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Drosophila and Galleria insect model hosts

New tools for the study of fungal virulence, pharmacology and immunology

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Over recent years we have witnessed the emergence of several non-vertebrate mini-hosts as alternative pathosystems for the study of fungal disease. These heterologous organisms have unique advantages, as they are economical, ethically expedient and facile to use. Hence, they are amenable to high-throughput screening studies of fungal genomes for identification of novel virulence genes and of chemical libraries for discovery of new antifungal compounds. In addition, because they have evolutionarily conserved immunity they offer the opportunity to better understand innate immune responses against medically important fungi. In this review, we discuss how the insects *Drosophila melanogaster* and *Galleria mellonella* can be employed for the study of various facets of host-fungal interactions as complementary hosts to conventional vertebrate animal models.

Introduction

The frequency, spectrum and associated cost of opportunistic invasive fungal infections have significantly increased over the past two decades accounted for by the rapidly growing populations of immunosuppressed and debilitated patients.[1] In spite of the parallel expansion of the antifungal armamentarium, patients who develop such infections have considerable mortality, often exceeding 50% despite the administration of potent antifungal therapy.[1] This substantial disease burden of opportunistic mycoses in humans underscores the need for better understanding of the molecular pathogenesis of these infections, from both the host and the pathogen fronts, and for identification of novel therapeutic targets.

Pathogenesis, pharmacology and immunology research has traditionally relied on mammalian models such as mice, rats, rabbits and guinea pigs, but has recently been complemented by the introduction of a variety of non-vertebrate pathosystems with tractable genetics and conserved innate immunity.[2,3] These easy-to-use hosts have found widespread applications in research of both infectious (bacterial and fungal)[2-8] and non-infectious diseases (i.e., dementia, stroke, cancer, diabetes),[9] as they lack the logistical and ethical constraints associated with conventional model hosts, and they are amenable to high-throughput testing and large-scale forward and reverse genetics with low cost.

The spark for the explosion in the use of mini-host models for studying fungal disease in particular, was the Nobel Laureate Jules Hoffmann's discovery that the Toll signaling pathway in *Drosophila melanogaster* is indispensable for effective antifungal host defense;[10] that breakthrough report in *Cell* was powerfully illustrated by the scanning electron microscopy picture of germinating Aspergillus hyphae covering the surface of a dead Toll-deficient fly[10] and paved the way for a new era in fungal disease research. Since then, besides the Drosophila fruit fly, several other elegant pathosystems have been exploited to study fungal pathogenesis, the efficacy of antifungal compounds, and innate antifungal immunity such as the greater wax moth *Galleria mellonella*, the nematode *Caenorhabditis elegans,* the soil-living amoebas *Acanthamoeba castellanii* and *Dictyostelium discoideum,* the silkworm *Bombyx mori*, the mosquito *Culex quinquefasciatus*, the German cockroach *Blattella germanica* and the plant *Arabidopsis thaliana* (**Table 1**).[11-36] Herein we outline the recent developments, challenges and comparative advantages of the insect hosts *Drosophila melanogaster* and *Galleria mellonella* in studying fungal virulence, pharmacology and immunology (**Table 2**).

Fungal Virulence Studies

The breadth of genetic information obtained from the completion of the Aspergillus, Candida and Cryptococcus genome sequencing[37-39] has created the need for testing fungal virulence traits in simple high-throughput in vivo assays for assessment of their contribution to pathogenesis. Identification of new virulence factors via large-scale screens may uncover novel targets for diagnosis and treatment of opportunistic mycoses. In this regard, Chamilos et al.[40] employed Drosophila to screen the virulence potential of 34 *Candida albicans* mutant strains defective in putative transcription factor genes. Of these, only one strain, defective in Cas5, a cell wall integrity regulator, was found to be avirulent; the lack of virulence was then confirmed in a mouse model of systemic candidiasis providing a proof of concept that Drosophila is promising for large-scale studies of genes involved in fungal pathogenesis in mammals.

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Table 1. Summary of non-vertebrate host models that have been adapted for the study of medically important fungi

Fungus	*Drosophila melano-gaster*	*Galleria mellonella*	*Bombyx mori*	*Caenorhabditis elegans*	*Acanthamoeba castellanii*	*Dictyostelium discoideum*	*Culex quinque-fasciatus*	*Blattella germanica*	*Arabidopsis thaliana*
Molds									
Aspergillus	+ [11]	+ [17]						+ [31]	
Zygomycetes	+ [12]								
Fusarium	+ [13]	+ [18]							+ [32]
Scedosporium	+ [13]								
Dimorphic fungi									
Histoplasma				+ [24]	+ [27]				
Blastomyces					+ [27]				
Sporothrix					+ [27]				
Yeasts									
Candida	+ [14]	+ [19]	+ [23]	+ [25]			+ [30]		
Cryptococcus	+ [15]	+ [20]		+ [26]	+ [28]	+ [29]			
Pneumocystis	+ [16]	+ [21]							
Dermatophytes									
Microsporum		+ [22]							

+ denotes that the corresponding non-vertebrate host has been used for studying the specific fungal pathogen. References are noted within the bracket.

