VOLATILE ORGANIC COMPOUNDS THAT ARE PRODUCED FROM HUMAN

PATHOGENIC FUNGI ARE TOXIC TO THE GENETIC MODEL *DROSOPHILA*

MELANOGASTER

by

HADEEL SAEED YASEEN ALMALIKI

A dissertation submitted to the

School of Graduate Studies

Rutgers, The State University of New Jersey

In partial fulfillment of the requirements

For the degree of

Doctor of Philosophy

Graduate Program in Plant Biology

Written under the direction of

Joan W. Bennett

And approved by

_____

_____

_____

_____

New Brunswick, New Jersey

October, 2020

ABSTRACT OF THE DISSERTATION

VOLATILE ORGANIC COMPOUNDS THAT ARE PRODUCED FROM HUMAN

PATHOGENIC FUNGI ARE TOXIC TO THE GENETIC MODEL *DROSOPHILA*

MELANOGASTER

By

HADEEL SAEED YASEEN ALMALIKI

Dissertation Director

Joan W. Bennett

Volatile organic compounds (VOCs) are organic compounds with low molecular mass and the ability to vaporize easily at room temperatures. Fungi produce many VOCs that vary in type and amount depending on producing species, as well as factors that influence growth such as temperature, substrate, moisture, pH, and other parameters. In nature, fungal VOCs are used as ecological signaling agents. In the built environment, for individuals who live in water damaged and mold infested houses, they have been implicated as possible contributors to "sick building syndrome" Finally, fungal VOCs have found practical applications in medical mycology as indirect assays for determining the presence of fungal growth.

In this thesis, I have postulated that the VOCs emitted by medically important fungi may contribute to the pathogenicity of human fungal pathogens.   Using a *Drosophila melanogaster* bioassay, third instar larvae were placed in a shared atmosphere with growing cultures of *Aspergillus fumigatus*, *Cryptococcus gatti*, *Cryptococcus neoformans*,

Candida albicans and *Saccharomyces cerevisiae*. In some cases, the human pathogenic fungi were pre-cultured at 25°C and in other cases they were pre-cultured at 37°C. Control larvae were incubated in the absence of fungal VOCs. The number of larvae, pupae and adults was counted over a 15-day period and the effect of fungal VOCs on stages of metamorphosis, time to eclosion, and fly viability was determined. In general, exposure of larvae to VOCs from human pathogenic fungi slowed the rate of metamorphosis, delayed eclosion, and caused toxicity. In some cases, morphological abnormalities were observed. These harmful effects were more pronounced when fungi were pre-grown at 37°C than at 25°C. Larvae grown in the presence of VOCs from *Saccharomyces cerevisiae* had metamorphotic and eclosion rates that were like controls. The VOCs from the environmental strain of *A. fumigatus* had the highest toxicity to the developmental stages of *Drosophila* when it was grown at either 25°C or 37°C. Exposure to VOCs from *Cryptococcus neoformans* caused more delays on fly metamorphosis and more toxicity than did exposure to VOCs from the six *A. fumigatus* strains tested. Using purge and trap gas chromatography-mass spectrometry (GC-MS), the VOCs from the most toxic and least toxic strains of *Aspergillus fumigatus* were assayed. The most toxic strains produced high levels of 1-octen-3-ol, an eight carbon alcohol that previously has been determined to be toxic in low concentrations to *Drosophila* flies, *Arabidopsis* plants, human embryonic cell cultures, and to cause nasal irritation in trials with human subjects. In conclusion, I postulate that VOCs from medically important fungal species may be acting as virulence factors during human infections, thereby enhancing the pathogenic effects of these species.

The second section of the dissertation focused on *Aspergillus fumigatus* strains that did or did not carry defects in their oxylipin pathways. Lipoxygenase (LOX) genes and oxylipins are involved in biosynthesis of several metabolites that affect various reproductive functions in filamentous fungi that are mediated by VOCs. A. wild type *A. fumigatus* strain and a "near wild type strain" with normal oxylipin genes were compared with *A. fumigatus* mutants blocked in the lipoxygenase pathway. The *Drosophila* bioassay with third instar larvae was used to detect delays in metamorphosis, eclosion and possible toxicity to flies. Exposure of larvae to VOCs produced by *A. fumigatus* wild type and overexpressed LoxB strain caused more delays on fly metamorphosis than did exposure to VOCs from *A. fumigatus* carrying blocks in the lipoxygenase pathway. There were no significant effects shown in the presence of arachidonic acid on the metamorphosis of the fruit fly compared with cultures lacking arachidonic acid. GC-MS analysis showed the wild type strain produced more abundant VOCs in higher concentrations than did the triple LOX mutant which released fewer VOCs in lower concentrations. VOCs produced by the *A. fumigatus* wild type strain AF293 included 1-octen-3-ol, 1-butanal, 1-octen, decanoic acid, lauric acid, myristic acid, and palmitic acid. These VOCs were not detected from the LOX triple mutant strain.

The final section of the dissertation studied the most toxigenic eight carbon compound 1-octen-3-ol. I hypothesize 1-octen-3-ol is of distinct importance as a toxigenicity factor. Toxigenicity is usually defined as the ability of a pathogenic organism to product injurious substances that damage the host. In order further to investigate this hypothesis, I tested low concentrations of chemical standards of three eight carbon

volatiles (1-octen-3-ol, 3-octanone and 3-octanol) characteristic of fungal metabolism against adult male *Drosophila* flies in order to determine their impact on fly survival. Two strains of *Drosophila,* one with red eyes and one with white eyes*,* that were wild type for immune pathway genes served as controls. In addition, strains that carried blocked mutations in the nitric oxide pathway, the Toll pathway, the Imd pathways, and a double mutant strains with blocks in both the Imd and Toll pathway were tested. Volatile phase 1-octen-3-ol and 3-octanone at 0. 5µL/L were toxic to almost all the strains after 24 hours. Oregon [R] (red-eyed, wild type) strain was less susceptible than wild type, white eyed strain (W[1118]) to all three volatile compounds at both concentrations. All strains showed high susceptibility to the high level of 1-octen-3-ol in the span of only two hours. Of the three compounds tested, 3-octanol at 0. 1µL/L was the least toxic to mutant and wild type strains. White eyed flies carrying the NOS mutation were more resistant to volatiles than were the white eyed control strains. The Relish [E20] white-eyed, mutant strain was more susceptible than the spz[6] red-eyed mutant strain to volatile phase 1-octen-3-ol at 0.1µL/L. The double mutant strain (red-eyed Relish [E20] spz[6)]) showed greater resistance to the presence of the volatile phase compounds than did either single mutant. This latter finding implies that the toxicity of 1-octen-3-ol and other VOCs may be related to aspects of the innate immune system that cause negative side effects on fly physiology; when the immune system is impaired, these negative side effects do not occur and the flies have increased survival in the presence of VOCs.

In summary, the *Drosophila* bioassay has been used to show that VOCs from several medically important fungi are toxigenic. Strains of *A. fumigatus* that were blocked

in lipoxygenase activity emitted fewer VOCs and had less toxicity. Presence of the eight-carbon volatile, 1-octen-3-ol was correlated with higher levels of toxicity in the *Drosophila* bioassay. VOCs from growing fungi can be considered toxigenic factors that contribute to pathogenic profiles of medically important fungi. To our knowledge, this is the first report that correlates fungal VOCs such as 1-octen-3-ol to the virulence of human medical pathogens.

ACKNOWLEDGEMENTS

working on a PhD at the same time. Thanks also to all the new friends I have made while in the USA.

TABLE OF CONTENTS

LIST OF TABLES

CHAPTER 4:

LIST OF FIGURES

CHAPTER 4:

CHAPTEER 1. LITERATURE REVIEW

The author has published most of this chapter in the following citation:

Al-Maliki HS, Martinez S, Piszczatowski P, Bennett JW. Drosophila melanogaster as a Model for Studying *Aspergillus fumigatus.* **Mycobiology. 2017 Dec 1;45(4):233-9.**

1.1. Introduction

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 (Agarwal 2009; Latgé and Steinbach 2009). 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 (Latgé 1999). Individuals with hematological malignancies, hematopoietic stem cell transplant recipients, and recipients of solid organ transplants are at highest risk of developing systemic aspergillosis (Latgé and Steinbach 2009; Marr, et al. 2002). 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.

Drosophila melanogaster is an important model organism that has been used to study a wide range of basic and applied biological research questions, including the impact of medically important filamentous fungi. These flies have a cellular immune system. The fruit flies' immune systems can eliminate pathogens if they are identified at the beginning of the infection(Hamilos, et al. 2012). Investigations about the interaction of *Drosophila* and fungal pathogens have demonstrated that this organism has an evolved antifungal immune system against some opportunistic fungi due to a long-term exposure and interaction in their natural environment. Our laboratory has

pioneered the application of the fruit fly model to study and evaluate the toxicity effects of volatile organic compounds (VOCs) produced by filamentous fungi isolated from indoor environments (Inamdar, et al. 2012; Yin, et al. 2015) (Zhao, et al. 2017). In this chapter, I discuss the advantages of *D. melanogaster* for toxicology research. I also discuss future challenges with respect to understanding the toxicological effects of VOCs of *A. fumigatus* and other VOCs from other fungal pathogens as possible virulence factors.

1.2. *Aspergillus fumigatus* as a pathogen

Fungal spores, including *Aspergillus* conidia, are present almost everywhere in the world. In an indoor environment, hundreds of different types of mold can grow wherever there are suitable conditions, with sufficient water being the most important variable for successful fungal growth. Thus, fungal growth is typically associated with high humidity and molds are able to grow in almost any place that has moisture. Respiratory disease is one of the most common diseases in the developed and developing world. Every year, the upper respiratory infections have been detected in six to eight American children (Heikkinen and Järvinen 2003). Indoor dampness and microbial growth are correlated with health problems, especially negative effects on the respiratory system such as asthma, wheeze, cough, respiratory infections and upper respiratory tract symptoms (Heseltine and Rosen 2009; National Academies of Sciences and Medicine 2017).

Previous studies have found that people who stay mostly outdoors, or live in places with well-ventilated buildings, have more diverse microbiomes compared to people who live with close or less open buildings (Clemente, et al. 2015; Hanski, et al. 2012).

Airborne molds and other biological particles contaminate the indoor air of our homes, offices, and schools in many parts of the world. Fungi, bacteria, viruses, pollen etc. are all biological contaminants of indoor environments. Fungi are able to grow in various environments due to their physiological and genetic plasticity. They produce spores in huge amounts and distribute in a wide range of areas. The spores of molds stay viable for a long time, even during unfavorable conditions. The metabolism of mold spores and products can cause allergic reactions consisting of hypersensitivity pneumonitis, allergic rhinitis and some types of asthma (Ayanbimpe, et al. 2012; Hospodsky, et al. 2015). Fungal metabolites include ergosterol, beta-glucans, cell fragments, endotoxins, mycotoxins and microbial volatile organic compounds, all of which are suspected as contributing to human health problems such as headache, as well as irritation of the eyes and mucous membranes of the nose and throat (Douwes 2009; Fischer, et al. 1999; Lambrecht and Hammad 2013; Lambrecht and Hammad 2014; Nielsen 2003). Species in the genus *Aspergillus* are among the most ubiquitous fungi that can grow in soil, plant debris, and indoor environments. They have an important role in global carbon and nitrogen recycling.

Fungi secrete acids and enzymes into the environment to breakdown the complex molecules to simpler compounds which are absorbed back by fungal cells. The genus *Aspergillus* genus was one of the first filamentous fungal groups described (Micheli 1729) and its members can serve as an example of the lifestyle of fungi. These fungi have a key role contributing in the decomposition process (including natural cycling and carbon cycle) and they also provide carbon dioxide in addition to other organic and inorganic compounds. Their small, hydrophobic conidia can spread easily into the air and have the ability to survive a broad range of environmental conditions. The genus encompasses over 300

species of which only a few are plant and human pathogens (Gugnani 2003; Machida and Gomi 2010) Morphologically, the spore-bearing structure in the genus *Aspergillus* is characterized by the "T" or "L" -shaped foot cells which are good morphological signs to know that a given isolate is an *Aspergillus* species. The foot cells bear a swollen conidiophore (vesicle) at its tip that carries phialides as a single or double series. The phialides of *Aspergillus* bear conidia or conidiophores as columnar or radiate unbranched chains which stay together and are linked by a cytoplasmic bridge. Fungal conidia are organized from the bottom, which have the youngest conidia, whereas the tip has the oldest conidia in the chains. The sporulating fungal colony has different textures and colors such as powdery, white, greenish yellow, brown or black. For example, *A. niger* species produce black conidia, while *A. flavus, A. nidulans,* and *A. fumigatus* bear green conidia. The colony color, the rate of fungal growth, and thermotolerance are used as the main factors to identify *Aspergillus* species (Fogarty 1994; Machida and Gomi 2010). Most species in the genus *Aspergillus* reproduce asexually, but a few species of this genus have a sexual state called the teleomorph. *Aspergillus* teleomorphs produce a round, closed structure which is called a cleistothecium that encloses   asci containing the ascospores. Most asci have eight ascospores. *Aspergillus* species that can reproduce sexually and form cleistothecia are both homothallic and heterothallic (Bennett 2009; Bennett 1986; Raper and Fennell 1965).

The sexual stage of *Aspergillus is* characterized by cleistothecia that differ in size, color, shape and appearance based on species (Fogarty 1994; Geiser, et al. 2008). Regular occurrence of a sexual stage, forming ascospores, is one of the features used to differentiate species. Sexual outcrossing produces genetic variation within populations which is an important for long- term survival and species evolution (Dyer and Paoletti

2005). Sexual reproduction requires a mating type locus which is called MAT in ascomycetes. A pure culture of a heterothallic mold bearing only one MAT locus will never produce the sexual stage. MAT loci include the genes that are important for ascocarp development, meiosis and other aspects of sexual reproduction (Galagan, et al. 2005).

The rarity of opposite MAT loci in many Aspergillus populations made early mycologists think that many *Aspergillus* species have only the asexual stage(Dyer and Paoletti 2005; Poggeler 2002).

The genus *Aspergillus* is economically important. Several species of *Aspergillus* are used for industrial production of enzymes, organic acids and pharmaceuticals. For example, *Aspergillus niger* has been used to produce citric acid, amylases, pectinases, phytases, and proteases. *A. terreus* is used to form statins which are in widespread use decrease cholesterols, while *A. oryzae* is used to produce soy sauce and sake, by fermenting soybeans and rice respectively(Abe and Gomi 2008; Machida and Gomi 2010). Other species of *Aspergillus* such as *A. flavus* and *A. parasiticus* have the ability to cause crop infections and form mycotoxins such as aflatoxin, which is a highly toxic carcinogen with immunosuppressive properties (Bennett and Klich 2003; Wild 2007; Williams, et al. 2004).

1.2.1 *Aspergillus* species as human pathogens

Many fungi are important pathogens to organisms such as insects, amphibians and plants (Casadevall 2005). However, normally, fungi do not cause disease in healthy people, and of a large number of known fungal species, only a few hundred are pathogens.

Nevertheless, as modern medical practices have created an increasing number of people who have compromised immune systems, the frequency of fungal disease has increased. Opportunistic species, including several aspergilli, have the capacity to infect those who are immunocompromised (Chakrabarti 2005; Reedy, et al. 2007; Richardson 2005; Yin, et al. 2015). *Aspergillus fumigatus* is considered the most important of the opportunistic filamentous fungal pathogens (Richardson 2005). Due to their ubiquity in the environment, the conidia of genus *Aspergillus* are inhaled daily by all of us, but only in severely immunocompromised individuals does conidial inhalation result in extensive hyphal growth in lung tissue, leading to invasive aspergillosis. All *Aspergillus* species capable of growth at human body temperature (37°C) have the ability to germinate and grow in lungs, and potentially cause invasive aspergillosis in immunocompromised patients, To date, approximately 40 of the more than 300 Aspergillus species have been documented as human pathogens (Klich 2006). Of these, *A*. *fumigatus* is the most common species associated with this terrible disease (Balajee, et al. 2007; Latge 1999; Latgé and Steinbach 2009).

It is hypothesized that other thermotolerant species of *Aspergillus* which do not cause severe infections are removed before, or when they get to, the lower respiratory tract, even in immunocompromised patients (Figure 1.1). *A. fumigatus* is a particularly thermotolerant organism, which may be one reason for its success as a pathogen. It has optimum temperature ranges from 37°C to 42°C, but it also has ability to tolerant temperatures over 55° which is the high limiting temperature range for eukaryotic organisms (Chang, et al. 2004).
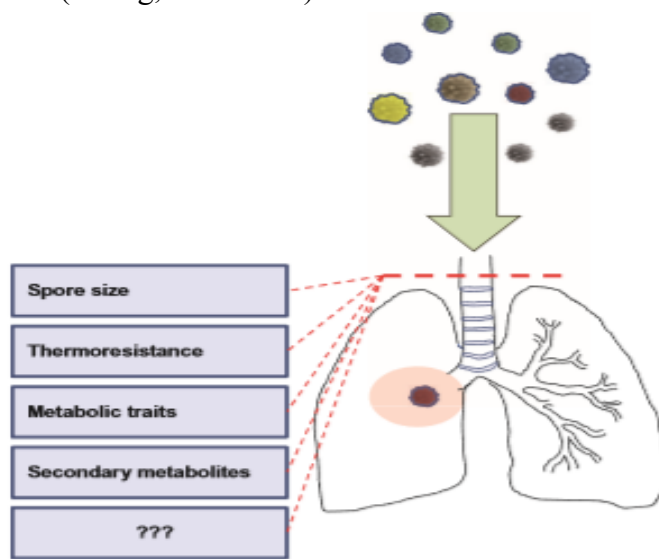
Figure 1.1 Spores of *Aspergillus* species and other fungi are inhaled by people, but *A. fumigatus* is the major pathogenic fungus for causing infections. (Chang et al., 2004).

1.2.2   *Aspergillus fumigatus*

A.fumigatus differ in their micromorphology and their cultural charactersitics (Samson, et al. 2009). *A. fumigatus* is responsible for causing more than 90% of human *Aspergillus* infections, followed by *A. flavus* and *A. niger* (Balajee, et al. 2009; Lass-Flörl, et al. 2005). The incidence of aspergillosis caused by different species of *Aspergillus* differs from country to country and based on the patient group that is under study. In

immunocompetent people, spores of the pathogenic strains of Aspergillus that get into human and other animals' respiratory systems are removed by their innate immune system (neutrophils and macrophages). Nevertheless, *Aspergillus* can cause different kinds of allergic reactions in immunocompetent people. In immunocompromised people, the degree of infection is based on the virulence factors of the fungal strain, the host immune status, as well as the pulmonary structure and function of the host.

Because it is widespread in the environment, and because it forms a huge number of asexual spores, it is not uncommon for humans to breathe hundreds of these pathogenic spores daily. They are small enough (2-3μm in dimeter) to reach the alveoli of lungs where they can break dormancy, germinate, and form a branched, septate vegetative mycelium that invades the pulmonary parenchyma into the lung tissues (Hohl and Feldmesser 2007; Latgé 1999; Zhang, et al. 2016). This is the primary route of human infection. Once the respiratory system is infected, for people with weak immune systems, fungal growth might spread to other organs (Paulussen, et al. 2017).

In healthy people, conidia are removed by mucociliary clearance and alveolar macrophages (Schaffner, et al. 1982). However, for people with weak immune systems, *A. fumigatus* is one of the most important pathogenic fungi. *A. fumigatus* causes a spectrum of pulmonary diseases, which can be classified into three groups based on the site of infection, and extent of colonization and invasion: allergic reactions; colonization with regulated invasiveness in immunocompetent individuals (saprophytic), systemic, and invasive infections in people who have weakness in their immune system such as those

with hematological malignancies or who have undergone organ transplantation (Brakhage and Langfelder 2002; Dagenais and Keller 2009; Latge 1999; Maturu and Agarwal 2015).

Aspergilloma is the saprophytic pulmonary form of *Aspergillus*. Aspergilloma occurs by saprophytic mold colonization and forms as a fungal ball in a pre-existing cavity within the parenchyma tissue of lung (Agarwal 2009). Aspergilloma happens in patients who have different diseases such as bronchiectasis, sarcoidosis, pulmonary abscess, pneumoconiosis, AIDS and hemoptysis (PS 1996). It can be life threatening in patients with histoplasmosis, fibrotic lung disease, sarcoidosis, and those with increasing *Aspergillus*-specific IgG titers (Kauffman 1996; Stevens, et al. 2000). Hemoptysis is one of the most important symptoms to indicate aspergilloma infection (Tomee, et al. 1995) . Allergic bronchopulmonary aspergillosis (ABPA) is the most common allergic form of aspergillosis. Aspergilloma and allergic bronchopulmonary aspergillosis are noninvasive forms of *Aspergillus* lung disease. Patients who have severe asthma with fungal sensitizations are diagnosed with allergic bronchopulmonary aspergillosis (ABPA) (Denning, et al. 2014; Steinbach 2008).

ABPA encompasses symptoms such as repeated fever, cough, wheezing, pulmonary infiltrated and fibrosis (Barnes and Marr 2006). ABPA findings include: asthma, peripheral blood eosinophilia, immediate skin reactivity due to high levels of *A. fumigatus* IgE in serum (>1 Mg/ml), and central bronchiectasis (Coop, et al. 2004; Laufer, et al. 1984; Wang, et al. 1978). ABPA is a hypersensitivity lung infection that occurs mostly in patients who have asthma or cystic fibrosis (Zmeili and Soubani 2007). The most serious form of aspergillosis is invasive and is diagnosed as acute invasive pulmonary

aspergillosis (IPA) and chronic pulmonary aspergillosis (CPA). Invasive aspergillosis has been found in severely immunocompromised patients, or those with chronic pneumonosis, such as chronic obstructive pulmonary disease. Hematopoietic stem-cell transplant recipients and those with hematological malignancies and chemotherapy are particularly susceptible to this life-threating disease (Kosmidis and Denning 2015a; Kosmidis and Denning 2015b; Lauruschkat, et al. 2018; Xu, et al. 2010; Zaas, et al. 2008).

Systemic aspergillosis also occurs in people who are taking corticosteroids and various forms of anticancer therapy (Clark and Hajjeh 2002; Garcia-Vidal, et al. 2008). The occurrence of IA has increased over the past 10-20 years as the number of immunocompromised patients has become more common. The mortality rates related with IA range between 60-90% with the incidence differing among different patient populations (Brakhage and Langfelder 2002; Singh and Paterson 2005). For example, *A. fumigatus* causes infection in approximately 15% of people who have allogeneic transplants (Paterson and Singh 1999). There are no vaccines against invasive aspergillosis, and available drugs have variable success rates between 40-90%. Moreover, fungal resistance against drugs is becoming increasingly common (Oliveira-Coelho et al., 2015).

Because of its medical importance, *A. fumigatus* (Strain 293) was one of the first species of fungi to have a sequenced and annotated genome. The fungus was sequenced using a whole genome random sequencing method augmented by using optical mapping. The genome of this strain is 29.4 megabases in size, with eight chromosomes, including 9,926 predicted genes (Nierman, et al. 2005).

1.3 Drosophila melanogaster as a genetic model

Drosophila melanogaster has been an important non-mammalian model system in biology since the early Twentieth Century. It has been widely used in genetic and developmental research due to its quick generation time, high reproductive rate, and inexpensive and uncomplicated maintenance. This "mini-model" has a fully sequenced genome, allowing the application of genome microarrays and RNA interface libraries (Lionakis and Kontoyiannis 2012). It also has a well- characterized immune system and a remarkable degree of conservation of biochemical pathways with humans and other animals (Hamilos, et al. 2012).

The life cycle of the fruit fly has four stages and takes 10-12 days, depending on the temperature (25°C-29°C). The first stage is the egg that develops into the embryo after one day. The second stage is the larval stage, which has three sub-stages or instars. The third instar larvae (mature larvae) metamorphoses into the third stage, or pupal stage, around the fifth day. Metamorphosis in the pupal case is such that most tissues in the larvae, and their replacements, will reorganize over the course of four days. The resultant proliferation and differentiation of cells form adult structures. In the early stages of embryos, most of specific structures of adults are created from imaginal discs such as the wings, legs, eyes, and genitalia (see Figure 1.2) (Hartwell, et al. 2004).
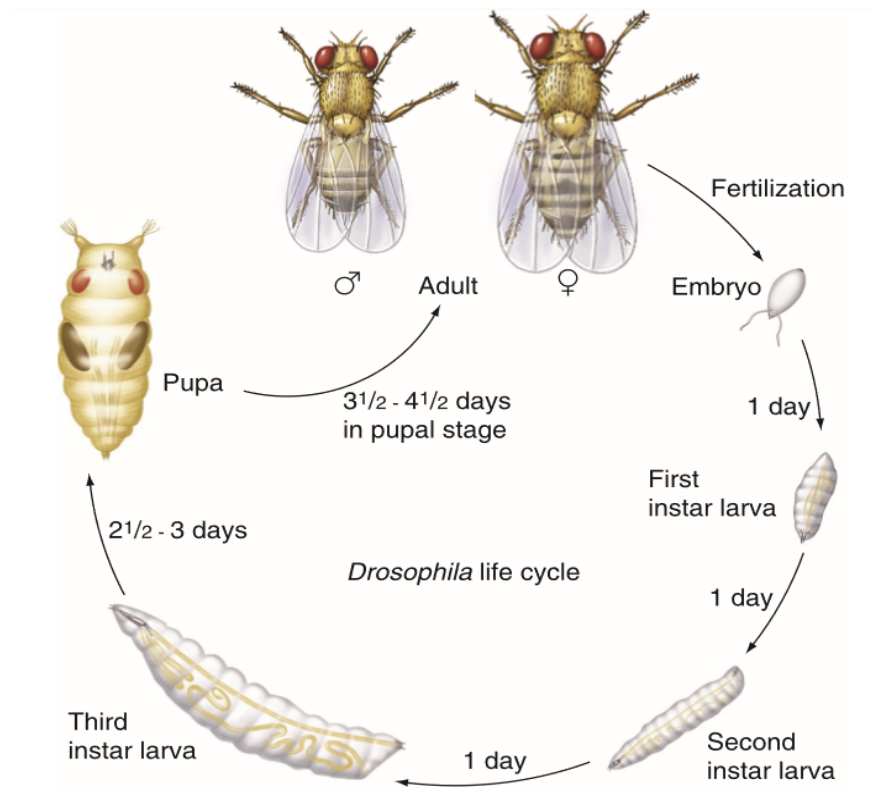
Figure 1.2. The life cycle of *Drosophila melanogaster* includes four stages. The first stage is the egg and embryo. The second stage is a series of molts which include three larval instars. The third stage encompasses the transition from third instar larvae to pupae, and is called pupation. The last stage of the cycle is when a pupa converts to an adult and is called eclosion (Hartwell et al., 2004).

Studies in *Drosophila* have offered many essential insights into developmental and cellular processes that flies share with humans. The nervous system of flies is complex containing of tens of thousands of neurons organized led into circuits which control complex behaviors (DeZazzo and Tully 1995; Guarnieri and Heberlein 2003; Sokolowski 2001). The fruit fly has neurotransmitters that are similar to those found in mammals and possesses many homologous neurotransmitters receptors and ion channels . *Drosophila*

melanogaster is one of the most useful models to study the interaction between neural function and genetic properties (Weber, et al. 2009). The fruit fly has a good host defense system against different types of pathogens. However, it does not have homologs for many immune response characteristics of mammals such as the adaptive immune response.

1.3.1 *Drosophila melanogaster* and its immune system

D. melanogaster grows and thrives at room temperature (25ºC); however, while the fruit fly has the ability to survive at 29ºC, it dies at 30º C (Krebs and Feder, 1998). The immune system of *Drosophila* is based on its innate immunity which can recognize different kinds of pathogens by producing a pattern of recognition receptors such as different families of antimicrobial peptides. This system includes two parts: humoral and cellular immune responses (Limmer, et al. 2011).

The cellular immune response is induced by three types of cells which appear in the blood in the body cavity of fruit flies. These cells increase in number when *Drosophila* flies are invaded by different microorganisms. The first type of cell is called the plasmocytes, which are similar to macrophages in mammalian cells, and which form about 95% of all hemocytes. These cells play an important role in phagocytosis. Secondly, lamellocytes are flat cells that are mostly present in the hemolymph of larval stages. These cells can kill pathogens by producing encapsulation. The third type of hemocytes is called crystal cells; these cells appear in larvae and form approximately 5% of all hemocytes. They have a dark color and kill pathogens through a melanization process. This process happens at the site of infection in the larvae and adult stage (Kocks, et al. 2005; Philips, et al. 2005; Rajak, et al. 2016). The second part of immune response is the humoral immune

response which is activated by three different pathways: the immune deficiency pathway (Imd), the Toll pathway, and JAK/STAT (Janus kinase/signal transducer and activator of transcription) pathway. In this response, *D. melanogaster* possesses seven different families of antimicrobial peptides (AMPs). They are released into a structure called the fat body (similar to the mammalian liver). These peptides are the most important immune response in fruit fly and are moved to the blood (Hoffmann 2003a). These small peptide families have activity against several types of microorganisms; the expression of the genes encoding these families depends on Toll and Imd pathways (Royet, et al. 2005). There are two types of AMPs that have activity against fungi (Drosomycins and Metchnikowin) while five types of AMPs defend against bacteria. For Gram-negative bacteria, the AMP Defensin is used; for Gram positive bacteria, the AMPs Attacins, Cecropins, Drosocin, and Diptericinsare used. These peptides accumulate together in the hemolymph of *Drosophila* to attack and kill the pathogenic microbes (Engström, et al. 1993; Kappler, et al. 1993). The Toll pathway plays an important role to defend against infections that are caused by fungi and Gram-positive bacteria, while the Imd pathway has activity to protect fruit flies from Gram negative bacterial infections (Mulinari, et al. 2006) (Figure 1.3. A &B).
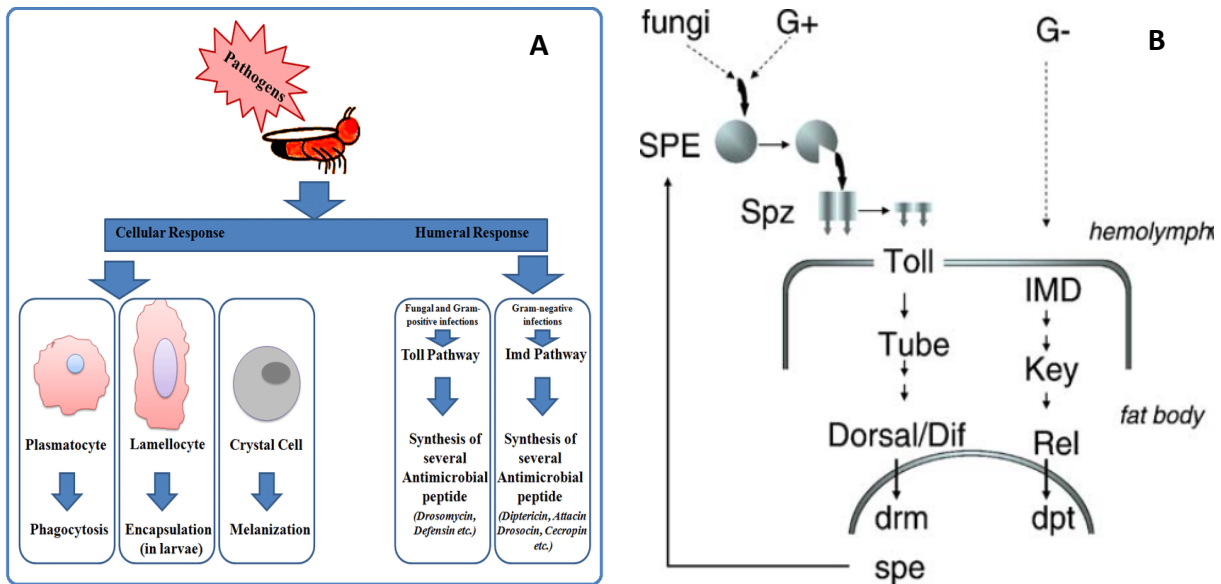
Figure 1.3. The innate immune system in *Drosophila melanogaster*. The diagram shows cellular and humeral responses in the fruit fly against invasion of different pathogens. The cellular response is activated by three types of hemocytes (Plasmocyte, Lamellocyte, and Crystal cells). The humeral response has two pathways: The Toll pathway is specifically to defend against fungal and Gram-positive infections. The Imd pathway is used to protect against Gram negative bacterial infections.

These pathways are similar to those found in mammals such as in Imd signaling which is homologous tumor necrosis factor receptor factor1 signaling in mammalian pathways, while Toll signaling is homologous to Toll/IL-1 receptor signaling pathway in humans. The innate immune system of the fruit fly has provided a model for scientists to screen drugs and vaccines that might provide treatments against deadly diseases such as HIV (Hoffmann 2003a; Rajak, et al. 2016). In addition to Imd and Toll, other pathways are also associated with the capacity of *Drosophila* to defend itself against bacteria and fungi. For example, the activation of p38 MAPK pathway is also important for resistance to bacterial and fungal infections independently of the Toll pathway (Hamilos, et al. 2012).

1.3.2 *Drosophila melanogaster* for studying aspergillosis

Drosophila melanogaster has been used to study fungal pathogens and is appropriate for analysis of the morbidity of a variety of other pathogenic microorganisms on a big scale (Ben-Ami, et al. 2010; Lionakis and Kontoyiannis 2010). *Drosophila melanogaster* has the ability to respond against pathogenic fungi through its innate immunity, using epithelial surfaces responses as self-defense. Flies also have physical barriers such as chitin that contribute to defense. However; if infection is reached, the host induces immune responses against the fungal pathogen (Chamilos, et al. 2007). Experimentally, feeding or rolling can be used to infect flies with *A. fumigatus* (Hamilos, et al. 2012). Injection is another standardized and reproducible way to obtain infection and leads to an accurate estimation of fungal inoculum. For example, fungi can be injected into the epithelial surfaces of the dorsal thorax of Toll-deficient fruit flies (Chamilos, et al. 2007).

The *A. fumigatus* mutant (*alb1*) has a hypovirulent phenotype in mice and gave low virulence in *Toll-deficient* flies. Infection is experimentally produced by feeding or rolling (Lamaris, et al. 2007). *Toll*-deficient flies have been used for studying infections caused by *Aspergillus* and other medically important filamentous fungi (Chamilos, et al. 2010; Lamaris, et al. 2007). Virulence factors are important for survival of fungi and other microbial pathogens at mammalian body temperature (Bhabhra, et al. 2004). Many of the virulence attributes that are important for *Aspergillus* pathogenicity in mammals are equally essential for successful infection in *Toll*-deficient fruit flies. In *Drosophila* and mouse models used to study invasive aspergillosis, lower rates of infections are associated with *Aspergillus* mutants that are deficient in siderophore biosynthesis (DeltasidA,

DeltasidD), PABA metabolism (H515), starvation stress response, secondary metabolite production (DgliP), or melanin biosynthesis ((Chamilos, et al. 2010; Lamaris, et al. 2007).

Moreover, several factors such as temperature and site of infection, as well as differences in mechanisms of local host defenses, may induce fungal virulence factors expression. For example, 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 fruit fly was used as a model in testing this hypothesis. In contrast to most environmental molds which grow poorly at mammalian body temperature, *A. fumigatus* thrives at 37°C. A gene required for thermotolerance in *A. fumigatus* gene has been detected and this Δ*CgrA* mutant was less virulent in immunosuppressed mice. As predicted, when tested in Toll deficient *Drosophila* at 25°C, the virulence was less pronounced (Bhabhra, et al. 2004). Using clinical isolates of *A. fumigatus* and *Aspergillus terreus* that vary in virulence, tested in Toll-deficient fruit flies, no significant differences in survival were observed. However, the survival rates of *Toll-deficient* flies have significant differences when infected by two dominant *A. fumigatus* clades (Ben-Ami, et al. 2010). In summary, flies are useful for studying aspergillosis, detecting differences in virulence, and revealing distinct *A. fumigatus* clades that have differences in their pathogenicity (Glittenberg, et al. 2011).

1.4 Fungal volatile organic compounds (VOCs)

Volatile organic compounds (VOCs) belong to a huge group of organic chemicals that can evaporate easily at room temperature due to their low molecular mass. Many VOCs are produced by living organisms during the processes of metabolism. Furthermore, many VOCs are manufactured industrially, and used for different purposes such as painting, air

refreshing and numerous other products. Inhalation of certain industrial compounds in the gas phase has shown demonstrable toxicity. Furthermore, dermal uptake of VOCs in indoor environments has shown the same rate or greater than inhalation uptake (Bennett and Inamdar 2015; Hung, et al. 2013; Inamdar, et al. 2012; Korpi, et al. 2009a; Morath, et al. 2012). Fungi emit mixtures of different VOCs, depending upon the temperature, moisture, pH and other environmental conditions of cultures, as well as the species and strain of fungus. Fungal VOC mixtures contain a range of sulfur, nitrogen, aromatic, hydrocarbon and fatty acid compounds. Many fungal VOCs have strong odors and can be easily recognized by humans.

1.4.1 Fungi and sick building syndrome

It has been hypothesized that fungal VOCs have negative effects on human health, possibly contributing to a condition known as the "sick building syndrome" (Ansarin, et al. 2013; Chambers, et al. 2016; Faber, et al. 2015; Morath, et al. 2012; National Academies of Sciences and Medicine 2017).

Aspergillus ssp. have been linked to many problems such as sick building syndrome, respiratory malfunctions, and other symptoms associated with poor indoor air quality. They are one of the most common genera found in damp indoor environments. Several *Aspergillus* species have isolated from water- impacted built environments, especially *A. versicolor* (Cetinkaya, et al. 2005; Hedayati, et al. 2010; Nielsen 2003). *A. fumigatus* also has been isolated from buildings with SBS and from homes of asthmatic children (Schwab and Straus 2004). The apparent relationship between building-related

illness and molds has promoted many research studies on the probable role of mycotoxins, particularly sterigmatocystin produced by *A. versicolor*, trichothecenes produced by *Stachybotrys chartarun*; and ochratoxins produced by several *Aspergillus* and *Penicillium* species (Bayman and Baker 2006; Straus 2009). However, even high concentration of spores and mycelial fragments rarely include enough mycotoxins to promote the wide array of reported symptoms (Kuhn and Ghannoum 2003; Robbins, et al. 2000).

Some skeptics suggest that there is no association between mold toxins and "sick building syndrome"(Charmaan and Terr 2003; Hardin, et al. 2003), which has led others to hypothesize that VOCs emitted from fungi may play an etiological role (Bennett and Inamdar 2015; Mølhave 2009; Mølhave, et al. 1993). Some of these fungal VOCs have odors similar, or identical to, industrial compounds which are known to cause headaches, dizziness, faintness, and irritation of the eyes and mucous membranes of the nose and throat (Araki, et al. 2012; Araki, et al. 2010; Takigawa, et al. 2009). Human volunteers exposed to 1-octen-3-ol at low concentrations had increased inflammatory markers in nasal secretions (Wålinder, et al. 2008).

1.4.2. Volatiles as biomarkers

VOCs have also been researched in the context of indirect detection of fungal growth. VOCs emitted into the environment can be used as rapid, cost-effective, and non-destructive indicators for recognizing the presence of indoor mold contamination (Cabral 2010; Gao, et al. 2002; Polizzi, et al. 2009). VOCs identified from fungi grown on building materials include: 2-methyl-1-butanol, 3-methyl-1-butanol, 3- methyl-furan, 3-octanone and 1-octen-3-ol (Claeson, et al. 2002; Claeson, et al. 2007; Fiedler, et al. 2001; Fischer, et

al. 1999; Matysik, et al. 2009; Van Lancker, et al. 2008). *Aspergillus* species release many different types of VOCs in indoor environments (Pennerman, et al. 2016).

Moreover, VOCs can be used in the diagnosis of pulmonary infections and lung cancer.

Early detection of invasive aspergillosis is required for efficient therapy of this infection so (Heddergott, et al. 2014b) did an in-depth analysis of "the volatome" of this pathogen. They found tha*t A. fumigatus* produces different spectra of VOCs; the variety and quantity of VOCs detected vary with the culture medium, method of analysis, and age of the mold culture (Table 1.1)., (Perl, et al. 2011) observed that *Aspergillus fumigatus* produced 3-octanone, isoamyl alcohol, 3- methyl- butanol, ethanol, cyclohexanone, and some uncharacterized volatiles compounds when cultures were grown on Columbia sheep blood agar and analyzed by using MCC-IMS and/or GC/MS analysis in headspace concentrations. Cyclohexanone and 3-octanone were found in higher amounts than other compounds. *A. fumigatus*, *C. albicans, C. neoformans ,* and *C.gattii* produced different VOCs in hospital air, with 1-pentanol, 1-octen-3-ol, 3- methyl-1-butanol, 3-octanol and 2-methyl-1-butanol produced in low concentrations while 2- heptanone and 2-methyl-1-propanol were found in high concentrations (Pantoja, et al. 2016). *A. fumigatus* and *C. albicans* emitted ethanol, acetaldehyde, acetone, methanethiol, 2-butenal, isoamyl alcohol, phenethyl alcohol, and cyclohexane as determined by ion mobility spectrometry and selected ion flow tube-mass spectrometry (SIFT-MS) (Perl, et al. 2011; Scotter, et al. 2005).

TABLE 1.1 *Aspergillus fumigatus* Volatile Organic Compounds (VOCs) have been analyzed by using different media and methods.

