Characterising *Tenebrio molitor*, mealworm, as a host for studying *Aspergillus fumigatus* pathogenesis.

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Statement of Authentication

The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I hereby declare that I have not submitted this material, either in full or in part, for a degree at this or any other institution.

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Abstract

Aspergillus fumigatus is an opportunistic fungal pathogen that is the leading cause of Invasive Aspergillosis (IA). Despite the availability of diagnostics and treatments, IA has high morbidity and mortality ranging from 30-95%. This is due to the complexity of infection as virulence depends on interactions that occur between fungal traits and host immunity. Model organisms can be utilised to investigate these interactions including invertebrates as they utilise innate immunity, which when impaired in humans represents an important IA risk factor.

In this project, *Tenebrio molitor* larvae were explored as an alternative invertebrate to study *A. fumigatus* pathogenesis. Several methods were utilised to characterise infection. This includes colony forming unit (CFU) counts and quantitative polymerase chain reaction (qPCR) to quantify infection over time. To visualise infection, histological examination and magnetic resonance imaging (MRI) were utilised, and haemocyte interaction assays to examine haemocyte-fungal interactions in detail. Inoculation of *T. molitor* larvae with *A. fumigatus* was observed to decrease survival over time, increase the fungal burden, and cause the presence of nodules within larval tissue. These nodules were accompanied by cells suspected to be haemocytes, which during interaction assays became activated upon exposure to *A. fumigatus* conidia. Similar observations have been observed in other invertebrates including *Galleria mellonella*. *T. molitor* larvae may thus be useful to study *A. fumigatus* pathogenesis, however further optimisations of these methods are necessary to confirm these findings and characterise infection, such as utilisation of fungal specific stains and additional methodologies including confocal microscopy and flow cytometry to assess phagocytosis.

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Chapter 1 Introduction

Microorganisms including bacteria, fungi, parasites, and viruses cause disorders known as infectious diseases which are responsible for significant morbidity and mortality worldwide. Millions of people are affected by these diseases each year resulting in 7.2 million doctor visits [1] and 3.4 million emergency department visits in the United States alone during 2018.[2] Depending on the causative agent and individual susceptibility, infectious diseases significantly impact public health. In 2016, approximately 10 million fatalities occurred due to infectious diseases which amounted to one-fifth of deaths globally.[3] The most common causes of mortality linked to infectious diseases were lower respiratory tract infections, diarrheal diseases, HIV/AIDs, malaria, and tuberculosis.[4]

As microbes are ubiquitous in the environment and constantly evolving with respect to other organisms; agents of infectious diseases are continuously emerging.[4] This was highlighted in late 2019 by the emergence of the coronavirus disease (COVID-19) pandemic which affected public health systems and economies worldwide. To date, over 262 million confirmed cases exist and approximately 5 million deaths reported from COVID-19 as documented by the World Health Organization (WHO).[5] With the emergence of COVID-19, fungal co-infections were observed in critically ill COVID-19 patients causing further complications. This is likely due to shared risk factors with serious fungal infections (e.g., corticosteroid therapy, chronic respiratory diseases etc.).[6]

Infectious diseases produce highly variable disease outcomes as it depends on interactions between host and pathogen. This encompasses host susceptibility which is their ability to limit or resist infection, and microbial pathogenicity which is the ability to cause disease and virulence which refers to the likelihood of causing disease. The development of infectious diseases occurs via stages: infection, disease, and recovery or death. Infectious diseases usually begin with the colonisation of host tissue involving microbial adherence and growth usually at the region of entry. This may then progress to disease involving disruption to the host such as via damage and invasion of tissue which leads to symptoms and signs of illness. The host may then recover completely from the infectious disease or incompletely, resulting in microbial persistence and thus chronic infection or latent infection where the microbe enters a quiescent stage for later reactivation.[4]

Factors that influence virulence vary and can include structural components (e.g., melanin in fungal spores) or biochemical such as the production of toxins.[4] An understanding of these factors and their interaction with the host is of interest in infectious disease research for disease management. During COVID-19, infection was managed by social distancing, mask wearing and vaccination as it was spread directly between individuals or via droplets (sneezing, coughing, talking). Infectious diseases can also result from exposure to a contaminated object, an environmental agent such as inhalation of spores, or via injury.[4]

1.1 Fungi and fungal Infections

Fungi are ubiquitous in the environment which has contributed to the large diversity of these eukaryotic microorganisms.[7] Around 12 million species are estimated to exist,[8] and of these only a few hundred are implicated in human infection. Most fungi

are saprotrophs that acquire nutrients via decomposition; though some are mutualistic or parasitic, obtaining nutrients from a live host. Despite their nutritional preference for dead organic matter, saprophytic fungal species have been implicated in human infection. Infections caused by saprophytic fungi are primarily opportunistic and limited to the immunocompromised as opportunistic fungi have low intrinsic virulence.[9-11]

Over one billion people worldwide are infected by fungi annually resulting in around 1.5 million fatalities each year.[12, 13] As fungal infections are most common at the skin where they can be treated using over the counter topical antifungals, fungi are often considered non-life threatening. Fungal skin infections are classified as superficial or subcutaneous infections.[9] Superficial infections occur in approximately 25% of the global population with infection in outer layers of skin (e.g., athlete's foot), hair and nails.[14] Subcutaneous infections occur at deeper skin layers often following skin trauma, such as by splinters or thorns which implant fungal components deep into the skin. Unlike fungal skin infections, invasive fungal infections are rare, life-threatening infections characterised by the systemic spread of a fungal pathogen. Infection is therefore severe affecting internal organs, and often challenged by difficult diagnosis and treatment. As mammalian immunity is well-developed, healthy individuals are generally resistant to systemic fungal infections which are mainly documented in individuals whose immune system is disrupted. Seriously ill or immunosuppressed individuals are thus most at risk.[10, 11, 14, 15]

Not all pathogenic fungi cause invasive infection. The ability of the fungus to survive within the host's environment is as important as the host's immune condition. This includes the ability to grow at minimum, human body temperature, which is uncommon amongst fungi.[16, 17] Furthermore, the ability to access host internal

tissues, to obtain nutrients for growth, and to evade host immune defences throughout infection.[18] Consequently, a limited number of fungal species cause these serious fungal infections.

1.1.1 Invasive fungal infections

Invasive fungal infections (IFIs) are becoming increasingly common due to the growing population of immunocompromised individuals. Not only are those with predisposing health conditions such as chronic obstructive pulmonary disease (COPD), diabetes mellitus and HIV/AIDS at risk of IFIs, but also a growing population of individuals undergoing life-changing medical interventions such as immunosuppressive or invasive treatments (e.g., organ transplants).[14, 19-21]. This is reflected in epidemiological data as of those affected by IFIs in a United States based study, COPD was a common comorbidity (44.9%) along with diabetes mellitus (28.7%), and 26.1% of IFI patients were receiving immunosuppressive therapy with corticosteroids most common (20.8% of all IFI cases).[21] IFIs can thus be considered an important complication of modern medicine due to high mortality rates and COVID-19 due to IFI co-infections.[6, 22]

The true burden of IFIs is unclear as diagnosis and treatment is difficult. Several challenges exist including the availability of diagnostic methods, sensitivity, patient suitability as invasive diagnostic methods may be too risky and processing times. As IFIs typically cause non-specific signs and symptoms, initial misdiagnosis or delayed treatment are another important challenge further complicated by the growing numbers of fungal species that infect humans. Delays in treatment can have deadly consequences as increased mortality rates have been observed.[23-25]

Accurate diagnosis of IFIs depends on specialist pathologists and a combination of diagnostic methods,[23, 25] though in many cases diagnosis such as of invasive aspergillosis remains “probable” due to the diagnostic challenges.[21] A “probable” diagnosis is utilised for immunocompromised patients excluding endemic mycoses. It depends on certain host factors, clinical features, and mycological evidence. Without mycological evidence, a “possible” diagnosis is utilised. A “proven” IFI diagnosis is applicable to any patient with microscopic evidence of fungi and microbiological culture yielding fungi. Beyond sensitivity and specificity limitations, these methods are not always viable with invasive methods necessary to recover samples (e.g., tissue) which risks patient death.[26-28]

Although epidemiological data concerning IFIs is scarce with limited monitoring of these infections,[18] efforts have been made to determine the global impact of IFIs using the few studies available. Accordingly, IFI mortality rates have been estimated to range from 20% up to 95%, often exceeding 50% despite diagnosis and treatment.[14] This is due to varied disease presentation which is influenced by fungal dissemination from lungs to other organs, patient health, and the timing of treatment. IFI mortality is unacceptably high based on these reports, which may have yet underestimated the impact of IFIs as of the studies utilised, most are geographically localised and of developed countries where healthcare is considerably better than in developing countries.[14] Developing countries generally have limited resources and therefore may lack the diagnostic technologies and drugs applied in developed countries to treat IFIs. Cheap phenotypic diagnostic methods are thus more common which are more time-consuming and less accurate.[29] Survival would thus be at risk, and hospitalisation extended when treatment is delayed.[30-32] The economic burden of IFIs is therefore significant due to necessary supportive care and antifungal

costs.[33, 34] In Australia, IFIs have resulted in median excess hospital costs of AU$30,957(US$21,203) per hospitalisation.[33]

The development of improved diagnostics and treatments for IFIs is important. Advancing our understanding of human fungal pathogens is an integral part of addressing this issue with only a handful of fungal species responsible for most fatalities. This includes members of genera *Aspergillus*, *Candida*, *Cryptococcus* and *Pneumocystis* which account for 90% of reported fungal deaths.[14] *Aspergillus* spp. are the most common moulds isolated from human deep site samples (>85%).[35] During COVID-19, additional concerns have arisen over fungi due to reports of co-infections resulting in COVID-associated fungal infections. This includes *Aspergillus* spp. infecting critical ill COVID-19 patients where the incidence of *Aspergillus*-related invasive lung infections was observed to range from 19.6% to 33.3%.[36] This highlights the risk of *Aspergillus* spp. to seriously ill individuals and potential rise in the incidence of serious fungal infections, especially with the increased usage of corticosteroids to treat COVID-19 patients. In India, excessive corticosteroid usage due to COVID-19 has led to a mucormycosis epidemic amongst the growing population of COVID-19 patients.[37]

1.1.2 Invasive Aspergillosis

Over 200 species belong to *Aspergillus*, a genus of moulds that produce septate hyphae and large quantities of pigmented conidia. Only a small subset of *Aspergillus* species is linked to human infections however, collectively called Aspergillosis.[11, 13] Key species includes *A. flavus*, *A. fumigatus*, *A. nidulans*, *A. niger* and *A. terreus*.[13] Aside from invasive aspergillosis (IA), infection may lead to allergic and non-invasive infections depending on the individual's immune status.[38, 39] IA is the most severe

form of Aspergillosis as mortality ranges from 30-95%. As an opportunistic infection, IA is highly dependent on host immune status.[14, 40] IA is thus common amongst patients using immunosuppressive medications (60.5%), and those with haematological malignancies (31.9%) and haematopoietic stem cell transplants (HSCT) (25.6%).[21] Low numbers of lymphocytes (50.8%) and neutrophils (29.6%) is also commonly observed prior to IA.[21]

Despite medical intervention, IA mortality remains high overall with poor prognosis associated with certain populations of individuals. This includes individuals affected by chronic obstructive pulmonary disease (COPD) which is common among IFI cases,[21] brain infections, intensive care patients and those affected by mismatched HSCT.[14] Approximately 10 million people are considered at risk of IA which affects around 300,000 people annually.[12] Not only is the health burden significant, but also the economic burden. According to an American study, IA costs an additional US$15,542 per hospitalisation resulting in costs up to US$600 million annually.[41]

1.2 *Aspergillus fumigatus*

Aspergillus fumigatus is the most clinically relevant *Aspergillus* species isolated in 60-70% of IA cases,[13] and most responsible for *Aspergillus*-related co-infection of COVID-19 patients.[36] *A. fumigatus* is a saprophytic fungus present throughout the environment despite its preference for compost.[42-44] Its ubiquity is due to the dispersion of hydrophobic asexual spores known as conidia, which are 2-3 µm in diameter produced via sporulation.[45] Conidia are essential for *A. fumigatus* propagation and thus they demonstrate resistance against various stressors. In the environment, conidia are protected from oxidative stress and sunlight by melanin in the cell wall.[46] Conidia can therefore safely disperse via air currents to both indoor and outdoor environments, with concentrations estimated to range from 1-100 conidia/m^3. However, in exceptional cases concentrations can reach up to 10^8 conidia/m^3.[40, 42] Consequently, hundreds of *A. fumigatus* conidia are unknowingly inhaled daily. Inhalation is thus the most common route of infection and lungs the most common site of IA. Ingestion or via wounds is also possible but uncommon.[47]

Although inhalation of *A. fumigatus* conidia is common, the interaction between conidia and the immune system is usually harmless and asymptomatic.[48] This has been demonstrated during lung biopsy samples of healthy adults where 37% had *Aspergillus* DNA present despite being culture negative.[49] Infection development is a function of the host's immune system with immune impairment putting individuals at risk of *A. fumigatus* infection which is the most virulent and successful agent of IA.[40]

1.2.1 Early interactions with host immunity

The early interactions between *A. fumigatus* conidia and the host immune system determines infection development as infection usually only progresses in

immunocompromised individuals and rarely in the immunocompetent.[40, 50] In the immunocompetent host, infection is rapidly cleared by host immunity to prevent tissue damage. The innate immune system is important for this response which includes epithelial barriers and phagocytic cells, mainly macrophages and neutrophils.[40] Owing to their small size, *A. fumigatus* conidia can access lung alveoli during infection.[44] However, conidia must first successfully bypass various defence mechanisms. This includes mucocillary clearance which removes almost all inhaled conidia. Consequently, subsequent defence mechanisms are necessary to complete conidial elimination. This may be provided by various cell types including those in airway epithelia. Based on the structure of the lungs, epithelial cells are likely one of the conidium's earliest points of contact.[51] Airway epithelial cells are non-professional phagocytes that can internalise and destroy *Aspergillus* conidia.[40, 52] Conidia (3%) can escape these cells however, via germination.[52] Inflammation and neutrophil recruitment thus follows,[40] with epithelial cells supporting innate immune effectors, macrophages and neutrophils.[53]

Macrophages and neutrophils are important innate immune effector cells which provide a rapid, non-specific immune response.[43, 54] These are professional phagocytes important for the early defensive response against *A. fumigatus* conidia. The impairment or reduction of macrophages, known as monocytopenia,[43] and neutrophils, known as neutropenia, are important risk factors for IA development.[54] These abnormalities can result for various reasons including as side-effects of immunosuppressive therapies such as myelotoxic chemotherapy and hematopoietic cell transplants[13, 44], or due to cellular defects such as in reactive oxygen species (ROS) production seen in individuals with chronic granulomatous disease (CGD) where increased susceptibility to IA is observed.[44] Conidial survival can be enhanced

by such defects which enable germination and growth of highly destructive hyphal filaments.[55, 56] The fungus can subsequently invade tissue and blood vessels, and potentially disseminate to infect other organs, most commonly to organs of the central nervous system (CNS) (Figure 1.1). [13, 57]

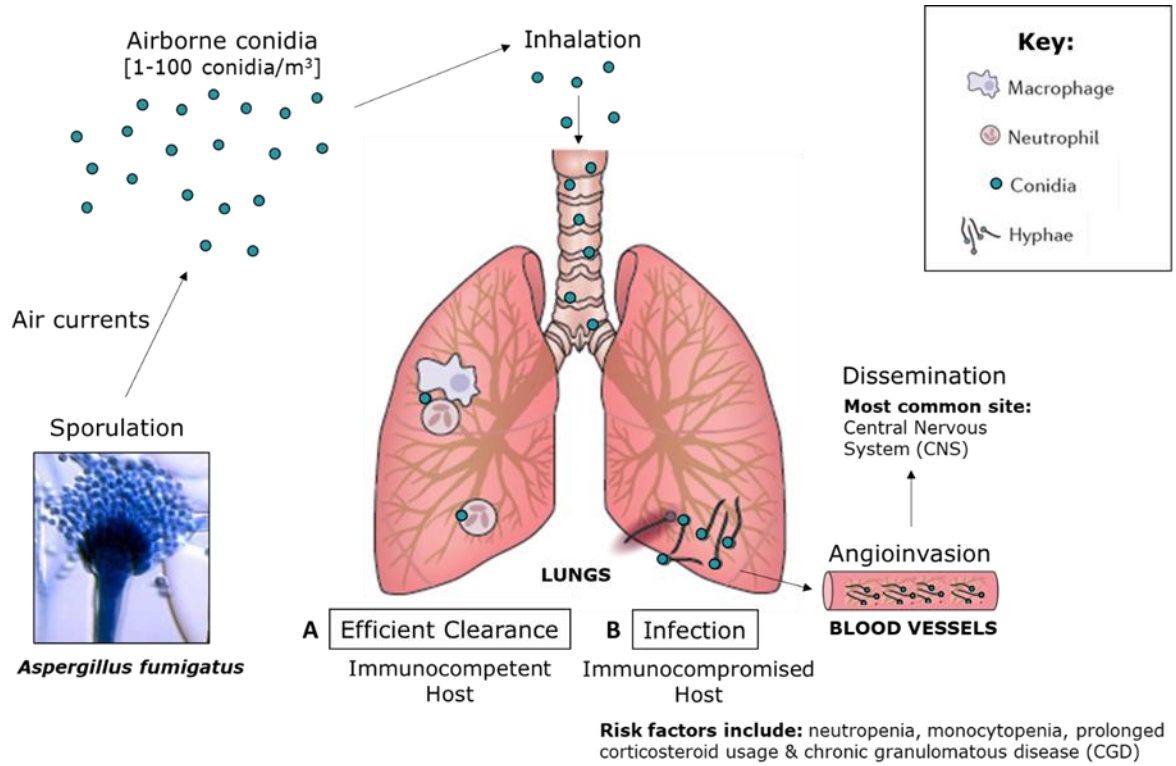

Figure 1.1 Development of invasive aspergillosis (IA) depends on host immune status. (A) Immunocompetent hosts successfully clear conidia to prevent infection. (B) Without conidial clearance such as due to immune defect, conidia can germinate into hyphae leading to tissue invasion and subsequent dissemination to other organs (Adapted from van de Veerdonk et al[44] to highlight IA).[44, 57]

Macrophages

Resident macrophages in lung alveoli are involved in the clearance of cellular debris and elimination of pathogens via phagocytosis and the secretion of pro-inflammatory cytokines that stimulate further immune responses.[48, 58] As phagocytic cells, macrophages rapidly engulf and kill pathogens within phagolysosomes.[48] This has been observed with swollen *A. fumigatus* conidia involving the production of reactive

oxygen species (ROS) by NADPH oxidase.[44, 56] In the lungs, elimination of conidia by macrophages is an almost complete process.[59, 60] If alveoli have a large conidial burden, macrophages recruit assistance from circulating neutrophils which respond against both conidia and hyphae.[48, 53] Although macrophages are important phagocytes, *A. fumigatus* conidia have been reported to resist macrophage phagocytosis by interrupting phagolysosome processes supporting conidial persistence,[61] germination and subsequent escape from the phagocyte.[62, 63] Neutrophils are thus considered the key early responder against *A. fumigatus*,[48, 64] which increase in response to infection despite host immune status.[65] Defects in neutrophils such as due to chronic granulomatous disease (CGD) or immunosuppression can thus increase host susceptibility to *A. fumigatus* as uncontrolled fungal growth can have deadly consequences.[66] Neutropenia is thus considered a primary risk factor for IA.

Neutrophils

Neutrophils are immune cells present in large numbers in the bloodstream (65-75% of immune cells).[67] During infection, neutrophils are rapidly recruited to the infection site via resident macrophage and epithelial cell signalling. Neutrophil pattern recognition receptors (PRRs) identify the pathogen via pathogen-associated molecular patterns (PAMPs) (e.g., β-glucan, α -glucan and chitin). Signalling thus ensues driving the antifungal immune response which is supported by opsonin proteins that increase pathogen susceptibility to phagocytes.[66, 68] Pentraxin 3 (PTX3) is an important opsonin released from neutrophil granules against *A. fumigatus*.[66]

Several mechanisms are utilised by neutrophils in response to infection. This includes phagocytosis which is observed against early *A. fumigatus* morphotypes,[69]

degranulation and neutrophil extracellular trap (NET) release.[66] Neutrophils may also employ clustering behaviour to restrict fungal growth.[70] This has been observed against *A. fumigatus* conidia and hyphae too large for internalisation.[66, 71]

1.2.2 Neutrophil elimination mechanisms

Phagocytosis

Phagocytosis is an important mechanism against *A. fumigatus* conidia.[71] This process requires internalisation of the pathogen involving pseudopod extension around the foreign body.[58] The internalised pathogen is contained within a vesicle called a phagosome. Neutrophil primary and secondary granules fuse with the phagosome, releasing granular contents to form the phagolysosome. The neutrophil then undergoes a respiratory burst resulting in reactive oxygen intermediates that along with antimicrobials contribute to the destruction of the phagocytosed pathogen.[58, 66, 67]

Degranulation

Neutrophils contain intracellular vesicles known as granules within their cytoplasm which store antimicrobial proteins released during host defence. Several granule types have been observed which differ in contents, function, morphology, and release. Primary (azurophilic) granules are the largest granules which are primarily antimicrobial. These contain proteases (e.g., elastase), peroxidases (e.g., myeloperoxidase) and membrane cytolytic molecules (e.g., defensins). Secondary (specific) granules follow and are multipurpose granules that assist with migration and have some antimicrobial function. These contain membrane cytolytic molecules, peroxidases, and iron-binding lactoferrins that can inhibit *A. fumigatus* germination. The smallest granules, tertiary (gelatinase), like secondary granules are involved in

migration with some antimicrobial function. These contain matrix metalloproteinases, sialidase, azurocidin and lysozyme which can hydrolytically damage the fungal cell wall. Another type of granule contained within neutrophils are secretory vesicles which are specialised for neutrophil migration.[69, 72]

Neutrophils deploy their granules in the process of degranulation. Upon pathogen recognition, signalling alters the neutrophil cytoskeleton in preparation for granule transportation by microtubules. Granules are transported similarly to the phagosome in degranulation, where granules are instead transported to the plasma membrane where fusion and subsequent release of granular contents occurs.[69] This occurs in response to pathogens too large to phagocytose such as hyphae, but also against conidia. PTX3 is also released from granules and involved in NETs. The release of granular contents by neutrophils may provide important antifungal activities as in a murine model with ROS-independent enzyme defects, *A. fumigatus* susceptibility was observed highlighting how various factors may contribute to pathogen defense.[66] Lactoferrin, an iron-binding glycoprotein contained in secondary granules, is considered an important inhibitor of *A. fumigatus* germination.[73] By scavenging extracellular iron, iron availability is limited which can restrict pathogen growth.[73, 74] Elastase has also been suggested to contribute to *A. fumigatus* clearance.[75]

Neutrophil extracellular traps (NETs)

Neutrophil release of neutrophil extracellular traps (NETs) occurs during NETosis which is a ROS-dependent cell death process.[71] It involves the disintegration of the nuclear envelop and granular membranes, and the secretion of nuclear DNA onto extracellular pathogens. The result is a mesh-like structure known as a NET which contains chromatin fibres, histones, and DNA.[66, 76] Pathogens caught by NETs are

immobilised and exposed to granular antimicrobial proteins including elastases and calprotectin that serve to eliminate the pathogen,[48, 66] with a risk to host tissue however.[76] NETs have been observed in human,[71, 77] and mouse neutrophils.[71, 78] NET release has been observed against fungi to limit hyphal growth,[79] such as against *C. albicans* hyphae in mice where higher ROS levels occurred compared to yeast form *in-vitro*.[78] In response to *A. fumigatus*, NETs have been deployed against all morphotypes; resting and swollen conidia, and hyphae. Neutrophils were observed to enlarge and burst appearing as cables, threads, and globular domains.[71]The response against conidia is weaker than hyphae due to hydrophobin linked previously to confer protection against adaptive immunity.[71, 80] Despite NET release against *A. fumigatus*, killing does not occur,[79, 81] unlike with *C. albicans*.[82] NETs are thus considered important inhibitors of fungal spread due to calprotectin.[71, 83] The importance of this inhibitory role was noted with *A. nidulans* where NET formation defects proved fatal.[76] Clearance of *A. fumigatus* and prevention of invasive infection may thus occur due to neutrophil phagocytosis,[71] ROS production and the release of proteases via degranulation (Figure 1.2).[48]

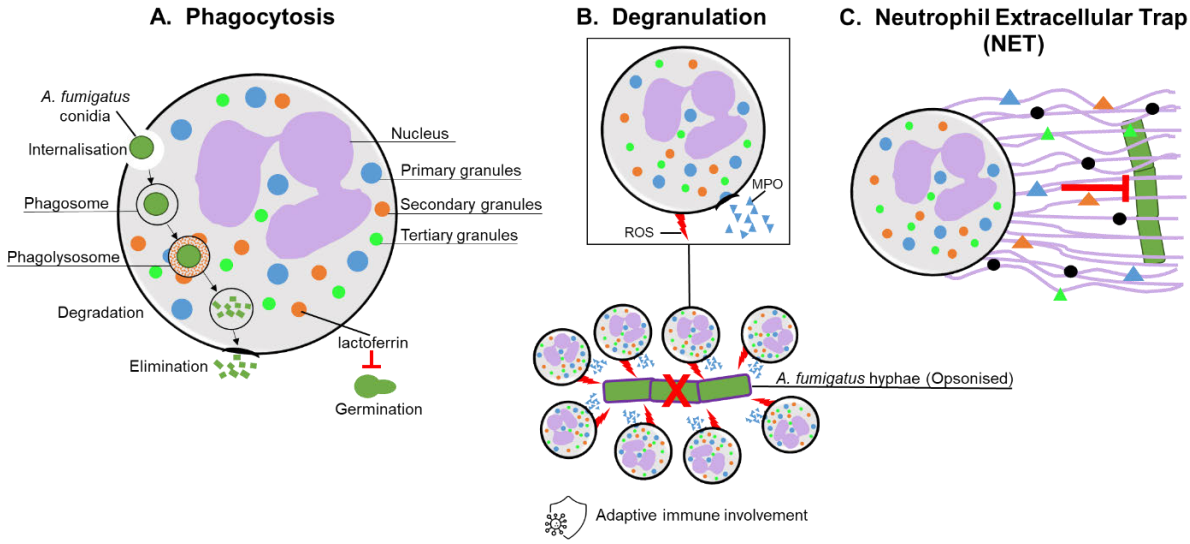

Figure 1.2 Neutrophil responses against *Aspergillus fumigatus* components.
(A) Phagocytosis of *A. fumigatus* conidia involves internalisation of conidia into a phagosome, fusion with primary and secondary granules to form the phagolysosome, and subsequent conidial destruction. Lactoferrin contained within secondary granules is an important inhibitor of conidial germination and thus conidial outgrowth and escape. (B) Opsonised *A. fumigatus* hyphae are eliminated by ROS generated by NADPH oxidase complex and the release of myeloperoxidase (MPO) by neutrophil primary granules.[73] (C) NETs produced in response to hyphae are fungistatic and prevent fungal dissemination. These contain chromatin fibres, histones, DNA, and proteins such as calprotectin which is important for *A. fumigatus* inhibition, and pentraxin 3 (secondary granules) which modulates host immunity by activating complement and facilitating pathogen recognition.[73, 84]

1.2.3 *A. fumigatus* virulence

Although the development of *A. fumigatus* infection is largely dependent on the host's immune status, the characteristics of the fungus are of equal importance as infection ultimately depends on the interactions between the host and fungus.[13, 57, 85] Understanding how infection progresses is difficult as the virulence of *A. fumigatus* is influenced by multiple factors and involves various fungal attributes. This has been identified by the differing virulence profiles between different *A. fumigatus* isolates of environmental and clinical origin.[86, 87]

As an opportunistic pathogen, *A. fumigatus* virulence is believed to have arisen from environmental pressures. Consequently, the fungus harbours a range of survival traits that contributes to their ubiquitous nature and predominance in compost especially during high temperatures. This ability to survive within the compost environment highlights the survivability of the fungus as conditions within compost are harsh and dynamic. High temperatures (up to 50°C), variations in pH, and nutrient availability which consequently results in competition are some challenges of compost.[88] Consequently, compost is likely where the fungus developed virulence traits that coincidentally benefit survival within immunocompromised hosts which although immune impaired, requires the fungus to overcome various immune challenges.[42] Virulence traits that benefit growth within human hosts includes thermotolerance which enables rapid growth of the fungus at human body temperature which is key for the success of human pathogens,[11, 57, 89] and its adaptability to challenging conditions which involves various stress response pathways.[42, 44]

Due to our immune system, *A. fumigatus* must overcome a range of host defensive responses. Beyond human body temperature[90] this includes host nutritional immunity, specifically the activity of lactoferrin which by scavenging iron is an important part of the first line of defence as it acts to starve invading pathogens.[74] Human pathogens thus have mechanisms to overcome such restrictions with *A. fumigatus* producing proteases that degrade host tissue for nutrient acquisition.[42, 44] The fungus can similarly adapt to oxygen deficiency (hypoxia) which can result from inflammation and fungal damage. During hypoxia, the fungus can switch its metabolism to fermentation negating the need for oxygen.[13, 44] Before the fungus can

even reach the growth stage, conidia must first access and adhere to host cells which is assisted by conidial morphology.[42]

Apart from the small size of conidia which enables access to the lower airway,[42] *A. fumigatus* conidia have several features implicated in infection. This includes the conidial cell wall consisting of an outer hydrophobin layer that allows immune evasion by preventing immune recognition and platelet activation.[91] Following the outer hydrophobin layer is melanin which has been implicated in the pathogenesis of various microbes with advantages for survival outside and inside the host.[92, 93] Within the host, melanin and the hydrophobin layer contribute to conidial survival by masking PAMPs necessary for pathogen identification by host immunity. Furthermore, melanin confers resistance against ROS utilised by host innate immune effector cells,[42, 44, 94] reduces internalisation by phagocytes,[95-97] and prevents apoptosis of macrophages favouring fungal persistence.[61] *A. fumigatus* conidia also have more sialic acid residues on their surface than other *Aspergillus* species and thus may have greater adherence.[42]

Following conidial persistence, germination can occur which involves various structural alterations. This includes to the hydrophobin layer which is dismantled to support nutrient exchange and growth.[91] Hyphal growth can thus occur with subsequent formation of a biofilm involving secretion of an adhesive molecule called galactosaminogalactan (GAG). Like melanin, GAG impairs immune recognition and protects the fungus from various immune effectors cells. This includes inducing neutrophil apoptosis and providing resistance against neutrophil extracellular traps (NETs), both of which are NADPH oxidase dependent.[44, 98] Other anti-ROS responses elicited by *A. fumigatus* includes the upregulation of cofilin. Cofilin is a transcriptional regulator of oxidative stress response genes such as *yap1*. Yap 1

regulates the production of superoxide dismutase and catalase which converts superoxide to hydrogen peroxide, and hydrogen peroxide to water respectively. This conversion of ROS into less harmful components is a protective response that inhibits hyphal damage by immune effectors that utilise NADPH oxidase such as neutrophils thus rendering important immune responses ineffective.[13]

A. fumigatus also releases various metabolites from conidia and hyphae for virulence including metabolites such as oxylipins which are sensory,[99-101] and mycotoxins implicated in evasion and suppression of host immune responses.[57, 101, 102] Various mycotoxins are recognised in *A. fumigatus*, including Asp-hemolysin, fumagillin, fumitremorgin A, gliotoxin and helvolic acid.[103] One of the most important mycotoxins produced by *A. fumigatus* is gliotoxin. It is produced by over 95% of isolates originating from both the clinic and the environment.[104] Gliotoxin production has been identified in individuals diagnosed with IA and in various animal models of aspergillosis where various immunosuppressive effects have been observed. This includes inhibition of conidial phagocytosis, T cells of the adaptive immune system, mast cell activation, monocyte apoptosis and NADPH oxidase which consequently inhibits ROS production. Gliotoxin may also lead to epithelial cell damage, and induction of ROS-facilitated apoptotic cell death.[105] Fumagillin is another important mycotoxin which inhibits immune cells such as neutrophils,[102] and may contribute to tissue damage during IA.[106, 107]

Despite the ability of *A. fumigatus* to overcome host immune responses and resist antifungals, successful infection primarily results from immune impairments which renders individuals susceptible to this opportunistic fungal pathogen. The host environment is thus a crucial factor for pathogenesis which depends on host-fungal interactions. Not enough is known about infection within the different host

environments. To gain new insights, *in-vivo* studies are necessary to elucidate key interactions between the host and fungus.[42, 44] As IA is a rare and unpredictable condition, studying IA within patient cohorts is difficult. Patient analogues, such as animals, have therefore been necessary to study pathogenesis in detail.