Three infection assays have been used for assessment of fungal virulence in insects: injection, rolling and ingestion assays. Although quantification of the infecting inoculum is feasible only in the injection assay, the availability of different routes of infection is permissive to comparative analyses of fungal virulence and host-pathogen interactions between an acute infection introduced directly into the hemolymph (injection assay) vs. more protracted infections originating from epithelial surfaces [i.e., skin (rolling assay) or gastrointestinal mucosa (ingestion assay)]. To that end, the *alb1*-deficient *Aspergillus fumigatus* mutant was found to be hypovirulent in Drosophila when introduced via epithelial surfaces but not by injection.[11]

Simple experimental protocols are available for both Drosophila and Galleria and may be adapted in any laboratory;[41,42] yet, differences between the two hosts do exist. Specifically, use of Drosophila requires more specialized equipment and experience than does Galleria. Further, because wild-type Drosophila are resistant to fungi, flies with perturbations in the Toll pathway need to be used, which entails fly genetic crossing; instead, wild-type Galleria larvae can be purchased from vendors, housed in Petri dishes in regular incubators and used directly without genetic crossing. Moreover, quantifying the infecting inoculum is more accurate in Galleria than in Drosophila. These advantages make Galleria an attractive host for future high-throughput screening studies of fungal virulence traits.

Besides screening for new virulence factors, insects have been extensively used to test the virulence of fungal strains previously known to be hypovirulent or avirulent in mammals.[11,14,15,17,19,20,43] These studies have revealed significant concordance in fungal pathogenicity between the phylogenetically disparate mammals and insects;[43-45] this finding has potential evolutionary implications as fungal virulence may have evolved as a countermeasure to environmental predation by non-vertebrate organisms that feed on fungi.[7,46] Consistent with this notion, *Histoplasma capsulatum* and *Cryptococcus neoformans* strains were reported to enhance their virulence after passage through amoebas;[27,28] thus, whether and how interaction of fungi with Drosophila or Galleria results in modulation of the expression of virulence factors merits investigation.

Despite the similarities in fungal virulence between mammalian and insect hosts, differences do exist; two examples are worthwhile mentioning. First, an *Aspergillus fumigatus* mutant strain lacking CgrA, a key thermotolerance regulator, was hypovirulent in mice but fully virulent in Toll-deficient Drosophila.[47] Therefore, because flies are infected and maintained at 29°C, certain aspects of fungal virulence in mammals may not be accurately modeled in this organism; Galleria, which can be maintained at 37°C, the mammalian physiologic temperature, may be used instead, taking into account however that increasing the temperature of Galleria to 37°C itself alters cellular and humoral immune responses.[48,49] Second, the *alb1*-deficient *Aspergillus fumigatus* mutant, which is hypovirulent in mice and flies,[11,50] was hypervirulent in Galleria, in which it appears to trigger dysregulated immunopathology.[51] Thus, the absence of virulence of a fungal strain in one host does not preclude its pathogenicity in another pathosystem. In fact, testing the virulence potential of fungal strains in different models could identify factors that regulate host-specific phenotypic expression of individual virulence traits.

Table 2. Comparative characteristics of the fruit fly *Drosophila melanogaster* and the greater wax moth *Galleria mellonella* heterologous hosts in the study of host-fungal interactions

Characteristic	*Drosophila melanogaster*	*Galleria mellonella*
Genetic tractability	+	-
Sequenced genome	Completed	-
Insect mutant strain availability	+	-
Gene microarrays	+	+
RNA interference libraries	+	-
Availability of phagocytic cell lines	+	+
Potential for harvesting of phagocytes for ex vivo studies	-	+
Adaptive immunity	-	-
Chemokine/cytokine production	-	-
Need for simple laboratory resources	±	+
Precision in fungal inoculum delivery with injection	±	+
Need for genetic crossing	+	-
Overall cost	Low	Low
Correlation of virulence factors with mammalian models	+	+
Potential for large-scale screening studies of fungal genomes	+	+
Survival at mammalian physiologic temperature (37°C)	-	+
Suitable for orally absorbed antifungal compound testing studies	+	+
Suitable for parenteral antifungal compound testing studies	±	+
Precision in parenteral delivery of drugs	-	+
Potential for pharmacokinetic studies	-	-
Potential for pharmacodynamic studies	-	+

+ denotes the presence and - denotes the absence of the corresponding characteristic.

