An analysis of the interaction of environmental and clinical isolates of *Aspergillus fumigatus* with vertebrate and invertebrate immune responses

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Declaration of Authorship

This thesis has not previously been submitted in part to this or any other university and is the sole work of the author.

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Oral Presentations

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Investigating the disease causing potential of environmental isolates of *Aspergillus fumigatus* using *Galleria mellonella* larvae. Departmental seminar, 4th June 2008.

Poster Presentations

Exposure of neutrophils to fumagillin leads to a reduction in NADPH oxidase complex formation and degranulation. 4th Advances against Aspergillosis, Rome, February 4-6th 2010.

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An assessment of the virulence of environmental isolates of *Aspergillus fumigatus* using *Galleria mellonella* larvae. SGM meeting, NUI Galway March 27th - 28th 2008.

Alterations in the humoral profile of *Galleria mellonella* mediates protection against infection with *Aspergillus fumigatus* SGM Autumn meeting, September 1st-2nd 2010.

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Abstract

The ubiquitous saprophytic filamentous fungus *Aspergillus fumigatus* is a significant pathogen among individuals undergoing chemotherapy, allogenic stem cell transplantation and in patients with underlying pulmonary conditions or chronic granulomatous disease patients. Central to the virulence of *A. fumigatus* is the production of various secondary metabolites, proteolytic enzymes and iron sequestering molecules which coupled to the rapidly growing nature of the fungus aid its persistence in host tissue. Assessment of the virulence of *A. fumigatus* isolates has previously centred on the use of murine models of aspergillosis however in recent years there has been an increasing body of evidence suggesting the use of invertebrates as a viable alternative.

The work presented here sought to further develop the use of the Greater wax moth *Galleria mellonella* as a novel *in vivo* tool to assess the pathogenicity of *A. fumigatus* isolates as an alternative to murine models of infection. The data presented here characterises the pathogenicity of clinical and environmental isolates of *A. fumigatus* in *G. mellonella*. Results here suggest that mycotoxins are produced at varying levels in the *A. fumigatus* isolates with culture filtrates demonstrating immunosuppressive properties. *In vivo* mycotoxin production by *A. fumigatus* ATCC 26933 in *G. mellonella* was characterised and demonstrated that high quantities of fumagillin were produced in the first 24 hours of invasive infection.

A study was performed to assess the effect of fumagillin on human neutrophils and *G. mellonella* haemocytes. Fumagillin inhibited phagocytosis and pathogen directed killing in both cell types and disrupted oxygen consumption through reduced translocation of p47^{phox} and its insect homologue. Exposure of neutrophils and haemocytes to fumagillin also disrupted degranulation. Experimental evidence presented here suggests that the inhibition to cellular processes is mediated by reduced

F-actin assembly. A proteomic analysis of *G. mellonella* haemolymph revealed evidence to suggest that fumagillin mediates an oxidative stress *in vivo* and disrupted the humoral immune response.

Recent literature has suggested that the immune system of *G. mellonella* could be primed to subsequent infection. Larvae primed with specific sub-lethal inocula of conidia demonstrated improved resistance to subsequent infection through increased haemocyte density, protein activity, and synthesis of proteins involved in oxygen transport, pathogen recognition and iron sequesterisation and increased expression of antimicrobial peptides.

This project has further developed the *G. mellonella* model as a model system to investigate the pathogenicity of *A. fumigatus* isolates and the immunomodulatory effects of fungal secondary metabolites due to an immune system which shows a high level of conservation with the human innate immune system.

Chapter 1

Introduction

1.1 *Aspergillus fumigatus* lifecycle.

Aspergillus fumigatus is an ubiquitous, saprophytic filamentous fungus whose primary ecological niches are soil and decaying vegetation. As a result it is a common element of air-borne microflora (Tekaia and Latgé, 2005). Currently there are over 180 identified *Aspergillus* species, with *A. fumigatus* (Figure 1.1 A) occurring as the most common mould pathogen in humans (Mabey *et al*., 2004). Other members of this genus include *A. nidulans*, *A. oryzae*, *A. niger*, *A. terreus* and *A. flavus.* These species are used as model organisms in molecular and cellular biology (*A. nidulans*), for commercial production in the biotechnology industry (*A. terreus*, *A. niger*, *A. oryzae*), or implicated in aflotoxin contamination of food in developing countries (*A. flavus, A. parasiticus*), (MacCabe *et al*., 2001; Adrio and Demain, 2003; Rashid *et al*., 2008; Andleeb *et al*., 2010; Zambare, 2010). Although *A. flavus*, *A. niger*, and *A. terreus* have been recorded as contributors to fungal pathogenesis, the most pathogenic species is *A. fumigatus* and it is responsible for over 90% of cases of invasive diseases caused by *Aspergilli*, (Thompson and Patterson, 2008). *A. fumigatus* is a major component in compost and due to its thermophilic nature is capable of growing below 20ºC and above 50ºC (Bhabhra and Askew, 2005). The primary function of *A. fumigatus* is the recycling of carbon and nitrogen in the environment and the physiological versatility to assimilate various sources of these elements is central to its persistence in the environment and pathogenicity within host tissue (Rhodes, 2006).

The life cycle of *A. fumigatus* begins with the asexual production of conidia (Figure 1.1 B and C) which measure 2-3µm in diameter and are easily dispersed by air thus contributing to the ubiquitous nature of *A. fumigatus* (Morris *et al*., 2000). The surface of *A. fumigatus* conidia is covered in hydrophobic rodlets (RodAp and RodBp) which enhance the buoyancy and distribution of conidia by air currents (Paris *et al*., 2003). Conidia may break dormancy and germinate upon exposure to appropriate nutrients. *A.*

fumigatus conidia switch from isotropic to polar growth concurrent with the emergence of germ tubes and septation with mitotic division (Momany and Taylor, 2000). The development of asexual conidia in *A. fumigatus* is highly regulated. Mah and Yu, (2006) demonstrated that conidiation in *A. fumigatus* and *A. nidulans* is similarly activated by gene homologues therefore underlying conservation of development in both *Aspergilli,* however it was shown that *A. fumigatus* possesses distinct regulatory mechanisms controlling asexual development. In recent years a sexual cycle for *A. fumigatus* has been established by O'Gorman *et al*., (2009) where "mating-type" (MAT) genes are responsible for the heterothallic breeding system leading to the production of cleistothecia and ascospores. Szewczyk and Krappmann, (2010) further characterised this development in understanding the life cycle of *A. fumigatus* through the verification of MAT variants as elicitors of the sexual cycle in unrelated clinical isolates. O' Gorman *et al*., (2009) annotated this sexual stage as *Neosartorya fumigata* and it is speculated that this development in the life cycle of *A. fumigatus* may play a role in virulence, resistance to harsh environmental conditions and the development of fungicide resistance.

1.2 Environmental exposure to *A. fumigatus*.

A study undertaken by Guinea *et al*., (2006) indicated that the presence of *Aspergillus* conidia in the air was dependent upon meteorological parameters such as temperature, rainfall and windspeed however *A. fumigatus* appears to be affected the least by these factors. This study quantified the prevalence of *Aspergillus* colony forming units (c.f.u) in the air with the greatest numbers being recorded in the Autumn with *A. fumigatus* comprising of 54% of *Aspergilli*. Interestingly quantification of the number of conidia in air reached levels of 70 c.f.u $(m^3)^{-1}$ during the Autumn which would coincide with increasing quantities of decaying vegetation. A more recent study

based in Delhi concerned with the quantification of *Aspergillus* species in air verified that aerial counts of *A. fumigatus* is dependent upon the time of year and varied from 1.25 to 15.6 c.f.u $(m^3)^{-1}$ with the prevalence of allergen content highest during the month of August (Vermani *et al*., 2010). While both studies demonstrate the ubiquity of *A. fumigatus* in air samples it is clear that the concentration of viable aerosolised conidia varies depending on geographical location, climate, decaying vegetation and other anthropocentric factors such as urbanisation and pollution.

The term "Sick Building Syndrome" has been used for over 25 years to describe illness where occupants of a particular building experience acute health complications as a result of spending time exposed to the micro-flora present indoors (Terr, 2009). The occurrence of water intrusion by means of flooding, leaks, condensation and poor insulation may contribute to the growth and persistence of moulds such as *A. fumigatus* in an indoor environment (Figure 1.2 B). Moulds such as *A. fumigatus* are capable of growth on surfaces as well as more concealed areas such as attics, sub-flooring, carpets and behind wallpaper. The saprophytic nature of *A. fumigatus* confers an ability to grow on materials which contain carbohydrates such as wood, plasterboard and wallpaper thus in conjunction with the availability of water and the thermotolerant nature of the fungus the rapid growth of *A. fumigatus* is possible (Li and Yang, 2004). Recent studies of flood damage have documented the increased prevalence of *Aspergillus, Penicilium* and *Paecilomyces* contamination in water damaged buildings in the aftermath of domestic flood damage caused by Hurricane Katrina (Chew *et al*., 2006). In this study the authors demonstrated levels of culturable mould from bioaerosol analysis ranging from 22,000 to 515,000 c.f.u $(m^3)^{-1}$ with spore counts ranging from 82,000 to 630,000 spores $(m^3)^{-1}$. The prevalence of *A. fumigatus* has been documented in hospitals (Warris *et al*., 2001; Panagopoulou *et al*., 2002; Smedbold *et al*., 2002; Falvey and Streifel, 2007). A recent article by Pini *et al*., (2008) identified the influence of hospital
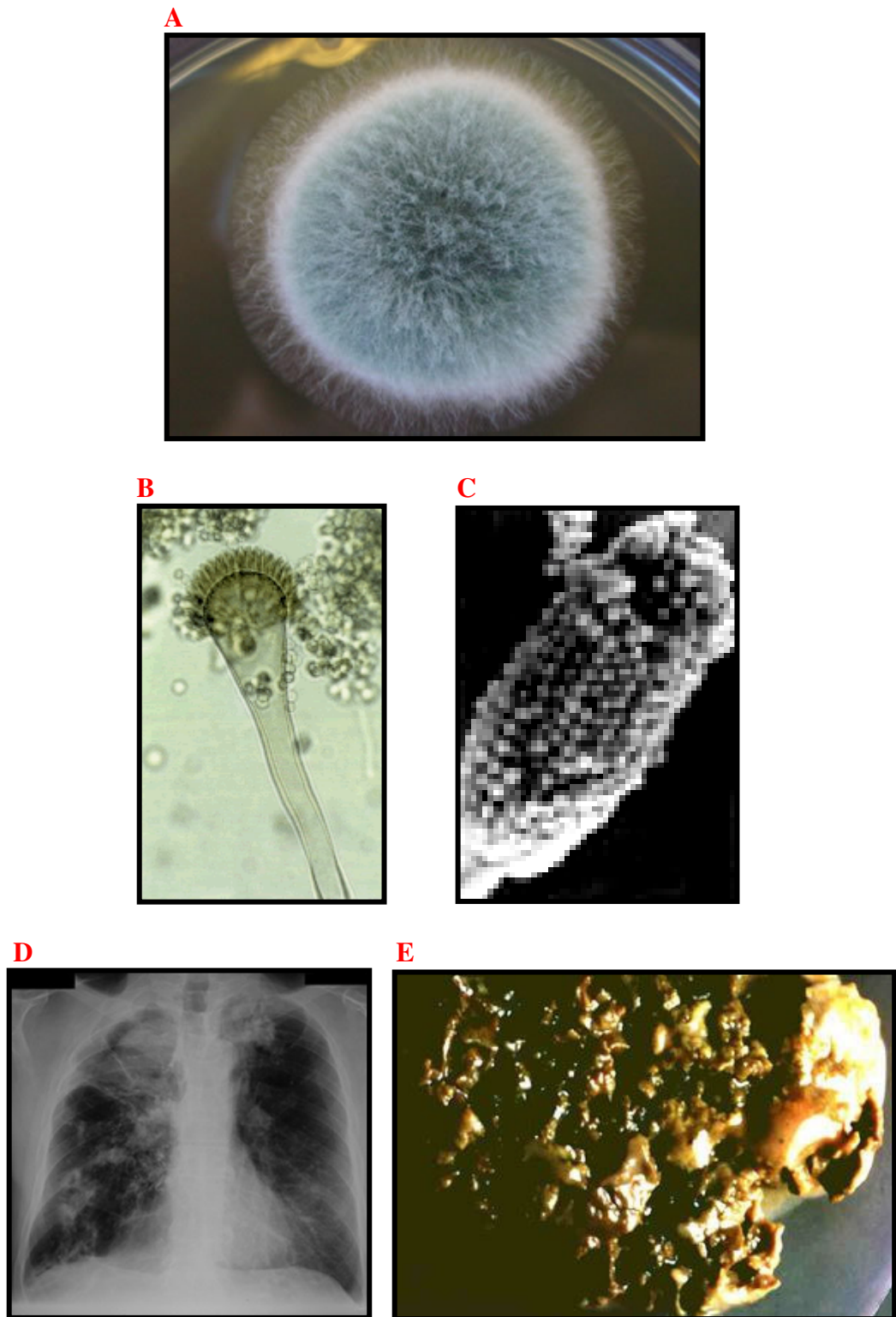
Figure 1.1 *A. fumigatus* **and the development of Aspergilloma.**
A. fumigatus grown on solid medium **A**, image of conidiophore **B**, and conidiophore with conidia dispersing **C**. *A. fumigatus* grows in a hyphal (vegetative) manner with the asexual production of conidia from the conidiophore which are wind dispersed. X-Ray Image of chest indicating the presence of fungal invasion **D**, and an aspergilloma removed from a lung by surgical excision **E**.

Images: www.aspergillus.org.uk

A
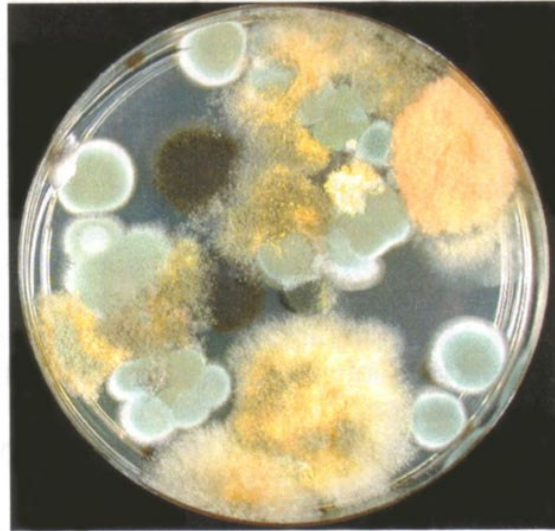
B

Figure 1.2 Sick Building Syndrome.
Fungal isolates are omnipresent in household dust **A**, Images from a water damaged house following flood damage **B**. The red arrow indicates extensive fungal growth on cellulose containing materials

Images: www.aspergillus.org.uk

renovation and construction work as a key factor in the prevalence of *A. fumigatus* in a nosocomial setting. It was found that cases of aspergillosis in the hallway of a haematology unit increased during a period of intense renovation work recording values of 2.98 cfu $(m^3)^{-1}$. Interestingly the values of 1.42 c.f.u $(m^3)^{-1}$ were recorded outside the hospital building further underlining the importance of regular surveillance of hospital units during periods of renovation. A subsequent study by Brenier-Pinchart *et al*., (2009) identified a correlation between high outdoor temperature and humidity with the occurrence of fungal flora in hospital wards. The requirement for particulate air filtration in high risk units coupled to partial air control and standard hygiene protocols in low risk wards as a means of controlling the level of fungal contamination was also emphasised.

The distribution of *A. fumigatus* conidia is not limited to aerosolisation. Several cases of *A. fumigatus* and other filamentous fungi have been reported in public water supplies and in hospital water systems thus confirming the widespread distribution and omnipresent nature of conidia (Gottlich *et al*., 2002; Warris *et al*., 2002). Warris *et al*., (2002) demonstrated the absence of mould contamination in hospital ground water supplies but high levels of fungal contamination were present in surface water. A more recent report of microbial contamination in a Belgian hospital showed that 55% of samples recovered from the water distribution system demonstrated fungal contamination with *Aspergillus* species present in 6% of samples. Interestingly *A. fumigatus* comprised 66.6% of *Aspergillus* isolates (Hayette *et al*., 2010). Warris *et al*., (2003) used genotypic analysis to demonstrate that *Aspergillus* isolated from patients could originate from water of airborne source with aerosolisation of water borne conidia speculated as a major source of exposure to patients. Anaissie *et al*., (2002) recommended minimal exposure of high risk patients to contaminated aerosolised water such as that resulting from showers as a means of restricting exposure. A subsequent

study has identified the application of effective "point of use" filtration in water systems as a potential measure to reduce the risk of filamentous fungi exposure for patients (Warris *et al.*, 2010). The beneficial implications of such measures to human health remain to be fully understood due to the cost and logistical implications of implementing such standard measures in hospital washrooms as filters would need to changed on a regular basis thus giving rise to the need for standard protocols for use.

1.3 Aspergillosis; routes of infection and clinical manifestations.

The successful colonisation by *A. fumigatus* of host tissue usually depends on the immunological status of the host. Successful infection relies on the presence of underlying factors such as existing pulmonary disorders, corticosteroid use, HIV infection, neutropenia, organ transplantation, allogenic stem cell transplantation or aggressive chemotherapy. Recent evidence suggests a role for single nucleotide polymorphisms present in affected hosts as a possible factor in predisposing patients to *A. fumigatus* infection (Mezger *et al.*, 2010). The most common region of *A. fumigatus* infection is pulmonary tissue however other tissues including skin, peritoneum, kidneys, eyes, bones, joints and the gastrointestinal tract have been reported as infected regions (Latgé, 1999; Bonfante *et al.*, 2005; Eggiman *et al.*, 2006; Tempkin *et al.*, 2006; Camus *et al.*, 2010; Golmia *et al.*, 2010). The size of airborne *A. fumigatus* conidia enables access to the lower regions of the respiratory tract (Paris *et al.*, 1997). In contrast larger conidia such as those of *A. flavus* and *A. niger* are less capable of such penetration (Ben-Ami *et al.*, 2010). In the majority of cases healthy immunocompetent individuals are not adversely affected by the presence of *A. fumigatus* due to the presence of a highly effective innate immune system (Latgé, 1999). In cases of infection the oxygenated nature of the lung coupled to the natural body temperature of 37ºC serves as an ideal environment for the growth of *A. fumigatus* with the fast growth rates associated with

more pathogenic strains (Paisly *et al*., 2005). Depending on the underlying health of affected individuals, as a result of the aforementioned factors affecting susceptibility to infection, three types of infection usually occur. These are allergic bronchopulmonary aspergillosis, aspergilloma and invasive aspergillosis.

1.3.1 Allergic Bronchopulmonary Aspergillosis (ABPA).

ABPA occurs as a chronic lung condition in non-immunocompromised individuals resulting from hypersensitivity to *A. fumigatus* allergens which manifest as poor mucocilary clearance, bronchial obstruction and mucoid impactions (Greenberger, 2002). The underlying predisposing conditions of ABPA are asthma and cystic fibrosis with 13% of the latter population affected by the fungus (Kraemer *et al*., 2006). The inhalation of spores from the environment stimulates an immune response involving Th2 CD4+ T-cells and IgE and IgG antibodies (Tillie-Leblond and Tonnel, 2005). It has been hypothesised that the abnormal nature of mucus in cystic fibrosis patients may promote the trapping of *A. fumigatus* in airways allowing for the growth of mycelia and further pulmonary colonisation by the fungus which in turn may promote T-helper cell responses which favour the development of ABPA (Kraemer *et al*., 2006). The activation of Th2 cells and resulting production of cytokines (Interleukin [IL] 4, 5 and 13) result in the synthesis of Ig E, mast cell degranulation and eosinophil responses. In clinical settings ABPA may be diagnosed through a combination of *Aspergillus* antigen reactivity skin tests, elevated Ig E, serum reactivity against *A. fumigatus*, eosinophil counts exceeding 1000 cell μl^{-1}, positive identification through sputum culturing and radiological imaging however many of these tests are non-specific therefore making the condition more difficult to treat (Agarwal, 2009).

The use of oral corticosteroids has been highlighted as a means of treating ABPA by suppressing the immune system and reducing levels of serum IgE. In recent

years the therapeutic use of an anti-Ig E antibody (omalizumab) has demonstrated success in terms of restoration of pulmonary function by reducing serum Ig E levels (van der Ent *et al*., 2007; Kanu and Patel, 2008). Recent reports using aerolised amphotericin B, voriconazole or itraconazole demonstrate the benefits of administering antifungal therapy as a viable replacement to the use of corticosteroids in ABPA patients (Hilliard *et al*., 2005; Seiberling and Wormaid, 2009; Hayes *et al*., 2010).

1.3.2 Aspergilloma.

Pulmonary aspergilloma (mycetoma) are roughly spherical mobile fungal balls in a pre-existing lung cavity. Aspergillomas consist of tangled hyphal growth mixed with mucous and cellular debris within cavities which are usually fibrotic (Gefter, 1992; Franquet *et al*., 2001). The development of a pulmonary aspergilloma represents a significant problem in terms of resulting mortality due to the subsequent development of invasive aspergillosis. Other species such as *Zygomycetes* and *Fusarium* are also implicated in the development of fungal balls however *A. fumigatus* is the most common constituent (Zmeili and Soubani, 2007). The pre-existence of underlying pulmonary complications is associated with the condition with tuberculosis the most common (Kawamura *et al*., 2000). The fungal ball is capable of moving within cavities however without invading surrounding lung tissue or blood vessels. The development of haemoptysis as a result of aspergilloma has been linked to local invasion of blood vessels or mechanical irritation caused by the movement of the fungal ball (Zmeili and Soubani, 2007). In some cases the occurrence of haemoptysis may be fatal (Goreelik *et al*., 2000; Sagan *et al*., 2010). Diagnosis is usually possible by chest radiography (Kern and Lopert, 2010) or CT scan (Greene, 2005). The treatment of pulmonary aspergillosis is a challenging issue with surgical removal of the fungal ball (Figure 1.1 E) proving invasive to the patient and may result in mortality. In spite of this surgical removal has

demonstrated success in the treatment of aspergilloma (Sagan and Gozdziuk, 2010). Antifungal therapy for the treatment of aspergilloma has previously focussed on the use of amphotericin B, itraconazole or voriconazole however the use of these treatments is complicated by nephrotoxicity, severe reactions to therapy or drug resistance (Sagan *et al*., 2010).

1.3.3 Invasive Aspergillosis (IA).

Invasive aspergillosis is a severe life threatening condition which commonly affects immunocompromised individuals undergoing chemotherapy, haematopoietic stem cell transplantation, corticosteroid therapy, HIV patients or individuals with chronic granulomatous disease (CGD) with mortality rates ranging from 40% to 90% depending on the immunological status of the host. Invasive aspergillosis occurs with the invasion of *A. fumigatus* hyphae into surrounding tissue where it may disseminate and spread to other organs (Dagenais and Keller, 2009). The occurrence of invasive aspergillosis in neutropenic individuals further highlights the role of neutrophils in host protection. The clinical manifestation of invasive aspergillosis is usually characterised by chest pain, coughing and haemoptysis however other symptoms such as weight loss and fever may also occur (Tomee and van der Werf, 2001).