1.3 Research using animals

Animals have been important for our understanding of biology since the times of Aristotle. Despite animal use dating far back in scientific history, the practice of using animals for biological research was only really appreciated during the 18th and 19th century where animal involvement became increasingly common in biological research. Consequently, animal studies have been fundamental for our understanding of biology, addressing questions concerning the basic principles of life, to the anatomy, physiology, pathology and pharmacology of both human and animal.[108] The importance of animals to enhance our understanding of biology is documented throughout history, including in the works of Antoine-Laurent de Lavoisier who used a guinea pig to study respiration,[109] and Louis Pasteur who used various animals including cows, rabbits, sheep, and pigs for vaccine development.[110] As the use of animals is a long-standing practice, their involvement in research can be considered integral to advance biological understanding as their usage has already contributed to various breakthroughs that have improved overall quality of life. This is evident by the availability of drugs, vaccines, cellular therapies, anaesthesia protocols and surgical techniques utilised in healthcare.[108, 111]

Due to the known contributions of animal studies to biology, increasing numbers of animal species have been established as research models, including mammals such as mice and a growing list of invertebrates. Those considered model organisms are

generally well-understood and manageable within the confines of the laboratory.[112] As biological similarities exist between humans and animals, model organisms have been useful to study the development of human diseases and related therapeutic agents.[111] This is especially true when human studies are not appropriate, for reasons including high risk, such as when dealing with potentially deadly agents, or low incidence, and during initial therapeutic testing.[113] By mimicking human infections in model organisms researchers can study important aspects of infection including progression, transmission, pathogen virulence and host immune responses, and evaluate diagnostics and treatments within a complex system that is like our own but without risk to the human population.[47, 111, 114]

Research using animals can be divided based on methodological approach into *in-vivo* and *in-vitro*. *In-vivo* experiments utilise a whole live organism while *in-vitro* experiments occur outside the living organism such as using isolated cells in tube experiments. Although *in-vitro* methods are usually more ethically sound than most *in-vivo* animal studies, *in-vitro* methods are usually too simple to study human infections especially those of opportunistic pathogens as what underlies these infections are complex interactions largely a product of the host environment. Consequently, important systemic interactions may not be studied directly using *in-vitro* methods.[47] A combination of *in-vivo* and *in-vitro* methods can be beneficial as *in-vitro* methods are useful to focus on specific cells (e.g., immune cells), molecules, or organs.[111]

1.3.1 Model organisms to study fungal pathogens

Model organisms are an important tool to study the pathogenesis of fungal pathogens such as *A. fumigatus* as research is not viable in sick patients where

priority is to treat and cure. High-throughput studies of fungal pathogenesis are thus not possible in patients leading to the appeal of alternative methods such as model organisms. Infection modelling using animals has provided insights towards our understanding of aspergillosis regarding pathogenesis, the immune response, virulence such as the impact of different strains and inoculum sizes, host susceptibility and transmission. Consequently, this has improved our ability to predict serious forms of infection and formed a basis to which diagnostic and treatment strategies can be developed.[47, 114, 115]

Research using various animal models have contributed to our understanding of infection, as in combination these can lead to approximations of the condition in humans. As each model has its limitations, one species alone is insufficient to answer all research questions. Data obtained from different animal species and infection routes is therefore vital to progress our knowledge on infection and subsequently develop treatment strategies.[114] Like fungal strain, model organisms are selected based on the research question as infection can differ depending on interactions between the host and pathogen.[38, 112] Consequently, various mammals and a growing list of insects, nematodes, fish, and frogs, have modelled aspergillosis due to some similarity with the human system. This includes in physiology and immunity,[38, 112] with invertebrates, although less like humans than mammals, having innate immune systems with structural and functional similarities despite their divergence long ago.[108, 112, 116]

1.3.2 Mammalian Models

Several mammalian species have been involved in aspergillosis research including mice, rats, guinea pigs and rabbits. Mice are most popular to study aspergillosis

utilised since 1967,[47, 99, 117] and in 85.8% of rodent studies modelling *A. fumigatus* induced IA.[38] The popularity of mice in research overall has contributed to their utility in studying *A. fumigatus* as a wealth of knowledge exists on this model species which has revealed the extent of their similarities with humans including in anatomy and immunity**.** In addition, mice also have favourable attributes that support their use in the laboratory. This includes their small size which enables large populations to be managed within the laboratory, and thus the preparation of replicates important to enhance statistical power. Furthermore, their relatively low cost and the consequent availability of materials and methods dedicated to the mice model system.[47]

Although mice have various similarities to humans, it is important to note that differences do exist like with other models which could greatly affect the insights gained from this model species. This includes in immunology with mice having more bronchus-associated lymphoid tissue and fewer neutrophils (10-25%) in their blood than humans which also differ in antimicrobial peptide expression.[78, 118] Caution should thus be taken with insights gained from all model species. Due to ethical considerations tied to mammalian systems, concerns are rising over the treatment of animals within the laboratory which is altering the selection of model organisms.[108]

As animal research is widely appreciated for contributions towards diagnostic and treatment development which has improved disease outcomes and thus quality of life, there is a need to continue generating scientific knowledge while also addressing concerns over the treatment of animals in the laboratory. As a result, various laws regulate the use of animals in research including the Cruelty to Animals Act. This act states that animals must only be used when the knowledge is necessary to save or prolong life, or alleviate suffering, and that animals must be anesthetized and killed immediately post-experiment if they are injured or in pain.

Along with such laws, principles guiding the use of animals has been established such as the principle of the 3R's which emerged from the United Kingdom and became universal. The 3R's refers to replacement, reduction, and refinement. Replacement, which is considered a major goal of animal research, aims to limit the use of mammals by encouraging non-mammalian substitutes such as invertebrates, microorganisms, cell cultures, organs, or cellular fractions whenever possible. Should replacement not be possible, reduction must be considered which states that methods should enable the use of a minimum number of animals to produce statistically significant results which is possible with detailed planning. Lastly, refinement states that methods to avoid animal suffering should be utilised (e.g., anaesthesia, pain relief and non-invasive techniques).[108, 119]

1.3.3 Invertebrates for biomedical research

Due to the development of these ethical guidelines, alternative non-mammalian species have grown in popularity which follows the replacement principle of the 3R's. This is demonstrated in PubMed research output as during 2008-2010, 44,000 research papers used invertebrates to study diseases, genes, and drugs.[120] Invertebrates including insects (e.g., *Galleria mellonella*) are clearly growing as research models, likely as they are ethically unconstrained and have similarities with mammals making them useful to study pathogens such as *A. fumigatus*.[112]

Although insects do not produce antibodies as they lack adaptive immunity, they do utilise a complex innate immune system that has cellular and humoral elements comparable to that of vertebrates. This makes them useful for early response studies such as in IA pathogenesis, along with their overall simplicity compared to mammals, cost efficiency, ease of manipulation and maintenance in the laboratory, and their

ability to provide rapid results as infection progresses faster which permits high-throughput research.[121] They are thus also beneficial during early stages of drug discovery and to investigate physiology as insects have similarities with higher animals such as in signalling molecules which highlights how certain factors are conserved in the animal kingdom.[111, 122]

Popular invertebrates utilised for biological research includes *Drosophila melanogaster* (fruit-fly) which led to the discovery of Toll-like receptors,[123] for genetic and disease research and *Caenorhabditis elegans* (roundworm) also utilised to study human diseases.[120] It is important to note that the use of alternatives such as invertebrates are unable to completely replace mammalian modelling. To minimise their use, mammals are utilised during later research stages for validation of developed hypotheses and models, and thus adopted for a more targeted role.[111, 124]

Invertebrates for *Aspergillus* research

Choosing the ideal research model is a critical part of research design. Invertebrates, although complex, are simple compared to mammals.[116] Determining which model/s is suitable to investigate a pathogen such as *A. fumigatus* therefore involves considering which virulence trait or host response is of interest, available finances, time, space, and the advantages and disadvantages of the model system as it influences the outcomes of the study.[125] *D. melanogaster* for example is a common model to study human genetics and diseases as a wealth of knowledge and methods dedicated to this organism exists. Consequently, the fly model has contributed to the understanding of various neurodegenerative diseases. *C. elegans*, have similarly been beneficial with it utilised to understand conditions such as diabetes, cancer, and immune disorders, and subsequently the corresponding therapeutics.[120] Despite

their contributions, both these models have limitations such as due to their size which prevents their accurate inoculation, and their inability to tolerate human body temperature which limits the insights that can be gained into human infections.[112] The larvae of the greater wax moth *Galleria mellonella* is growing in popularity as unlike the *D. melanogaster* and *C. elegans*, they can tolerate human body temperature and are larger enabling accurate inoculation, easier manipulation and maintenance in the laboratory.[112, 126] Each model species thus benefits research topics in different ways with *D. melanogaster*,[127] *C. elegans*,[128] and *G. mellonella* all utilised to study *Aspergillus* pathogenesis.[101, 102, 129-132]

G. mellonella larvae were first recognised for fungal research when susceptibility to fungal challenge was observed which enabled screening of pathogenic and non-pathogenic *Candida albicans* strains.[133] *G. mellonella* larvae has subsequently been utilised to study *C. albicans* biofilms,[134] filamentation,[135] antifungals,[136] and other fungal species including *A. fumigatus* with data that correlates with murine and human findings.[102, 126, 129, 137] *G. mellonella* larvae are thus a leading invertebrate to study *Aspergillus* pathogenesis. However, as it is not easily obtained in certain countries such as Brazil[138] and Australia where it is a plant pest, an alternative invertebrate with benefits like *G. mellonella* could expand research efforts towards understanding human fungal pathogens like *A. fumigatus*. The larvae of the beetle *Tenebrio molitor* may serve as one such alternative though limited research exists on their use to study human fungal pathogens like *A. fumigatus* [121].

To establish *T. molitor* larvae to study *A. fumigatus* pathogenesis which has already been utilised to study *C. albicans* and *Cryptococcus neoformans*,[138] its comparability to humans and existing model organisms must be understood to determine the extent to which it can mimic human infection. This includes assessments of

susceptibility and pathogenesis at various levels such as system, organ, tissue, cell, and subcellular levels as an extensive understanding of the species contributes to its standardisation as a research model. By having a thorough understanding, strengths and weaknesses of the model can be appreciated enabling accurate assessments and extrapolations of results thus preventing incorrect conclusions that hinder knowledge advancement and waste animal life.[139]

1.4 *Tenebrio molitor*

Tenebrio molitor is a species of darkling beetle from the order Coleoptera which consists of beetles and weevils. It is the most species-rich order in kingdom Animalia as it has approximately 400,000 species.[122] Some species are considered beneficial, such as those that pollinate flowers, while others are pests that interrupt human activities such as by damaging crops. *T. molitor* is a pest of stored grains and bran that is emerging as a beneficial species.[140] Not only are they a suitable food source for animals including birds, reptiles, fish, bats, and humans due to their high protein content, but they are also of increasing interest in environmental and biomedical research.[121, 138] Environmentally, interest into *T. molitor* larvae is due to their ability to degrade plastics,[141] while in biomedical research interest is due to the recognition of research characteristics comparable to that of *G. mellonella* larvae. This has suggested the use of *T. molitor* larvae as an alternative species to study human infections.[138]

Like *G. mellonella*, *T. molitor* is a typical holometabolous insect whose development is characterised by four stages: egg, larvae, pupae, and beetle. These are influenced by various environmental factors including temperature, humidity, population density, and availability of nutrients and oxygen. The larval stage, where they are known as

mealworms, is of interest to consumer and researcher. At this stage, *T. molitor* is slender and segmented emerging white before developing its characteristic yellow/brown appearance. Growth is up to 2.5 cm in length, and development via a series of stages, up to 20, known as instars which proceed via moulting.[121]

1.4.1 *T. molitor* for biomedical research

T. molitor has several favourable characteristics that have suggested its utility as a research model to study human pathogens (Figure 1.3). This includes larval size as they can be easily manipulated enabling accurate inoculation and maintained in large populations within the confines of the laboratory.[138] Smaller invertebrates like *D. melanogaster* which are only around 3 mm long,[142] are difficult to handle and manage especially without specialised equipment (e.g., “fly rooms”) and expertise. This consequently limits the suitability of the fruit fly model to certain laboratories.[112] The ability of *T. molitor* larvae to tolerate 37°C, human body temperature, is another important characteristic to study human pathogens that is not applicable in some insect research species.[138] Studying infection at 37°C can include temperature-dependent interactions in the model as temperature can influence microbial virulence and infection development.[143] Other characteristics include *T. molitor* larvae availability as they are readily sold as pet food in Australia, their overall low cost, and simple dietary requirements. As *G. mellonella* larvae have found use in pathogenesis research with similar characteristics,[112] *T. molitor* larvae may be a suitable research alternative. As an invertebrate, *T. molitor* only utilises innate immunity which has similarities to vertebrates,[121] and thus the potential to study infections where innate immunity is important to prevent pathogenesis.

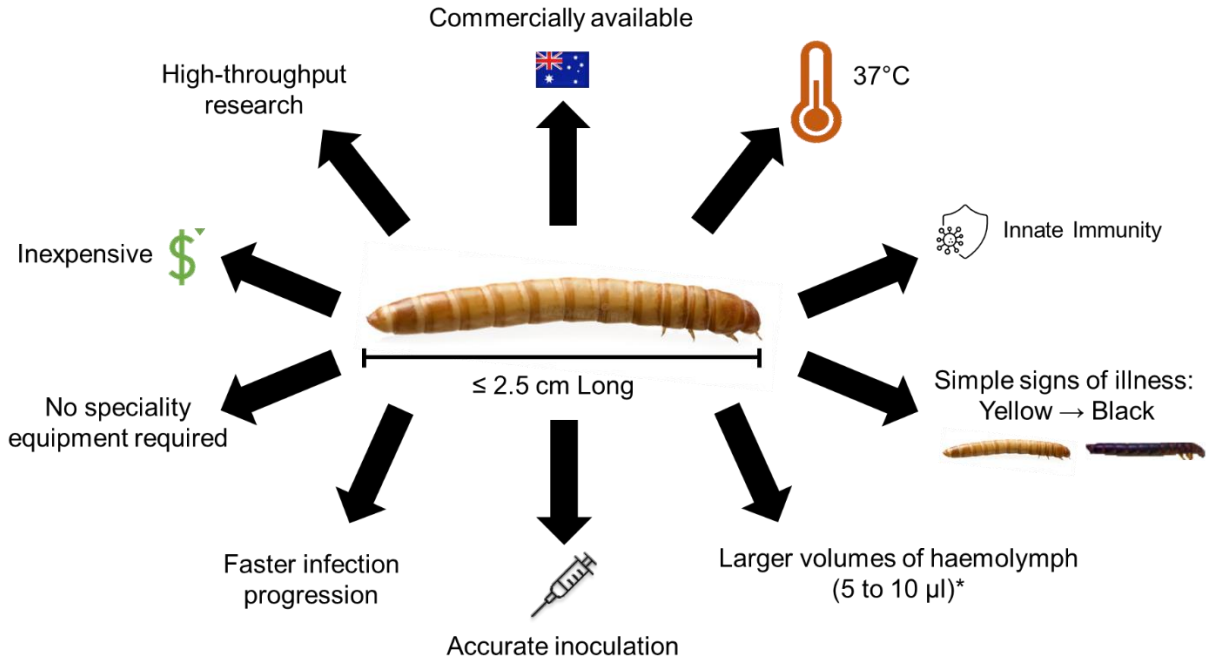

Figure 1.3 *Tenebrio molitor* larvae research characteristics.
T. molitor larvae are commercially available in Australia, can tolerate human body temperature (37°C), utilise innate immunity, have visible signs of illness, large volumes of haemolymph recoverable (*compared to *D. melanogaster*), can be accurately inoculated, have faster infection progression, no speciality equipment requirements, are inexpensive and viable for high-throughput research.[121, 138]

Although *T. molitor* lacks an adaptive immune system, longer lasting antimicrobial immune responses have been observed (lasting ≥14 days).[144, 145] This was observed against various stimuli including entomopathogenic fungi, likely to prevent reinfection.[146, 147] Similar long-term protective responses have been observed between parent and offspring as parental experience with pathogens enhanced offspring antimicrobial activity.[148] Thus, despite the reliance on innate immunity, insects like *T. molitor* have a well-developed defensive ability.

Insects and Entomopathogens

As microbes are ubiquitous in the environment, insects also have protective mechanisms against pathogenic microbes including fungi. Microbes are problematic

for insects when they penetrate the cuticle which is hydrophobic and antimicrobial, and thus in the case of fungi, should prevent adhesion and germination of fungal conidia. Entomopathogens can bypass the cuticle via physical or enzymatic means to cause infection.[149] Entomopathogens include the filamentous fungus *Beauveria bassiana* which infects hundreds of insects as the fungus can enzymatically degrade the cuticle to reach the internal space.[149, 150] Consequently, the insect innate immune system must respond to clear the pathogen from within. This must occur despite microbial virulence traits including masking of immunogenic surface components, and the secretion of immune modulators and metabolites for immune avoidance/suppression observed with entomopathogenic fungi.[149] In *T. molitor*, the antifungal response against *B. bassiana* includes the peptide Tenecin 3.[151]

1.4.2 Insect Innate Immunity

Like in vertebrates, the invertebrate innate immune system consists of cellular and humoral components which respond to pathogens that breach the first lines of defence. This includes the insect integument which is predominantly cuticle, and the digestive tract.[121] Insects utilise a range of mechanisms to manage infection. This includes humoral responses such as melanisation involving phenoloxidase which is utilised in defence against fungi and gram-positive bacteria in *D. melanogaster*.[152] Other humoral responses include haemolymph coagulation, synthesis of ROS, complement-like proteins,[58, 121] and antimicrobial peptides which are secreted into insect haemolymph from the fat body, epidermal cells, epithelia, and by haemocytes.[149] Haemocytes are part of the cellular response where they are involved in phagocytosis, and capsule responses which can ultimately lead to melanisation. These are initiated during pathogen exposure as like human immune

cells, insect haemocytes can distinguish self from non-self and thus recognise foreign invaders.[58, 121]

Several types of haemocytes are observed in insects which are morphologically and functionally distinct.[140] These circulate in the haemolymph or adhere to internal organs, such as the digestive tract and fat body where they vary in density depending on the insect's life and during pathogen challenge.[58, 153] At least eight types of haemocytes have been observed overall which includes granulocytes, plasmatocytes, prohaemocytes, oenocytoids, crystal cells, spherulocytes, lamellocytes and adipohaemocytes. These depend on the species, likely due to habitat preferences, with usually only a few types of haemocytes observed (Table 1.1).[58]

Table 1.1 Examples of haemocyte types reported in insects.

Order	Species	Types of haemocytes
Coleoptera	*Tenebrio molitor* (Mealworm)[140, 154]	• Granulocytes (50-60%) • Plasmatocytes (23-28%) • Oenocytoids (1-2%) • Prohaemocytes (10-15%)
Diptera	*Drosophila melanogaster* (Fruit-fly)[155]	• Plasmatocytes (95%) • Crystal cells (5%) • Lamellocytes (during stress)
Lepidoptera	*Galleria mellonella* (wax moth)[156]	• Granulocytes (52%) • Plasmatocytes (41%) • Oenocytoids (0.9%) • Prohaemocytes • Spherulocytes (6.1%)
	Role in immunity	
Adipohaemocytes	unknown	
Coagulocytes	coagulation[157]	
Crystal Cells	encapsulation[155]	
Granulocytes	Phagocytosis, encapsulation and nodulation[140]	
Lamellocytes	encapsulation[155]	
Oenocytoids	melanisation[140, 158]	
Plasmatocytes	Phagocytosis and encapsulation[140]	
Prohaemocytes	Precursor cells[140, 159]	
Spherulocytes	nodulation[158]	

Granulocytes and plasmatocytes account for the majority of *T. molitor* haemocytes at all development stages and are likely the main cellular responders (Figure 1.4).[154, 160] This is true in the Lepidopteran insect, *G. mellonella*, where granulocytes and plasmatocytes also predominate as phagocytes and are involved in most cellular responses (Figure 1.4).[161] *T. molitor* may thus be comparable to *G. mellonella* in immunity with mitosis, observed in circulating granulocytes to maintain cell numbers,[160, 162] stimulated in both insects by the same agent.[163] In *Drosophila* where granulocytes are absent, most cellular responses involve plasmatocytes and lamellocytes.[161] Insect innate immune responses may thus vary between species, with *T. molitor* also observed to differ from *Drosophila* in molecular recognition mechanisms.[164]

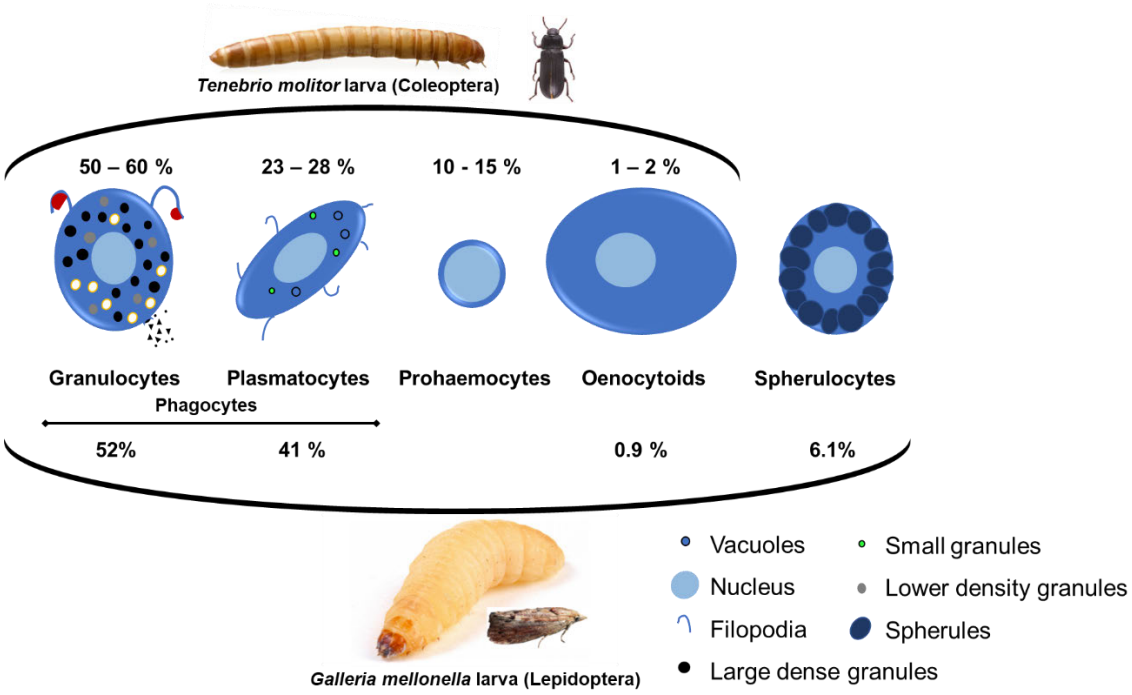

Figure 1.4 Haemocyte types observed in *T. molitor* larvae and *G. mellonella* larvae. Granulocytes and plasmatocytes are predominant haemocytes in both insects serving as phagocytic cells. Prohaemocytes are precursor cells and oenocytoids involved in melanisation. Spherulocytes are unique to *G. mellonella* and are believed to participate in transport of cuticular components.[140, 158-160]

Phagocytic Haemocytes

Granulocytes and plasmatocytes are phagocytic immune cells containing lysosomes that are comparable to mammalian macrophages and neutrophils.[58, 160, 165] During infection, haemocytes recognise pathogens in a similar way to human immune cells and consequently increase in density.[161] This was observed in the *Hyalophora cecropia* against *A. flavus* and *A. niger*.[163] Mechanisms utilised by haemocytes for clearance includes phagocytosis and capsule formation responses consisting of encapsulation and nodulation (Figure 1.5).[121]

In mammals, neutrophils are phagocytes important against *A. fumigatus*. The insect phagocytic equivalent are granulocytes and plasmatocytes as similarities have been

observed. This includes in effectors, genes, proteins, and receptors, including that of phagocytic cells as *G. mellonella* larvae phagocytes have proteins homologous to that of mammalian phagocytes.[58] Assessing the role of these phagocytes in *T. molitor* against *A. fumigatus* will be important as varied phagocytic roles have been observed between insects. In *Gryllus bimaculatus* (two-spotted cricket), granulocytes were identified as the predominant phagocyte,[166] while in *Manduca sexta* (tobacco hornworm), plasmatocytes are the main phagocytic cells.[167] In *D. melanogaster* where granulocytes are absent, plasmatocytes are also the main phagocytes.[165] In *T. molitor*, granulocytes have large amounts of lysosomes and thus may be the main phagocyte.[160]

Haemocyte Phagocytosis

Insect granulocytes and plasmatocytes increase in cell density like human neutrophils in response to infection. As observed in mammals, opsonisation supports phagocytosis by enhancing pathogen internalisation. This involves the complement-like protein, lectin, present in haemolymph which binds to pathogens. In response to bacteria, lectin binding has resulted in the breakdown of the bacterial surface due to lysozyme found in haemolymph, enabling recognition and phagocytosis by plasmatocytes.[58]

Phagocytosis involves alterations in haemocyte shape and the extension of filopodia-like filaments that surround the pathogen for internalisation. These morphological alterations occur similarly to human neutrophils in both plasmatocytes and granulocytes.[162, 166] Granules of the latter have been observed to become enlarged and “glittery” which were identified as phagosomes or lysosomal compartments. In a study using *Protaetia brevitarsis seulensis* larvae (white-spotted flower chafer),

granulocytes underwent the most changes upon exposure to fungi (*Saccharomyces cerevisiae*) and bacteria (*Escherichia coli*). When a pathogen is engulfed, which is size limited,[162] it is packaged into a vacuole and degraded upon lysosomal fusion. Degradation occurs due to ROS production, referred to as the respiratory burst which is necessary for phagocytic killing. Host tissue can become damaged during this response and inflammation induced when granulocytes which degranulate to release enzymes into the phagolysosome, release enzymes outside the cell.[58] Phagocytosis of fungi has been observed in *G. mellonella*, and of *A. flavus* in *Hyalophora sp.* with disintegration of the phagocyte cell membrane observed.[163]

Capsule Formation

Capsule formation is a non-phagocytic immune response in insects consisting of nodulation and encapsulation which are utilised when the pathogen burden is too large.[121] Unlike phagocytosis, these capsule responses can have deadly consequences for granulocytes and plasmatocytes.[58] Nodulation is a haemocyte mediated process without a human equivalent. It is considered a main insect cellular response involving the aggregation of haemocytes around a cluster of pathogens to form a nodule. Melanisation can then occur involving phenoloxidase, which follows a similar cascade to the human complement system and results in pathogen killing.[58] In *T. molitor*, granulocytes may be important for capsule responses due to their high phenoloxidase content compared to other circulating haemocytes.[160]