Medium	Method	VOCs	Reference
YEA	Thermal Desorption combined with GC/MS.	Camphene Trans –B-farnesene α-pinene B-phellandrene. Limonene 2-methyle-1-butanol. 3-methyl-1-butanol 2-methyl-1-propanol	(Fischer, et al. 1999)
MEA	Head-Space Micro Extraction (HS-SPME) analysis	1-octen-3-ol 2-Methyl-1-butanol 3-Methyl-1-butanol 3-Octanone Limonene Cyclopentanone	(Fiedler, et al. 2001)
Beech wood	Head-Space Micro Extraction (HS-SPME) analysis	2-Octanone 3-Octanone	(Fiedler, et al. 2001)
Gypsum board	GC/MS	Acetophenone 2-Pentylfuran 2-Heptanone 2-Hexanone 3-Methyl-1-butanol	(Gao, et al. 2002)
MEA	GC/MS	3-Octanone 3-Methyl-1-butanol	(Gao, et al. 2002)

		2-Pentylfuran	
Dichloran glycerol agar	Passive Sampling combined with GC/MS onto charcoal sorbets	2-Methyl-1 propanol Ethyl acetate 3- Methyl-1 butanol 2-Methyl-1-butanol 2-Ethyl-1-hexanol 2-Pentanone 3-Octanone Cyclohexanone Limonene	(Matysik, et al. 2009)
Columbia sheep	MCC-IMS	3-Octanone	(Perl, et al. 2011)
Columbia sheep	MCC-IMS	Isoamyl (3- Methyl-butanol) Cyclohexanone Ethanol	(Perl, et al. 2011)
MEA	SPME-GC-MS	3- Octanone 3-Octanol Diterpene Methyl benzoate 2-Methyl-2-bomene	(Polizzi, et al. 2012)
Brian's medium with different condition	SPME (Solid phase micro extraction) combined with GC/MS	1-octen-3-ol 3-Methyl-3-buten-1-ol 3-Octanone 3-Methyl-2-buten-1-ol 3-Methyl-1-butanol α-Pinene	(Heddergott, et al. 2014b)

Brain Heart Infusion (BHI) broth	Thermal desorption combined with GC/MS	-Phenyl-1-H-indene Azacyclotridecan-2-one 2-Ethyl-5-methyl-pyrazine 2-Nonanone	(Neerincx, et al. 2016)
PDA	Gas chromatography/mass spectrometry (GC/MS)	1-pentanol 1-octen-3-ol 3- methyl-1-butanol 3-octanol 2-methyl-1-butanol 2- heptanone 2-methyl-1-propanol	(Pantoja, et al. 2016)

1.4.3. Mushroom alcohol (1-octen 3-ol)

Mushroom alcohol, an eight carbon alcohol, is the most abundant VOC emitted by fungi (Combet, et al. 2006). It is used as a food flavoring ingredient and as a component of many perfumes. It is often detected in water-damaged buildings as a strong musty or "moldy" smell. Our laboratory has developed a method using the larvae of *Drosophila* flies to measure the toxicity of different fungal VOCs (Bennett and Inamdar 2015; Inamdar and Bennett 2014; Inamdar, et al. 2012; Inamdar, et al. 2014). The flies that were exposed to 1-octen-3-ol exhibited increased amounts of nitrate which is due to the breakdown of nitric oxide that lead to inflammatory response in hemocytes of *Drosophila* innate immune cells. When the brain of the exposed flies was observed, a dark purple hue was present showing the response of the nitric oxide pathway to 1-octen-3-ol. Exposure to this compound also promotes NOS expression in tracheal tissues

of larvae and caused remodeling of tracheal epithelial lining (Inamdar, et al. 2014). Mushroom alcohol also caused toxicity in *Arabidopsis thaliana* (Hung, et al. 2015) and human embryonic stem cells (Inamdar, et al. 2012).

1.4.4. Volatile organic compounds (VOCs) as diagnostic aids

Invasive aspergillosis may be diagnosed by the detection of VOCs that are emitted from *A. fumigatus* growing on the human host (Goeminne, et al. 2012). The VOCs produced by *A. fumigatus* can be used as indicators to diagnose systemic aspergillosis (Heddergott, et al. 2014b). Several other diagnostic tests that are used for aspergillosis involve fungal culturing and antigen detection from samples of respiratory, blood, or other tissues (Hope, et al. 2005; Kosmidis and Denning 2015b). VOC detection is non-invasive and offers enormous promise as a good, alternative diagnostic method (Chambers, et al. 2009; Heddergott, et al. 2014b). *A. fumigatus* produces 2- pentylfuran (2PF) in both on *A. fumigatus* cultures (Blood Agar) and in breath of patients who are infected by IA; it was not found in the breath of in the healthy people. 2- Pentylfuran (2PF) is a major metabolite found in the headspace of *A. fumigatus* cultures on various media such as malt extract agar and gypsum board and may be a specific marker for *A. fumigatus* (Bhandari, et al. 2011; Chambers, et al. 2009; Gao, et al. 2002).

In previous studies from our laboratory, we used third instar larvae of *D. melanogaster* to investigate the toxic effect of VOCs emitted from different fungal genera isolated after Hurricane Katrina (*Trichoderma, Aspergillus,* and *Penicillium*) and showed

a detrimental impact on survival of larvae (Inamdar, et al. 2012)). In addition, chemical standards of fungal VOCs, and some industrial solvents, have been tested to investigate the toxic effects of these gas phase chemicals on the *Drosophila* larvae and adult lifespan. Several eight carbon compounds (1-octen-3-ol, 3-octanol, and 3-octanone) had more toxic effect on the larvae and adult stages of fruit flies than the non-C8 compounds. For example, exposure to vapors of 1-octen-3-ol at 0.5 ppm prevented larvae from becoming adult files, 70% of larvae have transferred to adult flies when exposed to 1-octen-3-ol at 0.2 ppm. The toxic effects of common industrial compounds, consisting toluene, benzene, formaldehyde, and xylene were also tested. After 15 days of monitoring, benzene and toluene caused 50% mortality of flies while 18 and 20% of mortality were observed for formaldehyde and xylene receptively. In summary, vapors of chemical standards of C8 compounds produced by fungi showed more toxic effects on lifespan of *D. melanogaster* than the non-C8 compounds and industrial solvents tested (Inamdar, et al. 2012). In other experiments, a correlation was observed between the exposure to low concentrations of gas phase 1-octen-3-ol and neurotoxicity in *Drosophila.* Exposed flies exhibited Parkinson's disease-like symptoms. Because of *Drosophila*'s similarity to mammals in certain signaling pathways, it is possible to use them as genetic models in order to study the effects of the VOC's on pathways such as the c-Jun N Terminal Kinases (JNK) and the AKT pathway, a serine-threonine protein kinase, used to regulate cellular health and function (Inamdar, et al. 2010).

The presence of the JNK and the AKT pathway are important for the survival of *Drosophila*; especially when exposed to 1-octen-3-ol. The mutants exposed to the mushroom alcohol suffered from a truncated lifespan, while the wild type survived for its

normal lifespan. When the genes were overexpressed, there was an increase in the survival rate by roughly 5 days (Inamdar and Bennett 2014).   In other studies, on human embryonic stem cells, 1-octen-3-ol was more toxic than toluene by 80 times. Previously, several VOCs such as 1-octen-3-ol, 3-octanol, 3-octanone, 1-butanol, 1-propanol, 1-decanol, 2-methyl-1-propanol, 2-methyl-1-butanol, and 3-methyl-1-butanol have been tested to investigate their toxicity using mammalian cell culture systems and rodent models (Ema, et al. 2005; Kreja and Seidel 2002b; Nelson, et al. 1989).

In other studies, using the *Drosophila* model, chemical standards of three VOCs (1-octen-3-ol, (E)- 2- hexenal, and 1-hexanol) were tested. Third instar larvae were exposed in Petri dish to low concentrations of each compound and then the number of larvae, pupae, and adults were counted daily for a total of ten days. Flies that were exposed to 1-octen-3-ol experienced a significantly delayed development. Only a small number of the exposed pupae properly underwent metamorphosis and transformed into adult flies. In summary, the presence of the fungal VOCs inhibited growth and metamorphosis of the larvae (Yin, et al. 2015). The same fly toxicology system was used to test the volatiles emitted by several fungal species were isolated and identified from flooded buildings after Superstorm Sandy.   These isolates included *Penicillium* (11 species), *Fusarium* (four species), *Aspergillus* (three species), *Trichoderma* (two species), and one species each of *Metarhizium*, *Mucor, Pestalotiopsis*, and *Umbelopsis*. *Drosophila* larvae were exposed to volatile compounds released from these fungi. VOCs from *Aspergillus niger* 129B caused 80 percent mortality after 12 days of exposure while exposure to VOCs from *Trichoderma longibrachiatum* 117, *Mucor racemosus* 138a, and *Metarhizium anisopliae* 124 had little effect on viability and metamorphosis. Solid- phase microextraction- gas chromatography-

mass spectrometry analysis was used to identify volatile compounds produced from two of the most toxic, two of the least toxic, and two species of intermediate toxicity. It was shown that 1- octen- 3- ol, 3- octanone, 3- octanol, 2- octen- 1- ol, and 2- nonanone were produced in higher concentrations by *Aspergillus niger* 129B; while 3- methyl- 1- butanol and 2- methyl- 1- propanol were produced in lower concentrations by the least toxic species (Zhao, et al. 2017).

Research goals

My dissertation research goal has been to study the possible toxic effects of volatile organic compounds (VOCs) produced by human pathogenic fungi using *Drosophila melanogaster* as a model system.

There were four defined aims:

1- To test the effect of VOCs produced by wild type and mutant *Aspergillus fumigatus* strains, *Candida albicans, Cryptococcus sp.* and *Saccharomyces cerevisiae* on the metamorphosis of *Drosophila* third instar larvae.

2- To test the effects of VOCs produced by wild type and mutant *A. fumigatus* strains on the metamorphosis of different wild type and mutant *Drosophila* third instar larvae. The *A. fumigatus* mutants were blocked in different steps of the lipoxygenase pathway. The *Drosophila* mutants carried different defects in the fly innate immune system. In addition, the effect of VOCs emitted by *A. fumigatus* grown on medium supplemented with arachidonic acid was also tested in the *Drosophila* bioassay.

3- To test controlled levels of the volatile phase of chemical standards of single VOCs (1- octen- 3- ol, 3- octanone, and 3- octanol) on adult wild type and mutant *Drosophila* flies. The *Drosophila* mutant strains carried different defects in the fly innate immune system.

4- To determine the volatile profile emitted by the most toxic and least toxic wild type strains of A. *fumigatus* using gas chromatography-mass spectrometry. Further, to determine. the volatile profile emitted by an *A. fumigatus* strain carrying a triple defect in the lipoxygenase pathway genes.

CHAPTER 2. VOLATILE ORGANIC COMPOUNDS PRODUCED BY

ASPERGILLUS FUMIGATUS STRAINS AND OTHER PATHOGENIC FUNGI

ARE TOXIC TO THE GENETIC MODEL *DROSOPHILA MELANOGASTER*

Abstract

Volatile organic compounds (VOCs) are organic compounds with a small molecular mass and the ability to easily vaporize at room temperatures Fungi produce many VOCs that vary in type and amount depending on factors that influence growth, such as temperature, moisture, pH, and others. The effect of VOCs from several medically important fungi was tested on larvae of the genetic model *Drosophila melanogaster*.   The fungi used included six strains of *Aspergillus fumigatus*, and one strain each of *Candida albicans, Cryptococcus neoformans, Cryptococcus gattii,* and *Saccharomyces cerevisiae.*   The experiment was monitored for 15 days, and daily counts of larvae, pupae, and adults of *D. melanogaster* were completed. Of the *A. fumigatus* strains tested, the highest toxicity to the developmental stages of *Drosophila* was shown by strain 1607 when grown at either 25°C or 37°C. In contrast, there were no significant effects on fly viability when flies were exposed to VOCs from *A. fumigatus* strain 1592. In almost all cases, exposure to the VOCs from C*andida albicans, Cryptococcus neoformans, and Cryptococcus gattii* caused more delays on fly metamorphosis and more toxicity than did exposure to VOCs from *A. fumigatus*. VOCs from *S. cerevisiae* had no significant effect on the flies. VOCs produced from *A. fumigatus* strains caused some morphological abnormalities in *Drosophila* larval, pupal, and adult stages as compared to controls and other pathogenic fungi. The most common VOC produced by the most toxic and least toxic A. *fumigatus* strains was 1-octen-3-ol, however this compound in much greater concentrations by the most toxic strain.   In summary, these data suggest that VOCs from medically important fungal species could be

acting   as   virulence   factors   that   enhance   their   pathogenic   effects.

2.1. Introduction

Volatile organic compounds (VOCs) are low molecular organic substances which are easily vaporized at room temperature (Herrmann 2010) Industrial products are the best-known VOCs and include those used for such purposes as painting, cleaning, air refreshing and so forth; many of these industrial VOCs are known to have toxigenic effects and their emissions have been subject to government regulation (Bennett and Inamdar 2015; EPA 2011). Less is known, however, about the biogenic VOCs emitted by fungi and other organisms as part of their normal metabolism. Fungal VOCs are released as mixtures of chemical compounds with a variety of functional groups, such as hydrocarbons, acids, alcohols, aldehydes, aromatics, ketones, terpenes, thiols, and their derivatives. Different species growing on different substrates produce unique mixtures of VOCs (Bennett 2009; Hung, et al. 2015; Korpi, et al. 2009a). A German group has created an excellent data base of bacterial and fungal VOCs (Lemfack, et al. 2018; Lemfack, et al. 2014).

Fungal VOCs have characteristic odors, and the amounts and types of VOCs vary with certain factors such as 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 (Cabral 2010; Gao, et al. 2002; Pennerman, et al. 2016; Polizzi, et al. 2009).

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 symptoms such as headaches, dizziness, faintness, and irritation of the eyes and mucous membranes of the nose and throat (Araki, et al. 2012; Araki, et al. 2010; Takigawa, et al. 2009). *Aspergillus fumigatus* is a cosmopolitan filamentous fungus found in soils all over the world. As an opportunistic human pathogen, it causes diseases such as localized infections, aspergilloma (fungus ball), allergic bronchopulmonary aspergillosis, and invasive aspergillosis in immunocompromised patients (Agarwal 2009; Latgé and Steinbach 2009). It has been isolated from buildings whose occupants have complained of building-related illness, and also from the homes of asthmatic children (Schwab and Straus 2004).

Candida albicans is commensal on humans, but can become pathogenic in immunocompromised patients, especially those with cancer and AIDS (Calderone and Clancy 2011) (Gow and Yadav 2017). C. albicans has been isolated as one of the contaminants present in hospital air (Pantoja, et al. 2016). It readily colonizes, adheres, invades, and multiplies in host tissues of immunocompromised patients (Coronado-Castellote and Jimenez-Soriano 2013).

Cryptococcus neoformans and C. gattii are the causative agents of cryptococcosis, another opportunistic fungal disease with high mortality (Chen, et al. 2014; Ma and May 2009). Humans become infected through aerial means and disease occurs as a result of a new opportunistic infection or an activation of a latent infection. Individuals with cryptococcosis display large lesions in the tissues of the lung and brain. This pathogenic sign occurs more often with *C. gattii* than with *C. neoformans* (Datta, et al. 2009).

Cryptococcus neoformans is often transmitted in hospitals where it infects 1% to 5 % of transplant patients.   The mortality rate from cryptococcal disease is 20% to 42% (Singh and Paterson 2005).

Opportunistic pathogenic fungi such as *A. fumigatus*, *C. albicans, C. neoformans,* and *C. gattii* have been detected in a Brazilian hospital. When VOCs were assayed in hospital air*,* 1-pentanol, 1-octen-3-ol, 3- methyl-1-butanol, 3-octanol and 2- methyl-1-butanol were found in low concentrations while 2- heptanone and 2-methyl-1-propanol were present in high concentrations (Pantoja, et al. 2016). It is known that *A. fumigatus* and *C. albicans* emit ethanol, acetaldehyde, acetone, methanethiol, 2-butenal, isoamyl alcohol, phenethyl alcohol, and cyclohexane as determined by ion mobility spectrometry and selected ion flow tube-mass spectrometry (SIFT-MS) (Perl, et al. 2011; Scotter, et al. 2005).

The toxicity of some fungal VOCs has been studied in rodent models, cultured cell lines and, in a few cases, on human volunteers(Korpi, et al. 1999; Kreja and Seidel 2002a; Wålinder, et al. 2008). Our laboratory has developed *Drosophila melanogaster* as a model for studying the biological activity of fungal volatiles (Inamdar and Bennett 2014). Flies are well suited for toxigenic 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 (Lionakis and Kontoyiannis 2012). In this research, we tested the effects of VOCs produced by six different *A. fumigatus* strains, as well as one strain each of *Candida albicans*, *Cryptococcus neoformans* and *Cryptococcus gattii* on third instar larvae of *Drosophila*. The data reported here demonstrate that the VOCs of pathogenic fungi cause significant delays in

metamorphosis, along with significant lethality when the strains were pre grown at 37∘C than at 25∘C. We have used Gas Chromatograph – Mass Spectrometer (GC/MS). analysis to determine which VOCs are produced by the most toxic strain compared to the least toxic one.

2.2 Materials and Methods

2.2.1 Fungal Strains and Culture Conditions

Aspergillus fumigatus strains were obtained from Dr. Geromy Moore, Southern Regional Research Laboratories, U. S. Department of Agriculture, New Orleans, Louisiana, USA. The *A. fumigatus* strain numbers and their original sources were: SRRC 1607 (damp indoor environment); SRRC 46 (a penguin at the Brookfield Zoo, Chicago, IL) ; SRRC 323 (chicken lung); SRRC 51 (human chest cavity lining); SRRC 2569 (clinical isolate from the University of Manchester, Manchester, UK); and SRRC 1592 (rain forest soil). Other pathogenic fungi, and the nonpathogenic yeast *Saccharomyces cerevisiae*, were obtained from Dr. Chaoyang Xue, Public Health Research Institute Center, New Jersey Medical School-Rutgers, Newark, New Jersey USA. These strains are: *Saccharomyces cerevisiae* (BY4741), *Cryptococcus neoformans* (H99 serotype A), *Cryptococcus gattii* wild type (R265), and *Candida albicans* wild type (ATCC 90028).

Stock cultures were maintained on potato dextrose agar (PDA) (Difco) prepared following manufacturer's instructions by adding 39 g of the commercial mixture to 1 L of distilled water. The medium was mixed and dissolved by heating and then autoclaved. For all

Drosophila exposure experiments, the fungi were grown on 25 ml of PDA in 6 oz. *Drosophila* stock bottles (Genesee Scientific, CA). Fungi were incubated either at 25°C for 5 days, or at 37°C for 3 days, before using them in exposure studies with fly larvae.

2.2.2 Drosophila culture media and maintenance

White eyed *Drosophila* flies (W^{1118}; Y^{1}) with a wild type immune system were used for all experiments. Breeding stocks of *Drosophila* were maintained in Ward's Instant *Drosophila* medium (WARD's Natural Science, NY*)*. About every two weeks, the flies were transferred to new 6 oz. *Drosophila* stock bottles (Genesee Scientific, CA) so that there was a renewal of space and food for the breeding flies. The egg laying medium contained 376 ml of distilled water, 126 ml of grape juice, 15 g of agar, and 6 g of sucrose mixed in a 2-L flask, and then microwaved on high for 6 minutes until the agar was dissolved. This grape juice media was autoclaved and cooled to 60-65 ^{o}C and then 10 ml ethanol and 5 ml acetic acid were added. Finally, the medium was mixed thoroughly and poured into Petri plates. A simple sucrose medium, which consisted of 100 ml of distilled water, 5 g of sucrose and 1 g of agar mixed, dissolved by heating, and then autoclaved, was used for growing larvae and pupae (Figure 2.1)
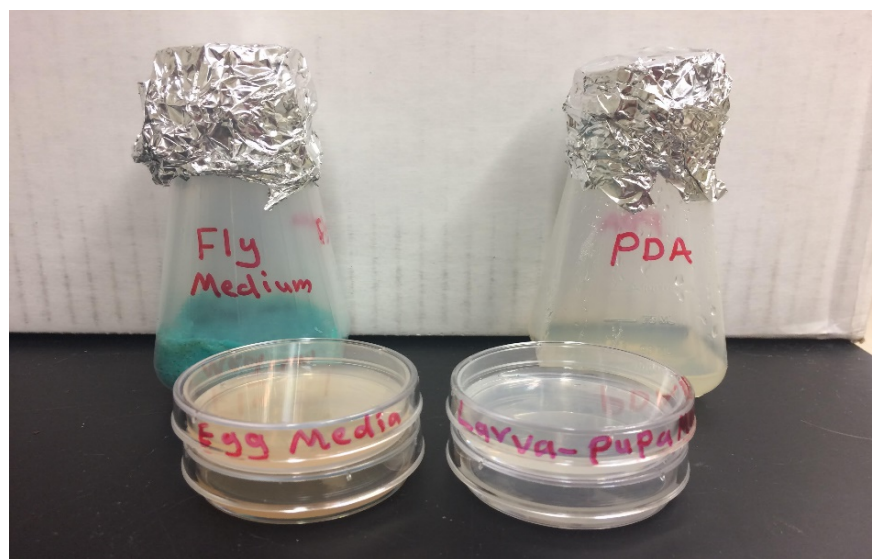
Figure 2.1 Egg laying medium and larval-pupal medium in Petri
plates. Ward's Instant *Drosophila* medium and potato dextrose
agar (PDA) in bottles.

2.2.3 Mating and egg laying of *Drosophila* flies

Eggs were collected by transferring mature adult flies into a bottle attached via masking tape to a Petri plate cover that had been punctured with a flame-hot pipe to make a tri-circular opening. The bottle, which held the mature adult flies, and the Petri plate cover were placed on top of a Petri plate containing the grape juice egg-laying medium. for 3 hours to give the flies time to mate (Figure 2.2). Then the adult flies were transferred back to their bottles with the Ward's Instant *Drosophila* Medium. Dried yeast, weighing about 0.06 g, was added to each grape juice agar plate. Plates were them placed in glass jars at room temperature for 4-5 days, during which time the flies completed three molts and developed into third instar larvae.

The bottle with adult flies is attached via masking tape to a Petri dish cover which has a tri-circular opening. The mated female flies lay their eggs on the surface of the medium in the Petri dish.

Petri dish containing grape juice agar for egg laying.
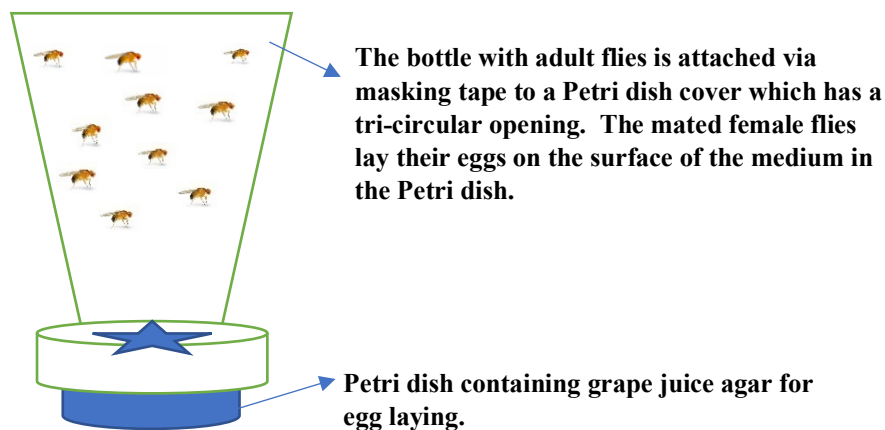
Figure 2.2 The experimental setup for mating the *Drosophila* flies and subsequent egg collection.

2.2.4 Exposure of *Drosophila* larvae to VOCs produced by fungi

Third instar fly larvae were exposed to VOCs following the method of (Inamdar, et al. 2012) with some modification. On the fifth day, the fungal strains grown at 25°C, the fungal strains grown at 37°C, and the third-instar larvae, were all ready at the same time to be used in the experiment. Using the same experimental setup as was used for the egg collection, a bottle was attached via masking tape to a Petri plate cover that had been punctured with a flame-hot pipe to make a tri-circular opening and paired with a Petri plate of larvae medium. Fifteen larvae were placed onto each larva -pupa medium, so that there were 15 larvae present in each "bottle-plate" setup Figure 2.3.

Each Petri plate was secured on top of the bottle with a strip of clear tape across the edges of the plate. The Petri plate pair was sealed with Parafilm to protect from any

possible contamination. Three replicates were made of each strain grown at each temperature, so per strain there were three bottles of fungi grown at 25°C and three bottles grown at 37°C, for a total of six bottles and two sets of three bottles for the controls without any culture. Once the larvae were placed and sealed, the bottle-plate setups were incubated at 25°C and were rotated at 50 rpm for 15 days. Each day, the number of larvae, pupae, and adult flies was monitored and recorded.

Larva-pupa medium with *Drosophila* larvae

The Petri dish cover has a hole in the center which is covered by membrane

Fungal cultures on medium
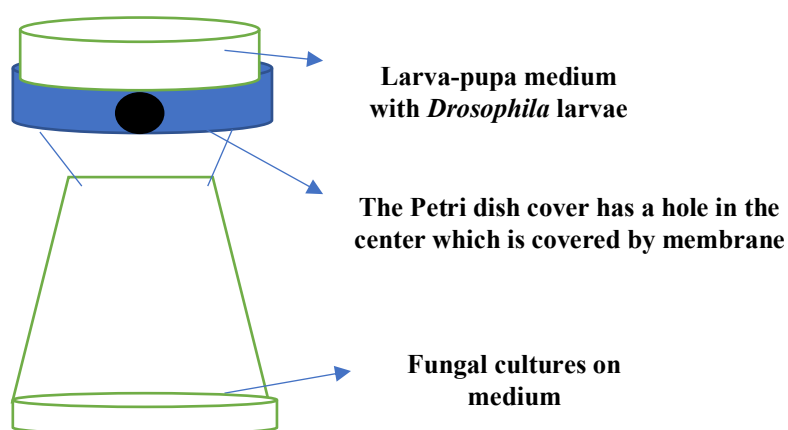
Figure 2.3. The Bottle -Plate microhabitat for exposing *Drosophila* larvae to fungal VOCs

2.2.5 Measurement of biomass/dry weight of *Aspergillus* strains

In order to determine if there was a significant difference in the amount of biomass between the different strains of *A. fumigatus*, the method of (Singh, et al. 2012) was followed with slight modifications.   Strains were cultured on PDA and incubated at 25°C for one week in order to prepare inocula. Spore suspensions were collected by using 2 ml of a mixture of 30% glycerol, 0.05% agar, and 0.05% tween 80, and refrigerated prior to

use. *A. fumigatus* spore suspensions ($1x10^3$ spores/ ml) were inoculated into Potato Dextrose Broth (PDB) in a small tube (50 ml) and incubated with shaking at 120 rpm for 5 days at 25 °C or 3 days at 37°C. The resultant mycelial pellets were filtered using Whatman filter paper No.1 and dried at 50°C for four days in an air incubator to measure the biomass/dry weight of fungus. The experiment was repeated twice and there were two sets of three replicates for each strain.

2.2.6 Gas chromatography-mass spectrometry analysis

Two strains of *Aspergillus fumigatus* were selected for VOC analysis: the most toxic strain 1607 and the least toxic strain 1592. They were grown either at 25 °C for 5 days or 37 °C for 3 days on PDA by using 250ml flasks and then closed with plastic stoppers and sealed it by Parafilm. Sterile PDA media was used as a control; another control was a blank consisting of air only. The fungal samples were analyzed using Purge and Trap-Thermal Desorption-GC-MS. The samples were purged with air at 100 mL/min at room temperature for 1 hour. Volatile and semi-volatile outgas products were trapped onto Tenax (modified poly(phenylene)oxide) traps. The Tenax traps were spiked with 1.0 ug of benzene-d6, toluene-d8, and naphthalene-d8 internal standards and purged with nitrogen for 90 minutes at 50 mL/min to remove water from the traps. Tenax traps were connected to a Short Path Thermal Desorption System (Scientific Instrument Services) and desorbed directly into the GC injector at 25^0C for 5 minutes with carrier gas flow. The GC temperature program was -20C to 26^0C @ 10C/min, injector temperature was 25^0C, and heated transfer line temperature was 28^0C. The split valve on the GC was set at a 10:1 ratio.

The mass spectrometer was operated in positive ion electron ionization (EI) mode (70 eV) scanning m/z 35-350 once per second. The compounds found in the PDA control were removed from the data obtained from the gas analysis of the *A. fumigatus* strains (Hung, et al. 2013) with some modifications.

Statistical Analysis

In each experiment, three replicates of 15 third instar larvae were exposed to VOCs from growing fungi, and the experiments were repeated twice (N=90). The differences in metamorphic stages and eclosion between controls and VOC-exposed strains were analyzed for significance by using the Student *t-test* on day 4 for the larvae stage, day 8 for the pupal stage and on day 15 for the adult stage.

2.3 Results

2.3.1 Effect of Volatile Organic Compounds Produced by *Aspergillus fumigatus* strains

Drosophila larvae were exposed to VOCs from six *A. fumigatus* strains grown on PDA that had been pre- incubated either for five days at 25°C or three days at 37°C. The experimental set ups were incubated on a shaker at 50 rpm. The number of larvae, pupa, and adults were monitored and counted daily for 15 days.

The toxic effects of *A. fumigatus* VOCs on the developmental stages of fruit flies were greater when the fungus had been originally grown at 37°C than at 25°C. Thirty-three percent of the larvae exposed to VOCs of *A. fumigatus* strains 1607 and 46 grown at 25°C

did not metamorphosize into pupae; and 28.8% of the larvae exposed to *A. fumigatus* strain

51were delayed. However, the larvae exposed to the VOCs released by *A. fumigatus* strains

2569 and 1592 had similar rates of metamorphic conversion to pupae and adults. VOCs

from *A. fumigatus* strains that were pre-grown at 37°C had higher toxicity levels and greater

delays in the developmental stages of fruit flies. On the fourth day of exposure, 55.5% of

the larvae had not transferred

to the pupa stage when they were exposed to VOCs of *A. fumigatus* strain SRRC

1607; 48% and 40% of the larvae exposed to VOCs of strains SRRC 46 and SRRC 323,

respectively, had not metamorphosed into pupae. However, the larvae exposed to the VOCs

released by *A. fumigatus* strains SRRC 51, SRRC 2569 and SRRC 1592 experienced fewer

delays (Figure2.4. A-B).

Strains were pre- grown at 25°C

A-Significant level of
larvae on day 4:
C. vs 1607 **
C. vs 46   **
C. vs 51   **
C. vs 323   *
C. vs 2569   NS
C. vs 1592 NS

A
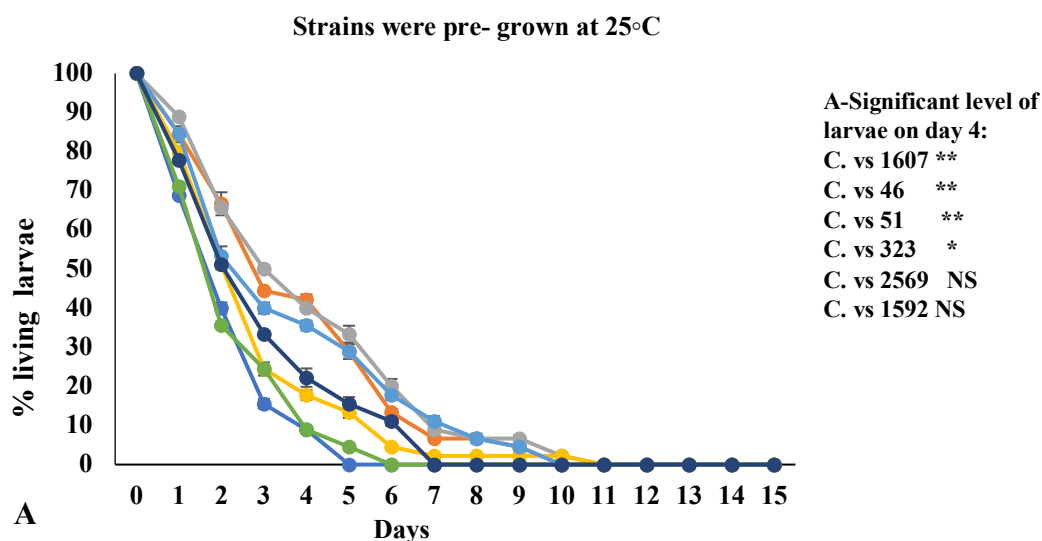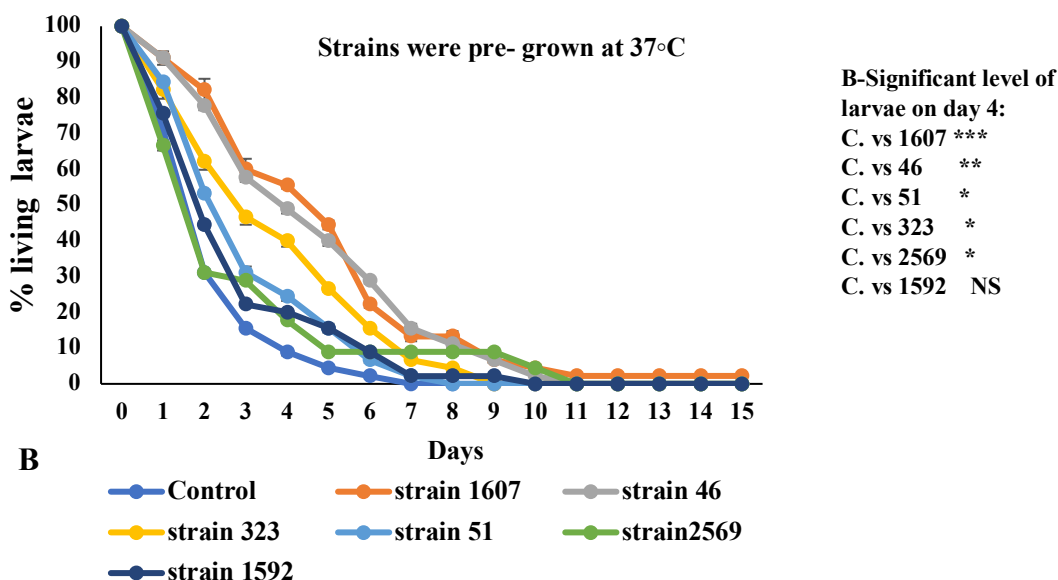
Figure 2.4 Exposure *Drosophila* larvae over 15 days of continuous exposure to VOCs of different strains of *Aspergillus fumigatus*. **A:** the percent of alive larvae that were exposed to VOCs from the strains were grown on PDA at 25°C for five days. **B**: the percent of alive larvae that were exposed to VOCs from the strains were grown on PDA at 37°C for three days. 15 instar larvae were exposed to three replicates of each strain and the experiment was repeated twice. (N=90). NS = Nonsignificant, * represents significant difference between control and *A. fumigatus* strains exposed larvae. Where *P<0.05 and **P<0.005.

Exposure to VOCs from *A. fumigatus* strains SRRC1607, 46, and 51, delayed the metamorphosis as compared to the other strains SRRC 323, 2569, and 1592, which showed fewer significant differences from controls. Exposure to a common atmosphere with *A. fumigatus* strain SRRC 1607 yielded the most delays in metamorphosis and the highest level of toxicity. VOCs from *A. fumigatus* strain SRRC1592 had no significant change on the pupae at both temperatures. Exposure to VOCs from *A. fumigatus* strain SRRC 1607

was the most effective in delaying eclosion into the adult stage, while exposure to VOCs from *A. fumigatus* strain SRRC1592 had no significant effect on the pupal metamorphosis **(**Figure 2.5 A-B). Almost all control flies completed metamorphosis and became adults after ten days. The number of adult flies is shown in (Figure 2.6 A-B).

Strains thst were pre- grown at 25 ◦C

A-Significant level of pupae on day 8:
C. vs 1607 **
C. vs 46   **
C. vs 51   *
C. vs 323   *
C. vs 2569   *
C. vs 1592 NS
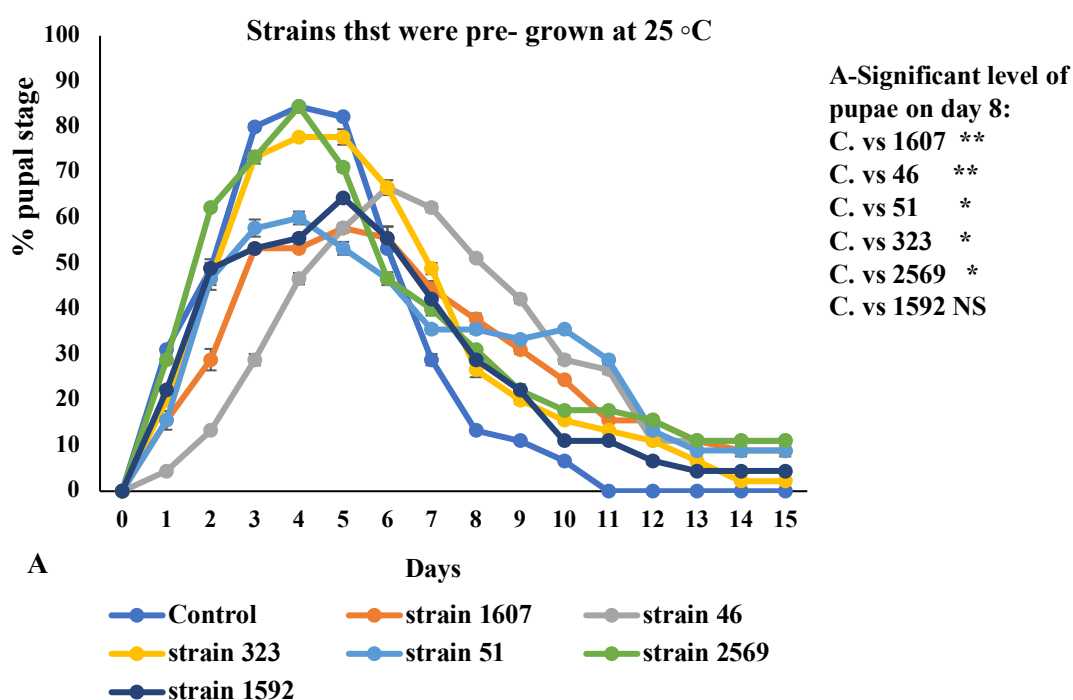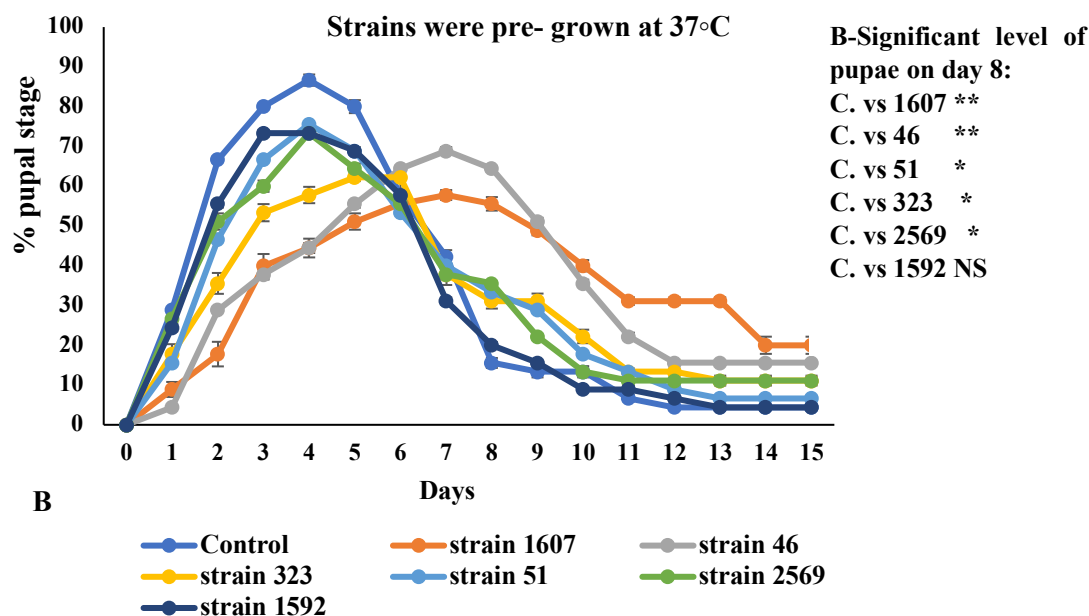
Figure 2.5 Exposure *Drosophila* larvae over 15 days of continuous exposure to VOCs of different strains of *Aspergillus fumigatus*. **A:** the percent of alive pupae that were exposed to VOCs from the strains were grown on PDA at 25°C for five days. **B:** the percent of alive pupae that were exposed to VOCs from the strains were grown on PDA at 37°C for three days. 15 instar larvae were exposed to three replicates of each strain and the experiment was repeated twice. (N=90). NS = Nonsignificant, * represents significant difference between control and *A. fumigatus* strains exposed larvae. Where *P<0.05 and **P<0.005.

Only 58% of the larvae transferred to adults while being exposed to the VOCs of *A. fumigatus* strain SRRC 1607 while the VOCs of strains SRRC 46, 51, and 323 respectively had less of an effect on adult metamorphosis. Exposure to VOCs from *A. fumigatus*, strains SRRC 2569 and 1592 had no significant effect when compared to the control flies. The number of adult flies at 7, 10 and 15 days are compared in Table 2.1.
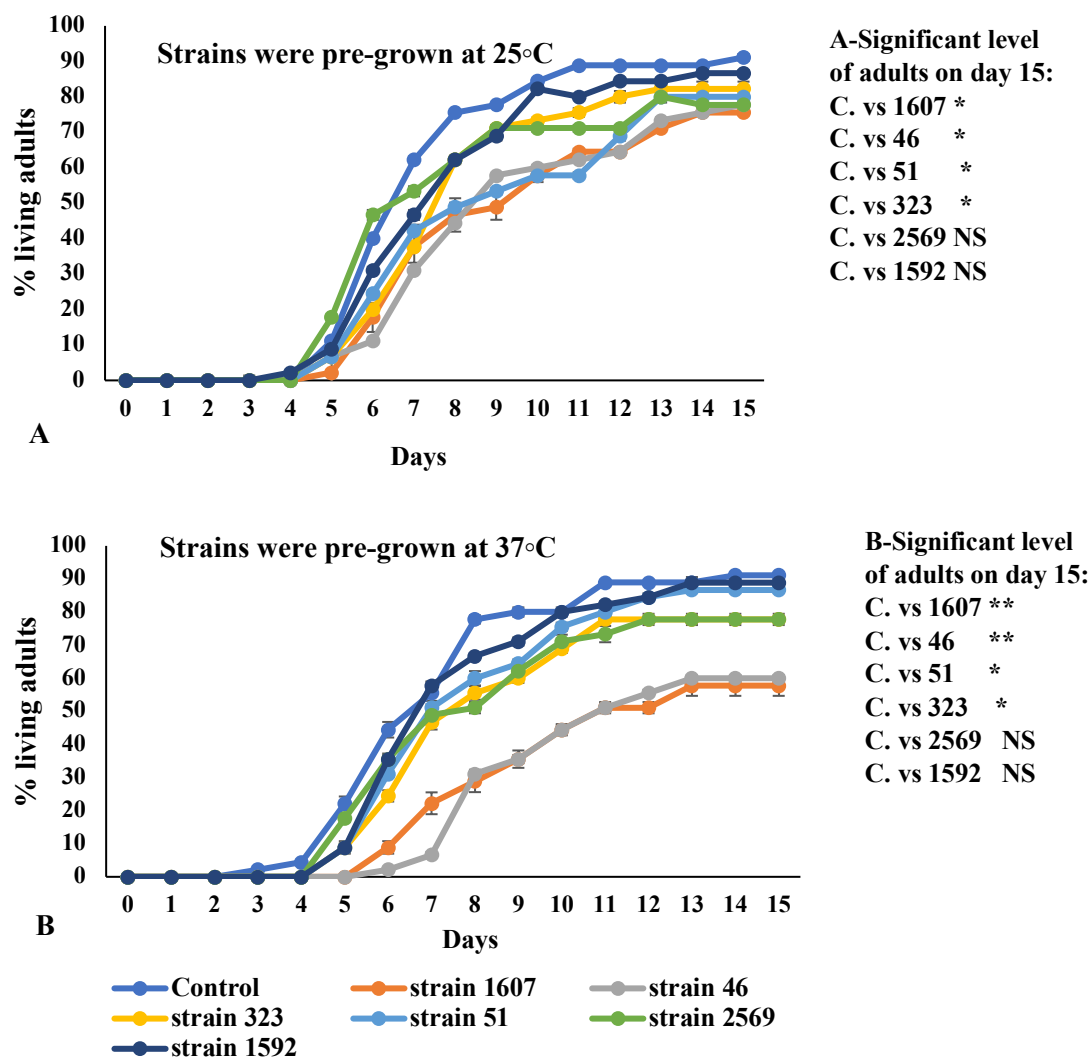
Figure 2.6 Exposure *Drosophila* larvae over 15 days of continuous exposure to VOCs of different strains of *Aspergillus fumigatus*. **A:** the percent of alive adults that were exposed to VOCs from the strains were grown on PDA at 25°C for five days. **B**: the percent of alive adults that were exposed to VOCs from the strains were grown on PDA at 37°C for three days. 15 instar larvae were exposed to three replicates of each strain and the experiment was repeated twice. (N=90). NS = Nonsignificant, * represents significant difference between control and *A. fumigatus* strains exposed larvae. Where *P<0.05 and **P<0.005.

