**Drosophila melanogaster as a Model for Studying Aspergillus fumigatus**

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Abstract *Drosophila melanogaster* is a useful model organism that offers essential insights into developmental and cellular processes shared with humans, which has been adapted for large scale analysis of medically important microbes and to test the toxicity of heavy metals, industrial solvents and other poisonous substances. We here give a brief review of the use of the *Drosophila* model in medical mycology, discuss the volatile organic compounds (VOCs) produced by the opportunistic human pathogen, *Aspergillus fumigatus*, and give a brief summary of what is known about the toxicity of some common fungal VOCs. Further, we discuss the use of VOC detection as an indirect indicator of fungal growth, including for early diagnosis of aspergillosis. Finally, we hypothesize that *D. melanogaster* has promise for investigating the role of VOCs synthesized by *A. fumigatus* as possible virulence factors.

Keywords *Aspergillus fumigatus*, *Drosophila melanogaster*, Volatile organic compounds

*Aspergillus fumigatus* is a cosmopolitan filamentous fungus found in soils all over the world. As an opportunistic human pathogen, it causes localized infections, aspergilloma (fungus ball), allergic bronchopulmonary aspergillosis, and invasive aspergillosis in immunocompromised patients [1, 2]. The likelihood of serious *Aspergillus* infection, with accompanying high morbidity and mortality, is based on three factors: the status of immunocompromised patients, the degree of exposure, and fungal virulence [3]. Individuals with hematological malignancies, hematopoietic stem cell transplant recipients, and recipients of solid organ transplants are at highest risk of developing systemic aspergillosis [2, 4]. In both humans and animals, aspergillosis can be caused by a number of different *Aspergillus* species, but *A. fumigatus* is the most prevalent etiological agent.

In this review, we briefly discuss the use of the invertebrate model, *Drosophila melanogaster* (the “fruit fly”) in medical mycology, specifically with reference to the study of *A. fumigatus* pathogenicity. We review what is known about volatile organic compounds produced by *A. fumigatus*, hypothesize that these volatile organic compounds (VOCs) may serve as pathogenicity factors, and present preliminary data showing that exposure of *Drosophila* to the VOCs produced by growing *A. fumigatus* retards metamorphosis and increases mortality.

**DROSOPHILA MELANOGASTER AS A MODEL IN MEDICAL MYCOLOGY**

Mammalian models are “the gold standard” for studying most human diseases. For aspergillosis, the mouse has been widely used to investigate virulence factors of the pathogen and their interactions with host factors, as well as to screen for treatment options for this infection [5]. In the majority of published research, immunosuppressed mice have been studied, but there is considerable variation in parameters involving induction of immunosuppression, inoculation route, strain of *A. fumigatus* and other experimental parameters [6].

However, the use of mammalian models to study *Aspergillus* pathogenicity has logistical limitations because they are expensive and labor-intensive. When large-scale screening of *Aspergillus* mutants is warranted, the costs can be prohibitive. Therefore, a number of invertebrate models, including *Caenorhabditis elegans*, *Drosophila melanogaster*, and *Galleria mellonella* have been developed as promising alternatives to study the pathogenesis of medically important fungi [7-13].
Among them, *Drosophila melanogaster* (the “fruit fly”) has many advantages as an experimental system [14]. In addition to short generation time, flies have a high reproductive rate; inexpensive and uncomplicated maintenance; and a long history of use in classical genetics. The fly is a powerful model for pathogenicity studies because the fly immune system is highly conserved, well-characterized, and shares traits with the innate immune system of mammals. Furthermore, flies have a fully sequenced and well annotated genome, allowing the application of genome microarrays and RNA interface libraries [15]. Research on *Drosophila* offers many essential insights into developmental and cellular biology as well as infectious disease. Consequently, this model species has been chosen to study several important human fungal pathogens such as *Aspergillus* sp. [16], *Candida* sp. [17], and *Cryptococcus* sp. [7].

Wild type flies are generally resistant to fungal infections. Flies have the ability to respond through their innate immunity against pathogenic fungi. Flies also have physical barriers such as cutin that contribute to defense. Therefore, in nature, only a few entomopathogenic fungi have the capacity to infect *D. melanogaster* through exoskeleton penetration [14, 18]; however; if infection is reached, microorganisms within the host induce immune responses against potential fungal pathogenicity [9]. In *D. melanogaster*, at least two essential pathways are involved in immune response activation: The Toll pathway and the immune deficiency (Imd) pathway. These parallel signaling cascades both contribute to the *Drosophila* response against microbes [9, 19, 20]. The Imd pathway provides protection from gram-negative bacteria as well as resistance against pathogenic fungi, whereas the Toll pathway works against gram-positive bacteria and fungi [14]. When flies carry mutations in the Toll signaling pathway, there is a dramatic lowering of survival after fungal infection while immune deficiency (Imd) impairs the antibacterial immune response of *Drosophila* [11]. Flies mutant for both the Imd and the Toll signaling pathways rapidly succumb to either fungal or bacterial infections, indicating that these pathways are essential for antimicrobial resistance in *Drosophila*.