Encapsulation is like nodulation but it occurs against large foreign structures and can occur without melanisation.[58] As suggested in *T.* molitor, granulocytes are also important for encapsulation in other insects including *G. mellonella* and *P. brevitarsis seulensis*.[162] During encapsulation, granulocytes contact the pathogen and lyse or

degranulate to promote plasmatocyte attachment.[168] Granulocytes and plasmatocytes have main roles in this response in *Lepidoptera* where they layer around the pathogen to form a capsule. In *Drosophila*, lamellocytes are primarily involved in encapsulation.[58]

NET-like structures

The production of NETs observed in human neutrophils has not been identified in insects. However, the production of NET-like structures has been described. NET-like structures produced by granulocytes has been observed in a species of cricket as part of encapsulation and was associated with cell death. Confirmation of whether it was composed of DNA like the NETs of human neutrophils was not achieved as *in-vitro* cultured haemocytes were studied.[166] In *G. mellonella*, NET-like responses were also described in haemolymph where the presence of extracellular nucleic acids led to the formation of NET-like coagulation fibres that entrapped bacteria. This process occurred unlike the NETs of human neutrophils.[169]

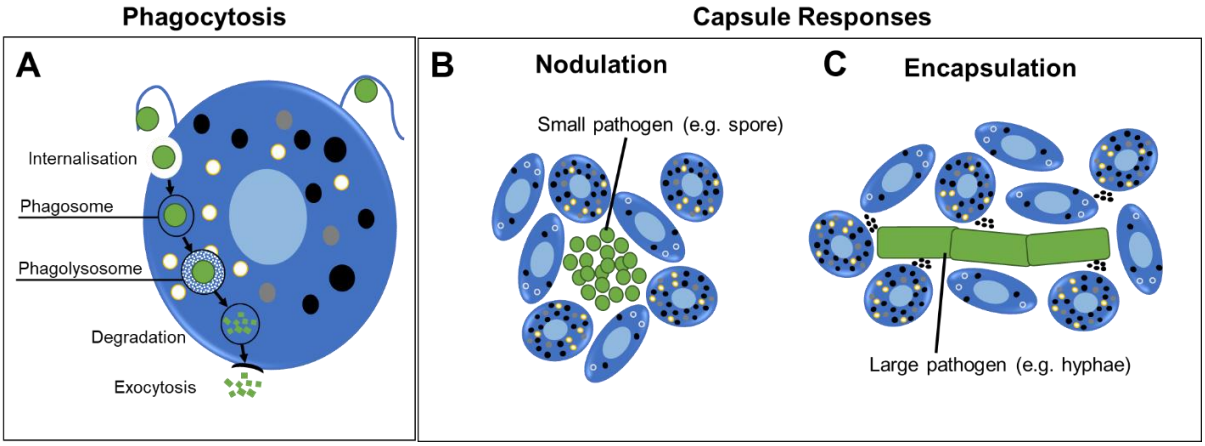

Figure 1.5 Mechanisms utilised by insect immune cells to control infection.
(A) Insect haemocytes respond to pathogens via phagocytosis, nodulation or encapsulation depending on pathogen size. Phagocytosis is carried out by granulocytes and plasmatocytes involving alterations in cell shape and the extension of filopodia-like projections that contact the pathogen resulting in internalisation into a vacuole. Phagosome fusion with cellular organelles then occurs which forms the phagolysosome resulting in pathogen destruction.[170] (B) Nodulation occurs against clusters of small pathogens. It involves haemocyte binding to surround the pathogen and subsequent melanisation. (C) Encapsulation occurs against large foreign invaders (e.g., hyphae). It involves granulocyte lysis or degranulation which attracts plasmatocytes that surround and isolate the pathogenic structure. Destruction then occurs involving phenoloxidase. [58]

1.4.3 Comparability of insect and mammalian phagocytes

Although insect and mammalian phagocytes differ such as in granular cells with insect granulocytes slightly larger and more granule-rich than neutrophils, important similarities do exist. This includes in phagocytosis where ROS is involved in both. Several studies have investigated this similarity as they rely on similar proteins. Consequently, similar responses have been observed such as against Phorbol 12-myristate 13-acetate (PMA) where superoxide production was induced in both.[58] Similar results have also been observed upon exposure to toxins produced by *A. fumigatus*. Gliotoxin suppresses both *G. mellonella* haemocytes and human neutrophils,[171, 172] and fumagillin inhibits phagocytosis, superoxide production and

degranulation in both via inhibition of F-actin conversion.[102, 173] Furthermore, similar responses have been observed against inhibitors of NADPH oxidase (Diphenyleneiodonium chloride) and cell function (cytochalasin b and nocodazole).[58] Insects like *T. molitor* may thus be useful to study phagocytic responses and gain insight into mammalian phagocytes.

1.4.4 *T. molitor* larvae to study *A. fumigatus*

Despite their comparability to *G. mellonella*, *T. molitor* larvae were not used for the study of human fungal pathogens prior to 2015,[138] despite studies into *Listeria monocytogenes*,[174] and *Staphylococcus aureus* infection [175]. In 2015,[138] *C. neoformans* and *C. albicans* infection was investigated using *T. molitor* larvae as an alternative to *G. mellonella* as the insect was readily available. The fungal species investigated are morphologically distinct, in that *C. neoformans* is a yeast while *C. albicans* is dimorphic and can thus grow as a yeast but also as a filamentous organism depending on the environment. Susceptibility of *T. molitor* larvae to both fungi was confirmed, with observations of growth within tissue including filamentation of *C. albicans* which is important for virulence.[138] As filamentation was observed in tissue, *T. molitor* larvae may be useful to study *A. fumigatus* infection as the invasion of tissue by hyphae is important for pathogenesis.

1.5 Project Aims

Although *T. molitor* larvae have been utilised to study several different microorganisms including bacteria (*E. coli*,[176] *L. monocytogenes*,[174, 177] and *S. aureus*)[154, 175, 176, 178, 179] and fungi (*B. bassiana*,[151, 180] C*. albicans*,[138, 176] *C. neoformans*,[138] *Metarhizium anisopliae*,[181] and *Sporothrix* spp.[182]), knowledge relating to this model system is currently limited especially when compared to *G. mellonella* which is a popular invertebrate to study infection. A greater understanding of *G. mellonella* thus exists which unlike *T. molitor* has been used to study infection by *Aspergillus* species.[101, 102, 126, 130-132] To establish *T. molitor* larvae as an invertebrate to study *A. fumigatus*, this project aims to characterise infection of larva by *A. fumigatus* clinical isolates as a continuation of previous research efforts.[183] This includes examination of the infection burden quantitatively using quantitative culture and quantitative polymerase chain reaction (qPCR), and qualitatively using histological techniques and magnetic resonance imaging (MRI). Furthermore, *T. molitor* larva haemocytes are also investigated *in vitro*. The utility of the larvae for *A. fumigatus* pathogenesis research is thus described and the applicability of several methods which may validate the use of *T. molitor* larvae for fungal research and as an alternative to *G. mellonella* larvae.

Chapter 2 General Methods

2.1 Potato dextrose agar (PDA)

Potato dextrose agar (PDA) (P2182, Sigma-Aldrich) was prepared as described by the manufacturer. 15.6 g of PDA was added to 400 mL distilled water. This was dissolved using a microwave and autoclaved. Plates were then poured once the molten agar had slightly cooled.

To inhibit bacterial growth, chloramphenicol was added to the agar prior to pouring. Once the molten agar had slightly cooled down, chloramphenicol selective supplement (29231, Sigma-Aldrich) was added as described by the manufacturer. 1.6 mL of supplement was added to 400 mL of PDA agar, mixed and plates poured.

2.2 PBS / PBST

A phosphate buffered saline (pH 7.4, PBS) (P4417, Sigma-Aldrich) tablet was dissolved in deionised water as described by the manufacturer and autoclaved. To make PBST, Tween-20 was added to have a 0.05% solution. This solution was sterilised by passing it through a 0.22 µm filter.

2.3 Spore suspension

A. fumigatus isolates (AF01-AF15) provided by Westmead Hospital (NSW, Australia) were grown on PDA for 2 to 3 days at 37°C until there was heavy sporulation on the cultures. To prepare a spore suspension, within a biosafety cabinet 4 mL of PBS

containing 0.05% Tween-20 (PBST) was added to the agar plate and a cotton swab was used to agitate the spores into suspension. To isolate the spores, the solution was collected and passed through a 40 µm cell strainer (15-1040, BIOLOGIX). Spore concentrations were determined using haemocytometer counts, typical yield was 1 x 10^8 conidia / mL.

2.4 *T. molitor* larvae selection and maintenance

T. molitor larvae utilised in experiments were purchased from BioSupplies, an Australian Pet Food supplier, and left to recover from transit for at least one day at room temperature (22°C). Individual larvae were chosen for uniformity based on weight (100 - 150 mg) and appearance, avoiding individuals with darkened cuticles as they may have increased pathogen resistance.[184] Chosen larvae were stored in Petri dishes in groups of 10 and provided with approximately 3 ml of fresh food consisting of wheat bran and LSA (linseeds, sunflower seeds, and almonds) in a 5:1 v/v ratio, and a small piece of carrot (approx. 500 mg) changed daily as described previously (Figure 2.1).[183]

Figure 2.1 *T. molitor* larvae experimental plate setup.

Selected larvae were stored in Petri dishes containing 3 mL of wheat bran and LSA (linseeds, sunflower seeds, and almonds, 5:1 v/v ratio) and a small piece of carrot (approx. 500 mg) changed daily. Bar represents 1 cm.

2.5 Inoculation of *T. molitor* larvae

T. molitor larvae were chilled in a Petri dish on ice for 5 minutes prior to inoculation. Larvae were held in position using tweezers and a sterilised 10 µL Hamilton syringe was used to inoculate larvae, with 5 x 10^4 conidia in 5 µL of PBS, into the ventral surface at the base of the 5th sternite;, a position identified previously to impact survival the least.[183] Once inoculated, mealworms were left to recover from injection and placed in their appropriate groups with food at 37°C incubation.

Chapter 3 Quantification

3.1 Introduction

Quantification of the microbial burden within a host organism is commonly utilised to investigate infection progression and virulence of the infectious agent. Two common methods used to monitor the progression of infection are colony forming unit (CFU) counts and quantitative polymerase chain reaction (qPCR). CFU counts involve serial dilution of a sample and growth of the diluted samples on agar. Quantification of colonies on agar gives an estimate of viable fungal biomass. Whereas qPCR is a molecular technique that by quantifying DNA provides an estimate of total fungal biomass. In this chapter, fungal biomass was estimated from infected *T. molitor* larvae over time using both CFU counts and qPCR. In a previous study, infection by an *A. fumigatus* inoculum caused the survival of *T. molitor* larvae to decline in a dose-dependent manner.[183] Therefore, fungal biomass should increase in infected larvae over time as infection progresses. The burden of two *A. fumigatus* clinical isolates derived from patients with and without IA is tested in this chapter to determine the relative virulence of these isolates in *T. molitor* larvae and whether it correlates with perceived virulence and larvae survival.

Virulence assays are an important part of infection research as *A. fumigatus* virulence can differ significantly depending on the isolate used. These can display high variability even in those of similar origin,[127, 185] and depending on the host

organism.[186] Due to the varied results across the different isolates and model systems, it is difficult to predict the virulence potential of an isolate. Virulence may thus be inconsistent between models highlighting how infection likely depends on interactions between fungal and host factors. Although certain *A. fumigatus* virulence factors have been described, the overall contribution to virulence is unclear.[187] Understanding isolate virulence within the selected model organism is important before host defence mechanisms or factors relating to host susceptibility can be studied.[114]

In addition to assessing *A. fumigatus* virulence using *T. molitor* larvae, potential isolate virulence is investigated *in-vitro* using skim milk agar (SMA) to assess proteolytic activity. *A. fumigatus* can produce over one hundred proteases which in the environment facilitate extracellular degradation necessary for saprophytism.[188, 189] Protease secretion may thus have a role in virulence by contributing to tissue invasion and pathogenicity.[57] Based on the substrate, protease secretion has been found to differ suggesting that secretion can occur in response to proteins available in the environment. During growth in casein medium, secretion of metalloproteases has been reported.[190]

3.2 Methods

3.2.1 Inoculation

T. molitor larvae were inoculated as described in section 2.3 with 5 µL PBST for controls, or 5 µL AF03 or AF11 spores at a concentration of 10^7 CFU / mL. Larval survival was recorded daily and five individuals from each group frozen daily over 5 days for later quantification of the fungal burden. Larval death was determined based on melanisation (blackened appearance) and the lack of movement. Survival data was plotted using the Kaplan-Meier method on IBM SPSS Statistics software.

3.2.2 Sectioning

For quantification using both CFU counts and qPCR, frozen larval samples were processed into two approximately equal halves. Using a scalpel, samples were decapitated and cut after the 8^{th} sternite to obtain the thorax with a section of the abdomen. This was then cut longitudinally down the centre to obtain two approximately equal halves that were processed separately (Figure 3.1). One half was utilised for CFU counts and the corresponding half for qPCR. Halves from five replicate samples were processed from each group (PBST, AF03 and AF11) at each time point (0-5 days).

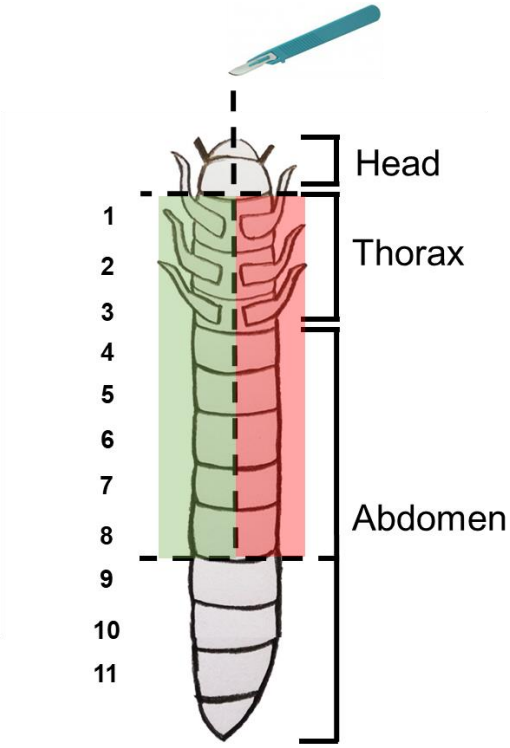

Figure 3.1 Sectioning of larval samples for quantification.

Samples were decapitated using a scalpel and a portion of the abdomen after the 8 ^{h} sternite removed. The portion containing a section of the thorax and abdomen was then divided longitudinally down the centre with one half for CFU and the other for qPCR (highlighted sections). Numbers indicate sternites.

3.2.3 Colony-forming unit (CFU) counts

Viable fungal biomass was quantified from one half of each sample. To recover fungal biomass from larval tissue, samples were placed in a 1.5 mL Eppendorf tube containing 200 µL sterile water and homogenised using a disposable micro-pestle. Once homogenised, additional water was added (300 µL) and the sample vortexed twice for 30 seconds. To ensure recovery of single colonies, the sample was serially diluted ten-fold to 10^{-4} in a final volume of 1 mL. 50 µL of each dilution was plated aseptically with a technical replicate on PDA containing chloramphenicol to inhibit

bacterial growth. After one to two days at 37°C, fungal colonies were counted, and CFU / mL calculated in Microsoft Excel.

3.2.4 Quantitative polymerase chain reaction (qPCR)

DNA Isolation

To quantify the fungal burden using qPCR, *A. fumigatus* DNA was recovered from *T. molitor* larvae halves using the Isolate II Genomic DNA Kit (Bioline) with a few alterations to the manufacturer's protocol. Samples were added to a 1.5 mL Eppendorf tube containing 180 µL lysis buffer GL and then homogenised using disposable micro-pestles. 25 µL proteinase K was added, the sample vortexed for 15 seconds and pulsed in the centrifuge before incubation at 56°C for 2 hours. A mixture of glass beads (710-1180 µm and 425-600 µm, 1:1) was added to the sample which was then agitated in 2 mL screw-cap tubes using a Mini-Beadbeater-16 (Biospec Products) for 1 minute twice, with 1 minute cooling in-between on ice. A total bead beating duration of two minutes was determined to be optimal based on an optimisation experiment to assess yield after one to four minutes of beadbeating (Table 3.1).

Table 3.1 Bead-beating durations tested during DNA isolation optimisation.

Inoculum	Mealworm samples	Bead-beating duration	Total duration (minutes)
PBST or AF11	1st Half	30 seconds, twice	1
	2nd Half	1 minute, twice	2
	1st Half	1 minute, twice	3
	2nd Half	1 minute, twice	4

Following beadbeating, DNA isolation was carried out as per manufacturer's instructions with 50 µL elution buffer utilised instead in the final step. 200 µL buffer G3 was added, samples vortexed for 40 seconds and incubated at 70°C for 10 minutes. Samples were vortexed once more and the beads removed. 210 µL of ethanol was added and the sample vortexed. The sample was then added to a binding column and centrifuged at 11,000 x g for 1 minute discarding the flow-through. This was repeated after the addition of 500 µL wash buffer GW1 and 600 µL wash buffer GW2 to the column. The column was then centrifuged to remove any residue, and the binding column containing the sample placed into a new tube. 50 µL elution buffer (70°C) was then added, the sample incubated at room temperature (22°C) for 1 minute and centrifuged at 11,000 x g for 1 minute to obtain the DNA sample.

qPCR Assay Plate setup

An Applied Biosystems® 7500Fast Real-Time PCR machine was utilised to determine the total fungal content of DNA isolated from *T. molitor* larval halves. 1 µL of each DNA sample diluted by 10^{-1} was added to the wells of a 96-well plate in replicates along with standards (10^1 to 10^5 genome equivalents) and controls. 19 µL of PCR Master-mix consisting of 10 µL 2x SensiFAST SYBR LO-ROX, 7 µL PCR water and 1 µL of each 5 µM primer; forward (Asp fum_F: 5'-GCAGTCTGAGTTGATTATCGTAATC-3') and reverse (Fungi 5.8_R : 5'-CAGGGGGCGCAATGTGC-3'); was added to each plated sample.[191] The plate was then sealed and cycled as follows: 95°C holding for 10 minutes, and 35 cycles of 95°C for 15 seconds and 62.5°C for 1 minute. Melt curve analysis was included in the program to ensure that there were no contaminants or primer dimers. The

resulting data was analysed using the 7500 software and exported to Microsoft Excel.

3.2.5 Skim milk agar

To further characterise virulence of the *A. fumigatus* isolates used in this study, the proteolytic activity of the isolates was assessed on skim milk agar (SMA). SMA was prepared using a mixture of store-bought ultra-heat-treated (UHT) liquid skim milk and bacteriological agar (A530G, Sigma-Aldrich). The agar (8 g) was dissolved in water (280 mL) and sterilised via autoclave. In a biosafety cabinet while the agar was still hot, liquid skim milk (120 mL) was added aseptically to the molten agar, mixed, and 20 mL of molten SMA was added to Petri dishes (100 mm diameter). Once set, plates were stored inverted at 4°C until required.

10 µL of *A. fumigatus* conidia at a concentration of 10^6 CFU / mL was added to the centre of a skim milk agar plate. Plates were incubated at 37°C and observed over 4 days for proteolytic clearance. Clearance was identified as the loss of agar "milkiness" (Figure 3.2). Both colony diameter and clearance diameter were recorded daily. Data from two replicate experiments were utilised.

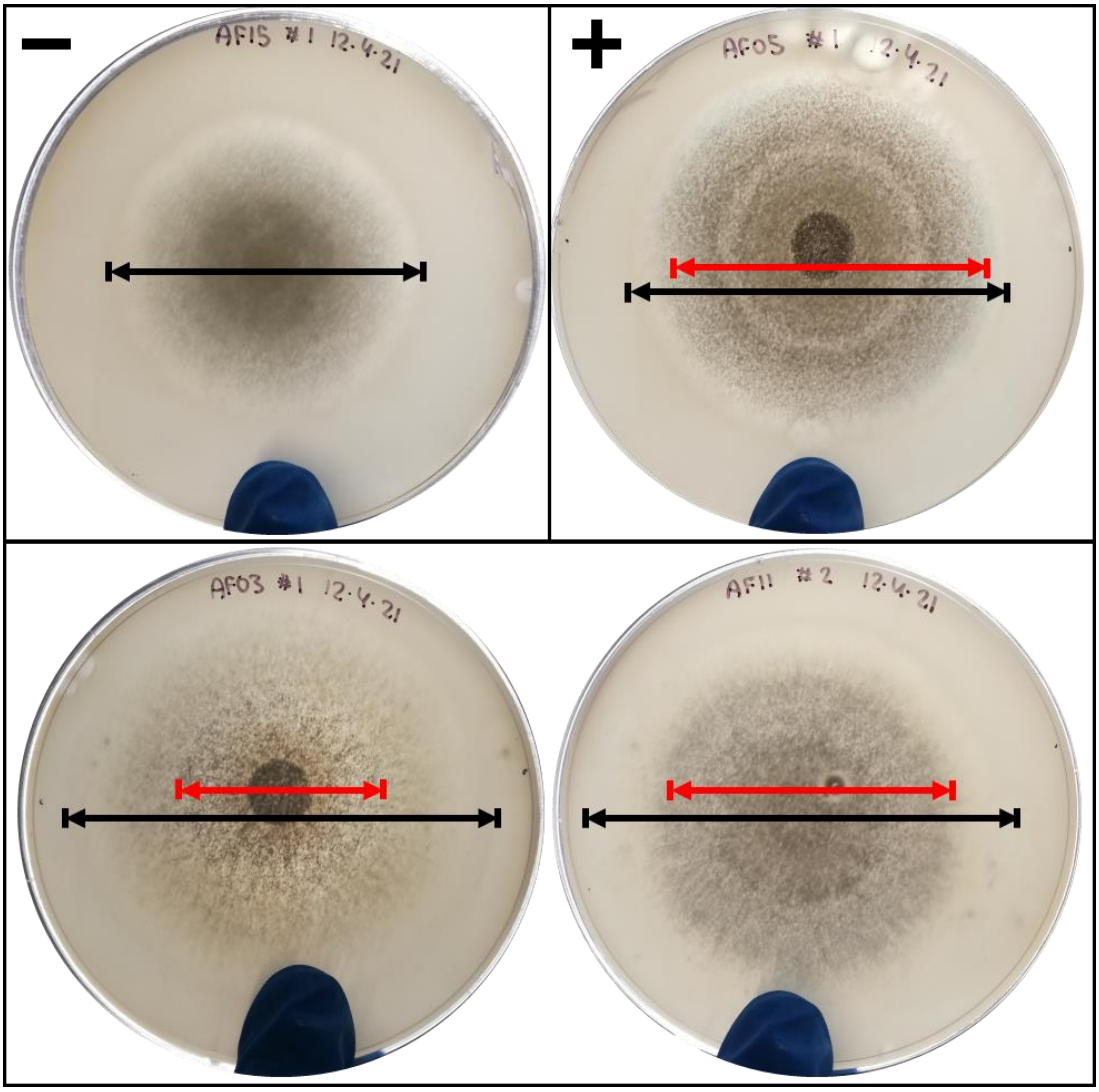

Figure 3.2 Colony (black) and clearance diameter (red) of *A. fumigatus* isolates grown on skim milk agar were measured from the base of the plate.

Blurriness was considered negative for clearance (-) while positive (+) was identified based on clarity of the colony from under the plate. A strong (-) and (+) plate are shown (top row).

3.3 Results

3.3.1 *T. molitor* larvae survival declines post-inoculation.

Survival of *T. molitor* larvae incubated at 37°C post-inoculation with the PBST control or $5x10^4$ *A. fumigatus* conidia was recorded daily over five days (Figure 3.3). The survival probability of both groups of *A. fumigatus* inoculated larvae, non-IA (AF03) and IA (AF11), decreased during the experimental period to a greater extent than PBST. Up until day five, AF03 inoculated larvae had a lower survival probability than AF11. After five days, the survival probability of the PBST group was 82.8%, compared to 61.2% for AF03 and 48.6% for AF11 inoculated larvae. Although the survival probabilities varied, these did not differ significantly (log-rank: $p = 0.428$). As five samples from each group were removed daily for later quantification of the fungal burden, the sample size was reduced over time which may affect the accuracy of the Kaplan-Meier survival probabilities.

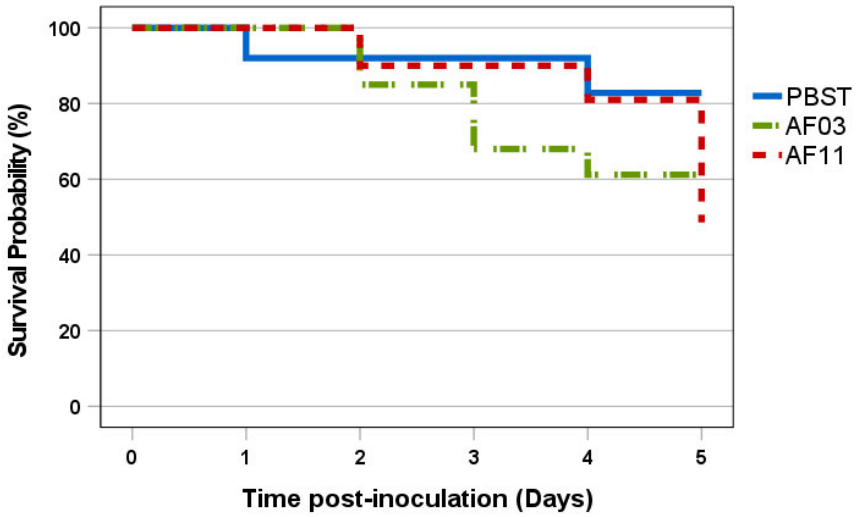

Figure 3.3 Kaplan-Meier survival curve of *T. molitor* larvae inoculated with *A. fumigatus* clinical isolates AF03 and AF11.

Thirty larvae were inoculated per group with 5 µL PBST, or 5 x 10^4 conidia (in 5 µL PBST) from *A. fumigatus* isolates AF03 (non-IA) or AF11 (IA). Five samples from each group were collected daily and frozen for subsequent assessment of the fungal burden. Survival did not differ significantly between treatment groups (log-rank: p = 0.428).

3.3.2 Viable fungal biomass decreases over time.

Viable fungal biomass was recovered from larval halves to assess infection progression. After 1 to 2 days of 37°C incubation, fungal colonies were quantified, and this showed a decrease in CFU with time post-inoculation. Figure 3.4 shows the average fungal CFU / mL recovered per larval half.

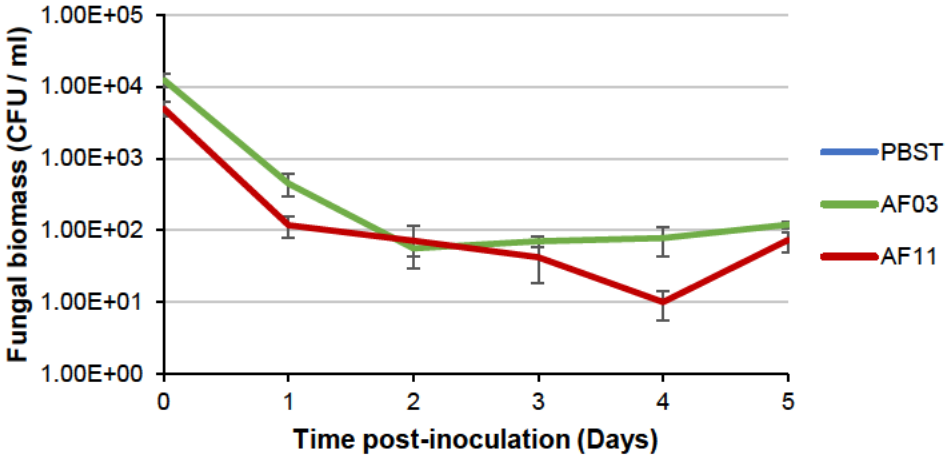

Figure 3.4 Viable fungal biomass recovered from *T. molitor* larvae.

Viable fungal biomass was quantified from one half of each larval sample inoculated with 5 µL PBST, or 5 x 10^4 conidia (in 5 µL PBST) of *A. fumigatus* isolates AF03 or AF11 using CFU counts. Mean CFU / mL calculated from at least four halves for each group at each time point is plotted. No CFU was observed for PBST. Error bars represent standard error.

As expected, no fungal growth was observed from PBST inoculated larvae with growth recovered from *A. fumigatus* inoculated larvae at each time point. Viable fungal biomass declined from the day of inoculation. For AF03, recovered biomass was lowest on day 2 where fungal biomass then remained at a stable level. Viable fungal biomass was lowest on day 4 for AF11 which then increased on day 5. This increase coincided with a relatively large drop in survival (Figure 3.3).

3.3.3 Total fungal biomass increases over time.

DNA isolation optimisation

qPCR was used to quantify fungal DNA in the corresponding *T. molitor* larval halves as CFU counts only provide an assessment of viable fungal biomass. Prior to qPCR quantification, DNA isolation was optimised involving the testing of several bead-

beating durations aiming to maximise fungal DNA yield. A bead-beating duration of 2 minutes produced the highest yield (Figure 3.5). Fungal DNA was not detected in PBST controls (not presented). As the optimisation experiment lacked replicate samples at each duration and a non-bead-beaten control sample, the results are variable.

