An Evaluation of *Galleria mellonella* as a Model for *Aspergillus ustus* Infections

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Invasive aspergillosis is a serious cause of mortality and morbidity in immunocompromised hosts. In particular, the emergence of multidrug-resistant fungi, such as *Aspergillus calidoustus*, poses a concern for both doctors and patients. Rapid screening *in vivo* of novel antifungals is essential for discovering new treatments to combat invasive aspergillosis and improve prognosis of patients. As such, this study proposed the use of the greater wax moth, *Galleria mellonella*, as a quick way to model *A. calidoustus* infection. Due to the dangers of working with *A. calidoustus* in a high school setting, *A. ustus* was used in experimentation. It was hypothesized that *G. mellonella* would be susceptible to infection by *A. ustus* and, thus, could be used to model *A. calidoustus* infection in humans. It was also hypothesized that larval mortality would be dose-dependent. Concentrations of 1 x 10^3, 1 x 10^4, and 1 x 10^5 spores of *A. ustus* per 10 µL were injected into the *G. mellonella* larvae, and survival was measured over 96 hours. Larval survival was found to be high, with only the larvae infected with 1 x 10^4 spores being significantly different from the untouched control larvae through a log-rank test, $X^2(1, N=10)=3.89$, $p=0.0486$. This did not support the hypothesis that larval mortality would be dose-dependent, and it implied that *G. mellonella* was not a viable model for *A. ustus* infections. However, factors such as low sample size, low temperature, and low spore concentrations may have affected the data, so *G. mellonella* should not be abandoned as a model for *A. calidoustus* infections without further research.

Introduction

Invasive aspergillosis has become a cause of serious mortality and morbidity for immunocompromised patients. Incapable of providing a proper immune response against fungal infection, patients with HIV/AIDS, leukemia, haematopoietic stem cell transplantation, and the like rely on a multitude of potent antifungals. However, some fungal isolates have been found to display reduced susceptibility to these antifungals, and doctors find it increasingly difficult to treat these infections. One species of particular interest is *Aspergillus calidoustus*, a rare infectious agent characterized by its intrinsic pan-resistance to all major classes of antifungals.[1] Despite often being associated with poor outcome in patients, *A. calidoustus* remains poorly understood, as there is little research on the subject.[2]

Aspergillus calidoustus is a fungal species that is commonly found in groundwater samples and soil.[3] It is an emerging causal agent of invasive aspergillosis and has demonstrated intrinsic reduced susceptibility to amphotericin B, itraconazole, and voriconazole both *in vitro* and *in vivo*.[4,2] Additionally, breakthrough infections in severely immunocompromised patients are common as a result of its innate resistance to azole antifungals, which are commonly used for prophylactic treatment.[5] Its multidrug resistance makes it more difficult to treat than other fungal species, and a higher mortality rate has been associated with *A. calidoustus* in comparison to other *Aspergillus* species in primary cutaneous aspergillosis (PCA) patients.[2]

A recent study compared the mechanisms of azole resistance in *A. calidoustus* to more common *Aspergilli*.[6] Akin to azole-resistant *A. fumigatus*, *A. calidoustus* was found to have point mutations on the *cyp51B* protein complex and the *cyp51C* protein complex, the latter of which was previously only associated with *Aspergillus flavus*. Both complexes contain lanosterol 14-α-demethylase, an important protein in the biosynthetic pathway of producing ergosterol, which forms the main component of the fungal cell wall. These mutations prevent the binding of azole antifungals onto the protein's surface, allowing for the continued production of ergosterol for fungal cell membrane integrity. Additionally, *A. calidoustus* was found to have coding regions for the overexpression of two *Cdr1B* efflux transporter proteins, which actively pump out the azole antifungal from the fungal cell.

Prior to its identification in 2008, clinical manifestations of *A. calidoustus* were incorrectly attributed to *A. ustus*.[7] Both are species of *Aspergillus* section *Usti* and display many similarities; however, they are chiefly distinguished by the temperature at which they can grow. *A. calidoustus* proliferates at 37°C, whereas *A. ustus* is not able to grow at that temperature and, thus, is incapable of infecting humans. However, *A. ustus* has displayed similar resistance patterns to *A. calidoustus*, such as elevated minimum inhibitory concentration (MIC) values for voriconazole.[8] The similarities between these two organisms imply that *A. ustus* can be used as a safer homologue for *A. calidoustus* infection, albeit at temperatures less than 37°C.