2.3.2 Measurement biomass/dry weight of *Aspergillus fumigatus* strains

Six strains of *A. fumigatus* were tested for dry weight measurement at different temperatures. All strains were cultured on PDB in a small flask and incubated at 120 rpm at different temperatures (5 days at 25 $^{\circ}$C and 3 days at 37°C). Fungal dry weight and mycelial ball size were measured after 5 and 3 days of incubation. There was no difference in biomass at the two different temperatures while 1607 strain has the lowest biomass compared to other strains (Figure 2.7).
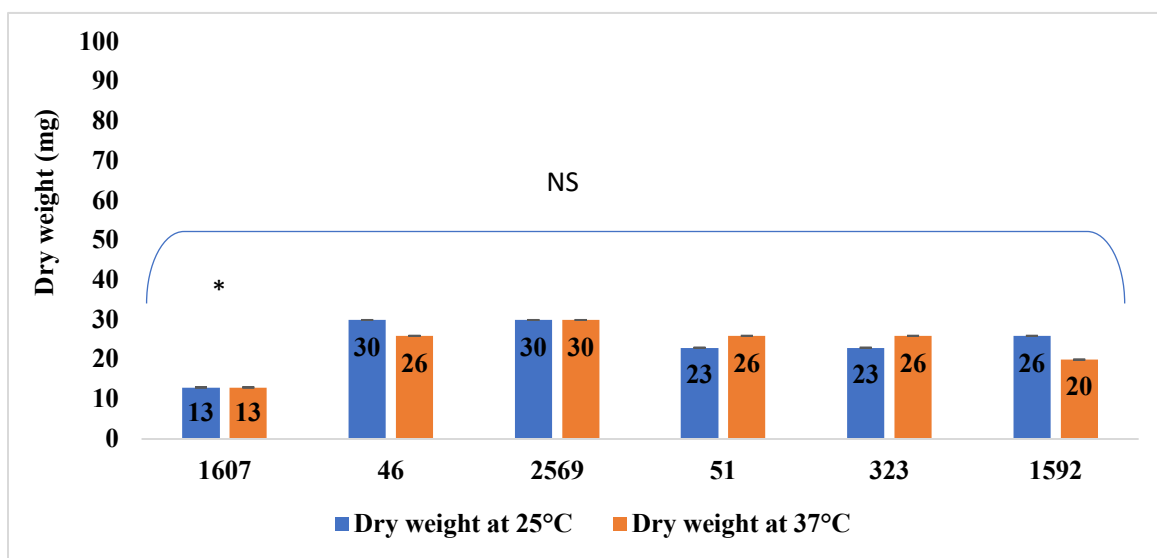
Figure 2.7: Measurement dry weight of *A. fumigatus* strains on potato dextrose broth at different temperatures 5 days at 25°C and 3 days at 37°C with shaker at 120 rpm. NS= Non significant between temperatures. * represents significant difference between strain SRRC 1607 and other strains. Where *P<0.05

2.3.3 Effect of *Candida albicans, Cryptococcus neoformans, Cryptococcus gattii,* and *Saccharomyces cerevisae* VOCs on fruit fly larvae

After fifteen days of exposure to the VOCs of the pathogenic fungi *C. neoformans*, *C. gattii, C. albicans,* the larvae experienced more of a toxic effect and a longer delay in metamorphosis when exposed to the fungal   strains were pre-grown at 37°C than at 25°C. Survival of exposed larvae showed no significant differences compared to the control when fungi were grown at 25°C**,** but at the pupal stage they exhibited a delay in metamorphosis. In the adult stage, *C. gattii* and *C. neoformans* exposures yielded the highest toxicity. Exposure of fly larvae to VOCs of *S. cerevisiae* had metamorphic patterns like the control (Figure 2. 8, 2.9, 2.10-A).

A-Significant level of larvae on day 4:
C. vs *S. cerevisiae*   NS
C. vs *C. albicans*   NS
C. vs *C. neoformans* *
C. vs *C. gattii*   *
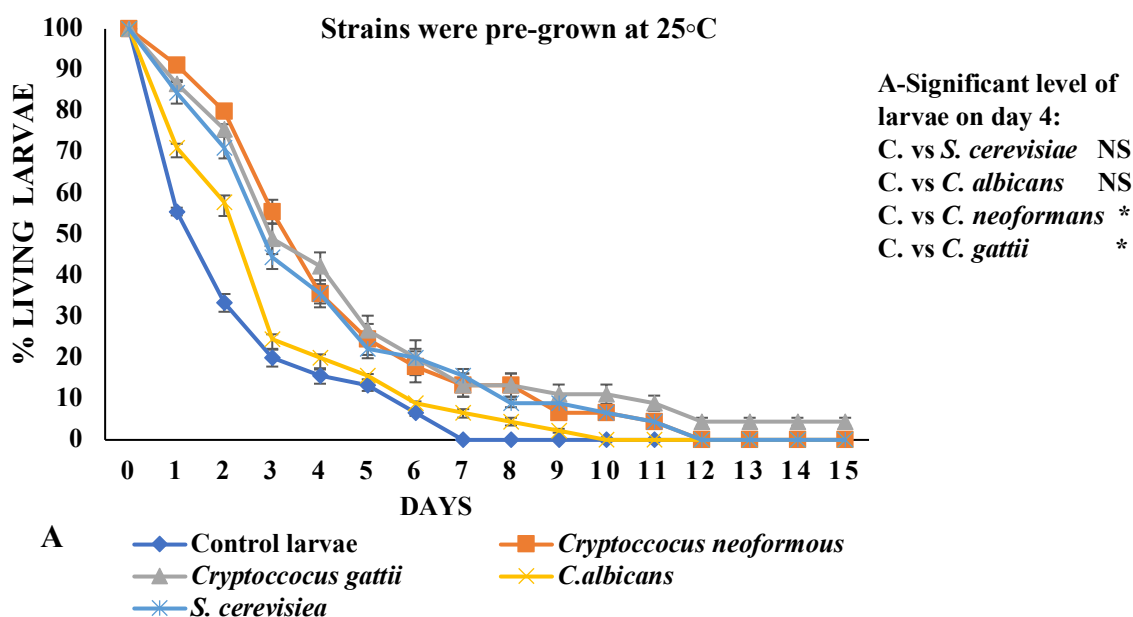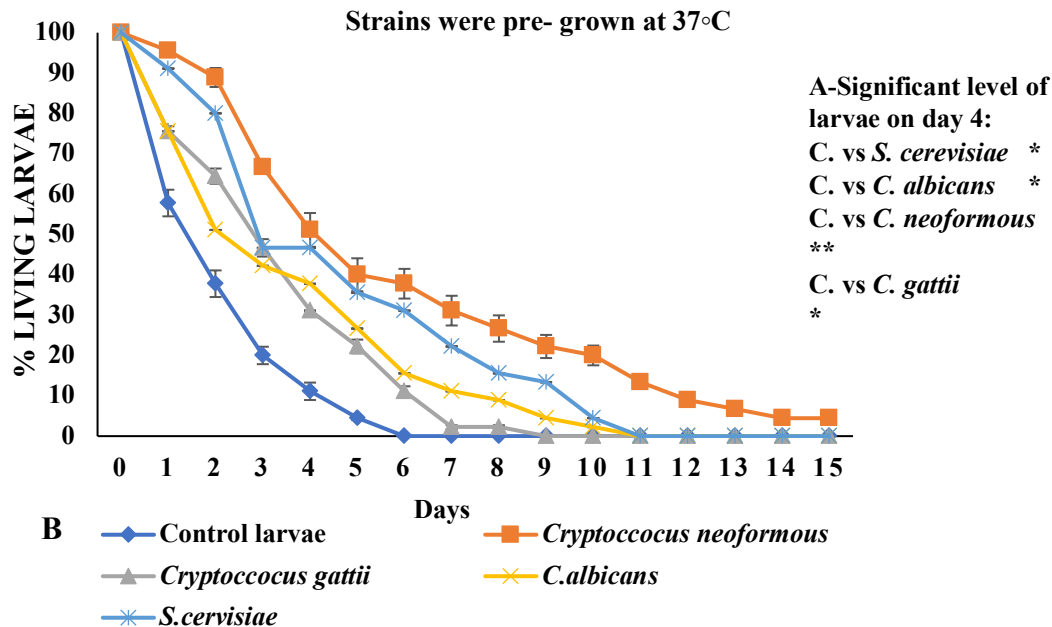
Figure 2.8. Exposure Drosophila larvae over 15 days of continuous exposure to VOCs of *Saccharomyces cerevisiae, Candida albicans, Cryptococcus neoformans, and Cryptococcus gattii*. **A**: the percent of alive larvae that were exposed to VOCs produced by strains were grown at 25◦C. **B**: : the percent of alive larvae that were exposed to VOCs produced by strains were grown at 25◦C. 15 instar larvae were exposed to three replicates of each strain and the experiment was repeated twice. (N=90). NS = Non-significant, * represents significant difference between control and fungus exposed larvae. Where *P<0.05 and **P<0.005.

There were more toxic effects on all stages of fruit fly's development when the fungi were pre-grown at 37 ◦C. *Cryptococcus neoformans* showed more toxic effects on the survival of larvae and adults while *C. gattii* exhibited more effects on the pupae compared to the controls. *S. cerevisiae* had the least toxic effects on the metamorphosis of fruit flies (Figure 2.8, 2.9, 2.10-B). *C. neoformans* had the highest mortality rate for larvae while *C. gattii* was the most toxic and had the deadliest effects on pupae and adults when grown at 37◦C. The percentage of adult flies after 7, 10 and 15 days is summarized in Table 2.2.
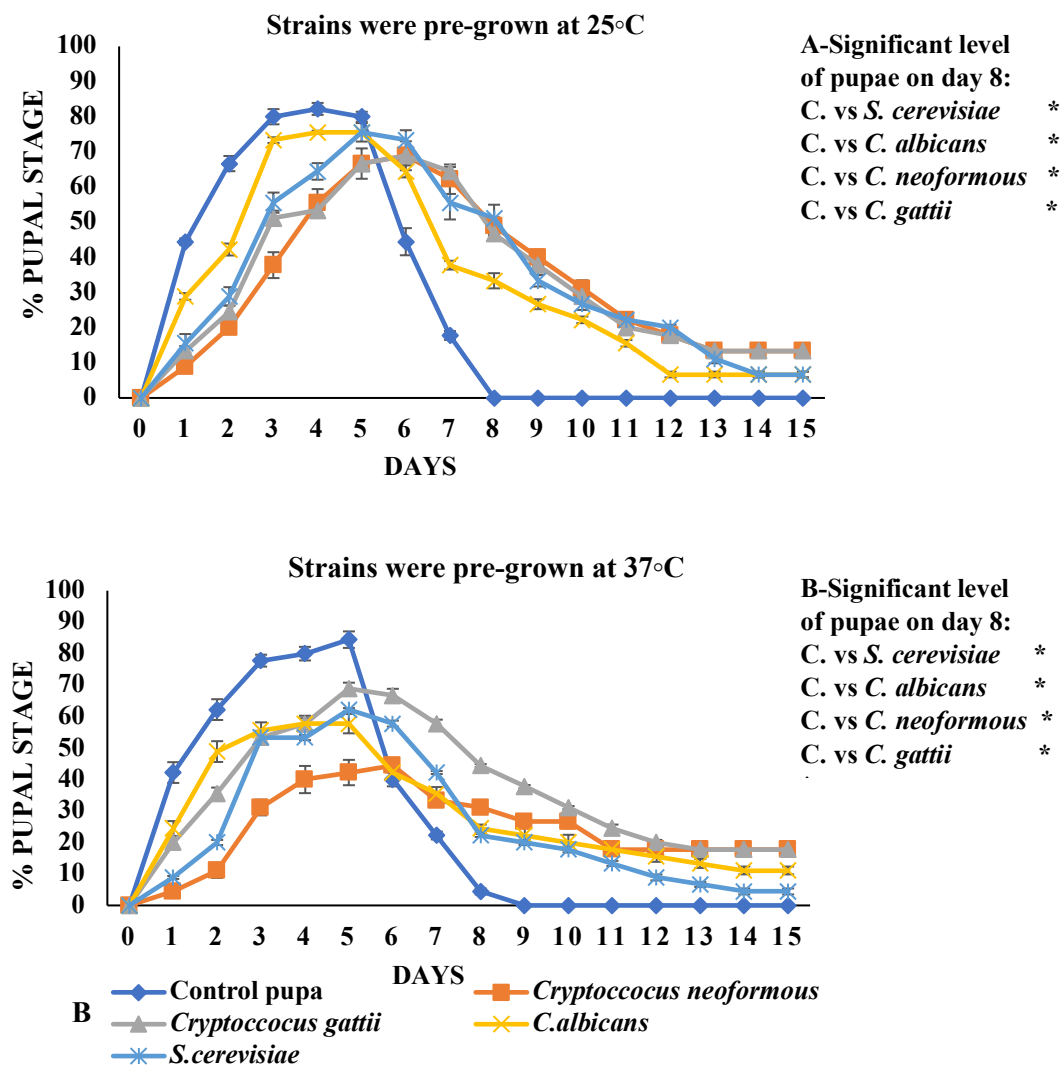
Figure 2.9 Exposure *Drosophila* larvae over 15 days of continuous exposure to VOCs of *Saccharomyces cerevisiae, Candida albicans, Cryptococcus neoformans,* and *Cryptococcus gattii*. **A:** the percent of alive pupae that were exposed to VOCs produced by strains were grown at 25∘C for five days. **B:** the percent of living pupa that were exposed to VOCs produced by strains were grown at 37∘C for three days. The number of larvae, pupae, and adults was monitored and counted daily for 15 days. 15 instar larvae were exposed to three replicates of each strain and the experiment was repeated twice. (N=90). NS = Nonsignificant, * represents significant difference between control and fungus exposed larvae. Where *P<0.05 and **P<0.005, ***p<0.0005.

Strains were pre-grown at 25∘C

A-Significant level of
adults on day 15:
C. vs *S. cerevisiae*   NS
C. vs *C. albicans*   *
C. vs *C. neoformous*   **
C. vs *C. gattii*   * *

Strains were pre-grown at 37∘C

A-Significant level of
pupae on day 15:
C. vs *S. cerevisiae*   *
C. vs *C. albicans*   *
*
C. vs *C. neoformous* **
*

Legend:
◆ control adult   ■ *Cryptoccocus neoformous*
▲ *Cryptoccocus gattii*   ✕ *C.albicans*
✳ *S. cerevisiae*
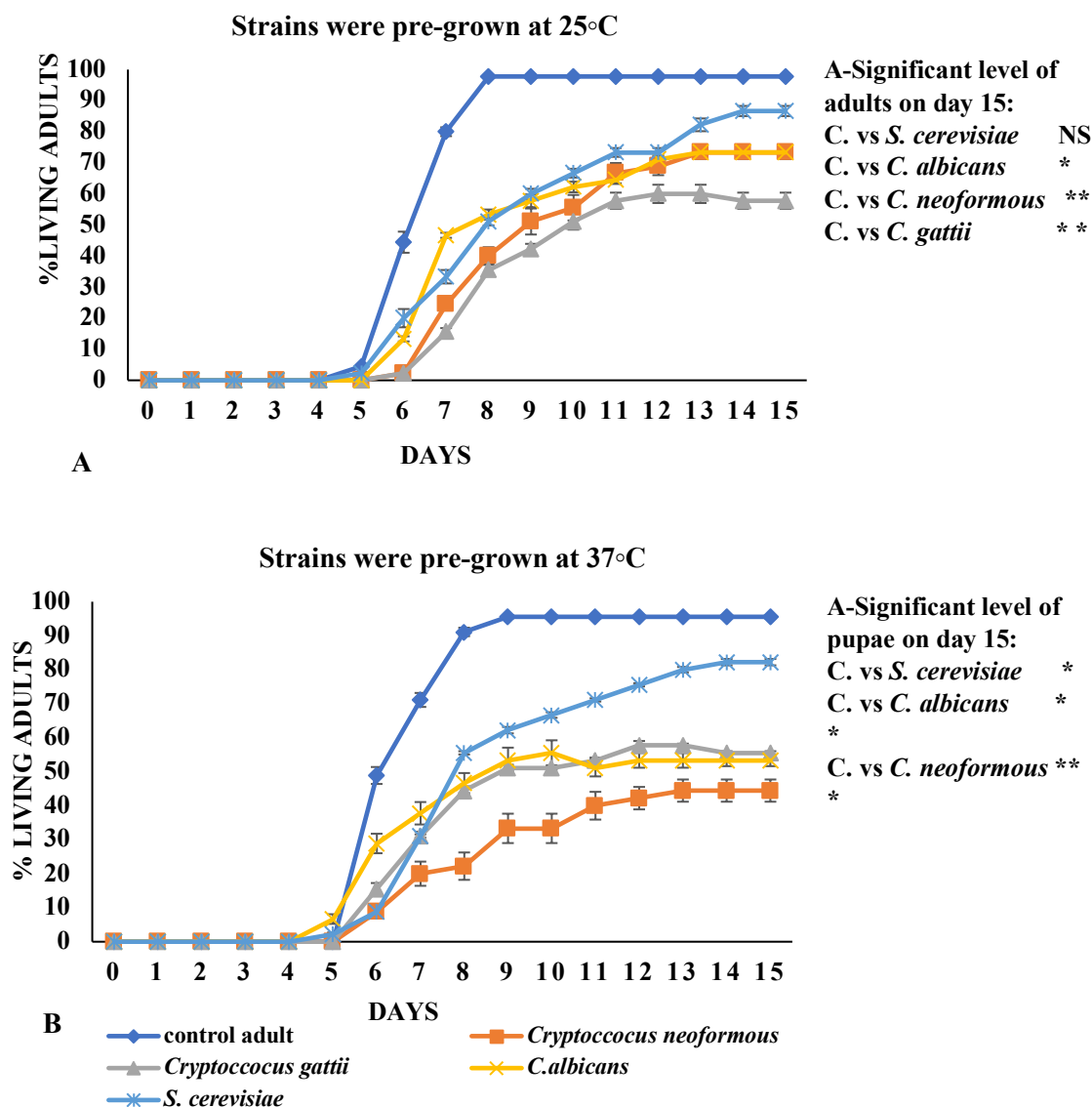
Figure 2.10 Exposure *Drosophila* larvae over 15 days of continuous exposure to VOCs of *Saccharomyces cerevisiae, Candida albicans, Cryptococcus neoformans,* and *Cryptococcus gattii*. **A:** the percent of living adults that were exposed to VOCs produced by strains were grown at 25∘C for five days. **B:** the percent of living adults that were exposed to VOCs produced by strains were grown at 37∘C for three days. The number of larvae, pupae, and adults was monitored and counted daily for 15 days. 15 instar larvae were exposed to three replicates of each strain and the experiment was repeated twice. (N=90). NS = Nonsignificant, * represents significant difference between control and fungus exposed larvae. Where *P<0.05 and **P<0.005, ***p<0.0005.

Table 2.1. Percent eclosed adult flies after 7, 10 and 15 days for controls (no fungi) and for flies reared in a common atmosphere with six different strains of *Aspergillus fumigatus* (N=90)

| Strain number | Fungi were pre-grown at different temperatures | | | | | |
| | Day 7 | | Day 10 | | Day 15 | |
	25∘C	37∘C	25∘C	37∘C	25∘C	37∘C
Control	62.2	55.5	84.4	80	95.5	95.5
SRRC 1607	37.7	22.2	57.7	44.4	75.5	57.7
SRRC 46	31.1	7	60	44.4	77.7	60
SRRC 51	42.2	51.1	57.7	75.5	80	86.6
SRRC 323	37.7	46.6	73.3	68.8	82.2	77.7
SRRC 2569	53.3	48.8	71.1	71.1	77.7	77.7
SRRC 1592	46.6	57.7	82.2	80	86.6	88.8

* 45 third instar larvae were exposed to the VOCs produced from different pathogenic fungi over 15 days. The number of viable adults was counted after 15 days of exposure to the VOCs produced by different *strains A. fumigatus that were pre-grown at different temperatures.

Exposure to volatile organic compounds produced from *A. fumigatus* strains and other pathogenic fungi (*C. albicans, Cryptococcus neoformans, and Cryptococcus gattii*) caused some morphological abnormalities in *Drosophila* larval, pupal, and adult stages as compared to controls. These abnormalities were more pronounced with exposure to VOCs from *A. fumigatus* than the other pathogenic fungi.   Dead larvae and pupae displayed dark

pigmentation (Figure 2.11 A-D). VOC-exposed   adults had wing and leg abnormalities (Figure 2.11 E-F).

A   Control larvae

Experimental larvae after 4 days of exposure to VOCs that are produce by *Candida albicans* and *Cryptococcous sp.*

B   Control larvae
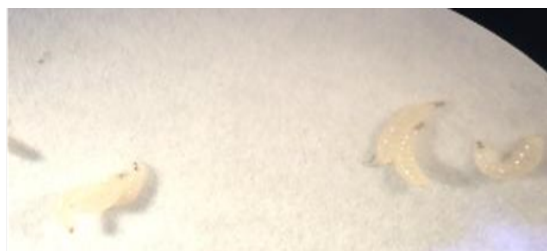
Experimental larvae after 10 days of exposure to VOCs that are produce by *Aspergillus fumigatus strains*

C   Control pupae

Experimental pupae 15 days of exposure to VOCs that are produced by *C. albicans* and *Cryptococcous sp.*

D   Control pupae

Experimental pupae 15 days of exposure to VOCs that are produced by *Aspergillus fumigatus strains*

E   **Control adults**   **F**   **Control adults**

Experimental adults 15 days of exposure to VOCs that are produced by *Candida albicans* and *Cryptococcous sp.*

Experimental adults 15 days of exposure to VOCs that are produced by *Aspergillus fumigatus strains*
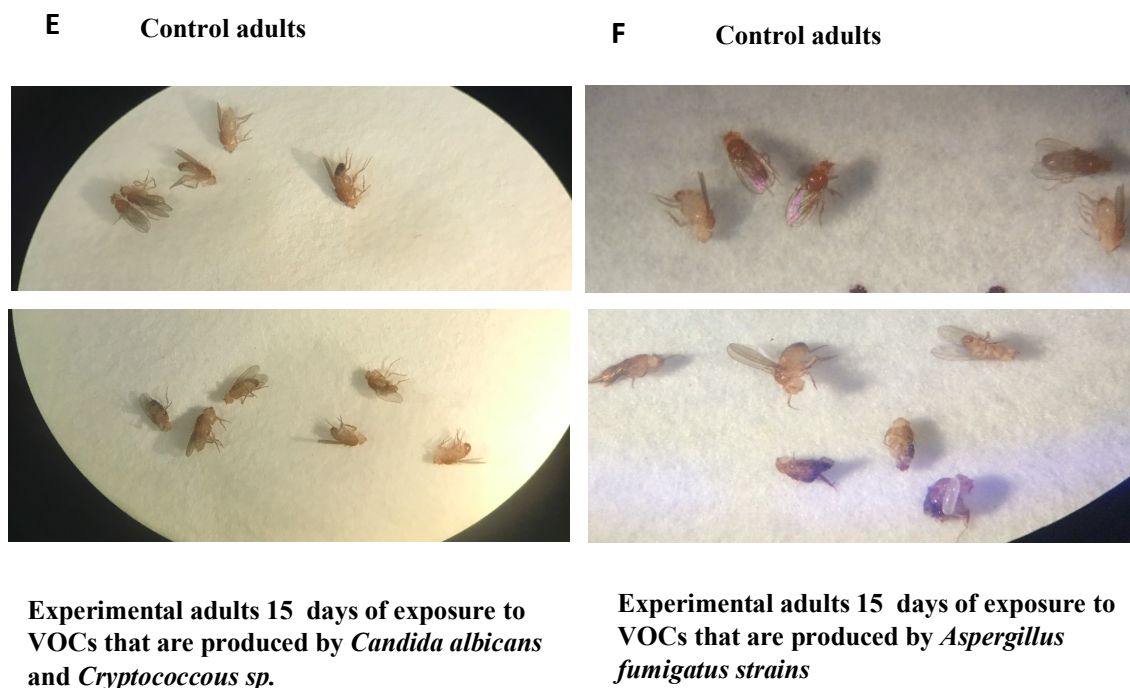
Figure 2.11 Morphological Effects of VOCs produced from *Aspergillus fumigatus* strains, *Candida albicans*, *Cryptococcous neoformans, and Cryptococcus gattii*. on the development stages of fruit flies.

A and B Control larvae and experimental larvae after seven days of exposure to VOCs.

C and D control pupae and experimental pupae after 15 days of exposure to VOCs.

E and F control adults and experimental adults after 15 days of exposure to VOCs.

Table .2.2. Percentage eclosed adults observed after seven, ten, and fifteen days from exposure to the VOCs produced from human pathogenic fungi that have grown at different temperatures (N=90).

Strains and Species	Pre-grown at different temperatures and days					
	Day 7		Day 10		Day 15	
	25◦C	37◦C	25◦C	37◦C	25◦C	37◦C
Control	80	71.1	97	95.5	97	95.5
Saccharomyces cerevisiae	33.3	31.1	66.6	66.6	86.6	82.2
Candida albicans	46.6	37.7	62.2	55.5	73.3	57.7
Cryptococcus neoformous	24.2	20	55.5	33.3	73.3	44.4
Cryptococcus gattii	15.5	31.1	51.1	51.1	57.7	55.5

* 45 third instar larvae were exposed to the VOCs produced from different pathogenic fungi over 15 days. The number of viable adults was counted after 15 days of exposure to the VOCs produced by different strains and species of fungi that were pre-grown at different temperatures.

2.3.4 Purge and Trap- Thermal Desorption-GC-MS

Two strains of *A. fumigatus* were selected for purge and trap- thermal desorption-GC-MS analysis. *A. fumigatus* 1607 strain had more toxic effects and delays on metamorphosis compared to *A. fumigatus* strain 1592, which was the least toxic strain tested. Purge and trap- thermal desorption GC-MS analysis was used to determine which VOCs were emitted by these two strains when pre-grown either at 25 ◦C for 5 days or 37 ◦C for 3 days on PDA These data are presented in Tables .2.3 and 2.4. Each strain produced its own profile of VOCs at different temperatures. 1-octen-3-ol, 2 butanone diacetyl, 1,3-octadeiene, 2-octen-1-ol, isopentyl alcohol, and isobutyl alcohol were the common VOCs produced by both

strains. *A. fumigatus* strain1607 released more different VOCs, at higher concentrations, when the strain was pre-grown at 37∘C than at 25∘C.   The most abundant VOC detected from both strains was 1-octen-3-ol. The eight-carbon volatile was made in greater concentrations by the toxigenic *A. fumigatus* strain 1607 than by the non-toxigenic strain 1592.

Table.2.3 VOCs produced by *Aspergillus fumigatus* strain 1607 which was pre-grown either for 5 or 3 days at 37∘C.

VOCs	Conc. ng/trap at 25∘C	Conc. ng/trap at 37∘C
1-octen-3-ol	521.4	1544.5
2-butanone+diacetyl	7.6	71.5
1-butanol	nd	15.7
acetic acid	67.7	nd
methyl, isobutyl ketone	6.3	6.53
2-octen-1-ol	nd	17.4
2,4-pentanedione	5.1	nd
1-octene	3	4.2
1,3-octadeiene	8.7	39.8
3-methylbutyric acid	3.8	nd
2-methylbutyric acid	3.8	nd
3-methyl-1,3-pentadiene	4.6	nd
hexanoic acid	8.9	nd
octanoic acid	1.5	nd
2-pentanone	nd	9.9
2-heptanone	nd	1.8
octanal	nd	5.7
cis-2-octenal	nd	8.5
Trans-2-octenal	nd	5.7
nonanoic acid	4.7	0.9
heptanal	nd	0.5
2-ethylfuran	1.5	4.3
acetoin	1.5	2.5
isopentyl alcohol	13.3	231.7
isobutyric acid	7.7	nd
styrene	10.8	8.8

2,4-dimethylfuran	nd	0.9
1-hepten-3-ol	nd	1.05
benzene-d6 (internal standard)	789.0	799.1
toluene-d8 (internal standard)	1000	1000
naphthalene-d8 (internal standard)	1015.9	1068.2

nd non detected

Table.2.4 VOCs produced by *Aspergillus fumigatus* strain 1592 which was pre-grown either for 5 days at 25∘C or 3 days at 37∘C

VOCs	Conc. ng/trap at 25∘C	Conc. ng/trap at 37 ∘C
1-octen-3-ol	164.5	192
Isopentyl alcohol	1.3	16.75
1,3-octadiene	2.9	3.4
Octanal	1.2	2.2
Cis-2-octenal	0.9	8
Trans-2-octenal	0.3	6.7
2-octen-1-ol	1.4	7.9
2-pentanone	nd	3.5
1-octene	nd	0.9
2-butanone	nd	9.9
hexanal	nd	10.2
trans-2-undecenal	nd	5.4
diacetyl	nd	6.1
nonanoic acid	nd	0.5
7-oxabicyclo.heptane, 3-oxiranly	nd	11.04
acetoin	nd	1.28
1-butanol	nd	13.2
Isobutyl alcohol	nd	0.8
methyl, isobutyl ketone	nd	4.2
Decanoic acid	nd	4.5
Lauric acid	nd	4.5
Myristic acid	nd	1.8
benzene-d6 (internal standard)	731.04	811.5
toluene-d8 (internal standard)	1000	1000
naphthalene-d8 (internal standard)	904.1	912.9

nd non detected

2.4 DISCUSSION

Filamentous fungi do not normally cause invasive infections in healthy people. However, when patients are immunocompromised, opportunistic species including *Aspergillus fumigatus*, *Cryptococcus sp.,* and *C. albicans* can cause morbidity and mortality. Fungal diseases, such as invasive aspergillosis, cryptococcosis, and invasive candidiasis, are becoming an ever more important cause of death because modern medical practice has increased the number of immunocompromised patients (Chamilos, et al. 2007). These pathogenic fungi emit VOCs as potent mixtures of hydrocarbons, acids, alcohols, aldehydes, aromatics, ketones, terpenes, thiols, and their derivatives (Korpi, et al. 2009a; Lambrecht and Hammad 2013). Many researchers have hypothesized that fungal VOCs have negative effects on human health with reference to processes like composting (Herr, et al. 2003) or with respect to their effects on people who live in damp houses with mold contamination (Heseltine and Rosen 2009).

The type and quantity of VOCs vary with the strain, substrate, age of the culture, temperature and other factors. Moreover, the VOCs detected are also dependent on the method used for separation and detection (Fischer, et al. 1999). The evidence for an association between VOCs and "sick building syndrome" has been reviewed (Mølhave 2009). The toxicity of specific compounds depends on the chemical nature of the VOC and the level and length of exposure (Bennett and Inamdar 2015; Morath, et al. 2012).

Drosophila melanogaster is a good model organism to use to study many human diseases, including those caused by medically important human fungal pathogens. In this work, the stages of larva, pupa, and adult fly were monitored over the 15 days to determine

how much the VOCs emitted by growing medically important fungi affected fly metamorphosis. Six strains of *A. fumigatus,* and one strain each of *C. neoformans, C. gattii, C. albicans,* and *S. cerevisiae* were tested. In general, exposure to VOCs from fungi delayed larvae from becoming pupae, and once at the pupal stage, more pupae were delayed in becoming adult flies. VOCs from fungi pre-grown at 37°C showed more effects on the development of the *Drosophila melanogaster* model than those fungi pre-grown at 25°C.

For the *Aspergillus* strains tested strain SRRC 1607 when pre-grown at 37°C was the most toxic to the model, since the larvae experienced the largest delay into pupae, and caused the largest mortality rate compared to the other strains. VOCs from *A. fumigatus* strain SRRC 1592 did not display any significant toxic effects on the metamorphosis. For the *C. neoformans, C. gattii,* and *C. albicans,* more toxic effects also were observed when fungi were pre-grown at 37°C than at 25°C. *S. cerevisiae,* which is a non-pathogenic, common yeast served as a biological control. VOCs from *C. neoformans* had more of a toxic effect on the larval and pupal stages while *C. gattii* had more of a toxic effect on the adult stage.

Two strains of *A. fumigatus* were chosen to analyze which VOCs produced by these strains at different temperatures by using GC-MS. Each strain produced a different VOCs signature. Among compounds analyzed,1-octen-3-ol, 2 butanone diacetyl, 1,3-octadeiene, 2-octen-1-ol, isopentyl alcohol, and isobutyl alcohol were produced in high concentrations by *A. fumigatus* SRRC 1607 when the strain was pre-grown at 37$\circ$C than at 25$\circ$C . The most abundant VOC detected was 1-octen-3-ol.

In earlier work, mixtures of VOCs emitted by growing cultures of *Aspergillus*, *Penicillium* and *Trichoderma* isolated from a flooded home had a detrimental effect on the

survival of exposed flies(Inamdar, et al. 2012). The fly toxicology assay also was used to test the VOCs produced by eleven 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 the toxic effects on flies ranged from 15 to 80%. The volatile metabolites that were released from *Aspergillus niger* were the most toxic, yielding 80% mortality for *Drosophila* after 12 days, while VOCs that were produced by *Trichoderma longibrachiatum*, *Mucor racemosus*, and *Metarhizium anisopliae* were relatively non-toxic. Using solid-phase micro extraction-gas chromatography-mass spectrometry (SPME) 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 3-methyl-1-butanol and 2-methyl-1-propanol were produced by the less toxic fungi (Zhao, et al. 2017).

In summary, this current work with fungal pathogens supported earlier work in our laboratory that showed that eight carbon compounds had more toxic effects than the non-C8 compounds on the larvae and adult stages of fruit flies (Inamdar, et al. 2012). The strains that were pre-grown at 37∘C, the temperature at which human infections occur, had more toxic effects on the fly model than strains that were pre-grown at 25∘. The data reported here suggest that VOCs from human pathogenic fungi may be a previously unknown virulence factor, contributing to the ability of these fungi to cause human disease. In future research, it will be important to test VOCs in mammalian models and to assess which individual compounds in the VOC mixture emitted by growing fungi are relevant to the pathogenicity. This research provides a reductionist approach towards studying the

possible contribution of fungal VOCs to the differential virulence of medically important fungi.

CHAPTER 3. EFFECT OF VOLATILE ORGANIC COMPOUNDS FROM WILD TYPE, OVER EXPRESSION AND OXYLIPIN DEFECTIVE MUTANTS OF *ASPERGILLUS FUMIGATUS* ON THE METAMORPHOSIS OF WILD TYPE AND IMMUNE DEICIENT *DROSOPHILA MELANOGASTER*

Abstract

Aspergillus is a large, economically important genus of filamentous fungi. Several species cause human disease, of which *Aspergillus fumigatus* is the most common filamentous pathogen. *A. fumigatus* is responsible for life -threatening infections in people with compromised immune systems including those affected by acute leukemia, hematopoietic cell transplant recipients, and solid- organ transplant recipients. We have previously reported that volatile organic compounds (VOCs) produced by *A. fumigatus*, specifically the VOC 1-octen-3-ol, cause delays in metamorphosis and time to eclosion in the *Drosophila melanogaster* model, as well as morphological abnormalities and death. Because 1-octen-3-ol is an oxylipin derived from *Aspergillus* oxygenases, here we examined the effect of *A. fumigatus* oxygenase mutants on *Drosophila* flies varying in immune genotypes. We compared *Drosophila* development when challenged with either two *A. fumigatus* strains with intact oxylipin pathways (*A. fumigatus* strains Af293 and CEA17Δ*ku80*) to a Af293 strain that overexpresses a lipoxygenase (*A. fumigatus OE::LoxB*) and its respective oxylipins, and to two strains deleted for all three COX-like oxygenases in both control backgrounds (Af293Δ*ppoABC* and CEA17Δ*ku80*Δ*ppoABC*) which should yield strains unable to synthesize 1-octen-3-ol. We also have grown *A. fumigatus* on medium containing arachidonic acid and tested the effects of the VOCs emitted by these cultures in the *Drosophila* model.

In the bioassay, third instar larvae of *Drosophila melanogaster* with and without mutations in their immune system were incubated in a shared atmosphere with the different wild type and mutant *A. fumigatus* strains for 15 days, and daily counts of larvae, pupae,

and adults of *D. melanogaster* were made. A comparison of the three Af293 strains (WT, *OE::LoxB* and Δ*ppoABC*) showed that *OE::LoxB* led to increased toxicity and metamorphosis delay compared to WT and, in contrast, the Δ*ppoABC* strain led to increased survival and less delay of metamorphosis. The CEA17Δku80 strain and its respective Δ*ppoABC* mutant showed less toxicity compared to the Af293 strains. In general, effects were more pronounced when fungi with intact oxylipin pathways were pre-grown at 37°C than when they were pre-grown at 25°C. When third instar larvae were exposed to VOCs from *Aspergillus* strains Af293 and *OE:loxB* pre-grown at 37°C the longest delay into pupal formation was observed, as well as the greatest mortality rate. Exposure to VOCs from Af293 wild type and *OE:loxB* also caused more accumulation of dark pigment on larvae bodies than did exposure to VOCs from CEA17Δku80 and the *Aspergillus* Δ*ppoABC* mutant strains.

Unexpectedly, flies carrying mutations in their innate immune system were more resistant to the toxic effects of volatiles than wild type flies, and flies carrying mutant immune genes displayed few differences in viability when exposed to VOCs from wild type or oxylipin mutants of *A. fumigatus*. No significant differences were detected on the metamorphosis of Drosophila when the fungi were grown in the presence of arachidonic acid compared with growth on media lacking arachidonic acid. GC-MS analysis showed the wild type strain *A. fumigatus* Af293 produced more abundant VOCs at higher concentrations then the oxylipin deficient mutant Af293Δ*ppoABC*. Major VOCs detected from wild type *A. fumigatus* Af293 included 1-octen-3-ol, 1-butanal, 1-octen, decanoic acid, lauric acid, myristic acid, and palmitic acid; these VOCs were not detected by the triple mutant strain. I hypothesize that VOCs from *A. fumigatus* enhance the toxigenic

effects of this medically important species and that higher concentrations of 1-octen-3-ol are correlated with increased toxicity. The increased resistance of flies carrying mutations in their innate immune response genes suggests that the toxigenic effects of fungal volatiles are related to the way in which the *Drosophila* innate immune system reacts to VOCs. Specifically, the toxigenic effects of the *Aspergillus* VOCs were not observed in mutant flies with blocks in the Toll pathway.

3.1 Introduction

The genus *Aspergillus* is a cosmopolitan group of filamentous fungi found all over the world in soils, composts and built environments (Bennett 2009; Brodhun and Feussner 2011). The various human diseases caused by *Aspergillus* species are called, collectively, "aspergillosis" and they range from localized and minor infections, to severe allergic bronchopulmonary aspergillosis (ABPA), and finally, to systemic and life-threatening invasive disease. Of species in the genus, *A. fumigatus* is the most single most common cause of aspergillosis, including ABPA and invasive infections. *A. fumigatus* is a common saprophyte that produces vast numbers of spores found in high concentrations in the atmosphere. *A. fumigatus* has the ability to grow at 37°C, a characteristic that contributes to its potential for becoming a human pathogen (Tekaia and Latge 2005) While not contagious, human aspergillosis is of growing importance in modern medical care (Latgé and Steinbach 2009; Latgé 1999).

The likelihood of *Aspergillus* infection leading to systemic aspergillosis is based on three factors: the degree of exposure; fungal virulence; and the immune status of patient (Dagenais and Keller 2009; Latgé 1999). Immunocompromised individuals such as acute

leukemia patients, hematopoietic cell transplant recipients, and solid- organ transplant recipients, exposed to high concentrations of airborne *A. fumigatus* spores are particularly vulnerable to systemic aspergillosis (Gregg and Kauffman 2015; Latgé 1999).

The virulence of *A. fumigatus* is associated with many traits that include production of melanin pigmentation in spores, as well as certain proteins and toxins that induce hyphal growth in mammalian tissues (Jahn, et al. 2000; Latgé 1999; Raffa and Keller 2019). Other fungal traits involved in fungal virulence include ability to grow in hypoxic tissues, siderophore biosynthesis, gliotoxin production, para-amino-benzoic acid metabolism and the starvation stress response (AL-Maliki, et al. 2017; Kowalski, et al. 2019; Lionakis and Kontoyiannis 2012). Of particular interest to our work is the finding that fatty acid signaling molecules, broadly known as oxylipins, are involved in *A. fumigatus* development and virulence traits (Tsitsigiannis, et al. 2005b).