Fungal Pharmacology Studies

The suboptimal in vivo efficacy of modern antifungal agents in immunocompromised patients and the increasing rates of drug resistance in fungi[1] emphasize the need for discovering new drug targets and devising novel therapeutic strategies such as combination antifungal therapy with different classes of drugs. The conventional methods of drug discovery involve either (a) computational selection of potential pathogen gene targets based on genome sequencing information and screening of chemical libraries for molecules that inhibit target gene function[52] or (b) screening of small molecule libraries for the capacity to induce a specific phenotype in purified protein targets or cultured cells;[53] subsequently, promising compounds are tested in vivo in mammalian hosts. Nevertheless, such host-free-based drug discovery methods are infrequently fruitful because they do not portray the complex and dynamic host-pathogen interactions that occur in vivo. Thus, non-vertebrates have been exploited as alternative strategies for the initial large-scale screening of molecules for antifungal activity before validation in mammals takes place; this approach was pioneered in *Caenorhabditis elegans* yielding compounds with potent anti-Candida activity.[54] Although less amenable to automated mass screening studies than *Caenorhabditis elegans,* insects may also be used; in fact, Drosophila was effectively employed to identify molecules that slowed disease progression in Fragile X syndrome through a screen of 2,000 compounds in *Fmr1*-mutant flies.[55]

Furthermore, the efficacy of licensed antifungal agents has been evaluated in Drosophila and Galleria demonstrating remarkable correlation between in vitro susceptibility testing results and in vivo drug efficacy in both insects and mammals.[11,13,14,20] Also, insects have been successfully used to demonstrate synergy between voriconazole and terbinafine against *Aspergillus fumigatus* (in Drosophila),[11] and between amphotericin B and 5-flucytosine against *Cryptococcus neoformans* (in Galleria);[20] these drug combinations are synergistic in vitro and in mammals, thus providing evidence that insects may be utilized as complementary "in vivo checkerboard assays," which are particularly time-consuming, laborious and expensive in conventional animal models. In addition, Galleria studies showed that combination of fluconazole with an inhibitor of the molecular chaperone Hsp90, which mediates resistance of fungi to azoles, was synergistic against candidiasis,[56] implying that insects are promising hosts for assessing the efficacy of innovative therapeutic strategies such as combination of antifungal agents with immune- or virulence-modulating drugs.

Despite their potential, pharmacology studies in insects also have limitations. Thus, although both Drosophila and Galleria can be used for testing orally absorbable compounds, the exact ingested drug dose per insect is impossible to quantify. Testing of parenteral antifungal compounds also has constraints as repeated drug injections lead to injury, especially in Drosophila. Precise quantification of the injected drug dose is

only feasible in Galleria, in which pharmacodynamic studies may be attempted, as demonstrated in the related silkworm *Bombyx mori*.[23] Yet, pharmacokinetic analyses are problematic in insects and reported methods for measurement of drug levels are technically challenging and often imprecise.[57] Importantly, critical pharmacological parameters such as drug absorption, distribution, metabolism, excretion, toxicity and drug-drug interactions are difficult to reliably study in insect models, and necessitate testing in mammalian hosts that are phylogenetically closer to humans.

Fungal Immunology Studies

Because insect innate immune responses at the epithelial, cellular and humoral levels are remarkably well characterized and highly conserved through mammals,[3] these mini-hosts have emerged as major tools for fungal immunology studies, with Drosophila being at the forefront. The fruit fly is amenable to forward and reverse genetics and large collections of Drosophila mutants and transgenic cell lines are commercially available (http://flybase.org). The Drosophila genome sequence has been completed and is among the most fully annotated eukaryotic genomes. Thus, gene microarrays have been generated, double-stranded RNA has been synthesized for all genes (www.flyrnai.org) and RNA interference technology is commercially available for conditional inactivation of any gene at the whole-animal or tissue levels (http://stockcenter.vdrc.at/control/main). In fact, Cronin et al. by performing such a genome-wide in vivo Drosophila RNA interference screen, discovered that the JAK-STAT signaling pathway regulates epithelial immune responses in the fruit fly.[58] In contrast to Drosophila, the Galleria genome has not been sequenced (**Table 2**). Nonetheless, the recent characterization of the Galleria immune gene repertoire and transcriptome by next generation sequencing and traditional Sanger sequencing[59] has led to the design of gene microarrays and paves the way for further use of Galleria for elucidation of innate antifungal immune mechanisms.