Invertebrate models of fungal infection have been shown to be in concordance with murine models of human fungal infections. Additionally, they are considerably less expensive, more ethical, and easier to handle than murine models. One organism commonly used as a model for fungal infections is *Galleria mellonella*, or the greater wax moth. The larvae of *G. mellonella* present a viable model of the human innate immune system's response to fungal infections as well as a model for the identification of novel antifungal compounds.[9] *G. mellonella* haemocytes are homologous to mammalian neutrophils, and a similar response to *A. fumigatus* toxin fumagillin during infection has been reported between both humans and the wax moth.[10] As such, *Galleria mellonella* shows potential for being an effective model for screening new and alternative antifungal compounds *in vivo* against *Aspergillus ustus* and, through comparison, *A. calidoustus*.

The purpose of this experiment was to evaluate the viability of *Galleria mellonella* as a model of *Aspergillus calidoustus* infection. Although studies have demonstrated *G. mellonella* larvae to be a practical model for *Aspergillus fumigatus* and *Aspergillus terreus* infection, no studies thus far have evaluated wax moth larvae as a model for species of *Aspergillus* section *Usti*.[11-12] Moreover, at the time of the study, no *in vivo* models of infection caused by species of *Aspergillus* section *Usti* had been completed in general. Thus, this research aimed to determine the lowest concentration of *A. ustus* conidia that resulted in infection and death of *G. mellonella.* This would aid in the future use of *G. mellonella* as a model to assess the efficacy of novel antifungals rapidly against *A. ustus* and *A. calidoustus*.

It was hypothesized that *Aspergillus ustus* would be able to infect the *G. mellonella* larvae in a dose-dependent manner, meaning that a greater inoculum concentration of spores would result in increased mortality of larvae over a 96 hour time period.

To test this, *Galleria mellonella* were inoculated with 1 x 10^5, 1 x 10^6, and 1 x 10^7 *A. ustus* spores per larva. Larvae were then monitored at 24 hour

increments over a period of 96 hours at 30°C in order to determine the capability of *A. ustus* to infect and kill *G. mellonella* larvae and thus indicate the viability of the wax moth larva as a model for screening novel antifungals against species of *Aspergillus* section *usti in vivo*.

Methods

Aspergillus ustus ATCC 10760 (VWR, Radnor, PA) were grown on potato dextrose agar plates for 48 hours at 30°C. Colonies were then transferred aseptically to potato dextrose agar slants and grown once more at 30°C. After 28 days, the fungal cultures were flooded with 0.85% saline solution, and colonies were gently scraped with a sterile micropipette tip to harvest conidia. The conidia-saline suspensions were then transferred to three sterile test tubes until each had a volume of ~ 3 mL. To evenly distribute conidia, suspensions were mixed by drawing up and down with a micropipette. A volume of 10 µL from each suspension was transferred to a Bright-Line™ hemocytometer (Sigma, St. Louis, MO), and conidia were counted to 1 x 10^7 conidia mL^{-1}. Two suspensions were then serially diluted 10-fold and 100-fold, respectively, to the concentrations of 1 x 10^6 and 1 x 10^5 conidia mL^{-1}.

Larval Inoculation and Mortality:

Larvae of the greater wax moth, *Galleria mellonella* (Carolina Biological, Burlington, NC) arrived in a plastic container filled with nutritive medium. Upon reaching the sixth instar stage, larvae were randomly separated into their respective experimental or control groups to begin experimentation (Figure 1). Larvae were picked up between the thumb and forefinger, and the needle of the hypodermic syringe (1 cc in 1/100 cc, 23 gauge, 1.0 inch needle) was inserted into the last left proleg. A volume of 10 µL of the *A. ustus* conidial suspensions were injected into the larvae, so that larvae received 1 x 10^3, 1 x 10^4, or 1 x 10^5 conidia. Larvae were then placed five each in a petri dish. Additionally, controls of five larvae injected with 10 µL of the 0.85% saline solution and five untouched larvae were placed into petri dishes.