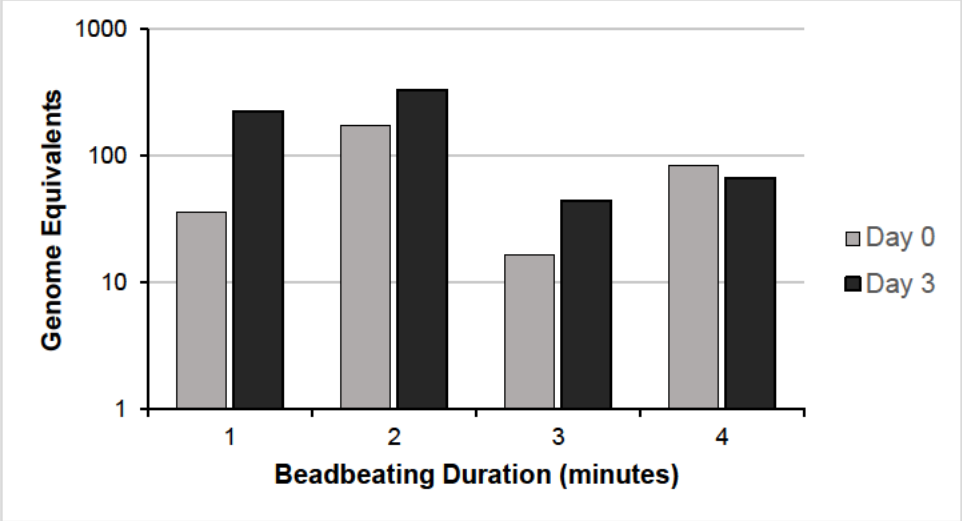

Figure 3.5 Effect of bead-beating duration on recovered fungal DNA.

Fungal DNA recovered from *T. molitor* larval halves inoculated with 5 x 10^4 AF11 conidia (in 5 µL PBST) were quantified using qPCR after 1 to 4 minutes of bead-beating. Data is presented as genome equivalents recovered from one larval half per beadbeating protocol, which includes a sample from 0- and 3-days incubation at 37°C.

qPCR troubleshooting

Initial qPCR quantification of fungal DNA isolated from *T. molitor* larvae produced an abnormal amplification curve also observed in PBST-inoculated controls. Figure 3.6 shows the qPCR amplification plots. PBST-inoculated samples were assessed during troubleshooting against a 10^5 DNA standard. Diluting samples by 10^{-1} and using 62.5°C cycling normalised the curve and was thus utilised during qPCR quantification. As a dilution was required to normalise the curve (Figure 3.6, C),

additional substances may have passed through the column into the eluate during DNA isolation.

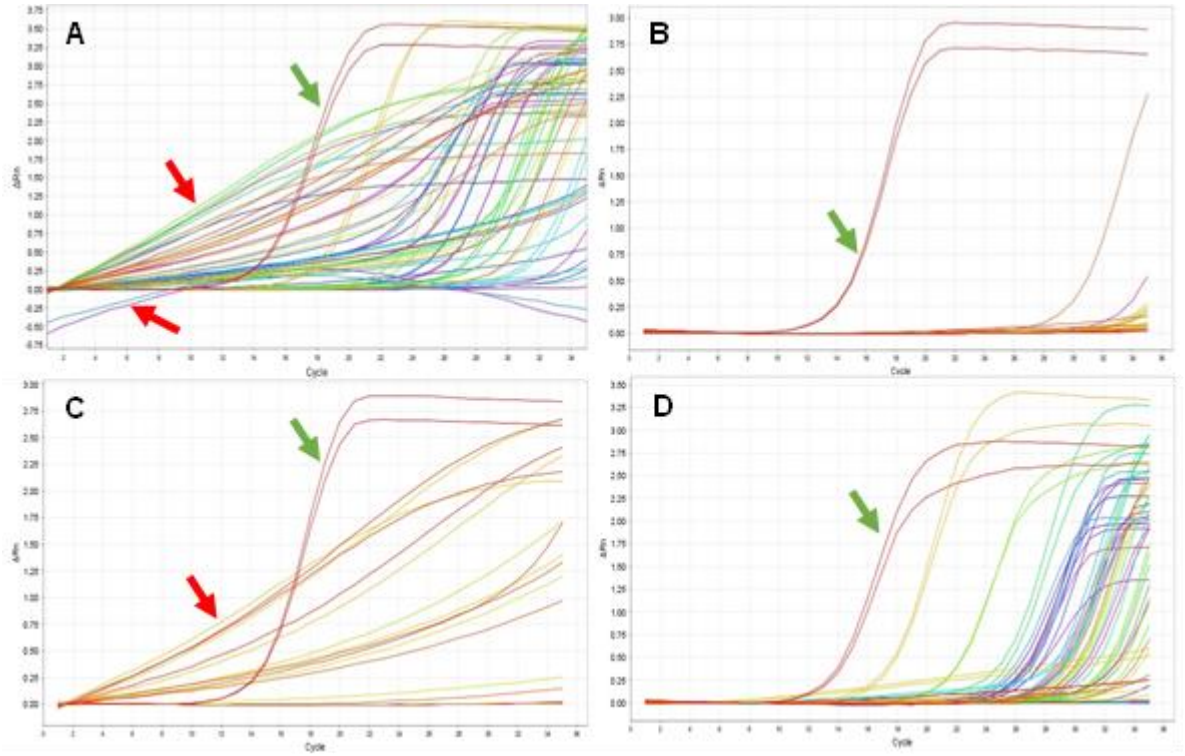

Figure 3.6 qPCR troubleshooting.

Linear qPCR amplification plots of fungal DNA recovered from *T. molitor* larvae halves from zero- and one-day post-inoculation. Abnormal (red arrows) amplification curves were observed for fungal DNA recovered from *T. molitor* larval halves inoculated with PBST, AF03 or AF11 (**A**). Normalised (green arrows) curve resulting from dilution by 10^{-1} and cycling at 62.5°C instead of 60°C (**B**). Abnormal curve from 62.5°C cycling alone (**C**). Normalised amplification plot including all samples resulting from 62.5°C cycling and a 10^{-1} sample dilution (**D**). Plots: A & D - all *T. molitor* larvae samples from 0- and 1-day post-inoculation, all DNA standards (10^1 to 10^5) and negative control, B & C - PBST-inoculated larval samples, 10^5 DNA standard and negative control.

Quantification of total *A. fumigatus* biomass using qPCR revealed an increasing trend of increased fungal DNA in larval samples inoculated with either AF03 or AF11 (Figure 3.7). Recovered fungal biomass from larval halves inoculated with AF11 increased with time, while AF03-inoculated samples although increasing had reduced fungal biomass after 3- and 5-days post-inoculation. Day 3 may have resulted from issues during DNA isolation as genome equivalents was similar in larvae inoculated with either isolate. The increasing trend identified by qPCR contradicts that suggested by viable fungal biomass quantified using CFU counts (Figure 3.4).

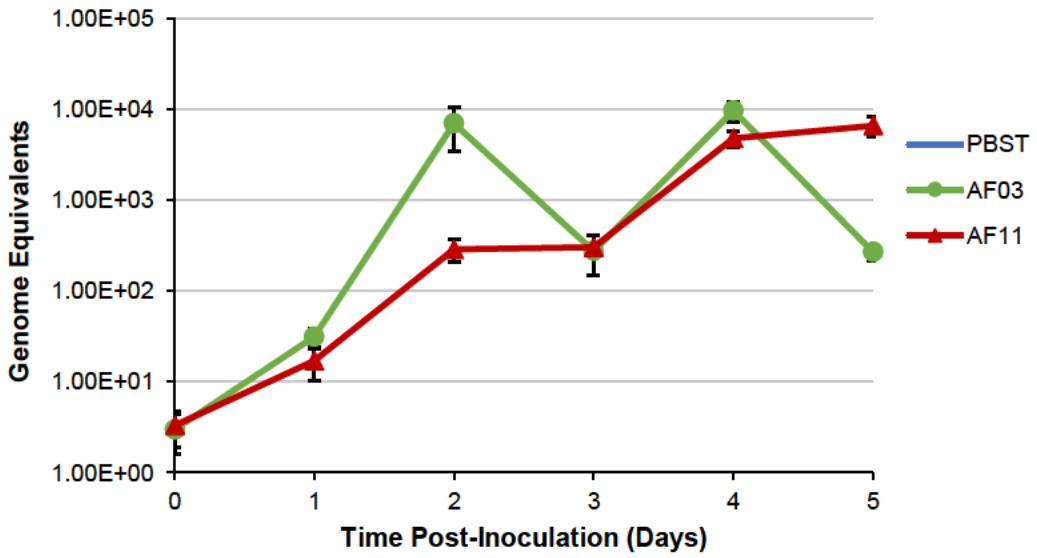

Figure 3.7 Genome equivalents recovered from *T. molitor* larvae using qPCR.

Genome equivalents were quantified using qPCR from one half of each larval sample inoculated with 5 µL PBST or 5 x 10^4 conidia (in 5 µL PBST) from *A. fumigatus* isolates AF03 or AF11. Mean genome equivalents recovered from at least three larval halves for each group at each time point is plotted. Error bars represent standard error.

3.3.4 *A. fumigatus* isolates differ in proteolytic ability.

A. fumigatus clinical isolates were plated on skim milk agar to assess isolate virulence via proteolytic activity. The proteolytic activity of both isolates, AF03 (non-IA) and AF11 (IA), utilised in fungal burden experiments were compared (Figure 3.8).

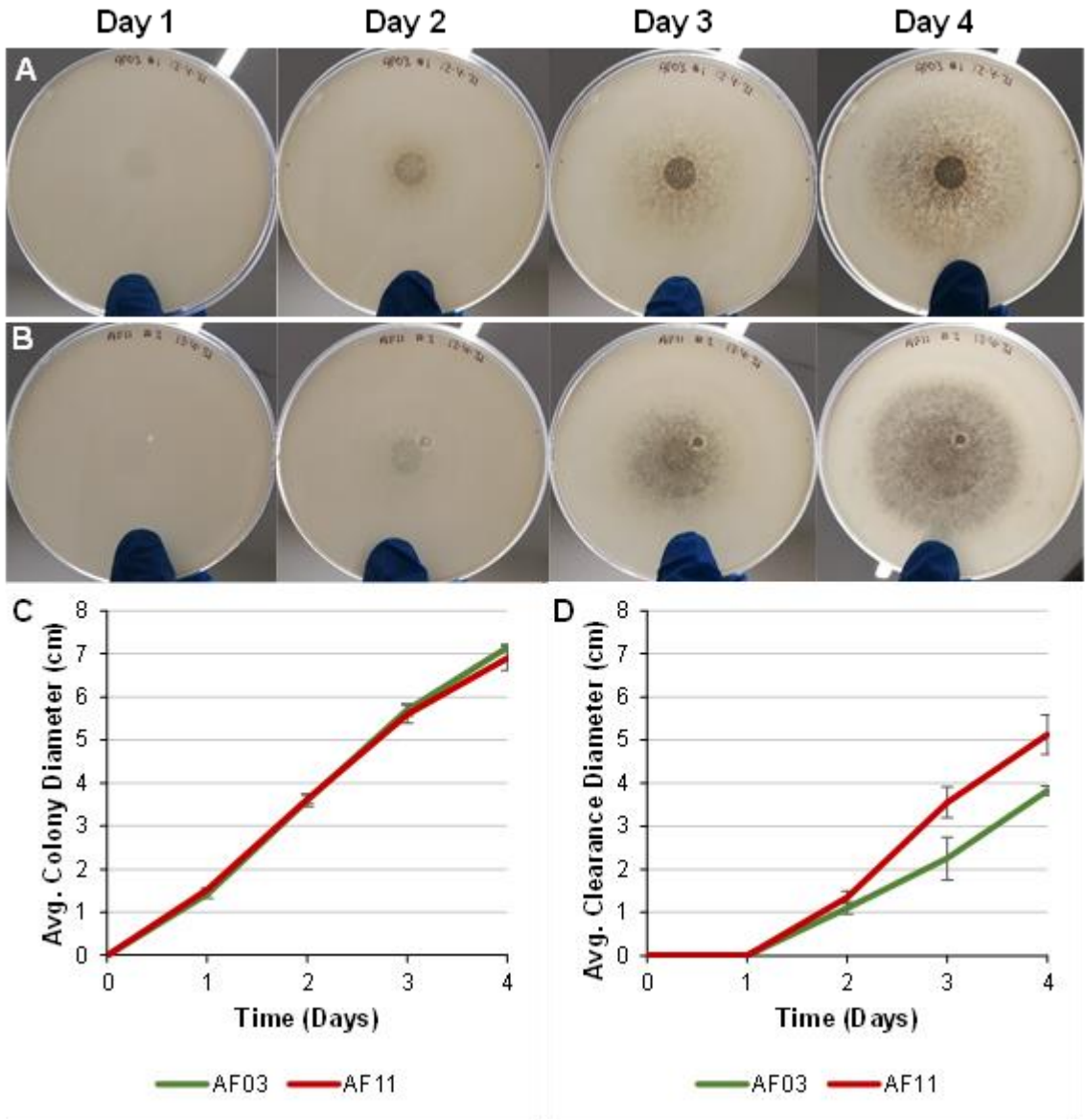

Figure 3.8 *A. fumigatus* isolates AF03 (A) and AF11 (B) grown on skim milk agar.

Average colony (C) and clearance (D) diameter (cm) is plotted from two independent experiments. Clearance was characterised by the loss of milky opacity. The diameters of each isolate grown at 37°C were recorded daily over 4 days. Error bars represent standard deviation.

Colony diameter similarly increased over time for both AF03 and AF11 with AF03 observed to form a more pigmented colony. Growth diameter overall differed between the isolates by less than or equal to 0.25 cm. For both isolates, clearance was first observed after 2 days. AF11 produced a larger zone of clearance with an average clearance diameter of 1.35 cm after 2 days compared to 1.1 cm for AF03. After 4 days, the clearance diameter of AF11 was 5.13 cm compared to 3.83 cm for AF03.

Proteolytic activity of *A. fumigatus* clinical isolates grouped into non-IA and IA is presented in Figure 3.9. Colony diameter was relatively similar between non-IA and IA with that of the former slightly larger. Clearance diameter was also larger for non-IA isolates. Clearance on skim milk agar was observed to vary overall between isolates regardless of isolate origin. All isolates produced some level of clearance. Some produced small intense clear zones while others could produce clearance that wasn't as clear but was larger in size.

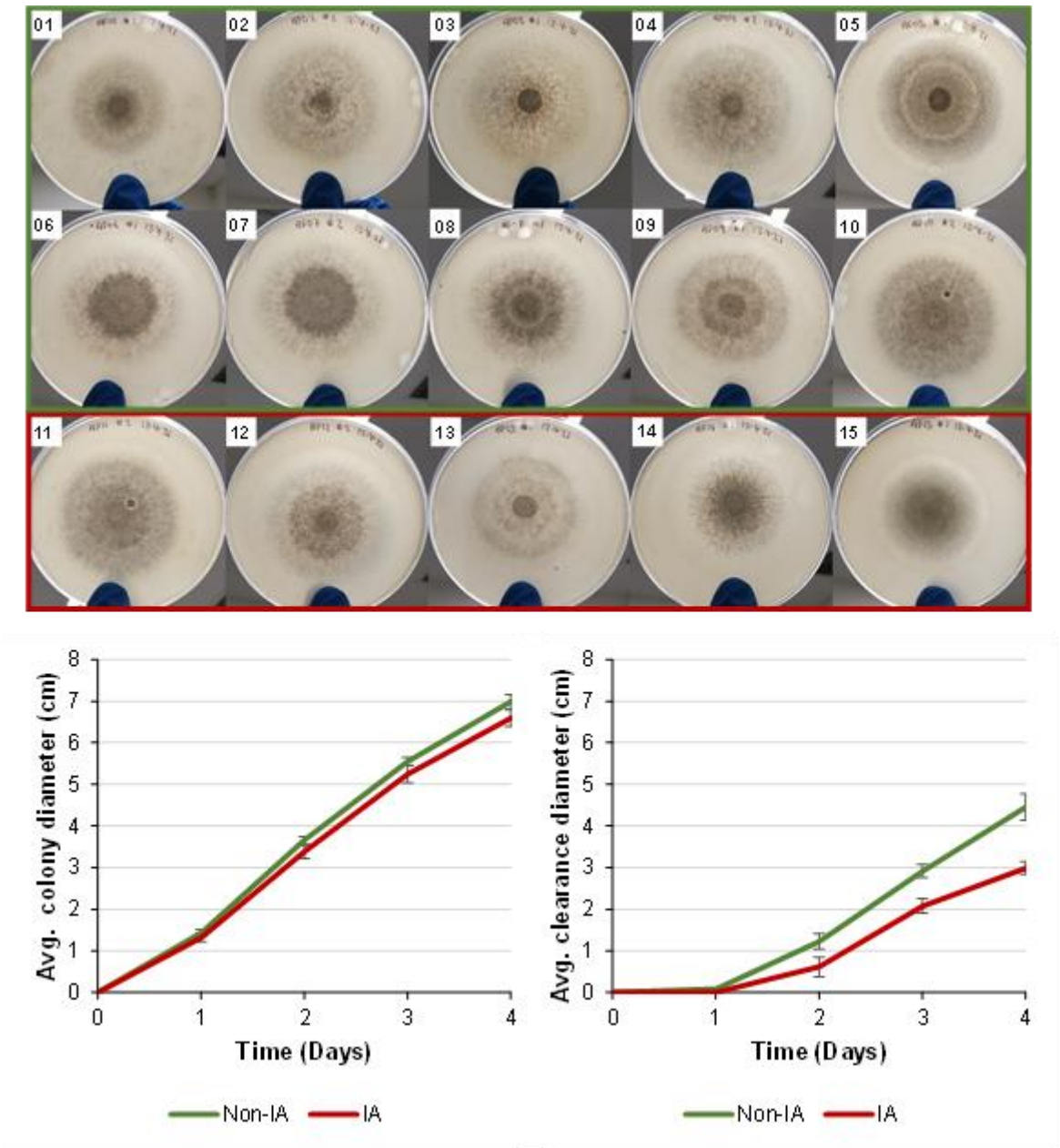

Figure 3.9 Average colony and clearance diameter of non-IA (AF01-AF10) and IA (AF11-AF15) *A. fumigatus* clinical isolates over 4 days at 37°C.

Pictured is one agar plate from each isolate on skim milk agar after 4 days arranged from AF01 to AF15, from left to right and top to bottom. Average diameter from non-IA and IA isolates is presented from two independent experiments. Error bars represent standard deviation.

3.4 Discussion

In this chapter, the burden of *A. fumigatus* recovered from inoculated *T. molitor* larvae was quantified to determine whether it correlates with perceived virulence and host survival. Larval survival was observed to decline post-inoculation with either *A. fumigatus* clinical isolate to a greater extent than PBST-inoculated control larvae (Figure 3.3). The fungal burden was thus expected to increase over time indicating progressive infection which is a trend that corresponds with declining survival and is relative to inoculum size.[192, 193]

Viable fungal biomass recovered from *T. molitor* larvae inoculated with either *A. fumigatus* inoculum decreased over time (Figure 3.4) while total fungal biomass as suggested by genome equivalents increased from the day of inoculation (Figure 3.7). The decline in viable fungal biomass suggests host clearance of fungi over time which contradicts larval survival data and total fungal biomass as both indicate the progression of infection. Reasons for the decline in viable fungal biomass may be due to the fungus becoming unculturable over time due to stressors within the larval environment, or due to difficulties in recovering fungal biomass as less vigorous homogenisation was utilised. Homogenisation during CFU counts was limited to a micro-pestle to prevent damage which could cause loss of fungal viability. qPCR in contrast involved additional more vigorous recovery steps including beadbeating as the aim was to damage the cell wall to recover DNA. The development of *A. fumigatus* conidia into hyphae is an important characteristic of infection as hyphae facilitate tissue invasion and dissemination.[13, 57] The decline in viable fungal biomass from the time of inoculation may therefore be due to the integration of hyphae into tissue with time. Fungal biomass may have thus been most recoverable on day 0 as

inoculated conidia had no time at 37°C and thus likely remained recoverable as conidia in haemolymph.

Although CFU quantification of other microbes such as yeast and bacteria has revealed microbial biomass that correlates with survival data,[194, 195] quantification of recovered *A. fumigatus* biomass using CFU counts has been previously reported to have limited sensitivity and poor correlation with growth especially once infection is established.[196, 197] This was observed here in the *T. molitor* larvae model where only total fungal biomass, as estimated by qPCR, displayed an increase over time which correlated with the declining survival of the larvae post-inoculation. qPCR may thus provide a more representative measure of the *A. fumigatus* burden as it displays greater sensitivity than CFU counts which has also been previously observed.[196-199] This includes various animal models including mice,[196, 198] rabbits,[200] guinea pigs,[197] and rats.[199]

In a murine model, an increase in biomass reported using CFU counts was not significant compared to qPCR which detected significant progression of *A. fumigatus* infection that peaked where significant mortality was recorded. CFU and qPCR data quantifying *C. albicans* in contrast which does not produce true hyphae in tissue, strongly correlated as infection progressed.[196] *A. fumigatus* hyphae may thus be the problem as they become integrated in tissue hindering recovery and hyphae are also prone to clumping which may lead to underestimations should sufficient single cells not be recovered.[196, 197, 201] Efforts to separate clumps using more vigorous homogenisation also risks underestimation due to potential damage to cells.[202] As multinucleate structures, hyphae can also produce overestimations during qPCR quantification. Hyphae are thus challenging structures. Although CFU counts and

qPCR data do not always correlate, a combination of these methods in certain contexts such as antifungal testing may be useful.[203]

Total fungal biomass as estimated by qPCR data was higher for AF11 on day 5 post-inoculation than AF03, which coincided with a relatively large drop in survival compared to that of other time points. This suggests a correlation between total fungal biomass and survival data for AF11-inoculated larvae. For AF03, total fungal biomass increased until day 2 but then declined, increased, and declined again on day 5. This may have been due to extraction efficiency. Up until day 5, larvae infected with AF03 had the lowest survival probability. As five larvae were removed from each group daily to be frozen for later quantification of the fungal burden, the accuracy of the Kaplan-Meier survival probabilities may have been affected as the sample size was reduced over time. Furthermore, the experiment was also not repeated, and the experiment terminated after day 5 post-inoculation.

Based on these data, AF03 may be the more virulent isolate as survival steadily declined whereas AF11 only had a lower survival probability on day 5 which is when the sample size was smallest and thus most inaccurate. This assessment of AF03 being more virulent is consistent with previous assessments in *T. molitor* larvae where several different *A. fumigatus* inoculums were tested including AF03.[183] AF03 was more virulent than AF11, and results were observed to vary between replicate experiments.[183] However, the differences observed here in survival were not significant (Figure 3.4).

Although isolate AF03 may be more virulent in the *T. molitor* larvae model system, in other hosts this may differ such as humans where it is not linked to IA. According to other research, the virulence *A. fumigatus* isolates can differ depending on the

host organism which highlights the importance of host factors for virulence.[186] Virulence assessments have produced varied results between different studies. For example, *A. fumigatus* clinical isolates were reported to be more virulent than environmental isolates in a murine model,[204] and in *G. mellonella* larvae.[185] The opposite has also been observed including in *G. mellonella*,[205] and in zebrafish where environmental isolates were more virulent.[206] No significant differences have also been observed in *D. melanogaster* when comparing colonising and invasive isolates from *A. fumigatus* and also those of *A. terreus*.[127] Isolate virulence can thus vary depending on the host organism and the source of the isolate. Screening for select virulence genes has similarly revealed no differences between clinical invasive and colonising isolates, and between clinical and environmental isolates. High genetic diversity has been observed between the latter however, and thus different virulence-related genes may be involved which highlights the multifactorial nature of infection.[187]

A. fumigatus as a saprophyte in the environment can produce a range of proteases that enable the fungus to acquire essential growth nutrients during infection. Despite links between protease secretion and damage to lung epithelial and blood cells *in vitro*,[189] the importance of proteases for virulence is controversial as deletion studies have revealed unaltered virulence in animal modelling with virulence remaining intact in a leukopenic murine model when using protease mutants.[40, 188, 189] In this study, the proteolytic activity of *A. fumigatus* isolates on skim milk agar was tested as an additional method to assess isolate virulence. Isolates of non-IA origins (AF01-AF10) and IA (AF11-AF15) were plated on skim milk agar for a rapid assessment of proteolysis. According to this qualitative assessment of virulence, proteolysis was observed to differ between isolates regardless of origin. Certain isolates performed

better with zones of varied clarity and size. AF03 produced a clearer centre compared to AF11 which produced a larger zone with slightly less clarity. Thus, the ability of these isolates to clear the agar varied, which was also seen when plating other non-IA and IA isolates. According to the averaged data, non-IA isolates produced larger clearance, however clearance was observed to differ between individual isolates overall regardless of clinical origin. Furthermore, only five IA isolates were available for testing compared to ten non-IA. It has been noted in other studies that SMA may not provide definitive estimates of protease production as clearance can also be caused by acid or glycosidic hydrolases which are formed by microbes including *Aspergillus spp*. during carbon starvation for survival.[207, 208] Alternative methods should thus be utilised to for a more conclusive assessment.

As *A. fumigatus* hyphae can challenge quantitative methods, the visualisation of infection is important. This may be possible using histological examinations and MRI of infected larvae.

Chapter 4 Visualisation of *A. fumigatus* infection in *T. molitor* larvae

4.1 Introduction

The identification of microbial growth within host tissue and its interactions with the immune system can provide important information about an infection. This can be identified during histological examinations which is the microscopic study of tissue anatomy. Important to this examination is the recovery of thin tissue sections. This involves several tissue processing steps, mounting on a glass slide and staining to highlight the different cellular components during microscopic analysis.[209] An understanding of normal tissue anatomy is essential as infection can be identified by abnormalities present in host tissue. Signs of tissue damage and immune reactions are rendered observable with the application of histological stains.[210] Special microbe-specific stains can be utilised to identify microbial components as these enhance visualisation of specific microbial structures within the histological section. These include Gram stain for bacteria, Ziehl-Neelsen (ZN) for mycobacteria and Grocott's Methenamine Silver (GMS) for fungi. When histological features are interpreted with consideration of data from other methods such as microbial culture, histological findings they can confirm the identity of the disease-causing microbe and reveal its interactions within the host enabling the study of pathogenesis.[211]

An important histological feature of *A. fumigatus* infection is the presence of hyphae invading the tissue. Where recovery of tissue samples is possible, observation of

hyphae contributes to the identification of the fungus and diagnosis of IA. This can be confirmed by *A. fumigatus* positive culture and observations of septate hyphae that branch at 45° angles.[212-214] Although standard histological staining with haematoxylin and eosin (H&E) can enable identification of *Aspergillus* hyphae,[215] fungus-specific stains greatly enhance identification. Useful stains for *Aspergillus* include Grocott's Methenamine Silver (GMS) and Periodic Acid Schiff (PAS) which can stain polysaccharides in the fungal cell wall,[216] and fluorescent stains such as Calcofluor White which stain chitin. Although the fungal species cannot be definitively identified, *Aspergillus* can be suspected with the observation of dichotomous branching and septate hyphae.[214] The presence of hyphae within blood or blood vessels can be observed during IA, and accompanied by signs of necrosis or haemorrhage.[213] Histological features observed in lung tissue includes hyphal growth in a radial arrangement, and distinct nodules of coagulation necrosis, haemorrhages, and inflammatory infiltrates.[217] When spread occurs from the lungs to other organs as has been observed post-mortem, similar signs are observed including in the heart and kidney.[218]

Although insects are different to mammals including their reliance on innate immunity and utilisation of an open circulatory system, histological features observed in *G. mellonella* larvae have suggested that the development of IA in insects is comparable to that in humans.[204] In *G. mellonella* larvae inoculated with *A. fumigatus* conidia, increased haemocyte density and antimicrobial peptides have been observed along with the formation of melanised nodules (Figure 4.1). Over time these nodules expand and spread from the inoculation site resulting in larger diffuse nodules at the inoculation site and smaller ones throughout larval tissue.[132, 204] Examination of these nodules has revealed signs of dense haemocyte infiltration,

germinating conidia and hyphae developing within melanised nodules highlighting the role of nodulation in restricting fungal growth.[132]

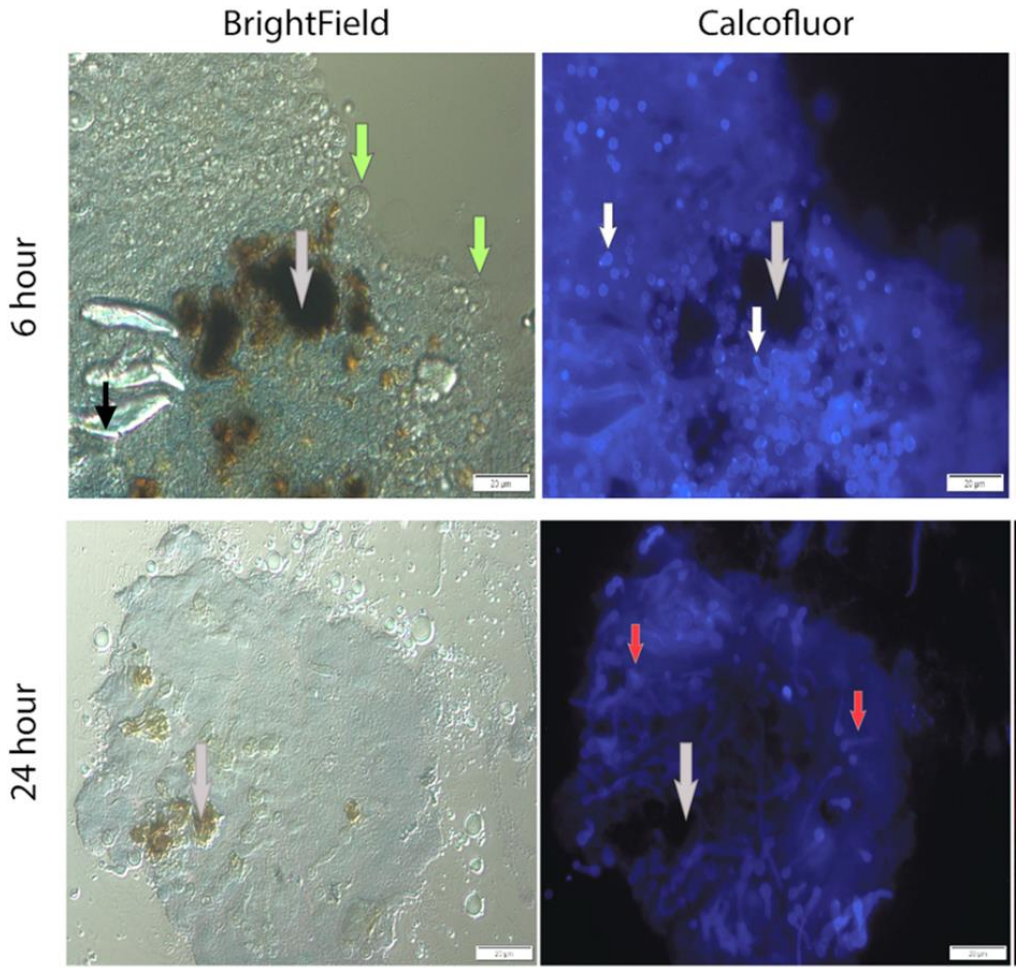

Figure 4.1 Example of *A. fumigatus* conidia and hyphae observed in nodules recovered from *G. mellonella* larvae tissue by Sheehan et al.[132]

Arrows: grey - melanised plaques, green - cellular infiltrates, white - germinating conidia, and red – hyphal infiltration.[132]

These melanised nodules have been compared to granulomas observed in lung tissue of a CGD mouse model of IA. The granulomas, although made up of

lymphocytes which are absent in larval nodules, were accompanied by significant hyphal invasion like the melanised nodules in larval tissue.[132, 219]

Aside from being involved in encapsulation and nodulation, melanisation can also be observed on lysed pathogens and pathogens prior to phagocytosis. Melanisation is thus a common part of the insect immune response being responsible for the formation of melanin around pathogens likely to damage it via oxidative damage or starvation.[220] Melanisation and tissue damage are thus histological features common during infection. These can thus be utilised to evaluate pathogenesis in larvae with these features influenced by time post-infection and the inoculum.[204] For example, inoculation of *G. mellonella* larvae with *A. fumigatus* revealed branching invasive hyphal growth while *A. lentulus* at the same time point and inoculum density was readily encapsulated by immune cells with melanised nodules evident. Survival analysis also confirmed the greater virulence of *A. fumigatus* in inoculated larvae.[192]

This chapter describes a histological examination of the development of *A. fumigatus* infection in *T. molitor* larvae tissue. Normal tissue anatomy is described, followed by histological features observed in inoculated larvae to determine the progression of *A. fumigatus* infection in the tissues of *T. molitor* larvae. In addition, larvae were scanned with MRI as a non-invasive method of investigating infection. The results presented are from optimisation experiments.