The development of novel, high specificity diagnostic methods to detect the development of invasive aspergillosis is critical to the administration of appropriate chemotherapy and improvement of patient health. Currently positive identification of galactomannan and β 1,3-glucan by ELISA from patient sera is regarded a reliable means to detect the development of IA (Chen and Kontoyiannis, 2010). The immunodetection of Aspf1 has also been commented upon as a useful means to detect IA in serological testing (Thornton, 2010). PCR based identification of I.A has focussed on the amplification of the 18s rRNA which is largely conserved in *Aspergilli*.

Antifungal treatment of invasive aspergillosis has previously centred on the use of amphotericin B (AmB) and its lipid derivatives however its limited efficacy and financial cost has limited its use. Furthermore the nephrotoxic effects of AmB have been documented with studies demonstrating rates of renal failure as high as 30% in patients (Bates *et al*., 2001). In recent years the use of itraconazole, voriconazole and posaconazole has been evaluated with voriconazole proving to be the recommended therapy for most patients due to efficacy and reduced toxicity (Steinbach and Stevens, 2003). A recent report by Bueid *et al*., (2010) highlights issues surrounding the use of azole resistance. In this study the authors report a growing shift towards itraconazole and posaconazole resistence with isolates still largely susceptible to voriconazole. Verweij *et al*., (2009) highlighted a potential resistance mechanism where genes encoding for a drug target protein *cyp51A* demonstrate a single mutation leading to reduced fungal susceptibility. The use of fungicides in the environment was identified as a selective agent in this case. Beuid *et al*., (2010) identified that 43% of azole resistant isolates carried the *cyp51A* mutation. It follows that other mechanisms of azole resistance may be developing.

The use of the echinocandin drugs caspofungin, micafungin, and anidulafungin have also been demonstrated as a combination therapy with azole antifungals. Initially caspofungin received regulatory approval in clinical trials however subsequent studies have endorsed the use of micafungin and anidulafungin. The echinocandins inhibit hyphal growth and demonstrate limited toxicity thus highlighting their utility in anti-fungal treatment (Patterson, 2006, Sable *et al*., 2008).

1.4 Immune responses to *A. fumigatus*.

The first line of defence against *A. fumigatus* is the anatomical barrier of the host. The initial ciliary action of the tracheal epithelium which is lined with mucus,

proteins, lipids, ions and water eliminates the majority of conidia (Latgé, 2001). The presence of hydrophilic surfactant proteins SP-A and SP-D (calcium dependent lectins secreted by type II pneumocytes) support this arm of the immune response by binding to conidial surface carbohydrates, enhancing agglutination and phagocytosis by alveolar macrophages and modifying cytokine release (Wright, 2005). Complement is activated on the conidial surface and results in the cleavage of C3. The cleavage products of this activation serve to opsonise conidia for phagocytosis by neutrophils and macrophages (Behnsen *et al*., 2008). The initial cellular immune response to *A. fumigatus* conidia is performed by macrophages. The phagocytosis of conidia is mediated by pattern recognition receptors (PRRs) on host cells. Furthermore the production of chemokines and proinflammatory cytokines TNF-α, IL-1β, IL-6, IL-8, macrophage inflammatory protein 1α and monocytes chemoattractant protein 1 results from engagement of PRRs with *A. fumigatus* (Dagenais and Keller, 2009). Conidia are rapidly phagocytosed by alveolar macrophages and destroyed within intracellular phagosomes through reactive oxygen intermediate production mediated by NADPH oxidase formation and the phagosome acidification with the effect of conidial killing six hours post-phagocytosis (Phillipe *et al*., 2003). Although previous studies implicated the primary role of neutrophils in the immune response to swollen and germinated conidia Bonnett *et al.,* (2006) demonstrated a role for neutrophils in the innate immune response to conidia. In that study BALB/c mice intranasally challenged with *A. fumigatus* conidia demonstrated significantly greater neutrophil recruitment coupled to the formation of "oxidase-active" aggregates with conidia than mice with impaired neutrophil function and recruitment. Zarember *et al*., (2007) demonstrated that lactoferrin degranulated by neutrophils contributes to the immune response by sequestering iron from the fungus thus ameliorating the local immune response. In addition to these processes serotonin stored

in the dense granules of platelets is released upon attachment with conidia with the effect of demonstrating fungicidal effects (Perkhofer *et al*., 2007).

The acquired immune response has also been implicated in protection against fungal pathogenesis. Following the maturation of dendritic and other antigen presenting cells Th1 cells become activated as evidenced by increased interferon-gamma production which inhibits the Th2 response of ABPA (Rivera *et al*., 2005). Interestingly the activation of Th1 cells appears to correlate with high concentrations of antigen detection while a Th2 response occurs in response to a low concentration therefore acting as a biomarker for the level of fungal pathogenesis within host tissue (Hosken *et al*., 1995).

1.5 Virulence factors of *A. fumigatus.*

The success of *A. fumigatus* is due in part to several factors which create a more favourable environment for fungal growth, evading host immune responses and restricting immune function.

The surface of *A. fumigatus* conidia is largely comprised of melanin. Melanin is a polymeric polyketide and forms a dense layer on the surface of conidia. Jahn *et al*., (1997) demonstrated that conidia lacking in melanin production demonstrated increased susceptibility to oxidative stress and killing by human monocytes *in vitro* coupled to attenuated virulence in a murine model. In addition the surface of conidia is covered in a thin layer of rodlets which are composed of hydrophobin proteins. These proteins are characterised as being small and moderately hydrophobic and demonstrate conserved spacing of eight cysteine residues (Wösten *et al*., 2000). Hydrophobins enhance the airborne dispersal of conidia. In *A. fumigatus* two hydrophobins encoded by *rodA* and *rodB* encode two homologous proteins. RodAp functions in the formation of rodlets with Δ*rodA* mutant strains demonstrating a granular structure and enhanced

susceptibility to oxidative stress and alveolar macrophage killing. In contrast RodBp functions in the formation of cell wall structure and modulates the surface properties of ∆r*odA* mutants (Paris *et al*., 2003). In addition to this, pathogenic species of *A. fumigatus* demonstrate sialic acid residues on the conidial surfaces through binding to fibrinogen and laminin has also been postulated as a virulence factor by enhancing attachment to the alveolar epithelium and previously damaged regions of the lung (Wasylnka *et al*., 2001; Dagenais and Keller, 2009). The composition of mycotoxins and other secondary metabolites on the surface of *A. fumigatus* has also received attention. A study by Hobson, (2000) demonstrated that conidial diffusates from *A. fumigatus* were capable of inhibiting neutrophil phagocytosis as evidenced by reduced pathogen phagocytosis. This work had been preceded by Mitchell *et al*., (1997) who characterised the presence of a water soluble component of the conidial surface of low molecular weight (<10 kDa) which disrupted superoxide production in neutrophils. Subsequent work has identified the presence of verruculogen, a tremorgenic mycotoxin on the conidial surface which decreased transepithelial electrical resistance and polarisation of the epithelium, (Khoufache *et al*., 2007). Panaccione and Coyle, (2005) describe the detection of four ergot alkaloids, fumigaclavine C, festuclavine, fumigaclavine A and fumigclavine B associated with conidial bodies. Ergot alkaloids have been demonstrated to interact with monoamine receptors thus negatively impacting on cardiovascular, reproductive, nervous and immune systems.

Upon germination *A. fumigatus* produces and secretes a variety of proteases which aid in the exacerbation of fungal pathogenesis by degrading extracellular protein and assisting in the scavenging of nutrients. The work of Kothary *et al*., (1984) demonstrated that elastase producing strains were more virulent in mice than non-elastase producing strains. This finding was further supported by evidence of advanced levels of tissue damage and hyphal development *in vivo*. An analysis of the elastase

activity index (EAI) of *A. fumigatus* isolates clearly demonstrates a strong correlation with pathogenicity. Interestingly this study demonstrated the presence of significant elastase activity in 30.9% of environmental *A. fumigatus* isolates and 96.9% of clinical strains. This feature highlights the issues surrounding elastase activity but also underlines the potential of environmental isolates to exhibit significant elastase activity (Blanco *et al*., 2002). In contrast, Alp and Arikan, (2008) found that there was not a direct correlation with the invasiveness of individual isolates and elastolytic activity therefore highlighting the need to fully understand the role of elastase and methods for detecting its production by *A. fumigatus* isolates.

As previously mentioned the activation of the complement pathway plays a key role in the protection of the alveolar epithelium against *A. fumigatus* (Section 1.3). A recent article by Behnsen *et al*., (2010) described the role of an *A. fumigatus* protease Alp1 which was found to degrade complement proteins C3, C4, C5 and C1q as well as immunoglobulin G. A Δ*alp1* strain was found to be incapable of degrading these components therefore highlighting this protein as a virulence factor in the initial pathogenesis of *A. fumigatus*. Phospholipase activity has also been characterised in *A. fumigatus* isolates as a means of degrading phospholipids thus leading to cell death. Birch *et al*., (2004) demonstrated that clinical *A. fumigatus* isolates (n = 53) produced 2.5 fold more phospholipase C activity than environmental isolates (n = 11). In contrast, environmental isolates produced higher levels of phospholipid acyl hydrolase activity. The total phospholipase activity was correlated with the advanced production of phospholipase C by clinical isolates thus highlighting this facet of the host tissue degradation in the secretome of *A. fumigatus*. The results of Alp and Arikan, (2008) strengthen the view that phospholipase activity is correlated with virulence as clinical isolates implicated in cases of invasive aspergillosis caused by *A. fumigatus* demonstrated high phospholipase activity.

The sequesterisation of iron is an important component is the pathogenesis of *A. fumigatus* as iron acts as a cofactor in enzymes and catalyses electron transport. *A. fumigatus* acquires iron through ferrous iron uptake, reductive iron assimilation and siderophores mediated iron uptake. Siderophores are of particular interest as they are produced in the presence of low levels of iron. Triacetylfusarine C functions to capture extracellular iron while ferricrocin and hydroxyferricrocin function in the storage of iron in conidia and hyphae, respectively (Haas, 2003; Schrettl *et al*., 2004; Haas *et al*., 2008). A deletion in the *sidA* gene resulted in the loss of siderophore synthesis coupled to reduced conidial germination and increased susceptibility to oxidative stress (Schrettl *et al*., 2007). In addition Seifert *et al*., (2008) demonstrated that siderophore production by *A. fumigatus* caused increased iron retention and ferritin synthesis in macrophage cell lines therefore placing a stress on the host innate cellular immune response.

1.6 Mycotoxin production by *A. fumigatus*.

The production of mycotoxins has been identified as a key facet in the modulation of the host immune system during fungal pathogenesis. *Aspergillus fumigatus* has been demonstrated to produce a range of secondary metabolites/mycotoxins with different harmful effects described. These include gliotoxin, fumagillin, helvolic acid, fumitremorgin, fumigaclavine C, aureperone C, restrictocin (Kamei and Wanatabe, 2005; Dagenais and Keller, 2009).

Fumagillin, (Figure 1.3) a cyclohexane derivative is produced by *A. fumigatus* upon hyphal development (Mitchell *et al*., 1997; Kamei and Watanabe, 2005). Fumagillin has been previously characterised as amoebicidal (Mc Cowen *et al*., 1951). Fumagillin has been identified as an inhibitor of angiogenesis through the covalent interaction of methionine aminopeptidase-2 (MetAP-2) a feature which is shared with its drug target analogue TNP-470 (Ingber *et al*., 1990). Fumagillin has been

demonstrated to retard the ciliary beat frequency of pulmonary epithelial cells at concentrations of 10 µg ml^{-1} however the physiological relevance of this concentration in terms of naturally occurring secondary metabolite secretions remains unclear (Amitani *et al*., 1995). In addition fumagillin has undergone clinical trials as an oral treatment for intestinal microsporidiasis in patients with HIV infection but adverse effects such as thrombocytopenia, neutropenia, hyperlipamaemia, and abdominal cramps were recorded (Molina *et al*., 2000). A recent report by Champion *et al*., (2010) further highlighted issues with the use of fumagillin for the treatment of intestinal microsporidiasis with patients reporting abdominal cramps. Successful therapeutic use of fumagillin has been reported when applied topically in cases of microsporicidal keratoconjunctivitis in humans (Diesenhouse *et al*., 1993). The fumagillin dicyclohexamine salt was found to induce sister chromatid exchanges at doses as low as 0.34 µg ml^{-1} in human peripheral blood lymphocytes. Furthermore an increase in chromosome aberrations coupled to a reduction in mitotic index, proliferation index, and nuclear division index were observed in the same cell lines showing the clastogenic and cytotoxic potential of fumagillin (Stevanovic *et al*., 2008).

Restrictocin blocks protein synthesis and exerts toxicity to mammalian cells however it is unclear as to the precise physiological relevance of restrictocin as strains deficient in its production did not demonstrate altered virulence (Smith, 1993). Helvolic acid, (Figure 1.3), has been demonstrated to affect the respiratory burst of macrophages Mitchell *et al*., 1997) and is capable of mediating ciliostasis of epithelial cells *in vitro* as well as causing cell damage at higher concentrations (Amitani *et al*., 1995; Khoufache *et al*., 2007). Fumitremorgin is an alkaloid produced by *A. fumigatus* and has been demonstrated to be tremorgenic. Recently, fumitremorgin been identified as a potent and specific inhibitor of breast cancer resistance protein (Kato *et al*., 2009) however its exact role in fungal pathogenesis remains to be fully understood.
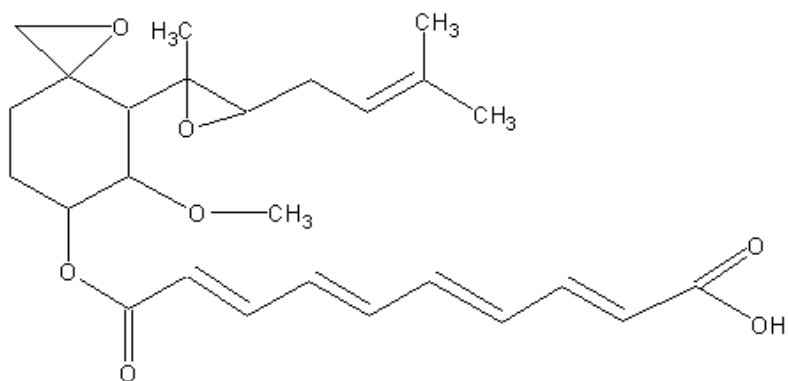
Fumagillin, $C_{26}H_{34}O_7$, M.W: 458.55
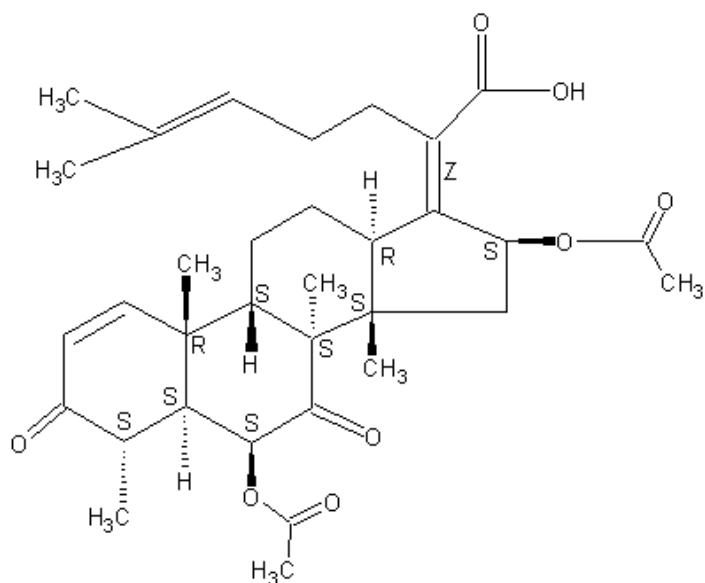
Helvolic acid, $C_{33}H_{44}O_8$, M.W:568.698
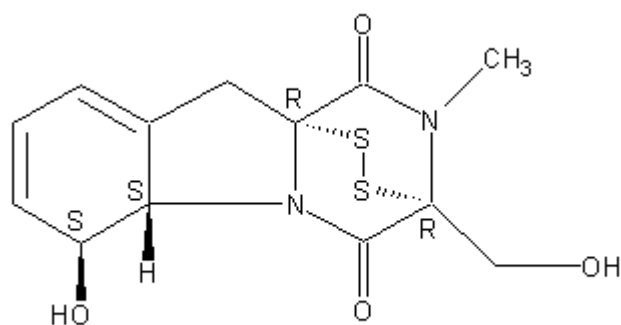
Gliotoxin, $C_{13}H_{14}N_2O_4S_2$, M.W: 326.393

Figure 1.3 Mycotoxins produced by *A. fumigatus*.
Fumagillin, Helvolic acid and Gliotoxin have are mycotoxins produced as secondary metabolites by *A. fumigatus* which mediate ciliostasis, epithelial cell damage and reduce cell function.

Molecular structures: www.aspergillus.org.uk

Gliotoxin, (Figure 1.3) is arguably the most comprehensively studied secondary metabolite/mycotoxin produced by *A. fumigatus*. Gliotoxin is an epipolythiodioxopiperazine (ETP) molecule with a molecular weight of 326 Da. The toxicity of gliotoxin is mediated by the presence of an internal disulphide bond which forms mixed disulphides with mixed thiol groups (Hurne *et al.*, 2000). There have been numerous articles published in the last few years describing the deletion of genes in gliotoxin biosynthetic cluster (Gardiner and Howlett, 2005). The gene *gliP* encodes a non ribosomal peptide synthetase (NRPS) which is capable of synthesising a gliotoxin precursor. Schrettl *et al.*, (2010) have recently demonstrated that expression of *gliT*, a member of the gliotoxin biosynthetic culture protects *A. fumigatus* from restricted growth caused by gliotoxin. Bernardo *et al.*, (2003) demonstrated a glutathione dependent reversible uptake mechanism for gliotoxin. Cells actively take up the oxidised form of gliotoxin and once within cells it binds to glutathione and remains in the reduced form. Upon the induction of apoptosis by gliotoxin glutathione levels are reduced and gliotoxin is released in its active form. There is mixed information as to the percentage of *Aspergillus* strains that produce gliotoxin and there appears to be an ill defined consensus as to the concentration that pathogenic species are capable of producing *in vivo* (Kwon-Chung and Sugui, 2009). Gliotoxin has been demonstrated to inhibit ciliary beat frequency in epithelial cell lines (Amitani *et al.*, 1995). Comera *et al.*, (2007) demonstrated that gliotoxin disrupted the organisation of the neutrophil actin cytoskeleton at concentrations of 100ng ml^{-1}. Other studies have demonstrated the inhibition of macrophage phagocytosis T-cell proliferation, mast cell activation and cytotoxic T-cell responses (Dagenais and Keller, 2009; Hof and Kupfahl, 2009). Gliotoxin inhibits the formation of the neutrophil NADPH oxidase complex by inhibiting the translocation of p47^{phox} thus inhibiting the translocation of other cytosolic components to the membrane bound flavocytochrome b$_{558}$ (Tsunawaki *et al.*, 2004). The

16

formation of the NADPH oxidase complex and its role in neutrophil function will be discussed in more detail (Section 1.7). A recent article by Bruns *et al*., (2010) demonstrated that bio-film growth by *A. fumigatus* results in the advanced synthesis of gliotoxin which may also play a key role of the *in vivo* pathogenesis of the fungus. This finding may have serious implications for the health of immunocompetent individuals with cystic fibrosis as patients with the condition have been demonstrated to present with complications resulting from fungal colonisation of the respiratory epithelia (Pihet *et al*., 2009).

1.7 Neutrophils.

Neutrophils are highly effective phagocytes of the innate immune system and are a critical component of protection against pathogenic microorganisms. Neutrophils internalise pathogens into intracellular compartments called phagosomes. Neutrophils contain primary azurophilic, secondary, gelatinase and lysosome granules which contribute to the killing of internalised bacteria and fungi. A particularly interesting facet of the neutrophil is the formation of the NADPH oxidase complex (Figure 1.4). The components of this complex are flavocytochrome b_{558} which is composed of $gp91^{phox}$ and $p22^{phox}$ as the two membrane components (Cross and Segal, 2004). In addition to this a number of cytosolic proteins are also required to activate the the complex. These include $p67^{phox}$, $p47^{phox}$, $p40^{phox}$ and the small GTP-binding protein rac in the GTP bound form. The translocation of the cytosolic components of the NADPH oxidase to the membrane component is required to produce a functional complex capable of producing superoxide (Wientjes *et al*., 1993; Sumimito *et al*., 1996). Upon neutrophil activation $p47^{phox}$ becomes heavily phosphorylated and following translocation to the membrane through its affinity with the membrane regions of $gp91^{phox}$, $p47^{phox}$ appears to act as an adaptor protein for the attachment of $p40^{phox}$ and

(A)

Cell membrane

Intracellular   Intravacuolar

rac

p67^{phox}

p47^{phox}

p40^{phox}

Flavocytocrome b$_{558}$

(B)

Cell membrane

Intracellular   Intravacuolar

NADPH $2O_2$

p47^{phox}

Rac p67^{phox}

p40^{phox}

Flavocytochrme b$_{558}$

H_2O_2

-OH

NADP+
H+

$2O_2^{-}$

HOCl
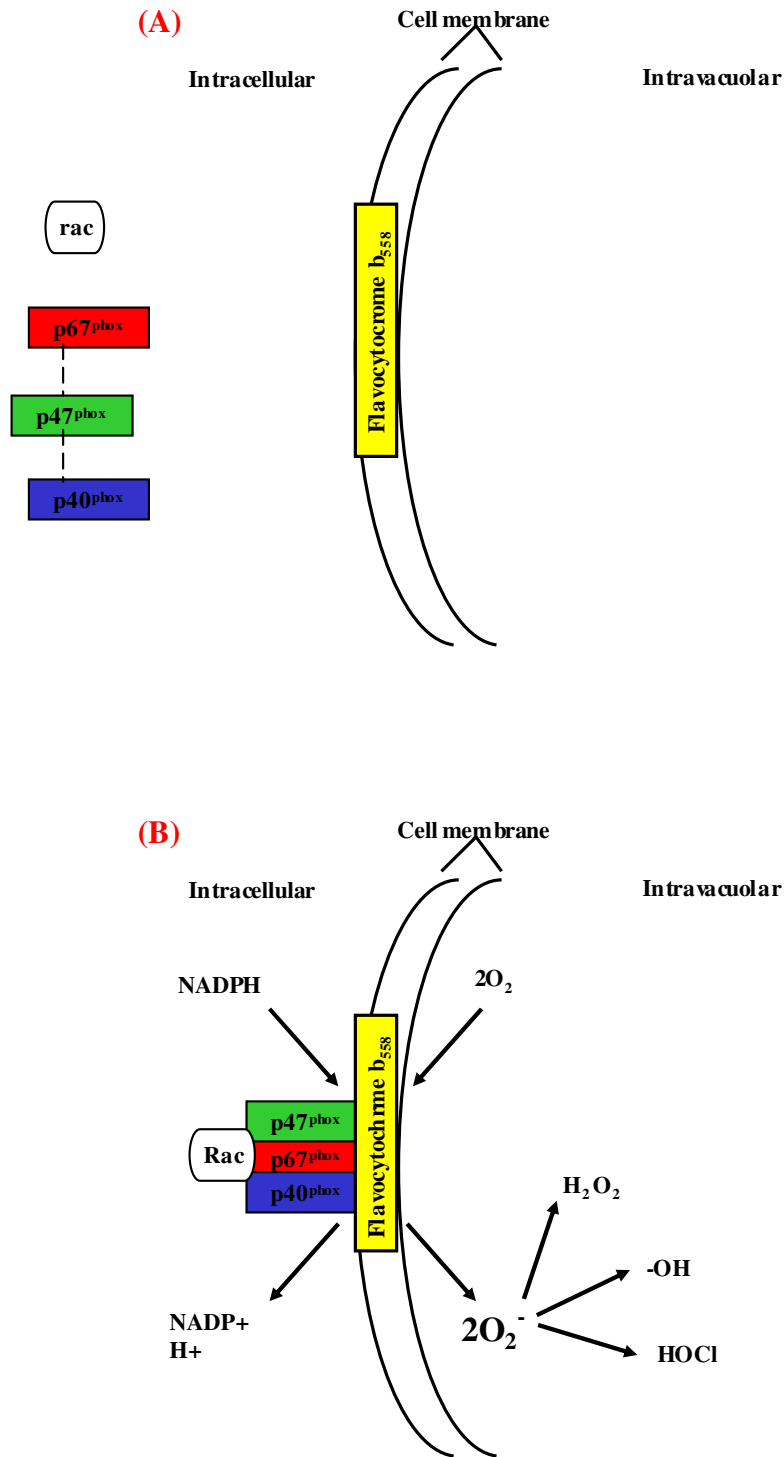
Figure 1.4 NADPH oxidase formation.
The NADPH oxidase complex consists of a cytosolic (p40^{phox}, p47^{phox}, p67^{phox} and rac) and a membrane flavocytochrome b$_{558}$ which consists of gp91^{phox} and p22^{phox} **(A)**. Upon neutrophil activation the cytosolic components translocate to the membrane and associate with flavocytochrome b$_{558}$ thus forming the active NADPH oxidase complex leading to the production of superoxide **(B)**.

subsequently p67^{phox} thus forming a functional NADPH oxidase complex (Wientjes and Segal, 2003). Molecular oxygen consumed during the phagocytosis of a pathogen is converted to superoxide (O_2^-) by the transferral of electrons from NADPH to the molecular oxygen molecule (Segal, 1996). The superoxide anion may then be involved in downstream reactions with hydrogen with the formation of hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-) or may interact with nitric oxide (NO) to form peroxynitrite (Pryor and Squadrito, 1995).