Because the Toll pathway of *D. melanogaster* is indispensable for protection against fungal infection, Toll-deficient flies have been used for studying infections caused by *A. fumigatus* [21]. Female *Drosophila* flies are larger in size and more resistant to injury by injection than male flies, and are often used in experiments. Flies are infected in one of three ways: by feeding, by rolling, or by injection of *Aspergillus* spores into the dorsal thorax [20, 22]. Using this model, a number of virulence factors have been elucidated. For example, flies infected with an *alb1*-deleted hypovirulent *Aspergillus* mutant had better survival rates than those infected with a wild-type of *Aspergillus* strain [23]. *A. fumigatus* mutants with altered siderophore biosynthesis, gliotoxin production (Δ*gliP*), PABA metabolism (Δ*h515*), starvation stress response, secondary metabolite production (Δ*gliP*), or melanin biosynthesis have lowered virulence or lack of pathogenicity [15]. In other experiments, the virulence of various isolates of *A. fumigatus* and *Aspergillus terreus*, defined by repetitive-sequence polymerase chain reaction (rep-PCR) and encompassing both colonizing and invasive isolates, were tested in Toll-deficient fruit flies. No significant difference was observed between species. However, the survival rates of Toll-deficient flies had significant differences when infected by two dominant *A. fumigatus* clades identified by rep-PCR. Therefore, using the fruit fly for studying aspergillosis infection revealed distinct *A. fumigatus* clades that had differences in their pathogenicity [21].

Thermotolerance is a factor that has long been hypothesized to play a role in the ability of *A. fumigatus* to be a human pathogen, and the *Drosophila* model was again useful in testing this hypothesis. In contrast to most environmental molds which grow poorly at mammalian body temperature, *A. fumigatus* thrives at 37°C. *A. fumigatus* gene has been detected which is required for thermotolerance and showed that this Δ*cgrA* mutant was less virulent in immunosuppressed mice. As predicted, when tested in Toll-deficient *Drosophila* at 25°C, the difference in virulence was less pronounced [24]. Finally, Toll deficient flies also have been used to screen for useful antifungal drugs against this pathogen [16].

**Fungal Volatile Organic Compounds with Emphasis on *Aspergillus fumigatus***

VOCs are low molecular weight organic substances that easily vaporize at room temperature [25]. The best-known VOCs are industrial products used for different purposes such as painting, cleaning, air refreshing and so forth; many of these industrial VOCs are known to have toxigenic effects and have been regulated [26]. Less is known, however, about the biogenic VOCs emitted by organisms as part of their normal metabolism. Growing fungi emit VOCs as mixtures of hydrocarbons, acids, alcohols, aldehydes, aromatics, ketones, terpenes, thiols, and their derivatives. Different species growing on different substrates produce different mixtures of VOCs [27, 28]. Fungal VOCs have characteristic odors, and the amounts and types of VOCs vary with temperature, moisture, pH, and other environmental conditions. Because fungi release mixtures of VOCs into their surroundings, the resultant VOC “signatures” can be used as rapid, inexpensive, and non-destructive indicators for recognizing the presence of indoor mold contamination [29-31]. For an overview of the volatiles produced by members of the genus *Aspergillus* see [32].

Most of the studies concerning the detection of mold VOCs have been conducted in the context of concerns about human health. Fungi have been implicated in poor indoor air quality especially with reference to a somewhat controversial health problem usually called “building related illness” or “sick building syndrome” [33, 34]. Although fungi produce VOCs regardless of whether they are indoors or
outdoors, fungus-related illness symptoms are usually reported from indoor environments. VOCs diffuse away outdoors whereas, when they become trapped indoors they may build up to toxic levels which are harmful to those who breathe in these fumes [35]. *A. fumigatus* has been isolated from buildings where occupants have complained of building-related illness and from homes of asthmatic children [36].