4.2 Methods

4.2.1 Inoculation

T. molitor larvae samples were inoculated with 5 µL PBS for controls, or 5 x 10^4 *A. fumigatus* conidia (in 5 µL) as described in Chapter 2. Inoculated larvae along with PBS and uninoculated controls were incubated with food at 37°C for several days. Five samples were removed from each group daily over 4 days and fixed.

4.2.2 Fixation

Larval samples were fixed using 10% neutral buffered formalin (NBF). 5 µL of NBF was injected into larvae at two locations, after the 4th and 8th sternite, as the larval exoskeleton hinders penetration of the fixative. Larvae were then stored vertically in 1 mL 10% NBF within Eppendorf tubes (1.5 mL) at 4°C to preserve tissue for subsequent processing. Due to COVID lockdowns (Sydney, Australia), larvae were left in fixative for over 3 months prior to tissue processing. This may have been beneficial as the cuticle has low permeability. In *G. mellonella* larvae, longer fixation (>4 weeks) was reported to produce better results.[221]

4.2.3 Manual Tissue Processing and Embedding

Fixed samples were rinsed in PBS three times for 15 minutes to dilute the fixative. Using a scalpel, samples were divided into four portions by cutting between sternites (Figure 4.2). Each portion contained approximately three sternites. Samples were then returned to fresh 10% NBF for 30 minutes prior to tissue processing.

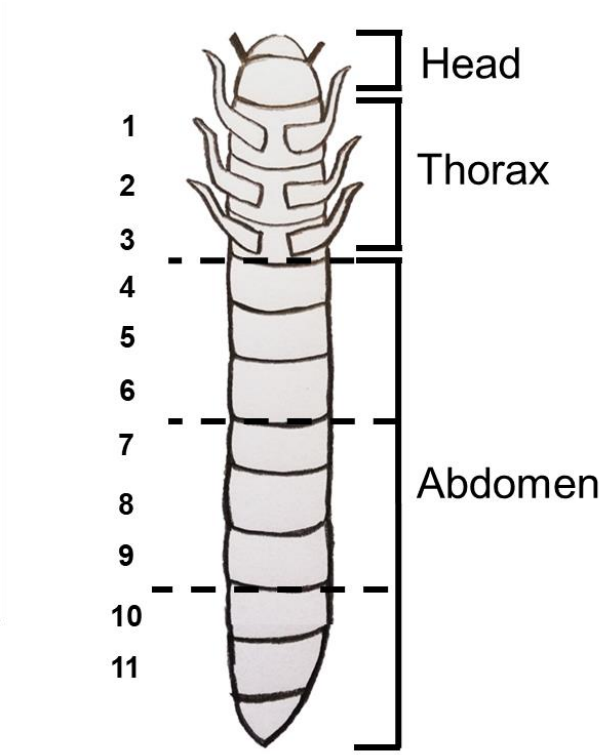

Figure 4.2 Fixed *T. molitor* larvae were divided into four portions prior to manual tissue processing.

Broken lines represent positions where larvae were divided. Sternites are numbered.

To recover thin sections of tissue for microscopic analysis, samples were histologically processed manually using the schedule described in Table 4.1. The tissue processing schedule utilised was adapted from a short protocol described for an automated tissue processor.[222] Samples were processed within 1.5 mL Eppendorf tubes. Fine-pointed tweezers were utilised to move samples between solutions, which were first heated in an oven when moving samples between wax baths. Processing steps included dehydration as wax is insoluble in water, clearing to replace alcohol with xylol/xylene as it is miscible in both alcohol and paraffin wax, and infiltration where molten paraffin wax enters tissue replacing xylol.[209] For infiltration steps, Paraplast Plus® (P3683, Sigma-Aldrich) paraffin wax pellets were

melted in 2 mL Eppendorf tubes until molten (approx. 1-2 hours) using a tube heater set to 59°C and subsequently maintained at temperature. Due to the toxicity of xylol, processing occurred within a fume hood. Xylol alternatives should be considered instead where possible however.[223-225]

Table 4.1 Manual tissue processing protocol for *T. molitor* larvae divided into four portions.

Steps	Reagent	Duration
Dehydration	1. 70% ethanol	Overnight (approx. 16 hours)
	2. 95% ethanol	20 mins, twice
	3. 100% ethanol	45 mins, twice
	4. 100% ethanol	60 mins, once
Tissue clearing	5. Xylol	45 mins, twice
	6. Xylol	60 mins, once
Paraffin wax Infiltration	7. Paraplast Plus (molten, mp: 56-57°C), 59°C	20 mins, three times and overnight (approx. 16 hrs)

Once infiltrated with paraffin wax, samples were embedded in wax blocks using a stainless-steel base mould (30 x 24 mm) and a labelled cassette. Using a 1000 µL pipettor and an oven-warmed barrier pipette tip, 1 mL of molten wax heated to 65°C in an oven was dispensed into an oven-warmed mould positioned on a heating block which provided a heated surface to slow the wax from setting. The four portions of the wax infiltrated sample were then placed in the mould containing a thin layer of wax using oven-warmed tweezers. The portions were spaced out in the mould so that each portion would have enough wax surrounding it whilst aiming to avoid overlap during microtomy. Portions were oriented non-specifically in the mould as information regarding orientation was lost during processing. If the wax set prematurely, the mould containing the sample was returned to the 65°C oven until

molten (approx. 30 minutes). Once sample arrangement was finalised, the mould was placed on an unheated edge of the heating block for 1 second to slightly set the wax to hold the samples in position. A labelled cassette was then placed on the mould and paraffin wax (65°C) carefully added to minimise shifting of the pieces from the desired positions. The wax was stored at a higher temperature during embedding as heat was rapidly lost when the glass beaker was removed from the oven. Wax that set prematurely resulted in difficulties during microtomy. The mould was then carefully placed on an iced water bath. Once set, approximately 30 minutes, the wax block was removed from the mould for later sectioning using a microtome.

4.2.4 Microtomy

Tissue blocks were sectioned using a rotary microtome (Leica RM2125RT) fitted with a disposable blade (Figure 4.3).[226] The microtome was set to a 4-5 µm thickness and at a clearance angle of approximately 3 to 5°. Slice thickness depended on the difficulty of the block to recover tissue sections. Prior to slicing, sample blocks were submerged in an iced water bath for a few minutes. Blocks were then defaced with the coarse cut to remove the cuticle and chilled once more. A new blade was then utilised for ribboning as the insect's cuticle commonly damaged the blade causing streaks on ribbons hindering sample recovery. The chilled block was sliced using a slow-even motion during ribboning and the block chilled regularly. The selected ribbon was carefully transferred to a 52°C water bath which smoothed out the ribbon. Within 15 seconds of floatation, tissue was collected using freshly opened positively charged slides[227] and water allowed to drip off from under the section to reduce bubble formation. Slides were then left in a vertical position to dry overnight at room temperature.

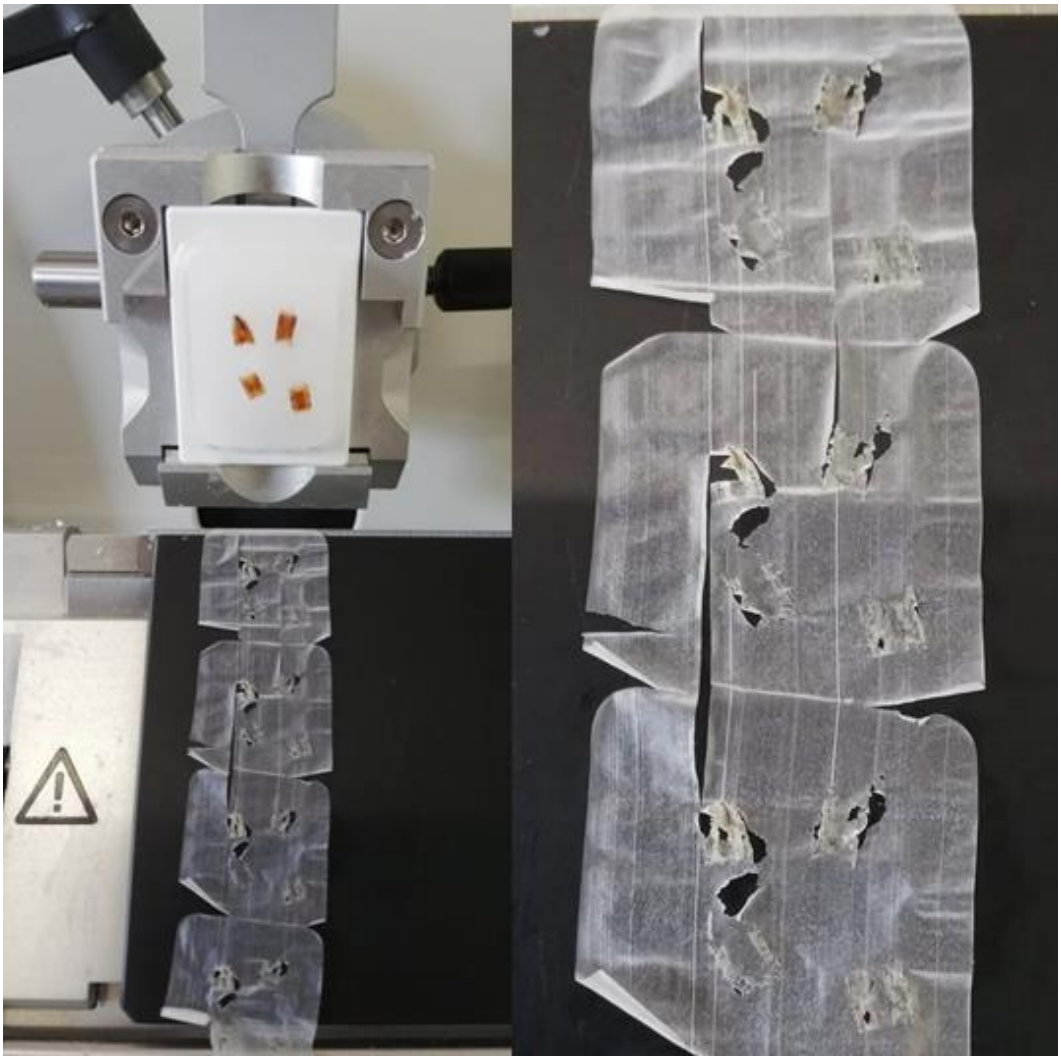

Figure 4.3 *T. molitor* larvae embedded in a paraffin wax block mounted on a microtome.

Streaks that were likely due to a damaged blade and holes around the samples were commonly observed during sectioning. The ribbon pictured is from early optimisation where sample blocks were sliced at a thickness of 7 µm. A thickness of 4 to 5 µm was subsequently utilised for H & E staining.

4.2.5 Haematoxylin and Eosin (H&E) Staining

Sectioned tissue was deparaffinised and rehydrated prior to staining. Dried slides were baked horizontally in an oven set to 59°C overnight and then processed in a fume hood as outlined in Table 4.2. Slides were deparaffinised by carefully pipetting

the solution onto the horizontal slides and carefully tipping it off during changes. During rehydration with deionised water, slides were sat vertically in a coplin jar.

Table 4.2 Dehydration and rehydration protocol of mounted *T. molitor* larvae tissue.

Step:	Solution, Duration
Deparaffinisation and Rehydration	1. Xylol, 1 minute, three times 2. 100% ethanol, 1 minute, three times 3. 95% ethanol, 1 minute, twice 4. 70% ethanol, 1 minute, twice 5. Deionised water, 1 minute, twice

Haematoxylin and Eosin (H&E) (ab245880, abcam) staining of rehydrated sections was carried out as described by the manufacturer using coplin jars. Rehydrated sections were first stained with Mayer's Haematoxylin (Lillie's Modification) for 5 minutes. Excess stain was then removed from slides using distilled water until it ran clear. The slide was then dipped in bluing reagent for 10 to 15 seconds and rinsed in distilled water. It was then dipped in 100% ethanol (anhydrous) and the excess blotted off. Slides were then placed in Eosin Y solution (Modified Alcoholic) and left for 2 to 3 minutes. Slides were then rinsed with 100% ethanol and dehydrated three times with 100% ethanol for 1 minute each. To clear the slide for mounting, xylol was pipetted to cover the tissue section and left for five minutes before mounting with a synthetic resin, DPX mountant for histology (06522, Sigma-Aldrich). Slides were observed and micrographs captured using brightfield microscopy (Olympus BX43, DP73 Camera).

4.2.6 Magnetic Resonance Imaging (MRI)

An uninoculated *T. molitor* larva sample and a sample inoculated with 5 x 10^5 conidia (AF11) incubated at 37°C for 72 hours, were scanned using the Bruker 14.1 T (600 MHz) MRI scanner equipped with a Micro2.5 probe. This was undertaken in the Biomedical Magnetic Resonance Facility (BMRF) on Campbelltown Campus, Western Sydney University, which is also a node of the National Imaging Facility (NIF). 3D Gradient Echo (FLASH) experiments were performed. Images were analysed using Image J.

4.3 Results

4.3.1 Uninoculated *T. molitor* larva tissue

To understand the changes that occur in the *T. molitor* larval host during *A. fumigatus* infection, uninoculated tissue stained with H&E was first observed to understand normal anatomy. Several structures were highlighted using H&E staining (Figure 4.4). This includes the cuticle (Ct), muscle fibres (M) which appear striated, fat body (FB) visible as a “loose” mass of tissue,[228] the gastrointestinal tract (GI) as an enclosed structure located centrally, and malpighian tubules[229] in the fat body (Figure 4.4, C-D) and near the gastrointestinal tract (Figure 4.4, B). Trachea (T) were also observable including near malpighian tubules.

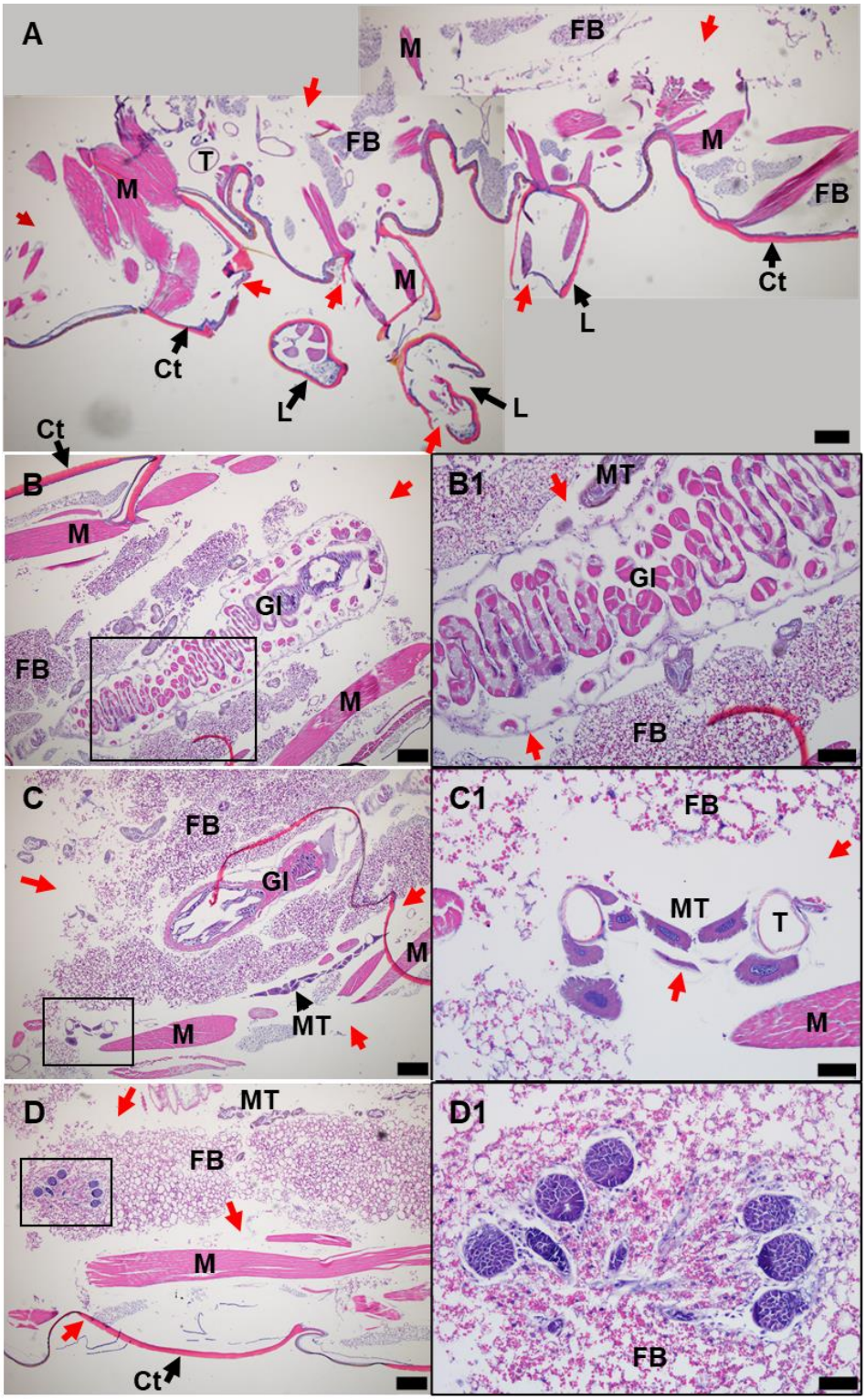

Figure 4.4 Uninoculated *T. molitor* larva tissue stained with H&E.

Fixed larvae were divided into four sections (A-D) and histologically processed. Various features can be observed including cuticle (Ct), gastrointestinal tract (GI), fat body (FB), malpighian tubules (MT), muscle fibres (M), trachea (T) and legs (L). Tissue loss is also observed (red arrows). Sections presented are from a single 4 µm slice containing one individual sample. Bars represent 200 µm (A-D), 100 µm (B1), and 50 µm (C1, D1).

4.3.2 *T. molitor* larvae tissue post-inoculation with *A. fumigatus*

T. molitor larvae inoculated with 5 x 10^4 conidia, as per the quantification experiments (Chapter 3), were observed histologically to characterise *A. fumigatus* infection (up to 96 hrs post-infection). Analysis of H & E-stained tissue sections revealed the presence of melanised nodules in all five *A. fumigatus* inoculated samples that were observed. Figure 4.5 displays micrographs from one portion of a larval block 96 hrs post-inoculation with AF03. The section, although displaying tissue loss and shifting from suboptimal tissue processing, has several structures visible including cuticle, fat body, malpighian tubules and muscle fibres. Within this tissue, several locations (≥12) of melanisation were observed in the fat body (FB); each of which were surrounded by purple-stained cells. Likely haemocytes, part of the immune response as this feature was absent in uninoculated tissue (Figure 4.4). A break in the cuticle was also observed (Figure 4.5, A). Due to the location, this may be the inoculation zone with larger infiltrations of purple-stained cells accumulating in fat body tissue next to and opposite the wound (Figure 4.5, B-C). This portion may thus include the 4^{th} to 6^{th} sternites. A section of the fat body was also darkened suggesting a melanisation event; however, no fungal structures were seen highlighting the importance of fungal-specific stains such as GMS. Purple-stained cells were also observed within and migrating to this site.

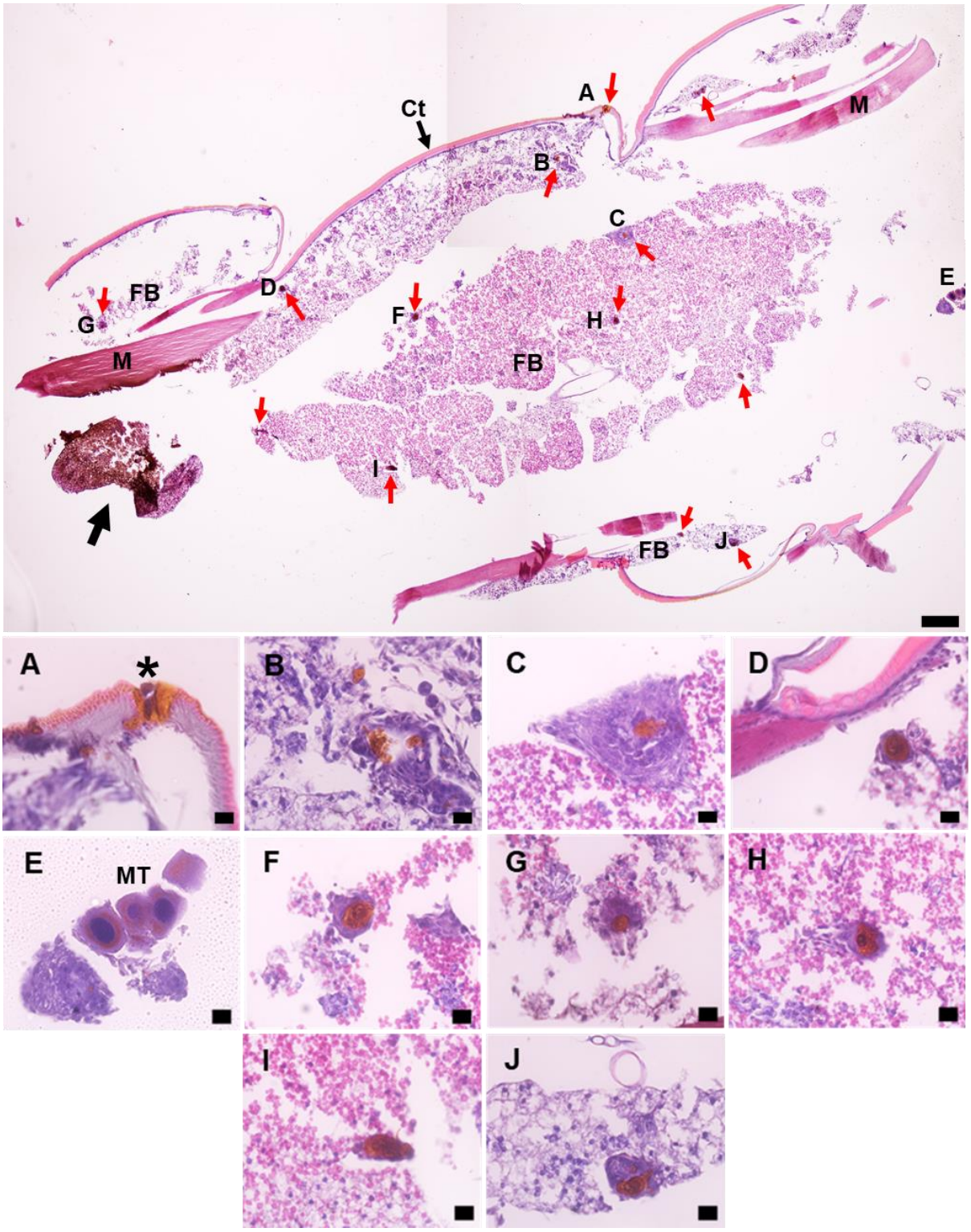

Figure 4.5 H & E-stained *T. molitor* larva tissue section 96 hours post-inoculation with 5 x 10^4 AF03 conidia showing several melanised nodules.

Melanised nodules (red arrows) are predominant within the fat body (FB) and accompanied by aggregating purple-stained cells (B-D, F-J) also near a malpighian tubule (MT) which has a densely stained nucleus surrounded by specks of melanisation (E). Melanisation is visible

at a break in the cuticle (Ct) (A - *). A section of fat body appeared darkened (large black arrow), and tissue loss evident throughout the section. Micrographs are from the least damaged section observed, sliced at 4 µm. Bars represent 200 µm (large image) and 20 µm (A-J).

Closer observation of the melanised nodules seen in AF03 inoculated larval tissue after 96 hours presented in Figure 4.5 reveals the presence of round conidia-like structures and elongated hyphal-like structures contained within melanised nodules (Figure 4.6, C-D, F, I). Both appear to have a diameter of approximately 2-3 µm which is the approximate size range of *A. fumigatus* conidia and hyphae.

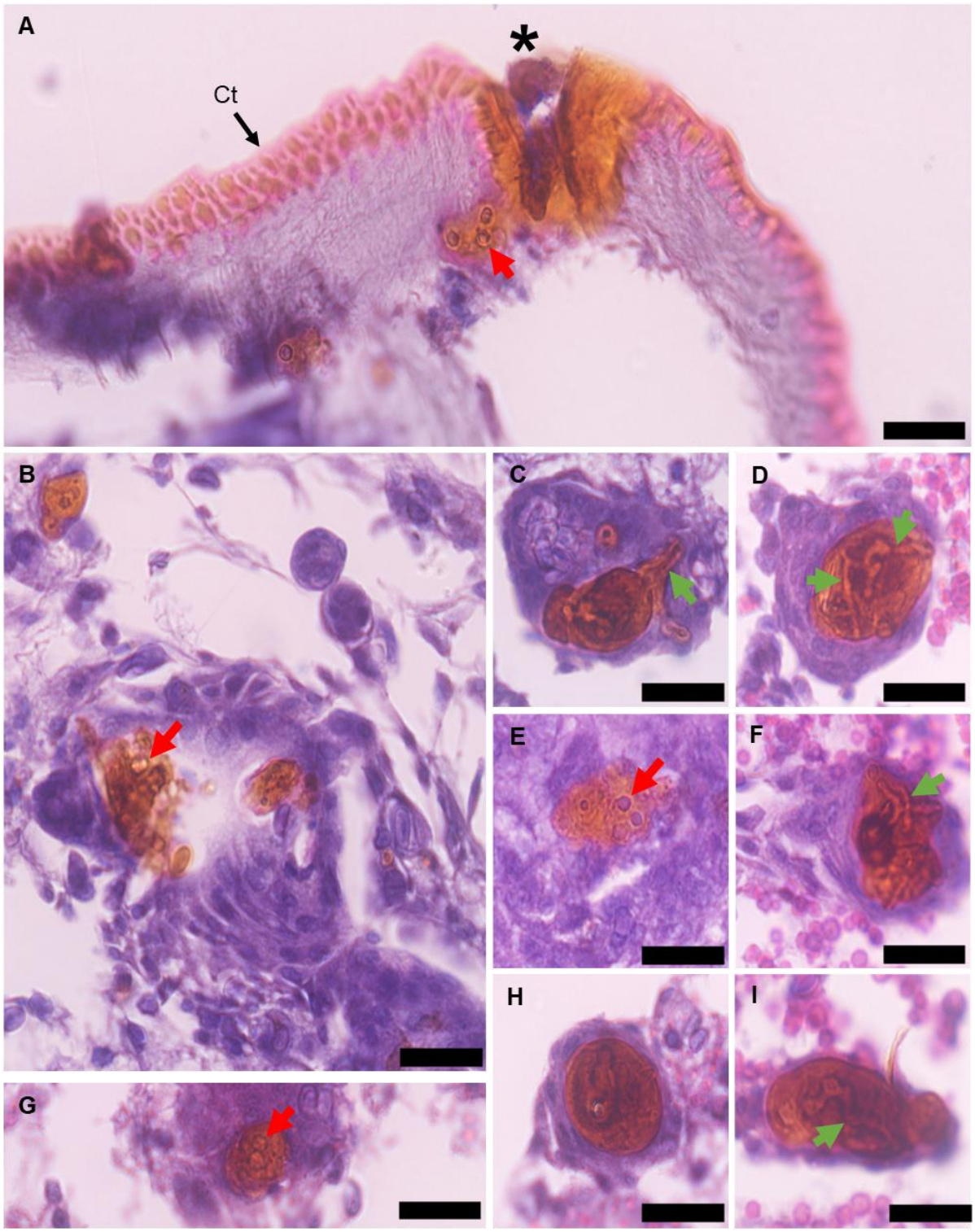

Figure 4.6 Melanised nodules contain conidia- and hyphal-like structures.