Insects mount highly efficient and orchestrated innate antifungal immune responses and are resistant to fungal microorganisms. The first line of defense consists of epithelial responses that prevent fungal colonization and infection. When physical barriers are breached and fungi invade within the insect body, insects induce a highly coordinated immune response that has both cellular and humoral constituents, mediated by a primitive phagocytic system and the generation of natural defensin-like molecules, respectively.[3] In Drosophila, as opposed to the requirement of intact Toll signaling for defense against systemic fungal challenge, the induction of protective antifungal immune responses at the epithelial level is Toll-independent. Consistent with that, ingestion but not injection of Cryptococcus results in mortality in wild-type Toll-sufficient Drosophila.[15] Instead of Toll, epithelial antifungal immune responses in the fruit fly are mediated by the dual oxidase (DUOX), JAK-STAT and immune deficiency (imd) pathways,[58,60,61] the conservation of which through mammals, and the similarity in the intestinal epithelium anatomy and regeneration time between flies and mammals[62] support the utility of Drosophila for examining immunological mechanisms of mucosal colonization and infection by yeasts. In addition, Drosophila shows promise for investigating the impact of gut microbiota on modulating mucosal innate immune responses and protecting against fungal mucosal colonization and invasion.[63]

Furthermore, the phagocytosis-defective *eater*-null Drosophila strain and the Drosophila S2 phagocytic cell line are valuable tools for studying cellular immune responses in the fruit fly; the former was used to show that phagocytosis is indispensable for fly survival against zygomycosis.[12] The latter was used by Stroschein-Stevenson and colleagues[64] to describe a novel protein called macroglobulin complement related, a member of the a2-macroglobulin/complement family, which was induced after exposure of S2 phagocytic cells to *Candida albicans*; the protein bound specifically on the surface of yeast cells and enhanced phagocytosis. In addition, Qin et al. recently utilized the S2 phagocytic cell line and identified evolutionarily conserved host factors associated with autophagy (e.g., Atg2, Atg5, Atg9, Pi3K59F), which were induced after exposure to *Cryptococcus neoformans.* The investigators then used a small interfering RNA approach to deplete the aforementioned autophagy molecules in murine RAW264.7 macrophages, and demonstrated their requirement for cryptococcal intracellular trafficking and replication within phagocytes.[65] Moreover, other researchers demonstrated that the S2 phagocytic cells exhibited decreased phagocytosis and impaired ability to damage hyphae of Zygomycetes compared with Aspergillus.[12]

In Galleria, the phagocytic system consists of six classes of hemocytes (i.e., prohemocytes, coagulocytes, spherulocytes, oenocytoids, plasmatocytes and granulocytes) and displays similarities in mechanisms of oxidative killing with mammals. Specifically, immunoblotting studies in hemocytes revealed the conservation of human protein homologs involved in generation of reactive oxygen species such as the subunits of the NADPH oxidase complex gp91phox, p47phox and p67phox,[66] which are mutated in patients with chronic granulomatous disease.[67] In contrast to Drosophila, the larger size of Galleria allows for hemocyte harvesting from the larval hemolymph, and Fluorescence-activated cell sorting (FACS) can be applied to (a) determine their density, which has been shown to inversely correlate with the pathogenicity of the infecting fungal strain[68] and to (b) evaluate their phagocytic capacity. To that end, studies have shown that hemocytes display substantially reduced rates of phagocytosis against Aspergillus germinating conidia compared with resting conidia.[69] Also, whereas hemocytes effectively inhibited germination of *Aspergillus fumigatus* conidia, they failed to do so against *Aspergillus flavus* spores.[70]

With regard to humoral immunity, the Toll signaling cascade, the fly counterpart of mammalian Toll/IL-1β receptor signaling, is crucial for host defense against systemic fungal insult via induction of potent antifungal peptide genes such as drosomycin and metchnikowin in the Drosophila fat body, which are then released into the fly hemolymph;[10,71] several Toll-deficient mutant Drosophila strains have been generated and used to study an array

of medically important fungi (**Table 1**). Further, besides Toll, other genes such as the transcription factor FOXO were recently recognized to also regulate drosomycin production[72] offering an opportunity to potentially decode novel antifungal effector mechanisms in mammals. Nonetheless, informative fungus-specific differences in Toll dependence for antifungal host defense do exist. For example, wild-type flies are highly susceptible to Zygomycetes injection,[12] demonstrating that Toll signaling is not sufficient for effective host defense against all fungal pathogens. In fact, despite Toll activation, Zygomycetes (but not Aspergillus) infection resulted in significant downregulation of a distinct set of genes that are important for innate immune activation, global stress responses and tissue repair in wild-type Drosophila.[12]

