
Infectious dose with *C. albicans*
Larvae response to injection

ısing this method.

Suggested control
Minimize time between worm arrival and inoculation
Ensure consistency when selecting worms
Order worms during moderate temperature, use immediately upon arrival
Select an infectious concentration which presents widest range of outcomes
Conduct PBS injection controls alongside worms with no injection

Reference
This study
Fuchs *et al.* 2010
This study
This study
Hirakawa *et al.* 2015

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Galleria mellonella	Snackworms.com		Buy twice as many worms a
10 uL, Model 1701 N SYR Cemented needle, 26G, type 2 syringe	Hamilton	80000	
Petri dish, 100X15 mm, 500 pack	Fisher	FB0875712	
Microcentrifuge tube, 1.7 mL, 500 pack	VWR	87003-294	
Phosphate Buffered Saline (Biotechnology grade), 500 mL	VWR	97062-818	
Ethanol absolute, ≥99.5% pure, 500 mL	Millipore Sigma	EM-EX0276-1S	
autoclaved ddH_2O			

as expected to use

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Author(s): Matthew J. Dunn, Andrew L. Wood-iff, Matthew Z. Anderson

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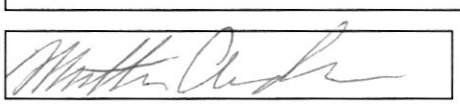
Name:	Matthew Z. Anderson
Department:	Microbiology; Microbial Infection and Immunity
Institution:	The Ohio State University
Title:	Asst. Professor
Signature:	
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We thank the reviewers for their thoughtful comments. We have taken the time and effort to 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.

Variable component	Suggested control	Reference
Larvae age at time of injection	Minimize time between worm arrival and inoculation	This study
Larvae size at time of injection	Ensure consistency when selecting worms	Fuchs *et al.* 2010
Larvae health at time of injection	Order worms during moderate temperature, use immediately upon arrival	This study
Infectious dose with *C. albicans*	Select an infectious concentration which presents widest range of outcomes	This study
Larvae response to injection	Conduct PBS injection controls alongside worms with no injection	Hirakawa *et al.* 2015

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.