Video Article

The *Galleria mellonella* Waxworm Infection Model for Disseminated Candidiasis

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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.

Video Link

The video component of this article can be found at https://www.jove.com/video/58914/

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[12,13,14]. 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[11,13,19,20,21]. 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. 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. 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.
   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.
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.
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.

1. Make yeast peptone dextrose (YPD) liquid and agar plates. Prepare 1x phosphate buffered saline (PBS) and 70% ethanol solutions.
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.
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.
   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.
4. After a final wash, resuspend cells in 1 mL of PBS and transfer them to a microcentrifuge tube.
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.
   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. 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.
6. Using the measured cell counts, make a new dilution of 2.5×10^7 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.
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.
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.

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.
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. 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.
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.

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.
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.
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.
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.
   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.
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.
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.
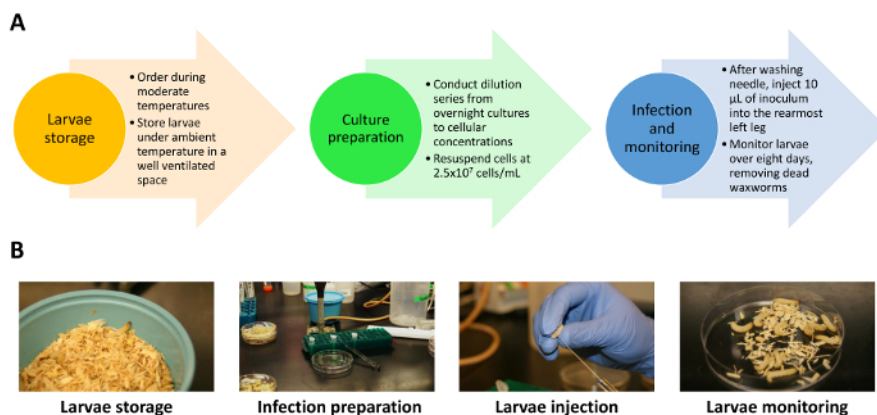
Figure 1. Overview of infection procedure. (A) A flowchart highlights the key elements of the infection procedure including ordering and storage of larvae, infection culture preparation, and injection. **(B)** Representative images show healthy storage conditions, preparation of the infection space, injection, and larvae maintenance following infection. Please click here to view a larger version of this figure.
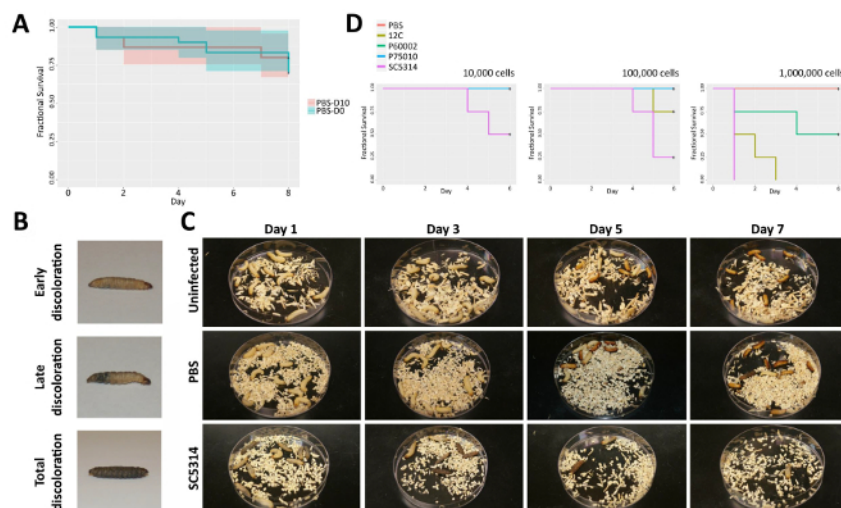

Figure 2. The effects of *G. mellonella* infection procedures. (A) PBS controls were infected either immediately upon arrival (PBS-D0, blue) or after 10 days (PBS-D10, red). **(B)** Representative images highlight larvae discoloration and death. **(C)** An image series of infection plates detail the 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. Please click here to view a larger version of this figure.
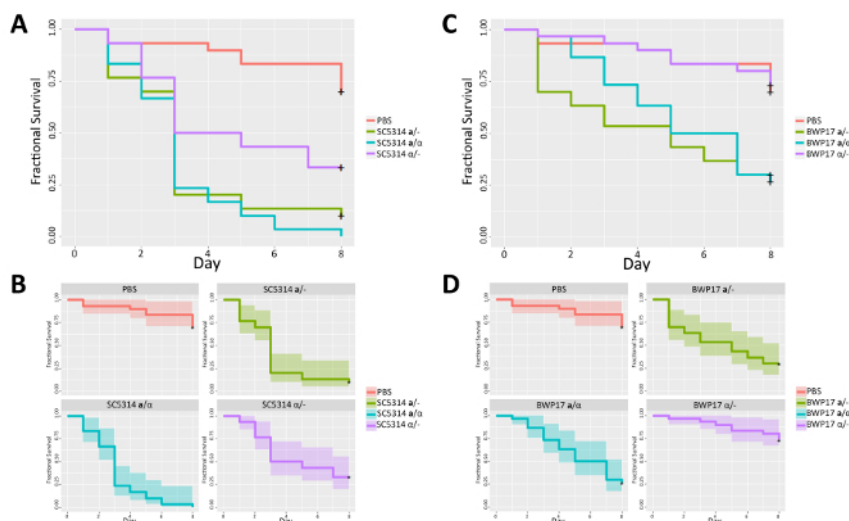
Figure 3. The *MTL*a allele promotes *C. albicans* killing of *G. mellonella*. *MTL* locus heterozygous (a/α) and homozygous (a/- and α/-) SC5314 **(A)** and BWP17 **(C)** derivatives are plotted for larvae mortality over eight days. Confidence intervals are plotted for each SC5314 **(B)** and BWP17 **(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. Please click here to view a larger version of this figure.

Candida strain	Parental strain	Genotype	MTL	Reference
MAY1	SC5314	*Candida albicans* clinical isolate	a/α	Wu et al. 2007
MAY6	P60002	*Candida albicans* clinical isolate	a/a	Wu et al. 2007
MAY11	P75010	*Candida albicans* clinical isolate	a/α	Wu et al. 2007
MAY15	12C	*Candida albicans* clinical isolate	a/a	Wu et al. 2007
MAY61	SC5314	*MTL*a::SAT1	α/-	This study
MAY71	SC5314	*MTL*α::SAT1^S tac1::TAC1-GFP-HYGR	a/-	This study
MAY313	BWP17	ura3Δ::λimm434/ura3Δ::λimm434 his1::hisG/his1::hisG arg4::hisG/arg4::RS-arg4-ura3-BN sir2::his1/sir2::dpl200	a/α	This study
MAY314	BWP17	ura3Δ::λimm434/ura3Δ::λimm434 his1::hisG/his1::hisG arg4::hisG/arg4::RS-ARG4-URA3-BN sir2::HIS1/sir2::dpl200	α/-	This study
MAY315	BWP17	ura3Δ::λimm434/ura3Δ::λimm434 his1::hisG/his1::hisG arg4::hisG/arg4::RS-ARG4-URA3-BN sir2::HIS1/sir2::dpl200	a/-	This study

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

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

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. albicansMTL***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.

Disclosures

The authors have nothing to disclose.

Acknowledgements

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