The commencement of degranulation has been demonstrated to occur with a lag phase of approximately twenty seconds (Segal *et al*., 1980). Myeloperoxidase (MPO) is a critical component of the degranulation process and comprises approximately 5% of the neutrophil proteome (Segal, 2005). MPO has been demonstrated to catalyse the H_2O_2 oxidation of halides that can kill microbes (Hampton *et al*., 1998). Data obtained from murine specimens have demonstrated that cases of MPO deficiency in neutrophils results in increased susceptibility to bacterial and fungal infections (Aratani *et al*., 2000). Other primary granules secreted by neutrophils include cathespin G, elastase, defensins and lysozymes. These contribute to the permeabilisation of bacterial and fungal cells following liberation and activation through pH alterations mediated by the depolarization of the phagosome due to NADPH oxidase formation (Segal, 2005). Secondary granules include lactoferrin which is thought to play an important role in protection against conidia and other fungal entities by sequestering iron (Bonnet *et al*., 2006). Defects associated with neutrophil granules include the inherited granule deficiency Chediak-Higashi syndrome which is a rare autosomal recessive disorder associated with a prominent defect in formation of neutrophil granules resulting in increased susceptibility to infection (Malech and Naseef, 1997). Chronic granulomatous disease (CGD) provides the most definitive evidence for the clinical importance of the NADPH oxidase as CGD patients are characterised by a predisposition to bacterial and

fungal infection. CGD is an immunodeficiency characterised by mutations in components of the NADPH oxidase complex thus resulting in the inability of the neutrophil to produced superoxide (Gallin and Zarember, 2007). The main genetic predisposing factors in CGD are mutations in gp91^{phox} and p47^{phox} which comprise 60% and 30% of CGD cases. Defects in the formation of p67^{phox} and p22^{phox} account for the remainder of CGD manifestations (Roos *et al*., 2003).

1.8 Insects as models to study microbial pathogenesis

Insects and vertebrates diverged approximately 500 million years ago however many similarities between both metazoans still exist at the biochemical and cellular level (Vilmos and Kurucz, 1998; Salzet, 2001). Insects do not possess an adaptive immune response however many similarities exist between the insect immune response and the innate immune response of mammals, therefore studies of infections requiring the activation of a strong innate immune response may be studied in insects. Furthermore in recent years insects have been used as a viable means to screen the efficacy of antimicrobial chemotherapeutic agents *in vivo* (Hamamoto *et al*., 2004; 2009). Insects offer the benefits of a high throughput model and viable alternative to mammals due to lower costs, less laborious maintenance and speed of results. In addition employing insects for *in vivo* experimentation does not result in as many bioethical issues as using mice therefore making them a favorable model in modern laboratory practice.

1.8.1   The insect cuticle as an initial defence barrier to infection.

The insect cuticle forms an initial mechanical barrier to infection in a manner that is analogous to human skin. The cuticle is largely comprised of chitin with the outer layer of the cuticle covered in a waxy layer of lipids, fatty acids, and sterols (Böröczky

et al., 2008). During entamopathogenic infection the insect cuticle is often broken down by chitinases (St Leger *et al*., 1987; Santi *et al*., 2010). Pathogenic microbes rarely degrade the epidermis however upon infection of broken tissue or during invasive pathogenesis microbes have been demonstrated to secrete extracellular proteases which facilitate the colonisation of host tissue (Walsh, 1998; Cowell *et al*., 2003). An intact insect cuticle is generally successful in preventing microbial infection however injury to the cuticle does activate immune responses comprising antimicrobial peptide expression, initiation of the phenoloxidase cascade and cellular activation (Kavanagh and Reeves, 2004; Lemaitre and Hoffmann, 2007).

1.8.2   Clotting and melanisation

The insect body cavity is known as the haemocoel which contains haemolymph and this structure is surrounded by the fat body. Both of these anatomical features function in a manner analogous to blood and the liver respectively (Søndergaard, 1993; Reeves and Kavanagh, 2004). Insect haemolymph is a protein and nutrient rich environment which functions in the transport of macromolecules and contains signal molecules, haemocytes and antimicrobial peptides which confer immunity to microbial challenge. The function of haemocytes will be discussed in greater detail in Section 1.8.3. Upon physical injury the activation of two clotting mechanisms have been identified. The first is the polymerisation of vittellogenein-like proteins and lipophorins by a calcium-dependent transglutaminase which function in a manner similar to mammalian von Willebrand factor (Sritunyalucksana and Soderhall, 2000; Theopold *et al*., 2002). The second mechanism is initiated by the release of cytoplasmic L-granules. Gram negative bacteria activate the factor C component while the factor G component is activated by the presence of a fungal burden. The subsequent conformational change activates the proclotting enzyme which catalyses the conversion of soluble coagulogen
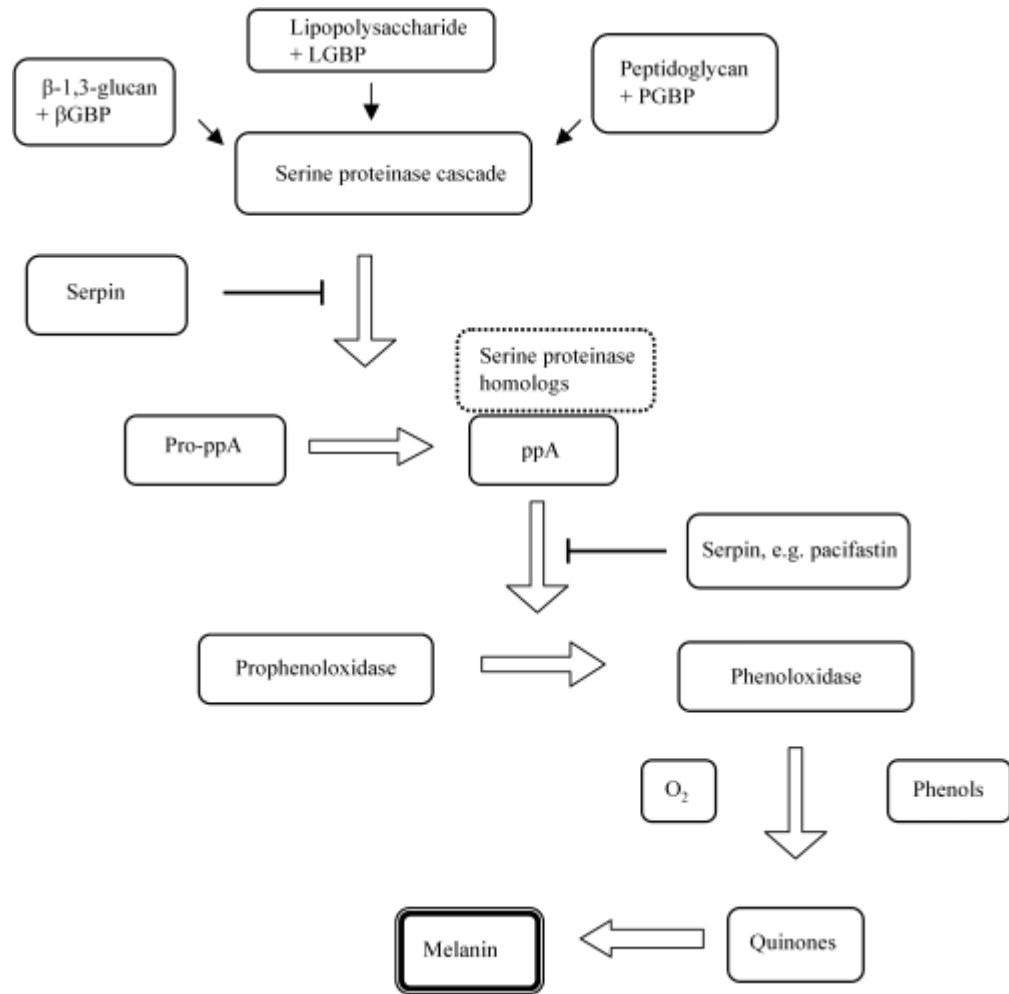
Figure 1.5 The invertebrate phenoloxidase pathway.
The system is activated by pattern recognition receptors for β1-3, glucan (βGBP), peptidoglycan (PGBP) and lipopolysaccharide (LGBP). A cascade of serine proteases results in the cleavage of prophenoloxidase-activating enzyme (Pro-ppA) to form ppA and thus cleave PPO to form PO. Some serine proteases can directly activate the pathway. The active phenoloxidase catalyses the o-hydroxylation of phenols to form quinines which polymerise to form melanin.

Image: Cerenius and Soderhall, (2004)

to insoluble coagulin (Sritunyalucksana and Soderhall, 2000). The implications of blood clotting in insects has been discussed by Haine *et al*., (2007) who identified that infection mediated through injury in *Periplaneta americana* results in the localization of haemocytes to the site of infection and restricts microbial spread in the haemocoel. The cumulative effect of these measures is the compartmentalization of the open haemocoel thus limiting the development of microbial pathogenesis.

The process of melanisation (Figure 1.5) is of central importance in insect immunity and conveys protection against a broad range of microorganisms as well as serving as a biomarker of the immunological status of insects. Activation of the phenoloxidase cascade results in the formation and deposition of melanin on the microbe. The native protein of phenoloxidase pathway is prophenoloxidase, a zymogen located within haemocytes. The process of phenoloxidase activation and subsequent melanin formation is initiated by the binding of soluble pattern recognition receptors which bind lipopolysaccharide, peptidoglycan and 1,3-glucan. Soluble pattern recognition receptors in insects include lectins, peptidoglycan recognition protein and 1,3-glucan binding protein. In addition the insect lipoprotein Apolipophorin III has been demonstrated to activate the phenoloxidase pathway (Halwani *et al*., 2000; Park *et al*., 2005). This recognition leads to the serine protease mediated cleavage of prophenoloxidase by proPO-activating enzyme (PPA) (Cerenius and Soderhall, 2004). The active phenoloxidase protein catalyses the *o*-hydroxylation of monophenols to form diphenols, and oxidation of *o*-diphenols to *o*-quinones which polymerise to form melanin (Soderhall and Cerenius, 1998). Melanin and its precursors have been demonstrated as mediating direct toxicity against microbes (St Leger *et al*., 1988) however the controlled activation of the PO pathway is necessary as melanin at excessive levels is toxic to host tissue. Prophenoloxidase has been demonstrated to have sequence similarity with the thiol-ester region of vertebrate complement proteins C3

and C4 (Nair *et al.*, 2005) thus indicating the possibility of a conserved evolutionary role in the initial host protection against microbes.

1.8.3   Cellular Responses of Insects

The insect haemolymph contains a cellular element in the form of haemocytes (Figure 1.6) which function in a manner similar to phagocytes of the vertebrate innate immune system. In the majority of cases haemocytes circulate freely through the haemolymph however it is estimated that up to 30% of haemocytes are associated with the fat body, trachea or wall of the haemocoel (Ratcliffe, 1985). Haemocytes have been further classisfied as prohameocytes, plamatocytes, granulocytes, coagulocytes, spherulocytes, and oenocytoids (Price and Ratcliffe, 1974). Prohaemocytes are 6-13 μm in diameter, have a high nuclear to cytoplasmic ratio and are believed to be the precursor of two differentiated haemocyte types (Ribeiro and Brehélin, 2006). Plasmatocytes are larger (up to 20 μm long). Plasmatocytes spread on coverslips and upon contact develop numerous pseudopodia thus leading some to suggest that they are involved in phagocytic reactions (Ling and Yu, 2006). Granulocytes measure 5-8 μm in diameter, possess pseudopodia and have been well demonstrated to phagocytose and to perform the initial interaction of the cellular immune response with pathogenic microorganisms. The release of their granular content acts as a chemoattractant for plasmatocytes thus ameliorating the immune response (Gillespie *et al.*, 1997). Oenocytoids appear as large binucleate cells, have a low nuclear to cytoplasmic ratio, and have been demonstrated to contain phenoloxidase (Ribeiro and Brehélin, 2006). Spherulocytes are small rounded cells however their functions remain unknown. Coagulocytes and Adipohaemocytes are characterised by their roles in clotting and the presence of fat droplets, respectively (Kavanagh and Reeves, 2004). Dean *et al.*, (2004) described the presence of a haemocyte with "extreme phagocytic ability" with a
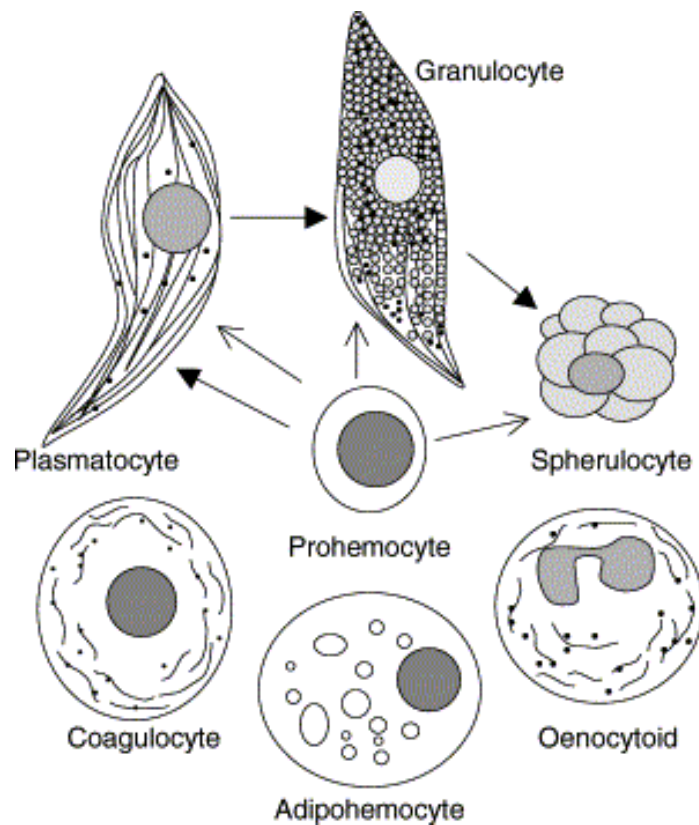
Figure 1.6 Different insect haemocyte subtypes.
Haemocytes predominantly function in cellular mediated protection against microbes.
The prohaemocyte is the precursor cell prior to cellular differentiation

Image: Kavanagh and Reeves, (2004)

capacity to phagocytose up to 500 bacteria per haemocyte therefore indicating that there may be other sub-classes of haemocytes which remain to be fully characterised in insects.

At a functional level phagocytosing haemocytes and vertebrate phagocytes both behave in a similar manner. Activation of the ProPO cascade is required for granulocytes to bind to non-self matter while calcium is required for plasmatocyte binding. Lectin mediated recognition in phagocytosis is conserved in vertebrates and invertebrates thus allowing for a diversity of pattern recognition sites specific to the infective microorganism (Kavanagh and Reeves, 2004). The discovery of proteins homologous to human p67^{phox} and p47^{phox} and subsequent production of superoxide demonstrated the parallels of the vertebrate innate immune system and insect immunity (Bergin et al., 2005). Furthermore Renwick et al., (2007) demonstrated that gliotoxin was capable of inhibiting the translocation of haemocyte p47 and p67 in a manner similar to neutrophils thus confirming a significant parallel in the field of comparative immunology.

1.8.4 Antimicrobial Peptide Synthesis

The production of antimicrobial peptides (AMPs) is a key element in the protection of insects against microbial infection. The main sites of AMP synthesis in insects are the haemocytes, fat body, salivary and reproductive glands. These molecules act to cause the lysis of microbial surfaces and are synthesized rapidly upon microbial sensing (Lowenbrger, 2001). The production of AMPs is largely due to the activation of the Toll and immunodeficiency (IMD) pathways which are NF- like. The Toll pathway is similar to the Toll like receptor (TLR)/IL-1 receptor pathway present in vertebrates and was originally described by Lemaitre et al., (1996). The Toll pathway, characteristic of a Gram positive bacteria or fungal response and is initiated by the

product of Spaetzle cleavage which is believed to bind the Toll receptor. Transduction through the MyD88 Tube molecules and Pelle leads to phosphorylation and activation of Cactus which in turn liberates Dif. Thereafter, Dif translocates to the nucleus to induce transcription (Salzet, 2001).

The IMD pathway is comparable to the vertebrate tumour necrosis factor (TNF)-pathway and is mostly involved in the recognition of Gram negative bacterial infections. In the process peptidoglycan recognition protein, (PGRP) can recognise peptidoglycan and lipopolysaccharide and initiate the pathway. FADD associates with IMD. Meanwhile, TAK1 acts downstream of IMD/FADD where it exerts its function on signalosome to phosphorylate Relish. DREDD then associates with FADD to cleave Relish and produce REL-49 and REL-68, the latter translocating to the nucleus to act as a transcription factor (Hultmark, 2003). The regulation of the Toll and IMD pathways in insects in critical to the synthesis of appropriate antimicrobial defence and ensures a tailored immune response depending on the nature of infection. Gregorio *et al*., (2002) demonstrated that the absence of Toll and IMD in double knockout mutants of *Drosphila* resulted in the dysregulation of AMP gene activation and increased susceptibility to infection.

1.9 *Galleria mellonella* as a model to study fungal pathogenesis

The Greater wax moth *Galleria mellonella* (Figure 1.7) is a common pest of beehives. The larval stage ofis naturally found in beehives where they feed on pollen, honey, wax and other organic material. *G. mellonella* colonises already weakened colonies whereby adult females fly at night and may deposit up to 102 eggs per minute in the cracks and crevices of the beehive. The larvae hatch from their eggs after a few days and commemnce feeding activity whereby thry tunnel through the honeycomb. In this action a protective silk trail during the prepupal stage of the lifecycle is spun which

effectively traps bees in the beehive and renders it unusable (Jafari *et al*., 2010, Schmolz *et al*., 1999). Following pupation male insects have been demonstrated to live an average of 21 days with females living for 12 days (Warren and Huddleston, 1962).

Recent studies have demonstrated that *G. mellonella* larvae may be used as an effective model to study mycological virulence and the efficacy of anti-fungal drugs *in vivo* (Table 1.1). Cotter *et al*., (2000) demonstrated that they are a useful model to asses the relative pathogenicity of yeast species with statistically significant results obtained within 48 hours of infection. This initial concept in the study of fungal virulence was developed further by Brennan *et al*., (2002) with the discovery of a direct correlation between the virulence of *C. albicans* mutants in *BalbC* mice and *G. mellonella* larvae. An interesting development in the use of *G. mellonella* to assess the virulence of fungal pathogens was described by Scully and Bidochka, (2005) with the discovery that repeated serial passage of *A. flavus* results in the host specialization of the fungus as evidenced by increased fungal development in *G. mellonella* coupled to decreased growth on artificial media. Such findings are of particular importance given the requirement for a comprehensive understanding of virulence factors in emerging pathogens.

The use of *G. mellonella* in *A. fumigatus* research has become recognised as a valuable alternative to the murine model. Reeves *et al*., (2004) demonstrated that *A. fumigatus* ATCC 26933 was pathogenic in *G. mellonella*. In addition it was demonstrated that the level of *in vivo* mycotoxin production by *A. fumigatus* could be evaluated through organic extraction and RP-HPLC analysis. Renwick *et al*., (2006) further developed this by using *G. mellonella* to assess the virulence of conidia undergoing germination and hyphal development. The authors demonstrated that an increased level of conidial development during the germination and outgrowth phase detrimentally affects the viability of larvae which is further supported by the finding
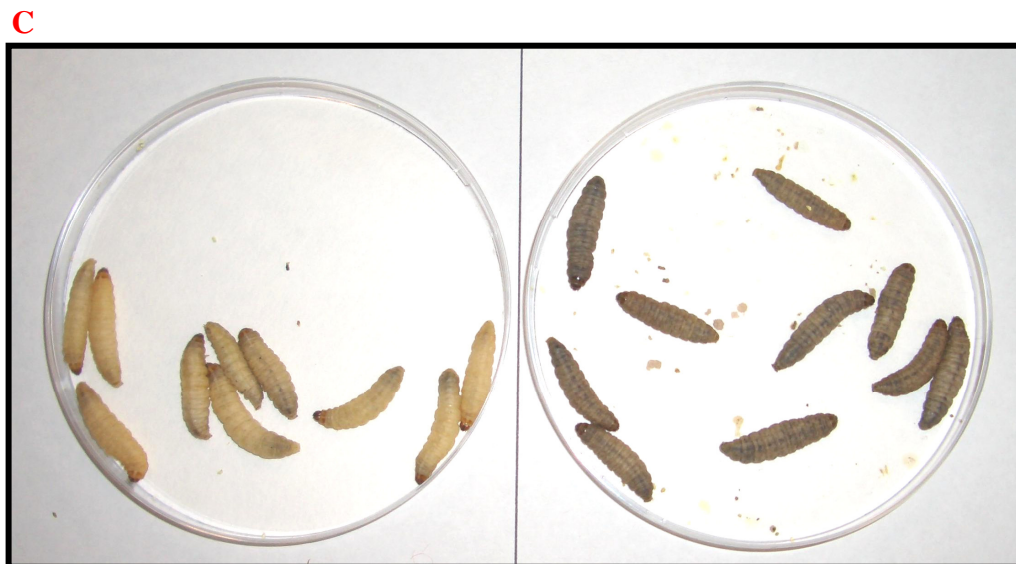
Figure 1.7 *Galleria mellonella*

G. mellonella larvae **A,** and close up of larval pro-legs **B.** Uninfected healthy larvae (left) and heavily melanised dead larvae right **C.**

Yeasts	
Cotter *et al*. 2000	Differentiation of virulence of a range of yeast species
Brennan *et al*, 2002	Comparison of virulence of *C. albicans* mutants with virulence in mice
Mylonakis *et al*., 2005	Development of a model to study *Cryptococcus* pathogenesis
Fan *et al*., 2007	Analysis of the virulence of *eca 1* mutants of *C. neoformans* in *G. mellonella*
Velagapudi *et al*., 2009	Sexually reproduced spores from *Cryptococcus* as effective infectious propagules
Filamentous fungi	
Reeves *et al*., 2004	*In vivo* analysis of gliotoxin as a virulence factor of *A. fumigatus*
Scully and Bidochka, 2005	Host specialisation of *A. flavus* following repeated larval infection
Reeves *et al*., 2006	Assessment of alteration of virulence of Δ*pes1* isolate in *A. fumigatus*
Vickers *et al*., 2007	Demonstration of efficacy of anti-Cts-D antibody in vivo
Jackson *et al*., 2009	Assessment of virulence of melanin mutants of *A. fumigatus*
Wodja *et al*., 2009	Heat shock and natural infection by *B. bassiana* induce an anti-fugal response
Assessment of anti-fungal activity	
Cowen *et al*., 2009	Identification of Hsp90 as a drug target in an in vivo model
Rowan *et al*., 2009	Evaluation of anti-fungal efficacy of silver-based compounds

Table 1.1 Publications using *G. mellonella* for *in vivo* analysis.
Selected examples of the utilisation of *G. mellonella* larvae with fungi and anti-fungal agents

that larval haemocytes are incapable of phagocytosing larger conidial bodies. Recent work by Cheema and Christians, (2010) demonstrated the differential pathogenicity of the mating types of *A. fumigatus*. Additionally Slater *et al*., (2010) identified that *A. fumigatus* isolates containing gene deletions of *cpcA, sidA, sidC, sidD, sidF* and *paba* demonstrated avirulence or attenuated virulence in *G. mellonella* and showed a strong correlation with murine data.