Our laboratory has developed a *Drosophila* model for studying fungal volatile organic compounds (VOCs) and used it successfully to document the toxicity of VOCs from fungi isolated from flooded homes in the aftermath of Hurricanes Katarina and Sandy ((Inamdar and Bennett 2014; Inamdar, et al. 2013; Zhao, et al. 2017). Several VOCs are oxylipin derivatives that have been associated with fungal/insect interactions (Ferrari, et al. 2018). Here, we have applied this *Drosophila* bioassay to investigate two of the factors associated with the ability of *A. fumigatus* to become a pathogen: the immune status of the host and the possible the role of volatile oxylipins from *A. fumigatus* as virulence factors. In a preliminary study, we have showed that *A. fumigatus* volatiles were toxigenic in the

Drosophila model and we hypothesized that they may be playing a role in the pathology of the mycoses caused by this common filamentous species (AL-Maliki, et al. 2017).

Flies are well suited for experimental studies because they are inexpensive, have a short generation time, a long history of use in genetics, commercial sources of mutant stocks and a fully sequenced and well annotated genome (Lionakis and Kontoyiannis 2012). The typical sequence of developmental stages during the time course of metamorphosis from egg, larval instars, pupal case formation and eclosion into adults are an asset for assessing the toxicological impact of outside agents. The fly immune system is highly conserved, well-characterized, and shares traits with mammalian system. *Drosophila* has been used successfully in numerous studies on the underlying mechanisms of human diseases (Tolwinski 2017a).

The term "oxylipin" describes a large group of oxygenated fatty acids and their derivatives. Plants, animals, and fungi produce oxylipin compounds from polyunsaturated fatty acids (PUFAs). Oxylipins are synthesized by enzyme families such as cyclooxygenases (COX), lipoxygenases (LOX) or p450 monooxygenases (Patkar, et al. 2015). In fungi, both LOX enzymes, and COX-like oxygenases called Ppo enzymes have been studied in detail in *Aspergillus* spp. Oxylipins have been shown to play an important role in cross-kingdom interactions, especially with respect to plant and human pathogenesis (Dagenais, et al. 2008; Fischer, et al. 2017; Pohl and Kock 2014; Tsitsigiannis, et al. 2005a; Tsitsigiannis and Keller 2007). In fungi, oxylipins often function in development and reproduction (Pohl and Kock 2014).

Volatiles oxylipins are generated from oxylipin degradation products (Kowalski, et al. 2019) as well as PUFA (linoleic acid and arachidonic acid) breakdown (Assaf, et al.

1997; Husson, et al. 2001). While there has been a great deal of research on non-volatile phase fungal oxylipins as described above, in most of these studies the volatile phase molecules have been ignored. Many of these compounds, especially 8-carbon volatiles such as 1-octen-3-ol, are responsible for the characteristic moldy odors associated with fungal growth and have been hypothesized as contributing to a poorly defined health issue usually called "sick building syndrome" (Bennett and Inamdar 2015; Douwes 2009; Mølhave, et al. 1993; Wålinder, et al. 2008). More recently, some of these fungal VOCs have been shown to repel nematodes (Ferrari et al., 2018). Different mold species growing on different substrates produce unique combinations of VOCs, but 1-octen-3-ol is almost always present (Bennett 2009; Hung, et al. 2015; Korpi, et al. 2009a; Pennerman, et al. 2016).

In this chapter, we have tested the effect of VOCs produced by different strains of wild type, over expression, and triple oxylipin deletion mutant strains of *A. fumigatus* (strain A293 and two Af293 mutants: Δ*ppoA*Δ*ppoB*Δ*ppoC*, *OE:loxB* and strain CEA17Δku80 and one CEA17Δ*ku80* mutant Δ*ppoA*Δ*ppoB*Δ*ppoC*) on third instar larvae of *Drosophila*. We postulated that VOCs from triple mutant strains with three deleted oxylipin biosynthesis genes would cause fewer negative effects on metamorphosis compared to the wild type of *Aspergillus fumigatus*. Further, we tested the effects of VOCs produced by an over expression strain (Af293 OE:loxB) and postulated that it would be have a greater impact on fly metamorphosis than the wild type strain. We tested the different *Aspergillus* strains against *Drosophila* larvae with wild type immune systems and with those carrying mutant blocks in three different aspects of the fly innate (humoral) immune system: nitric oxide synthase, Toll and Imd. Finally, GC/MS analysis (using the

purge and trap method) was used to determine the volatile profile of wild type (Af293) and Af293 triple mutant strains of *A fumigatus.*

The long-term goal of this project is to establish the possible role of fungal VOCs, particularly those derived from the oxylipin pathway, on the potential virulence of *A. fumigatus.* The project had four specific aims:

1) To study the effects of volatiles emitted by wild type and oxylipin mutant strains of *A. fumigatus* in a *Drosophila* model using flies with intact immune systems,

2) To study the effects of volatiles emitted by wild type and oxylipin mutant strains of *A. fumigatus* in a *Drosophila* model using flies with defective innate immune systems,

3) To study the effect of volatiles emitted by *A. fumigatus* grown on medium with and without arachidonic acid in a *Drosophil*a model using flies with an intact immune system, and

4) To use GC-MS to determine the composition of the suite of volatiles produced by wild type *A. fumigatus* and strains carrying triple mutations in the oxylipin pathway genes in order to determine the specific compounds correlated with toxigenic effects .

3.2 Methods and Materials

3.2.1 Fungal Strains and Culture Conditions

All strains of *Aspergillus fumigatus* were obtained from Dr. Nancy Keller, Department of Medical Microbiology and Immunology, Department of Bacteriology, University of Wisconsin – Madison USA. The strain numbers, genotypes and literature citations for these strains are listed in Table 3.1. Stock cultures were maintained on potato dextrose agar (PDA) (Difco) or on *Aspergillus* glucose minimal medium (GMM). PDA medium was

prepared following manufacturer instructions by adding 39 g of the commercial mixture to 1 L of distilled water. It was mixed and dissolved by heating and then autoclaved.

GMM contained: 6.0 g $NaNO_3$, 0.52 g KCl, 0.52 g $MgSO4 \cdot 7H_2O$, 1.52 g KH_2PO_4, 1 ml trace elements [2.2 g $ZnSO_4 \cdot 7H_2O$, 1.1 g H_3BO_3, 0.5 g $MnCl_2 \cdot 4H_2O$, 0.5 g $FeSO_4 \cdot 7H_2O$, 0.16 g $CoCl_2 \cdot 5H_2O$, 0.16 g $CuSO_4 \cdot 5H_2O$, 0.11 g $(NH_4)6Mo_7O24 \cdot 4H_2O$, 5.0 g Na4 EDTA in100 ml distilled H_2O], then 10 g glucose, 15.0 g agar, pH 6.5, in 1 liter distilled H_2O. All were dissolved and autoclaved separately by using 250 ml flask. After being autoclaved, all ingredients were mixed and separated in two 500 ml flasks. Arachidonic acid was purchased from Sigma-Aldrich(>95%). AA(Arachidonic acid) 5 mM quantity was dissolved into 2 ml of ethanol (95%) and added to GMM; the control contained was added no

arachidonic acid. Then 25 ml of the mixtures were poured into in 6oz *Drosophila* stock bottles (Genesee Scientific, CA). All strains of *A. fumigatus* were inoculated and incubated either at 25 °C for 5 days or 37 °C for 3 days before using them for exposure studies with fly larvae.

Table 3.1. *Aspergillus fumigatus* strains were used in the experiments

Strains	Strain name	Genotype	Reference
A) A .fumigatus **1- AF293**	AF293	Wild type	(Rosowski, et al. 2018)
2- Triple mutant strain **Af293 ΔppoA; ppoB; ppoC**	TMN31.10	*ΔppoC::A. nidulans argB; argB1pyrG1; ΔppoA(Af4g10770)::A. p. pyrG; ΔppoB (Afu4g00180)::six*	Unpublished papers
3- Overexpressed strain **Af293 OE::lox B**	TGJF1.5	*pyrG1; gpdA(p):loxB A. parasiticus pyrG*	(Fischer, et al. 2017)
A) A.fumigatus CEA10 **1-CEA17Δku80**	As a control strain	pyrG1, *Δku80*::*A.fumi*.pyrG,	(Ferrari, et al. 2018)
2- Triple mutant strain **CEA17Δku80 ΔppoA; ppoB; ppoC**	TMN32.1	*pyrG1, ΔakuB::pyrG, pyrG1,ΔargB (AFUB_064280)::A.p.pyrG; ΔA.p.pyrG; ΔppoA(AFUB_067850)::A.p.pyrG; ΔppoC(AFUB_037060)::A.fumi. argB; ΔppoB(AFUB_100690)::six*	Unpublished papers.

3.2.2 *Drosophila* strains culture media and maintenance

Wild type and mutant *Drosophila* flies were obtained from the Bloomington *Drosophila*

Stock Center (BDSC) at Indiana University. Strain number, phenotypes, genotypes and

literature citations for these strains are listed in Table 3.2 The same methods outlined in Chapter 2 were used for preparing media, for fly maintenance, and exposure studies.

Table 3.2 Strain number, phenotype, genotype, and literature citations for *Drosophila* strains used in VOC exposure studies.

ID number at BDSC	Type of mutation	Genotype	Reference
4783	Wild type, white-eyed strain	y1, w1118, a yellow body and white-eyed strain	(Inamdar, et al. 2013)
24283	NOS (nitric oxide synthase), white-eyed mutant strain	w [1118]; Mi(Gabbs, et al. 2015)Nos[MB04018]	(Inamdar, et al. 2013)
9457	White-eyed mutant strain Relish [E20] gene (lack this gene) to induce Imd pathway	w[1118]; Rel[E20] e[s]	(Kambris, et al. 2006)
5	Red-eyed, wild type	Oregon-R-C	(De Gregorio, et al. 2002)
10719	Red-eyed mutant strain Spz[6] gene(lack this gene) to induce Toll pathway	w[1118]; PBac{w[+mc]=PB}fc`z6[c01763]	(Kambris, et al. 2006)
55718	Relish[E20] &spz[6] (double , red-eyed mutant strain) in the both pathways	Rel[E20] spz[4]/TM6C, Sb[1] Tb[1]	(De Gregorio, et al. 2002)

3.2.3 Arachidonic acid supplementation of fungal medium

Third instar larvae of white eyed *Drosophila* with no mutations in their immune pathway

genes were exposed to VOCs produced by two strains of *A. fumigatus* (Af293 and CEA17)

lacking mutations in their oxylipin pathway. The *A. fumigatus* strains were cultured on

Aspergillus glucose minimal medium (GMM) with and without arachidonic acid wherein

a5 mM quantity was dissolved into 2 ml of ethanol (95%) and added to GMM.

3.2.4 Gas chromatography-mass spectrometry (GC-MS) analysis

For headspace volatile analysis, wild type *Aspergillus fumigatus* strain AF293 and the

triple lipoxygenase mutant Af293ΔppoA; ΔppoB; ΔppoC triple mutant (Strain TMN31.10)

were grown in 250 ml flasks on PDA at either at 25 °C for 5 days or 37 °C for 3 days on

PDA. The flasks were closed with plastic stoppers and sealed with parafilm. Headspace

samples taken from sterile PDA media was used as one control; another control was a blank

consisting of air only. VOC capture and analysis were conducted as described previously

using Purge and Trap-Thermal Desorption-GC-MS (Lee, et al. 2015).

The samples were purged with air at 100 mL/min at room temperature for 1 hour. Volatile

and   semi-volatile   outgas   products   were   trapped   onto   Tenax   (modified

poly[phenylene]oxide) traps. The Tenax traps were spiked with 1.0 ug of benzene-d6,

toluene-d8, and naphthalene-d8 internal standards and purged with nitrogen for 90 minutes

at 50 mL/min to remove water from the traps. The internal standards were used to

normalize the peak areas. Tenax traps were connected to a Short Path Thermal Desorption

System (Scientific Instrument Services) and desorbed directly into the GC injector at 25^0C

for 5 minutes with carrier gas flow. GC temperature program was -20C to 260C @ 10C/min, injector temperature was 250C, and heated transfer line temperature was 280C. The split valve on the GC was set at a 10:1 ratio. The mass spectrometer was operated in positive ion electron ionization (EI) mode (70 eV) scanning m/z 35-350 once per second. The linear regression coefficient was used to calculate the concentrations in the samples from peak areas obtained in the chromatographs. Compounds were identified by comparison of spectra obtained from the *Aspergillus* samples with those from a reference library (NIST 08 Mass Spectra Library, National Institute of Standards and Technology). GC-MS analysis was conducted in duplicate for each strain.

Statistical Analysis

The Student t-test was used to determine significant differences in metamorphic stages between flies exposed to *A. fumigatus* VOCs from strains with wild type immune systems (Af293 and CEA17Δku80); and flies exposed to *A. fumigatus* strains with mutations in the oxylipin pathway: (overexpression loxB (E:loxB) and the triple mutant strains (Af293 ΔppoA; ΔppoB; ΔppoC, and CEA17Δku80 ΔppoA; ΔppoB; ΔppoC). Fifteen third instar larvae were exposed to three replicates of each strain and the experiment was repeated twice. (N=180). For larval counts, the t-test was conducted on day 4, for pupal counts on day 8; and for adults on day 15.

3. Results

Using the *Drosophila* bioassay*,* metamorphic stages were compared when fly larvae were grown in the presence of VOCs emitted by wild type *Aspergillus fumigatus* or strains with mutations in the oxylipin pathway. In each of the sections below, one of six different strains of wild type or mutant *Drosophila* was grown in a shared atmosphere with VOCs from five different *A. fumigatus* strains, namely: Control, Af293; Af293 OE:loxB; Af293 ΔppoABC;   CEA17Δku80; or CEA17Δku80ΔppoABC In these exposure studies, the effects of VOCs from the wild type strain (Af293) were compared with an overexpression strain (Af293 OE:loxB) and a triple blocked oxylipin mutant   (Af293 ΔppoABC). Similarly, an *A. fumigatus* control strain (CEA17Δku80) with wild type oxylipin genes was compared with a triple blocked oxylipin mutant (CEA17Δku80 ΔppoABC).   We hypothesized that VOCs from strains blocked in the oxylipin pathway would have fewer effects on *Drosophila* metamorphosis, and that the overexpression strain Af293 OE:loxB would be more toxigenic. For each set of experiments, *A. fumigatus* strains were pre-grown at either 25°C or 37°C prior to being placed in a shared atmosphere with *Drosophila* third instar larvae at 25°C.

3.3.1   Metamorphic stages of a white eyed *Drosophila* strain (W*1118*) with a wild type immune system when incubated in a common atmosphere with VOCs produced by wild type and mutant *A. fumigatus*

Third instar larvae of white eyed *Drosophila* with a wild type immune system (strain W[1118]) were exposed to VOCs from the five different *A. fumigatus* strains grown on PDA and pre-incubated either for five days at 25°C or three days at 37°C. The viability

data for fungi pre-incubated at 25°C are shown in Figure 3.1 (ABCDEF) and the data for those pre-incubated at 37°C are shown in Figure 3.2 (ABCDEF).

For those fungi originally grown at 25°C, all flies exposed to *A. fumigatus* VOCs showed significant delays in morphogenesis. The VOCs from the overexpression Af293 LoxB strain caused more delays than VOCs from the wild type (Figure. 3.1 E and F). However, larvae exposed to VOCs from the triple mutant ppoABC strain had eclosion rates like the controls. Similarly, for those fungi originally grown at 37°C, VOCs from the overexpression strain were more toxic than wild type Af293 after 15 days, while the triple mutant was less toxigenic (Figure 3.2, E and F). For the CEA17Δku80 strain pre-grown at both 25°C and 37°C, there were no significant differences in viability between flies exposed to the control and the triple mutant at either temperature (Figures 3.1 EF and 3.2 EF).

strains were pre-grown at 25◦C

% LIVING LARVAE

Significance level :
C. vs Af293 wt. **
C. vs Af 293ABC   *
C. vs Af 293 OE   ***
Af293 wt. vs Af293 ABC *
Af 293 OE vs Af293 ABC   *

A

strains were pre-grown at 25◦C

% LIVING LARVA

Significance level :
C. vs CEA17Δku80 **
C. vs CEA17Δ ku80ΔppoABC**
CEA17 vs CEA17Δppo ABC NS

B

% PUPAL CASE

Significance level :
C. vs Af293 wt. **
C. vs Af 293ABC   **
C. vs Af293 OE   **
Af293 wt. vs Af 293 ABC *
Af 293 OE vs Af293 ABC   NS

C

% PUPA L CASE

Significance level :
C. vs CEA17Δku80 **
C. vs CEA17Δ ku80ΔppoABC *
CEA17 vs CEA17Δppo ABC NS

D

% ADULT ECLOSION

Significance level :
C. vs Af293 **
C. vs Af293 ABC *
C. vs Af 293 OE ***
Af293 vs Af293 ABC *
Af 293OE vs Af293 ABC **

E

- Control adults
- Af293Δ ppo ABC
- AF293
- Af293 OE:loxB

DAYS

% ADULT ECLOSION

Significance level :
C. vs CEA17Δku80 **
C. vs CEA17Δ ku80ΔppoABC **
CEA17 vsvs CEA17Δppo ABC NS

F

- Control adult
- CEA17 Δku80
- CEA17Δku80 Δ ppo ABC

DAYS

Figure 3.1. Mature third instar larvae W[1118] strain (white-eyed with wild type immune genes) over 15 days of continuous exposure to VOCs produced by *A. fumigatus* pre-grown on PDA at 25◦C for 5 days. **A:** the percent of living larvae stage exposed to wild type, overexpressed and triple mutant strains. **B**: the percent of living larvae stage exposed to control and triple mutant strains. **C**: the percent of pupal case exposed to wild type, overexpressed and triple mutant strains. **D:** the percent of pupal case exposed to control and triple mutant strains. **E** the percent of adult eclosion exposed wild type, overexpressed and triple mutant strains. **F** the percent of adult eclosion exposed control and triple mutant strains.   (N=180). NS = nonsignificant, * represents significant difference between *A. fumigatus* wild type strains and *A. fumigatus* LoxB and triple mutant strains exposed larvae. Where *P<0.05, **P<0.005 and ***P<0.0005.

Figure 3.2. Mature third instar larvae W[1118] strain (white-eyed with wild type immune genes) over 15 days of continuous exposure to VOCs produced by *A. fumigatus* pre-grown on PDA at 37°C for 3 days. **A:** the percent of living larvae stage exposed to wild type, overexpressed and triple mutant strains. **B**: the percent of living larvae stage exposed to control and triple mutant strains. **C**: the percent of pupal case exposed to wild type, overexpressed and triple mutant strains. **D:** the percent of pupal case exposed to control and triple mutant strains. **E** the percent of living adult eclosion exposed wild type, overexpressed and triple mutant strains. **F** the percent of living adult eclosion exposed control and triple mutant strains. (N=180). NS = nonsignificant, * represents significant difference between *A. fumigatus* wild type strains and *A. fumigatus* LoxB and triple mutant strains exposed larvae. Where *P<0.05, **P<0.005 and ***P<0.0005.

3.3. 2 Metamorphic stages of a white eyed *Drosophila* strain with a nitric acid synthase (NOS) mutation (w^{1118} m^{24283}) when incubated in a common atmosphere with VOCs produced by wild type and mutant *A. fumigatus*

In this set of experiments, Drosophila larvae of strain $w^{1118}m^{24283}$ carrying a heterozygous mutation in the nitric acid synthase (NOS pathway) were used in the bioassay. Third instar larvae with the NOS mutation were exposed to the VOCs emitted by the same set of five *A. fumigatus* strains with and without mutations in the oxylipin pathway (Figures. 3.3 and 3. 4). The viability data for fungi pre-incubated at 25°C are shown in Figure 3.3 and the data for those pre-incubated at 37°C are shown in Figure 3.4. In general, the negative effects on the *Drosophila* larvae were greater when the flies were co-incubated in a common atmosphere with *A. fumigatus* strains that were pre-grown at 37°C than at 25°C.
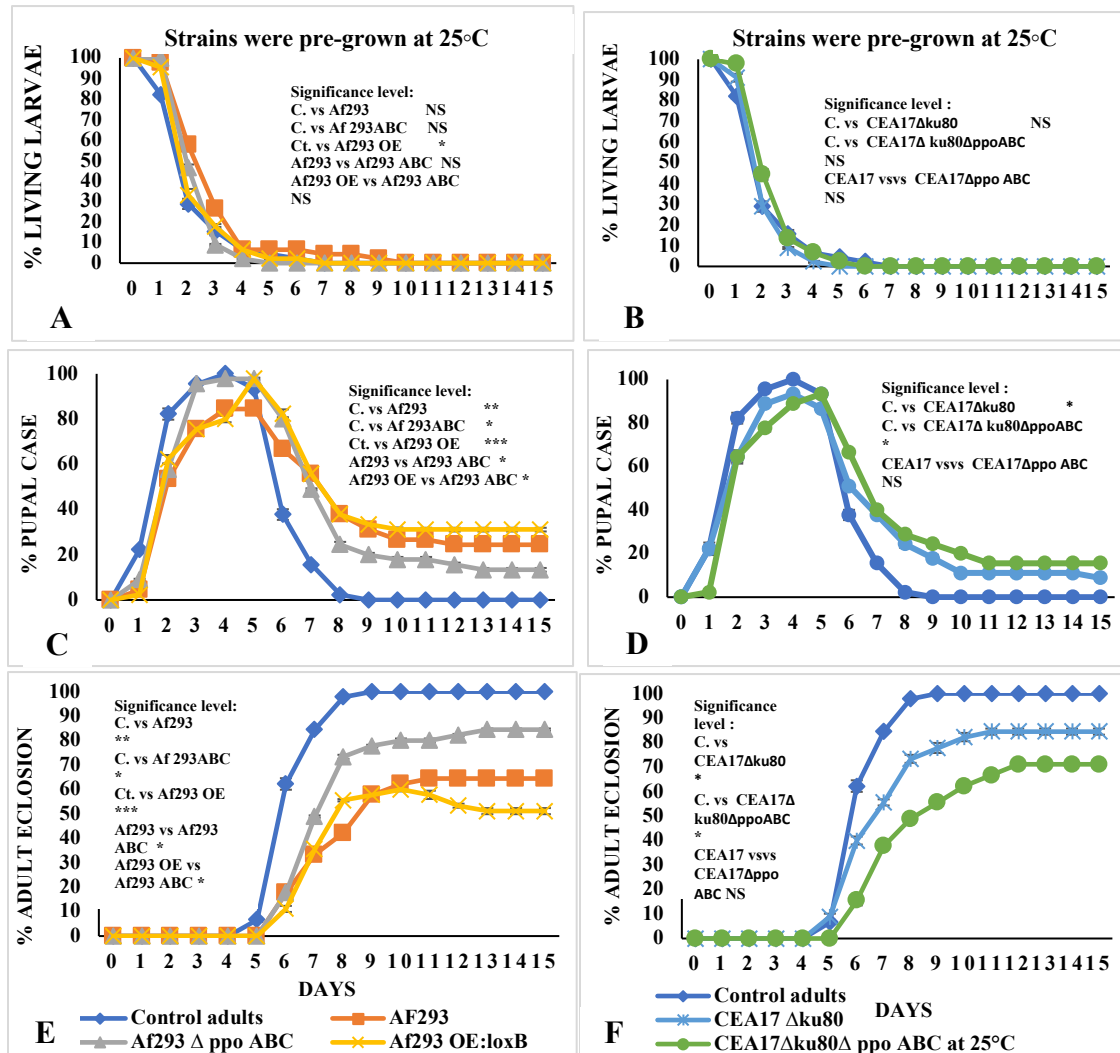
Figure 3.3. Mature third instar larvae of W^{1118}m^{24283} (white eyed with heterozygous mutation for NOS) over 15 days of continuous exposure to VOCs produced by *A. fumigatus strains* that were pre-grown on PDA at 25°C for 5 days. **A:** the percent of living larvae stage exposed to wild type, overexpressed and triple mutant strains. **B**: the percent of living larvae stage exposed to control and triple mutant strains. **C**: the percent of pupal case exposed to wild type, overexpressed and triple mutant strains. **D:** the percent of pupal case exposed to control and triple mutant strains. **E** the percent of adult eclosion exposed wild type, overexpressed and triple mutant strains. **F** the percent of adult eclosion exposed control and triple mutant strains. (N=180). NS = nonsignificant, * represents significant difference between *A. fumigatus* wild type strains and *A. fumigatus* LoxB and triple mutant strains exposed larvae. Where *P<0.05, **P<0.005 and ***P<0.0005.

Figure 3.4. Mature third instar larvae of W^{118}m^{24283} (white eyed with heterozygous mutation for NOS) over 15 days of continuous exposure to VOCs produced by *A. fumigatus strains* that were pre- grown on PDA at 37°C for 3 days. **A:** the percent of living larvae stage exposed to wild type, overexpressed and triple mutant strains. **B**: the percent of living larvae stage exposed to control and triple mutant strains. **C**: the percent of pupal case exposed to wild type, overexpressed and triple mutant strains. **D:** the percent of pupal case exposed to control and triple mutant strains. **E** the percent of living adult eclosion exposed wild type, overexpressed and triple mutant strains. **F** the percent of living adult eclosion exposed control and triple mutant strains. (N=180). NS = nonsignificant, * represents significant difference between *A. fumigatus* wild type strains and *A. fumigatus* LoxB and triple mutant strains exposed larvae. Where *P<0.05, **P<0.005 and ***P<0.0005.

When *Drosophila* larvae of strain $w^{1118}m^{2483}$ carrying a heterozygous mutation for NOS were grown in a shared atmosphere with VOCs emitted by wild type and mutants of *A. fumigatus*, there were fewer negative effects on metamorphosis than were observed for white eyed flies with intact immune systems. The timing of the transition from larvae to pupae was almost the same for VOC-exposed and non-exposed control flies (Figure 3.3A, Figure 3.4A). However, the time to eclosion was delayed in flies exposed to fungal VOCs. It is noteworthy that the VOC effects were not as negative as those observed for *Drosophila* strain W[1118] which had a wild type immune system. For example, after 15 days of exposure to VOCs from *A fumigatus* strain Af293 that had been pre-grown at 37°C., only 44% of W[118] flies had eclosed into adults; when Drosophila strain $w^{1118}m^{2483}$ was exposed to VOCs from the same fungal strain, eclosion after 15 days was 53% . Similar greater eclosion rates for the NOS mutant flies than for the wild type W[1118] flies also were observed with exposure to VOC from the other *A. fumigatus* strains tested (Table 3.3).

3.3.3 Metamorphic stages of a white eyed *Drosophila* strain with a blocked mutation in the Imd pathway (w[1118]Relish[E20]) when incubated in a common atmosphere with VOCs produced by wild type and mutant *A. fumigatus* strains

White eyed *Drosophila* strain 9457 carrying a blocked mutation in the Imd pathway (w[1118]Relish[E20]) was more resistant to the toxic effects of fungal VOCs that was the white eyed strain with an intact immune system. This differential effect on viability was particularly striking for flies exposed to VOCs from the *A. fumigatus* overexpression strain Af293 OE:loxB pre-grown at 37°C. After 15 days of continuous exposure to VOCs from

the *A. fumigatus* overexpression strain showed only 31% eclosion into adults ; *Drosophila* strain w[1118] with an intact immune system showed 44%, while flies carrying the mutation in the Imd pathway showed 60% and 58% respectively (Figure 3.5 . Table 3.3).   In summary, fly strains carrying a mutation in the Imd pathway were less susceptible to the toxic effects of VOCs produced by the five *A. fumigatus* strains than was the wild type or the strain carrying a heterozygous mutation in the NOS gene.

84
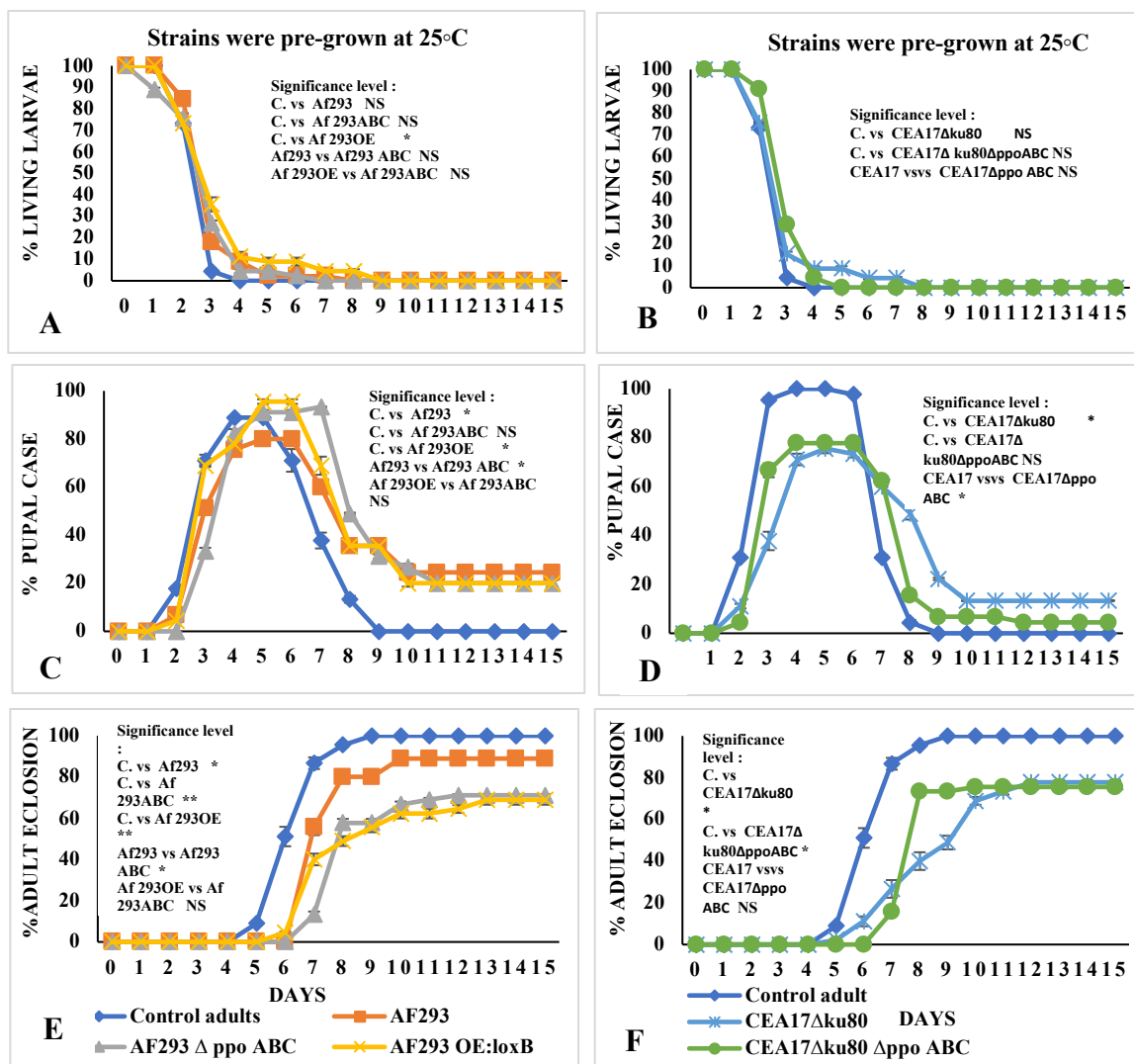
Figure 3.5. Mature third instar larvae of *Drosophila* Relish[E20] mutant (strain 9457, white-eyed Imd pathway) over 15 days of continuous exposure to VOCs of continuous exposure to VOCs produced by *A. fumigatus* strains that were pre- grown on PDA at 25°C for 5 days. **A:** the percent of living larvae stage exposed to wild type, overexpressed and triple mutant strains. **B**: the percent of living larvae stage exposed to control and triple mutant strains. **C**: the percent of pupal case exposed to wild type, overexpressed and triple mutant strains. **D:** the percent of pupal case exposed to control and triple mutant strains. **E** the percent of adult eclosion exposed wild type, overexpressed and triple mutant strains. **F** the percent of adult eclosion exposed control and triple mutant strains. (N=180). NS = nonsignificant, * represents significant difference between *A. fumigatus* wild type strains and *A. fumigatus* LoxB and triple mutant strains exposed larvae. Where *P<0.05, **P<0.005.
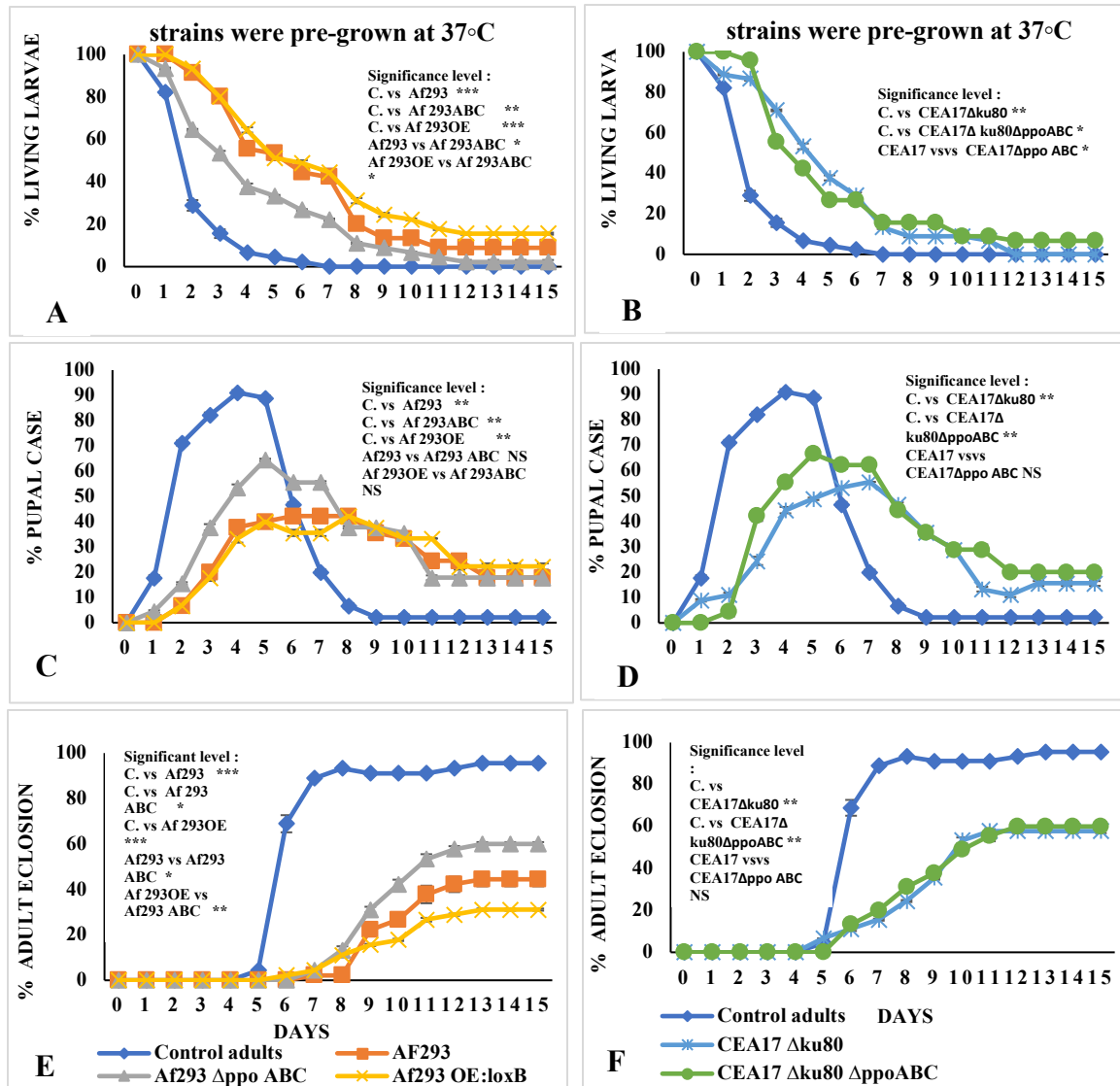
Figure 3.6. Mature third instar larvae of *Drosophila* Relish[E20] mutant (strain 9457, white-eyed Imd pathway) over 15 days of continuous exposure to VOCs produced by *A. fumigatus* strains that were pre- grown on PDA at 37°C for 3 days. **A:** the percent of living larvae stage exposed to wild type, overexpressed and triple mutant strains. **B**: the percent of living larvae stage exposed to control and triple mutant strains. **C**: the percent of pupal case exposed to wild type, overexpressed and triple mutant strains. **D:** the percent of pupal case exposed to control and triple mutant strains. **E** the percent of adult eclosion exposed wild type, overexpressed and triple mutant strains. **F** the percent of adult eclosion exposed control and triple mutant strains. (N=180). NS = nonsignificant, * represents significant difference between *A. fumigatus* wild type strains and *A. fumigatus* LoxB and triple mutant strains exposed larvae. Where *P<0.05, **P<0.005 .

3.3.4 Metamorphic stages of a red eyed wild *Drosophila* strain (Oregon[R]) when incubated in a common atmosphere with VOCs produced by wild type and mutant *A. fumigatus* strains
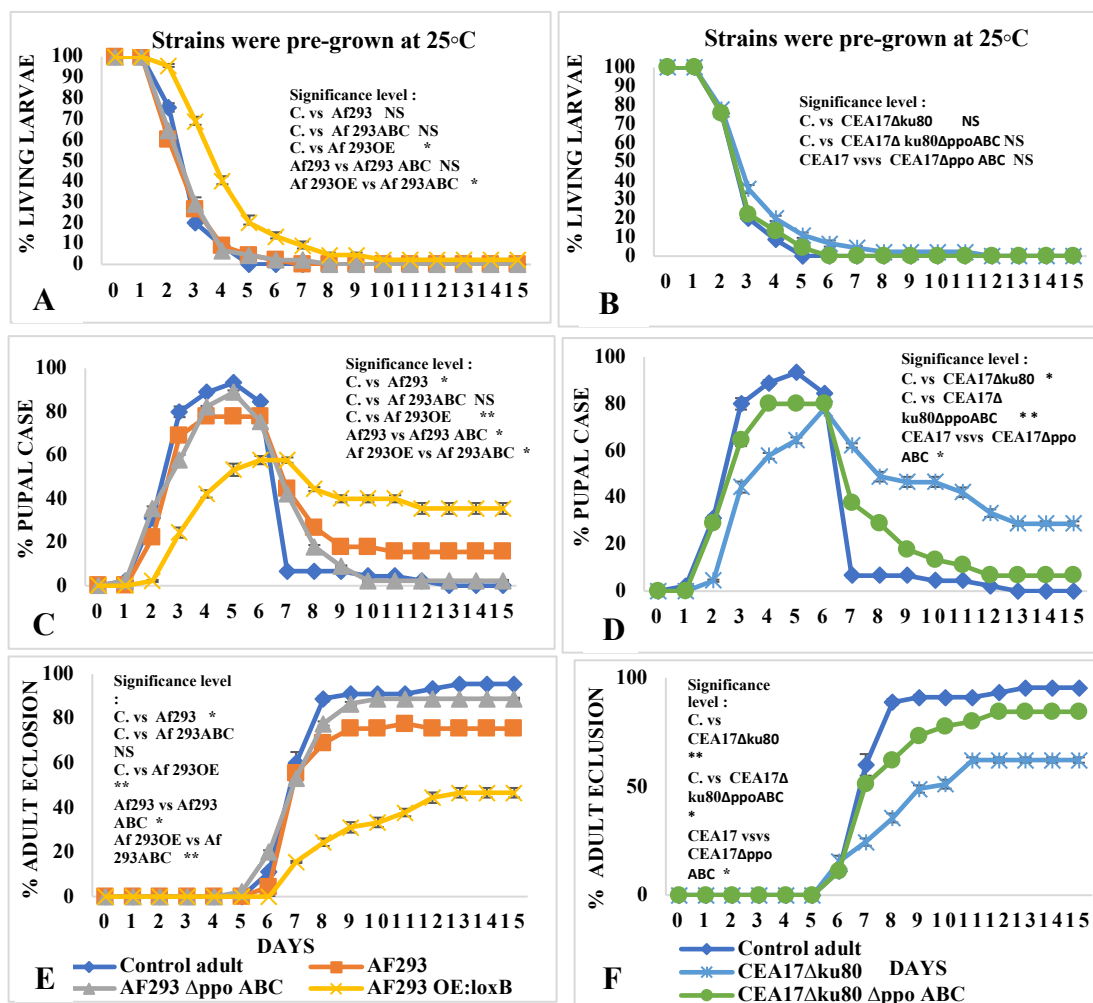
Figure 3.7. Mature third instar larvae of red eyed, wild type Drosophila (strain OregonR) over 15 days of continuous exposure to VOCs *A. fumigatus* that were pre- grown on PDA at 25°C for 5 days. **A:** the percent of living larvae stage exposed to wild type, overexpressed and triple mutant strains. **B**: the percent of living larvae stage exposed to control and triple mutant strains. **C:** the percent of pupal case exposed to wild type, overexpressed and triple mutant strains. **D:** the percent of pupal case exposed to control and triple mutant strains. **E** the percent of adult eclosion exposed wild type, overexpressed and triple mutant strains. **F** the percent of adult eclosion exposed control and triple mutant strains. (N=180). NS = nonsignificant, * represents significant difference between *A. fumigatus* wild type strains and *A. fumigatus* LoxB and triple mutant strains exposed larvae. Where *P<0.05, **P<0.005.