Figure 1. Experimental Design Diagram

Hypothesis: *A. ustus* would be able to infect *G. mellonella*, and infection of *G. mellonella* would be dependent on concentration of conidial inoculum					
Independent Variables: Concentration of *A. ustus* conidial inoculum (1.0 x 10^3 - 1.0 x 10^5 CFU/larva)					
Inoculum Concentration (CFU/larva)	1 x 10^3 CFU/larva	1 x 10^4 CFU/larva	1 x 10^5 CFU/larva	Saline solution only	Untouched larvae
# of Trials (# of Larvae)	5	5	5	5	5
Controls: Untouched *G. mellonella* larvae and *G. mellonella* larvae injected with 10 µL of saline solution					
Dependent Variable: *G. mellonella* survival (in days following inoculation)					
Constants: *A. ustus* would be grown at 30°C; all *G. mellonella* would be kept at 30°C following inoculation; all *A. ustus* would be grown on potato dextrose agar slants; all *G. mellonella* lifespan data would be collected starting on the same day; all *A. ustus* would be of the same strain and same purchase location; all *G. mellonella* larvae would be fed the same food; and all *G. mellonella* larvae would be of the same stage of life					

Each petri dish was incubated for 96 hours at 30°C. Larval mortality was monitored every 24 hours by counting the number of living and dead larvae in each petri dish. Larvae were considered dead if they appeared black or brown or did not respond to touch with forceps (Figure 2). Larval behaviors such as the formation of cocoons and pupation were recorded as well.

Safety Precautions:

All experimentation was completed under a fume hood in order to prevent the spread of *A. ustus* spores in the laboratory. Moreover, aseptic technique was practiced in order to prevent contamination of lab equipment and surfaces. Nitrile gloves and goggles were worn at all times throughout experimentation to prevent unwanted exposure to organisms and chemicals. *G. mellonella* larvae were housed in plastic containers with holes at the top and lidded petri dishes in order to prevent their escaping into the laboratory. Additionally, *G. mellonella* larvae were all disposed of by being frozen, being sealed in a plastic bag, and being placed in the trash.[13] *A. ustus* cultures were subjected to a 10% bleach solution, and Lysol wipes were used to clean the lab area after experimentation was completed.

Statistical Analysis:

Survival rates of *G. mellonella* larvae were evaluated using Kaplan-Meier survival curves and compared using the log-rank (Mantel-Cox) test at $\alpha = 0.05$. All statistical analyses were completed using GraphPad Prism 7.

Figure 2. Types of dead larvae found during experimentation. Larvae whose deaths were attributed to three different causes are shown above. From left to right, this image shows larvae who died from being pierced by a needle, being victim to cannibalization, and being infected by *A. ustus*. Each is an example of a larva that was considered "dead" during experimentation.

Results

Survival data of *G. mellonella* was collected for larvae infected with 1 x 10^3 spores, 1 x 10^4 spores, and 1 x 10^5 spores, as well as larvae injected with a saline solution and untouched larvae over a 360 hour time frame (Tables 1 -5). The number of living larvae, dead larvae, cocoons/pupa, and wax moths in each group were recorded on each day at approximately the same time, excluding weekends. Larvae infected with 1 x 10^5 spores either died or completed metamorphosis by 360 hours, while none of the larvae infected with 1 x 10^4 spores completed metamorphosis, and four-fifths perished. All of the larvae infected with 1 x 10^3 spores spun cocoons, but none of them completed metamorphosis. Both untouched larvae and larvae injected with a saline solution had one death and four larvae complete metamorphosis.

Table 1. Survival data of *G. mellonella* infected with 1 x 10^5 spores over 360 hours

	24 hours	48 hours	72 hours	96 hours	168 hours	192 hours	216 hours	240 hours	264 hours	360 hours
Living larvae	3	0	0	0	0	0	0	0	0	0
Dead larvae	0	2	2	2	2	2	2	2	2	2
Cocoons / Pupa	2	3	3	3	3	3	3	2	1	0
Wax moths	0	0	0	0	0	0	0	1	2	3

Survival data of larvae infected with 1 x 10^5 spores show two larval deaths and the formation of cocoons at 48 hours. Afterwards, no other larvae died, and by 360 hours, the remaining three living larvae exited the cocoons as wax moths.