G. mellonella larvae have been employed to evaluate the antifungal activity of amphotericin B, flucytosine and fluconazole following infection with *Cryptococcus neoformans* (Mylonakis *et al*., 2005). In addition, Rowan *et al*., (2009) demonstrated that novel silver compounds could be used as an effective means to assess *in vivo* antifungal activity. Inoculation of larvae with [Ag_2(mal)(phen)$_3$], $AgNO_3$ or 1,10-phen resulted in a significant increase in haemocyte density 4 hours post inoculation coupled to the increased expression of *gallerimycin* as identified by RT-PCR. A recent publication by Kelly and Kavanagh, (2010) identified that administration of Caspofungin at concentrations of 0.19 μg ml^{-1} mediated protection against subsequent infection with *Staphylococcus aureus* or *C. albicans*. In this case increased *impi* and *transferrin* gene expression was observed coupled to the increased synthesis of Apolipophorin III and ferritin subunits. These two publications provide evidence for the use of *G. mellonella* as *in vivo* tools however they also raise the issue of immunological priming which is an important factor to consider when using larvae.

The immunological status of *G. mellonella* may also be assessed depending on the administered treatments. Bergin *et al*., (2003 and 2006) demonstrated that larval haemocyte density and quantification of fungal load may be used as indicators of immune state and mycological progression. In these studies mildly pathogenic fungi resulted in a significant increase in circulating haemocyte density while highly pathogenic infections caused a loss in haemocyte density. In addition it was found that

higher fungal loads inversely correlated with this decrease in haemocyte density. Furthermore the application of 2-dimensional electrophoresis and RT-PCR may was demonstrated to be a useful indicator in understanding the larval immune status. Proteins identified through the application of proteomic methodologies in *G. mellonella* demonstrated homology with mammalian proteins or inclusion within a family of functionally related proteins in some cases. In addition to this Mowlds and Kavanagh, 2008; Mowlds *et al*., (2008) and Mowlds *et al*., (2010) demonstrated that pre-incubation temperature, physical stress and exposure to glucan all effectively primed the immune system of *G. mellonella* through increased haemocyte density, elevated expression of antimicrobial peptide genes and alterations in the humoral proteome. Clearly the use of *G. mellonella* is a novel and highly beneficial approach to the study of microbial pathogenesis and chemotherapy however the characterisation of alterations to the immunological profile of larvae is warranted in order to fully utilise this model.

1.10 Aims of study

The primary aim of this project was to strengthen the current understanding of fungal pathogenesis mediated by *A. fumigatus* and its metabolites and to further develop the application of *G. mellonella* as an alternative model in innate immune system research. The aims of this study were:

1. To further develop the body of knowledge that exists of the pathogenicity of clinical and environmental *A. fumigatus* isolates in the *G. mellonella* model of infection coupled to characterisation of these isolates through biochemical and microbiological methodologies.

2. Contribute to the understanding of the impact of secondary metabolite production *in vivo* and *in vitro* through an analysis of the effect of one of these

mycotoxins on the cellular immune response of human neutrophils and *G. mellonella* haemocytes.

3. Analyse the humoral profile and activity of insect haemolymph in response to mycotoxin exposure while undertaking a parallel study with human serum using a variety of biochemical methodologies.

4. Characterise the immune response of *G. mellonella* to *A. fumigatus* infection with non-lethal inocula through changes in cellular responses, proteomic alterations and priming of antimicrobial peptide synthesis.

Chapter 2

Materials and Methods

2.1 General laboratory practice and sterilisation procedures.

2.1.1 Chemicals and reagents.

All reagents were of the highest purity and were purchased from Sigma Aldrich Ltd, Somerset, U.K unless otherwise stated.

2.1.2 Statistical analysis.

All experiments are the mean of at least three independent determinations ± standard error unless otherwise stated. Larval viability data were analysed using the Log rank test or Chi-Squared test. Differential expression of protein by 2-D electrophoresis was analysed by ANOVA. Experimental data were tested for statistical significance using a Students t-test (Minitab 14 package). For all experimentation a p-value of ≤ 0.05 was deemed statistically significant.

2.1.3 Sterilisation procedures.

All micro-centrifuge tubes and pipette tips were stored in a sealed container and autoclaved at 121ºC for 15 minutes prior to use. All microbiological media with the exception of minimal essential medium / 5% (v/v) foetal calf serum (Section 2.2.7) were sterilised at 105ºC for 30 minutes prior to use. Semi-solid agar media were allowed to cool until hand hot, (approx 45-50ºC) and poured under sterile conditions using 90mm petri-dishes. All work involving *A. fumigatus* was performed using a class II safety cabinet.

2.1.4 Cell enumeration and haemocytometry.

Neutrophil, haemocyte and fungal cell counts were performed using an improved Neaubeur haemocytometer and all determinations were calculated from the average of three counts.

2.1.5 *Galleria mellonella.*

Sixth - instar larvae of the greater wax moth *G. mellonella* were purchased from Livefoods Direct Ltd, Sheffield, United Kingdom and were stored in the dark at 15ºC to prevent pupation. For experimental use all larvae were of a mass of 0.2–0.4 g, and were excluded if there was evidence of localised melanisation. Larvae were injected through the last left pro-leg (Figure 2.1 **(A)**), with a Myjector U-100 insulin syringe, (Terumo Europe N.V., 3001 Leuven, Belgium), while applying mild pressure to the insect to allow the opening of pro-legs (Figure 2.1 **(B)**). During experiments larvae were kept in 9cm petri-dishes with 0.45 mm Whatmann filter paper inserted on the lids. Throughout all experimentation great care was taken to ensure the most accurate delivery of injection. In addition all in *vivo* assays were performed on three separate occasions using a group size of 10 larvae per dish and average results were calculated. For viability studies larvae were injected with sterile PBS as a positive control for the injection of larvae.

2.2 *Aspergillus fumigatus* **strains and culture conditions.**

2.2.1 Malt Extract agar.

Malt extract agar was prepared by adding 50 grammes of extract per litre of deionised water and autoclaving at 105ºC for 30 minutes. Once the agar was hand hot it was poured into Petri-dish plates.

2.2.2 *Aspergillus* **Trace Elements.**

$Na_2B_4O_7.7H_2O$ (0.04 g), $CuSO_4.(5H_2O)$ (0.7 g) $FeSO_4.7H_2O$ (1.2 g), $MnSO_4$ (0.7 g), $NaMoO.2H_2O$ (0.8 g) and $ZnSO_4.7H_2O$ (10 g) were added to 800 ml of distilled water and dissolved. The solution was made up to 1 L with distilled water and

(A)

(B)

Figure 2.1 *Galleria mellonella* **pro-legs and inoculation.**
Pro-legs of larvae are exposed, **(A),** when mild pressure is applied to the larvae. Larvae were injected through the last left pro-leg with a 20 µl volume each time.

subsequently autoclaved. The solution was aliquoted in 50 ml volumes and stored at -20ºC.

2.2.3 *Aspergillus* Salt Solution.

KCl (26 g), $MgSO_4.7H_2O$ (26 g), KH_2PO_4 (76 g) and *Aspergillus* Trace elements 50 ml were added to 800 ml distilled water and dissolved. The solution was made up to 1 L with distilled water and autoclaved. Thereafter the solution was stored at room temperature.

2.2.4 Ammonium Tartrate.

Ammonium Tartrate (92 g) was dissolved in 1L of distilled water and autoclaved. The solution was stored at room temperature

2.2.5 *Aspergillus* Minimal Medium (AMM).

Aspergillus minimal medium was prepared by adding Ammonium Tartrate, (10mls), *Aspergillus* Salt Solution, (20 mls), and Glucose, (10 g), to 800 mls of distilled water. AMM was adjusted to pH 6.8 and made up to 1 L using distilled water. The medium was autoclaved and stored at room temperature.

2.2.6 *Aspergillus* Minimal Medium (Agar).

AMM agar was prepared in the same way as liquid AMM however following the volume being adjusted to 1 L, 20 g of Agar was added and dissolved into solution and autoclaved. Following sterilisation AMM agar was cooled to approximately 50ºC and poured in clean Petri – dish under sterile conditions.

2.2.7 Minimal Essential Medium / 5% (v/v) Foetal Calf Serum.

All flasks were autoclaved at 121ºC for 15 minutes prior to use. Minimal Essential Medium supplemented with 5 % (v/v) Foetal Calf serum and aliquoted into each flask in a sterile environment and re-plugged with cotton wool ahead of inoculation with *Aspergillus fumigatus*.

2.2.8 Phosphate Buffered Saline 0.01% (v/v) Tween.

One PBS tablet was dissolved in 200 mls deionised water and was autoclaved at 121ºC for 15 minutes. PBS was stored at room temperature. For PBS-T, PBS was supplemented with 0.01% Tween-80 (Merck), and was vortexed until mixed homogenously. PBS-T was filtered through a 0.22 µm filter (Millipore), prior to use.

2.2.9 *Aspergillus* strain culture.

A. fumigatus ATCC 26933 obtained from American Type Culture Collection Maryland USA, was grown and maintained on Malt Extract Agar (Oxoid Ltd Basingstoke UK) at 37ºC in thermally controlled incubator for a minimum of 4 days. In some experiments conidia were withdrawn from 37ºC incubation and stored at room temperature to monitor the effect of conidial maturation on the virulence of *Aspergillus* isolates on *G. mellonella* larvae. Environmental and clinical isolates of *A. fumigatus* (EAF 1-15 and CAF 1-15 respectively) and environmental *A. flavus*, *A. terreus* and *A. niger* were a kind gift from Professor Ricardo Araujo, Faculty of Medicine of Porto, Portugal. Environmental strains were isolated by air and surface sampling. Clinical isolates were isolated from patients with invasive aspergillosis. *Aspergillus* isolates AF293, Δ*ftmA*, Δ*laeA*, and Δ*psoA* were a kind gift from Professor Elaine Bignell, (Imperial College London). All strains were stored in 50% (v/v) glycerol and 10 µl aliquot of this was used was stabbed in the centre of a Petri-dish containing Malt Extract

Agar prior to starting a new culture for every experiment. Plates were inspected on a daily basis for the level of growth. Once conidial development was achieved the plates were wrapped in parafilm and stored at 4ºC.

2.2.10 Harvesting of *Aspergillus* conidia.

Aspergillus conidia were harvested in a Class II safety cabinet. Plates were initially washed with 10 mls PBS-T and centrifuged at 1,500 x g for 5 minutes at room temperature. The supernatant was removed and the conidial pellet was washed twice in sterile PBS to remove excess Tween 80. The concentration of conidia in the suspension was calculated by haemocytometry.

2.2.11 Liquid Culture of *A. fumigatus*.

For *Aspergillus* culture in MEM/5% (v/v) FCS or AMM sterile medium was inoculated in a sterile laminar flow hood and conidial inoculations were 5 x 10^7 cells per 100 ml culture.

2.2.12 Milk Agar.

Dry skimmed milk (5 g) and Agar (8 g) was mixed to a homogenous solution for 60 minutes and was subsequently sterilised by autoclaving at 105ºC. Following sterilisation the agar was allowed to cool to hand warm and was spread on a 9 cm Petri-dish.

2.3 *Candida albicans* strain growth and harvest.

2.3.1 YEPD broth / agar.

YEPD agar was prepared by dissolving Glucose (2% w/v), Yeast extract (1% w/v), and bacteriological peptone (2% w/v), in deionised water and autoclaving at 105ºC for 30 minutes.

For agar plates 2% (w/v) agar was added and autoclaved as described. In some cases erythromycin was added to the hand warm agar prior to pouring to control bacterial contamination. This was prepared by dissolving 5 mls of a 5 mg ml^{-1} solution of erythromycin in DMSO when the agar solution was hand warm. Once in the agar solution the plates were spread as per normal and stored at 4ºC. All erythromycin supplemented plates were used within 3 days.

2.3.2 *Candida albicans* liquid culture and cell harvest.

C. albicans MEN was a kind gift from Dr. D. Kerridge, Cambridge, UK. An individual colony of cells was transferred to sterile YEPD broth using a sterile inoculating loop. The flask was re-plugged with cotton and incubated at 30ºC shaking at 200 rpm overnight. To obtain a suspension of cells, 50 ml culture was transferred to a sterile 50 ml tube and centrifuged at 500 x g for 5 minutes. The cellular pellet was washed twice in sterile PBS and the final cell concentration in a 10 ml volume of PBS was ascertained by haemocytometry.

2.4 *Pseudomonas aeruginosa* maintenance, growth and cell culture.

2.4.1 Luria Bertani (L.B) Broth.

Difco L.B broth, (12.5 g) was dissolved in 500 mls deionised water. The broth was aliquoted 50 ml volumes in a conical flask and sterilised by autoclaving.

2.4.2 *P. aeruginosa* isolation agar.

Pseudomonas aeruginosa isolation agar powder (38 g) was dissolved in 1 L deionised water and sterilised by autoclaving. The agar was poured once hand hot.

2.4.3 Liquid culture of *P. aeruginosa.*

P. aeruginosa PA01 cultures were obtained by transferring a single bacterial colony to L.B broth with a sterile inoculating loop and was grown overnight at 37ºC and 200 r.p.m.

2.4.4 Calculation of *P. aeruginosa* culture cell concentration.

An overnight bacterial culture was spectrophometrically measured at an O.D. of 600nm using L.B broth as a blank and the absorbance was measured. Cells were diluted to an O.D. of 1 and serially diluted by factors of ten using sterile PBS and 100 µl was plated out on *Pseudomonas aeruginosa* isolation agar. The dilution which produced 80-100 cells per 100 µl spread was used to calculate the concentration of cells per ml of culture at 0.D 1.

2.5 *Aspergillus* plate growth measurement and conidial size analysis.
2.5.1 Plate analysis of *Aspergillus* growth.

To compare the relative levels of growth of *A. fumigatus* isolates conidia from different isolates were harvested. Conidia were diluted in sterile PBS and 5×10^3 cells in a 5 µl volume were spotted on the centre of the AMM agar plate under sterile conditions. The droplet was allowed to dry and plates were incubated at 37ºC for 24, 48, and 72 hours. Radial growth was measured and calculated in terms of colony area (mm^2).

2.5.2 Comparative analysis of conidial size using PAMAS counter.

Conidia were extracted and a 10 ml suspension of 1×10^6 ml^{-1} conidia was prepared. All samples were checked for the absence of spore agglutination prior to analysis. Conidial size analysis was performed using a PAMAS counter (SVSS-C) with analysis and size distribution software. The size variation of conidia was monitored at 1-8 µm for *A. fumigatus* strains and from 1-16 µm for *A. flavus*.

2.6 1-D and 2-D Sodium Dodecyl Sulphate–Polyacrylamide gel electrophoresis (SDS PAGE).

2.6.1 1.5 M Tris–HCl.

Tris – HCl (1.5 M) was prepared by dissolving 36.3 g Trizma Base, (Tris Base) in 170 mls deionised water and adjusted to pH 8.9 using HCl. Following pH adjustment 1.5 M Tris – HCl was filter sterilised through a 0.22 µM cellulose filter (Millipore) and stored at 4ºC.

2.6.2 0.5 M Tris – HCl.

Tris-HCl (0.5 M) was prepared by dissolving 12.1 g Trizma Base in 175 mls deionised water and adjusted to pH 6.8 using HCl. Following pH adjustment 0.5 M Tris – HCl was filter sterilised through a 0.22 µM filter (Millipore) and stored at 4ºC.

2.6.3 10% w/v Sodium Dodecyl Sulphate (SDS).

Sodium Dodecyl Sulphate (SDS), (10% w/v) was prepared by adding 10 g SDS to 100 mls of deionised water and left to stir until all SDS had been solubilised. The solution was sterilised through a 0.22 µM filter and stored at room temperature. The solution was inspected for solubility prior to use.

2.6.4 10% Ammonium Persulphate (APS).

APS (10% w/v) was prepared by adding 0.1 g APS into 1ml of deionised water and vortexed briefly to achieve solubility. APS (10% w/v) was prepared fresh every day and kept on ice when not in use.

2.6.5 10X electrode Running Buffer.

10 X running buffer, (electrode buffer), was prepared by dissolving Tris Base (30 g), Glycine (144 g), and SDS (10 g) using a magnetic stirrer in 800 mls distilled water. The mixture was allowed to solubilise overnight at room temperature and adjusted where required to pH 8.9. The buffer was made up to 1 litre and stored at room temperature. Electrode running buffer (10X) stock was diluted to different 1X, 2X and 5X concentrations by making 1/10, 1/5, and 1/2 dilutions with distilled water when required.

2.6.6 5X Solubilisation Buffer for 1-D SDS–PAGE.

Solubilisation buffer was prepared by dissolving the following constituents to solubility.

- Glycerol   8 mls
- Deionised water   4 mls
- 10% (w/v) SDS   1.6 mls
- 0.5 M Tris – HCl   1 ml
- Bromophenol Blue (0.5% w/v)   200 µl
- 2 – Mercaptoethanol   400 µl

The buffer was gently mixed for 1 hour at 4ºC and aliquoted in 500 µl volumes prior to storage at -20ºC.

2.6.7 Preparation of SDS–PAGE minigels.

All glass plates were washed in warm soapy water and cleaned thoroughly with 70% ethanol prior to use. SDS–PAGE minigels (cast using the Mini-Protean II gel casting apparatus), were made with a definite percentage concentration of acrylamide which was varied depending on the sample characteristics. A 12.5% acrylamide gel was normally used in experimental procedure however for samples where molecular weight was generally low the percentage of acrylamide was increased to 15% or 20% so as to aid greater separation and definition of bands.

SDS–PAGE mingels contain a stacking buffer where proteins are loaded in wells and once an electrical current is applied they are sequentially separated depending on their size. The separating gel separates the proteins from each other depending on size therefore aiding visualisation. All gels were used within 24 hours of setting and were allowed to polymerise for at least one hour prior to being loaded.

- *Seperating gel composition*

Solution component	% Acrylamide		
	12.50%	15%	20%
1.5 M Tris-HCl	3 mls	3 mls	3 mls
Deionised water	3.8 mls	2.8 mls	800 µl
30% Acrylamide	5 mls	6 mls	8 mls
10% v/v SDS	120 µl	120 µl	120 µl
10% v/v APS	75 µl	75 µl	75 µl
TEMED	3 µl	3 µl	3 µl

These volumes were sufficient to make 3 minigels and volumes were adjusted accordingly where a larger volume was required.

- *Stacking gel composition*

The following components were mixed together and applied on top of the separating gel; deionised water (3.4 mls), 30% acrylamide 830 µl, 0.5 M Tris – HCl (630 µl), 10% v/v SDS (50 µl), 10% v/v APS (50 µl), and N,N,N',N'-Tetramethylethylene-diamine (TEMED) (5 µl). Combs were placed in the gel matrix before it set to create wells for sample loading. These volumes were sufficient to make 2 minigels and volumes were adjusted accordingly where a larger volume was required.

2.6.8 1-D SDS-PAGE sample loading and voltages.

SDS-PAGE gels (12.5%, 15% or 20% w/v acrylamide) were immersed in 1X running buffer. Samples varied in sample volume and protein concentration depending on the experiment but were aliquoted using a sterile gel loading tip (Fisher). The gels were electrophoresed at 60V initially and the voltage was increased to 100 V once the protein had travelled sufficiently through the gel. Once the blue tracking dye had moved to the bottom of the gel the gels were transferred to a clean staining dish.

2.7 2-D SDS-PAGE preparation and analysis.

2.7.1 Isoelectric focussing (IEF) Buffer.

The following constituents were dissolved in deionised water and were stored in 2 ml aliquots at -20ºC until required.

- Urea   8 M
- Triton X-100 (BDH)   1% (v/v)
- CHAPS   4% (w/v)
- Tris HCl   10 mM
- Thiourea   2 mM

Prior to use DTT (65 mM) was added to the buffer and solubilised by vortexing

2.7.2 Equilibration Buffer.

- Tris-base   50 mM
- Urea   6 M
- SDS   2% (w/v)
- Glycerol   30% (v/v)

The solution was adjusted to pH 6.8 and aliquoted in 40 ml volumes prior to storage at -20ºC. For equilibration the buffer was modified as either reducing or alkylating. For reduction, DTT (0.02 g ml^{-1}) was added and dissolved thoroughly. For alkylation, IAA (0.04 g ml^{-1}) was dissolved thoroughly in the buffer and a few grains of bromophenol blue were added to the buffer.

2.7.3 Agarose sealing solution (1% w/v).

Agarose (1 g) was dissolved in 100 mls 10X running Buffer and heated until boiling. A few grains of bromophenol blue was added to the mixture and was allowed to cool to hand hot prior to applying to the top of the gel.

2.7.4 Gel preparation for 2-D electrophoresis.

Glass plates were washed thoroughly with warm soapy water, rinsed and dried with 70% ethanol to remove any residual contamination left on the glass. Glass plates were 200 mm wide and were 200 mm in length on the front and 223 mm in length at the back. Gels (12.5 % acrylamide) were poured to approximately 190 mm x 160 mm in surface area and 1.5mm thick

- Tris – HCl (1.5 M)   60 mls

- Deionised water   76 mls

- Acrylamide (30% w/v)   100 mls

- SDS (10% w/v)   2.4 mls

- APS (10% w/v)   1.5 mls

- TEMED   60 µl

2.7.5 2-Dimensional Electrophoresis.