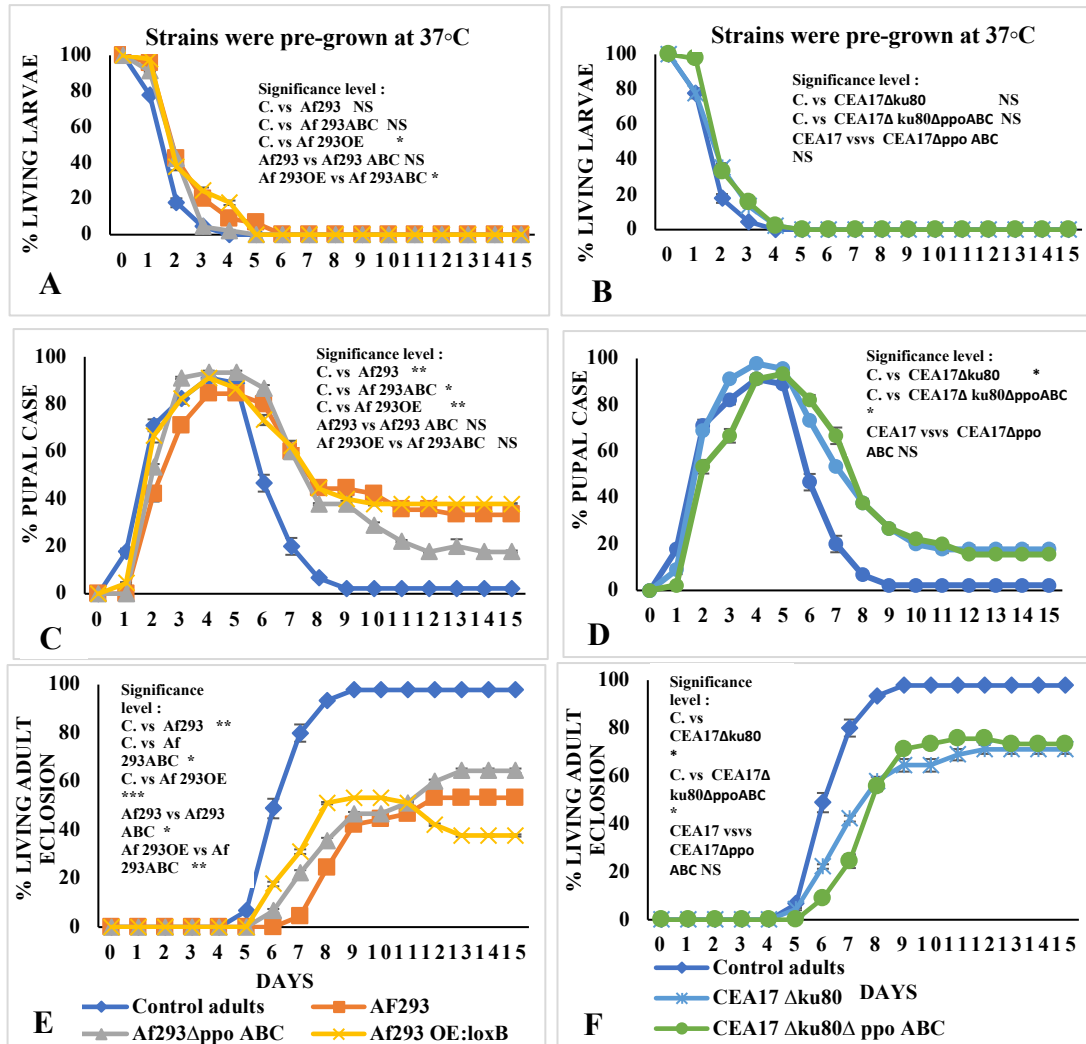Few differences between exposure to the VOCs of fungal cultures pre-cultured at 25°C or 37°C were observed in the exposure studies on *Drosophila* Oregon[R]. When exposed to VOCs from wild type *A. fumigatus* strain Af293 pre-grown at either temperature, only 60-62% of larvae eclosed into adults after 15 days. An even greater toxicity was observed with exposure to the over expression stain Af OE:loxB, with significant delays observed in pupal formation, and with less than 50% eclosion after 15 days.   In contrast, when exposed to VOCs from *A. fumigatu*s strains in which the three lipoxygenase genes were absent (Af293ΔppoABC or *CEA17Δku80 ΔPPOABC*), toxicity was reduced and 82-89% of larvae eclosed into adults after 15 days.
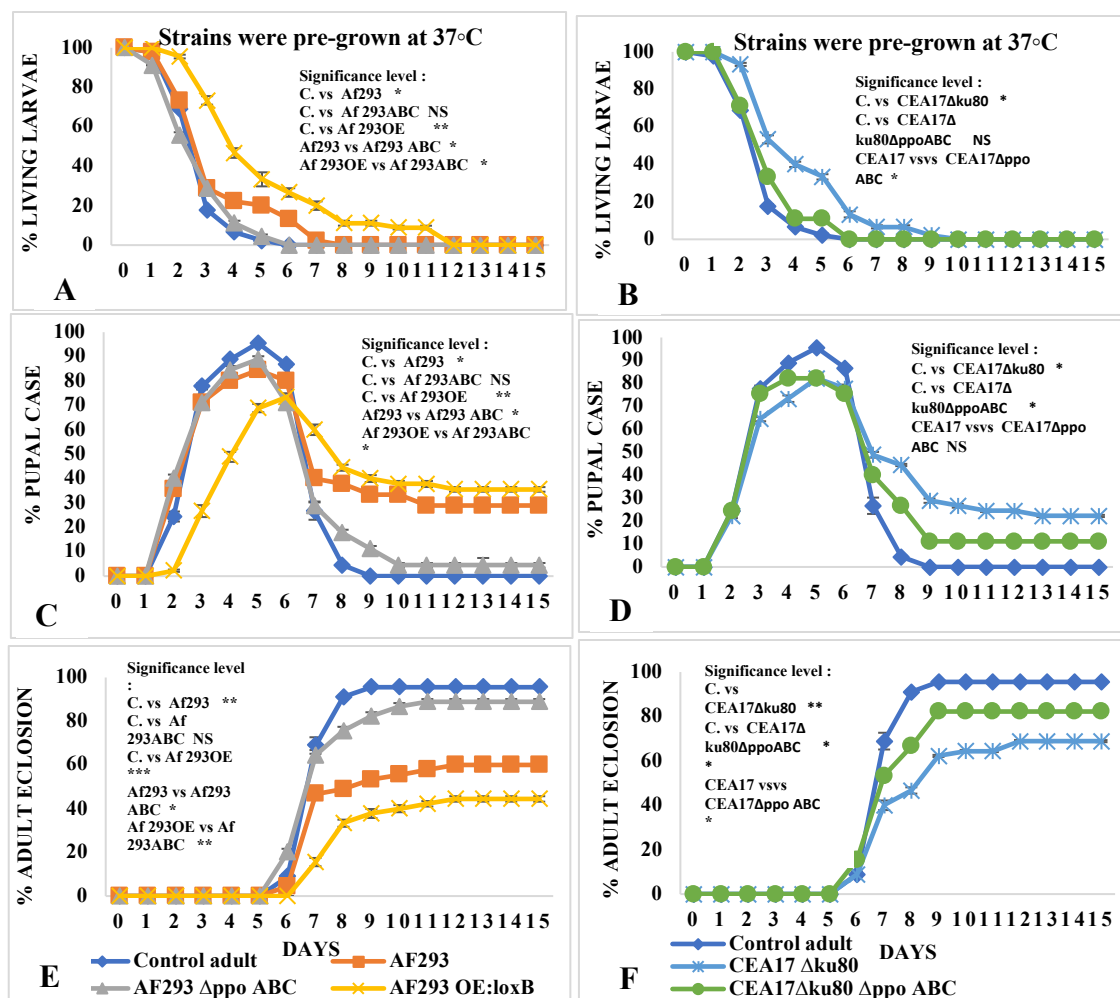
Figure 3.8. Mature third instar larvae of red eyed, wild type Drosophila (strain Oregon[R]) over 15 days of continuous exposure to VOCs *A. fumigatus* that were pre- grown on PDA at 25°C for 5 days. **A:** the percent of living larvae stage exposed to wild type, overexpressed and triple mutant strains. **B**: the percent of living larvae stage exposed to control and triple mutant strains. **C**: the percent of pupal case exposed to wild type, overexpressed and triple mutant strains. **D:** the percent of pupal case exposed to control and triple mutant strains. **E** the percent of adult eclosion exposed wild type, overexpressed and triple mutant strains. **F** the percent of adult eclosion exposed control and triple mutant strains. (N=180). NS = nonsignificant, * represents significant difference between *A. fumigatus* wild type strains and *A. fumigatus* LoxB and triple mutant strains exposed larvae. Where *P<0.05, **P<0.005.

3.3.5 Metamorphic stages of a red eyed *Drosophila* strain with a mutation in the Toll pathway (w^{1118}Spz^6) when incubated in a common atmosphere with VOCs produced by wild type and mutant *A. fumigatus* strains

With few exceptions, there were few differences observed in the metamorphic stages of flies exposed to VOCs from the *A. fumigatus* strains pre-cultured at 25°C or 37°C. (See Figures. 3.9 and 3.10). Moreover, in all cases, few delays in metamorphosis were observed from exposure to VOCs from any of the *Aspergillus* strains tested. Eclosion at 15 days ranged from 83% (for larvae exposed to VOCs from *A. fumigatus* CEA17Δku80 pre-grown at 25°C), to 96% for larvae exposed to VOCs from *A. fumigatus* CEA17Δku80Δppo ABC pre-grown at 37°C.

90
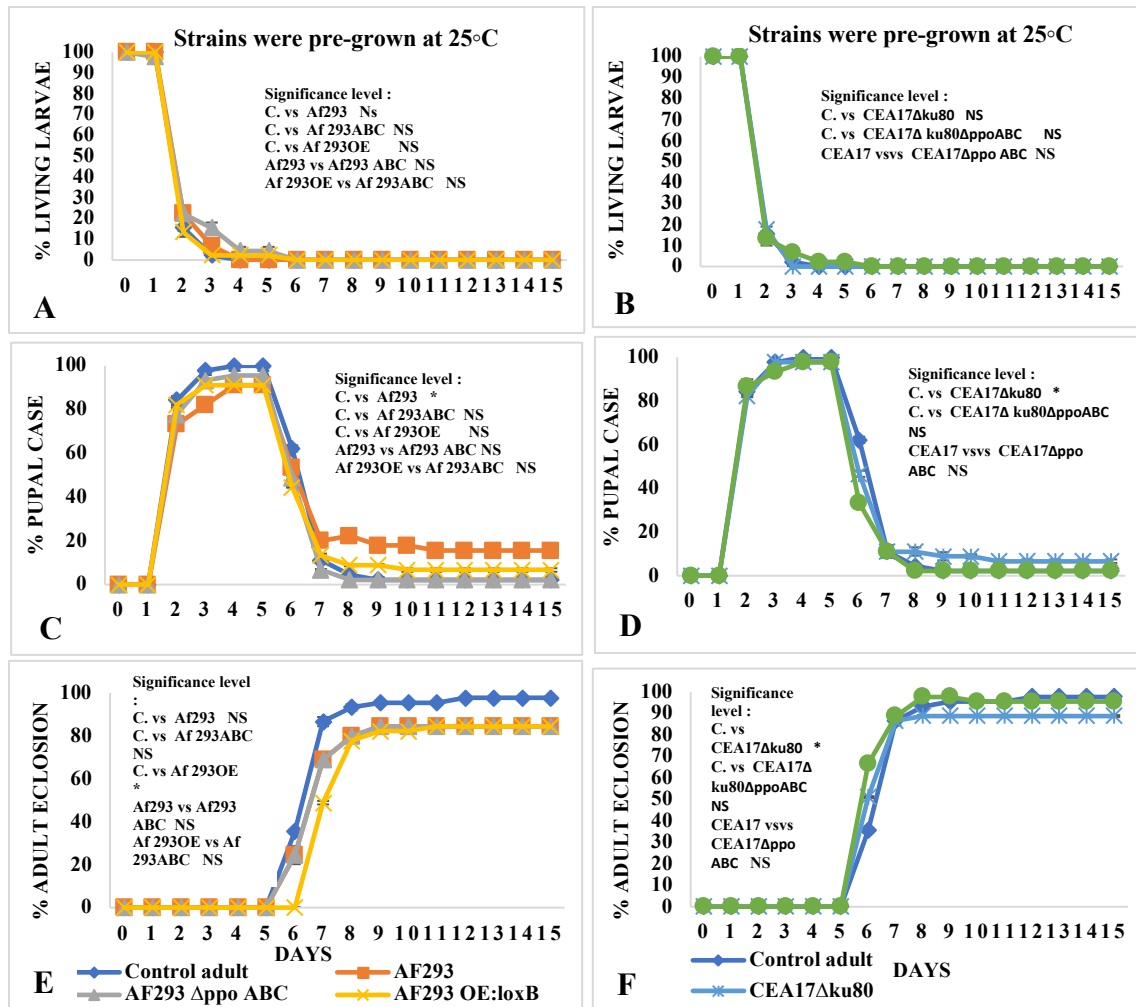
Figure 3.9. Mature third instar larvae of red eyed, Toll deficient *Drosophila* (strain Spz[6]) over 15 days of continuous exposure to VOCs *A. fumigatus* that were pre- grown on PDA at 25°C for 5 days. **A:** the percent of living larvae stage exposed to wild type, overexpressed and triple mutant strains. **B**: the percent of living larvae stage exposed to control and triple mutant strains. **C**: the percent of pupal case exposed to wild type, overexpressed and triple mutant strains. **D:** the percent of pupal case exposed to control and triple mutant strains. **E** the percent of adult eclosion exposed wild type, overexpressed and triple mutant strains. **F** the percent of adult eclosion exposed control and triple mutant strains. (N=180). NS = nonsignificant, * represents significant difference between *A. fumigatus* wild type strains and *A. fumigatus* LoxB and triple mutant strains exposed larvae. Where *P<0.05, **P<0.005.

Figure 3.10. . Mature third instar larvae of red eyed, Toll deficient *Drosophila* (strain Spz[6]) over 15 days of continuous exposure to VOCs *A. fumigatus* that were pre- grown on PDA at 37°C for 3 days.**A:** the percent of living larvae stage exposed to wild type, overexpressed and triple mutant strains. **B**: the percent of living larvae stage exposed to control and triple mutant strains. **C**: the percent of pupal case exposed to wild type, overexpressed and triple mutant strains. **D:** the percent of pupal case exposed to control and triple mutant strains. **E** the percent of adult eclosion exposed wild type, overexpressed and triple mutant strains. **F** the percent of adult eclosion exposed control and triple mutant strains.   (N=180). NS = nonsignificant, * represents significant difference between *A. fumigatus* wild type strains and *A. fumigatus* LoxB and triple mutant strains exposed larvae. Where *P<0.05, **P<0.005.
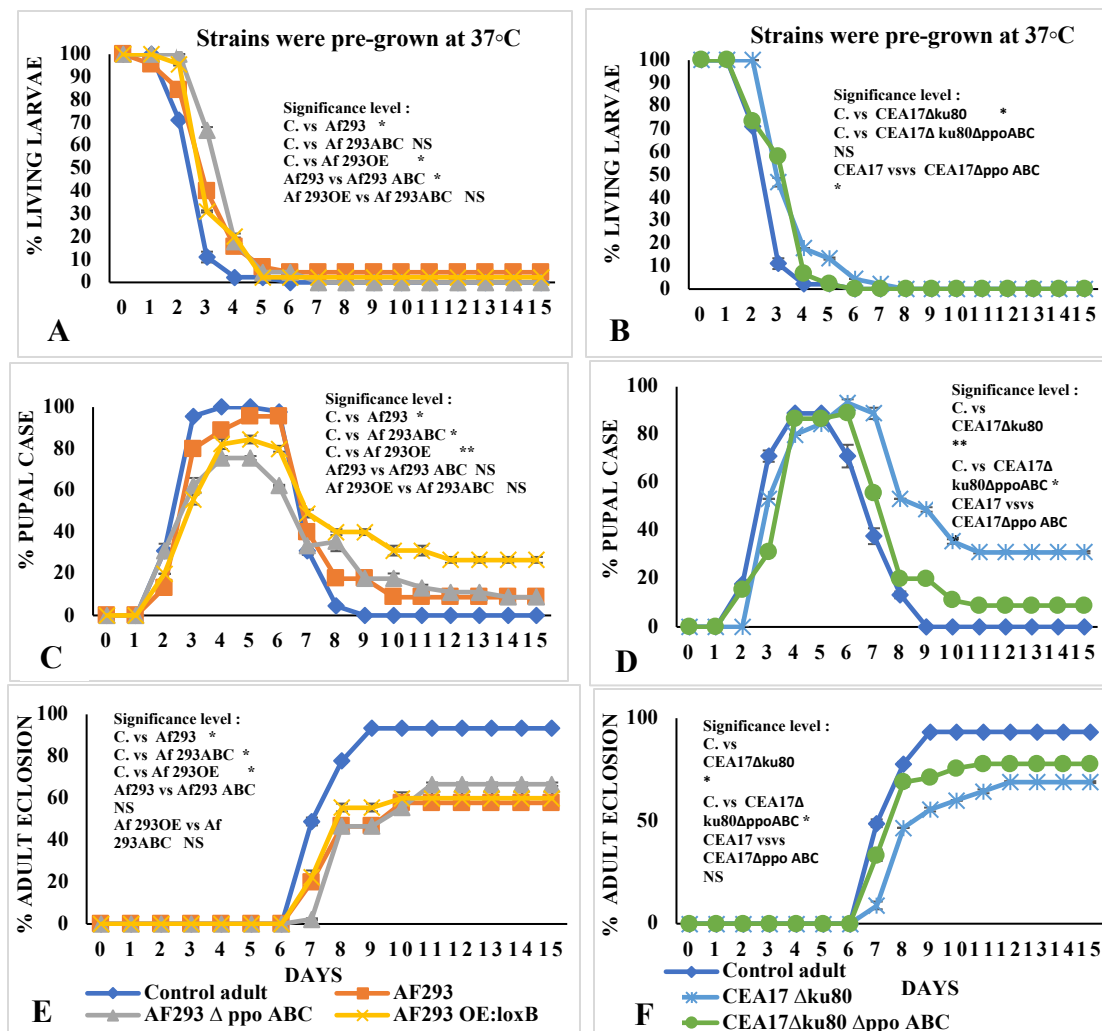
3.3.6 Metamorphic stages of a red eyed *Drosophila* strain with double mutations in the Imd and Toll pathways (Relish^{E20}Spz^6**) when incubated in a common atmosphere with VOCs produced by wild type and mutant *A. fumigatus* strains .**

These data were similar to those observed in Section 3.3.5. Few delays in metamorphosis were observed for any of the exposures to VOCs of the different *A. fumigatus* strains at either temperature. Eclosion at 15 days ranged from 85-96%.   The larvae carrying the double mutation in their Imd and Toll immune pathways were more resistant to the effect of fungal VOCs than was the wild type Oregon^R strain (See Figures 3.11 and 3. 12; Table 3.3).
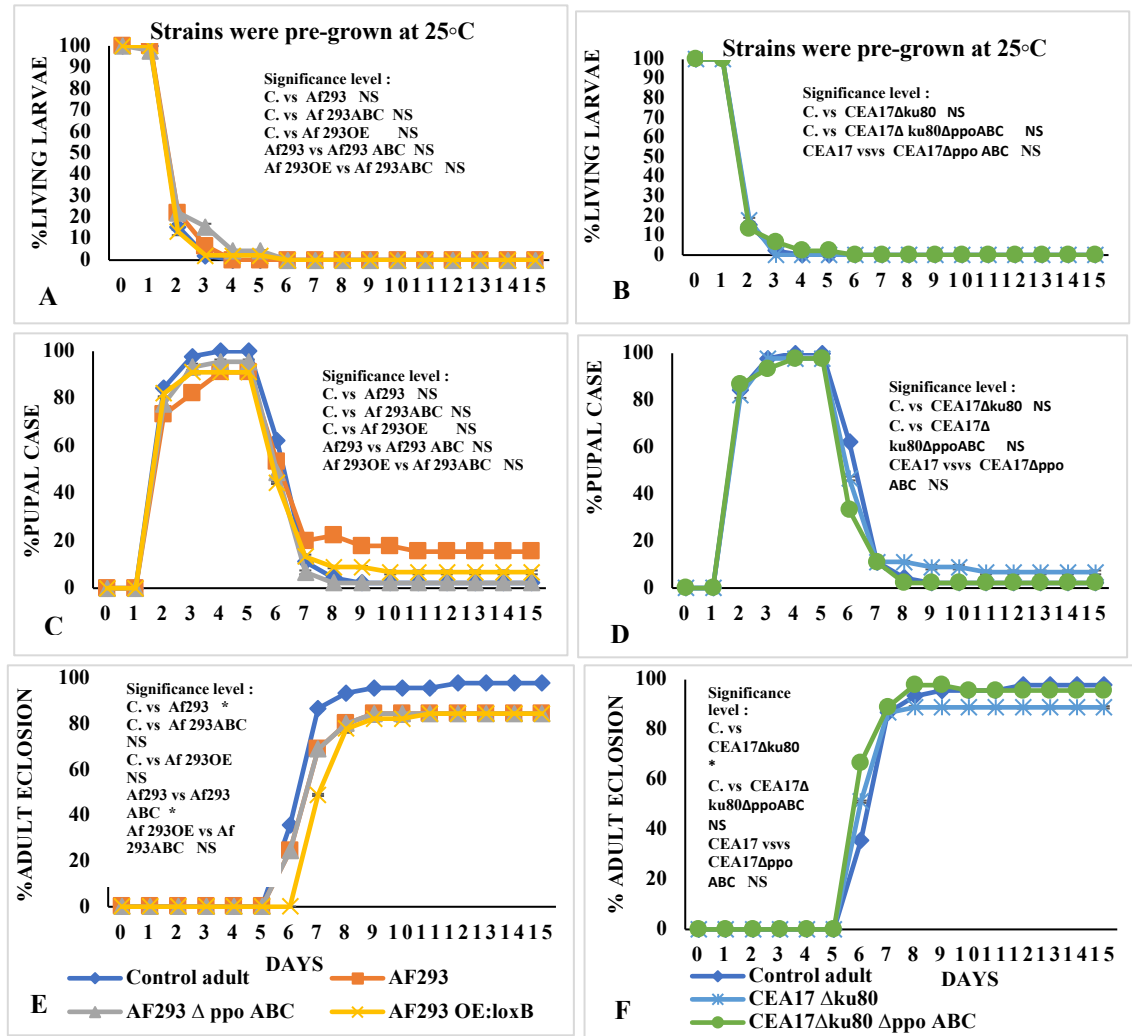
Figure 3.11. Mature third instar larvae of red eyed, Imd and Toll deficient *Drosophila* (Relish^{E2}Spz^{6}) over 15 days of continuous exposure to VOCs *A. fumigatus* that were pre- grown on PDA at 25°C for 5 days. **A:** the percent of living larvae stage exposed to wild type, overexpressed and triple mutant strains. **B**: the percent of living larvae stage exposed to control and triple mutant strains. **C**: the percent of pupal case exposed to wild type, overexpressed and triple mutant strains. **D:** the percent of pupal case exposed to control and triple mutant strains. **E** the percent of adult eclosion exposed wild type, overexpressed and triple mutant strains. **F** the percent of adult eclosion exposed control and triple mutant strains. (N=180). NS = nonsignificant, * represents significant difference between *A. fumigatus* wild type strains and *A. fumigatus* LoxB and triple mutant strains exposed larvae. Where *P<0.05.

Figure 3.12. Mature third instar larvae of red eyed, Imd and Toll deficient *Drosophila* (Relish^{E2}Spz^6) over 15 days of continuous exposure to VOCs *A. fumigatus* that were pre- grown on PDA at 37°C for 3 days. **A:** the percent of living larvae stage exposed to wild type, overexpressed and triple mutant strains. **B**: the percent of living larvae stage exposed to control and triple mutant strains. **C**: the percent of pupal case exposed to wild type, overexpressed and triple mutant strains. **D:** the percent of pupal case exposed to control and triple mutant strains. **E** the percent of adult eclosion exposed wild type, overexpressed and triple mutant strains. **F** the percent of adult eclosion exposed control and triple mutant strains.   (N=180). NS = nonsignificant, * represents significant difference between *A. fumigatus* wild type strains and *A. fumigatus* LoxB and triple mutant strains exposed larvae. Where *P<0.05.

Table 3.3 Percent eclosion of adult flies after 15 days of continuous exposure to VOCs from wild type and mutant strains of *Aspergillus fumigatus* pre-grown either at 25 ^oC or 37^oC

Drosophila strains	*Aspergillus* strains pre-grown at different temperatures											
	Control		Af293		Af293 OE:loxB		Af293 Δppo ABC		CEA17Δku80		CEA17Δku80Δppo ABC	
	25^oC	37^oC	25^oC	37^oC	25^oC	37^oC	25^oC	37^oC	25^oC	37^oC	25^oC	37^oC
W[1118] wild type, white-eyed strain	97	95	46	44	40	31	60	60	64	57	51	60
NOS (w[1118]m[24283])mutant strain	100	97	64	53	51	37	84	64	84	71	71	73
w[1118] Relish[E20], Imd pathway mutant strain	100	93	88	57	68	60	68	66	77	68	75	77
Oregon[R] wild type,red-eyed strain	95	95	60	62	44	44	88	88	68	75	82	95
Oregon[R] spz[6], Toll pathway mutant strain	97	91	84	86	84	84	84	95	88	82	95	90
Relish[E20] and spz[6] double mutant strain	97	91	84	88	84	84	84	91	88	82	95	88

3.7. Summary of exposure studies

The *A. fumigatus* strain emitting VOCs with the greatest toxigenic effects in the *Drosophila* bioassay was the over expression Af293OE:lox B. The next most toxigenic VOCs were emitted by the wild type Af293 strain and the control CEA17Δku80 strain. The least toxigenic VOCs were emitted by the triple oxylipin mutants Af293Δppo ABC and CEA17Δku80Δppo ABC.

In general, larvae from the red eyed flies were more resistant to fungal VOCs that were larvae of flies carrying the white eyed mutation. Interestingly, white eyed *Drosophila* fly larvae that carry mutations in the NOS and Imd immune pathways were more resistant to the toxigenic effects of fungal VOCs than were the white eyed w[118] flies with a wild type immune system. Furthermore, larvae of red eyed flies carrying the Toll mutation Relish[E20] or a double mutation in both the Imd and Toll pathway (Relish[E20]spz[6]) were the most resistant of all fly strains tested to the negative effects of exposure to *A. fumigatus* VOCs.

3.3.8 Exposure of *Drosophila* larvae W[1118] strain to VOCs produced by different strains of *A. fumigatus* grown on GMM medium with and without arachidonic acid

A published study has determined that exposure of *A. fumigatus* to arachidonic acid caused changes in fungal germination and development (Tsitsigiannis, et al. 2005b). We tested the effects VOCs emitted by *A. fumigatus* grown on GMM medium with and without added arachidonic acid (5 mM) on white eyed *Drosophila* larvae strain W[1118] with wild type immune genes. The numbers of larvae, pupa, and adults were monitored and counted daily for 15 days. The percent larvae on day 4, the percent pupae on day 7 and the percent eclosed adults on day 10 are shown in Table 3.4. There were no significant differences noted

between fungi pre-grown at 25°C or 37°C. A significant delay in the larvae and pupal stages was noted for larvae exposed to VOCs produced by the wild type Af293 on medium lacking arachidonic acid., however this delay was no longer detected for the number of adult flies at day 10. (Table 3.4).

Table 3.4. The percentage of *Drosophila* adults observed on four, seven, and ten days from exposure to the VOCs produced from *Aspergillus fumigatus* strains that have grown at different temperatures.

Strains and Species	Pre-grown at different temperatures and days											
	Day 4(Larvae stage)				Day 7 (Pupae stage)				Day 10(adult stage)			
	25°C		37°C		25°C		37°C		25°C		37°C	
	+AA	-AA	+AA	-AA	+AA	-AA	+AA	-AA	+AA	-AA	+AA	-AA
Control	11.1	8.8	2.2	0	13.3	11.1	17.7	13.3	95.5	97.7	95.5	93.3
Aspergillus fumigatus Af293 (*)	45	50	68.8*	43	70 *	47.7	53.3	48.8	70	75	60	55.5
Aspergillus fumigatus Af293 OE:loxB (NS)	60	57.7	60	68.8	35	47.7	68.8	66.6	66.6	60	53.3	45.5
Aspergillus fumigatus Af293 Δ ppoABC (NS)	23	36.6	33	37.7	22.2	24.4	26.6	22.2	80	82.2	77.7	80
Aspergillus fumigatus CEA17Δku80 (NS)	36.6	33.3	50	48.8	53.3	48.8	44.4	55.5	77.7	75	64	67.7
Aspergillus fumigatus CEA17Δku80 ΔPPOABC (NS)	24.4	22.2	37.7	33.3	37.7	33.3	40	37.7	80	82.2	77.7	78.8

3.3.9 GC-MS analysis of VOC profiles of *Aspergillus fumigatus* strains wild type (Af29) and triple mutant strains (Af293Δppo ABC)

Two strains of *Aspergillus fumigatus* were selected for analysis of their VOC profiles. The VOCs emitted by the Af293 wild type strain caused delays in metamorphosis and high toxicity on *Drosophila*. In contrast, when the ppo genes were deleted from this strain (Af293Δppo ABC), metamorphic delays and toxicity were greatly reduced. We used purge and trap GC-MS analysis to determine the VOC profiles of the two strains of fungi grown on PDA and sampled at 25∘C for 5 days or 37∘C for 3 days. The wild type strain produced more abundant VOCs with higher concentrations compared to the triple mutant, which released fewer VOCs with low concentrations. The single most abundant VOCs produced by the wild type strain was isopentyl alcohol. Other abundant VOCs included isobutyl alcohol, acetoin, 2-methyl butanol and 1-octen-3-ol. More 1-octen-3-ol was produced when the fungus was pre-grown at 25ºC than at 37ºC. Myristic acid and palmitic acid were produced by Af293 wild type strain while these VOCs were not detected from the triple mutant strain (Tables 3.5 and 3.6).

Table 3.5. VOCs released from the triple lipoxygenase mutant strain Af293Δppo ABC which was grown either for 5 days at 25◦C or 3 days at 37◦C by using purge and trap-thermal desorption method.

VOCs	Conc. at 25◦C ng/trap	Conc. at 37 ◦C g/trap
1-octen-3-ol	42.8	16.7
Isopentyl alcohol	547.2	875.6
2-butanone+diacetyl	37	93.7
ethyl acetate	18.7	29
Isobutyl alcohol	124.9	65.7
2-methylbutanal	99.7	5.8
Acetoin	76.5	25.2
farnesene	7.9	3.7
3-methylbutyric acid	1.8	1.7
2-methylbutyric acid	1.9	5.2
Isobutyric acid	1.2	nd
2-ethylfuran	nd	7.4
Acetic acid	nd	4.8
1-octene	nd	1.6
2-heptanone	nd	1.8
1-butanol	nd	8.2
2-hetanone	0.6	nd
heptanal	0.6	0.9
1-butanol	3.7	nd
Octanoic acid	1.4	0.75
Nananoic acid	1.01	0.5
Decanoic acid	10	7.6

Lauric acid	8.97	8.4
Myristic acid	5.2	6.8
Palmitic acid	2.02	4.6
benzene-d6 (internal standard)	977.5	751.4
toluene-d8 (internal standard)	1000	1000
Naphthalene-d8 internal standard)	859.8	859.6

Table 3.6. VOCs released from the triple lipoxygenase mutant strain Af293ΔppoABC which was grown either for 5 days at 25∘C or 3 days at 37∘C by using purge and trap-thermal desorption method.

VOCs	Conc. at 25 ∘C ng/trap	Conc. at 37 ∘C ng/trap
1-octen-3-ol	3.5	nd
Isopentyl alcohol	67.9	296.3
2-butanone+diacetyl	3.4	20.6
ethyl acetate	1.8	11.4
acetoin	nd	17.6
Isobutyl alcohol	11.5	35.6
2-methylbutanal	1.9	29.6
Acetic acid	136	95.9
Isobutyric acid	1.4	11.1
2-methylbutyric acid	nd	3.4
3-methylbutyric acid	nd	3.2
3-methyl-1,3-pentadiene	nd	1.4
Propionic acid	nd	2.3
2-heptanone	nd	0.8
heptanal	nd	1.3
Hexanoic acid	Nd	45.6
Heptanoic acid	nd	4.2
Nananoic acid	nd	3.7
Octanoic acid	nd	2.7
benzene-d6(internal standard)	976.1	930.7
toluene-d8 (internal standard)	1000	1000

Naphthalene-d8(internal standard)	762.5	911.02

nd non detected.

3.4 Discussion

3.4.1 Introduction.

In this chapter, we continued to take advantage of the *Drosophila* model for studying the effects of fungal VOCs. Fly larvae and fungi were grown together in microcosms whereby they shared a common atmosphere but had no direct physical contact. Using this VOC-exposure system, we applied the power of mutational analysis in two distinct ways. In the first approach, third instar *Drosophila* larvae that were wild type except for carrying a white eyed gene, were exposed to VOCs from a collection of *A. fumigatus* strains, some of which carried mutations in pathways associated with the biosynthesis of oxylipins. In the second approach, the VOCs from wild type and oxylipin mutants of *A. fumigatus* were tested against wild type flies as well as flies that carried different immune system mutations.   These data are discussed in separate sections below.

3.4.2 Overview of oxylipins

"Oxylipin" is the collective word for all oxygenated lipids. Because many of these compounds are highly bioactive and play important physiological roles in animals, plants, and fungi there is a vast literature on their biosynthesis, metabolic functions, and importance in health. Well known oxylipins metabolically derived from C_{20} fatty acids include the eicosanoids (*eicano* is Greek for 20) such as prostaglandins, thromboxanes and

leukotrienes. (Note: These compounds derive their names from their earliest source of isolation. Prostaglandins were originally shown to be synthesized in prostate glands, thromboxanes from platelets [thrombocytes], and leukotrienes from leukocytes**.)**

Eicosanoids have a truly extraordinary range of physiological effects in mammals. Many are pro-inflammatory and associated with various disease states including atherosclerosis and Alzheimer's disease(Gabbs, et al. 2015). Prostagladins contract and relax smooth muscle; dilate and constrict blood vessels, control blood pressure, and modulate inflammation. Leukotrienes play a key role in inflammation and are involved in allergies, allergic rhinitis, and asthma. (PAPATHEOFANIS and LANDS 1985). Eicosanoids use receptor-dependent pathways to control signaling; in mammals their dysregulation is central to numerous pathological states including cancer and inflammation (Kozak and Marnett 2002).

Lipoxygenase enzymes (LOX) are dioxygenases which incorporate one molecule of oxygen at a certain position of unsaturated fatty acids such as arachidonic and linoleic acids. Atypical lipoxygenases that incorporate two molecules of oxygen are called fatty acid cyclooxygenases (COX) and initiate the biosynthesis of prostaglandins and thromboxanes. In mammalian cells, arachidonic acid (20:[n-6]) is the main eicosanoid precursor (Sharma and Mohammed 2006).

To summarize a vast biochemical literature, oxylipins are formed by lipoxygenase (LOX), cyclooxygenase (COX) and cytochrome p450 enzymes (Brodhun and Feussner 2011; Patkar, et al. 2015). Moreover, oxylipins can also be produced non-enzymatically by autoxidative processes associated with reactive oxygen species (ROS). Oxylipins from

animals and plants are best characterized where many of them function as chemically and functionally varied intra- and inter-specific chemical signaling agents (Andreou, et al. 2009; Davis, et al. 2013; Wadman, et al. 2009; Watson, et al. 2009).

Major plant oxylipins tend to be derived from C_{18} polyunsaturated fatty acids such as linoleic or α-linolenic acid formed by an initial peroxidation reaction catalyzed by lipoxygenases (LOXs) (Grechkin 1998; Mosblech, et al. 2009). LOX-catalyzed pathways include leukotrienes, green leaf volatiles, jasmonic acids and various other important plant signaling compounds. Jasmonic acid and green leaf volatiles are often produced in response to attack by microbial pathogens, physical damage by animals and abiotic stress. (Delker, et al. 2006; Scala, et al. 2013). An excellent summary of LOX catalyzed pathways is given in (Joo and Oh 2012). The role of oxylipins in cross-kingdom interactions, especially with respect to plant pathogenesis has received extensive study (Fischer and Keller 2016; Pohl and Kock 2014; Tsitsigiannis and Keller 2007; Tsitsigiannis, et al. 2005b).

3.4.3 Oxylipins from fungi

Prostaglandins, prostaglandin-like molecules, leukotrienes and thromboxanes also are produced by fungi where they were first identified in the pathogens *Candida albicans* and *Cryptococcus neoformans* (Noverr, et al. 2001). Perhaps the best understood family of fungal oxylipins are the psi factors ("precocious sexual inducer" factors), originally described from *Aspergillus nidulans* where they alter the ratio of asexual to sexual sporulation (Champe, et al. 1987). Using a genomic analysis, the genes to produce psi factors were predicted to be three oxygenases and named PpoA, PpoB and PpoC

(precocious sexual inducer-producing oxygenases) (Tsitsigiannis, et al. 2005b; Tsitsigiannis, et al. 2004). Subsequently, similar genes have been described from many other fungi and this group of COX-like oxygenases are now called, generically, Ppo enzymes.

Using a crude *E. coli* lysate, (Brodhun, et al. 2010) showed that expression of *ppoC* is sufficient to catalyze the breakdown of linoleic acid into a wide range of compounds including 1-octen-3-ol, 2-octen-1-ol, 2-octenal, and 3-octanone. (Dagenais, et al. 2008) demonstrated the ΔPpo mutants of *A. fumigatus* was more susceptible to killing by murine alveolar macrophages. A homologue of this gene is necessary for production of 1-octen-3-ol in *Aspergillus luchuensis* (Kataoka, et al. 2020). In *A. fumigatus*, the Δppo C mutant is hypervirulent in a murine model of pulmonary aspergillosis, suggesting that the synthesis of certain oxylipins may be detrimental, perhaps by activating a forceful host immune response via an augmented tolerance to oxidative stress (Singh and Del Poeta 2011; Tsitsigiannis, et al. 2005b).

Far less is known about smaller, volatile oxylipin degradation products (Fischer, et al. 2017). C_8 oxylipins such as 1-octen-3-ol, 3-octanone, and 3-octanol can be derived from the longer-chain oxylipins by breakdown of polyunsaturated fatty acids such as arachidonic acid oleic acid, linoleic, and linolenic acids (Assaf, et al. 1997; Husson, et al. 2001). Many of these volatile oxylipins function in fungal development and reproduction (Pohl and Kock 2014). For example, mushroom alcohol (1-octen-3-ol) is an extremely common oxylipin-derived molecule that contributes a large part of the characteristic odor of mushrooms and molds. Despite its ubiquity, and its importance as a food and flavoring agent, the details of the metabolic origin of 1-octen-3-ol are poorly understood (Assaf, et

al. 1997; Inandar A 2020). A useful table of lipoxygenase and cyclooxygenase genes in selected fungal genomes has been developed by (Ferrari, et al. 2018) This group showed that COX and LOX genes in *Podospora anserine* produced oxylipins that participate in the production of some C8 volatiles which   play a role in deterring nematodes (Ferrari, et al. 2018). A phylogenetic analysis of fatty acid dioxygenases from fungi, mammals and plants that show the relationship of putative enzyme sequences including those of the PPoC-group has been generated by (Andreou, et al. 2009).

Fungal VOCs, including many volatile oxylipins, have been hypothesized to contribute to negative health effects in people who have live near compost facilities (Herr, et al. 2003) or in water damaged buildings (Douwes 2009). A limited number of controlled studies has shown that VOCs such as 1-octen-3-ol, ethanol and certain air pollutants are harmful for human health (Pantoja, et al. 2016; Perl, et al. 2011; Scotter, et al. 2005; Wild 2007; Wright, et al. 2002).It is well known that *A. fumigatus* emits numerous VOCs, some of which have been used as diagnostic aids for early diagnosis of aspergillosis (Heddergott, et al. 2014a; Korpi, et al. 2009a; Lemfack, et al. 2014). The toxicity of certain fungal VOCs has been studied in rodent models, cultured cell lines and, in a few cases, on human volunteers (Korpi, et al. 1999; Kreja and Seidel 2002a; Wålinder, et al. 2008).

3.4.4 *Drosophila* model for studying fungal VOCs

Because mammalian models are expensive and ethically problematic, we used a *Drosophila* model (AL-Maliki, et al. 2017; Rand, et al. 2014; Wasserkort and Koller 1997)

Previous research has used *Drosophila* to study aspergillosis in experiments involving direct physical contact (Chamilos, et al. 2007). In our approach, fly larvae were placed in a shared atmosphere with growing fungi but there was no physical contact between the two organisms. Previously, we showed that VOCs emitted by living fungal cultures, and by chemical standards of 1-octen-3-ol and other C_8 volatiles, caused toxicity and death in *Drosophila* larvae and adults (Inamdar, et al. 2013; Inamdar, et al. 2014). Here, we hypothesized that VOCs produced by human pathogenic fungi would be toxic to *Drosophila* and in Chapter 2 of this dissertation we showed that this was, indeed, the case.

In order to determine if volatile C_8 oxylipins, and other volatiles emitted by *A. fumigatus,* play a role in pathogenesis, we have tested the toxicity of VOCs produced wild type, loxB, and triple mutant (Δppo ABC genes) strains on the metamorphosis of wild type *Drosophila* flies. In general, VOCs from *A. fumigatus* wild type strain Af293, and the over expression strain loxB derived from it, had more toxic effects on all stages of fruit fly metamorphosis than VOCs from the control CEA17 Δku80 stain and its derivative triple mutant strain. Of the *Aspergillu*s strains tested, exposure to VOCs emitted by strains Af293 and OE:loxB, pre-grown at 37ᵒC, were the most toxic to the *Drosophil*a model, *i.e.* the larvae experienced the longest delays in pupal formation, as well as the lowest eclosion rates. Furthermore, as hypothesized, VOCs from the triple mutant Δppo ABC strains had the least significant toxic effects on the metamorphosis on all fruit fly strains tested. Using purge-trap thermal desorption GC-MS, we found that the triple mutant strain of *A. fumigatus* did not produce 1-octen-3-ol, 1-butanal, 1-octen, decanoic acid, and lauric acid all of which

were detected from the wild type strain. Furthermore, the concentrations of VOCs produced by the triple mutant strain were lower than those produced by the wild type.

3.4. 5 Innate immune system

The ability to set off the immune system and repel pathogenic invaders is an essential part of organismal health. Research in many fields has shown that cellular and molecular mechanisms associated with the immune response are highly conserved. There have been numerous studies on the relationship of the fruit fly and mammalian immune systems (see, for example, (Lemaitre and Hoffmann 2007; Wang and Ligoxygakis 2006). Most of the genes involved in the *Drosophila* host defenses against infectious disease are quite similar to genes used mammalian innate immune defenses (Hoffmann 2003a). In addition to an epithelial barrier and efficient wound healing, *Drosophila* fights infection using phagocytosis by plasmatocytes an ancient forms of host defense (Ulvila, et al. 2011).