In Galleria, the Drosophila drosomycin analog is gallerimycin.[73] Besides gallerimycin, several natural antifungal peptides with homology to mammalian antimicrobial peptides (e.g., galiomicin, cecropins, moricins) and peptides that inhibit fungal virulence factors [e.g., insect metalloproteinase inhibitor (IMPI)] have been identified in Galleria using proteomic approaches;[74-76] actually, some of these peptides have been cloned in order to develop novel antifungal agents,[75] and their transgenic expression was reported to confer resistance to fungal pathogens in agriculture.[77] Of interest, pre-exposure of Galleria to non-pathogenic fungi or non-lethal inocula of *Candida albicans* or *Aspergillus fumigatus* leads to induction of protective antimicrobial peptides against subsequent lethal fungal re-challenge;[76,78] in fact, Galleria was recently shown that is able to assess the extent of the infecting fungal inoculum and differentially activate cellular and/or humoral immune responses.[78]

In summary, because Drosophila and Galleria have differential susceptibility to infection by some fungi (e.g., wild-type Galleria is susceptible to Candida or Cryptococcus injection whereas wild-type Drosophila is not),[14,15,19,20] and because an insect may exhibit differential susceptibility to a specific fungus depending on the route of fungal inoculation (e.g., Cryptococcus ingestion but not injection kills wild-type Drosophila,[20] and Candida injection but not ingestion kills adult Toll-deficient flies[14]), comparative analyses of immune responses using more than one insect hosts and more than one fungal inoculation assays could be enlightening for dissecting fungus- and tissue-specific innate immune mechanisms.[79] Finally, insects may be modeled to investigate important understudied areas in human antifungal immunity such as the impact of immunosenescence[80] and sex hormones[81] on induction of antifungal immune responses and fungal infection susceptibility.[82,83]

Despite the aforementioned advantages, studying fungal immunology in insects has shortcomings; first, although antifungal innate immune signaling is substantially conserved between insects and mammals, important differences in innate immune sensing of fungi do exist. Specifically, besides Toll-like receptors, C-type lectins such as Dectin-1 and Dectin-2 are critical for fungal recognition and downstream antifungal effector function in mammals; conversely, no homologs for C-type lectin pattern recognition receptors exist in insects.[84] In addition, fungal sensing in insects entails two independent processes; the first, which also operates in mammals, involves direct recognition of invariant fungal molecular patterns by pattern recognition receptors (i.e., GNBP3 in Drosophila and PGRP-1 in Lepidoptera),[85,86] and the second involves direct sensing of fungal secreted virulence factors by the Drosophila Toll cascade-activating Persephone protease.[85] In contrast to insects, such a host sensor system dedicated to the detection of fungal virulence activity has not been identified in mammalian innate antifungal immunity thus far. Furthermore, insects do not mount adaptive immune responses and lack specialized immune cells including natural killer cells, dendritic cells, T lymphocytes and γδ T cells that secrete cytokines, chemokines and other immunomodulatory factors in mammals. Lastly, because insects have no orthologs for key genes involved in human antifungal immunity against mucocutaneous mycoses such as *AIRE* (autoimmune regulator), *CARD9* (Caspase recruitment domain-containing protein 9), *STAT1* (Signal Transducer and Activator of Transcription 1), *STAT3* (Signal Transducer and Activator of Transcription 3), *DOCK8* (Dedicator of cytokinesis 8), *IL17RA* (interleukin 17 receptor A) and *IL17F* (interleukin 17F),[87] they are not suitable for studying all facets of immunopathogenesis of human fungal disease.

Concluding Remarks

Drosophila melanogaster and *Galleria mellonella* have emerged at the forefront of host-fungal interaction research and show promise for identification of novel fungal virulence genes, testing the efficacy of antifungal drugs, and deciphering conserved antifungal innate immunity mechanisms. Because no single non-vertebrate organism fully reproduces all aspects of mammalian fungal infection, comparative research in these hosts is required and should be complemented by studies in mammalian models of infection. The use of a combination of vertebrate and non-vertebrate in vivo pathosystems should improve our understanding of fungal pathogenesis, pharmacology and immunology and should lead to better outcomes of opportunistic fungal infections in humans.

Conflicts of Interest

The author declares that no conflict of interest exists.

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