Protein was extracted from haemolymph of *G. mellonella* larvae and 300 µg of protein was obtained via quantification by the Bradford method. The required aliquot of haemolymph was added to 100 µl of IEF buffer and allowed to solubilise fully with regular vortexing for 1 hour. Ampholytes (Immobline 3-10) (2 µl) were added directly to the sample and a further 150 µl of IEF buffer with a few grains of bromophenol blue added was added to the sample. The sample was briefly vortexed and applied to a 13cm Immobline DryStrip pH 3-10 (G.E. Healthcare). Prior to isoelectic focussing the strips were covered in Plus One drystrip cover fluid, (GE Healthcare). Focussing was performed on an Ettan IPGphor II (Amersham Biosciences, NJ, USA) system using the following programme.

1. Step   50 Volts   12 hours

2. Step   250 Volts   15 minutes

3. Gradient   5000 Volts   2 hours

4. Step   5000 Volts   5 hours

5. Gradient   8000 Volts   2 hours

6. Step   8000 Volts   1 hour

Following IEF IPG strips were frozen at -70ºC or were transferred directly to equilibration.

Strips were initially equilibrated in 10 mls reducing equilibration buffer for 15 minutes. Strips were transferred to the alkylation buffer for 15 minutes. Following equilibration IPG strips were rinsed briefly in 1X electrode running buffer. Strips were placed on top of homogenous 12.5% SDS-PAGE gels and sealed with 1% w/v agarose sealing solution. A piece of straw with filter paper inserted was placed to the left of the IPG strip and an aliquot of molecular weight marker was placed into the filter. The top of the gel was sealed with molten cooled sealing solution and allowed to set. The second dimension of protein separation was achieved by placing the gels in ProteanXi-II vertical electrophoresis cells as per manufacturer's instructions. The inner chamber was filled to the top with 5X electrode running buffer while the outer chamber was filled with 2X running buffer. Gels were initially electrophoresed for 1 hour at 40 volts and at 80 volts for the remaining time. Gels were monitored regularly to assess the level of electrophoresis and were transferred to a staining dish which was previously washed with soap and subsequently with 70% ethanol to prevent any contamination to the gels during staining.

2.7.6 Comparative analysis of protein expression.

2-D electrophoresis gel images (In triplicate) were analysed using Progenesis SameSpots software (Nonlinear Dynamics) in order to assess the fold change in protein expression in larvae injected with different treatments. In all experiments untreated larvae (U.T) incubated under the same conditions as injected larvae were used as control. The level of differential expression was analysed by ANOVA with p-values of ≤ 0.05 considered statistically significant for changes in expression.

2.8 General Protein Methodology.

2.8.1 Bradford Protein assay.

Bovine Serum Albumin standards (5 - 60 µg ml^{-1}), were made in 800 µl deionised water and 200 µl Biorad Bradford protein assay reagent (Bio-Rad Munich Germany) and read on a spectrophotometer at 595 nm (Eppendorf Biophotometer). A standard curve was prepared. Bradford protein assay reagent was prepared by diluting the stock 1/5 using deionised water. Protein samples were added (20 µl) to 980 µl of Bradford protein assay reagent. The samples were inverted, allowed to stand for 5 minutes and read spectrophotometrically.

2.8.2 Acetone precipitation of protein samples.

Acetone precipitation was used to concentrate protein from a dilute sample volume. The required volume of protein which corresponded to a calculated quantity of protein from Bradford assay quantification was aliquoted to a pre-chilled micro-centrifuge tube and 100% ice-cold acetone was added to the tube at ratio of 1:3, (sample volume: 100% Acetone) ratio. Protein was left to precipitate overnight at -20ºC and centrifuged at 20,000 x g for 30 minutes at 4ºC to pellet protein.

2.8.3 Colloidal Coomassie staining of 1-dimensional and 2-Dimensional SDS-PAGE gels.

Upon finishing, electrophoresis gels were removed and immediately placed in a fixing solution of 50% (v/v) ethanol and 3% (v/v) phosphoric acid. After this time gels were washed 3 times in deionised water. Gels were pre-incubated for approximately 60 minutes in a solution of 34% (v/v) methanol, 3% (v/v) phosphoric acid and 17% (w/v) ammonium sulphate. After 60 minutes a spatula tip of CBB, (Serva), was spread across the gels and the gels were sealed with parafilm for 3-5 days until maximal staining. At

this point gels were washed several times in deionised water to remove any excess or residual staining.

2.8.4 Coomassie Brilliant Blue (CBB) Stain.

The following constituents were mixed in a sealed Duran bottle and stored in the dark at room temperature.

- Methanol   45% (v/v)

- Deionised Water   44.8% (v/v)

- Acetic acid   10% (v/v)

- CBB (Brilliant Blue)   0.2% (w/v)

2.8.5 Coomassie Destain.

The following constituents were mixed in a sealed Duran bottle and stored in the dark at room temperature.

- Methanol   25%

- Deionised Water   65%

- Acetic Acid   10%

2.9 Western Blotting.

2.9.1 TBS (10X).

One litre of TBS was prepared by dissolving 0.5 M Tris–HCl, (78.7 g in 1 litre), and 1.5 M Tris HCl, (86.65 g in 1 litre). Prior to being made up to 1 litre volume the buffer was adjusted to pH 7.6 using NaOH. The buffer was autoclaved and stored at room temperature.

2.9.2 1X TBS-Tween.

1X TBS-Tween was prepared by adding 100 mls 10X TBS to 900 mls distilled water. Tween–20 (500 µl) was added to the solution and mixed thoroughly. The solution was used immediately.

2.9.3 Transfer Buffer.

Transfer Buffer was prepared by dissolving 3.03 g Tris - Base and 14.4 g Glycine in 1 litre of 20% (v/v) methanol, 80% (v/v) deionised water. The buffer was allowed to dissolve thoroughly with a magnetic stirring bar and was made fresh on the day.

2.9.4 Blocking Buffer.

Blocking buffer was made by dissolving 3 g Dried Skimmed Milk, (Marvel), and 1 g Bovine Serum Albumin, (BSA) in 100 ml 1X TBS-Tween-20. Blocking buffer was stored at 4ºC and used within 24 hours.

2.9.5 DAB Buffer.

DAB buffer was prepared by dissolving 10 mg 3,3' –Diaminobenzidine tetrahydrochloride hydrate in 15 ml 0.1 M Tris Base, (pH 7.6) supplemented with 7 µl Hydrogen Peroxide. DAB buffer was briefly vortexed to ensure complete solubility of DAB powder prior to use.

2.9.6 Western Blotting method.

Transfer of protein onto nitrocellulose paper was performed by electro-blotting using a wet blotter (Mini Trans Blot Cell, Bio-Rad). Prior to transfer filter paper, nitrocellulose paper, (Whatman) were cut to the size of the gel, (approximately 85 x 65

mm), and soaked with sponge inserts for 30 minutes. An assembly was constructed within the gel holder cassettes in the following order;

- Sponge insert

- Whatman paper

- Nitrocellulose membrane

- SDS-PAGE gel

- Whatman paper

- Sponge insert

The SDS-PAGE gels were transferred carefully from the glass plate to the surface of the nitrocellulose membrane to avoid tearing of the gel. The assembly was gently rolled to remove any air bubbles trapped between layers. The cassette was closed and placed into the tank with a cooling block. The apparatus was run at 100 V for 70 minutes and the nitrocellulose membrane was transferred to a staining dish of Ponceau S solution for 5 minutes with gentle shaking to ascertain the quality of the transfer. The nitrocellulose membrane was rinsed in deionised water prior to further processing. The blots were initially blocked in blocking buffer for approximately 1 hour and washed for 2 x ten minute washes in 1X TBS-T. The primary antibody was applied using different dilutions of the antibody stock overnight at 4ºC under mixing conditions. The blots were removed and subjected to 3 x twenty minute washes in 1X TBS-T and transferred to the secondary antibody buffer. Blots were incubated for 2 hours at room temperature with gentle mixing. Blots were washed 3 times in TBS-T for 20 minute intervals changing the 1X TBS-T buffer each time. Blots were washed then developed using ECL or DAB reagents.

2.9.7 Development of reactive protein bands analysis.

Blots were immersed in ECL reagent (Perkin Elmer) for 1 minute and developed on a developing film using a 1/3 dilution of developer, and fixed using a 1/4 dilution of fixer (Kodak).

Alternatively DAB buffer (15 mls) was poured over the Western blot after sufficient washing of the nitrocellulose paper. Immunoreactive bands developed within a few seconds and the buffer was removed. The developed blot was washed 3 times in deionised water.

Reactive protein bands were analysed by densitometry using the Image J image analysis software.

2.10 Peptide extraction from *G. mellonella* haemolymph.

The extraction of peptides from *G. mellonella* larval haemolymph was performed as described by Cytrynska *et al*., (2007).

2.10.1 Extraction (Acid/Methanol) buffer.

Extraction buffer was prepared to a volume of 10 mls by preparing HPLC grade methanol, HPLC grade water and glacial acetic acid in a 9:0.9:0.1 ratio. The buffer was allowed to mix thoroughly and was chilled on ice prior to use.

2.10.2 Peptide Extraction.

Peptide extraction buffer was chilled on ice for approximately one hour prior to use. All micro-centrifuge tubes were pre-chilled on ice to prevent any subsequent breakdown of crude extract or activation of proteolytic cascades.

Larvae (10) were bled into a micro-centrifuge tube and immediately centrifuged at 500 x g for 5 minutes at 4ºC to pellet haemocytes. The supernatant was removed and

transferred to a clean micro-centrifuge tube. The cell free haemolymph was centrifuged at 20,000 x g for 30 minutes to remove any remaining cell debris or tissue present from the original bleed. Haemolymph protein (7.5 mg) was diluted in extraction buffer by diluting 100µl of cell free haemolymph in 900 µl extraction buffer and the contents of the micro-centrifuge tube were mixed thoroughly to precipitate protein. The precipitated proteins were pelleted by centrifugation at 20,000 x g for 30 minutes at 4ºC. The supernatant (900 µl) was transferred to a fresh micro-centrifuge tube and stored at -20ºC overnight and subsequently centrifuged to ensure that all high molecular weight protein was removed from the extraction buffer. Two 400 µl volumes were removed from each treatment and lyophilised until dryness and stored at -70ºC until required.

Extraction samples were resuspended in equivalent volumes of SDS-PAGE solubilisation buffer or apyrogenic water supplemented with 0.1% (v/v) TFA. Samples were subsequently subjected to 1-D SDS–PAGE or RP–HPLC.

For lipid removal the extraction procedure was performed in a method adapted from Schoofs *et al*., (1990). Following reconstitution in 200 µl 0.1% (v/v) TFA the extract was further extracted in an equivalent volume of n-hexane and vortexed at room temperature. The upper lipid fraction was removed and the lower aqueous layer was extracted further with an equivalent volume of ethyl-acetate. The water fraction containing peptides was removed and freeze dried prior to reconstitution with 200 µl 0.1% (v/v) TFA and HPLC analysis.

2.11 Organic extraction from *A. fumigatus* infected larvae and *Aspergillus* culture filtrates.

2.11.1 Mycotoxin extraction from *G. mellonella.*

Extraction of mycotoxins from *G. mellonella* was performed using an adaptation of the method of Reeves *et al*., (2004). Larvae (25) were chosen at random and were

crushed in a pestle and mortar using liquid nitrogen. The crushed larvae were transferred to a 5 ml volume of 6 M HCl. The pestle and mortar was washed with two 10 ml volumes 6 M HCl. Chloroform (50 ml) was added and the mixture was incubated with constant mixing at room temperature for 30 minutes. The mixture was centrifuged at 500 x g for 5 minutes and the chloroform layer was transferred to a separate tube. The chloroform extraction step was repeated with 50 ml chloroform and all organic extracts were pooled. Chloroform was evaporated at 37ºC using a rotary evaporator (Buchi rotavapor–R) which resulted in an insoluble lipid pellet (approximately 1 ml). This lipid pellet was extracted with four 1 ml volumes of HPLC grade methanol and these extracts were pooled and concentrated to a volume of 200 µl. Prior to RP-HPLC analysis all samples were vortexed briefly followed by centrifugation at 10,000 x g to separate out any particulate matter and transferred to a HPLC sample vial.

2.11.2 Mycotoxin extraction from *A. fumigatus* culture filtrates.

Culture filtrates were extracted using mira-cloth to exclude cellular fragments followed by filtration through a 0.22 µm filter. The culture filtrates were organically extracted for 2 hours in a 50ml sterile tube at room temperature at a 1:1 ratio using chloroform to extract fungal metabolites. Following centrifugation at 500 x g for 10 minutes the lower chloroform layer was removed and transferred to a fresh tube. Chloroform was evaporated at 37ºC using a rotary evaporator (Buchi rotavapor – R) and the toxin pellet was resuspended in 200 µl HPLC grade methanol.

Prior to RP-HPLC analysis all samples were vortexed briefly followed by centrifugation at 10,000 x g to separate out any particulate matter and transferred to a HPLC sample vial.

2.12 Reverse Phase High Performance Liquid Chromatography (RP–HPLC).

2.12.1 Buffer A.

HPLC grade water (1 L) was placed in a darkened Duran bottle. 1 ml Triflouroacetic acid (1 ml) was added and the bottle was inverted to mix the contents thoroughly. This was made fresh on the day

2.12.2 Buffer B.

HPLC grade Acetonitrile (1 L) was placed in a clear Duran bottle. Triflouroacetic acid (1 ml) was added to the bottle and the bottle was inverted to mix the contents thoroughly. This was made fresh on the day.

2.12.3 RP-HPLC analysis of mycotoxins.

Organic extracts were analysed by reverse phase HPLC with multiple wavelength detection at 254, 351, and 220 nm Agilent 1200 series using a C-18 RP-HPLC column at a flow rate of 1 ml min^{-1}. The elution profile (Figure 2.2 A), was 5 min of Buffer B (Section 2.12.2) at 5% followed by a linear gradient mobile phase with Buffer B to 100% acetonitrile for 20 minutes. The column was eluted fully with 100% Buffer B for 3 minutes and was re-equilibrated with 95% Buffer A (Section 2.12.1), 5% Buffer B for 5 minutes prior to further analysis. The concentration of gliotoxin or fumagillin in a given sample was ascertained by performing standards with commercially available mycotoxins resuspended and diluted in HPLC-grade methanol (Figures 2.3 and 2.4).

2.12.4 RP-HPLC analysis of haemolymph peptides and peak fractionation.

Peptide samples which had been lyophilised and resuspended in HPLC grade water supplemented with 0.1% formic acid were initially loaded in a 10 µl volume on a

(A)

Time (mins)

(B)

Time (mins)
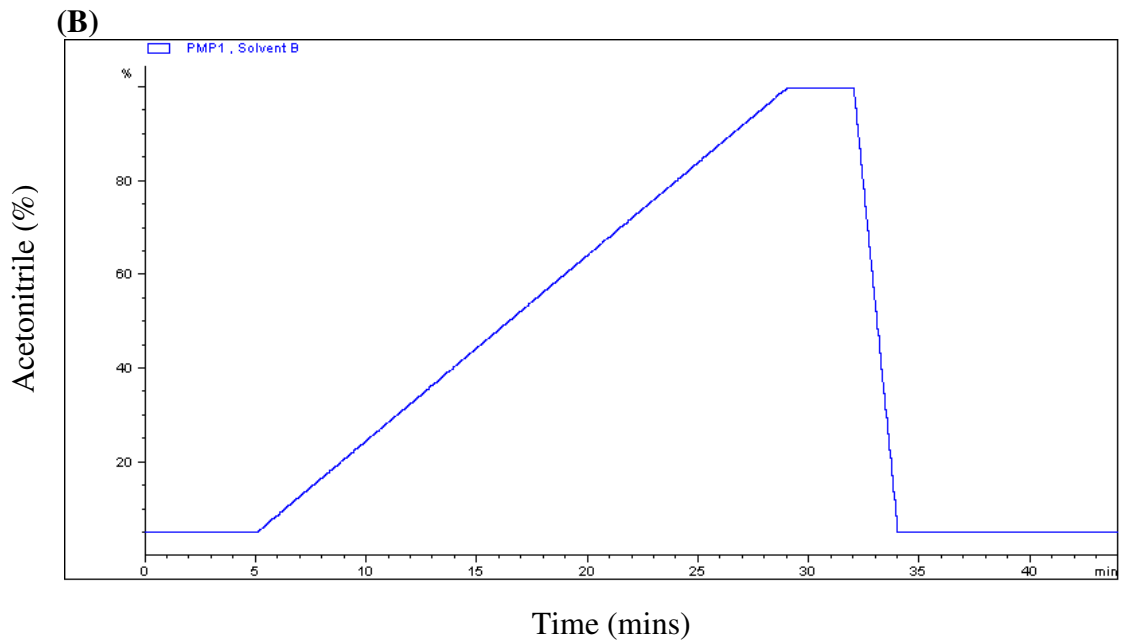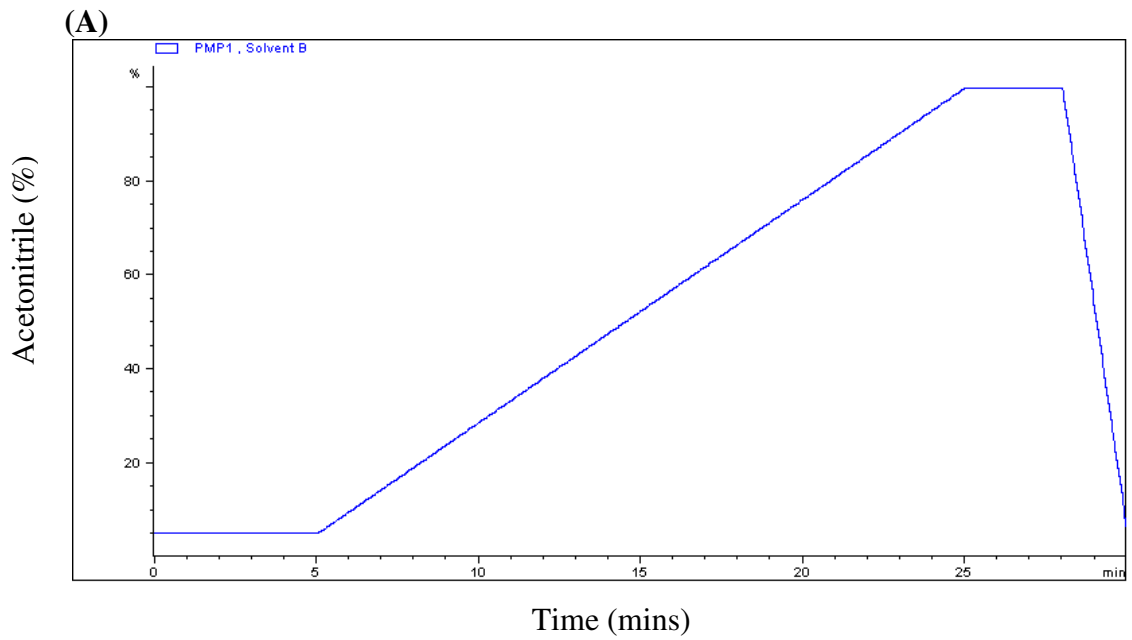
Figure 2.2 RP-HPLC acetonitrile gradient profiles.
Acetonitrile increased as a gradient from 5% acetonitrile after 5 minutes to 100% acetonitrile after 25 minutes during culture filtrate mycotoxin analysis, **(A)**. For analysis of *G. mellonella* haemolymph peptides **(B),** the gradient was between 5 and 29 minutes and was fully eluted for 3 minutes before allowing the column to re-equilibrate

Time (mins)
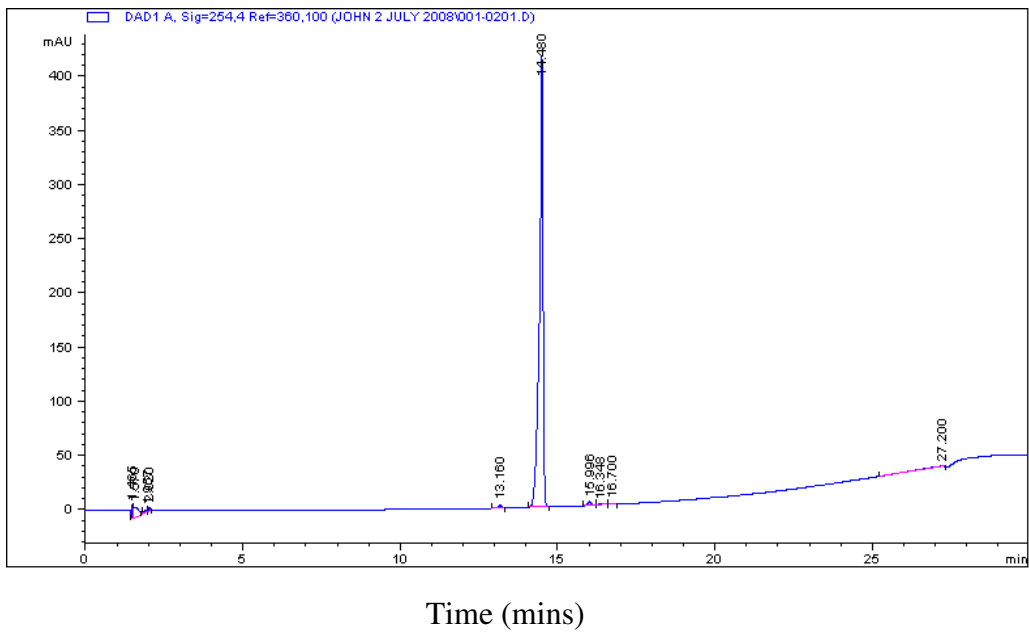
Figure 2.3 Detection of purified gliotoxin by RP-HPLC.
Gliotoxin detection was performed at 254 nm with a retention time of approximately 14.48 minutes. Image shows gliotoxin detection of 4 µg 20µl^{-1}.

Time (mins)
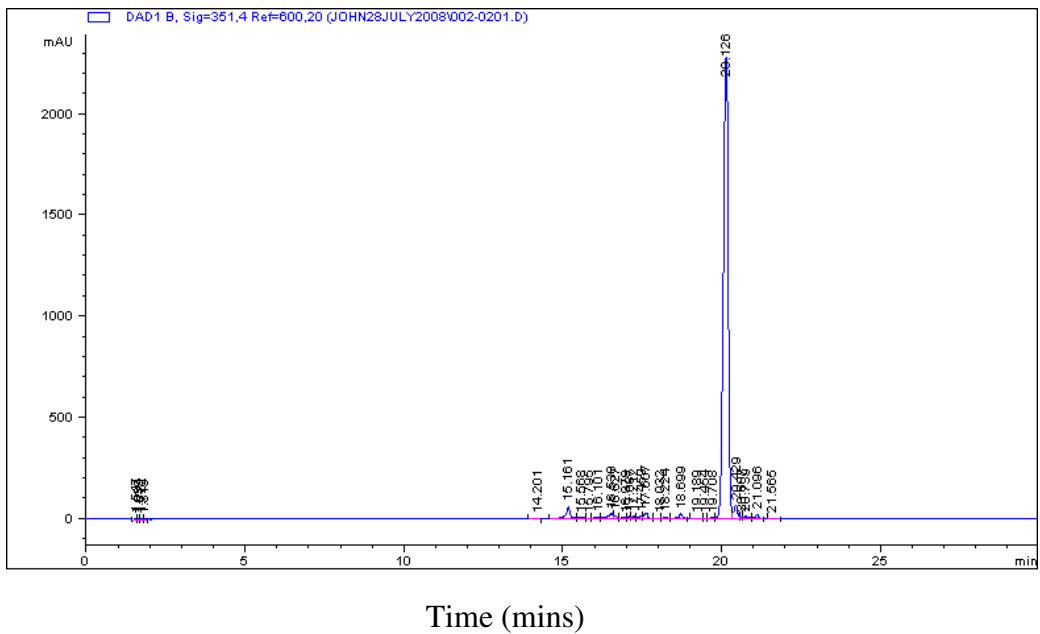
Figure 2.4 Detection of purified fumagillin by RP-HPLC.
Fumagillin detection was performed at 351 nm with a retention time of approximately 20.130 minutes. Image shows detection of 4 µg 20µl^{-1}.