Humoral immunity is the part of immunity mediated by molecules found in extracellular fluids. The term encompasses secreted antibodies, complement and certain antimicrobial peptides ("AMPs"), and it can involve the activation of cytokine production (Kleino and Silverman 2014). The *Drosophila* humoral response generates the production of antimicrobial peptides by the fat body (Lemaitre and Hoffmann 2007). Eight families of antimicrobial peptides have been identified in *Drosophila*. Within a few hours of microbial infection, these peptides reach concentrations in the insect blood (hemolymph) that make it a hostile environment for the invading pathogens (Hoffmann 2003a).

The discovery that the Toll pathway is required for protection against fungal infection in *Drosophila* was crucial in studies of both mammalian and *Drosophila* immunity. *Drosophila* Toll is activated by an endogenous ligand called Spaetzle which resembles certain mammalian growth factors such as nerve growth factor (Lewis, et al. 2013).

In mammals, "pattern recognition receptors" ("PPRs") recognize danger signals. Mammalian Toll - like receptors ("TLRs") were discovered in database searches after the identification of Toll and, to date, eleven members have been found (Shizuo and Takeda 2004). These vertebrate receptors are related to the Toll family and are activated by pathogen-associated molecules such as bacterial endotoxin. Mammalian pattern recognition receptors recognize microbial molecules such as cell wall components that are common to most pathogens. These molecules are sometimes called pathogen-associated molecular patterns ("PAMPs"). Different surface and intracellular components of microorganisms are recognized by different Toll-like receptors. They elicit the activation of an intracellular signaling cascade whereby different microbial cell wall components are recognized by different Toll-like receptors. For example, mammalian Toll-like receptor-2 discriminates peptidoglycan, lipoprotein, lipoarabinomannan and zymosan. Toll-like-receptor 4 recognizes lipopolysaccharide, lipoteichoic acid and Taxol (Valanne, et al. 2011). For clarity, we will avoid the use of the numerous acronyms developed by immunologists in our text below.

3.4.6 *Drosophila* Toll and Imd

The Toll gene was discovered in *Drosophila* as part of research in developmental biology and is essential for establishing dorsoventral polarity in fly embryos (Hashimoto, et al.

1988). Later it was found that Toll is a transmembrane receptor that also functions in innate immunity. Toll deficient *Drosophila* are highly susceptible to fungal infection (Lemaitre, et al. 1996b). Toll does not directly recognize microbial determinants but is activated by the binding of the ligand Spatzle, which occurs after fungal determinants (mainly glucans) are detected by circulating pattern recognition receptors that induce, by an unknown mechanism, the activation of proteolytic cascade that lead to the cleavage of Spaetzle and subsequent Toll activation (El Chamy, et al. 2008). Thus, in *Drosophila* and other insects, the presence of Gram positive bacteria and fungi are detected indirectly by Spätzle. Spaetzle is synthesized in an inactive form (Brennan and Anderson 2004; El Chamy, et al. 2008; Lewis, et al. 2013). The antifungal peptide induced by infection by the Toll signaling pathway is called drosomycin (Lemaitre, et al. 1996b).

After the discovery of Toll, a second signaling cascade, namely the immune deficiency (Imd) pathway, was found by subsequent genetic screens looking for *Drosophila* mutants that lacked innate responses to infection. Flies carrying *Toll* mutations are more resistant to fungi and Gram-positive bacteria than are Imd mutant flies, while the Imd pathway responds to Gram negative bacteria. It is similar to the mammalian TNF-receptor signaling pathway and is activated through membrane-bound pattern recognition receptors. Imd pathway initiation leads to the expression of the NF-kB factor Relish, its translocation to the nucleus, and the subsequent transcriptional activation of a group of target genes. The Imd pathway is triggered by the presence of meso-diaminopimelic acid type peptidoglycan, found as part of the cell ways of most Gram-negative bacteria and some Gram- positive bacteria as well. Adult flies carrying this mutation have impaired production of most antimicrobial peptides, although the antifungal peptide drosomycin remains inducible

(Lemaitre, et al. 1996b) Relish is the key transcription factor activated in the Imd signaling pathway and is responsible for the induction of the humoral immune response including both antibacterial and antifungal peptides (Hedengren, et al. 1999).

In summary, both Toll and Imd are important in initiating pathogen recognition; synergistic interactions between the Toll and Imd pathways allow *Drosophila* to survive microbial infections (Brennan and Anderson 2004; Valanne, et al. 2011). However, when immune responses are incorrectly timed, or when they are overactive, they can be harmful to the health and survival of mammals and insects (Kleino and Silverman 2014). Reactive oxygen species (partially reduced metabolites of oxygen that can damage cells constituents) are often released during immune reactions. ROS can not only target invading pathogens, they can also cause "collateral damage" by attacking host tissues (Sedghi, et al. 2017). In order to prevent the negative aspects of the immune response, the *Drosophil*a Imd pathway is modulated such that Relish activation is tightly controlled through several poorly understood regulatory check points (Kleino and Silverman 2014).

For many years, the research in fungal immunology has focused on defining the molecular interactions between the pattern recognition receptors ("PRRs") of mammals such as Toll like receptors and their respective pathogen-associated molecular patterns ("PAMPs"). Most of the models for fungal-fly immune reactions are based on the recognition of conserved cell -wall molecules such as peptidoglycan and beta-glucan. Enormous progress has been made in understanding how mammalian pattern recognition receptors identify fungal associated molecular pattens such as beta-1,3-glucans, beta-glucanases and DNA (Hultmark 2003). It has been suggested that pathogen sensing may similar to plant systems whereby one part of the system recognizes molecules common to many classes of microbes

(pathogen associated molecular patterns) and a second part responds to virulence factors detected through their effects on host targets "danger signals.")   (El Chamy, et al. 2008).

3.4.7 VOCs and the *Drosophila* innate immune system

In earlier work in our laboratory, adult flies exposed to low concentration of volatile phase 1-octen-3-ol, in the presence of nitric oxide synthase inhibitors such as L-NAME and minocycline, survived longer than flies exposed to 1-octen-3-ol alone, indicating that the toxicity of 1-octen-3-ol is partly mediated via excessive nitric oxide activity. It is known that *Drosophila* nitric oxide synthase dNOS) regulates a number of biological processes including host immune response (Mukherjee, et al. 2011; Stasiv, et al. 2001). When in excess, NOS enzymes promote tissue injury in the human nervous system as well as the *Drosophila*'s hemocytes (Carton, et al. 2009a; Foley and O'Farrell 2003; Laskin, et al. 2011a). When the genes were overexpressed in the *Drosophila* model, there was an increase in the survival rate by roughly 5 days (Inamdar and Bennett 2014). Nitric oxide is thought to acts upstream of the Imd pathway. In parallel research, paraquat has been used in a fly model of Parkinson's disease. Paraquat exposure leads to *Drosophila* neurotoxicity and activation of Relish, indicating a link between exposure to this environmental toxin and the modulation of innate immunity.   Knockdown mutants of Relish are resistant to paraquat (Maitra, et al. 2019).

In our current work, we hypothesized that the toxic effects we observed when fly larvae were exposed to *Aspergillus* VOCs might be mediated in a similar fashion to the NOS and

paraquat experiments, i.e. by the host immune response. Therefore, we repeated our *Aspergillus* VOC exposure experiments using flies that carried mutations in different immune response pathways.

In our experiments, when wild type flies were exposed to *Aspergillus* VOCs, significant developmental delays and death were observed. When flies with a mutation in one of their immune pathways were exposed to same fungal VOCs, the toxic effects were diminished . The absence of toxic effects was particularly striking for the Toll pathway mutants where the flies carrying the spaetzle mutation were similar to unexposed controls in both the timing of metamorphosis and the successful completion of eclosion. In other words, the death of the *Drosophila* flies was not caused by the VOCs themselves, but by an immune pathway reaction in wild type flies that causes damage to *Drosophila*.

As stated earlier, in *Drosophila,* the cellular immune response to pathogenic attack by bacteria and fungi largely is controlled by two signaling cascades , namely the Toll and the immune deficiency (Imd) pathways. These pathways both activate members of the Nuclear Factor Kappa B (NF-kB) family of transcription factors (Shia, et al. 2009). Mutant *Drosophila* flies that lack the ligand, Spaetzle for Toll pathways provide models for understanding the kinds of molecules that can elicit the innate immune response in flies. Because *Drosophila* Toll plays a key role in recognizing fungal infections (Lemaitre and Hoffmann 2007), it seems counterintuitive that our data show that spätzle mutants were more resistant to fungal volatiles than were wild type flies.

The innate immune system is responsible for the initial and rapid host defense as well as mediating inflammatory responses. However, sometimes the immune system overreacts. When overproduced, cytokines (signaling molecules that mediate and regulate immunity and inflammation) can cause damage. It is known that certain oxylipins may be detrimental, possibly by activating a forceful host immune response via an augmented tolerance to oxidative stress (Singh and Del Poeta 2011; Tsitsigiannis, et al. 2005b). In mammals, necrotic cells, in the absence of any microbial input, can provoke detrimental inflammatory responses (Sloane, et al. 2010).

There are data from other systems that show that volatile toxicity associated is associated with the immune system. For example, a human promyelocytic leukemia cell line exposed to volatiles such as toluene, xylene, and dichloromethane displayed gene expression profiles involving both apoptosis-related and immune response genes (Sarma, et al. 2010). In Sprague Dawley rats, benzene targets various components of the immune system (Robinson, et al. 1997). Furthermore, volatile anesthetics are known to attenuate some of the negative effects of cell damage associated with chronic inflammation (Sedghi, et al. 2017). Perhaps most relevant to our studies, however, is the work by Eom et al (2016) on inhalation toxicity of indoor air pollutants in *Drosophila*. The inhalation toxicity of toluene and formaldehyde was investigated using a transcriptomics analyses. Gene ontology (GO) categories of the most significantly differentially expressed genes with exposure to toluene or formaldehyde include "innate immune response" and "response to oxidative stress", and "Toll-signaling pathway" was with exposure to formaldehyde (Eom, et al. 2017). Two immune response-related mutants (imd and ref(2)p) were tested under

the same conditions as wild type flies. As stated by (Eom, et al. 2017) "counter-intuitively, the immune response-related mutants appeared to be unaffected by toluene and formaldehyde."

To date, the overwhelming bulk of research on fly recognition of pathogens has focused on the physical contract of the fly with cell wall components and other macromolecular components of microbial pathogens.   The fact that *Drosophila* flies respond to VOCs shows that much smaller molecules also can be detected.   Which small gas phase molecules are involved in the ability of *Drosophila* to recognize fungi? In mammals, fungal associated molecular patterns are a subset of pathogen-associated molecular patterns ("PAMPS") and known to   include fungal cell wall constituents and intracellular components like DNA(Salazar and Brown 2018). Our data suggest that the small volatile molecules that are characteristic of the odor compound emitted by fungi and other microbes also can be detected as "danger signals" by the *Drosophila* immune system. Volatile organic molecules like 1-octen-3-ol and isobutanol may be functioning as pathogen-associated molecular signals, lead to dysregulation of inflammatory responses and fly toxicity.

In mammals, numerous studies have shown that inappropriate signaling of cytokines in the tumor necrosis factor superfamily can lead to pathologies like septic shock, asthma, rheumatoid arthritis and autoimmunity (Perkins 2007). Moreover, industrial VOCs are known to be associated with negative health effects in human. For example, house painters, who experience chronic exposure to VOCs , show enhanced inflammatory responses (Wieslander, et al. 1997). Given the remarkable conservation of pathways that initiate the

immune response in flies and in vertebrates, we hypothesize that some of the negative health effects in humans that have been attributed to fungal VOCs , often studied under the rubric "sick building syndrome may be due to their impact on the human innate immune response (Medzhitov and Janeway 1998).

We recognize that these data on the effect of volatile compounds on *Drosophila* metamorphosis constitute a descriptive study. We do not know which volatile compound or compounds cause the activity leading to toxicity in wild type flies. Nor is the mechanism of *Drosophila* cell death clear although it may involve the generation of reactive oxygen species (ROS), an immediate and common response to pathogen assault. Our experiments show, nevertheless, that for the flies exposed to VOCs from *A. fumigatus*, possession of a healthy immune system is a liability. We hypothesize that fungal volatiles can serve as molecular signals and trigger the inflammatory process. Although our data are not sufficient to identify the specific mechanisms involved in fly toxicity, they provide guidance for future investigations.

Our work paves the way for future research on the role of volatile phase oxylipins and other VOCs in the pathogenic process. If fungal volatiles are detected by the innate immune systems of susceptible human beings our findings also may provide new hypotheses for studying the symptomology of sick building syndrome. Finally, our *Drosophila* studies reflect the fact that, yet again, research using non-vertebrate model systems can generate new hypotheses that have relevance to human health.

3.4.8 Summary

Our data show that fungal-associated gas phase molecules, in the absence of physical contact with fungal cell wall components or other macromolecular cellular constituents, can delay metamorphosis and cause toxicity in wild type *Drosophila*. This pathogenic effect was less pronounced in flies carrying NOS and Imd mutations and almost absent in flies carrying a mutation in the gene for spaetzle. Because spaetzle is a circulating, cytokine-like endogenous molecule that acts as a ligand to activate Toll receptors and thereby set off an effective immune response, these data imply that the mortality we have observed in wild type flies is associated with an overreaction of the fly innate immune system. Further, these results may have relevance for human conditions associated with immune reactions to fungi such as allergies, asthma and possibly "sick building syndrome." Since the volatiles are detected without physical contact between fungi and flies, our data also suggest that flies can perceive fungal pathogens at a distance through their characteristic volatile emissions.

It is well known that the immune systems can overreact and attack healthy cells. Most studies that demonstrate how the immune system response can backfire, over react, and cause tissue damage have involved physical contact between the host, and the pathogen or its macromolecular cellular components. In this current study, only gas phase molecules are in contact with the *Drosophila* larvae. The ability to detect and respond to gas phase molecules enables flies to sense the presence of potential pathogens at a distance. Our study provides strong evidence for the involvement of the *Drosophila* innate immune system in causing the fly toxicity associated with exposure to fungal volatiles.

ACKNOWLEDGEMENTS

CHAPTER.4 THREE VOLATILE PHASE EIGHT CARBON COMPOUNDS CAUSE DIFFERENT EFFECTS ON SURVIVAL OF WILD TYPE AND MUTANT STRAINS OF *DROSOPHILA MELANOGASTER*.

Abstract

Volatile organic compounds (VOCs) are environmental agents with low molecular mass that exist as gases at room temperature. It has been hypothesized that fungal VOCs have negative effects on human health, causing headaches, gastrointestinal issues, as well as respiratory problems in people exposed to indoor mold odors. Previous work in our laboratory has employed a *Drosophila* bioassay to determine the possible toxicity of fungal VOCs. Here we exposed both wild type and mutant strains of *Drosophila melanogaster* at two concentrations (0.1µL/L and 0.5µL/L) to vapors of 3-octanone, 3-octanol, or 1-octen-3-ol, to determine if mutations in certain immune pathways, and the NOS pathway, would show differential responses to fungal VOCs. Adult male fruit flies of a single strain were exposed to the different VOCs and their viability was monitored for 24 hours.

  All strains showed high susceptibility to 0.5µL/L of 1-octen-3-ol in the span of only two hours. Volatile phase 1-octen-3-ol and 3-octanone at 0. 5µL/L were toxic to all the strains after 24 hours. Oregon [R] (red-eyed, wild type) strain was less susceptible than wild type, white-eyed strain (W[1118]) to all three compounds at both concentrations. Of the three compounds tested, 3-octanol at 0. 1µL/L was the least toxic to mutant and wild type strains. The NOS mutant was more resistant to VOC exposure than was the white eyed strain carrying no defects in the immune system The Relish [E20] white-eyed mutant strain was more susceptible than the spz[6] red-eyed mutant strain to volatile phase 1-octen-3-ol at 0.1µL/L. Unexpectedly, the double mutant strain (red-eyed Relish [E20] spz[6)]) showed greater resistance to the presence of the volatile phase compounds than did either single mutant or the wild type, white-eyed strain (W[1118]). The implications of these findings are discussed.

4.1 Introduction

Volatile organic compounds, also known as VOCs, are compounds with low molecular mass and high vapor pressures that exist in gas form when in room temperature conditions. VOCs produced by molds give damp indoor areas their trademark musty smell. Fungi emit mixtures of many kinds of VOCs that vary with the temperature, moisture, pH and other environmental conditions of cultures, as well as the species and strain of fungus (Morath, et al. 2012). Many fungal VOCs have strong odors and can be easily recognized by humans. It has been hypothesized that fungal VOCs have negative effects on human health, possibly contributing to a condition known as the "sick building syndrome" (Ansarin, et al. 2013; Burge 2004; Chambers, et al. 2016; Cooley, et al.

Figure 4. 1. The line-angle representation of the three volatile organic compounds (VOCs) used in this research. **A.** 3-octanone, **B.** 3-octanol, and **C.** 1-octen-3-ol

1998; Faber, et al. 2015). Sick building syndrome involves headaches, gastrointestinal issues, as well as respiratory problems. Symptoms affecting the nervous system have also been reported by people exposed to fungal VOCs for prolonged periods of time (Burge 2004; Kilburn 2004; National Academies of Sciences and Medicine 2017).

Most studies on exposure to toxic environmental volatiles have focused on the effects of industrial chemicals on human health rather than on biogenic agents. It is well

known that inhalation of certain industrial compounds in the gas phase has demonstrable toxicity. Furthermore, dermal uptake of VOCs in indoor environments is also possible (Bennett and Inamdar 2015; Hung, et al. 2013; Inamdar, et al. 2012). The link between fungal metabolites and their possible detrimental effects on human health indoor environments remains unclear(Heseltine and Rosen 2009).

The VOCs of fungi occur in different chemical classes such as acids, alcohols, aldehydes, esters, ethers, hydrocarbons, ketones, sulfur, and terpene compounds (Korpi, et al. 2009b). These metabolites allow fungi to communicate with one another and respond flexibly to environmental pressures (Leeder, et al. 2011). Eight carbon volatiles are particularly characteristic of fungal metabolism (Combet, et al. 2006).

Our laboratory has developed a bioassay using larval or adult *Drosophila* flies to measure the toxicity of different fungal VOCs (Bennett and Inamdar 2015; Inamdar, et al. 2012; Inamdar, et al. 2014). Moreover, the *Drosophila* model has been used widely in other studies researching the underlying mechanisms of human diseases (Tolwinski 2017b). Here, we have focused on the impact of VOCs on adult *Drosophila* flies carrying different mutations in the fly immune system. We tested the effect of three volatile-phase, eight carbon compounds characteristic of fungal metabolism: 1-octen-3-ol (alcohol), 3-octanol (alcohol), and 3-octanone (ketone) (Figure 4. 1). We analyzed how exposure to these VOCs impacted the viability of adult male flies carrying different mutations in immune pathways and compared them to the red eyed and white eyed wild type flies without defects in their immune pathways. We hypothesized that different fungal VOCs with known toxicity in the *Drosophila* bioassay would have different impacts on wild types and mutant strains.

Unexpectedly, we found that flies carrying two immune pathway mutations were more resistant to the volatiles than were the single mutants.

4.2 Materials and Methods

4.2.1 Drosophila culture media and maintenance

Six strains of *Drosophila* flies were obtained from the Bloomington *Drosophila* Stock Center (BDSC) at Indiana University. All strains are listed in Table 4.1. The NOS and Relish[E20] (Imd) mutant strains carried the white-eyed genotype. The spz[6] (Toll) and double mutant Relish[E20] spz[6] strains carried the red-eyed genotype. *Drosophila* stocks were maintained in Ward's Instant *Drosophila* medium (WARD's Natural Science, NY). About every two weeks, the flies were transferred to fresh 6 oz *Drosophila* stock bottles (Genesee Scientific, CA) so that there was an adequate supply of breeding flies and a renewal of space. To prepare egg-laying medium, 376 ml of distilled water, 126 ml of grape juice, 15 g of agar, and 6 g of sucrose were used. These ingredients were mixed, put into a 2-L flask, microwaved on high for 10 minutes until the agar was dissolved, autoclaved, and cooled to 60-65 ^{o}C before 10 ml ethanol and 5 ml acetic acid were added. Finally, the medium was mixed thoroughly and poured into Petri plates.

Table.4.1 *Drosophila* wild type and mutant strains used in this study.

ID number at BDSC	Type of mutation	Genotype	Reference
4783	Wild type, white-eyed strain	y1, w1118, a yellow body and white-eyed strain	(Inamdar, et al. 2013)
24283	NOS (nitrate oxide synthase), white-eyed	w [1118]; Mi{ET1}Nos[MB04018]	(Inamdar, et al. 2013)
9457	Relish [E20] gene to induce Imd pathway, white-eyed	w[1118]; Rel[E20] e[s]	(Kambris, et al. 2006)
5	Red-eyed, wild type	Oregon-R-C	(De Gregorio, et al. 2002)
10719	Spz[6] gene to induce Toll pathway, red-eyed strain	w[1118]; PBac{w[+mc]=PB}spz6[c01763]	(Kambris, et al. 2006)
55718	Relish[E20] &spz[6], double mutant at both pathways, red-eyed strain	Rel[E20] spz[4]/TM6C, Sb[1] Tb[1]	(De Gregorio, et al. 2002)

4.2.2 Eggs collection and flies mating

Eggs were collected by transferring mature adult flies onto Ward's Instant *Drosophila* Medium. The adult flies were left together for 3 hours to give them time to

125

mate. Afterwards, the flies were transferred back to their original bottles with the Ward's

Instant *Drosophila* Medium.   Dried yeast, weighing about 0.06 g, was added to each

bottle containing the eggs. Any dead flies remaining in the egg-collection bottles were

removed using thin, soft-tipped art brushes that prevented the accidental removal of eggs.

Egg-collection bottles then were stoppered with a cotton stopper and placed into an

incubator at 25°C for 12- 14 days to allow the eggs to undergo metamorphosis and

eclosion. After the first day of eclosion into adults, the flies were incubated for an additional

48 hours to allow the sexual dimorphic features of adult flies to mature so as easily differentiate

between the males and the females. At the end of the 48-hour period, using CO_2 to

incapacitate the flies temporarily, the males were segregated from the females using

phenotypic sex- indicators such as the sex-comb in males and long, white abdomens in

females (Figure 4.2). Only males were used due to the female *Drosophila*'s increased

resistance to VOCs (Lemaitre, et al. 1996a; Lemaitre, et al. 1997) .
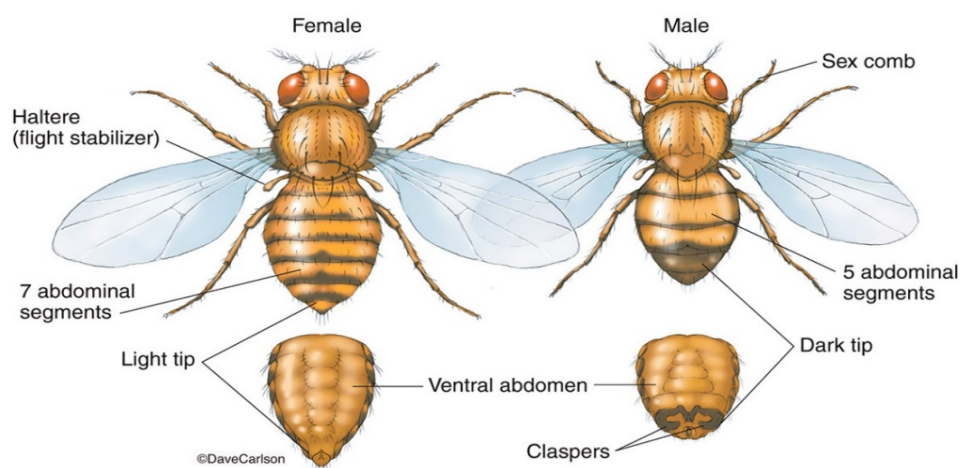
Figure 4.2 Fruit fly - genus *Drosophila*: Illustration comparing the male and female fruit fly *https://www.carlsonstockart.com/photo/fruit-fly-drosophila-male-female-illustration/* , 03/2020

4.2.3 Chemical Standards of Volatile Compounds

Three chemical standards of eight carbon compounds characteristic of fungal metabolism were used, all obtained from Sigma-Aldrich: 1-octen-3-ol (**98%**, MW: 128.21 g/mol), 3-octanone (**≥97%**, MW: 128.21 g/mol), and 3-octanol (**≥98%**, MW: 130.23 g/mol).

Two concentrations for each of these chemicals were tested in the *Drosophila* bioassay, 0.1 µl/L and 0.5 µl/L. The formula below was used to calculate the amount of liquid chemical standard needed to deliver the appropriate concentration of VOC for each of the compounds tested. The volume of the vials used was determined by filling the vial up with water and then measuring out the total amount of water in the vial with a graduated cylinder. The total volume of each vial was 35mL or 0.035L. The volume needed for 3-octanone was converted using the following calculations:

$$P = 1\text{atm}, V=0.035\text{L}, R=0.08206 \frac{L \times atm}{mol \times K}, T= 298\text{K}$$

The same calculations were used to calculate the necessary amount of VOC needed for each experiment. For 3-octanone, we calculated 0.545µL, for 3-octanol 0.54µL, and for 1-octen-3-ol 0.55µL. A volume of 0.1µL was also used for every VOC to compare the toxicity of the volatiles at a lower volume.

4.2.4 Exposure of Adult Flies to VOCs

Each vial contained a thin layer (3 mL) of *Drosophila* medium, a cotton ball with the appropriate volume of VOC, and a cotton stopper. The *Drosophila* media was made by adjusting the original recipe of 1.00g of agar with 5.00g of sucrose and 100mL of

H_2O to the appropriate ratio for necessary number of vials. Ingredients were put into an Erlenmeyer flask with a magnetic stirring rod and heated until the medium was clear. Vials and media were then autoclaved following the standard protocol. After the vials and media were autoclaved, 3mL of media was transferred into each vial and left on a rack to cool and solidify. After the vials had cooled, the inside of the vial was dried with sterile cotton, and a clean cotton ball was attached with a piece of tape. A sterile cotton stopper was used to stopper the vial (Figure 4.3.a) (Inamdar, et al. 2014) with some modification.

Adult flies were knocked out using CO_2. Exposures were conducted according to the method of Inamdar et al., 2012 with modification. Before putting the flies into the vial, they were re-checked to make sure only males were used for the experiment. After 10 adult males were put into a vial, the appropriate VOC volume was pipetted onto the cotton ball and quickly stoppered. Each vial was then labeled with a piece of scotch tape and two layers of parafilm were wrapped around the stopper to prevent further evaporation of the VOCs (Figure 4.3.b). The vials were placed on a tube rack in an incubator at 25°C rotated at 50 rpm. Vials were checked every two hours for a period of 24 hours. The number of dead flies was recorded.
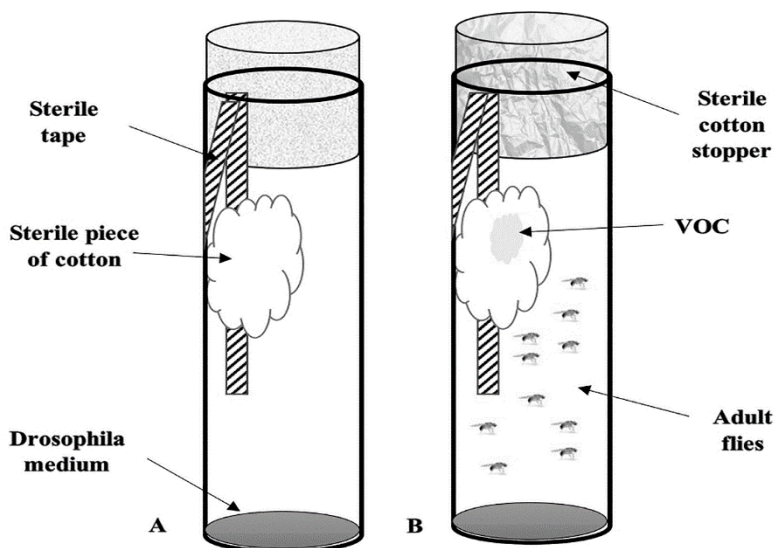
Figure 4.3. Exposure vials set-up. **a.** Piece of cotton inside vial where VOC is to be added is taped (striped block) with tape sticking to inside and outside of vial. **b.** Adult male *Drosophila* were added to vial along with measured VOC. Vials were then labeled and wrapped with double parafilm to prevent further evaporation of VOC.

Statistical analysis

The significant differences in viability after 24 hours were calculated between controls and flies exposed to different VOCs by using the student *t-test* . More details are presented in the figure legends.

-

4.3 Results

4.3.1 Effects of 1-octen 3-ol, 3-octanone, and 3-octanol on different strains of the fruit fly at different amounts of compounds

The effect of exposure to the vapor phase of two concentrations (0.1 µl/L and 0.5 µl/L) of 1-ocen-3-ol, 3-octanone, and 3-octanol on viability of adult on white eyed *Drosophila* flies that were otherwise wild type (Strain W[1118], see Figure 4.4A, B) , and on white eye flies carrying a mutation in the gene for nitric oxide synthase (NOS) (Figure 4.5A,B). *Drosophila* possesses a single NOS gene (dNOS) that exhibits 47% sequence similarity to mammalian neuronal NOS(Regulski and Tully 1995). The dNOS gene is involved in diverse biological processes including host immune response (Coleman 2001). Earlier work in our laboratory had demonstrated that exposure to volatile phase 1-octen-3-ol induced a nitric oxide (NO) a mediated inflammatory response in hemocytes, *Drosophila* innate immune cells suggesting a role for inflammation in VOC-mediated toxicity (Inamdar and Bennett 2014).
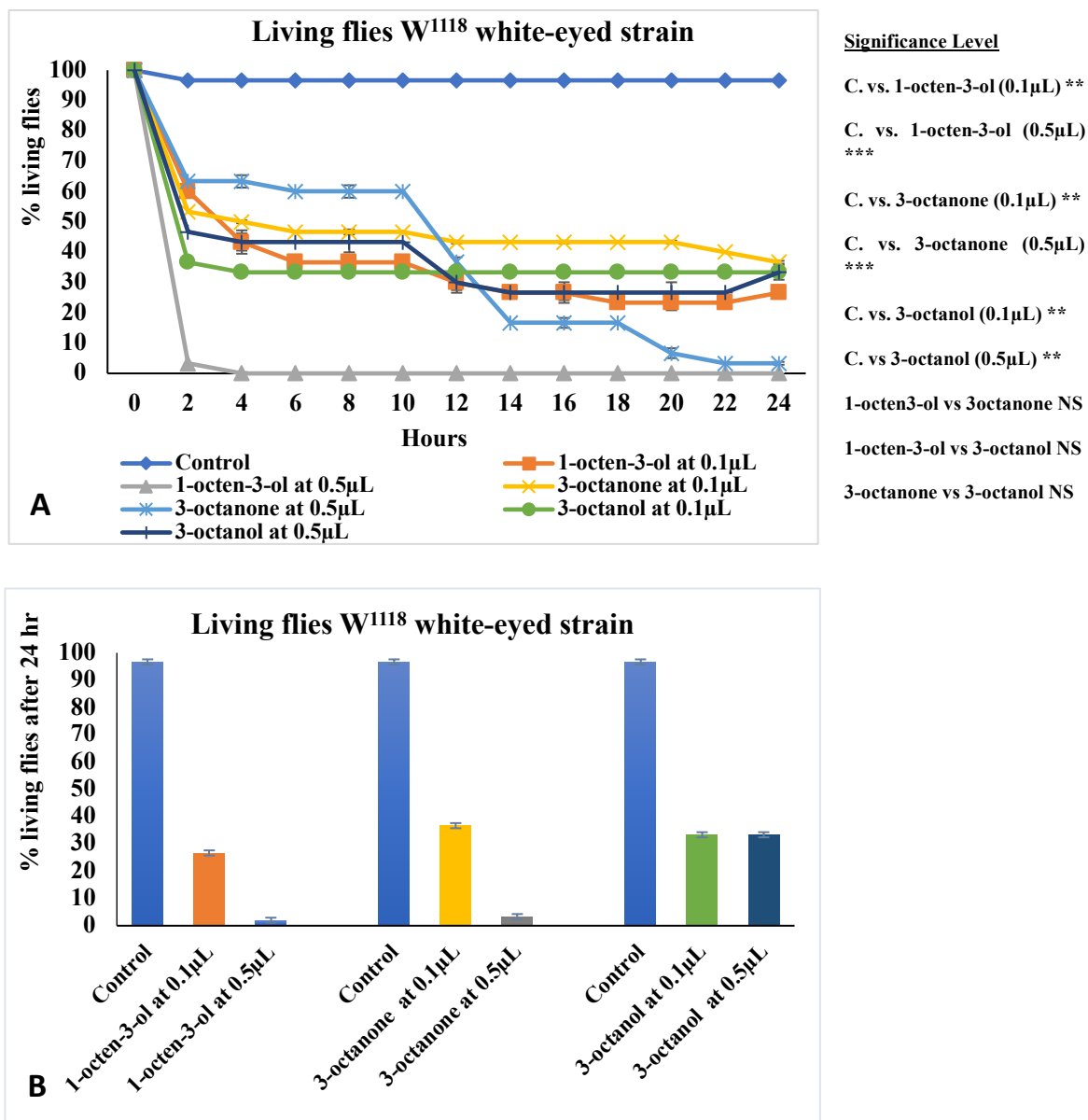
Figure 4.4. Viability of adult wild type, white-eyed W[1118]strain (N=30) after exposure to gas phase 1-octen-3-ol, 3-octanone, and 3-octanol at either 0.1 or 0.5 µL over 24 hours. **A)** Percent viability determined at two hours intervals over 24 hours. **B)** Percent viability after 24 hours where * p<0.05, ** p<0.005, and *** p<0.0005. NS stands for non-significant.
.

All of the white eyed wild type flies were far more sensitive to vapors of 0.1 µL and 0.5

µL of 1-octen-3-ol, 3-octanone, and 3-octanol than were the wild type red eyed flies

Oregon[R] (Compare Figure 4.4A for white eyed flies with Figure 4.7A for red eyed flies).

Within four hours, all white eyed wild type flies exposed to vapors of 0.5 μL 1-octen-3-ol were dead (Figure 4.4A). At the end of 24 hours, more than 50% of flies were dead after exposure to all three volatile compounds at both concentrations (Figure 4.4B).
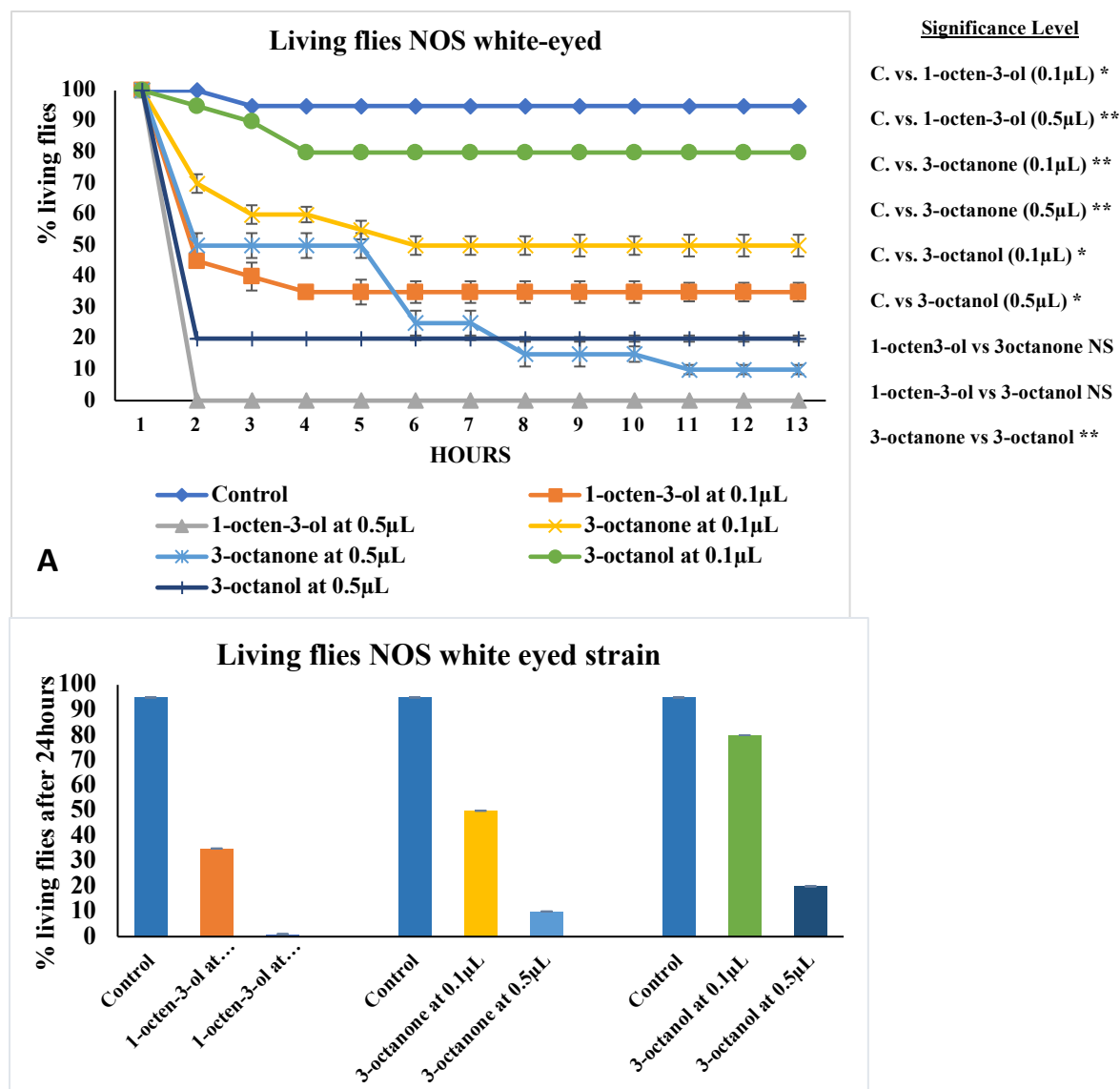
Figure 4.5. Viability of adult mutant m[24283] strain (N=30) after exposure to gas phase 1-octen-3-ol, 3-octanone, and 3-octanol at either 0.1 or 0.5 μL over 24 hours. **A)** Percent viability determined at two hours intervals over 24 hours. **B)** Percent viability after 24 hours where * $p<0.05$, ** $p<0.005$, and *** $p<0.0005$. NS stands for non-significant.

White eye flies with the introduction of the heterozygous NOS marker (*Drosophila* Strain m^{24283}), flies were more resistant to vapors of the three compounds. After 4 hours, 15% of NOS mutants exposed to 0.5 µL of 1-octen-3-ol were still alive; improvements in viability also were exhibited in the presence of 0.1 µL and 0.5 µL of 3-octanone. At the end of 24 hrs., for strain W^{1118}, 37% of flies exposed to 0.1µL of 3-octanone were alive, but 50% of those carrying the m^{24283} marker had survived (Figure 4.5-A-B). The greatest differential between the white eyed w^{1118} and the heterozygous NOS mutant m^{24283} was for exposure to 3-octanol at 0. 1µL, where the strain carrying the NOS mutation displayed 80% survival after 24 hrs as compared to less than 20% for the wild type white eyed flies (See Figures 4.4A and 4.4B).

In order further to investigate the effect of immune pathway defects on the response to volatile phase 1-octen-3-ol, 3-octanone and 3-octanol, exposure studies were continued with several additional *Drosophila* immune pathway mutants. *Drosophila* Strain Relish [E20] has white eyes and a mutation in the Imd pathway of the fly humoral immune response pathway. The same experimental protocol for exposure of adult flies was repeated against Strain Relish [E20].

These data are presented in Figure 4.6 A and B. Exposure of *Drosophila* strain white-eyed Relish [E20] to the three volatiles at the two concentrations gave similar viability data as those obtained for the control white eyed W^{1118}.However, in a few cases the mutant strain exhibited more resistance to the presence of volatile compounds than did the wild-type white-eyed train (W^{1118}). Exposure to the two concentrations of 1-octen-3-ol yielded the greatest loss of viability with 100% death after 24 hours for 0.5 µL and more than 95%

death after exposure to the lower concentration of 0.1 µl/L. (Figure 4.6A, B). Exposure

to vapors of 0.5 µL of 3-octanone also yielded 73% mortality after 24 hrs. The increased

reistance of white-eyed Relish[E20] was most apparent with 0.1 µL of 3-octanone and 3-

octanol. At 0.1 µL of 1-octen-3-ol, no difference in viability was observed between W[1118]

and Reslish [E20]. Overall, Relish [E20] was more similar to Wild type W[1118] than was the strain

carrying the NOS mutation.

Living flies Relish ^{E20} white-eyed mutant

% living flies

HOURS

Control — 1-octen3-ol at 0.1μL
1-octen-3-ol at 0.5μL — 3-octanone at 0.1μL
3-octanone at 0.5μL — 3-octanol at 0.1μL
3-octanol at 0.5 μL

A

Significance Level

C. vs. 1-octen-3-ol (0.1μL) **

C. vs. 1-octen-3-ol (0.5μL) ***

C. vs. 3-octanone (0.1μL) **

C. vs. 3-octanone (0.5μL) **

C. vs. 3-octanol (0.1μL) *

C. vs 3-octanol (0.5μL) **

1-octen3-ol vs 3octanone **

1-octen-3-ol vs 3-octanol *

3-octanone vs 3-octanol NS

Living flies Relish^{E20} white-eyed mutant strain

% living flies after 24 hr

Control, 1-octen3-ol at 0.1μL, 1-octen-3-ol at 0.5μL, Control, 3-octanone at 0.1μL, 3-octanone at 0.5μL, Control, 3-octanol at 0.1μL, 3-octanol at 0.5 μL

B

Figure.4.6 Viability of adults double mutant (Relish^{E20}&spz^6) red-eyes strain (N=30) after exposure to gas phase 1-octen-3-ol, 3-octanone, and 3-octanol at either 0.1 or 0.5 µL over 24 hours. **A)** Percent viability determined at two hours intervals over 24 hours. **B)** Percent viability after 24 hours where * p<0.05, ** p<0.005, and *** p<0.0005. NS stands for non-

Similarly exposure experiment of the effect of the vapor phase of two concentrations (0.1 µL and 0.5 µL) of 1-ocen-3-ol, 3-octanone, and 3-octanol on viability of adult red eyed adult *Drosophila* (Oregon[R]) over 24 hours is given in Fig. 4.7A. Vapors of 1-octen-3-ol and 3-octanone at the higher concentration (0.5 µL) were toxic to the flies. Within the first two hours of exposure to 0.5 µL of 1-octen-3-ol, only about 20% of the flies were viable. For 1-octen-3-ol at the lower concentration of 0.1 µL, 90 % of the flies were alive within 2 hours of exposure, and at the end of 24 hours, about 75% were viable. Similarly, 3-octanone vapors at 0.5 µL yielded a sharp decrease in viability after two hours of exposure; only about 35% of the flies were viable after 24 hrs. However, 3-octanone vapors were less toxic at the lower concentration. Exposure to vapors of 3-octanol had few effects on viability at either concentration.