In general, VOC profiles are obtained by cultivating pure cultures of a given species in the laboratory on artificial media. The suite of volatile compounds produced is then separated and identified by some form of gas chromatography coupled with mass spectrometry (GC-MS). Because the methods for cultivation and for chemical identification differ so much between studies, except to note that distinctive, often unique, volatile profiles are detected by different species of fungi growing on different substrates. Furthermore, the amount and kind of volatiles change over time, depending on the age of the culture. While the representatve studies summarized below do not attempt to link the VOCs detected to any purported toxicological effects, they do show that *A. fumigatus* has a large metabolic repertoire of volatiles.

In a study of possible health hazards associated with a composting facility, 13 mold species commonly isolated from composts, including *A. fumigatus*, were grown in pure culture on yeast extract medium and assayed by GC-MS. High quantities of trans-β-farnesene were detected. In addition, camphene, α-pinene, limonene, 2-methyl-1-butanol, 3-methyl-1-butanol (isopentanol), and 2-methyl-1-propanol (isobutanol) were identified from *A. fumigatus* [37]. When five different species of *Aspergillus*, including *A. fumigatus*, were cultured on malt extract and gyspum board, the main VOCs detected were 1-octen-3-ol, dimethyl disulfide, 2-heptanone, 3-methyl-1-butanol, terpineol, and 2-methyl-1-propanol [29].

Volatile profiles are sometimes used as means for differentiating species and genera of fungi. For example, *A. fumigatus* could be distinguished from 11 other species commonly isolated from indoor air using headspace solid-phase microextraction-gas chromatography-mass spectrometry analysis because *A. fumigatus* produced trans-β-farnesene in high concentration compared to other compounds such as isoprene, 3-methyl-1-butanol, and 2-methyl-1,3-pentadiene [38]. Similarly, *A. fumigatus* could be distinguished from four different *Candida* species when fungi were grown on Columbia sheep blood agar and assayed using multi-capillary column-ion mobility spectrometry and/or GC-MS analysis. *A. fumigatus* produced 3-octanone, isoamyl alcohol (3-methyl-butanol), ethanol, cyclohexanone, and several unknown compounds, with higher amounts of cyclohexanone and 3-octanone than the other compounds detected [39].

Exhaled human breath has been used successfully to diagnose many different diseases [40]. Since there is no good early detection for invasive aspergillosis, and since early diagnosis is required for efficient therapy of this potentially lethal infection, several laboratories have attempted to find specific VOC markers for *A. fumigatus*. The compound 2-pentylfuran was found from the breath of aspergillosis patients and in cultures of *A. fumigatus* on blood agar [41] and seemed to be specific to the fungus [42]. However, an intensive survey on the "volatome" of *A. fumigatus* cultured in vitro consisted largely of terpenes, along with 1-octen-3-ol, 3-octanone and pyrazines, with no 2-pentylfuran detected. The researchers hypothesized that the 2-pentylfuran observed in earlier tests was the result of a nonspecific inflammatory reaction [43]. In another approach, thermal desorption combined with GC-MS to examine VOCs emissions of *Pseudomonas aeruginosa* and *A. fumigatus* in mono- and co-cultures. Distinct VOC-marker combinations were observed between head space analysis of the mono- and co-cultures. VOCs released by *A. fumigatus* in mono-culture included 1-methylethyl-pyrazine, azacyclotridecan-2-one, 3-phenyl-1-H-indene, and 2-nonanone [44].

In summary, *A. fumigatus* produces a large number of different VOCs. The type and quantity of VOCs vary with the strain, substrate, age of the culture, temperature, and other variables. Moreover, the VOCs detected are also dependent on the method used for separation and detection. Most studies to date are surveys of VOCs that are found when fungi are grown in pure culture under laboratory conditions. While this research shows that *A. fumigatus* makes a wide array of VOCs, representing many chemical families, it is difficult to link the descriptive data obtained from these controlled *in vitro* studies into either definitive tools for early diagnosis of aspergillosis or evidence for specific VOCs as etiological agents of "sick building syndrome."

**NEGATIVE PHYSIOLOGICAL EFFECTS OF FUNGAL VOLATILE ORGANIC COMPOUNDS**

In this section, a few examples of known toxicological effects of fungal VOCs are presented. It is known that many industrial VOCs are common air pollutants. Fungal VOCs often have odors similar or identical to industrial compounds and have been associated with headaches, dizziness, faintness, and irritation of the eyes and mucous membranes of the nose and throat [45-47]. Many researchers have hypothesized that fungal VOCs have negative effects on human health with reference to processes like composting [48] or with respect to people who lives in damp houses with mold contamination [49]. The evidence for an association between VOCs and "sick building syndrome" has been reviewed [50]. Toxicity of specific compounds depends on the chemical nature of the VOC and the level and length of exposure [26, 51].