C-18 Agilent HPLC column as before with diode array detection at 254, 220, and 280nm. Samples were maintained at 4ºC in a thermally controlled sample tray to prevent peptide breakdown. The elution profile, (Figure 2.2 B) was 5 min of Buffer B at 5% followed by a linear gradient mobile phase with Buffer B to 100% acetonitrile for 24 minutes. The column was eluted fully with 100% Buffer B for 3 minutes and was re-equilibrated with 95% Buffer A, 5% Buffer B for 15 minutes prior to further analysis.

For peak fractionation a 100 µl aliquot of sample was loaded onto the column and fractionated by individual peak. Peak volumes were collected in glass vials on a sample collection tray and were stored at -20ºC prior to lyophilisation and tryptic digestion.

2.13 In-gel trypsin digestion and LC/MS analysis.

2.13.1 Preparation of micro-centrifuge tubes.

All micro-centrifuge tubes were washed by vortexing and sonication in 100% acetonitrile prior to use. Acetonitrile was discarded prior to using the tube. Scalpels and pipette tips were immersed and sonicated in 2 x volumes of methanol prior to use to remove keratin or dust contamination.

2.13.2 Trypsin digestion of 2-D SDS-PAGE protein spots for LC/MS analysis.

Processing of bands and spots for LC-MS analysis was achieved by following the method of Shevchenko *et al.,* (2006). Gel pieces were cut and transferred to a micro-centrifuge tube and all residual water was removed. Gel pieces were sliced to approximately 1-2 mm max in thickness using a scalpel where required to ensure full exposure to the de-staining and digestion process. Gel pieces were de-stained by the addition of 100 mM Ammonium bicarbonate: Acetonitrile 1:1. The samples were vortexed occasionally and left on a rotating table to allow mixing of the sample. In

some cases gel pieces had not de-stained thoroughly within the first 30 minutes so all the buffer was removed and fresh buffer was added for another 30 minutes until destaining was complete.

Acetonitrile (100 – 200 µl) was added to the sample until the gel pieces shrunk and became white. At this point the acetonitrile was removed and the sample was stored at -20ºC or processed further immediately. Tryptic digestion was achieved with the addition of approximately 50 µl of trypsin buffer, (13 ng µl^{-1} trypsin enzyme prepared in 10 mM ammonium bicarbonate, 10% (v/v) acetonitrile). At this point all samples were placed on ice to prevent trypsin auto-digestion and allow for the total penetration of trypsin buffer into the gel piece. After 30 minutes all samples were checked and more trypsin buffer was added where necessary. After a further 60 minutes all samples were checked for rehyration in the presence of trypsin buffer and were topped with 10 mM ammonium bicarbonate/10% (v/v) acetonitrile to prevent drying out. At this point all samples were transferred to an incubator held at 37ºC overnight.

Following digestion the samples were centrifuged and the supernatant was transferred to clean micro-centrifuge tube. The original gel piece was extracted further with 5% Formic Acid: 100% Acetonitrile in a 1:2 ratio. Samples were incubated at 37ºC in a rotary shaker and all supernatant was transferred to the tryptic digest supernatant. All samples were dried in a vacuum centrifuge and resuspended with sonication in 10-20 µl 0.1% formic acid. All digests were passed through 0.22 µM cellulose acetate filter tubes and transferred to mass spectrometry vials. Analysis of digested peptides was achieved using an Agilent 6340 Ion Trap LC/MS using acetonitrile elution.

2.13.3 Trypsin digestion of 1-D SDS-PAGE protein bands for LC/MS analysis.

Protein bands of interest resolved by 1-D SDS-PAGE were excised from the gel using a sterile scalpel and immediately transferred to a clean tube where the gel was

chopped into approximately 1 x 2 mm pieces. Ammonium bicarbonate (100 mM/acetonitrile, (1:1) was added to the gel pieces to destain and incubated at room temperature for one hour with mild agitation and occasional vortexing. All the liquid was removed and 10 mM DTT (made using 100 mM ammonium bicarbonate) was added to cover the gel piece and incubated at 56ºC for one hour. The liquid was removed and 50 mM IAA solution (made using 100 mM ammonium bicarbonate) was added to the gel piece for 30 minutes at room temperature. The IAA buffer was removed and 100 mM ammonium bicarbonate was added to each tube and incubated at 37ºC for 15 minutes. The buffer was removed and the gel pieces were shrunk with 100% acetonitrile and digested with trypsin as described for the extraction of peptide digests for 2-D electrophoresis. Analysis of digested peptides was achieved using an Agilent 6340 Ion Trap LC/MS using acetonitrile elution according to the following:

Time (mins)	% Acetonitrile
0	5
1	5
7	70
8	100
9	100
10	5

2.13.4 Bioinformatic analysis of LC/MS results.

Resulting data were analysed using the mascot search engine, (www.matrixscience.com). The mass error tolerance was 1 Da allowing for a maximum of no more than two missed cleavages with a MASCOT score cutoff of 62. Verification of protein sequences was confirmed by blasting the protein sequence on the Uniprot,

(www.uniprot.org), and NCBI, (www.ncbi.nlm.nih.gov), databases. Identified proteins are listed by their gi number as accessed through www.ncbi.nlm.nih.gov. For comparative analysis of sequence similiarity between different proteins the ClustalW-multiple sequences alignment tool (accessed through www.ebi.ac.uk/Tools/msa/clustalw2/) was used.

2.14 Purification of neutrophils from fresh blood.

2.14.1 Heparin.

Sterile PBS (2.5 mls) was added to a 2.5 KU tube of Heparin sodium salt, Grade I-A from porcine intestinal mucosa and was kept in the fridge or on ice prior to use.

2.14.2 Saline solutions (1X and 2X).

Saline (1X) was prepared by dissolving 9 g of NaCl to 1 litre of deionised water. Saline (2X) was prepared by dissolving 18 g of NaCl to 1 litre of deionised water. Both solutions were autoclaved and used at room temperature.

2.14.3 Dextran 10% (w/v).

Dextran 10% (w/v) was prepared by adding 20 g of Dextran Sulphate to 180 mls 1X Saline, (1.62 g NaCl in 180 mls deionised water). The solution was allowed to dissolve thoroughly on a magnetic stirrer. Once dissolved the solution was filter sterilised through a 0.22 µm filter and kept at room temperature.

2.14.4 5 mM PBS – Glucose (PBS-G).

PBS – Glucose (5 mM) was prepared by dissolving 0.9 g of glucose in 1 L of PBS, and was filter sterilised through a 0.22 µM filter and kept at room temperature.

2.14.5 Purification of neutrophils from fresh blood (method).

1X and 2X Saline and 10% Dextran solution were supplemented with 50 µl Heparin solution/100 mls of buffer. Blood was obtained from consenting healthy donors and collected in heparinised tubes to prevent coagulation. Initially the majority of red blood cells were allowed to sediment by mixing 20 mls of the fresh blood with 25 mls of 1X Saline and 5 mls 10% Dextran solution in a 50 ml tube. The contents of the tube were allowed to sediment and the supernatant was transferred to a fresh tube. The supernatant was under-laid with 5 mls of Lymphoprep (Axis Shield) and centrifuged at 500 x g for 10 minutes. The supernatant was discarded and 25 mls of deionised water was added to the tube. Following a brief mixing step a further 25 mls of heparinised 2X solution was added to the tube to prevent lysis of neutrophils. Following centrifugation at 200 x g for 5 minutes at room temperature the supernatant was removed and the neutrophil pellet was resuspended in 5 mM PBS-Glucose. Neutrophils were counted and tested for viability using the Trypan Blue exclusion assay.

2.14.6 Trypan Blue exclusion assay.

The trypan blue assay was used to ascertain the viability of neutrophils and haemocytes, (Eichner *et al*., 1986). The basis of the assay is that unhealthy cells will not form an intact membrane and form pores which allow the leakage of the dye into the cell which can be observed under light microscopy. Cells were suspended in trypan blue at the following volumes: 20 µl cells, 60 µl PBS and 20 µl 0.4% (v/v) Trypan Blue solution, (made by dissolving trypan blue powder in PBS and sterilising through a 0.22 µm filter). Following 3 minutes standing at room temperature the cells were counted and recorded with regard to the percentage viability of the cell suspension.

2.15 Extraction of haemocytes from *G. mellonella* haemolymph and quantification of haemocyte density.

2.15.1 Insect Physiological Saline (IPS).

IPS was made by dissolving the following in 800 mls of deionised water; 8.76 g Sodium Chloride, 0.36 g Potassium Chloride, 15.76 g Tris HCl, 3.72 g EDTA, and 4.72 g Sodium Citrate. The solution was allowed to mix but was adjusted to pH 6.9 to ensure solubility of EDTA. The solution was brought up to 1 litre with deionised water and was filter sterilised through a 0.22 µm filter and stored in 50 ml tubes at room temperature until required.

2.15.2 Extraction of haemocytes from *G. mellonella* larvae.

Larvae were bled through the head with a sterile 23G needle head and haemolymph was squeezed through the head region into 10mls of IPS. Haemocytes were pelleted by centrifugation at 500 x g for 5 minutes and washed in 5 mls IPS to remove any excess haemolymph. Following a further centrifugation step at 500 x g for 5 minutes, the remaining supernatant was removed and the pelleted cells were resuspended gently in 5 mM PBS-Glucose.

2.15.3 Haemocyte density from *G. mellonella* larval haemolymph.

Larvae were bled through the anterior region into a pre-chilled micro-centrifuge tube with a few grains of phenyl-2-thiourea (PTU) to prevent melanisation. A 10 µl aliquot of haemolymph was diluted by a factor of ten in ice cold PBS and the number of cells was counted using an improved Neaubeur haemocytometer. The haemocyte density was expressed per µl of haemolymph.

2.16 Phagocytic cell mediated microbial killing assays.

2.16.1 Neutrophil mediated killing using *C. albicans* as a target.

Neutrophils (1×10^7 ml^{-1}), were isolated as described previously, (Section 2.14.5), and were incubated in the presence of fumagillin, (1 or 2 µg ml^{-1}) for 25 minutes. A solvent control using the equivalent solvent concentration used in the highest concentration of fumagillin was used to assess the effect of solvent on neutrophils. Neutrophils were washed twice with 5 mM PBS-G. *C. albicans* MEN, (5×10^6 ml^{-1}) were obtained from a stationary phase overnight culture and were opsonised in PBS supplemented with pooled human serum, (1/50 dilution in PBS) for 45 minutes at 37ºC. C. *albicans* cells were washed twice in sterile PBS to remove any non-specifically bound serum protein following opsonisation and pelleted at 500 x g. *C. albicans* cells and neutrophils were mixed in a 1 ml volume and immediately transferred to a sterile thermostatically controlled stirred chamber in a 2:1 ratio. A 100 µl aliquot was immediately removed from the chamber (t = 0) and serially diluted in ice cold MEM to quench phagocytosis. The concentration of yeast cells was diluted to give 100 cells in a 100 µl volume which was then spread on a sterile YEPD agar plate supplemented with erythromycin (Section 2.3.1). Plates were incubated overnight at 30ºC. The percentage of viable cells and subsequent reduction in viability was calculated based on the number of colony forming units at t = 0 and any subsequent changes in viability were expressed as a percentage of the t = 0 value.

2.16.2 Haemocyte mediated killing using *Candida albicans* as a target.

Haemocytes (5×10^6) were extracted from *G. mellonella* (Section 2.15.2) and incubated in the presence of fumagillin, (2 µg ml^{-1}) or with a solvent control (0.002% (v/v) methanol) for 25 minutes at 37ºC. The cells were pelleted by centrifugation and washed twice in IPS. *C. albicans* (2.5×10^6 ml^{-1}) were taken from a stationary phase

culture and incubated in the presence of a 1/10 dilution of cell free haemolymph to opsonise the target. *C. albicans* cells were pelleted by centrifugation at 500 x g for 5 minutes and resuspended in a 1 ml volume in the presence of pre-treated haemocytes and transferred directly to a thermostatically controlled stirred chamber. The assay was performed in the same manner as section 2.15.2. The percentage of viable cells and subsequent reduction in viability was calculated based on the number of colony forming units at t = 0 calculated as 100% viability and any subsequent changes in viability were expressed as a percentage of that value.

2.16.3 Neutrophil mediated killing using *P. aeruginosa* as a target.

P. aeruginosa PA01 cells were grown as per section 2.4.3 and diluted to a concentration of 5×10^6 ml^{-1} in 1 ml PBS supplemented with pooled human serum (2% v/v). The *P. aeruginosa* suspension was mixed at 37ºC for 25 minutes to opsonise the bacteria. Neutrophils (1×10^7) were prepared and pre-treated in the presence of fumagillin in the same manner as described in section 2.15.1, and were pelleted. The opsonised bacterial suspension, (containing 5×10^6 ml^{-1} bacterial cells), was transferred to the neutrophil pellet and resuspended by gently mixing with a Pasteur pipette. The neutrophil/opsonised bacterial suspension was transferred to a closed 50 x 12 mm specimen tube (Samco). A 100 µl aliquot was immediately removed from the tube and serially diluted in ice cold PBS to a concentration of 1×10^3 cells ml^{-1} to quench phagocytosis at t = 0. The tubes were mixed by gentle rotation on a daisy wheel and a 100 µl aliquot was withdrawn at 30 and 60 minutes and serially diluted in the same manner. Cells (100 µl) were spread on *P. aeruginosa* isolation agar (Section 2.4.2). Plates were incubated overnight at 37ºC and the percentage of viable cells and subsequent reduction in viability was calculated in the same manner as Section 2.16.1.

2.16.4 Neutrophil and haemocyte mediated phagocytosis of a pathogenic target.

In order to examine the effect of the phagocytic ability of neutrophils or haemocytes *A. fumigatus* conidia were used as a target as conidia could be easily visualised by microscopy. Neutrophils or haemocytes (5×10^6) were isolated and incubated in the presence of 2 µg ml^{-1} fumagillin or a solvent control, (0.002% v/v methanol) for 25 minutes. Cells were pelleted and incubated with 1×10^7 conidia which were incubated in the presence of a 1/10 dilution or serum or cell-free haemolymph to a final volume of 1 ml. The mixture was transferred to a thermally controlled chamber and a 100 µl aliquot was removed from the chamber at 0, 30, 60, and 120 minutes and serially diluted in ice cold minimal essential medium to quench subsequent phagocytosis. Three hundred phagocytic cells were observed by microscopy using a Neaubeur haemocytometer and the number of cells which clearly showed internalised conidia was recorded over this time. The internalisation of conidia was verified by making adjustments to the microscope focus as internalised conidia are not visible at the same focus as external conidia.

2.17 Degranulation and MPO activity analysis.

2.17.1 Neutrophil /haemocyte cell degranulation.

Neutrophils or haemocytes, (5×10^7), were pre-treated for 25 minutes in the presence of fumagillin at 37ºC. Neutrophils were centrifuged at 500 x g for 5 minutes to pellet the cells and this pellet was washed twice to remove excess fumagillin. Cells were finally resuspended in 1 ml 5 mM PBS-G supplemented with protease inhibitors, (10 µg/ml leupeptin, pepstatin A, aprotinin, and TLCK). Cells were transferred to a thermostatically controlled stirred chamber and were stimulated with 1 µg ml^{-1} PMA. After 6 minutes the contents of the chamber were transferred to a pre-chilled tube and were centrifuged at 700 x g for 5 minutes with soft acceleration and braking to prevent

cell rupture. The supernatant was removed and transferred to a pre-chilled tube. The samples were analysed for protein concentration using the Bradford assay, and were concentrated by acetone precipitation prior to electrophoresis and immunoblotting

2.17.2 Myeloperoxidase peroxidation activity assay.

Quantification of MPO activity was ascertained using a standard activity assay Invitrogen EnzCheck® and used according to manufacturer's specifications. Neutrophils were stimulated and protein was extracted as previously described (Section 2.17.1). A 50 µl aliquot of degranulated protein sample (standardised to 10 µg protein following quantification by Bradford assay) was assessed for peroxidation activity by addition to 100 µl 2X Amplex® Ultra red reagent. The non-fluorescent substrate is oxidised by H_2O_2 redox intermediates of MPO to form a florescent product. Florescence intensity was read at excitation 530 nm and emission at 590 nm. All measurements were calculated with the absorbance for the blank subtracted from all values. A standard curve of activity was calculated based on the MPO standard supplied by the manufacturer. Fluorescence was recorded using a Synergy HT micro-plate reader (BioTek).

2.18 Cellular fractionation of neutrophils and haemocytes, determination of oxygen consumption and quantification of F-actin assembly.

2.18.1 Breaks Buffer.

Breaks Buffer (10 mM KCl, 3 mM NaCl, 4 mM $MgCl_2$ and 10 mM PIPES) was adjusted to pH 7.2 and was filter sterilised prior to use using 0.22 µM filter. Prior to use Breaks Buffer was chilled on ice and protease inhibitors were added, (10 µg ml^{-1} leupeptin, pepstatin A, aprotinin, and TLCK).

2.18.2 Neutrophil membrane solubilisation buffer.

Neutrophil membrane solubilisation buffer (2% v/v Triton X–100, 150 mM NaCl, 1% w/v Sodium deoxycholate) was made using Breaks Buffer to solubilise. The reagents were dissolved and filter sterilised with a 0.22 µM filter. Prior to use neutrophil membrane solubilisation buffer was chilled on ice and protease inhibitors were added, (10 µg ml^{-1} leupeptin, pepstatin A, aprotinin, and TLCK).

2.18.3 Haemocyte membrane solubilisation buffer.

A minimal volume of Breaks Buffer was supplemented with 36 mM CHAPS and allowed to dissolve with continuous mixing for 30 minutes. The buffer was filter sterilised, chilled on ice and protease inhibitors were added, (10 µg ml^{-1} leupeptin, pepstatin A, aprotinin, and TLCK).

2.18.4 Fractionation of neutrophils.

Neutrophils were fractionated using an adaptation of the method of Renwick *et al.*, (2007). Neutrophils, (1 x 10^8), were incubated in the presence of fumagillin, (2 µg ml^{-1}) and incubated with regular mixing at 37ºC for 25 minutes. Cells were pelleted at 500 x g for 5 minutes and washed twice in 5 mM PBS-G to remove excess mycotoxin. Neutrophils were stimulated with PMA (1 µg ml^{-1}), and allowed to mix gently at 37ºC for 6 minutes. Neutrophils were immediately pelleted by centrifugation at 500 x g for 5 minutes and resuspended in Breaks Buffer, with protease inhibitors added. Neutrophils were lysed using a sonication probe (Bendelin Senopuls), three times for 10 seconds at 20% power and centrifuged at 200 x g for 10 min at 4ºC.

The post nuclear supernatant was transferred to pre-chilled Beckman SW60 Ti rotor and centrifuged at 100,000 x g for 60 minutes at 4ºC to separate membrane and pellet fractions. Tubes were carefully transferred to ice and the supernatant (cytosolic

fraction) and pellet (membrane fraction) were separated into pre-chilled micro-centrifuge tubes. The membrane pellet was washed twice in Breaks Buffer with centrifugation at 20,000 x g for 5 minutes at 4ºC to remove any cytosolic residual protein. The washed membrane pellet was solubilised for 60 minutes at 4ºC with mild agitation in membrane solubilisation buffer, (Section 2.18.2). The solubilised membrane fraction was centrifuged at 7,000 x g for 10 minutes at 4ºC to pellet any debris. Cytosolic and membrane protein concentration was assessed using the Bradford assay and protein was concentrated by acetone precipitation prior to 1–D SDS-PAGE.

2.18.5 Fractionation of haemocytes.

Haemocytes, (1 x 10^8) were extracted from *G. mellonella* larvae, and were incubated in the presence of 2 µg fumagillin ml^{-1}. Cells were stimulated, pelleted, washed, and lysed in the same way as neutrophils, (Section 2.18.4). The post nuclear supernatant was transferred to a pre-chilled Beckman SW60 Ti rotor and centrifuged at 100,000 x g for 60 minutes at 4ºC to separate membrane and pellet fractions. Tubes were carefully transferred to ice and the supernatant (cytosolic fraction) and pellet (membrane fraction) were separated into pre-chilled micro-centrifuge tubes. The membrane pellet was washed twice in Breaks Buffer with centrifugation at 20,000 x g for 5 minutes at 4ºC to remove any cytosolic residual protein. Membrane pellets were solubilised with the addition of 500 µl haemocyte membrane solubilisation buffer and gentle mixing for 45 minutes. The membrane pellet was placed in a sonication bath for five 1 minute intervals with cooling on ice in between. The solubilised membrane fraction was centrifuged at 7,000 x g for 10 minutes and the supernatant was transferred to a pre-chilled micro-centrifuge tube. Cytosolic and membrane protein content was assessed using the Bradford assay. Protein was concentrated using acetone precipitation prior to 1–D SDS-PAGE.

2.18.6 Determination of oxygen consumption of neutrophils and haemocytes.

Oxygen consumption was measured using a Clarke type oxygen electrode. The reaction chamber was thermostatically controlled at 37ºC. The electrode was covered in saturated Potassium chloride and a semi-permeable layer of plastic was placed over the detection electrode. The upper chamber sat on top of this layer and was tightened down. The level of oxygen from 100% to 0% was calibrated by the addition a few grains of sodium dithionate.

Neutrophils, (1×10^7 ml^{-1}), and haemocytes, (5×10^7 ml^{-1}), were incubated in the presence of fumagillin for 35 minutes and washed twice in sterile PBS-G. Cells were resuspended in 1 ml of PBS-G and immediately transferred to the electrode chamber. Cells were allowed to stabilise for approximately 3 minutes before stimulation with addition of 1 µg ml^{-1} PMA, (Figure 2.3). The rate of oxygen consumption was observed over a ten minute period and the consumption of oxygen was calculated based on the following equation.

$$\frac{\text{Units travelled in x minutes} \times 230}{\text{Total units}} = \text{nmol ml}^{-1} \text{ oxygen consumed}$$

- x is the number of minutes passed since PMA stimulation.

- 230 is the molarity of dissolved oxygen present in water at 37ºC

- Total units are the distance in small squares on the graph paper which covered the span of 0–100% oxygen availability.