The viability data for *Drosophila* (Oregon[R]) are summarized in bar graph form in Figure 4.7 B. At 24 hrs., 97% of control flies were alive but only 17% of those exposed to 1-octen-3-ol at 0.5 µL were still viable. Of flies exposed to 0.5 µL 3-octanone and 3-octanol, respective viabilities at 24 hours were 33% and 83% (Figure 4.7 B). At the lower concentration, at 24 hrs, approximately 70 % of the flies exposed to 1-octen-3-ol at 0.1 µL were still viable, as were over 80% of the flies exposed to 3-octanone at 0.1 µL (Figure 4.7 B).
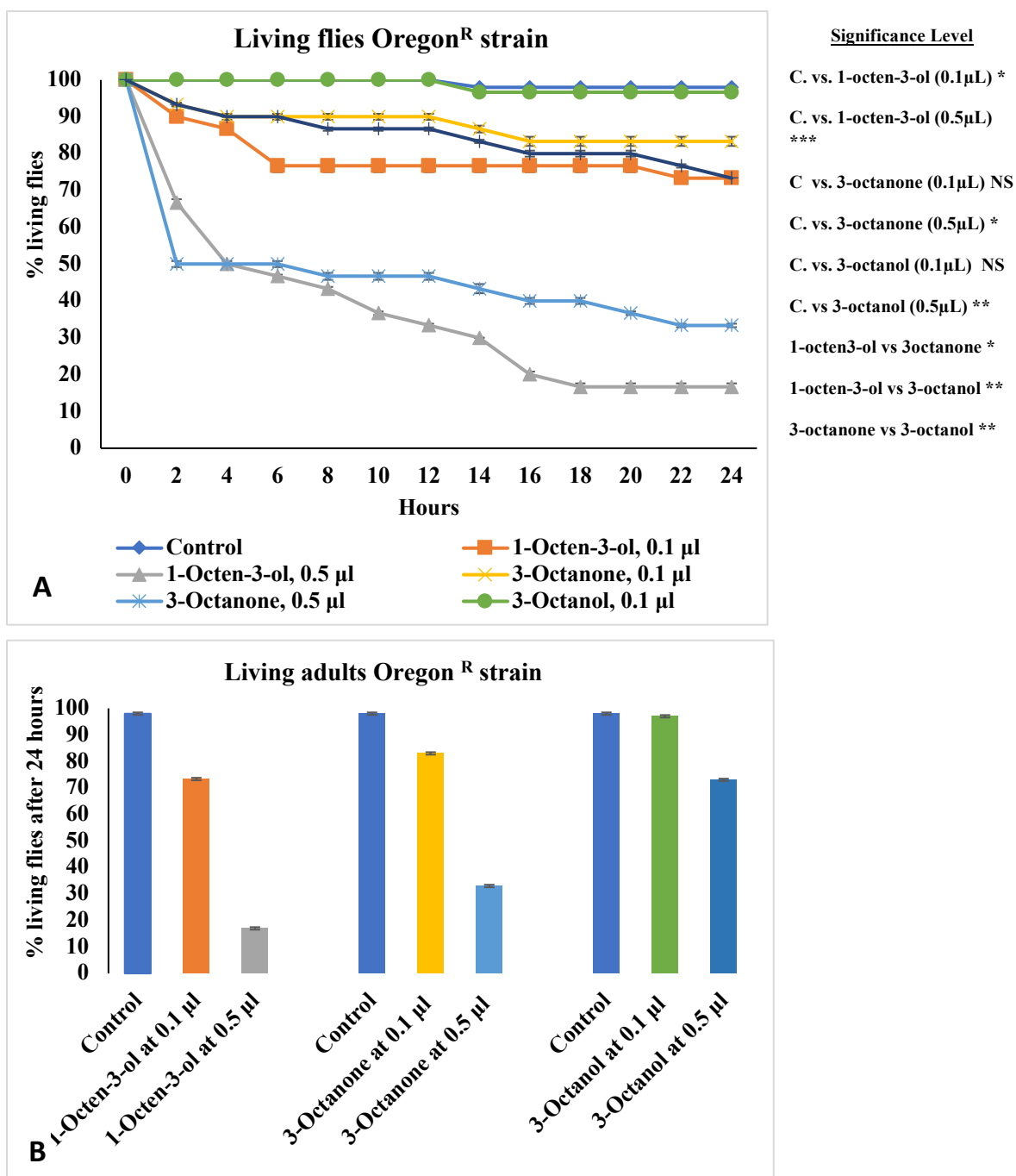
A mutant fly with a different defect in the *Drosophila* immune response was used for the next set of experiments. *Drosophila* Strain 10719 (spz [6] red-eyed) carries a mutation in the Toll pathway of the humoral immune response. The spz[6] mutant flies (red-eyed), like the Relish [E20] strain (white eyed), was more susceptible to 0.1 µL of 1-octen-3-ol, but less susceptible to 0.1 µL of 3-octanone than is the wild type white eye fly W[1118] (Figure 4.8A , B). It is more susceptible to the effects of volatile compounds compared to the red eyed wild type (Oregon [R]) strain. As is the case in the other exposure studies, 1-octen-3-ol was the most toxic compound tested (Figure 4.8-A). The control at hour 24 had 10% mortality while there was 100% mortality for the mutant exposed to both 0.5 µL of both 1-octen-3-ol and 3-octanone, with 67% for 0.5 µL 3-octanol. For the lower amount of 1-octen-3-ol viability was 73**%**; for 3-octanone was 33%; and for 3-octanol 50% (Figure 4.8 B).
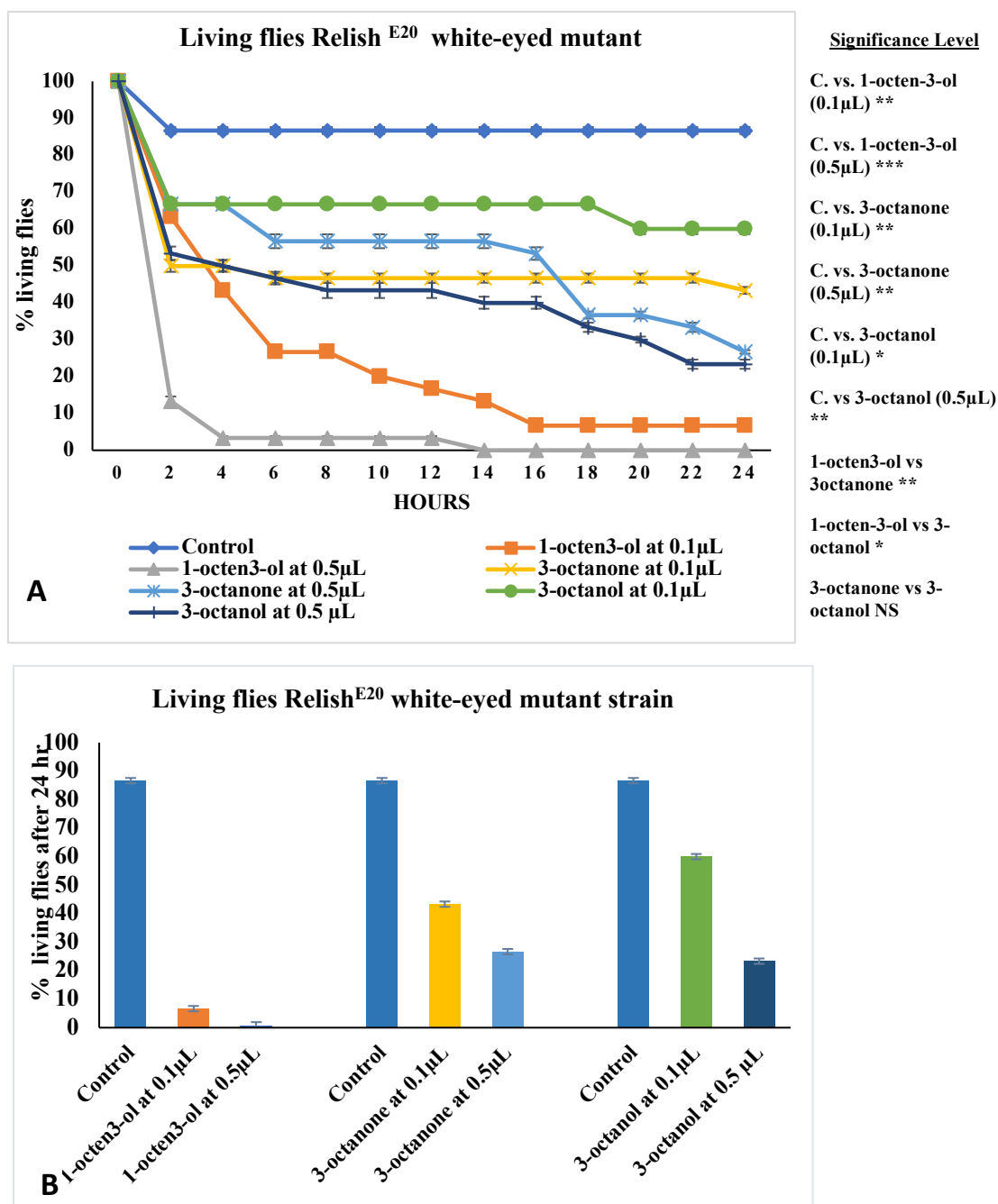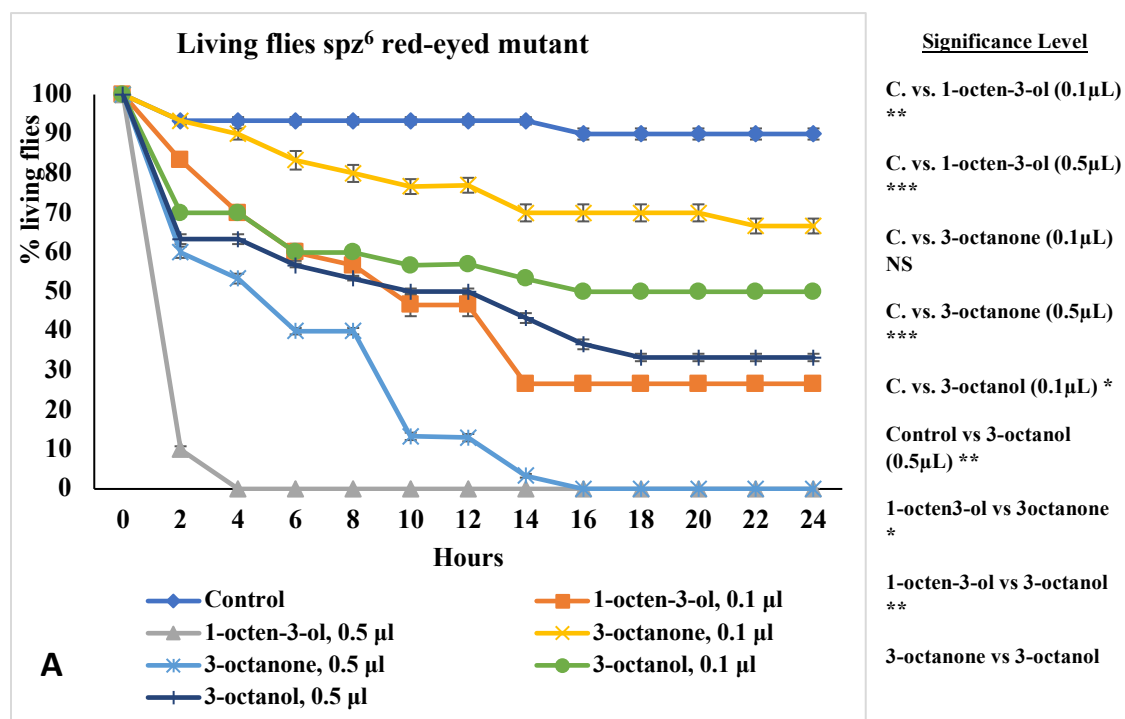
Figure 4.8 Viability of adult mutant spz^6 red-eyed strain (N=30) after exposure to gas phase 1-octen-3-ol, 3-octanone, and 3-octanol at either 0.1 or 0.5 µL over 24 hours. **A)** Percent viability determined at two hours intervals over 24 hours. **B)** Percent viability after 24 hours where * p<0.05, ** p<0.005, and *** p<0.0005. NS stands for non-significant.
.

The most interestingly data were obtained with the double mutant *Drosophila* strain 55718(spz^6 and Relish ^{E20} red-eyed strain). This strain was significantly less susceptible to the toxic effects of the three volatile compounds mortalities than were the other wild type and mutant strains. It was the single most resistant strain of those tested (Figure 4. 9-A).

 Although, like the other strains, it was susceptible to 1-oceten-3-ol at the higher concertation of 0.5 µL, at the lower concentration of 0.1 µL, it was the single most resistant strain tested. After 24 hrs, lethality was 43% at 0.5 µL of 3-octanone; 30% for   0.1 µL

of 3-octanone; and 17% for 0.1 μL 1-octen-3-ol; 13% for 0.5 μL of 3-octanol; and finally,

3% at 0.1 μL 3-octanol (Figure 4. 9-B).

Significance Level

C. vs. 1-octen-3-ol (0.1μL) **

C. vs. 1-octen-3-ol (0.5μL) ***

C. vs. 3-octanone (0.1μL) **

C. vs. 3-octanone (0.5μL) **

C. vs. 3-octanol (0.1μL) NS

C. vs 3-octanol (0.5μL) *

1-octen3-ol vs 3octanone **

1-octen-3-ol vs 3-octanol ***

3-octanone vs 3-octanol *
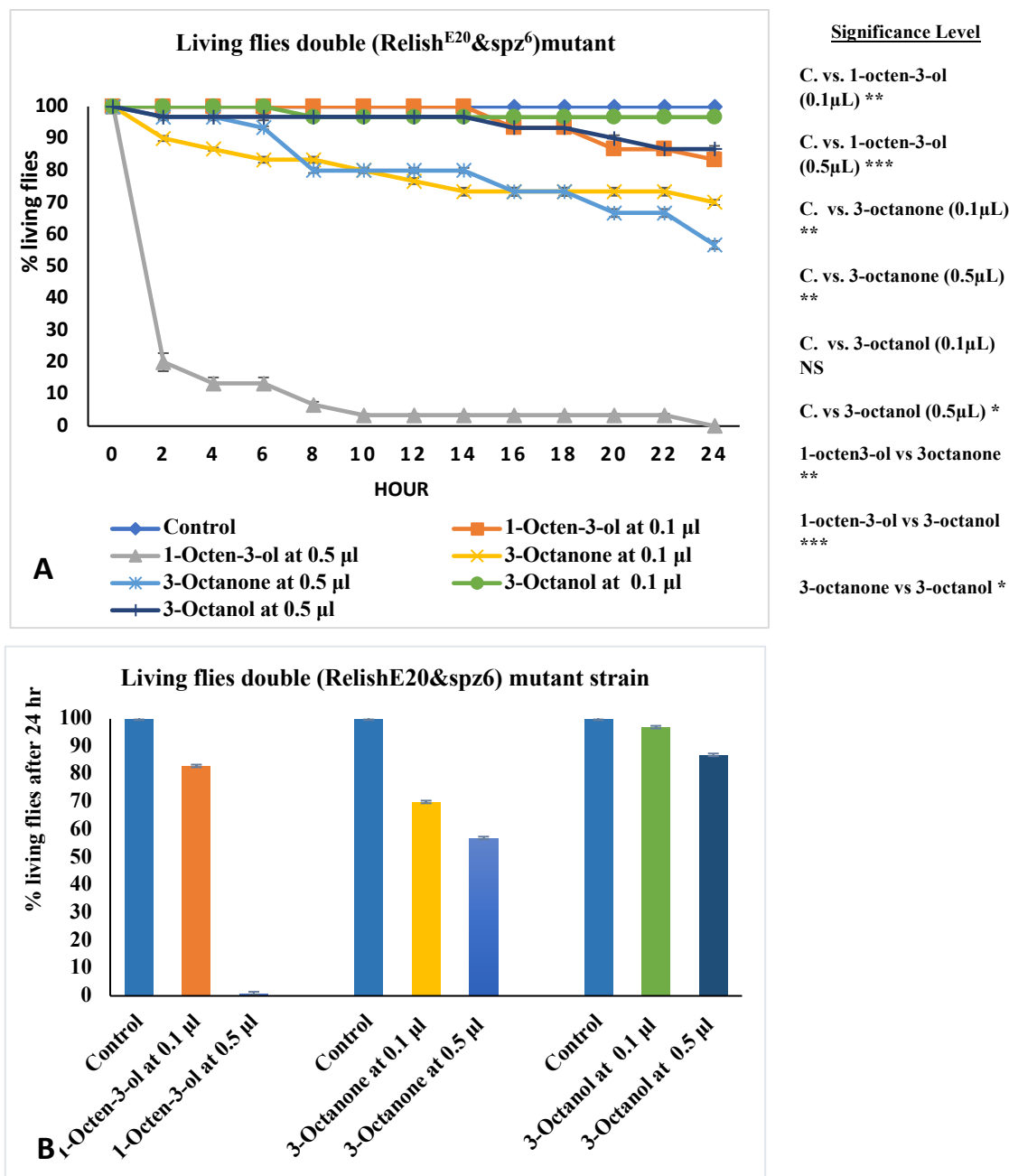
Figure.4.9 Viability of adults double red-eyed mutant (Relish^{E20}&spz^6) strain (N=30) after exposure to gas phase 1-octen-3-ol, 3-octanone, and 3-octanol at either 0.1 or 0.5 μL over 24 hours. **A)** Percent viability determined at two hours intervals over 24 hours. **B)** Percent viability after 24 hours where * $p<0.05$, ** $p<0.005$, and *** $p<0.0005$. NS stands for non-significant.

Data comparing the viability rate of the six strains, for each of the three compounds, at both concentrations, are presented in bar graph form in Figure 4.10 A, B, C.   These summary data show that 1-octen-3-ol was the most toxic of the three volatiles tested and that 3-octanol was the least toxic. Furthermore, these data also illustrate that the double mutant strain carrying defects in both the Imd and Toll pathways is more resistant to the toxic effects of the volatiles than are the single mutants and the wild type.

A
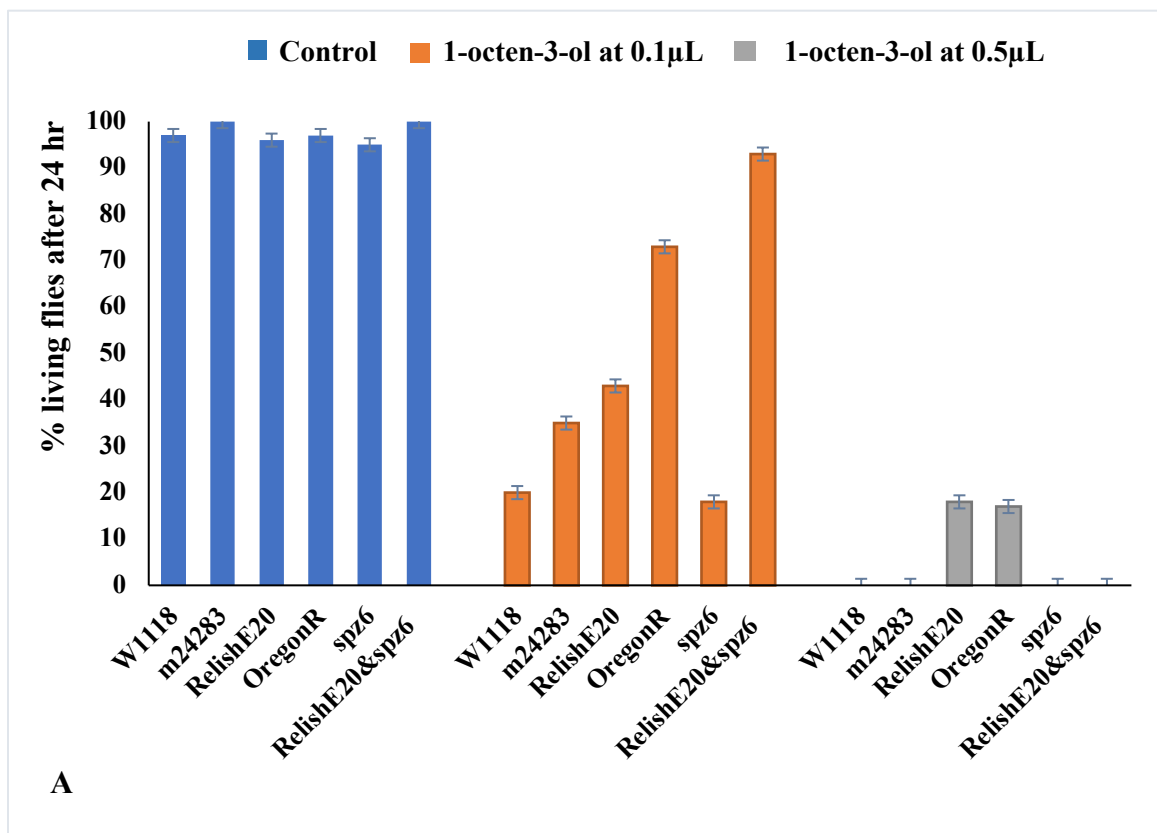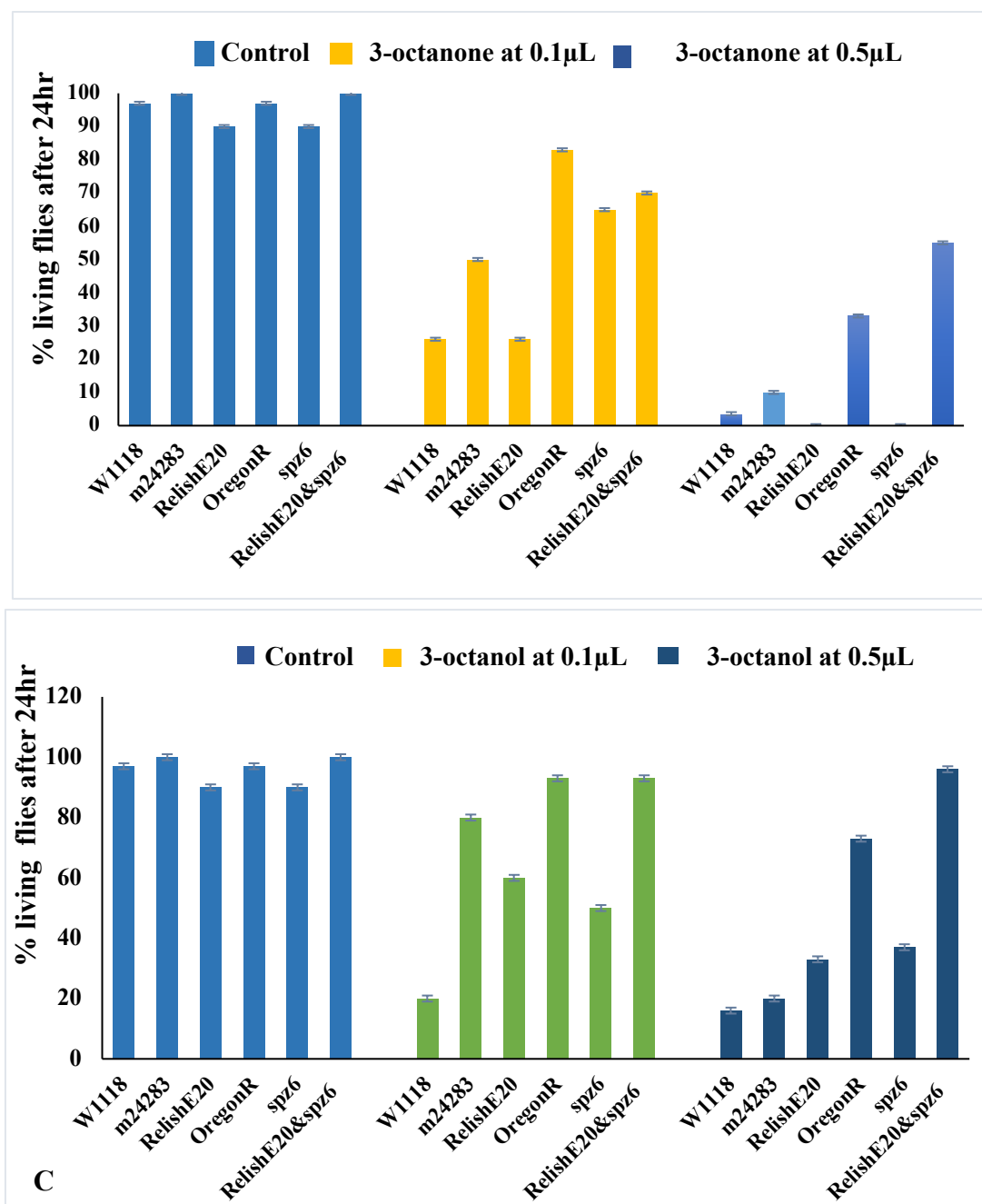
Figure 4.10. Viability of White -eyed wild-type (w^{1118}), wild type, red-eyed Oregon^R, ,NOS (m^{24283} white-eyed), Imd pathway(Relish ^{E20} white-eyed), Toll pathway(spz^6 red-eyed), and the double mutant (spz ^{6} and Relish ^{E20} red-eyed) strains (N=30) after exposure to gas phase at either 0.1 or 0.5 µL over 24 hours. **A)** percent viability of flies determined after24 hours upon exposure to 1-octen-3-ol. **B)** percent viability of flies determined after 24 hours upon exposure to 3-octanone. **C)** percent viability of flies determined after 24 hours upon exposure to 3-octanol.

4.4 Discussion

Using *Drosophila melanogaster* as an experimental model in previous studies led to think about the relationship of the immune system and the potential survival of flies when exposed to different VOCs. *Drosophila melanogaster* possesses the capacity for cellular and humoral immune responses as part of its innate immunity system (Limmer, et al. 2011). The cellular immune response is controlled by plasmocytes, lamellocytes, and crystal cells found in the body cavity of fruit flies. When *Drosophila* flies are invaded by microorganisms, these cell types increase in number at the site of infection in larvae and adults (Kocks, et al. 2005; Philips, et al. 2005; Rajak, et al. 2016; Shia, et al. 2009).

Nitric oxide (NO) is a fundamental signaling agent which serves as a potent mediator of inflammation and cytotoxicity(Coleman 2001; Pacher, et al. 2007). The oxidation of L-arginine into citrulline and NO is mediated by the enzyme nitric oxide synthase (NOS) in the presence of NADPH and other cofactors (Mayer 1994). The NO signaling pathway is well conserved among many different species. In *Drosophila*, there is a single NOS gene (dNOS) that shares 47% sequence similarity to mammalian neuronal NOS (Regulski and Tully 1995; Stasiv, et al. 2001). dNOS activity is reported in fly brains, airway linings and other tissues (Muller 1994). Like mammalian NOS, in addition to the immune response, dNOS regulates diverse biological processes (Coleman 2001; Mukherjee, et al. 2011; Roeder, et al. 2009).

The second part of immune response is called the humoral immune response, which is activated by three different pathways: the immune deficiency pathway (Imd), the Toll pathway, and the JAK/STAT (Janus kinase/signal transducer and activator of transcription) pathway (Shia, et al. 2009). The Toll pathway accounts primarily for the response to

infections by fungi and gram-positive bacteria (Rutschmann, et al. 2002), while the IMD pathway is primarily, although not exclusively, involved in defense against Gram-negative bacteria, and most of the antimicrobial peptides controlled by this pathway have detrimental activity directed towards gram-negative bacteria (Hoffmann 2003b).

In earlier work using a *Drosophila* model, we demonstrated truncated life span and neurotoxicity associated with exposure to 1-octen-3-ol, the volatile organic compound (VOC) responsible for much of the musty odor found in mold-contaminated indoor spaces (Inamdar et al, 2010; Inamdar et al, 2012). A Swedish study using human volunteers showed that 2 hr. exposure to low concentrations of volatile phase 1-octen-3-ol induced activation of the inflammatory markers in nasal secretions (Roeder, et al. 2012; Wålinder, et al. 2008). Using the *Drosophila* model for viability assays complemented with biochemical and immunological assays, we showed that exposure low concentrations of vapor phase 1-octen-3-ol induced a nitric oxide (NO) mediated inflammatory response in hemocytes, *Drosophila* innate immune cells. A similar effect was observed with vapor phase toluene but not 1-butanol. Exposure to 1-octen-3-ol also induced NOS expression in larval tracheal tissues (Inamdar and Bennett 2014). However, when flies carrying heterozygous mutants for NOS, or flies tested with pharmacological inhibitors of NOS (L-NAME, D-NAME and minocycline) were exposed to low concentrations of volatile phase 1-octen-3-ol, they showed improvements in life span as compared to wild type flies. These findings suggested a possible mechanistic basis for some of the reported adverse health effects attributed to mold exposure and demonstrated the utility of this *in vivo Drosophila*

model to complement other genetic model systems for understanding the role of inflammation in VOC-mediated toxicity.

In this chapter, I have investigated the possible inverse association between the toxicity of fungal volatile metabolites and defects in the *Drosophila* immune system. Chemical standards of three eight carbon VOCs associated with moldy odors were used: 1-octen-3-ol (alcohol), 3-octanol (alcohol), and 3-octanone (ketone) (Figure 4.1). I analyzed how exposure to two concentrations (0.1µL/L and 0.5µL/L) of these VOCs impacted the viability of adult, male *Drosophila* strains carrying different genetic backgrounds. Each mutant strain had a different defect in the fly innate immune pathway.

I used two strains that had wild type immune systems, the red eyed Oregon^R and the white eyed strain W^{1118}. The red-eyed wild type Oregon^R flies were more resistant to the toxic effects of the three volatiles tested than were the white-eyed wild type flies *W^{1118}*. The white eyed strain served as the control for the NOS mutant strain which is heterozygous for a NOS pathway mutation *(m^{24283})*, and the single Imd pathway mutant (W^{1118,} Relish^{E20}). The red eyed strain served as the control for the single Toll pathway mutant (spz^6); and the red-eyed double Imd-Toll mutant (spz^6 and Relish^{E20}).

Compared to wild type white eyed W^{1118}, the NOS flies were more susceptible to the effects of all three volatiles at both concentrations, except in the first hours of exposure to 0. 5µL of octanone. The viability data for the single Imd mutant Relish^{E20} as compared to wild type W^{1118}, were varied. It more susceptible to 0. 1µL 1-octen-3-ol, and less

susceptible to 0. 1μL 3-ocanone than was wild type W[1118]. The Strikingly the double mutant, Oregon[R] spz[6] and Relish[E20], was significantly more resistant than were either single mutant or the red-eyed, wild type Oregon[R]

Exposure to environmental agents such as industrial chemicals, mycotoxins and VOCs is known to have negative effects on the human nervous system and fruit fly hemocytes by causing neurogenic inflammation (Carton, et al. 2009b; Foley and O'Farrell 2003; Laskin, et al. 2011b; Meggs 1993). It is well known that different VOCs such as 2-methyl-1-propanol, 2-methyl-1-butanol, 3-methyl-1-propanol, 1-octen-3-ol, 3-octanol and 3-octanone can be found in indoor air (Korpi, et al. 2009a). Further, fungal VOCs such as 1-octen-3-ol, ethanol and other biogenetic and industrial gas phase compounds can be harmful to human health (Pantoja, et al. 2016; Perl, et al. 2011; Scotter, et al. 2005; Wright, et al. 2002). In particular, exposure to environmental agents such as industrial chemicals, mycotoxins and VOCs can have an effect on the human nervous system and fruit fly hemocytes by causing neurogenic inflammation (Carton, et al. 2009b; Foley and O'Farrell 2003; Laskin, et al. 2011b; Meggs 1993).

Both the Imd and Toll pathways produce different antimicrobial peptides that participate in attacking different microbial infections. Drosomycin transcription is enabled by the Toll pathway, while Diptericin transcription is enabled by the Imd pathway, and a few AMSs are produced by both pathways (De Gregorio, et al. 2002; Williams, et al. 1997). These antimicrobial peptides participate in different immune response reactions such as coagulation, opsonization, iron sequestration, and wound healing. In general, flies with mutations in these immune pathways are more susceptible to bacterial infections (Tzou, et al. 2002). However, is likely that the toxic effects of volatile compounds are mediated by

different lethal mechanisms than those caused by direct bacterial infection. When the genes for infection response are intact, volatile compounds such as 1-octen-3-ol may interact with the fly immune system in such as way as to trigger physiological mechanisms that cause toxicity and hence death of the flies. Our data suggest that when the genes for immune responses are impaired, the fly does not respond with these toxic responses and hence have improved survival.

Work in another lab has shown that certain genes induced by septic injury have no impacts on the double mutant strains flies, suggesting there are unidentified genes involved in signaling pathways activated by infection (De Gregorio, et al. 2002; Yamamoto-Hino and Goto 2016). The data presented here on the *Drosophila* response to certain volatile agents also may be useful in unraveling the complex and paradoxical ways in which flies respond to harmful environments. Further, I hypothesize that at least some of the toxic effects of fungal volatile compounds may be due to negative side effects associated with activation of the innate immune response.

CHAPTER 5. CONCLUDING

Fungal volatile organic compounds, also known as VOCs, are environmental agents with low molecular mass that exist as gases at room temperature. Previously, it has been hypothesized that fungal VOCs have negative effects on human health, usually causing headaches, gastrointestinal issues, as well as respiratory problems in people exposed to fungal VOCs indoors. Prolonged periods of exposure to VOCs have also contributed to negative effects in the human nervous system. Most studies on the health effects of exposure to volatile agents have focused on industrial chemicals not on biogenic agents such as those released naturally by plants, fungi and other microorganisms.

Our laboratory has pioneered the application of the fruit fly model to study and evaluate the toxicity effects of volatile organic compounds produced by filamentous fungi isolated from indoor environments. In this thesis, I have used *Drosophila melanogaster* as a model to study the toxicological effects of VOCs produced by human pathogenic fungi such as *A. fumigatus*, *Candida albicans* and *Cryptococcus* species. I hypothesized that VOCs produced from human pathogenic fungi would cause more toxic effects on the fruit fly when the fungal strains were pre-grown at 37∘C rather than at 25∘C. First of all, I tested six strains of *A. fumigatus* and found that VOCs from strain SRRC1607 had greatest toxic effects on the developmental stages of *Drosophila* . These effects were more pronounced when the strain was grown at 37°C rather than at 25∘C. In contrast, there were few effects on fly viability when flies were exposed to VOCs from *A. fumigatus* strain SRRC 1592 grown at either temperature. For yeast strains tested, the fewest delays in metamorphosis and eclosion rates were observed when flies were grown in a shared atmosphere with VOCs from *S. cerevisiae* pre-grown at 25°C. Exposure to the VOCs from *Cryptococcus neoformans* caused more delays on fly metamorphosis and more toxicity

than did exposure to VOCs from *Candida albicans and Cryptococcus gattii.* In general, exposure to VOCs produced from *A. fumigatus* strains was associated with more pigmentation and morphological abnormalities in *Drosophila* larval, pupal, and adult stages as compared to controls and other pathogenic fungi. The most common VOC produced by the most toxic and least toxic *A. fumigatus* strains was 1-octen-3-ol, however this compound was found in much greater concentrations when the strain was grown at 37◦C rather than at 25◦C. I suggest that VOCs from these medically important specie are acting as virulence factors that enhanced their pathogenic effects.

In further studies, the toxicity of VOCs produced by *A. fumigatus* wild type and several fungal strains with mutations in their oxylipin pathways were tested in the *Drosophila* model. We compared *Drosophila* development when challenged with either two *A. fumigatus* strains with intact oxylipin pathways (*A. fumigatus* strains Af293 and CEA17Δ*ku80*) to a Af293 strain that overexpresses a lipoxygenase (*A. fumigatus OE::loxB*) and its respective oxylipins, and to two strains deleted for all three COX-like oxygenases in both control backgrounds (Af293Δ*ppoABC* and CEA17Δ*ku80*Δ*ppoABC*) which should yield strains unable to synthesize 1-octen-3-ol. GC-MS analysis showed the wild type strain *A. fumigatus* Af293 produced more abundant VOCs at higher concentrations then the oxylipin deficient mutant Af293ΔppoABC. Major VOCs detected from wild type *A. fumigatus* Af293 included 1-octen-3-ol, 1-butanal, 1-octen, decanoic acid, lauric acid, myristic acid, and palmitic acid. The *A. fumigatus* strain in which the gene loxB was overexpressed caused more toxic effects and delays on the fly metamorphosis than the triple mutant strains. No significant differences were detected on the

metamorphosis of the fruit flies when the fungi were grown in the presence of arachidonic acid compared with growth on media lacking arachidonic acid.

In order to determine the effects of individual volatile compounds, chemical standards of three eight carbon volatiles were tested on adult *Drosophila* files with and without mutations in their immune pathways. Of the three compounds tested, 3-octanol at 0. 1μL was the least toxic to mutant and wild type strains. The Relish [E20] mutant strain was more susceptible than the spz[6] mutant strain to volatile phase 1-octen-3-ol at 0.1μL/L. The double mutant strain (Relish [E20] spz[6)] showed greater resistance to the presence of the volatile phase compounds than did either single mutant. *Drosophila* carrying mutant immune genes displayed few differences in viability when exposed to VOCs from wild type or oxylipin mutants of *A. fumigatus*.

In summary, I hypothesize that VOCs from *A. fumigatus* enhance the toxigenic effects of this medically important species and that higher concentrations of 1-octen-3-ol are correlated with increased toxicity. Furthermore, and surprisingly, flies carrying mutations in their innate immune system were more resistant to the toxic effects of volatiles than were the wild type flies. Specifically, the toxigenic effects of the *Aspergillus* VOCs were not observed in mutant flies with blocks in the Toll pathway, The increased resistance of flies carrying mutations in their innate immune response genes suggests that the toxigenic effects of fungal volatiles are related in an unexpected way to the *Drosophila* innate immune system.

References

- Abe, Keietsu, and Katsuya Gomi. 2008 Food products fermented by Aspergillus oryzae. The Aspergilli: Genomics, Medical Aspects, Biotechnology, and Research Methods.:429-439.
- Agarwal, R. 2009 Allergic bronchopulmonary aspergillosis. Chest 135(3):805-826.
- AL-Maliki, H. S., et al. 2017 *Drosophila melanogaster* as a Model for Studying *Aspergillus fumigatus*. Mycobiology 45(4):233-239.
- Andreou, Alexandra, Florian Brodhun, and Ivo Feussner. 2009 Biosynthesis of oxylipins in non-mammals. Progress in lipid research 48(3-4):148-170.
- Ansarin, K., L. Sahebi, and S. Sabur. 2013 Obstructive sleep apnea syndrome: complaints and housing characteristics in a population in the United States. Sao Paulo Med J 131(4):220-7.
- Araki, A., et al. 2012 The relationship between exposure to microbial volatile organic compound and allergy prevalence in single-family homes. Science of the Total Environment 423:18-26.
- Araki, A., et al. 2010 Relationship between selected indoor volatile organic compounds, so-called microbial VOC, and the prevalence of mucous membrane symptoms in single family homes. Science of the Total Environment 408(10):2208-2215.
- Assaf, S., Y. Hadar, and C. G. Dosoretz. 1997 1-octen-3-ol and 13-hydroperoxylinoleate are products of distinct pathways in the oxidative breakdown of linoleic acid by *Pleurotus pulmonarius*. Enzyme and Microbial Technology 21(7):484-490.
- Ayanbimpe, Grace Mebi, Wapwera Samuel Danjuma, and Mark Ojogba Okolo. 2012 Relationship between fungal contamination of indoor air and health problems of some residents in Jos. Air Quality-Monitoring and Modeling.
- Balajee, S. A., et al. 2007 *Aspergillus* species identification in the clinical setting. Stud Mycol 59:39-46.
- Balajee, S. A., et al. 2009 Molecular identification of *Aspergillus* species collected for the Transplant-Associated Infection Surveillance Network. J Clin Microbiol 47(10):3138-41.
- Barnes, P. D., and K. A. Marr. 2006 Aspergillosis: Spectrum of disease, diagnosis, and treatment. Infectious Disease Clinics of North America 20(3):545-+.
- Bayman, P., and J. L. Baker. 2006 Ochratoxins: a global perspective. Mycopathologia 162(3):215-23.
- Ben-Ami, R., et al. 2010 Interstrain variability in the virulence of *Aspergillus fumigatus* and *Aspergillus terreus* in a Toll-deficient *Drosophila* fly model of invasive aspergillosis. Medical Mycology 48(2):310-317.
- Bennett, J. W. 2009 *Aspergillus*: a primer for the novice. Medical Mycology 47(sup1):S5-S12.