Table 2. Survival data of *G. mellonella* infected with 1 x 10^4 spores over 360 hours

	24 hours	48 hours	72 hours	96 hours	168 hours	192 hours	216 hours	240 hours	264 hours	360 hours
Living larvae	5	5	3	2	1	0	0	0	0	0
Dead larvae	0	0	2	3	3	3	3	3	3	4
Cocoons/ Pupa	0	0	0	0	1	2	2	2	2	1
Wax moths	0	0	0	0	0	0	0	0	0	0

Survival data of larvae infected with 1 x 10^4 spores show two larval deaths at 72 hours and one larval death at 96 hours. Additionally, none of the larvae completed metamorphosis during the 360 hour period.

Table 3. Survival data of *G. mellonella* infected with 1 x 10^3 spores over 360 hours

	24 hours	48 hours	72 hours	96 hours	168 hours	192 hours	216 hours	240 hours	264 hours	360 hours
Living larvae	2	0	0	0	0	0	0	0	0	0
Dead larvae	0	0	0	0	0	0	0	0	0	0
Cocoons / Pupa	3	5	5	5	5	5	5	5	5	5
Wax moths	0	0	0	0	0	0	0	0	0	0

Survival data of larvae infected with 1 x 10^3 spores show all larvae forming cocoons by 96 hours. None of the larvae exited the cocoon and became a wax moth during the 360 hour time span.

Table 4. Survival data of *G. mellonella* injected with a saline solution over 360 hours

	24 hours	48 hours	72 hours	96 hours	168 hours	192 hours	216 hours	240 hours	264 hours	360 hours
Living larvae	3	1	1	0	0	0	0	0	0	0
Dead larvae	0	0	0	1	1	1	1	1	1	1
Cocoons / Pupa	2	4	4	4	4	4	4	3	1	0
Wax moths	0	0	0	0	0	0	0	1	3	4

Survival data of larvae injected with a saline solution show four larvae forming cocoons by 48 hours and one larva dying at 96 hours. The four larvae who formed cocoons all completed metamorphosis and exited the cocoons as wax moths by 360 hours.

Table 5. Survival data of untouched *G. mellonella* over 360 hours

	24 hours	48 hours	72 hours	96 hours	168 hours	192 hours	216 hours	240 hours	264 hours	360 hours
Living larvae	4	3	1	0	0	0	0	0	0	0
Dead larvae	0	0	0	0	1	1	1	1	1	1
Cocoons / Pupa	1	2	4	5	4	4	4	2	1	0
Wax moths	0	0	0	0	0	0	0	2	3	4

Survival data of untouched larvae show all larvae forming cocoons by 96 hours. At 168 hours, one larva exited its cocoon and was discovered dead. The remaining four larvae all exited their cocoons as wax moths by 360 hours.

The data were then used to create Kaplan-Meier survival curves to model *G. mellonella* larvae mortality over 96 hours (Figure 3). Of the three inocula concentration groups, larvae infected with 1 x 10^4 spores had the lowest survival rate, whereas larvae infected with 1 x 10^3 spores appeared to have the greatest survival rate. However, larvae infected with 1 x 10^3 spores became censored 48 hours after infection. All five of the larvae in this group formed cocoons, preventing the further collection of mortality data.

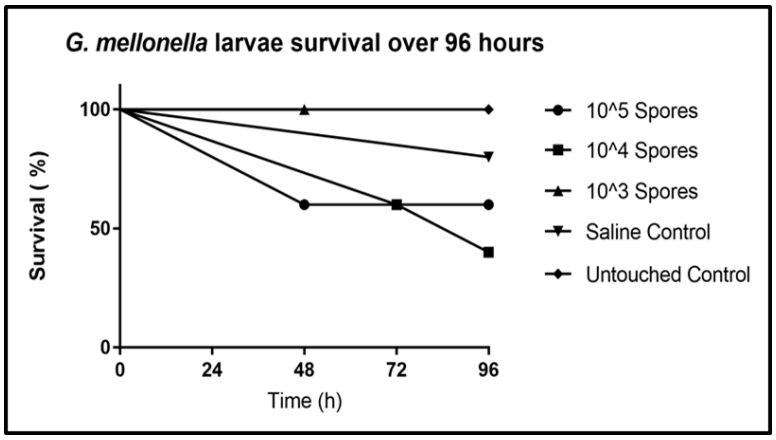

Figure 3. Kaplan-Meier survival estimator of mortality of *G. mellonella* larvae over 96 hours. Kaplan-Meier survival curves show *G. mellonella* larval mortality at different concentrations of spore inocula. Five larvae in each group were either infected with a spore solution, injected with a saline control, or left untouched. No larva in the untouched control died during the 96 hour period, while a single larva died in the saline control. Larvae injected with 1 x 10^3 spores were censored at 48 hours. Only the group of larvae injected with 1 x 10^4 spores had less than a 50% survival rate, but they began to die later (72 hours) than larvae injected with 1 x 10^5 spores (48 hours).