Only a limited number of controlled studies have clearly demonstrated toxigenic effects of fungal VOCs. One of the best-known examples concerns an endophytic species called *Muscodor albus* which emits a suite of VOCs that can kill many bacteria and fungi. When adapted for agricultural purposes, the process is called "mycofumigation" [52, 53].
The single fungal VOC that has received the most attention is 1-octen-3-ol, also known as “mushroom alcohol.” This compound is responsible for the characteristic odor of mushrooms and molds, and is one of the most abundant and commonly detected VOCs associated with mold growth. Using mammalian cell cultures, toxicogenic effects have been detected for 1-octen-3-ol as well as a number of other individual biogenic VOCs [27, 54, 55]. In a study with human volunteers in Sweden, a 2 hr exposure to low concentrations of 1-octen-3-ol caused an increased inflammatory response in nasal secretions [56].

The larvae of Drosophila flies also can be used to test the toxicological effects of VOCs produced by growing fungi. Mixtures of VOCs emitted by growing cultures of Aspergillus, Penicillium, and Trichoderma isolated from a flooded home had a detrimental effect on survival of exposed flies [57]. The fly toxicology assay was also used to test the VOCs produced by 11 species of fungi isolated from flooded homes after a hurricane event in New Jersey in 2013. Drosophila larvae were exposed to a shared atmosphere with growing cultures of each of the molds, and toxic effects on flies ranged from 15% to 80%. The volatile metabolites that released from Aspergillus niger were most toxic yielding 80% mortality to Drosophila after 12 days, while VOCs produced by Trichoderma longibrachiatum, Mucor racemosus, and Metarhizium anisopliae were relatively non-toxicogenic. Using solid-phase microextraction-gas chromatography-mass spectrometry 1-octen-3-ol, 3-octanone, 3-octanol, 2-octen-1-ol, and 2-nonanone were found in a high concentration by the most toxic species, while 3methyl-1-butanol and 2-methyl-1-propanol were produced by the less toxic fungi [58]. Similar toxicogenic effects were observed when flies are exposed to the vapors of chemical standards of selected fungal VOCs. The eight carbon compounds have more toxic effects than the non-C8 compounds on the larvae and adult stages of fruit flies with 1-octen-3-ol, 3-octanol, and 3-octanone compounds causing 100% mortality in 24 hr. The toxic effect of common industrial compounds, including toluene, benzene, formaldehyde, and xylene were monitored at the same concentration and toxic effects on 15 days of exposure, benzene and toluene caused 50% mortality of flies with under 20% of mortality for formaldehyde and xylene. In short, the chemical standards of the fungal C8 compounds display more toxicity than the non-C8 compounds and industrial solvents [57]. In another study, Drosophila larvae exposed to vapors of low concentrations of 1-octen-3-ol, (E)-2-hexenal, or 1-hexanol had not metamorphosed into pupae while VOCs had reached adult flies [59].

Interestingly, exposure to 1-octen-3-ol had particular impacts on dopaminergic neurons in the brain of adult Drosophila, causing Parkinson’s-like symptoms in fruit flies [60, 61]. It also specifically affected the caspase-3 dependent apoptotic signaling pathway [62]. Moreover, flies exposed to 1-octen-3-ol exhibited increased amounts of nitrate, associated with the breakdown of nitric oxide in hemocytes, leading to an inflammatory response. Exposure to this compound also promotes nitric oxide synthase expression in tracheal tissues of larvae thereby causing remodeling of tracheal epithelial lining [63].

**TOXICITY OF VOLATILE ORGANIC COMPOUNDS FROM ASPERGILLUS FUMIGATUS IN THE DROSOPHILA MELANOGASTER MODEL**

We hypothesized that VOCs produced by A. fumigatus may contribute to the pathogenicity of this species and have conducted preliminary trials to test our hypothesis using the fly model. Aspergillus fumigatus strain 1607 was obtained from Dr. Geromy Moore, Southern Regional Research Laboratory, New Orleans, Louisana, USA. This strain had originally been isolated from a damp indoor environment. It was inoculated on potato dextrose agar and cultured at either 37°C for 3 days or at 25°C for 5 days. Drosophila larvae were exposed to VOCs emitted by growing cultures of A. fumigatus using published protocols [57, 58]. In brief, adult Drosophila flies were placed on egg media for 3 hr in order to lay eggs. After 3 hr, the flies were transferred back to Ward’s Instant medium. The eggs were incubated at 25°C for 4–5 days to develop into third instar larvae. Fifteen larvae were transferred into a fresh larva-pupa media and the plate was placed over a membrane-covered fungal colony plate. These larvae were exposed to a shared atmosphere with cultured A. fumigatus using a “double Petri plate” exposure system. The cover of the fungal culture Petri plate was replaced with a sterile cover with a circular hole, created using a flamed spatula, and covered with 0.2-μm filter membrane so as to allow passage of VOCs but to prevent transmission of spores or hyphae. The Petri plate containing A. fumigatus was affixed to the Petri plate bottom containing Drosophila larvae, sealed using Parafilm, and placed in an orbital shaker at 50 rpm. The plates were observed daily and the numbers of larvae, pupae or adult fly were counted for 15 days. The experiment was conducted in triplicate.