Nodules were observed surrounded by layers of purple stained cells. Round (red arrows) and long filamentous structures (green arrows) approx. 2-3 µm diameter appear within melanised

nodules. Micrographs are compiled from one larva inoculated with 5 x 10^4 conidia and incubated at 37°C for 96 hours. Ct - cuticle, * - break in the cuticle. Bars represent 20 µm.

Melanised nodules were also evident in the fat body after 24 hrs of 37°C incubation (Figure 4.7).

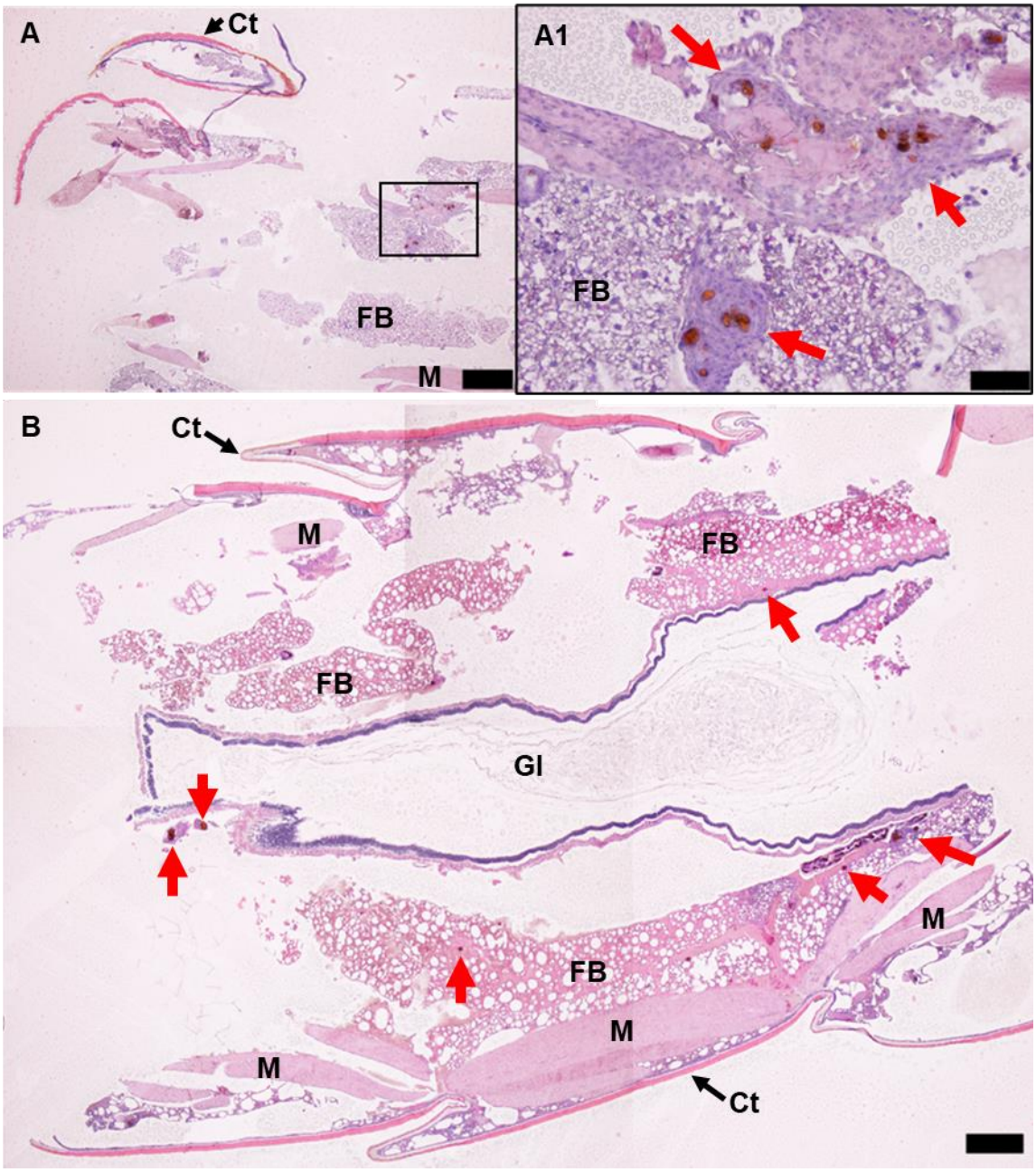

Figure 4.7 Melanised nodules evident in *T. molitor* larvae tissue inoculated with 5 x 10^4 AF03 conidia 24 hrs post-inoculation stained with H & E.

Sections are from two separate larval samples (A & B) differing in quality. Melanised nodules were observable regardless of section quality (red arrows). Purple stained cells can be seen accumulating around melanised nodules (A1). Ct – cuticle, FB – fat body, M – muscle fibres, GI – gastrointestinal tract. Bars represent 200 µm (A & B), and 50 µm (A1).

Melanisation may thus be a key response against *A. fumigatus*, with conidia and hyphae similarly observed within these structures after 24 hours (Figure 4.8).

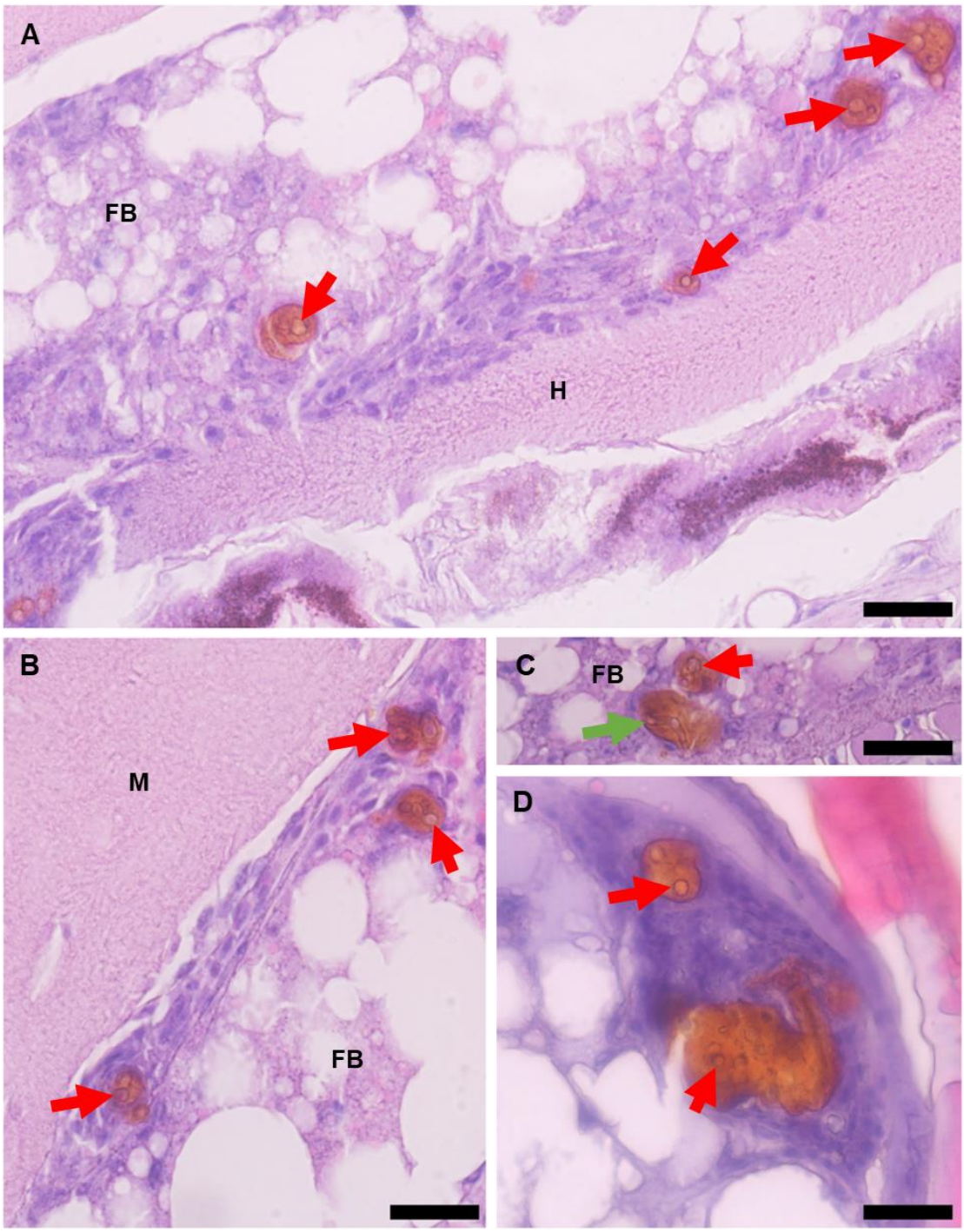

Figure 4.8 Melanised nodules from a section of *T. molitor* larva tissue 24 hrs post-inoculation with AF03 conidia stained with H & E.

Melanised nodules are accompanied by purple-stained cells. Structures visible within melanised nodules appear predominantly conidia-like (red arrows) with a hyphal-like structure

also visible (green arrow). These are approx. 2-3 µm in diameter. Nodules are compiled from a sample inoculated with $5x10^4$ conidia and incubated at 37°C. FB – fat body, M – muscle fibres, H – haemolymph. Bars represent 20 µm.

Unlike uninoculated larvae where the fat body appeared evenly stained, fat body of *A. fumigatus* inoculated larvae were observed to be uneven, with purple accumulations evident around and away from melanised nodules (Figure 4.9). These are likely haemocytes that are responding to infection.

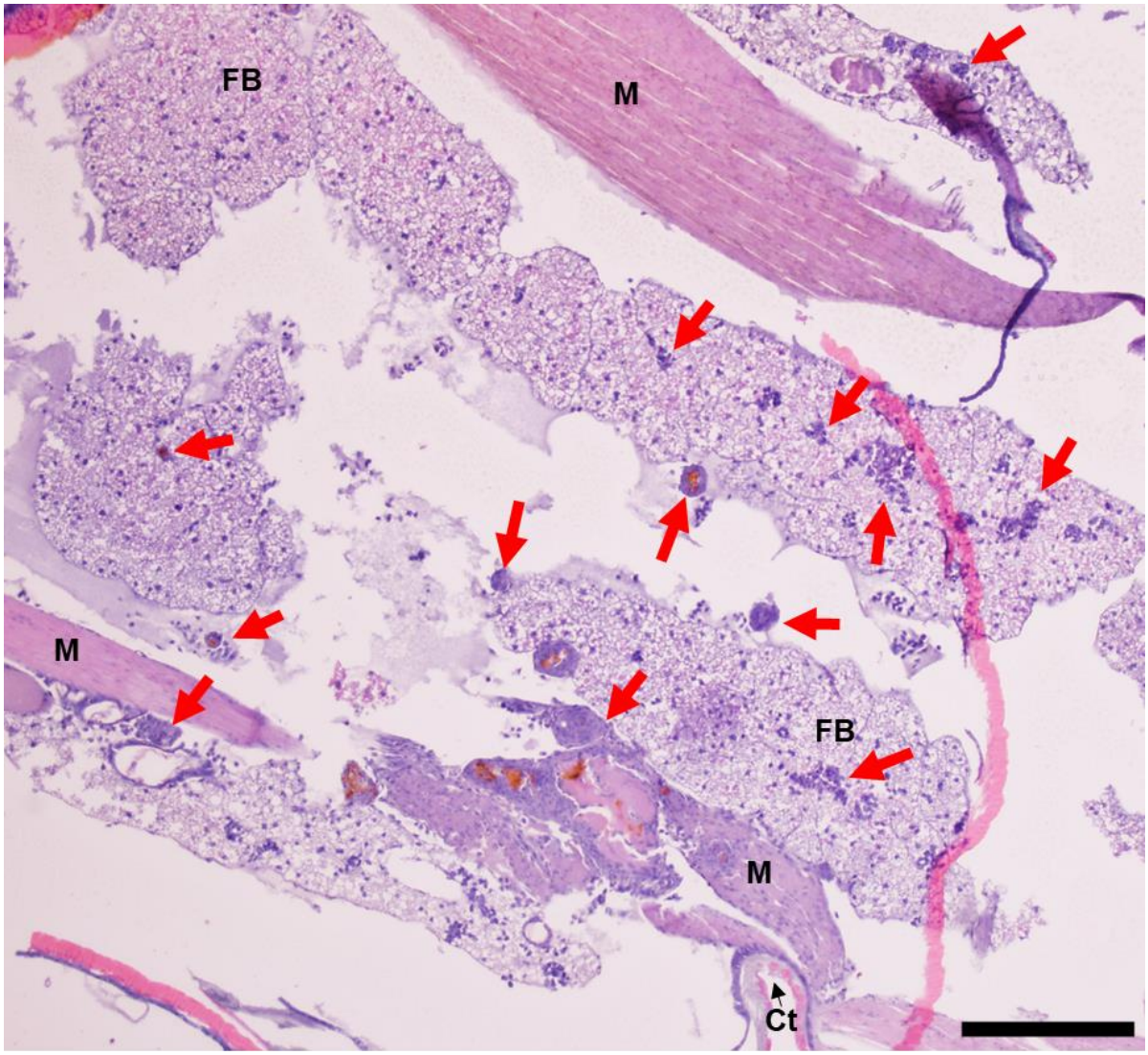

Figure 4.9 Cellular aggregates spread throughout fat body post-inoculation with *A. fumigatus* as revealed using H & E.

Section presented is from an AF03 inoculated larval sample 24 hours post-inoculation at 37°C. Cellular aggregates can be observed throughout the tissue section (red arrows). Bar represents 100 µm.

T. molitor larvae were scanned using MRI due to the challenges experienced during histology to track infection in whole larvae (Figure 4.10). Signs of infection were not observed, however larval morphology could be seen. Marking fungal elements may thus be important to track infection.

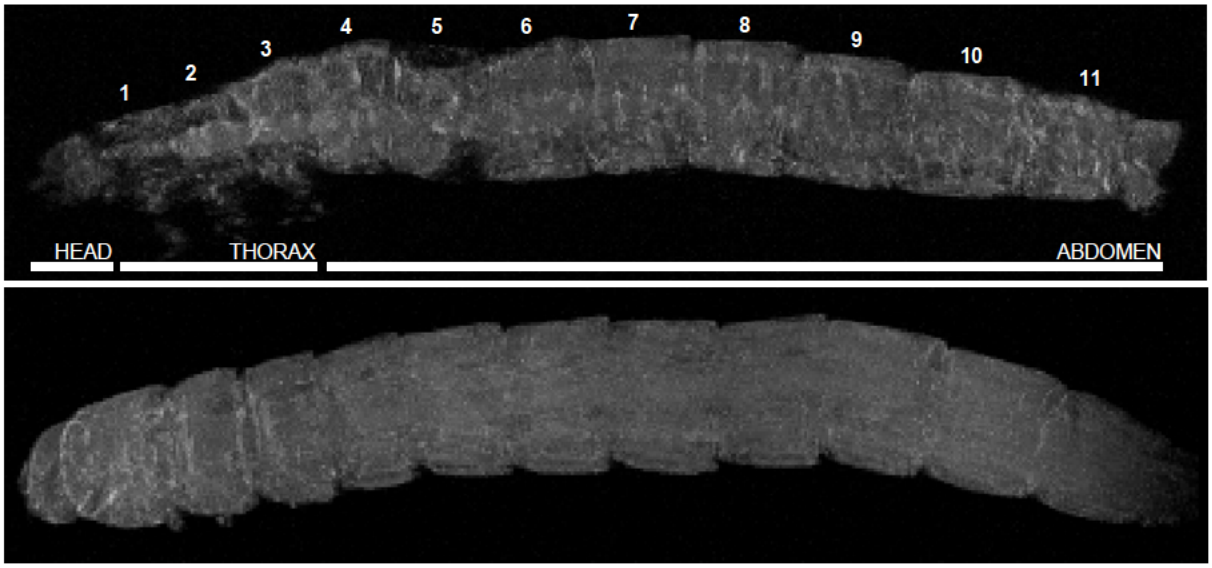

Figure 4.10 *T. molitor* larvae were scanned using MRI during early optimisation experiments.

An uninoculated *T. molitor* larva (top) and larva inoculated with 5 x 10^5 AF11 conidia (bottom) were scanned using MRI. Samples were incubated at 37°C for 72 hours. Images were analysed using Image J. Sternites are numbered.

4.4 Discussion

In this chapter, *T. molitor* larvae inoculated with *A. fumigatus* conidia were histologically processed to investigate interactions occurring within larval tissue during infection. *A. fumigatus* could not be visualised freely within the tissue due to stain limitations, such as Calcofluor white which stained both fungi and *T. molitor* larvae tissue (data not shown). Infection was thus identified based on the larval immune response which provided observable features related to infection. The main feature observed was the presence of melanised nodules accompanied by the aggregation of purple-stained cells suspected to be haemocytes (Figure 4.5). *T. molitor* larvae thus mounted an immune response against the *A. fumigatus* inoculum which was visualised histologically after 24 and 96 hours. The immune features are indicative of a nodulation or encapsulation response, both of which are characterised by the aggregation of haemocytes and presence of melanisation.[230]

The response observed here by *T. molitor* larvae against *A. fumigatus* has been documented histologically in *G. mellonella* larvae. In a study by Durieux et al[204] nodules containing conidia and hyphae were identified throughout larvae using GMS staining. In that study, the size of nodules and their abundance was identified relative to the infection duration.[204] Similar observations were documented by Sheehan et al[132] who observed most melanisation at the inoculation zone, and via dissection of melanised plaques observed cellular infiltration and germinating *A. fumigatus* conidia throughout the melanised nodules.[132] *T. molitor* larvae and *G. mellonella* larvae may thus utilise a similar response against *A. fumigatus*.

As larger nodules were observed in this study containing hyphae after 96 hours (Figure 4.6), *A. fumigatus* may resist the *T. molitor* larvae immune responses to

continue growing and persist over time, which may thus also account for the presence of smaller nodules with increasing distance from the inoculation zone suggestive of dissemination (Figure 4.5). In *G. mellonella*, production of fumagillin and gliotoxin by *A. fumigatus* has been linked to virulence and thus may inhibit the insect's immune response.[101, 102] This has also been observed in human immune cells.[171, 173, 231] This may also be true in *T. molitor* larvae, however additional research is necessary to confirm this.

The nodulation response observed in this study against *A. fumigatus* has also been documented against other pathogens. This includes in *G. mellonella* larvae in response to *Paracoccidioides lutzii* where inoculum size influenced melanisation rate,[232] *Exophiala dermatitidis*,[233] and *C. albicans*.[234, 235] Differing melanisation, aggregation and invasion was observed between two *C. albicans* isolates highlighting how host responses may be useful to distinguish between isolates.[234] Nodules have also been observed in *G. mellonella* larvae inoculated with *S. aureus*.[195] Nodulation may thus be a non-specific response important for defence against a range of pathogens. *T. molitor* larvae, like *G. mellonella* where differing *C. albicans* virulence was observed,[234] may thus also be useful to investigate virulence.

Although nodulation was observed in *T. molitor* larvae in response to *A. fumigatus* as noted in other research, additional research is important. Due to technical issues regarding manual processing utilised here, only a few sections were of a quality that retained larval morphology. *T. molitor* larvae should thus be further investigated histologically, which when processing in smaller portions should include pre-embedding.[236] Special stains are also important such as GMS to highlight fungal elements in tissue. These were not tried as focus was on the optimisation of tissue processing. GMS staining previously helped identify *C. neoformans* within *T. molitor*

larvae tissue,[138] and *A. fumigatus* in *G. mellonella* larvae tissue.[204] Additionally, application of stains such as Giemsa can confirm the presence of haemocytes and thus host immune responses.[234] In this histological assessment, haemocytes were suspected to be the purple-stained cells using H & E which were observed to aggregate around melanised nodules and in nearby tissue (Figure 4.9). Haemocytes in *G. mellonella* were previously highlighted using haemocyte stains such as Giesma and Alcian blue which revealed the distribution of haemocytes which was subcuticular, in the fat body and with discrete aggregates around intestinal and tracheal boundaries. Haemocytes in that study were also observed to increase around the inoculation zone, the fat body and at nodules depending on the isolate.[234] Histological examination of infected *T. molitor* larvae tissue, should various stains and optimal tissue processing be utilised, may thus provide important information regarding pathogenesis as documented in *G. mellonella* larvae.[234] Alternative methods may also be helpful such as *in-vitro* assessments of *T. molitor* larvae haemocytes in response to *A. fumigatus* which is explored in Chapter 5.

As manual tissue processing demonstrated technical difficulties, MRI was considered as a non-invasive method to assess infection. However, preliminary observations using several *T. molitor* larvae have not yet revealed any signs of infection (Figure 4.10). This may be due to the small size of *T. molitor* larvae where things would thus occur on a much smaller scale especially compared to humans where MRIs are commonly utilised. Furthermore, MRI is usually utilised to assess the response induced by microbes in tissue such as inflammation rather than the microbe itself.[237, 238] Detecting tissue damage in *T. molitor* larvae would be challenging as like the causative microbe it would be relatively small. Further

optimisations are thus necessary such as using stains to track the fungus. Methods to track microbes using MRI have been reported.[239]

Chapter 5 *T. molitor* larvae cellular response

5.1 Introduction

Cellular immune responses are an important part of the insect innate immune system which includes phagocytosis, nodulation, and encapsulation. These are performed by cells known as haemocytes which are invertebrate immune effector cells. Four types of haemocytes have been identified in *T. molitor* which include: granulocytes, plasmatocytes, prohaemocytes and oenocytoids (Figure 5.1).[140, 154, 160] Granulocytes are phagocytic cells representing 50-60% of *T. molitor* haemocytes. These are oval shaped cells that are approximately 8-11 µm in size and contain characteristic dense granules. The second most common haemocytes are plasmatocytes which represent 23-28% of haemocytes. These are large and elongated cells, approximately 9-15 µm long, that may be involved in encapsulation. Prohaemocytes are small oval cells, approximately 5-8 µm in size with a large nucleus. These account for 10-15% of haemocytes and are likely precursor cells. The least common haemocyte are oenocytoids which are elliptical cells, approximately 15 µm in size that contain a small nucleus. Oenocytoids account for 1-2% of haemocytes and likely produce enzymes involved in melanisation important for nodulation and encapsulation, and wound healing which involves coagulation.[140, 160] During the pupal stage, the relative abundance of these cells exhibits a slight variation as oenocytoids are more numerous than prohaemocytes.[154]

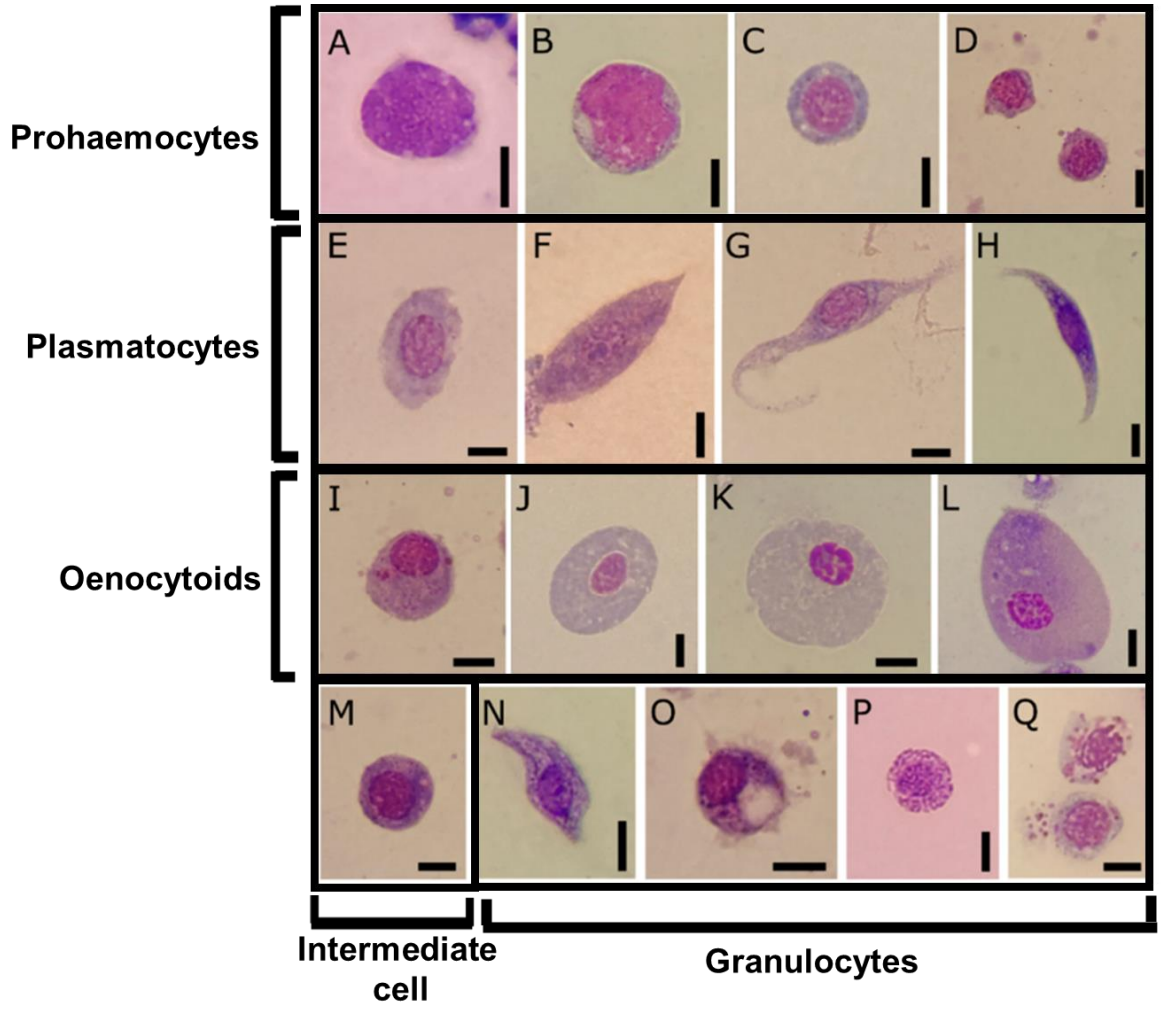

Figure 5.1 Example of *T. molitor* haemocytes stained with May-Grunwald Giemsa from an article by Vommaro et al.[160]

Four haemocyte types were identified each of which had variable morphology. Scale bars represent 5 µm.

These four haemocytes types have been documented in other coleopteran species, along with additional haemocytes not found in *T. molitor* such as adipohaemocytes and spherulocytes.[240, 241] Haemocytes vary in their morphology and function between insect species, and further differ in their response against different pathogens and at different stages of infection.[242] In this chapter, haemocytes were extracted from *T.*

molitor larvae to evaluate the interactions that occur during exposure to *A. fumigatus in-vitro*. Phagocytic responses were of particular interest as neutrophil phagocytosis in humans is considered a crucial early response against conidia that prevents infection from progressing.[68] Results of methods development and optimisation experiments to observe interactions are described.

The roles of phagocytic cells differ between insect species. In *T. molitor*, granulocytes have large amounts of lysosomes and therefore may be the main phagocyte.[160] Granulocytes in *Gryllus bimaculatus* (two-spotted cricket) were identified as the predominant phagocyte.[166] However, in *Manduca sexta* (tobacco hornworm) the main phagocytes are plasmatocytes,[167] which are also the main phagocytes in *D. melanogaster* where granulocytes are absent.[165] In *G. mellonella*, plasmatocytes are considered the main phagocytes while granulocytes indirectly contribute.[243]

The internalisation of pathogens during phagocytosis is a size limited process.[162] Pathogens are packaged into vacuoles and degraded upon lysosomal fusion. Degradation occurs due to ROS production, referred to as the respiratory burst, which is necessary for phagocytic killing. Host tissue can become damaged during this response and due to inflammation induced when granulocytes, which degranulate to release enzymes into the phagolysosome, release enzymes outside the cell.[58] Should the phagocytic response fail to eliminate pathogens, encapsulation and nodulation responses may follow.[243]

5.2 Methods

5.2.1 Haemocyte Extraction

Haemocytes were extracted from unharmed *T. molitor* larvae (100-150 mg) stored with food and a carrot. To extract *T. molitor* haemocytes, larvae were cold anaesthetised on ice for 5 minutes. On a chilled surface under a dissection microscope, larvae were positioned on their dorsal surface using tweezers. Using clean dissection scissors, the first pair of legs was cut as previously described,[154] resulting in the formation of a haemolymph bubble. Using a 20 µL pipette set to 10 µL, the haemolymph was collected and dispensed into 500 µL of ice-cold PBS stored on ice. Haemolymph was collected several times from individual larvae to maximise yield. Any haemolymph with orange/brown discolouration was not used. Once the haemolymph of fifteen mealworms had been pooled, it was then centrifuged at 200 x *g* for 5 minutes at 4°C. The supernatant was carefully removed, and the cells washed twice with 500 µL ice-cold PBS. The resulting pellet was resuspended in 20 µL ice-cold Grace's Medium (BioWhittaker®, 04-457F, Lonza). Cells (5 µL) were quantified using a haemocytometer and Trypan Blue (1:1, v/v ratio) to determine viability.