Larvae infected with 1 x 10^5 spores were the first group to succumb to infection, 48 hours after experimentation began. Two of the five larvae died at this time point, but afterwards no other larvae in this group perished. At 72 hours post-infection, mortality was first recorded for larvae injected with 1 x 10^4 spores. Unlike larvae infected with 1 x 10^5 spores, percent survival continued to decrease at 96 hours for this group. The Kaplan-Meier curve for the untouched control displayed 100% survival for all the larvae over the 96 hour time frame. In the saline control group, however, mortality was observed at 96 hours.

A log-rank test (Mantel-Cox) was run to compare the Kaplan-Meier curves at alpha = 0.05. Results were found to be non-significant, $X^2(4, N = 25) = 7.42$, $p = 0.1153$. However, each inoculum concentration was also compared individually with the untouched control. Only the Kaplan-Meier curve of larvae infected with 1 x 10^4 spores was found to be significantly different from that of the untouched control, with $X^2(1, N = 10) = 4.464$, $p = 0.0486$.

The differences between the untouched larvae and larvae infected with the 1 x 10^5 spores and 1 x 10^3 spores were both non-significant, with $X^2(1, N = 10) = 2.25$, $p = 0.1336$ and $X^2 (1, N = 10) = 0$, $p = 0.9999$, respectively.

Because some of the larvae formed cocoons during experimentation, the number of moths that completed pupation was counted after 15 days. The remaining larvae infected with 10^5 spores successfully formed cocoons and developed into viable wax moths. However, neither of the remaining larvae inoculated with 1 x 10^4 spores completed the pupal stage, nor did any of the five larvae inoculated with 1 x 10^3 spores. Interestingly, one of the untouched larvae came out of its cocoon and died for no apparent cause, but the remaining four larvae all became moths. The remaining larvae of the saline control all finished pupation and transformed into moths as well.

Discussion

Antifungal resistance is currently a major concern for both caregivers and patients. In particular, infections by multidrug-resistant species, such as *A. calidoustus,* are difficult to treat because of their resistance to current treatments. Moreover, it is necessary that doctors be able to rapidly screen novel antifungals against such species *in vivo*. A promising model for antifungal testing *in vivo* is the greater wax moth, *Galleria mellonella*. As such, the goal of this research was to determine whether *Galleria mellonella* could be a viable model for *Aspergillus calidoustus* infections through the use of the genetically and morphologically similar *A. ustus*. It was hypothesized that *A. ustus* would be capable of infecting *G. mellonella*, with an increasing spore dosage correlating to an increase in wax moth mortality over 96 hours. Mortality data of wax moths infected with increasing doses of *A. ustus* spores (1 x 10^3,1 x 10^4, and 1 x 10^5) was collected over a 96 hour period.

A log-rank test (Mantel-Cox) found that there were no significant differences between the Kaplan-Meier survival curves for each group when they were all compared at alpha = 0.05, $X^2 (4, N = 25) = 7.42$, $p = 0.1153$ (Figure 3). Considered individually, however, a significant difference was found between survival curves of larvae infected with 1 x 10^4 spores and larvae of the untouched control, $X^2 (1, N = 10) = 3.89$, $p = 0.0486$. The other experimental groups and the saline control were not found to be significantly different from the untouched control ($p > 0.05$). Therefore, the hypothesis that larval mortality would be dose-dependent was not supported by the log-rank test or the Kaplan-Meier curves, as the data showed that the greatest mortality occurred in larvae infected with 1 x 10^4 spores rather than those infected with 1 x 10^5 spores. Even at this concentration, larval mortality was only slightly greater than 50% after 96 hours.