Drosophila larvae exposed to VOCs from growing cultures of A. fumigatus exhibited delays in metamorphosis (Fig. 1). After four days (Fig. 1A), the effect of A. fumigatus VOCs on the developmental stages of fruit flies was greater when the fungus was pre-grown at 37°C than at 25°C. Sixty percent of the larvae exposed to VOCs of A. fumigatus grown at 25°C had not metamorphosed into pupae while 80% of the larvae exposed to those grown at 37°C were delayed. The metamorphosis is reflected in the number of pupae (Fig. 1B). The number of adult flies is shown in Fig. 1C. Almost all control flies completed metamorphosis and became adults after 1 wk. In contrast after 10 days, fewer than 20% of the flies exposed to the shared atmosphere with A. fumigatus VOCs had reached adult stage, and at the end of 15 days, fewer than 30% of the original larvae had become adults, indicating significant
toxicity. Because the exposure experiments were performed at room temperature (25°C), it is not surprising that there were no significant differences in the number of flies that become adults after 15 days between those exposed to A. fumigatus cultures originally pre-grown at 25°C or 37°C.

**DISCUSSION AND SUMMARY**

Pathogenicity is the ability of an organism such as A. fumigatus to cause disease and it is well known that disease is not an inevitable outcome of all pathogen-host interactions. Pathogenicity varies with strain of pathogen, host susceptibility, and environmental conditions, often expressed as different levels of virulence. The Drosophila model has been successfully used for over 25 years in elucidating aspects of fungal virulence. Our data suggest that VOCs may be a hitherto unknown virulence factor in A. fumigatus, contributing to the ability of this common mold to cause disease. In future research, it will be important to test VOCs in mammalian models and to assess which compounds in the VOC profile are relevant to the pathogenicity.

Filamentous fungi do not normally cause invasive infections in healthy people. However, when patients are immunocompromised, opportunistic species including Aspergillus fumigatus have the capability to cause morbidity and mortality. Fungal diseases like invasive aspergillosis are becoming an increasingly important cause of death because modern medical practice increases the number of immunocompromised patients. Drosophila melanogaster is a good model organism in which to study many human diseases, including those caused by medically important human fungal pathogens. Toll deficient flies already have provided an important model system for studying A. fumigatus infections.

In this report, we present preliminary data showing the fly model has utility for studying the pathogenic effects of volatile phase metabolites produced by A. fumigatus. We used flies with an intact immune system in our research and found that when larvae of these flies were grown in a shared atmosphere with A. fumigatus VOCs, significant delays in metamorphosis were observed, along with significant lethality. The preliminary data presented here indicate that medical mycologists should view the VOCs produced by A. fumigatus not only as useful diagnostic tools for early detection of aspergillosis, but also as possible virulence factors in the development of human pathogenesis.

There are many future challenges with respect to understanding the toxicology effects of A. fumigatus VOCs as possible virulence factors. The complete genome of A. fumigatus has been sequenced [64, 65] and will provide a useful platform for future studies to help pin point the individual genes which are responsible for the success of this common filamentous fungus as a human pathogen, and to pinpoint the possible role of fungal VOCs in its virulence.

**ACKNOWLEDGEMENTS**

We would like to thank Gulalai Ahmed and Rawan Alsehali for their help in the experiments. We also thank The Higher

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**Fig. 1.** Development of mature third instar Drosophila larvae over 15 days of continuous exposure to volatile organic compounds (VOCs) of Aspergillus fumigatus (strain 1607) that was previously grown at 25°C or 37°C. A, Percent of living larvae; B, Percent of pupa; C, Percent of living adults. The number of larvae, pupa, and adults was counted daily for 15 days, and 15 third instar larvae were exposed to A. fumigatus VOCs in each trial. The experiment was conducted in triplicate (n = 180). Significant difference between control and experimental, **p < 0.005.
Committee for Education Development in Iraq (HCED) for supporting H. S. AL-M. with a graduate fellowship.

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