5.2.2 Haemocyte-fungal interaction assay

For haemocyte interaction assays, AF11 conidia were resuspended in 5 µL Grace's Medium for a multiplicity of infection (MOI) of 10 (10^4 haemocytes, 10^5 conidia). The assay was then divided amongst 1.5 mL centrifuge tubes for incubation at 37°C, 5% CO_2 for varying durations (up to four hours). After incubation, cells were fixed with 10% neutral buffered formalin (1:3, v/v ratio) and stored overnight at 4°C. Prior to staining, the fixative was removed via 200 x *g* centrifugation at 4°C for five minutes.

5.2.3 Staining

Giemsa

Fixed cells were stained using 4 µL Giemsa Stain (Modified Solution, 48900, Sigma) diluted 1:20 with PBS. The sample was then mounted on a slide using round cover slips (10 mm #1, G401-10, ProSciTech) and observed using brightfield microscopy (Olympus BX43, DP73 Camera).

Calcofluor White

Fixed cells were stained in a 1:1 ratio v/v, with Calcofluor White and 10% KOH in a 1:1 v/v ratio. The sample was mounted on a slide, cover slip added and observed using fluorescence microscopy (Olympus BX43, DP73 Camera).

Phalloidin and DAPI

Fixed cells were rinsed twice with 30 µL PBS, resuspended in 30 µL of 1X Phalloidin-iFluor 488 Conjugate (ab176753, abcam) and incubated in the dark at room temperature for thirty minutes. 30 µL 1X DAPI readymade solution (MBD0015, Sigma) was then added, and the sample incubated in darkness for an additional fifteen minutes. The sample was then centrifuged at 200 x *g* for 5 minutes at 4°C to remove the stains and rinsed twice with 30 µL PBS. Cells were then resuspended in 5 µL of PBS and mounted on slides using round cover slips. Slides were observed using fluorescence microscopy (Olympus BX43, DP73 Camera).

5.3 Results

Haemocytes from fifteen *T. molitor* larvae were recovered and pooled. Viability was determined using the trypan blue exclusion test with cells that were stained blue considered non-viable. The viability of pooled haemocytes ranged from 30% to 79% (Figure 5.2). Haemocyte viability thus varied between experiments which was also noted with haemocyte counts (115 to 4088 viable haemocytes per µl – not presented). According to Figure 5.2, average viability was 54% (standard deviation = 12.26). The lowest viability determined (30%) corresponded with the lowest haemocyte counts.

Staining of cells with Giemsa revealed the varied morphology of haemocytes as observed using brightfield (Figure 5.2). Four types were identified based on shape and size as previously characterised.[160] Granulocytes were commonly identified as round cells approximately 10 µm in diameter, and prohaemocytes as cells approximately 5 µm in diameter with a large nucleus relative to the cytoplasm. Other cell types were rarely identified or difficult to distinguish. Plasmatocytes were identifiable when they had an elongated spindle shape and oenocytoids based on their large size and relatively small nucleus. Granulocytes and plasmatocytes commonly had several filopodia.

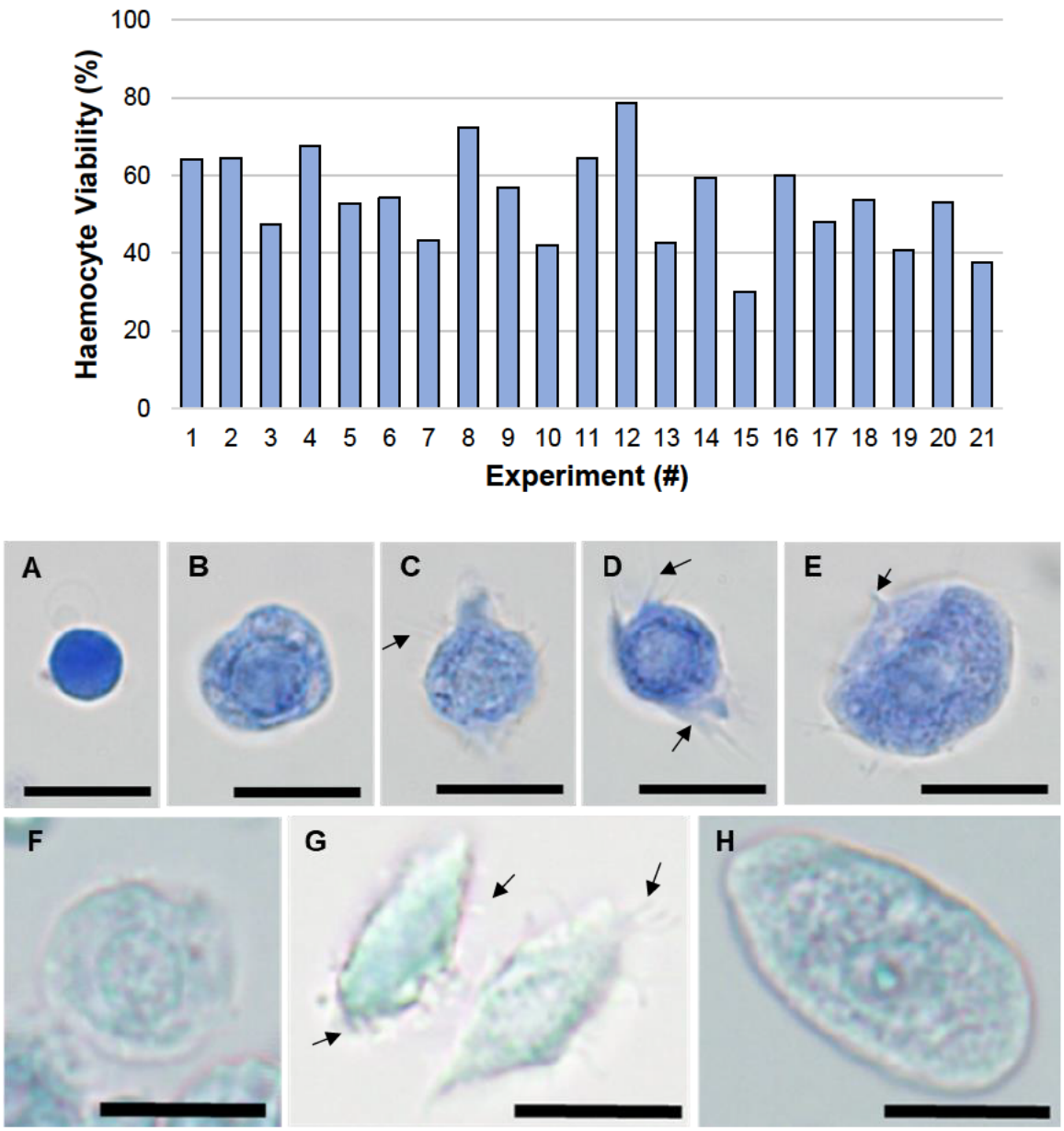

Figure 5.2 Viability of pooled *T. molitor* larvae haemocytes and micrographs of Giemsa-stained haemocytes.

Haemocytes were pooled from fifteen larvae per experiment and viability determined using the trypan blue exclusion method. Average haemocyte viability was 54% (standard deviation = 12.26). Fixed haemocytes were stained with Giemsa and observed using brightfield. Prohaemocytes (A), granulocytes (B-D, F), plasmatocytes (G) and oenocytoids (E, H) were identified based on size and shape. Filopodia were common on granulocytes and plasmatocytes, and also observed on oenocytoids (black arrows). Images are compiled from two separate preparations, A-E and F-H. A-E were captured at 1000x and F-H at 400x. Bars represent 10 µm.

Interaction experiments between *T. molitor* larvae haemocytes and *A. fumigatus* conidia (AF11) were performed *in-vitro* at MOI 10 and 37°C incubation, 5% CO_2 to examine haemocyte responses. Cells were stained with Giemsa and observed using brightfield microscopy. Figure 5.3 shows micrographs from two experiments representative of 0- and 25-minutes incubation. Haemocytes and conidia were commonly observed to be positioned around each other including next to and overlapping, and near a pink/purple stained substance which was commonly observed with Giemsa staining.

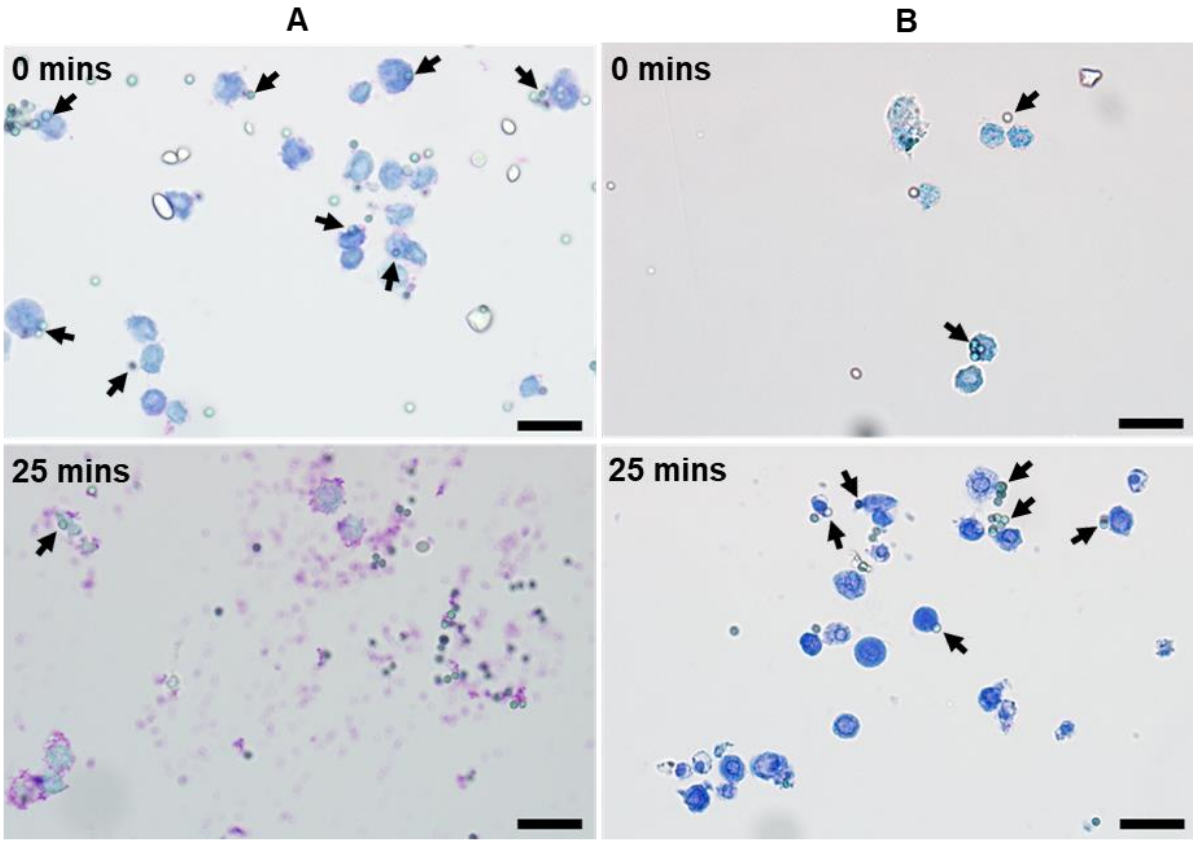

Figure 5.3 *T. molitor* haemocytes and *A. fumigatus* conidia after 0 and 25 minutes of interaction.

Haemocytes and conidia were observed in close proximity to each other (black arrows). Images are from two experiments (A - 72.3% viability, and B – 56.9% viability) using pooled haemocytes and AF11 spores at MOI 10, and 37°C, 5% CO_2 incubation. Cells were fixed and stained with Giemsa. All images were taken at 400x. Bars represent 20 µm.

Experiment “B” (Figure 5.3) which utilised pooled haemocytes with the greatest haemocyte counts, had little to no pink/purple stained substance visible in all durations observed (<25 mins). This may be due to factors such as fewer conidia allocated to the pooled haemocytes due to technical error. In experiment “A” (Figure 5.3), pink/purple stained substance was present at each incubation duration observed. As the number of observable haemocytes varied between assays as pooled haemocytes and conidia were divided amongst several tubes, results may also be variable. For example, in figure 5.3 experiment “A” has fewer haemocytes to conidia present at twenty-five minutes, while “B” has approximately equal numbers of both present.

Observation of experiment ‘A’ presented in Figure 5.3 after 0- and 5 minutes incubation using oil immersion further revealed the interaction between larval haemocytes and AF11 conidia (Figure 5.4). Pink-stained secretions with Giemsa staining are visible around haemocytes which appear more prevalent at the later time point and at locations with conidia. Filopodia are also visible at both time points. As samples were observed using brightfield and Giemsa staining, distinguishing internalisation of conidia from positioning on haemocytes was not clear.

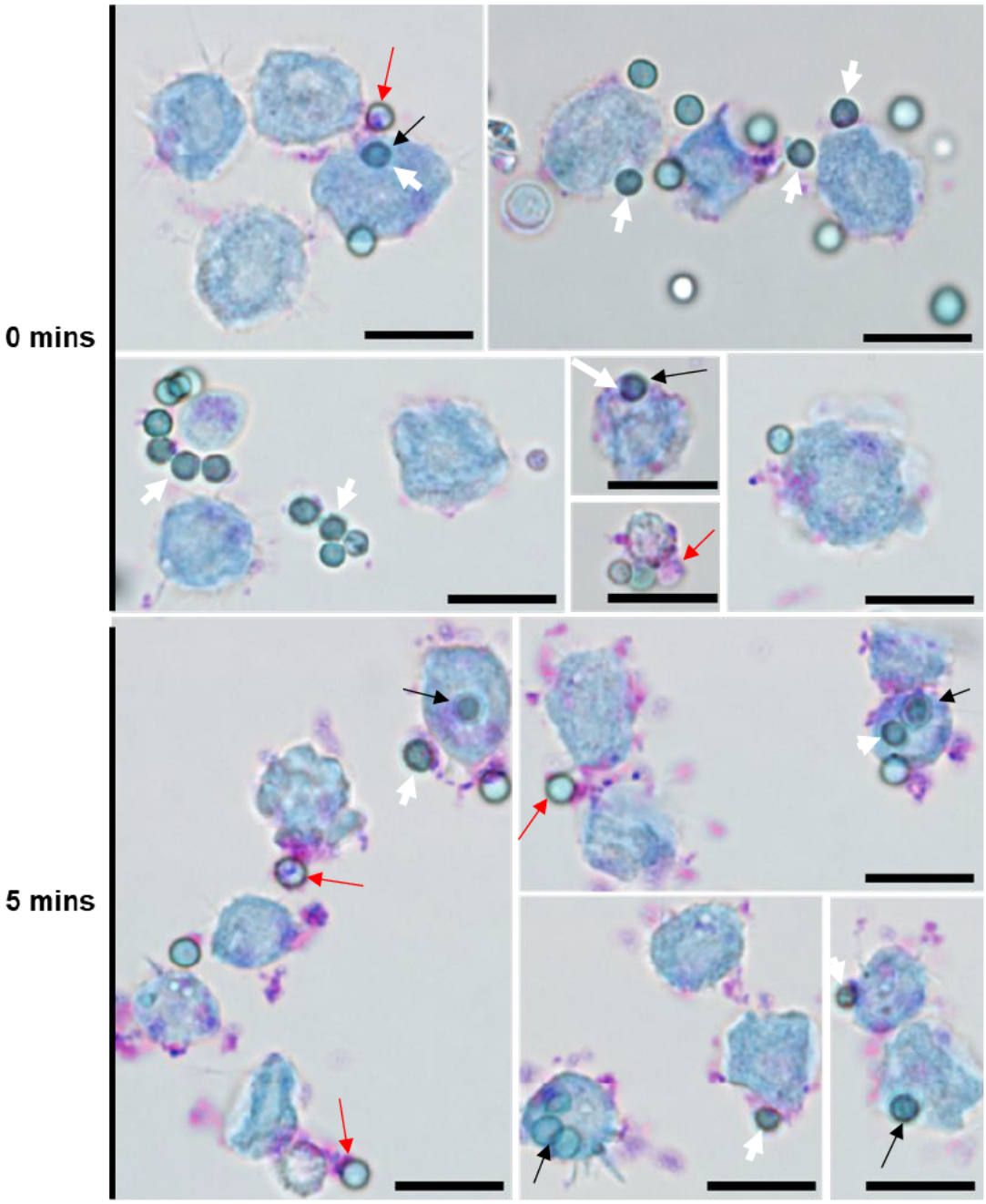

Figure 5.4 *T. molitor* haemocytes and *A. fumigatus* conidia after 0 and 5 minutes.

Haemocytes and AF11 conidia at MOI 10 were incubated at 37°C, 5% CO_2. Conidia can be seen associating with haemocytes including positioned on/in haemocytes (black arrows) and with pink/purple stained substance (red arrows). "Darkened" conidia are also visible (white arrows). Images are compiled from one experiment divided into tubes per incubation duration. Images were taken at 1000x. Bars represent 10 µm.

During longer interaction assays incubated for 1.5 hours and 2 hours, bulges were common on the surface of Giemsa-stained haemocytes post-interaction with AF11 conidia at MOI 10. These bulges were sized similarly to conidia however could also be indicative of blebbing. As conidia-free haemocytes were not prepared for this duration and staining of conidia is not used, the identity of these bulges is unclear. Specific staining of conidia would be important to confirm internalisation events.

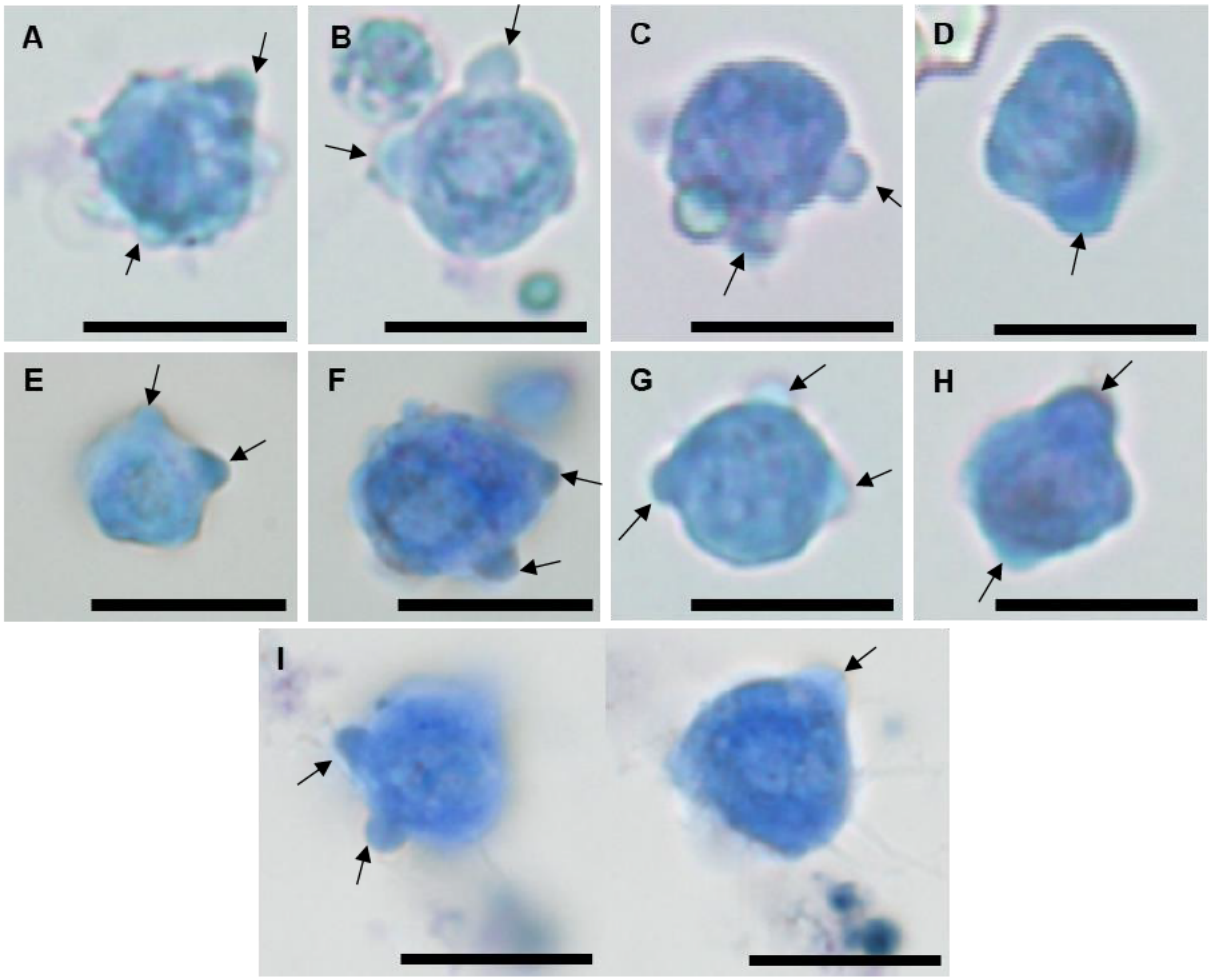

Figure 5.5 *T. molitor* larvae haemocytes with round bulges (black arrow).

All images are post-interaction with AF11 conidia at MOI 10 and 37°C, 5% CO_2 incubation. A-H – 2 hours incubation. I - one-and-a-half hours incubation showing the same cell from two planes. A-D, G-H – taken at 400x, E-F & I – taken at 1000x. Bars represent 10 µm.

Round structures were observed within haemocytes recovered from *T. molitor* larvae that had been injured with a Hamilton syringe 2 hours prior to haemocyte collection. Larvae had been injured as part of early optimisation to see if it would increase haemocyte counts. This was not investigated further as the haemocyte recovery method utilised did not involve recovering a set amount of haemolymph. These round structures on haemocytes from injured larvae were observed after 0- and 15-minutes incubation with AF11 conidia at MOI 10 (Figure 5.6, A-C). As this was not observed in other assays testing shorter incubation durations, this observation may be representative of an internalisation event that occurred within larvae prior to haemocyte collection. The presence of these round conidia-like structures (approx. 2 to 3 μm diameter) within haemocytes was also observed in an interaction assay after three hours incubation however (Figure 5.6, F), along with cells with bulges (Figure 5.6, D-E). This was only performed once and should be investigated further using confocal microscopy and stains for *A. fumigatus* conidia.

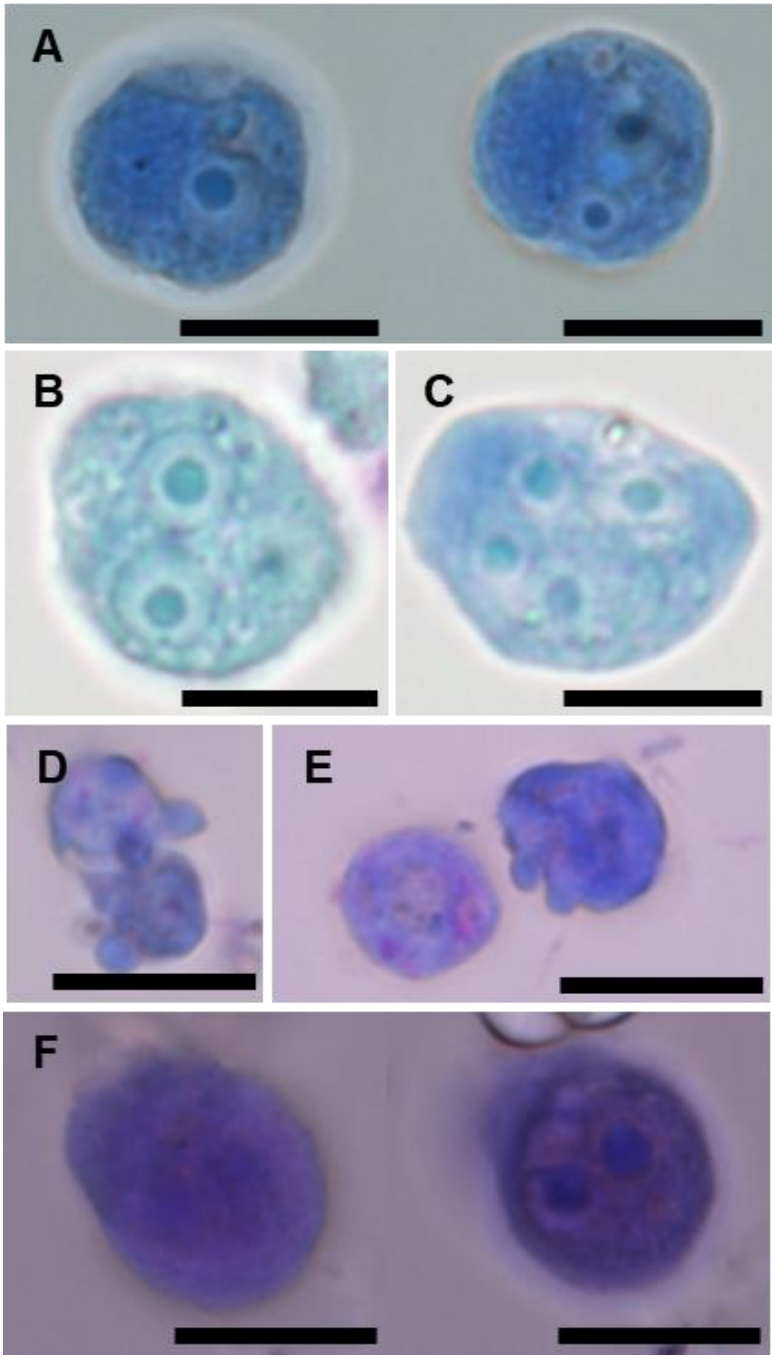

Figure 5.6 *T. molitor* larvae haemocytes containing round structures.

Haemocytes extracted 2 hours post-injury with a Hamilton syringe after the 7th sternite contained round structures at 0 minutes (A-B) and 15 minutes (C) incubation at 37°C, 5% CO_2. Haemocytes were exposed to AF11 conidia at MOI 10, fixed and stained with Giemsa. Haemocytes after 3 hours incubation showed round bulges on surface (D-E) and a haemocyte containing round structures visible on a specific plane (F). A,D-F – taken at 1000x, B-C – 400x. Bars represent 10 µm.

To investigate the interactions between haemocytes and conidia, calcofluor white staining was initially utilised (Figure 5.7). Both *A. fumigatus* and haemocytes were stained, with the appearance of a blue background around the cells (Figure 5.7, A-B). Distinguishing fungal components and haemocytes using this fluorescent stain was thus difficult as it stains chitin present in both. Granular details within the cells were observable however (Figure 5.7, C).

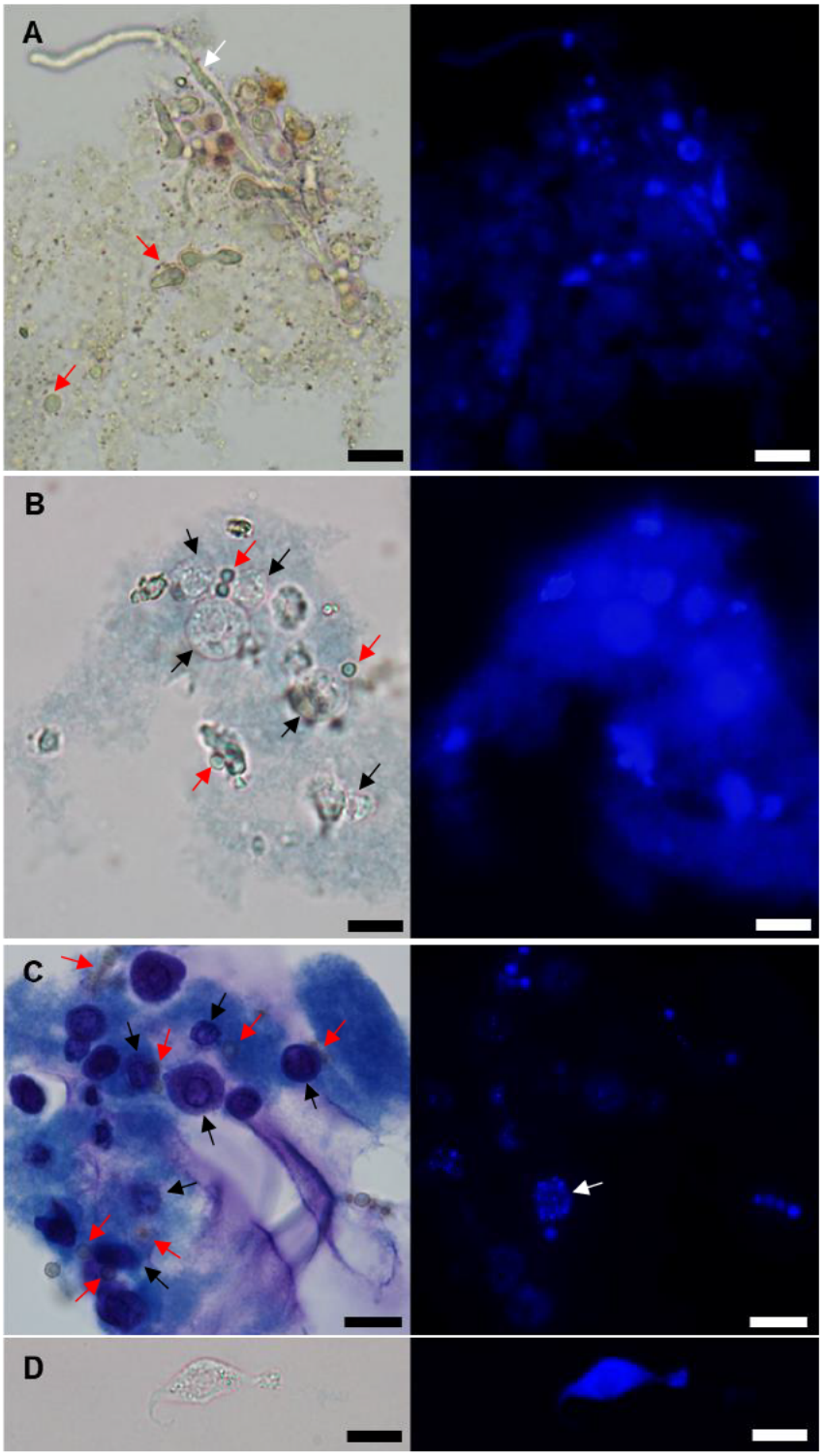

Figure 5.7 *T. molitor* larvae haemocytes and *A. fumigatus* stained with Calcofluor White.