2.18.7 Quantification of F-actin assembly.

To investigate the effect of fumagillin on F-actin assembly neutrophils or haemocytes (5×10^7 ml^{-1}) were incubated in the presence fumagillin (2 µg ml^{-1}) or the 0.002% (v/v) methanol for 25 minutes and then stimulated with 1µg PMA ml^{-1} for 6
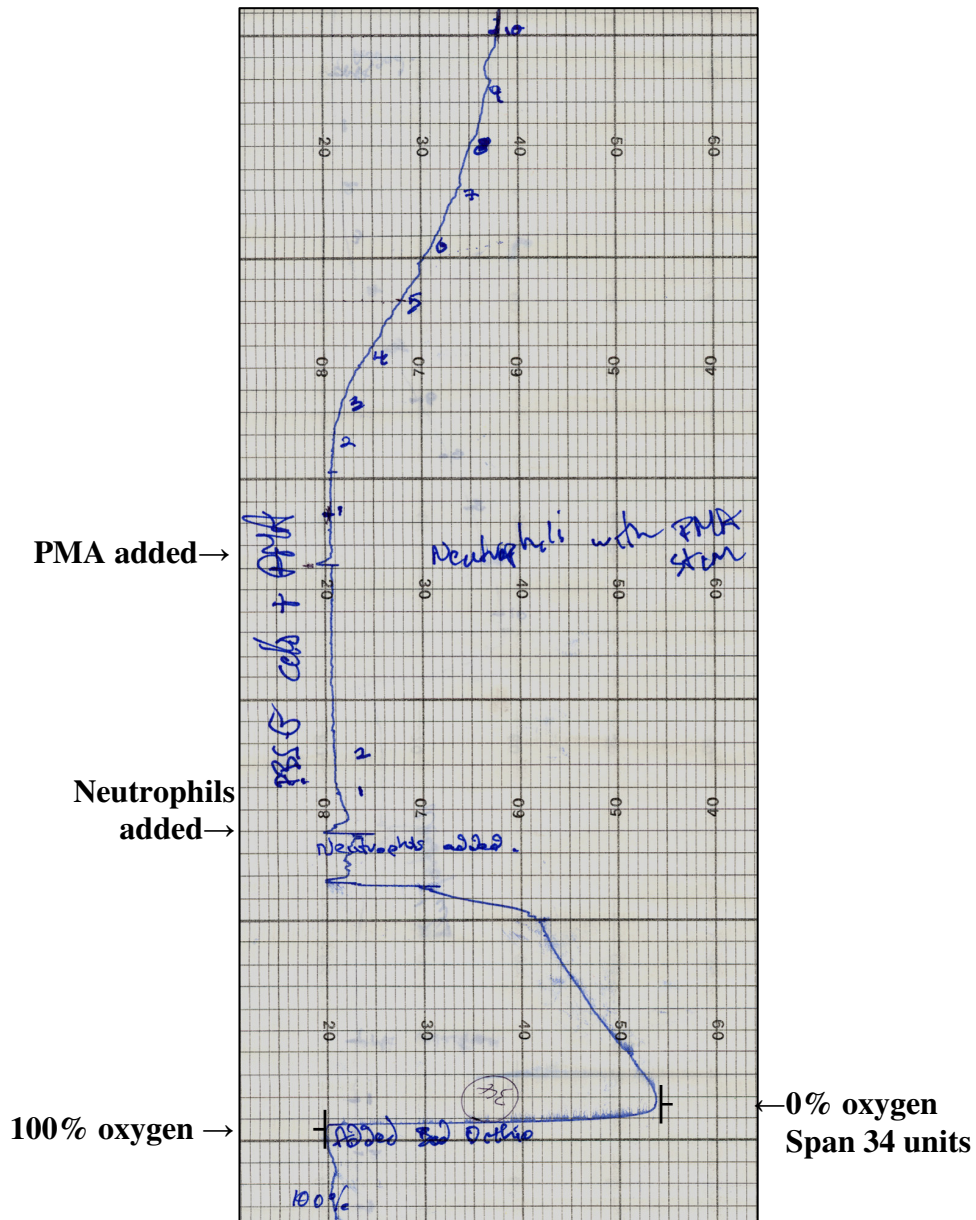
Figure 2.5 Calibration of oxygen consumption using a Clarke-oxygen electrode.
The image above represents the span of oxygen at 100% oxygen and with the addition
of sodium dithionate oxygen drops to 0% giving a span of units for the consumption of
oxygen over time. The chamber is washed with 5 mM PBS-G and neutrophils are added
and allowed to adjust to the chamber for a few minutes. PMA (1 µg ml $^{-1}$) was added
and the amount of oxygen consumed was monitored over a ten minute period

minutes and washed and lysed as per section 2.18.4. The supernatant was transferred to a pre-chilled micro-centrifuge tube and protein was concentrated using acetone precipitation prior to electrophoresis and immunoblotting.

2.19 Confocal Immunoflouresence microscopy.

2.19.1 Confocal microscopy of neutrophil translocation and degranulation.

Neutrophils (1×10^6 ml^{-1}) were pre-incubated in the presence of fumagillin and washed twice in sterile PBS-G. Cells were allowed to adhere to saline-preparation slides for 5 minutes at room temperature in a sterile laminar flow hood. Cells were incubated at 37ºC in the presence of 1 µg PMA ml^{-1} for 6 minutes. Cells were washed twice in PBS and fixed in 4% paraformaldehyde for 10 minutes. Cells were washed twice and permeabilised in 0.2% Triton X-100. Cells were blocked for 1 hour in 10 mM NaBH$_4$ for one hour and washed with PBS prior to incubation with anti-p47^{phox} or anti MPO antisera at a dilution of 1/2500 or 1/650 respectively. Following overnight incubation the slides were washed in PBS and incubated with tetramethyl rodamine iso-thiocyanate (TRITC) goat anti-rabbit IgG secondary antibody (The Jackson Laboratory, Bar Harbour, Maine, USA) for 1 hour in the dark at a concentration of 1/3000 against anti-p47^{phox} and 1/1000 for anti-MPO probed slides. Cells were washed again and a cover-slip was placed over the cell area. The perimeter of the cover-slip was sealed to prevent the slides from drying out. The controls for this experiment included cells alone and cells exposed to secondary antibody. Cells were viewed with an Olympus Flouview 1000 confocal microscope under the supervision of Dr. Ica Dix.

2.19.2 Observation of neutrophil F-actin assembly.

For observation of changes in F-actin distribution cells were pre-treated, fixed, and permeabilised in the same manner as previously described (Section 2.19.1) however

there was only one probing step whereby cells were stained with 0.8 µM rhodamine coupled phalloidin overnight at 4ºC and washed 3 times in PBS. Cells were viewed with an Olympus Flouview 1000 confocal microscope under the supervision of Dr. Ica Dix.

2.20 *G. mellonella* haemolymph activity assays.

2.20.1 Cacolydate Buffer supplemented with L-DOPA.

Sodium Cacolydate (2.14 g), NaCl (26.298 g), $CaCl_2$ (1.12 g), $MgCl_2$ (2.39 g) was added to 800 mls deionised water. The solution was adjusted to pH 6.5 and made up to 1 L using deionised water. The solution was autoclaved and stored at room temperature. L-3,4-Dihydroxyphenylalanine (L-DOPA) (150 mg) was added to cacolydate buffer (50 mls) to give a working concentration of 3 mg/ml. The solution was wrapped in aluminium foil to prevent light degradation and left to mix for approximately 1 hour or until all the L-DOPA was solubilised.

2.20.2 Phenoloxidase Activity using *G. mellonella* haemolymph.

Phenoloxidase activity was determined by an adaption of the method used by Liu *et al*., 2004. After the relevant treatment and duration of incubation 10 larvae were bled into a pre-chilled micro-centrifuge tube. Following centrifugation at 800 x g for 2 minutes at 4ºC the cell free extract was removed to a fresh pre-chilled tube. The cell free haemolymph protein extract (1.5 mg protein approximates to 20 µl of cell free haemolymph as ascertained by Bradford assay) was added to 1 ml cacolydate buffer supplemented with L-DOPA in a plastic cuvette and inverted briefly. The mixture was left to stand for 3 minutes at room temperature and was read spectrophotometricaly at 490 nm.

2.20.3 Comparative analysis of protein binding of haemolymph proteins to conidia.

Conidia were isolated as previously described and were concentrated to a pellet of 5 x 10^8 cells. *G. mellonella* larvae were bled through the anterior region into a pre-chilled micro-centrifuge tube and centrifuged immediately at 800 x g for 2 minutes at 4°C. A 150 µl aliquot of cell free haemolymph was removed and quantified for protein concentration by Bradford assay. Approximately 100 µl of cell free haemolymph (approximately 7.5 mg of protein) standardised to contain the same amount of protein in the assay for comparative analysis was diluted in ice-cold IPS and transferred to the conidia to resuspend the pellet. The 5 x 10^8 conidia/haemolymph suspension was mixed gently for 40 minutes at 4°C and immediately centrifuged at 500 x g for 5 minutes to pellet conidia. Conidia were washed 3 times with 1 ml sterile IPS to remove any non-specifically bound proteins and a 10 µl aliquot was withdrawn and enumerated by haemocytometry to ensure that no loss of conidia had occurred. Protein was then lysed from the conidial surface by the addition of 5X solubilisation buffer and brief sonication in a water bath followed by boiling at 95°C for 5 minutes. The samples were centrifuged at 1000 x g for 5 minutes at 4°C and the protein supernatant was transferred to a pre-chilled micro-centrifuge tube. Samples were frozen immediately at -20°C or subjected to 1-D SDS-PAGE.

For analysis of serum proteins bound to conidia whole blood was centrifuged at 500 x g for 5 minutes. Serum was pooled from multiple donors and the quantity of protein was ascertained. A diluted protein solution (7.5 mg ml^{-1}) was prepared using sterile PBS and the binding protocol was performed as described for the haemolymph study. Conidia were washed in PBS prior to protein extraction in 5X solubilisation buffer.

2.21 RT-PCR analysis of larval antimicrobial peptide expression.

2.21.1 RNase treatment of water and lab apparatus.

All water was treated with diethyl pyrocarbonate (DEPC) at a concentration of 0.1% (v/v). DEPC treated water was left to stir at room temperature for 4 hours and left overnight at 37ºC. Water was autoclaved twice and was subsequently used in the preparation of all buffers. Pestles and mortars were left to soak in a large beaker of DEPC treated water prior to being wrapped in aluminium foil and autoclaved twice prior to use. RNase free filter tips (Fisher) were used and all tubes were taken from a previously unopened bag and autoclaved twice prior to use. For RNA visualisation all gel casting, and gel electrophoresis equipment was washed in DEPC water supplemented with 2% (v/v) hydrogen peroxide. Gloves were worn at all times and changed at regular intervals.

2.21.2 RNA extraction from *G. mellonella*.

Larvae were injected and incubated at 30ºC for 4, 24 or 48 hours. Three larvae per treatment were selected at random from a plate of ten and were crushed to a fine powder using liquid nitrogen and a pestle and mortar. TRI-Reagent (3 mls) was added, mixed and allowed to rise to room temperature for 15 minutes. The mixture was transferred to a pre-chilled micro-centrifuge tube and centrifuged at 12,000 x g for 10 minutes at 4ºC. The supernatants were transferred to a fresh tube and the pellets were discarded. Two hundred micro-litres of chloroform were added and mixed by vortexing. The solution was allowed to stand at room temperature for 10 minutes and centrifuged for 10 minutes at 12,000 x g for 4ºC. The top "clear layer" was transferred to a fresh tube and 500µl of molecular grade 2-propanol was added. The tube was inverted 10 times and left to stand for ten minutes at room temperature. The samples were centrifuged at 12,000 x g for 10 minutes at 4ºC. The supernatant was discarded and the

pellet was washed in 100 µl 75% (v/v) molecular grade ethanol and was centrifuged at 12,000 x g for 10 minutes at 4ºC. The supernatant was removed and the resulting pellet was allowed to air dry prior to resuspending in 80 µl molecular grade RNase free water. RNA was aliquoted in 20 µl volumes and stored at -70ºC or used immediately.

2.21.3 DNase digestion of RNA.

Contaminating DNA was removed by DNase treating the RNA extract with a Deoxyribose I (AMP-D1) kit. RNA (4 µg) quantified by spectrometry, was brought up to a volume of 8 µl using DEPC treated water. This was followed by the addition of 1 µl 10X reaction buffer and 1 µl DNase 1 (1 unit/µl) to the RNA mixture and incubation at room temperature for 15 minutes. The enzyme activity was stopped by adding 1µl of Stop Solution (50 mM EDTA). Samples were incubated at 70ºC for ten minutes and cooled on ice. RNA was further processed for cDNA synthesis or frozen at -70ºC.

2.21.4 cDNA synthesis.

cDNA was synthesised using the Superscript III First Strand Synthesis System for RT-PCR kit from Invitrogen (CA, USA). RNA concentration was determined and all samples contained equal amounts of RNA prior to cDNA synthesis following adjustment to 8 µl using DEPC treated water. The 10 mM dNTP (1 µl) mix and 1 µl 50 mM oligo (dT) were added to the RNA. The solution was incubated at 65ºC for 5 minutes and then cooled to 4ºC. A master-mix was prepared (Per reaction: 2 µl 10X RT Buffer, 4 µl 25 mM $MgCl_2$, 2 µl 0.1 mM DTT, 1 µl RNaseOUT) according to the number of reactions necessary. Mastermix (9 µl) was added to each of the RNA samples and mixed gently followed by incubation at 42ºC for 2 minutes and then 4ºC for 5 minutes. Superscript III RT (200 U $µl^{-1}$) was added to each reaction tube and incubated as follows: 42º for 50 minutes, 70º for 5 minutes, and held on ice. RNase H (1 µl) was

added to each tube and incubated for 20 minutes at 37ºC. cDNA was aliquoted and stored at -20ºC.

2.21.5 PCR primers and cycle conditions.

The following PCR primer pairs used to amplify regions of four genes involved in the immune system and resistance to microbial challenge were used (Bergin *et al*., 2006). *G. mellonella* β–*actin* was used as a control housekeeping gene to demonstrate equal loading of RNA. The concentration of RNA was checked using a ThermoFisher Nanodrop 1000 V3.7 spectrophotometer for all samples prior to the commencement of PCR reactions to and adjusted to give equal loading of RNA in each reaction.

Primer Name	Oligonucleotides	Fragment Size b.p
β – actin F	GGGACGATATGGAGAAGATCTG	400
β – actin R	CACGCTCTGTGAGGATCTTC	
Transferrin F	CCCGAAGATGAACGATCAC	535
Transferrin R	CGAAAGGCCTAGAACGTTTG	
IMPI F	ATTTGTAACGGTGGACACGA	409
IMPI R	CGCAAATTGGTATGCATGG	
Galiomycin F	CCTCTGATTGCAATGCTGAGTG	359
Galiomycin R	GCTGCCAAGTTAGTCAACAGG	
Gallerimycin F	GAAGATCGCTTTCATAGTCGC	175
Gallerimycin R	TACTCCTGCAGTTAGCAATGC	

The PCR cycle conditions were 98ºC, 2 min, [94ºC - 1 minute, 55ºC – 1 minute, 72ºC – 1.5 minutes] x 30 cycles and 72ºC – 10 minutes as optimised by Bergin *et al*., (2006).

2.21.6 DNA gel electrophoresis of PCR products.

DNA was visualised by running samples on a 1% agarose gel. Agarose (1 g) (Melford Labs Ltd., Ipswich England) was dissolved in 100 ml 1 X TAE Buffer, (1/50 dilution of 50X stock: 24.2% (w/v) Tris-Base, 5.71% (v/v) Acetic acid, 0.05 M EDTA (pH8), with the addition of 4 µl of 5 mg ethidium bromide ml^{-1}. Samples (8 µl) were mixed with 2 µl of Blue Orange 6X loading dye (Promega). The sample was placed into the wells of the agarose gel. Samples were run at 80 V Embi-Tech and viewed using a UV-tranilluminator (Alpha Innotech). Densitometric analysis of resulting bands were performed using Imaje J software and all values were expressed against untreated larvae incubated over the same period of time and with the same temperature condions.

2.21.7 RNA electrophoresis.

Prior to use the gel rig and tank was washed in RNase ZAP, (Ambion) and rinsed thoroughly with sterile DEPC treated water and all apparatus was allowed to air dry in a sterile safety cabinet that was previously sterilised in 70% ethanol. A 1% (v/v) agarose gel was prepared. Agarose (1 g) was boiled in 100 mls 1X FA buffer made from a 1/10 dilution of 10X stock: (200 mM 3-[N-morpholino]propanesulfonic acid (MOPS), 50 mM sodium acetate, 10mM EDTA adjusted to pH 7) In DEPC treated water. RNA sample (4 µl) was mixed with 3 µl formamide and 3 µl 6X loading dye. Samples were run at 80 V Embi-Tech and viewed using a UV-transilluminator (Alpha Innotech).

Chapter 3

Characterisation of the virulence of clinical and environmental *Aspergillus fumigatus* isolates

3.0 Introduction.

G. mellonella larvae have been validated in recent years as a highly advantageous tool to determine the pathogenicity of bacteria and fungi, (Cotter *et al*., 2000; Bergin *et al*., 2005; Mylonakis *et al*., 2005; Joyce and Gahan, 2010). Additionally this model has been used to determine the pathogenicity of genetically manipulated micro-organisms as an alternative to murine screening, (Reeves *et al*., 2006; Jackson *et al*., 2009). *G. mellonella* larvae have also been used to detect mycotoxins and insecticidal toxins produced *in vivo* (Fuguet and Vey, 2004; Reeves *et al*., 2004). Despite recent advances in published literature in the use of this organism it is evident that there are many facets of the larval immune response and ability to cope with microbial burden that remain to be elucidated.

The use of *G. mellonella* as a novel means to determine the relative virulence of *Aspergillus* spp. was initially characterised by Reeves *et al*., (2004). This work correlated the production of gliotoxin with larval pathogenicity. Since then understanding the process of *A. fumigatus* mediated larval pathogenicity has been expanded to include the onset of hyphal development (Renwick *et al*., 2006) and more recently differential virulence based on mating type (Cheema and Christians, 2010). Therefore, a study of the effect of differentially sourced or genetically manipulated isolates of *A. fumigatus* on *G. mellonella* is necessary in order to establish the model as a comprehensively used means of measuring fungal pathogenesis.

Gliotoxin produced by *A. fumigatus* has been widely studied and identified as a potent inhibitor of phagocytosis and superoxide production in macrophages (Eichner *et al*., 1986) and restricts the formation of the neutrophil NADPH oxidase complex (Tsunawaki *et al*., 2004). Due to its potent toxicity and production by *A. fumigatus* quantitative analysis of gliotoxin may be an indicator of potential isolate pathogenicity. Fumagillin, another secondary metabolite produced by *A. fumigatus* is produced upon

hyphal development and has been characterised as a potent amoebicide, (Mitchell *et al*., 1997, McCowen *et al*., 1951). Amitani *et al*., (1995) described fumagillin as an inhibitor of ciliary beat frequency of pulmonary epithelial cells. As a result of this previous research an analysis of the production of fumagillin *in vivo* and its implication in fungal pathogenesis requires further characterisation.

The work presented in this Chapter was to characterise the relative pathogenicity of clinically and environmentally sourced *A. fumigatus* isolates following characterisation of the larval response to conidial infection at different temperatures and with strains containing deletions for secondary metabolite biosynthesis. Due to the significance of secondary metabolite biosynthesis in the pathobiology of *A. fumigatus* an analysis of *in vitro* and *in vivo* mycotoxin production coupled to the analysis of immunosuppressive properties of cell free culture filtrate was performed. As the virulence and persistence of *A. fumigatus* is dependent on strong growth and an ability to assimilate nutrients from a variety of sources an investigation of these parameters was performed to further understand the link between virulence traits and pathogenicity of *A. fumigatus* isolates.

3.1 Analysis of parameters affecting *A. fumigatus* infection in *G. mellonella* larvae.

In order to perform an analysis of the pathogenicity of different strains it was necessary to examine the effect of inoculum concentration and incubation temperature on larval viability. Mowlds and Kavanagh, (2008) established that the immune status of *G. mellonella* may be influenced by incubating larvae at 37ºC as evidenced by increased haemocyte density and antimicrobial peptide gene expression relative to larvae incubated at 30ºC. In this section the variation in larval viability mediated by changes in the concentration of conidial inoculum and incubation temperature were investigated. In addition a study investigating the virulence of mutant strains of *A. fumigatus* compared to a wild type strain was performed.