- Bennett, J. W., and A. A. Inamdar. 2015   Are Some Fungal Volatile Organic Compounds (VOCs) Mycotoxins? Toxins (Basel) 7(9):3785-804.
- Bennett, JW. 1986   Taxonomy of fungi and biology of the aspergilli. Biotechnology Series[BIOTECHNOL. SER.]. 1986.
- Bennett, JW, and M Klich. 2003   Mycotoxins. Clin. Microbiol. Re 2003:16.
- Bhabhra, R., et al. 2004   Disruption of the *Aspergillus fumigatus* gene encoding nucleolar protein CgrA impairs thermotolerant growth and reduces virulence. Infect Immun 72(8):4731-40.
- Bhandari, S., et al. 2011   Determining the limits and confounders for the 2-pentyl furan breath test by gas chromatography/mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci 879(26):2815-20.
- Brakhage, A. A., and K. Langfelder. 2002   Menacing mold: the molecular biology of *Aspergillus fumigatus*. Annu Rev Microbiol 56(1):433-55.
- Brennan, Catherine A, and Kathryn V Anderson. 2004   *Drosophila*: the genetics of innate immune recognition and response. Annu. Rev. Immunol. 22:457-483.
- Brodhun, Florian, and Ivo Feussner. 2011   Oxylipins in fungi. The FEBS journal 278(7):1047-1063.
- Brodhun, Florian, et al. 2010 PpoC from *Aspergillus nidulans* is a fusion protein with only one active haem. Biochemical Journal 425(3):553-565.
- Burge, P Sherwood. 2004   Sick building syndrome. Occupational and environmental medicine 61(2):185-190.
- Cabral, João PS.2010 Can we use indoor fungi as bioindicators of indoor air quality? Historical perspectives and open questions. Science of the total environment 408(20):4285-4295.
- Calderone, Richard A, and Cornelius J Clancy. 2011   *Candida* and candidiasis: American Society for Microbiology Press.
- Carlson, Dave.Fruit Fly- Genus *Drosophila*. Pp. Illustration comparing the male and female fruit fly: CarlsonStockArt.com.
- Carton, Y., F. Frey, and A. J. Nappi. 2009a Parasite-induced changes in nitric oxide levels in *Drosophila paramelanica*. J Parasitol 95(5):1134-41.
- Carton, Y., F. Frey, and A.J. Nappi. 2009b Parasite-Induced Changes in Nitric Oxide Levels in *Drosophila paramelanica*. Volume 95: BIOONE.
- Casadevall, Arturo. 2005   Fungal virulence, vertebrate endothermy, and dinosaur extinction: is there a connection? Fungal Genetics and Biology 42(2):98-106.
- Cetinkaya, Z., et al.2005   Assessment of indoor air fungi in Western-Anatolia, Turkey. Asian Pac J Allergy Immunol 23(2-3):87-92.
- Chakrabarti, A. 2005 Microbiology of systemic fungal infections. J Postgrad Med 51 Suppl 1(5):S16-20.
- Chambers, E. C., M. S. Pichardo, and E. Rosenbaum. 2016 Sleep and the Housing and Neighborhood Environment of Urban Latino Adults Living in Low-Income Housing: The AHOME Study. Behav Sleep Med 14(2):169-84.
- Chambers, S. T., et al. 2009 Detection of 2-pentylfuran in the breath of patients with *Aspergillus fumigatus*. Med Mycol 47(5):468-76.

- Chamilos, G., et al. 2007   Role of mini-host models in the study of medically important fungi. Lancet Infectious Diseases 7(1):42-55.
- Chamilos, Georgios, et al. 2010   Exploring the concordance of *Aspergillus fumigatus* pathogenicity in mice and Toll-deficient flies. Medical mycology 48(3):506-510.
- Champe, Sewell P, Prema Rao, and Amy Chang. 1987   An endogenous inducer of sexual development in *Aspergillus nidulans*. Microbiology 133(5):1383-1387.
- Chang, Y. C., et al. 2004   THTA, a thermotolerance gene of *Aspergillus fumigatus*. Fungal Genet Biol 41(9):888-96.
- Charmaan, JA, and AI Terr. 2003   Ja cobs RL Toxic mold: phantom risk as science. Ann. Allergy Asthma Immunol 91(3):217.
- Chen, S. C., W. Meyer, and T. C. Sorrell. 2014   *Cryptococcus gattii* infections. Clin Microbiol Rev 27(4):980-1024.
- Claeson, A. S., et al. 2002   Volatile metabolites from microorganisms grown on humid building materials and synthetic media. J Environ Monit 4(5):667-72.
- Claeson, A. S., M. Sandstrom, and A. L. Sunesson. 2007   Volatile organic compounds (VOCs) emitted from materials collected from buildings affected by microorganisms. J Environ Monit 9(3):240-5.
- Clark, T. A., and R. A. Hajjeh. 2002 Recent trends in the epidemiology of invasive mycoses. Curr Opin Infect Dis 15(6):569-74.
- Clemente, J. C., et al. 2015   The microbiome of uncontacted Amerindians. Sci Adv 1(3):e1500183.
- Coleman, J. W. 2001 Nitric oxide in immunity and inflammation. Int Immunopharmacol 1(8):1397-406.
- Combet, Emilie, et al. 2006   Eight-carbon volatiles in mushrooms and fungi: properties, analysis, and biosynthesis. Mycoscience 47(6):317-326.
- Cooley, J. D., et al. 1998   Correlation between the prevalence of certain fungi and sick building syndrome. Occupational and Environmental Medicine 55(9):579-584.
- Coop, Christopher, MAJ Ronald W England, and LTC James M Quinn. 2004   Allergic bronchopulmonary aspergillosis masquerading as invasive pulmonary aspergillosis. Allergy and asthma proceedings, 2004. Vol. 25, pp. 263. OceanSide Publications.
- Coronado-Castellote, L., and Y. Jimenez-Soriano 2013   Clinical and microbiological diagnosis of oral candidiasis. J Clin Exp Dent 5(5):e279-86.
- Dagenais, T. R., et al. 2008   Defects in conidiophore development and conidium-macrophage interactions in a dioxygenase mutant of *Aspergillus fumigatus*. Infect Immun 76(7):3214-20.
- Dagenais, T. R., and N. P. Keller. 2009   Pathogenesis of *Aspergillus fumigatus* in Invasive Aspergillosis. Clin Microbiol Rev 22(3):447-65.
- Datta, K., et al. 2009   Spread of *Cryptococcus gattii* into Pacific Northwest Region of the United States. Emerging Infectious Diseases 15(8):1185-1191.
- Davis, Thomas Seth, et al. 2013   Microbial volatile emissions as insect semiochemicals. Journal of chemical ecology 39(7):840-859.

- De Gregorio, E., et al. 2002   The Toll and Imd pathways are the major regulators of the immune response in *Drosophila*. EMBO J 21(11):2568-79.
- Delker, C, et al. 2006 Jasmonate biosynthesis in Arabidopsis thaliana-enzymes, products, regulation. Plant Biology 8(03):297-306.
- Denning, D. W., et al. 2014   Fungal allergy in asthma-state of the art and research needs. Clin Transl Allergy 4(1):14.
- DeZazzo, J., and T. Tully. 1995   Dissection of memory formation: from behavioral pharmacology to molecular genetics. Trends Neurosci 18(5):212-8.
- Douwes, Jeroen. 2009 Building dampness and its effect on indoor exposure to biological and non-biological pollutants. *In* WHO guidelines for indoor air quality: dampness and mould. World Health Organization.
- Dyer, PS, and M Paoletti. 2005   Reproduction in *Aspergillus fumigatus*: sexuality in a supposedly asexual species? Medical mycology 43(sup1):7-14.
- El Chamy, Laure, et al. 2008 Sensing of 'danger signals' and pathogen-associated molecular patterns defines binary signaling pathways' upstream 'of Toll. Nature immunology 9(10):1165-1170.
- Ema, M., et al.   2005   Evaluation of developmental toxicity of 1-butanol given to rats in drinking water throughout pregnancy. Food Chem Toxicol 43(2):325-31.
- Engström, Ylva, et al. 1993   κB-like motifs regulate the induction of immune genes in *Drosophila*: Elsevier.
- Eom, H. J., et al. 2017 Inhalation toxicity of indoor air pollutants in *Drosophila melanogaster* using integrated transcriptomics and computational behavior analyses. Sci Rep 7:46473.
- EPA. 2011   Volatile Organic Compounds (VOCs). . Technical Overview; .
- Faber, S., et al.   2015   A cleanroom sleeping environment's impact on markers of oxidative stress, immune dysregulation, and behavior in children with autism spectrum disorders. BMC Complement Altern Med 15(1):71.
- Ferrari, R., et al. 2018 Cyclooxygenases and lipoxygenases are used by the fungus *Podospora anserina* to repel nematodes. Biochim Biophys Acta Gen Subj 1862(10):2174-2182.
- Fiedler, K., E. Schutz, and S. Geh. 2001   Detection of microbial volatile organic compounds (MVOCs) produced by moulds on various materials. Int J Hyg Environ Health 204(2-3):111-21.
- Fischer, G. J., et al. 2017   Lipoxygenase Activity Accelerates Programmed Spore Germination in *Aspergillus fumigatus*. Front Microbiol 8:831.
- Fischer, G. J., and N. P. Keller. 2016 Production of cross-kingdom oxylipins by pathogenic fungi: An update on their role in development and pathogenicity. J Microbiol 54(3):254-64.
- Fischer, G., et al. 1999   Species-specific production of microbial volatile organic compounds (MVOC) by airborne fungi from a compost facility. Chemosphere 39(5):795-810.
- Fogarty, WM. 1994   Enzymes of the genus *Aspergillus*. *In* Aspergillus. Pp. 177-218: Springer.

- Foley, Edan, and Patrick H O'Farrell. 2003   Nitric oxide contributes to induction of innate immune responses to gram-negative bacteria in *Drosophila*. Genes & development 17(1):115-125.
- Gabbs, Melissa, et al. 2015   Advances in our understanding of oxylipins derived from dietary PUFAs. Advances in nutrition 6(5):513-540.
- Galagan, J. E., et al. 2005   Sequencing of *Aspergillus nidulans* and comparative analysis with *A. fumigatus* and A. oryzae. Nature 438(7071):1105-15.
- Gao, P. F., et al.   2002   Determination of unique microbial volatile organic compounds produced by five aspergillus species commonly found in problem buildings. Aihaj 63(2):135-140.
- Garcia-Vidal, C., et al. 2008 Epidemiology of invasive mold infections in allogeneic stem cell transplant recipients: biological risk factors for infection according to time after transplantation. Clin Infect Dis 47(8):1041-50.
- Geiser, D. M., et al. 2008   A review of molecular phylogenetics in *Aspergillus*, and prospects for a robust genus-wide phylogeny. *Aspergillus* in the Genomic Era:17-32.
- Glittenberg, M. T., et al. 2011Wild-type *Drosophila* melanogaster as an alternative model system for investigating the pathogenicity of *Candida albicans*. Disease Models & Mechanisms 4(4):504-514.
- Goeminne, P. C., et al. 2012 Risk factors for morbidity and death in non-cystic fibrosis bronchiectasis: a retrospective cross-sectional analysis of CT diagnosed bronchiectatic patients. Respir Res 13(1):21.
- Gow, N. A. R., and B. Yadav. 2017 Microbe Profile: *Candida albicans*: a shape-changing, opportunistic pathogenic fungus of humans. Microbiology 163(8):1145-1147.
- Grechkin, Alexander. 1998   Recent developments in biochemistry of the plant lipoxygenase pathway. Prog Lipid Res 37:317-352.
- Gregg, K. S., and C. A. Kauffman.   2015   Invasive   Aspergillosis: Epidemiology, Clinical Aspects, and Treatment. Semin Respir Crit Care Med 36(5):662-72.
- Guarnieri, D. J., and U. Heberlein.2003   *Drosophila melanogaster*, a genetic model system for alcohol research. Int Rev Neurobiol 54:199-228.
- Gugnani, H. C. 2003 Ecology and taxonomy of pathogenic aspergilli. Front Biosci 8(1-3):s346-57.
- Hamilos, Georgios, George Samonis, and Dimitrios P Kontoyiannis. 2012 Recent advances in the use of *Drosophila melanogaster* as a model to study immunopathogenesis of medically important filamentous fungi. International journal of microbiology 2012.
- Hanski, I., et al. 2012 Environmental biodiversity, human microbiota, and allergy are interrelated. Proc Natl Acad Sci U S A 109(21):8334-9.
- Hardin, B. D., B. J. Kelman, and A. Saxon. 2003   Adverse human health effects associated with molds in the indoor environment. J Occup Environ Med 45(5):470-8.
- Hartwell, LH, et al. 2004   *Drosophila melanogaster*: Genetic Portrait of the Fruit Fly. Genetics: From Genes to Genomes:75-107.

- Hashimoto, Carl, Kathy L Hudson, and Kathryn V Anderson. 1988 The Toll gene of *Drosophila*, required for dorsal-ventral embryonic polarity, appears to encode a transmembrane protein. Cell 52(2):269-279.
- Hedayati, M. T., S. Mayahi, and D. W. Denning.2010 A study on Aspergillus species in houses of asthmatic patients from Sari City, Iran and a brief review of the health effects of exposure to indoor *Aspergillus*. Environ Monit Assess 168(1-4):481-7.
- Heddergott, C, AM Calvo, and JP Latgé.2014a The volatome of *Aspergillus fumigatus.* Eukaryotic cell 13(8):1014-1025.
- Heddergott, C., A. M. Calvo, and J. P. Latge 2014b The volatome of *Aspergillus fumigatus.* Eukaryot Cell 13(8):1014-25.
- Hedengren, Marika, et al. 1999 Relish, a central factor in the control of humoral but not cellular immunity in *Drosophila*. Molecular cell 4(5):827-837.
- Heikkinen, Terho, and Asko Järvinen. 2003 The common cold. The Lancet 361(9351):51-59.
- Herr, C. E., et al. 2003 Ranking and frequency of somatic symptoms in residents near composting sites with odor annoyance. Int J Hyg Environ Health 206(1):61-4.
- Herrmann, Andreas. 2010 Volatiles–An Interdisciplinary Approach. The chemistry and biology of volatiles:1-10.
- Heseltine, Elisabeth, and Jerome Rosen. 2009 WHO guidelines for indoor air quality: dampness and mould: WHO Regional Office Europe.
- Hoffmann, J. A. 2003a The immune response of *Drosophila*. Nature 426(6962):33-8.
- Hoffmann, Jules A. 2003b The immune response of *Drosophila*. Nature 426(6962):33-38.
- Hohl, T. M., and M. Feldmesser. 2007 *Aspergillus fumigatus*: principles of pathogenesis and host defense. Eukaryot Cell 6(11):1953-63.
- Hope, W. W., T. J. Walsh, and D. W. Denning. 2005 Laboratory diagnosis of invasive aspergillosis. Lancet Infect Dis 5(10):609-22.
- Hospodsky, D., et al. 2015 Characterizing airborne fungal and bacterial concentrations and emission rates in six occupied children's classrooms. Indoor Air 25(6):641-52.
- Hultmark, Dan. 2003 *Drosophila* immunity: paths and patterns. Current opinion in immunology 15(1):12-19.
- Hung, R., S. Lee, and J. W. Bennett. 2013 *Arabidopsis thaliana* as a model system for testing the effect of Trichoderma volatile organic compounds. Fungal Ecology 6(1):19-26.
- Hung, R., S. Lee, and J. W. Bennett. 2015 Fungal volatile organic compounds and their role in ecosystems. Applied Microbiology and Biotechnology 99(8):3395-3405.
- Husson, F., et al. 2001 Biogeneration of 1-octen-3-ol by lipoxygenase and hydroperoxide lyase activities of *Agaricus bisporus*. Process Biochemistry 37(2):177-182.

- Inamdar, A. A., and J. W. Bennett. 2014   A common fungal volatile organic compound induces a nitric oxide mediated inflammatory response in *Drosophila melanogaster*. Sci Rep 4:3833.
- Inamdar, A. A., et al. 2013   Fungal-derived semiochemical 1-octen-3-ol disrupts dopamine packaging and causes neurodegeneration. Proc Natl Acad Sci U S A 110(48):19561-6.
- Inamdar, A. A., P. Masurekar, and J. W. Bennett. 2010   Neurotoxicity of Fungal Volatile Organic Compounds in *Drosophila melanogaster*. Toxicological Sciences 117(2):418-426.
- Inamdar, A. A., et al. 2012   A model to evaluate the cytotoxicity of the fungal volatile organic compound 1-octen-3-ol in human embryonic stem cells. Mycopathologia 173(1):13-20.
- Inamdar, A. A., et al. 2014   *Drosophila melanogaster* as a model to characterize fungal volatile organic compounds. Environ Toxicol 29(7):829-36.
- Inamdar A, Morath S, Bennett JW.   2020   Fungal volatile organic compounds: more than just a funk smell? . Annual Rev Microbiology in press:in press.
- Jahn, Bernhard, et al. 2000   Interaction of human phagocytes with pigmentless*Aspergillus* conidia. Infection and immunity 68(6):3736-3739.
- Joo, Young-Chul, and Deok-Kun Oh.   2012   Lipoxygenases: potential starting biocatalysts for the synthesis of signaling compounds. Biotechnology advances 30(6):1524-1532.
- Kambris, Z., et al. 2006   *Drosophila* immunity: a large-scale in vivo RNAi screen identifies five serine proteases required for Toll activation. Curr Biol 16(8):808-13.
- Kappler, C., et al. 1993   Insect immunity. Two 17 bp repeats nesting a kappa B-related sequence confer inducibility to the diptericin gene and bind a polypeptide in bacteria-challenged *Drosophila*. EMBO J 12(4):1561-8.
- Kataoka, Ryousuke, et al. 2020   *Aspergillus luchuensis* fatty acid oxygenase ppoC is necessary for 1-octen-3-ol biosynthesis in rice koji. Journal of bioscience and bioengineering 129(2):192-198.
- Kauffman, C. A. 1996Quandary about treatment of aspergillomas persists. Lancet 347(9016):1640-1640.
- Kilburn, K. H. 2004   Role of molds and mycotoxins in being sick in buildings: Neurobehavioral and pulmonary impairment. Advances in Applied Microbiology, Vol 55 55:339-359.
- Kleino, Anni, and Neal Silverman. 2014   The *Drosophila* IMD pathway in the activation of the humoral immune response. Developmental & Comparative Immunology 42(1):25-35.
- Klich, M. A. 2006   Identification of clinically relevant aspergilli. Med Mycol 44(Supplement_1):S127-S131.
- Kocks, C., et al. 2005 Eater, a transmembrane protein mediating phagocytosis of bacterial pathogens in *Drosophila*. Cell 123(2):335-46.
- Korpi, A., J. Jarnberg, and A. L. Pasanen 2009a   Microbial volatile organic compounds. Crit Rev Toxicol 39(2):139-93.

- Korpi, Anne, Jill Jrnberg, and Anna-Liisa Pasanen. 2009b Microbial Volatile Organic Compounds. Critical Reviews in Toxicology, 2009, Vol.39(2), p.139-193 39(2):139-193.
- Korpi, ANNE, JP Kasanen, and AL Pasanen. 1999 Sensory irritation of microbially produced volatile organic compounds in mice during repeated exposures. Proceedings of the 3rd international conference: Bioaerosols, fungi and mycotoxins: health effects, assessment, prevention and control. Fungal Research Group Foundation, Inc. Albany, NY: Boyd Printing, 1999, pp. 106-111.
- Kosmidis, C., and D. W. Denning. 2015a Republished: The clinical spectrum of pulmonary aspergillosis. Postgrad Med J 91(1077):403-10.
- Kosmidis, Chris, and David W Denning. 2015b The clinical spectrum of pulmonary aspergillosis. Thorax 70(3):270-277.
- Kowalski, Caitlin H, et al. 2019 Fungal biofilm morphology impacts hypoxia fitness and disease progression. Nature microbiology 4(12):2430-2441.
- Kozak, KR, and LJ Marnett. 2002 Oxidative metabolism of endocannabinoids. Prostaglandins, Leukotrienes and Essential Fatty Acids (PLEFA) 66(2-3):211-220.
- Kreja, L., and H. J. Seidel.2002a Evaluation of the genotoxic potential of some microbial volatile organic compounds (MVOC) with the comet assay, the micronucleus assay and the HPRT gene mutation assay. Mutation Research-Genetic Toxicology and Environmental Mutagenesis 513(1-2):143-150.
- Kreja, L., and H. J. Seidel 2002b On the cytotoxicity of some microbial volatile organic compounds as studied in the human lung cell line A549. Chemosphere 49(1):105-110.
- Kuhn, D. M., and M. A. Ghannoum.2003 Indoor mold, toxigenic fungi, and Stachybotrys chartarum: infectious disease perspective. Clin Microbiol Rev 16(1):144-72.
- Lamaris, G. A., et al. 2007 Virulence studies of *Scedosporium* and *Fusarium* species in *Drosophila melanogaster*. J Infect Dis 196(12):1860-4.
- Lambrecht, B. N., and H. Hammad. 2013 Asthma: the importance of dysregulated barrier immunity. Eur J Immunol 43(12):3125-37.
- Lambrecht, B. N., and H. Hammad 2014 Allergens and the airway epithelium response: gateway to allergic sensitization. J Allergy Clin Immunol 134(3):499-507.
- Laskin, Debra L, et al.2011a Macrophages and tissue injury: agents of defense or destruction? Annual review of pharmacology and toxicology 51:267-288.
- Laskin, Debra L., et al. 2011bMacrophages and Tissue Injury: Agents of Defense or Destruction? Annual Review of Pharmacology and Toxicology 51(1):267-288.
- Lass-Flörl, Cornelia, et al. 2005 Epidemiology and outcome of infections due to *Aspergillus terreus*: 10-year single centre experience. British journal of haematology 131(2):201-207.
- Latge, J. P. 1999 *Aspergillus fumigatus* and aspergillosis. Clin Microbiol Rev 12(2):310-50.
- Latgé, J.P., and W.J. Steinbach. 2009*Aspergillus fumigatus* and aspergillosis. . Washington, DC: ASM Press 568.

159

- Latgé, Jean-Paul. 1999   *Aspergillus fumigatus* and aspergillosis. Clinical microbiology reviews 12(2):310-350.
- Laufer, P., et al. 1984 Allergic bronchopulmonary aspergillosis in cystic fibrosis. J Allergy Clin Immunol 73(1 Pt 1):44-8.
- Lauruschkat, C. D., H. Einsele, and J. Loeffler. 2018   Immunomodulation as a Therapy for *Aspergillus* Infection: Current Status and Future Perspectives. J Fungi (Basel) 4(4).
- Lee, Samantha, et al. 2015   Age matters: the effects of volatile organic compounds emitted by *Trichodermaatroviride* on plant growth. Archives of microbiology 197(5):723-727.
- Leeder, Abigail C., Javier Palma-Guerrero, and N. Louise Glass.   2011   The social network: deciphering fungal language. Nature Reviews Microbiology 9:440.
- Lemaitre, B., et al. 1996a   The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in *Drosophila* adults. Cell 86(6):973-83.
- Lemaitre, B., J. M. Reichhart, and J. A. Hoffmann. 1997   *Drosophila*   host defense: Differential induction of antimicrobial peptide genes after infection by various classes of microorganisms. Proceedings of the National Academy of Sciences of the United States of America 94(26):14614-14619.
- Lemaitre, Bruno, and Jules Hoffmann. 2007 The host defense of *Drosophila melanogaster*. Annu. Rev. Immunol. 25:697-743.
- Lemaitre, Bruno, et al.   1996b The dorsoventral regulatory gene cassette spätzle/Toll/cactus controls the potent antifungal response in *Drosophila* adults. Cell 86(6):973-983.
- Lemfack, M. C., et al. 2018   mVOC 2.0: a database of microbial volatiles. Nucleic Acids Res 46(D1):D1261-D1265.
- Lemfack, M. C., et al. 2014   mVOC: a database of microbial volatiles. Nucleic Acids Res 42(Database issue):D744-8.
- Lewis, Miranda, et al.2013   Cytokine Spätzle binds to the *Drosophila* immunoreceptor Toll with a neurotrophin-like specificity and couples receptor activation. Proceedings of the National Academy of Sciences 110(51):20461-20466.
- Limmer, S., et al. 2011   Virulence on the Fly: *Drosophila melanogaster* as a Model Genetic Organism to Decipher Host-Pathogen Interactions. Current Drug Targets 12(7):978-999.
- Lionakis, M. S., and D. P. Kontoyiannis. 2010   The growing promise of Toll-deficient *Drosophila melanogaster* as a model for studying *Aspergillus* pathogenesis and treatment. Virulence 1(6):488-499.
- Lionakis, Michail S, and Dimitrios P Kontoyiannis. 2012   *Drosophila melanogaster* as a model organism for invasive aspergillosis. *In* Host-Fungus Interactions. Pp. 455-468: Springer.
- Ma, H., and R. C. May. 2009 Virulence in *Cryptococcus* species. Adv Appl Microbiol 67:131-90.
- Machida, Masayuki, and Katsuya Gomi. 2010   *Aspergillus*:   molecular biology and genomics: Horizon Scientific Press.

- Maitra, Urmila, et al. 2019   Innate immune responses to paraquat exposure in a *Drosophila* model of Parkinson's disease. Scientific reports 9(1):1-17.
- Marr, K. A., T. Patterson, and D. Denning. 2002   Aspergillosis. Pathogenesis, clinical manifestations, and therapy. Infect Dis Clin North Am 16(4):875-94, vi.
- Maturu, V. N., and R. Agarwal. 2015 Acute Invasive Pulmonary Aspergillosis Complicating Allergic Bronchopulmonary Aspergillosis: Case Report and Systematic Review. Mycopathologia 180(3-4):209-15.
- Matysik, S., O. Herbarth, and A. Mueller. 2009   Determination of microbial volatile organic compounds (MVOCs) by passive sampling onto charcoal sorbents. Chemosphere 76(1):114-9.
- Mayer, B. 1994   Regulation of nitric oxide synthase and soluble guanylyl cyclase. Cell Biochem Funct 12(3):167-77.
- Medzhitov, Ruslan, and Charles A Janeway. 1998   Self-defense: the fruit fly style. Proceedings of the National Academy of Sciences 95(2):429-430.
- Meggs, W. J. 1993   Neurogenic inflammation and sensitivity to environmental chemicals. Environmental health perspectives 101(3):234-238.
- Micheli, Pier Antonio. 1729   Nova plantarum genera.
- Mølhave, Lars. 2009 Volatile organic compounds and sick building syndrome: Chapt 8. *In* Environmental Toxicants, Human Exposures and Their Health Effects. Pp. 241-256: John Wiley & Sons Ltd.
- Mølhave, Lars, et al. 1993   Sensory and physiological effects on humans of combined exposures to air temperatures and volatile organic compounds. Indoor Air 3(3):155-169.
- Morath, Shannon U., Richard Hung, and Joan W. Bennett. 2012   Fungal volatile organic compounds: A review with emphasis on their biotechnological potential. Fungal Biology Reviews 26(2-3):73-83.
- Mosblech, Alina, Ivo Feussner, and Ingo Heilmann. 2009   Oxylipins: structurally diverse metabolites from fatty acid oxidation. Plant Physiology and Biochemistry 47(6):511-517.
- Mukherjee, Tina, et al. 2011   Interaction between Notch and Hif-α in development and survival of *Drosophila* blood cells. Science 332(6034):1210-1213.
- Mulinari, S., U. Hacker, and C. Castillejo-Lopez. 2006   Expression and regulation of Spatzle-processing enzyme in *Drosophila*. FEBS Lett 580(22):5406-10.
- Muller, U. 1994   Ca2+/calmodulin-dependent nitric oxide synthase in Apis mellifera and *Drosophila melanogaster*. Eur J Neurosci 6(8):1362-70.
- National Academies of Sciences, Engineering, and Medicine. 2017 Microbiomes of the built environment: a research agenda for indoor microbiology, human health, and buildings: National Academies Press.
- Neerincx, A. H., et al. 2016 Identification of *Pseudomonas aeruginosa* and *Aspergillus fumigatus* mono- and co-cultures based on volatile biomarker combinations. J Breath Res 10(1):016002.
- Nelson, B. K., et al. 1989   Behavioral Teratology Investigation of L-Propanol Administered by Inhalation to Rats. Neurotoxicology and Teratology 11(2):153-159.

- Nielsen, K. F. 2003   Mycotoxin production by indoor molds. Fungal Genetics and Biology 39(2):103-117.
- Nierman, W. C., et al.2005   Genomic sequence of the pathogenic and allergenic filamentous fungus Aspergillus fumigatus. Nature 438(7071):1151-6.
- Noverr, M. C., et al. 2001   Pathogenic yeasts *Cryptococcus neoformans* and Candida albicans produce immunomodulatory prostaglandins. Infect Immun 69(5):2957-63.
- Pacher, P., J. S. Beckman, and L. Liaudet. 2007   Nitric oxide and peroxynitrite in health and disease. Physiol Rev 87(1):315-424.
- Pantoja, L. D. M., R. F. do Nascimento, and A. B. D. Nunes.2016   Investigation of fungal volatile organic compounds in hospital air. Atmospheric Pollution Research 7(4):659-663.
- PAPATHEOFANIS, FRANK J, and WILLIAM EM LANDS.1985 active products formed from radioactive precursor. Many published. Biochemistry of Arachidonic Acid Metabolism 1:9.
- Paterson, D. L., and N. Singh.1999   Invasive aspergillosis in transplant recipients. Medicine (Baltimore) 78(2):123-38.
- Patkar, R. N., et al. 2015   A fungal monooxygenase-derived jasmonate attenuates host innate immunity. Nat Chem Biol 11(9):733-40.
- Paulussen, C., et al. 2017   Ecology of aspergillosis: insights into the pathogenic potency of *Aspergillus fumigatus* and some other *Aspergillus* species. Microb Biotechnol 10(2):296-322.
- Pennerman, KK, et al. 2016   Fungal volatile organic compounds (VOCs) and the genus *Aspergillus*. *In* New and Future Developments in Microbial Biotechnology and Bioengineering. Pp. 95-115: Elsevier.
- Perkins, Neil D. 2007 Integrating cell-signalling pathways with NF-κB and IKK function. Nature reviews Molecular cell biology 8(1):49-62.
- Perl, T., et al. 2011   Detection of characteristic metabolites of *Aspergillus fumigatus* and *Candida* species using ion mobility spectrometry - metabolic profiling by volatile organic compounds. Mycoses 54(6):E828-E837.
- Philips, J. A., E. J. Rubin, and N. Perrimon.2005   *Drosophila* RNAi screen reveals CD36 family member required for mycobacterial infection. Science 309(5738):1251-3.
- Poggeler, S. 2002   Genomic evidence for mating abilities in the asexual pathogen *Aspergillus fumigatus*. Curr Genet 42(3):153-60.
- Pohl, Carolina H, and Johan LF Kock. 2014 Oxidized fatty acids as inter-kingdom signaling molecules. Molecules 19(1):1273-1285.
- Polizzi, V., et al. 2012Identification of volatile markers for indoor fungal growth and chemotaxonomic classification of *Aspergillus* species. Fungal Biol 116(9):941-53.
- Polizzi, V., et al. 2009JEM Spotlight: Fungi, mycotoxins and microbial volatile organic compounds in mouldy interiors from water-damaged buildings. J Environ Monit 11(10):1849-58.
- PS, Hasleton.1996   Spencer's pathology of the lung. Anatomy of the lung. Hasleton PS and Curry A (edrs) 1:6-7.

- Raffa, Nicholas, and Nancy P Keller.2019   A call to arms: Mustering secondary metabolites for success and survival of an opportunistic pathogen. PLoS pathogens 15(4).
- Rajak, Prem, et al. 2016   Key Players of Innate Immunity in *Drosophila melanogaster*. BN Seal Journal of Science Volume VIII Issue 1 September 2016:45.
- Rand, Matthew D, et al. 2014 Developmental toxicity assays using the *Drosophila* model. Current protocols in toxicology 59(1):1.12. 1-1.12. 20.
- Raper, Kenneth Bryan, and Dorothy I Fennell.   1965   The   genus *Aspergillus*. The genus Aspergillus.
- Reedy, J. L., R. J. Bastidas, and J. Heitman. 2007   The virulence of human pathogenic fungi: notes from the South of France. Cell Host Microbe 2(2):77-83.
- Regulski, M., and T. Tully.1995   Molecular and biochemical characterization of dNOS: a Drosophila Ca2+/calmodulin-dependent nitric oxide synthase. Proc Natl Acad Sci U S A 92(20):9072-6.
- Richardson, Malcolm D. 2005   Changing patterns and trends in systemic fungal infections. Journal of Antimicrobial Chemotherapy 56(suppl_1):i5-i11.
- Robbins, C. A., et al. 2000   Health effects of mycotoxins in indoor air: a critical review. Appl Occup Environ Hyg 15(10):773-84.
- Robinson, Simon N, et al. 1997   Immunotexicological effects of benzene inhalation in male Sprague-Dawley rats. Toxicology 119(3):227-237.
- Roeder, T., K. Isermann, and M. Kabesch. 2009   *Drosophila*   in   asthma research. Am J Respir Crit Care Med 179(11):979-83.
- Roeder, Thomas, et al. 2012 A *Drosophila* asthma model–what the fly tells us about inflammatory diseases of the lung. *In* Recent Advances on Model Hosts. Pp. 37-47: Springer.
- Rosowski, E. E., et al. 2018 Macrophages   inhibit   *Aspergillus   fumigatus* germination and neutrophil-mediated fungal killing. PLoS Pathog 14(8):e1007229.
- Royet, J., J. M. Reichhart, and J. A. Hoffmann.2005 Sensing and signaling during infection in *Drosophila*. Curr Opin Immunol 17(1):11-7.
- Rutschmann, Sophie, Ahmet Kilinc, and Dominique Ferrandon. 2002.Cutting Edge: The Toll Pathway Is Required for Resistance to Gram-Positive Bacterial Infections in *Drosophila*,The Journal of Immunology 168(4):1542-1546.
- Salazar, Fabian, and Gordon D Brown. 2018 Antifungal   innate   immunity: a perspective from the last 10 years. Journal of innate immunity 10(5-6):373-397.
- Samson, Robert A, János Varga, and Paul S Dyer. 2009   Morphology   and reproductive mode of *Aspergillus fumigatus*. *In Aspergillus fumigatus* and Aspergillosis. Pp. 7-13: American Society of Microbiology.
- Sarma, Sailendra Nath, Youn-Jung Kim, and Jae-Chun Ryu. 2010 Gene expression profiles of human promyelocytic leukemia cell lines exposed to volatile organic compounds. Toxicology 271(3):122-130.
- Scala, Alessandra, et al. 2013 Green leaf volatiles: a plant's multifunctional weapon against herbivores and pathogens. International journal of molecular sciences 14(9):17781-17811.

- Schaffner, Andreas, Herndon Douglas, and Abraham Braude.1982 Selective protection against conidia by mononuclear and against mycelia by polymorphonuclear phagocytes in resistance to *Aspergillus*: observations on these two lines of defense in vivo and in vitro with human and mouse phagocytes. The Journal of clinical investigation 69(3):617-631.
- Schwab, Christopher J, and David C Straus.2004 The roles of *Penicillium* and *Aspergillus* in sick building syndrome. Advances in Applied Microbiology 55:215-240.
- Scotter, Jennifer M, et al. 2005 Real-time detection of common microbial volatile organic compounds from medically important fungi by Selected Ion Flow Tube-Mass Spectrometry (SIFT-MS). Journal of microbiological methods 63(2):127-134.
- Sedghi, Siavash, et al. 2017 Volatile anesthetics and immunity. Immunological investigations 46(8):793-804.
- Sharma, JN, and LA Mohammed.2006 The role of leukotrienes in the pathophysiology of inflammatory disorders: is there a case for revisiting leukotrienes as therapeutic targets? Inflammopharmacology 14(1-2):10-16.
- Shia, A. K., et al. 2009 Toll-dependent antimicrobial responses in *Drosophila* larval fat body require Spatzle secreted by haemocytes. J Cell Sci 122(Pt 24):4505-15.
- Shizuo, Akira, and K Takeda 2004 Toll-like receptor signaling. Nat Rev Immunol 4(7):499-511.
- Singh, A., and M. Del Poeta. 2011 Lipid signalling in pathogenic fungi. Cell Microbiol 13(2):177-85.
- Singh, K., et al. 2012 Comparative transcriptome analysis of the necrotrophic fungus *Ascochyta rabiei* during oxidative stress: insight for fungal survival in the host plant. PLoS One 7(3):e33128.
- Singh, N., and D. L. Paterson. 2005 *Aspergillus* infections in transplant recipients. Clin Microbiol Rev 18(1):44-69.
- Sloane, Jacob A, et al. 2010 A clear and present danger: endogenous ligands of Toll-like receptors. Neuromolecular medicine 12(2):149-163.
- Sokolowski, M. B. 2001 *Drosophila*: genetics meets behaviour. Nat Rev Genet 2(11):879-90.
- Stasiv, Y., et al. 2001 The *Drosophila* nitric-oxide synthase gene (dNOS) encodes a family of proteins that can modulate NOS activity by acting as dominant negative regulators. Journal of Biological Chemistry 276(45):42241-42251.
- Steinbach, William J. 2008 Clinical aspects of the genus *Aspergillus*: Boca Raton, FL: Taylor & Francis Group, LLC.
- Stevens, David A, et al. 2000 Practice guidelines for diseases caused by *Aspergillus*. Clinical Infectious Diseases:696-709.
- Straus, D. C. 2009 Molds, mycotoxins, and sick building syndrome. Toxicol Ind Health 25(9-10):617-35.
- Takigawa, T., et al. 2009 A longitudinal study of environmental risk factors for subjective symptoms associated with sick building syndrome in new dwellings. Sci Total Environ 407(19):5223-8.

- Tekaia, Fredj, and Jean-Paul Latge. 2005 *Aspergillus fumigatus*: saprophyte or pathogen? Current opinion in microbiology 8(4):385-392.
- Tolwinski, Nicholas S. 2017a Introduction: *Drosophila*—A Model System for Developmental Biology: Multidisciplinary Digital Publishing Institute.
- Tolwinski, Nicholas S. 2017b Introduction: *Drosophila*-A Model System for Developmental Biology. Journal of developmental biology 5(3):9.
- Tomee, J. F., et al. 1995 Serologic monitoring of disease and treatment in a patient with pulmonary aspergilloma. Am J Respir Crit Care Med 151(1):199-204.
- Tsitsigiannis, D. I., et al.2005a *Aspergillus* cyclooxygenase-like enzymes are associated with prostaglandin production and virulence. Infect Immun 73(8):4548-59.
- Tsitsigiannis, D. I., and N. P. Keller 2007 Oxylipins as developmental and host-fungal communication signals. Trends Microbiol 15(3):109-18.
- Tsitsigiannis, D. I., et al. 2005b Three putative oxylipin biosynthetic genes integrate sexual and asexual development in *Aspergillus nidulans*. Microbiology 151(Pt 6):1809-1821.
- Tsitsigiannis, D. I., R. Zarnowski, and N. P. Keller. 2004 The lipid body protein, PpoA, coordinates sexual and asexual sporulation in *Aspergillus nidulans*. J Biol Chem 279(12):11344-53.
- Tzou, Phoebe, Ennio De Gregorio, and Bruno Lemaitre. 2002 How *Drosophila* combats microbial infection: a model to study innate immunity and host–pathogen interactions. Current opinion in microbiology 5(1):102-110.
- Ulvila, Johanna, LEENA-MAIJA VANHA-AHO, and MIKA RÄMET. 2011 *Drosophila* phagocytosis–still many unknowns under the surface. Apmis 119(10):651-662.
- Valanne, Susanna, Jing-Huan Wang, and Mika Rämet. 2011 The *Drosophila* toll signaling pathway. The Journal of Immunology 186(2):649-656.
- Van Lancker, Fien, et al..2008 Use of headspace SPME-GC-MS for the analysis of the volatiles produced by indoor molds grown on different substrates. Journal of Environmental Monitoring 10(10):1127-1133.
- Wadman, Mayken W, et al. 2009 Characterization of oxylipins and dioxygenase genes in the asexual fungus *Aspergillus niger*. BMC microbiology 9(1):59.
- Wålinder, Robert, et al. 2008 Acute effects of 1-octen-3-ol, a microbial volatile organic compound (MVOC)—An experimental study. Toxicology letters 181(3):141-147.
- Wang, Josephine LF, et al. 1978 Serum IgE and IgG antibody activity against *Aspergillus fumigatus* as a diagnostic aid in allergic bronchopulmonary aspergillosis. American Review of Respiratory Disease 117(5):917-927.
- Wang, Lihui, and Petros Ligoxygakis.2006 Pathogen recognition and signalling in the *Drosophila* innate immune response. Immunobiology 211(4):251-261.
- Wasserkort, Reinhold, and Theodor Koller. 1997 Screening toxic effects of volatile organic compounds using *Drosophila melanogaster*. Journal of Applied Toxicology: An International Forum Devoted to Research and Methods

Emphasizing Direct Clinical, Industrial and Environmental Applications, 1997. Vol. 17, pp. 119-125. Wiley Online Library.

- Watson, Susan B, Gary Caldwell, and Georg Pohnert. 2009 Fatty acids and oxylipins as semiochemicals. *In* Lipids in aquatic ecosystems. Pp. 65-92: Springer.
- Weber, B., et al. 2009 Increased volatile anesthetic requirement in short-sleeping *Drosophila* mutants. Anesthesiology 110(2):313-6.
- Wieslander, G, D Norbäck, and C Edling.1997 Airway symptoms among house painters in relation to exposure to volatile organic compounds (VOCS)—a longitudinal study. The Annals of occupational hygiene 41(2):155-166.
- Wild, C. P. 2007 Aflatoxin exposure in developing countries: the critical interface of agriculture and health. Food Nutr Bull 28(2 Suppl):S372-80.
- Williams, J. H., et al. 2004 Human aflatoxicosis in developing countries: a review of toxicology, exposure, potential health consequences, and interventions. Am J Clin Nutr 80(5):1106-22.
- Williams, M. J., et al. 1997 The 18-wheeler mutation reveals complex antibacterial gene regulation in *Drosophila* host defense. EMBO J 16(20):6120-30.
- Wright, L., et al. 2002 Metabolites released by *Cryptococcus neoformans var. neoformans* and *var. gattii* differentially affect human neutrophil function. Microbes Infect 4(14):1427-38.
- Xu, H., et al. 2010 Clinical itraconazole-resistant strains of *Aspergillus fumigatus,* isolated serially from a lung aspergilloma patient with pulmonary tuberculosis, can be detected with real-time PCR method. Mycopathologia 169(3):193-9.
- Yamamoto-Hino, M., and S. Goto. 2016 Spatzle-Processing Enzyme-independent Activation of the Toll Pathway in *Drosophila* Innate Immunity. Cell Struct Funct 41(1):55-60.
- Yin, G., et al.2015 Effects of Three Volatile Oxylipins on Colony Development in Two Species of Fungi and on *Drosophila* Larval Metamorphosis. Curr Microbiol 71(3):347-56.
- Zaas, A. K., et al. 2008 Plasminogen alleles influence susceptibility to invasive aspergillosis. PLoS Genet 4(6):e1000101.
- Zhang, Z. L., et al. 2016 Fumigant toxicity of monoterpenes against fruitfly, *Drosophila melanogaster*. Industrial Crops and Products 81:147-151.
- Zhao, G., et al. 2017 Volatile organic compounds emitted by filamentous fungi isolated from flooded homes after Hurricane Sandy show toxicity in a *Drosophila* bioassay. Indoor Air 27(3):518-528.
- Zmeili, O. S., and A. O. Soubani. 2007 Pulmonary aspergillosis: a clinical update. QJM 100(6):317-34.