Haemocytes and AF11 conidia at MOI 10 were incubated for 1 or 2 hours at 37°C, 5% CO_2, fixed and stained. Hyphae (white arrow) and conidia at various stages (red arrows) 2 hours post-haemocyte interaction with germinated conidia (A). Haemocytes (black arrows) and conidia (red arrows) in proximity after 1 hour incubation with resting conidia (B-C). Haemocyte

granules can be observed using fluorescence (C). Plasmatocyte stained with calcofluor white (D). A,B,D – observed at 400x, C – observed at 1000x. Bars represent 10 µm.

T. molitor larvae haemocytes post-incubation with *A. fumigatus* conidia were stained with phalloidin and DAPI to further investigate haemocyte morphology as changes have been observed in response to pathogens.[162] These stain actin filaments and nuclei respectively. Haemocyte nuclei were stained blue by DAPI, and filopodia stained green with phalloidin. Filopodia and the outer surface of haemocytes were most intensely stained green (Figure 5.7). The round bulges observed previously were also stained green indicating actin. Orange/brown structures were observed free in solution, including as clusters near haemocytes with actin encircling them. These structures were not stained and are likely melanised. Figure 5.9 shows additional micrographs of orange/brown structures including those potentially attached by actin filaments, near haemocytes and free floating as clusters. Large masses of orange/brown were also observed which during Phalloidin and DAPI staining show nuclei and actin filaments as part of the mass (not shown). The orange/brown structures may thus be due to the haemocyte melanisation response, with the smaller structures observed indicative of clusters of melanised conidia.

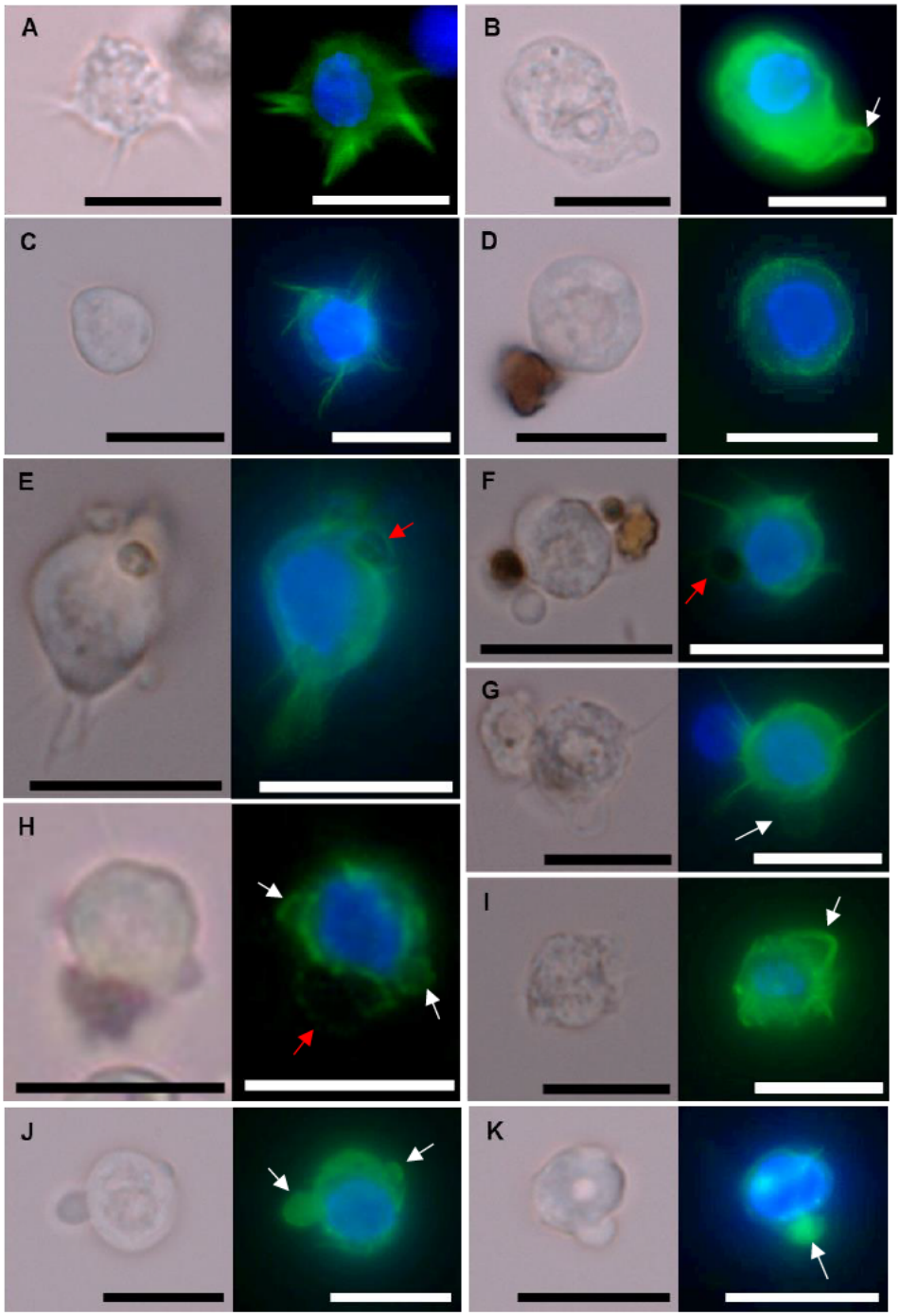

Figure 5.8 Phalloidin and DAPI stained *T. molitor* larvae haemocytes from interaction experiments.

Haemocytes were observed with filopodia (A, C, E-G, I-K) and with round actin-stained structures (B, G-K) (white arrows). Orange/brown structures observed near haemocytes (D, F) and connected by actin (F, H) (red arrows). All micrographs are from interactions with AF11 conidia at MOI 10 and incubation at 37°C, 5% CO_2. A – B; 0 minutes, C; 1 hour, D; 2 hours, E-I; 3 hours, and J-K; 4 hours incubation. A-G & I-K – taken at 1000x, H – taken at 400x. Bars represent 10 µm.

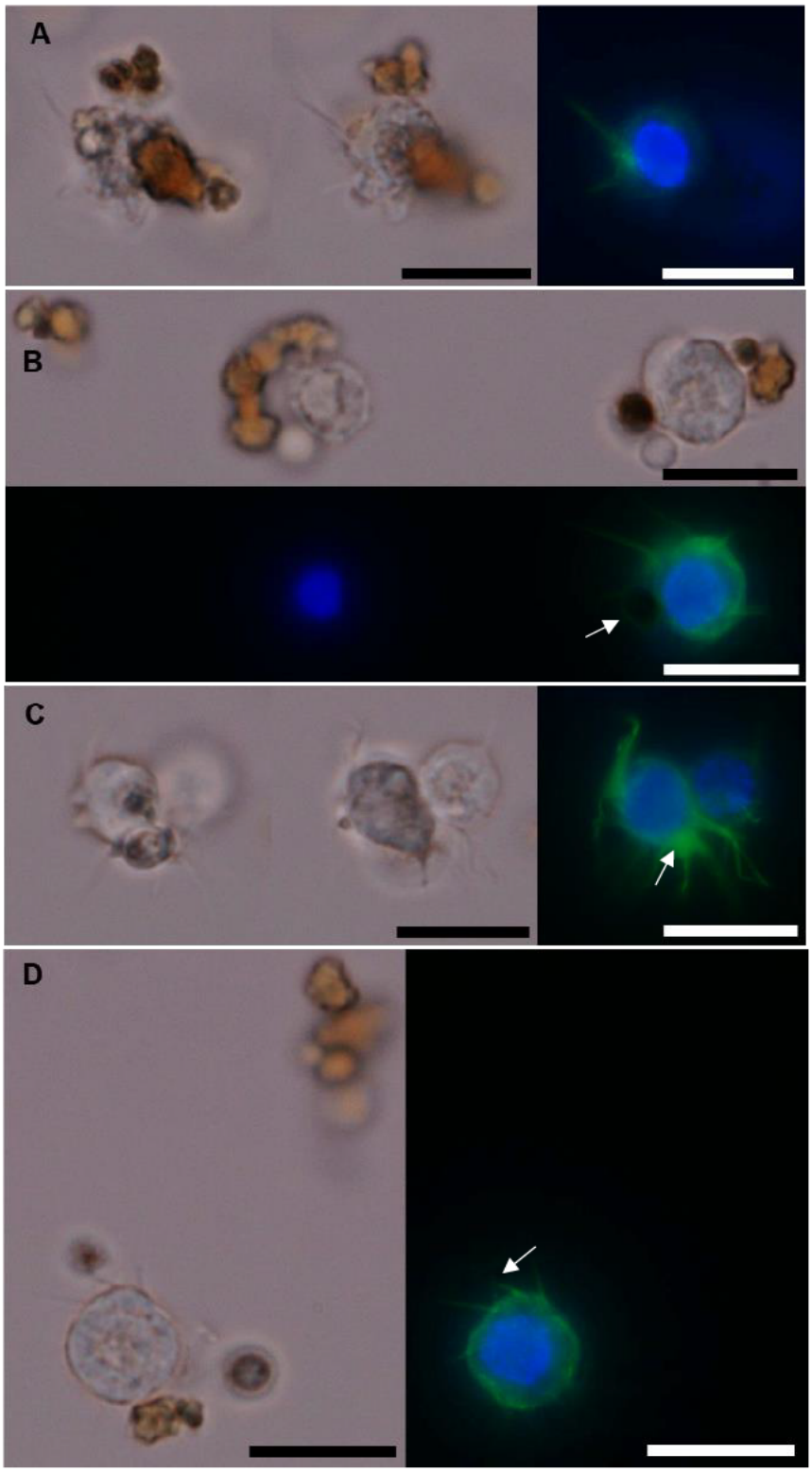

Figure 5.9 Orange/brown structures located near *T. molitor* larvae haemocytes stained with phalloidin and DAPI.

Haemocytes were incubated at 37°C, 5% CO_2 with AF11 conidia at MOI 10 for 3 hours. Orange/brown structures were observed freely in solution and near haemocytes. Structures are also attached by actin (A-D) (white arrow). Images taken at 1000x. Bars represent 10 µm.

5.4 Discussion

In this chapter, the interactions between *T. molitor* larvae haemocytes and *A. fumigatus* were explored to understand the mechanisms utilised by haemocytes in response to *A. fumigatus*. As shown in Figure 5.2, four types of haemocytes were identified based on shape and size. Of the cells recovered, granulocytes appeared to be the most abundant haemocyte type which was also identified in previous research.[154] Haemocytes have been reported to have different morphologies within the four types due to intermediate cell stages.[160] Identification of haemocyte types can thus be challenging, with haemocyte types observed to have variable function between the different insect species. For example, in the larvae of a Coleopteran, *Carabus lefebvrei* (ground beetle), which utilises the same four haemocyte types as *T. molitor*, plasmatocytes were the main phagocytes in addition to oenocytoids, while granulocytes were non-phagocytic.[240] Distinguishing between haemocyte types is thus important to identify the immune response. Haemocyte types were identified here using Giemsa stain and brightfield microscopy which stained haemocytes light-blue (Figure 5.2). Details within cells were difficult to distinguish however using this stain. Additional staining with May-Grünwald may enhance identification of haemocyte types.[160]

During early interaction assays, *T. molitor* larvae haemocytes and *A. fumigatus* conidia often became associated (Figure 5.4). Haemocytes were commonly observed with filopodia-like structures composed of actin as highlighted by phalloidin staining (Figure 5.8). These observations may thus be representative of early interactions that occur between haemocytes and conidia. *T. molitor* haemocytes in previous studies have been observed to aggregate during interaction with bacteria which despite phagocytosis were localised around haemocytes. In that study,

filopodia became attached to bacteria causing the haemocyte surface area to decrease which was suggested to be for easier migration during infection.[154] Similar observations have been observed in other insects and against different pathogens prior to phagocytosis. This includes in other Coleopteran insects such as *Rhynchophorus ferrugineus* (red palm weevil) during granulocyte interactions with yeast,[244] and *Allomyrina dichotoma* (Japanese rhinoceros beetle) where morphological changes indicated granulocyte activation. This included the presence of filopodia, and granules that increased in size and number.[241] In human alveolar macrophages, filopodia and lysosomes were also early signs of phagocytosis during exposure to *A. fumigatus* conidia.[245]

An unknown substance stained pink/purple by Giemsa was also observed. This was common during interaction assays where it was visible around haemocytes, and directed at *A. fumigatus* conidia. This may thus be part of the early interaction as it occurred alongside haemocytes that were associated with conidia and had filopodia visible (Figure 5.4). In *Melanoplus sanguinipes* (migratory grasshopper), granulocytes were reported to secrete mucopolysaccharide strands that attached to *B. bassiana* conidia which was suspected to be an important early immune response.[246] This may thus have been occurred here however cytochemical stains are necessary determine the identity of the secretion. As granulocytes were the predominant haemocyte, Giemsa may have stained granular components released during degranulation in response to conidia, which is also an important mechanism utilised by neutrophils. Degranulation of polymorphonuclear leukocytes to *A. fumigatus* conidia was observed previously relative to conidia stage as swollen conidia produced more degranulation likely to enhance clearance.[247]

Conidia-sized bulges were observed on the surface of haemocytes were observed after 1.5 hours incubation and onwards (Figure 5.5). The identity of these was unclear, with DAPI and phalloidin staining revealing round actin loops (Figure 5.8). During fluorescent staining of human alveolar macrophages, actin was observed to encompass ingested *A. fumigatus* conidia and form phagocytic cups.[245] In *G. mellonella*, haemocytes that internalised amastigotes during *Leishmania braziliensis* infection had round amastigote projections after 2 hours, sized approximately 1 to 5 µm, bulging on the side that were considered inside the haemocyte.[248] Blebs, which are a sign of cell disintegration, also have a similar appearance. Apoptosis previously induced in *T. molitor* haemocytes altered haemocyte morphology and viability. The aggregation of f-actin was observed as intensely stained foci, and numerous round membrane blebs also observed which disrupted filopodia and resulted in round haemocytes.[249] Intensely stained foci were visible here on haemocytes that had round structures on their surface (Figure 5.8, K), and bleb-like structures (Figure 5.8, B, G-J). Filopodia were also visible however (Figure 5.8, G). Thus, the round bulges on the haemocyte surface may be internalised conidia, or apoptotic bodies resulting from stress or to eliminate damaged cells.[250] A potential source of stress may be *A. fumigatus* gliotoxin which is an important virulence factor that can inhibit human neutrophil phagocytosis and alter the actin cytoskeleton.[171]

Internalisation events suggestive of phagocytosis was observed in several cells which appeared as round structures within vacuoles (Figure 5.6). The ability of *T. molitor* larvae haemocytes to internalise pathogens may have thus been observed, however without proper staining of the inoculum the identity of the contents is unclear. Additional assessments of phagocytosis are thus important using methods such as flow cytometry, confocal microscopy, and using stains for conidia and

vacuolar acidification to identify antimicrobial processes. Haemocytes with round-internalised structures were observed after 3 hours incubation, but also in haemocytes from larvae injured 2 hours prior to haemocyte extraction and after 15 minutes incubation in an interaction assay. As internalisation events were not observed in assays under 30 minutes, internalisation in the latter may have occurred within larvae prior to haemocyte extraction. Phagocytosis has been observed after varied amounts of time. Phagocytosis was reported in *A. dichotoma* granulocytes after 30 minutes exposure to yeast.[241] In neutrophils, *A. fumigatus* conidia phagocytosis was observed after 15 minutes as quantified by flow cytometry and assessed by confocal.[68] In the amoeba, *Protostelium aurantium*, which is a model for macrophages, swollen conidia were taken up after 2 hours.[186]

Additional methodologies will thus be important to assess phagocytosis of *A. fumigatus* conidia by *T. molitor* haemocytes. Inoculating larvae with conidia and then recovering haemocytes after interaction events may also be useful to assess the cellular response as it would occur during more natural conditions within the organism. Lower MOI should also be utilised. In previous research using polymorphonuclear leukocytes, low numbers of *A. fumigatus* conidia per immune cell resulted in a high percentage of ingestion, whereas higher numbers of conidia resulted in immune cell lysis.[247] This may thus also apply to *T. molitor* haemocytes with MOI 10 utilised here. In murine alveolar macrophages, *A. fumigatus* was at MOI 7 and phagocytosis observed after 1 hour.[251] Conidial stage also affects phagocytosis. In *G. mellonella*, *A. fumigatus* virulence was related to conidial progression with resting conidia avirulent unless a high inoculum was utilised. Phagocytosis was also size limited and observed for conidia less than 3 µm in diameter.[131] For larger sized conidia, a different response would thus be required for

clearance. In *Spodoptera littoralis* (cotton leafworm), both phagocytosis and nodulation were observed against entomopathogenic fungi.[252]

Orange/brown structures not stained by DAPI and phalloidin were also observed using brightfield microscopy (Figure 5.9). These were located separate to and around haemocytes; they varied in darkness as some were almost black and others orange. These may be part of the melanisation response utilised by haemocytes. In *Armigeres subalbatus* (mosquito), granulocytes phagocytosed both melanised and un-melanised bacteria including as early as 5 minutes, with the extent of melanisation varied.[230] Melanised bacteria were also located freely in the hemocoel, and phenoloxidase which is involved in melanisation detected in the melanotic capsules.[230] In *T. molitor*, phenoloxidase has been detected in all haemocytes with the strongest reaction in granulocytes which may thus have an important role in melanisation responses.[160] Like phagocytic responses, melanisation has been observed to depend on the pathogen. In a previous study, *Micrococcus luteus* was predominantly melanised while *E. coli* predominantly phagocytosed.[230] Melanisation may thus be an alternative response to phagocytosis when pathogens are too large. *A. fumigatus* conidia observed in the longer interaction assays may have thus been larger conidia that could not be phagocytosed.

Several staining protocols were tested, however further optimisation is required to enable unambiguous observation of interactions between haemocytes and conidia. Haemocytes were stained with DAPI and phalloidin during interaction assays which revealed nuclei and actin, respectively (Figure 5.8). Some haemocytes appeared poorly stained by phalloidin and lacked filopodia. This may be due to exposure to *A. fumigatus*, with *T. molitor* larvae haemocytes observed previously to have reduced filopodia and an altered f-actin cytoskeleton during exposure to *S. aureus*.[154]

Calcofluor white staining was also utilised including in assays with germinated conidia to identify *A. fumigatus* within haemocytes (Figure 5.7). Calcofluor white has been used in a previous study for differential staining of conidia located outside the cell.[251] Calcofluor White staining was not ideal in these experiments as both haemocytes and fungal components were stained and thus did not provide good contrast to identify fungal components from haemocytes. Calcofluor white did appear to enable visibility of granules however at 1000x (Figure 5.7).

Haemocytes utilised here from *T. molitor* larvae were recovered by removing a portion of the first pair of legs as performed previously.[154] Haemocyte density (not presented) and viability (Figure 5.2) varied between experiments. Several factors may have contributed to this variability including the collection of haemolymph. To maximise haemocyte yield, haemolymph was not collected to a set amount as individual larvae differed in haemolymph recovery rate. In other research, the immediate mixing method of haemolymph influenced haemocyte viability with a short duration of vortexing enabling high cell viability.[253] This should be utilised in future haemocyte recovery experiments. An anticoagulant buffer was also not utilised with some clumps evident that contained both haemocytes and conidia. This may have contributed to observations of low haemocyte numbers with haemocytes confirmed within them using DAPI and phalloidin (not presented).

Chapter 6 General Discussion

Invertebrates have become an important means to study infection due to the growing recognition of animal ethics and the need to reduce the use of animals in research.[108] *G. mellonella* larvae, as documented in the literature, is a popular inveterate model to study human pathogens including fungi such as *A. fumigatus*, *C. albicans* and *C. neoformans*.[112] Several benefits have led to its popularity to study infection. This includes their large size compared to other invertebrates such as *D. melanogaster* and without the need for specialised equipment.[112] Furthermore, the ability to tolerate 37°C, which is an important environmental factor involved in virulence in humans and for the study of human pathogens.[143] *T. molitor* larvae can similarly tolerate 37°C, can be managed using non-specialised equipment and can be directly inoculated due to size. They are also relatively cheap with simple dietary requirements and large populations can be purchased in Australia unlike *G. mellonella*. Despite these similar research benefits, *T. molitor* larvae are not widely used for infection research; limited research using this invertebrate has been conducted on some bacterial[174, 175, 179] and fungal pathogens.[138]

In 2015, the potential of *Tenebrio molitor* larvae as an invertebrate host to study human fungal pathogens was reported using two fungal species; *C. albicans* and *C. neoformans*.[138] This prompted testing of *T. molitor* larvae as a model to study *A. fumigatus* pathogenesis. In this project, *A. fumigatus* infection of *T. molitor* larvae was investigated to follow the course of infection in larvae. This further established

the potential of this invertebrate to study fungal pathogenesis. Several methods were utilised to characterise *A. fumigatus* infection of *T. molitor* larvae. Quantitative methods consisting of CFU counts and qPCR were utilised, and qualitative methods including histological examination, MRI and haemocyte interaction assays. These methods have been utilised in other models to understand infection.

6.1 Use of Quantitative Methods to Analyse Infection

Quantitative assessments of fungal biomass were utilised to monitor infection via estimations of the fungal burden over time. The data generated highlighted how CFU counts may not be ideal to study *A. fumigatus* infection in *T. molitor* larvae due to the lack of correlation with survival data. qPCR utilised here correlated with the declining survival data. Results thus suggest qPCR as the better method to monitor *A. fumigatus* infection progression over time in *T. molitor* larvae.

CFU counts and qPCR quantification has been utilised previously to assess *A. fumigatus* infection. qPCR was more sensitive than CFU counts to monitor infection over time. In a rat model of IA, qPCR detected an increase in the fungal burden in lung tissue while CFU counts did not.[199] Similar findings were observed during quantification of the fungal burden in the lungs of guinea pigs.[197] In a mouse model assessing antifungal efficacy on the *A. fumigatus* burden, qPCR better reflected untreated infection but during treatment both methods were found to be effective.[198] Both CFU counts and qPCR may thus be useful to monitor infection progression during treatment. This could be utilised here if testing antifungals as CFU counts are a relatively simple method.

In another study using mice, similar results were observed during quantification of the *A. fumigatus* burden in all five organs. CFU counts only detected a slight

increase compared to qPCR. In contrast, CFU counts and qPCR strongly correlated during infection with *C. albicans*.[196] *A. fumigatus* may thus form hyphae that do not give reliable CFU data compared to yeast. Monitoring *A. fumigatus* infection using CFU counts is not ideal, likely as hyphae are multinucleate structures that may form clumps and invade tissue. Alternative methods were thus investigated here to monitor infection in *T. molitor* larvae.

Isolate virulence was also investigated using *T. molitor* larvae based on survival data and plating of isolates on skim milk agar to assess protease activity. No significant difference was observed between the two isolates based on Kaplan-Meier survival probabilities (Figure 3.3). A slight difference was observed in survival of larvae inoculated with the non-IA isolate, AF03, which appeared to be more virulent in *T. molitor* larvae than the IA isolate, AF11. This result was observed in a previous survival experiment.[183] Results on skim milk agar varied between isolates. Based on clearance diameter, non-IA isolates performed better (Figure 3.9). However, the secretion of proteases according to other research may not be an important virulence factor.[254, 255]

6.2 Use of Qualitative Methods to Analyse Infection

Histological examination was utilised here to characterise *A. fumigatus* infection in tissue. Fungi were observed histologically in a previous study of *T. molitor* larvae tissue, however the method did not retain morphology as only the internal content of larvae were processed.[138] A study by Perdoni et al[234] described whole tissue processing which preserved the morphology of the *G. mellonella* larval host to enable infection to be observed relative to tissue. Whole tissue processing was thus utilised here. Although tissue processing was not optimal and produced blocks of

various qualities due to technical variabilities from manual processing, the data generated using this methodology enabled observation of both fungal and host factors. Although *A. fumigatus* was not directly observed due to lack of fungal specific staining, nodules were observed dispersed throughout tissue which differed in size suggesting fungal dissemination (Figure 4.5). Nodules were identified by regions of melanisation which were accompanied by cellular aggregation.

Nodulation is a haemocyte-mediated response characterised by haemocyte aggregation, pathogen entrapment and melanisation. Nodulation is used in response to large numbers of small pathogens while encapsulation is utilised against pathogens too large to be phagocytosed.[242] Nodules have been reported previously in *G. mellonella* inoculated with *A. fumigatus* conidia. Hyphae and conidia were identified within nodules that were accompanied by cellular infiltrates by using GMS staining of whole larvae tissue and Calcofluor White staining of dissected tissue.[132, 204]

Choice of stain is thus a key part of histological examinations to characterise infection. By using different stains,[234] insights into host-pathogen interactions can be enhanced. In histologically processed mouse lung tissue infected with *A. fumigatus*, quantification of the infection burden was possible using GMS and an image analysis program enabling comparisons of the relative fungal burden of experimental groups.[256] Future examinations of *A. fumigatus* infection of *T. molitor* larvae tissue should include GMS for fungal elements useful during infection of insect[138, 204] and mammalian[256, 257] tissue. To observe host responses, stains such as Giemsa may be useful by staining haemocytes.[234] Recovering quality tissue sections are important to enable a spatial understanding of infection.

As manual tissue processing often resulted in suboptimal sections, MRI was tested as an alternative methodology to investigate *A. fumigatus* infection of *T. molitor* larvae tissue. Infection was not identified during initial optimisation experiments. MRI scans are useful for diagnosis of infectious diseases such as in humans based on observations of tissue responses.[237] Larvae are significantly smaller and so changes in tissue, especially during early infection, would be microscopic. Thus, MRI may not be sensitive enough to characterise infection in *T. molitor* larval tissue. However, it has been shown in mouse tissue that labelling of bacteria can enable identification of small bacterial colonies and information on host morphology and inflammation.[239] MRI may thus be a useful method, but it will need significant experimental optimisation.

6.3 Haemocyte Interaction Assays

Haemocyte extraction was utilised to look at finer scale host-pathogen interactions that occur within larvae *in-vitro*. Immune cells are commonly isolated from human volunteers for *in-vitro* experiments including for studying phagocytosis of *A. fumigatus*.[68, 258] Early interactions were observed between haemocytes and conidia. These were observed to associate with haemocytes of which some had filopodia that are made of actin as revealed by phalloidin staining (Figure 5.8). These have been identified in previous research as part of the early haemocyte response.[154, 244] Haemocytes also appeared to produce Giemsa stained secretions. During early interactions of haemocytes from a grasshopper, *Melanoplus sanguinipes*, with *B. bassiana* conidia, haemocytes that adhered to conidia secreted acid mucopolysaccharide likely for entrapment of invading fungi.[246] In addition, melanised particles were also observed in that study.[246] Melanised structures were observed in this study during longer assays using brightfield microscopy (Figure 5.9). These

appeared conidia-like based on shape and size. Melanisation in previous research was found to encapsulate *Escherichia coli*, some of which were identified within phagocytic cells.[230] Melanisation appears to be a key response against pathogens. Additional research is important to confirm interactions that occur between *T. molitor* larvae haemocytes and *A. fumigatus*. Preliminary data was presented here with the method requiring further optimisation and methods. This includes the application of stains for *A. fumigatus* elements, confocal microscopy, and flow cytometry to measure phagocytosis.

Of the methods utilised to characterise *A. fumigatus* infection of *T. molitor* larvae, histological examination of tissue appeared most suitable for monitoring infection. Histological preparation of tissue enabled interactions between fungi and host tissue to be observed after a set time. This method does require the preparation of many individuals should you want to investigate interactions that occur over time. As demonstrated by Perdoni et al,[234] different stains can be utilised to highlight the different components of tissue which differed between a non-biofilm and biofilm *C. albicans* isolates. Although fungal specific staining was not utilised here, infection could still be visualised using H & E based on the host response. GMS should ideally be utilised in future. Should manual tissue processing be utilised, further optimisations are required such as prior to embedding incorporating a 1:1 solution of clearing solution and wax [259] to attempt to remedy the holes present within samples. Ensuring wax remains molten also appeared important during embedding for the quality of the block as it often set too quickly. This may have accounted for the varied quality of tissue blocks. Returning wax filled moulds to the oven for a longer duration should be utilised in future experiments. Furthermore, as the samples were processed in portions, only the thorax was identifiable based on the legs whilst those

containing the abdomen appeared similar. Pre-embedding divided samples in an agar medium should be utilised post-fixation in future experiments. This would ensure that the orientation and arrangement of tissue can be preserved for microscopic analysis.[236]

Chapter 7 Conclusion

In this study, several methods were utilised to characterise *A. fumigatus* infection of *T. molitor* larvae to establish this invertebrate as a host to study *A. fumigatus* pathogenesis. Based on qPCR quantification, the fungal burden was observed to increase over time while CFU counts revealed a decrease in fungal biomass which may be due to hyphal formation. Histological examination of inoculated tissue, although did not confirm the presence of *A. fumigatus*, enabled observation of fungal-like elements located within melanised nodules. These nodules were surrounded by cells suspected to be haemocytes, which during interaction assays became activated during exposure to *A. fumigatus*. MRI was also investigated however infection was not observed. *A. fumigatus* infection was thus suggested however, confirmation is necessary and the further optimisation of methods to monitor infection progression in *T. molitor* larvae. This includes using fungal stains such as GMS to identify *A. fumigatus* within histological sections, confocal microscopy, and flow cytometry to investigate phagocytosis.

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