Thus it appears that *G. mellonella* is not an appropriate model of *A. ustus* infections. The data support that *G. mellonella* is little affected by its inoculation with *A. ustus* spores. It should be noted, however, that the spore concentrations tested in this study were significantly lower than those used in prior studies. Maurer et al. (2015) assessed whether spore dosage and temperature affected *G. mellonella* prognosis after infection with *A. terreus*. Testing with spore concentrations of 1 x 10^5 - 1 x 10^7 per larva, the authors found that larval survival was inversely related to spore concentration and incubation temperature. At the optimal growth temperature for *A. terreus*, 37°C, a dose of 1 x 10^5 spores resulted in 50% larval mortality. Similarly, infection by *A. ustus* with a dose of 1 x 10^4 spores produced >50% mortality in larvae in this research at its optimal growth temperature of 30°C. Infection with 1 x 10^6 or 1 x 10^7 spores of *A. ustus* per larva, therefore, may decrease survival of *G. mellonella* and increase its viability as a model for *Aspergillus ustus* infections.

As Maurer et al. concluded, incubation temperature also plays a role in the capacity of *G. mellonella* larvae to succumb to infection.[12] In the case of *A. terreus*, a higher temperature (37°C versus 30°C) was associated with increased larval mortality. However, *A. ustus* is unique among *Aspergilli* in that is incapable of growing at temperatures greater than 30°C. As such, 30°C would have been the optimal temperature for *A. ustus* pathogenesis. However, Kwadha, Ong'amo, Ndegwa, Raina, and Fombong reported that the temperature range of 29-33°C is optimal for the development of *G. mellonella* larvae and that temperatures above this range can impair development.[14] To determine whether temperature contributes to larvae immune response to fungal infection, García-Rodas, Casadevall, Rodríguez-Tudela, Cuenca-Estrella, and Zaragoza tested for the level of phagocytosis of larval haemocytes on *Cryptococcus neoformans* at 30°C and 37°C, finding that the larval immune system was impaired at the latter temperature.[15]

Therefore, larvae infected with *A. ustus* spores at 30°C may have had a primed immune system. The increased phagocytic activity of larval haemocytes could have lowered the ability of *A. ustus* spores to kill *G. mellonella* larvae and thereby reduced the apparent viability of the *G. mellonella* model for such fungal infections. Future research could focus on using the more virulent *A. calidoustus*, which is capable of growth at 37°C, at which the larval immune system is impaired. Additionally, a greater number of larvae could be used to evaluate the efficacy of the *G. mellonella* model, as this study only tested with groups of 5 larvae. Larger groups, with sample sizes of 10 or 20 larvae, would make the results more comprehensive and conclusive. Furthermore, a macroscopic dissection of larvae and histological analysis would be completed in the future to better understand *A. ustus* and *A. calidoustus* pathogenesis.

It should also be acknowledged that larvae were in various instar stages during experimentation. Although the majority of chosen larvae were in the 5th fifth instar stage, a few larvae may have been in earlier or later stages. For instance, one larva infected with 1×10^4 spores committed an act of cannibalism, eating another larva. Such behavior generally occurs when larvae are in starved conditions; however, fifth instar larvae stop eating, so this behavior implies that some larvae were in an earlier stage. Moreover, many larvae formed cocoons during experimentation. Although this was handled by counting the number of wax moths that completed metamorphosis, the formation of cocoons may have skewed the data by making it impossible to determine whether larvae were living or dead, especially since there were so few larvae in the study. As such, future research should use larvae that are stored at reduced temperatures of around 15°C to halt larval development past the instar stages and to prevent the formation of cocoons.

In conclusion, this study was the first to evaluate *G. mellonella* as a potential model for infections caused by *A. ustus*, a species of *Aspergillus* section *Usti*. The data support that *G. mellonella* is not an appropriate model for invasive aspergillosis caused by *Aspergillus ustus*. However, low sample size, low spore concentrations, and low temperature may have decreased the viability of the larvae as such a model. Experimentation with *A. calidoustus* and immunocompromised *G. mellonella* larvae at 37°C has yet to be completed, so *G. mellonella* should not be abandoned as an easy-to-use and inexpensive model for *A. calidoustus* infections.

Acknowledgements

The author would like to thank his parents for support and financial aid during the research process, and he would like to thank Dr. Michael Wyatt and Dr. Michelle Wyatt for their aid in acquiring the *Aspergillus ustus.* The author would also like to thank Mrs. Michelle Spigner and Rahithya Meda for their help in infecting the wax moth larvae with *A. ustus* spores, as well as his peers for reviewing his paper. Finally, the author would like to thank Dr. Steinbach and Dr. Shwab of Duke University for their aid in providing the methodology used during experimentation.

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