3.1.1 Determination of virulence of *A. fumigatus* in *G. mellonella* larvae.

A. fumigatus (ATCC 26933) conidia were harvested as described (Section 2.2.10). Larvae were injected with PBS or increasing concentrations of conidia, (1 x 10^4, 1 x 10^5, 1 x 10^6 or 1 x 10^7) 20µl^{-1} and were incubated at 30ºC or 37ºC. Larvae were assessed for viability at 24, 48 and 72 hours (Figure 3.1). Inspection of larvae at 24 and 48 hours showed that larvae melanised and died at a much faster rate at 37ºC than 30ºC when injected with the same conidial inoculum. Larvae injected with 1 x 10^4 conidia did not die when incubated at 30ºC or 37ºC. Larvae injected with 1 x 10^5 conidia remained viable when incubated at 30º; however at 37ºC there was a 16.6 ± 5.7% reduction in viability at the 72 hours. Larvae injected with 1 x 10^6 conidia remained viable at 30ºC. Larvae incubated at 37ºC showed evidence of melanisation and reduced movement 24 hours post-infection which culminated in a 30 ± 5.7% and 90 ± 3.3% reduction in viability at 48 and 72 hours. Larvae inoculated with 1 x 10^7 conidia demonstrated a 33 ± 3.3% loss of viability 24 hours post-infection and demonstrated no viability at 48 hours when incubated at 37ºC. Larvae injected with the same concentration of conidia and
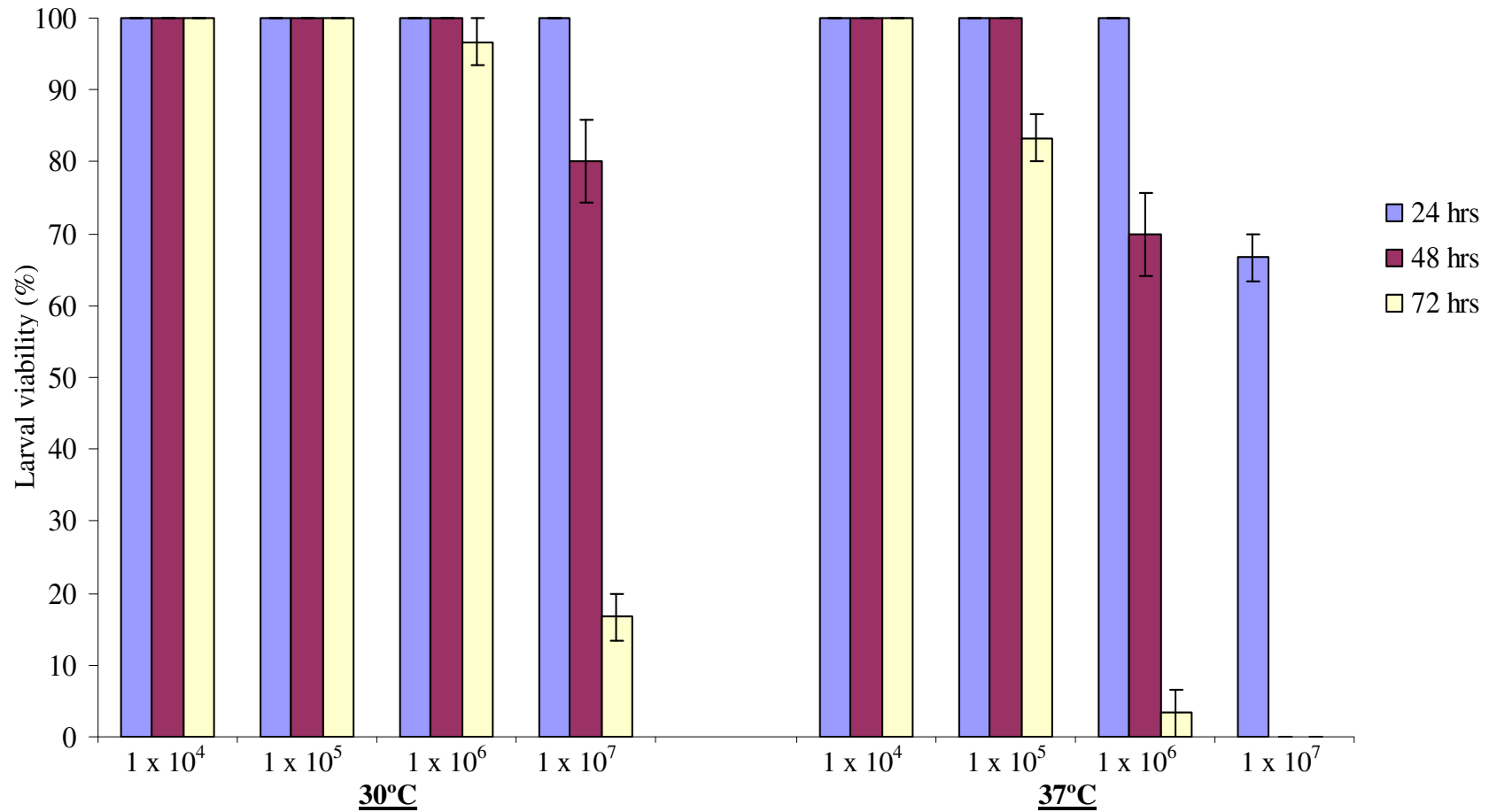
Figure 3.1 Analysis of the pathogenicity of different concentrations of *A. fumigatus* in *G. mellonella* at 30ºC and 37º.
Larvae were infected with conidia of *A. fumigatus* ATCC 26933 at different concentrations. Larvae were incubated at 30ºC or 37ºC and viability was recorded 24, 48 and 72 hours post infection. As an additional control larvae were injected with PBS however this did not affect larvae (data not shown).
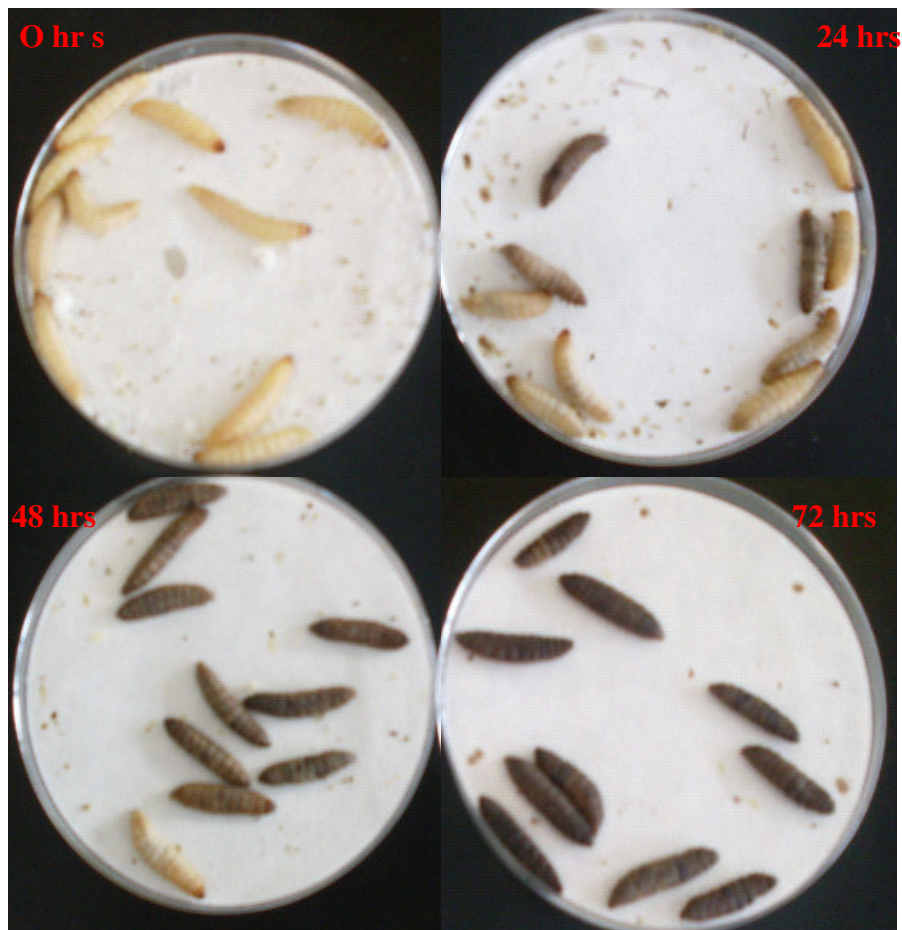
Figure 3.2 *G. mellonella* larvae at different stages of *A. fumigatus* pathogenesis.
Larvae were injected with 1 x 10^7 *A. fumigatus* ATCC 26933 conidia and incubated at
30ºC. Larvae demonstrated localised melanisation 24 hours post infection. There is
reduced larval movement coupled to advance melanisation after 48 hours while the
majority of larvae do not demonstrate viability at 72 hours as evidenced by lack of
movement following mild physical stimulation.

incubated at 30ºC demonstrated a more gradual reduction in viability recording values of 100%, 80 ± 5.7% and 16.6 ± 3.3% at 24, 48 and 72 hours, respectively. From the data presented it is clear that incubating larvae at 30ºC following infection with 1×10^7 conidia resulted in a similar level of larval mortality as incubating at 37ºC following infection with 1×10^6 conidia. As mentioned previously (Section 3.1), Mowlds and Kavanagh, (2008) demonstrated that incubation of larvae at 37ºC causes a significant increase in haemocyte density and expression of antimicrobial peptides. Considering this finding and the similarities in larval viability when injected with 1×10^6 conidia and incubated at 37ºC, or incubated at 30ºC following infection with 1×10^7 conidia it was decided that further investigations of the relative pathogenicity of *A. fumigatus* in *G. mellonella* would be performed at 30ºC. The visual progression of fungal pathogenesis using this condition in *G. mellonella* is presented in Figure 3.2. It was hoped that such an approach would negate any effect that incubation temperature would have on altering the larval immune response independent of infection.

3.1.2 Analysis of the effect of *A. fumigatus* secondary metabolite mutants on larval viability.

Conidia from *A. fumigatus* isolates containing gene deletions for *ftmA*, *psoA* and *laeA* from the "parent" Af293 strain were prepared as described and *G. mellonella* larvae were inoculated with 1×10^7 conidia. Larvae were incubated at 30ºC and viability was monitored at 24, 48 and 72 hours (Figure 3.3). Larvae injected with the Af293 strain demonstrated 70 ± 5.7%, 3.3 ± 3.3% and 0% viability at 24, 48 and 72 hours, respectively. Larvae injected with the Δ*ftmA* isolate demonstrated 100%, 90 ± 10% and 63.3 ± 12% viability at 24, 48 and 72 hours, respectively. Injection of larvae with Δ*psoA* resulted in 96.6 ± 3.3%, 73.3 ± 6.6% and 30 ± 5.7% viability at 24, 48 and 72 hours respectively. Inoculation of larvae Δ*laeA* mutant resulted in 93.3 ± 6.6%, 43.3
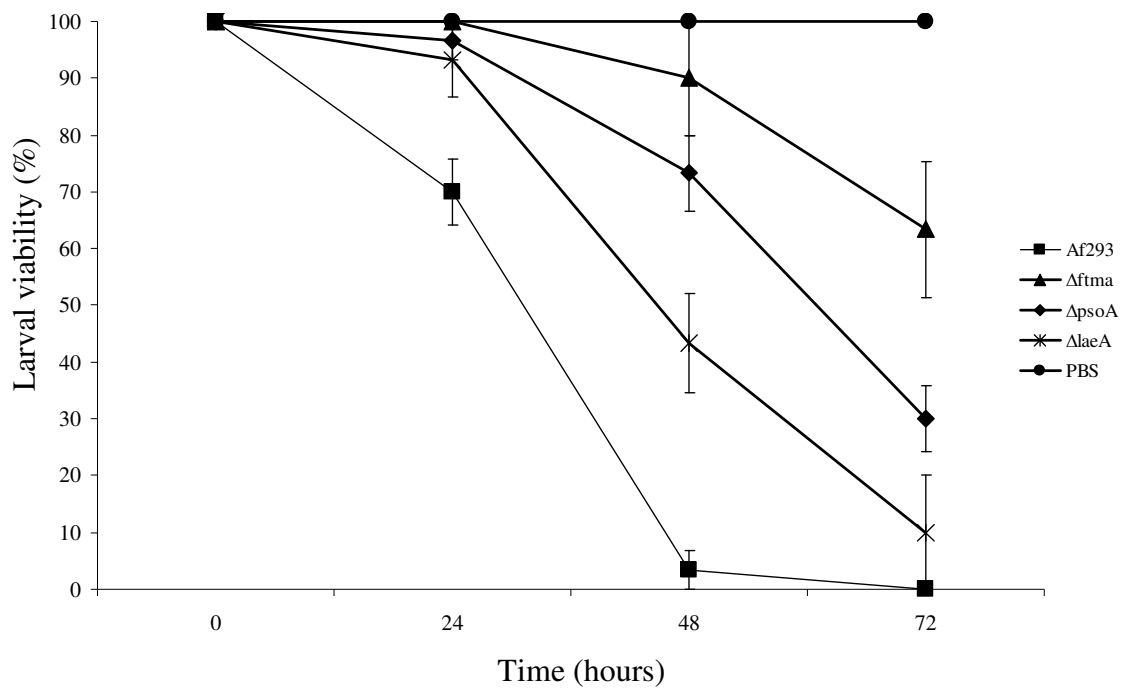
Figure 3.3 Evaluation of pathogenicity of *A. fumigatus* wild type and mutant isolates in *G. mellonella.*

Larvae were inoculated with *A. fumigatus* Af293 and mutant stains (Δ*ftmA*, Δ*psoA* and Δ*laeA*) and were incubated at 30ºC. Larval viability was ascertained 24, 48 and 72 hours post inoculation.

± 8.8% and 10 ± 10% viability at 24, 48 and 72 hours respectively. Statistical analysis of these data revealed that at 24 hours the mutant strains were less virulent than the wild-type, (p ≤ 0.02). Furthermore analysis of the 48 hour survival data revealed that larvae infected with the mutant strains demonstrated significantly greater viability than those infected with Af293 (p ≤ 0.001). At 72 hours larvae infected with the Δ*ftmA* and Δ*psoA* strains demonstrated significantly more viability relative to the Af293 strain (p = 0.001 and 0.004 respectively). Injection of larvae with Δ*laeA* resulted in slightly increased viability however this value was deemed less significant (p = 0.076).

3.1.3 Analysis of the relative pathogenicity of clinical and environmental isolates of *A. fumigatus* in *G. mellonella*.

The relative pathogenicity of 15 clinical and 15 environmental isolates of *A. fumigatus* was analysed in *G. mellonella* larvae. In order to assess the effect of conidial maturation in *A. fumigatus* isolates plates were allowed to age for 3, 14 or 28 days from the onset of conidiation at room temperature. Plates were harvested and conidia were injected at a concentration of 1 x 10^7 per larva and incubated at 30ºC. Larvae were assessed for viability at 24, 48, and 72 hours. Figures 3.4, 3.5, 3.6, 3.7, 3.8 and 3.9 demonstrate that clinical and environmental isolates show differential pathogenicity which was variable depending on the extent of conidial maturation. It was hoped to compare the pathogenicity of environmental and clinical isolates as grouped data from the *G. mellonella* viability data. Furthermore an analysis of alterations to the pathogenicity of these isolates as a result of conidial ageing was performed. Figure 3.10 shows the mean larval viability from this study with bars indicating standard deviation.

Larvae injected with clinical isolates of *A. fumigatus* demonstrated no significant difference in relative pathogenicity in comparisons made between differentially aged conidia 24 hours post-infection, (p ≥ 0.05). At 48 hours 3 day old conidia were
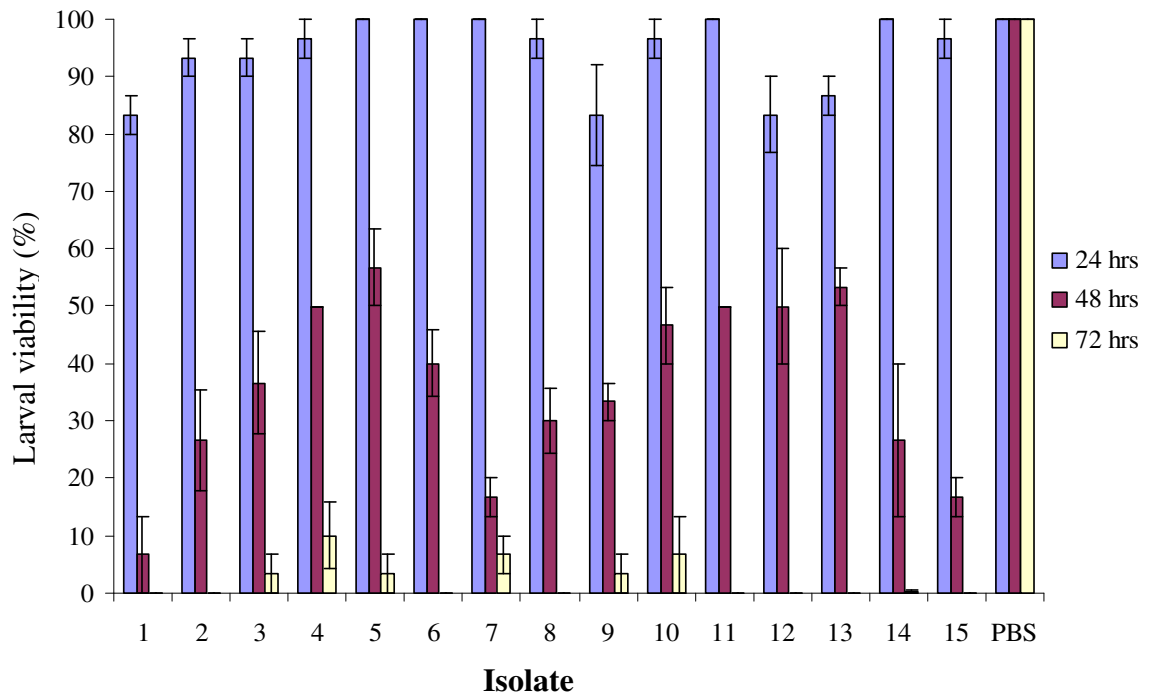
Figure 3.4 Pathogenicity of 3 day old conidia from clinical strains.
Clinical isolates of *A. fumigatus* (n = 15) were matured for 3 days. Conidia were harvested and injected (1×10^7) into *G. mellonella* larvae. Larvae were incubated at 30ºC and viability was recorded 24, 48 and 72 hours post infection.
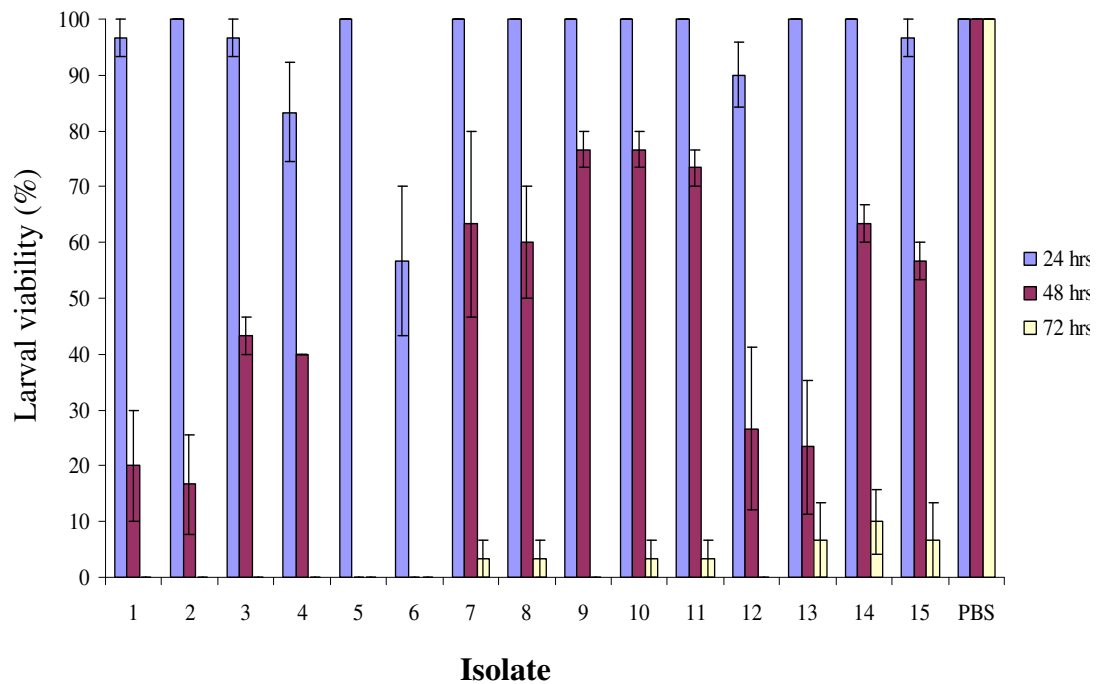
Figure 3.5 Pathogenicity of 3 day old conidia from environmental strains.
Environmental isolates of *A. fumigatus* (n = 15) were matured for 3 days. Conidia were harvested and injected (1×10^7) into *G. mellonella* larvae. Larvae were incubated at 30ºC and viability was recorded 24, 48 and 72 hours post infection.
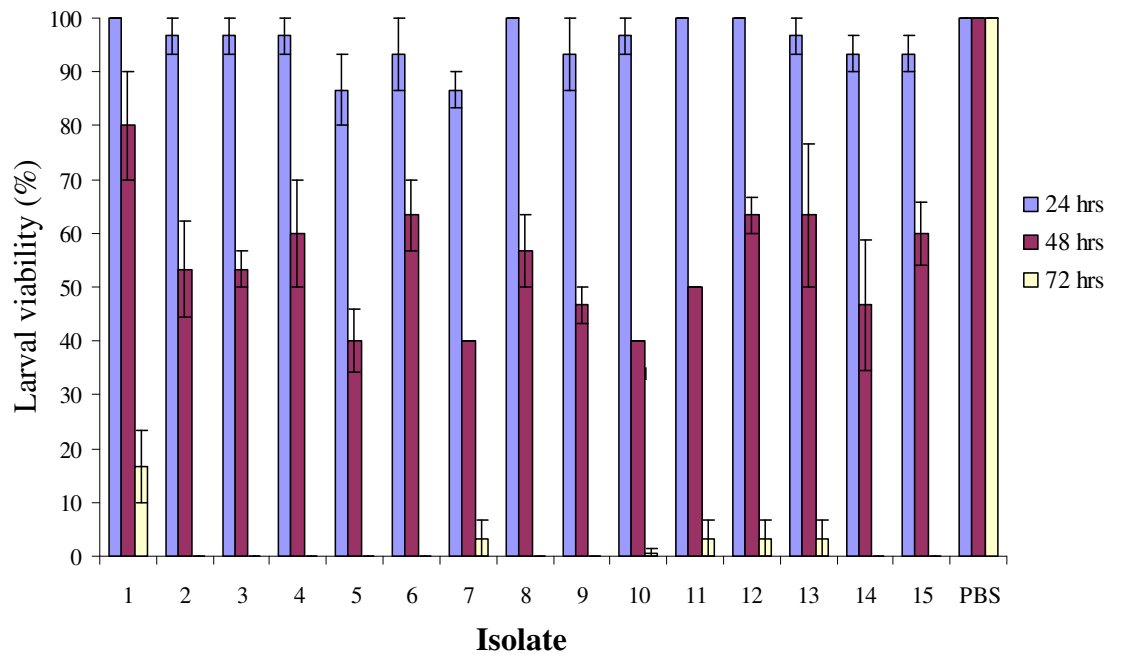
Figure 3.6 Pathogenicity of 14 day old conidia from clinical strains.
Larvae were infected with 14 day old conidia grown from clinical isolates (n = 15).
Larvae were incubated at 30ºC and viability was recorded 24, 48 and 72 hours post-infection.
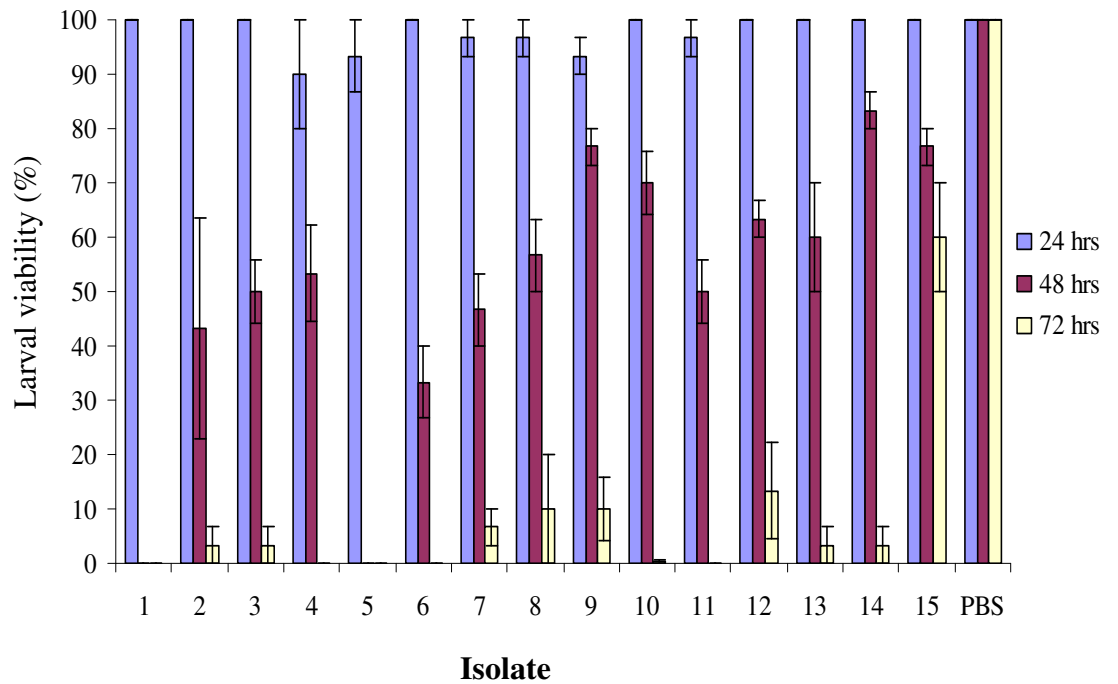
Figure 3.7 Pathogenicity of 14 day old conidia from environmental strains.
Larvae were infected with 14 day old conidia grown from environmental isolates (n = 15). Larvae were incubated at 30ºC and viability was recorded 24, 48 and 72 hours post-infection.
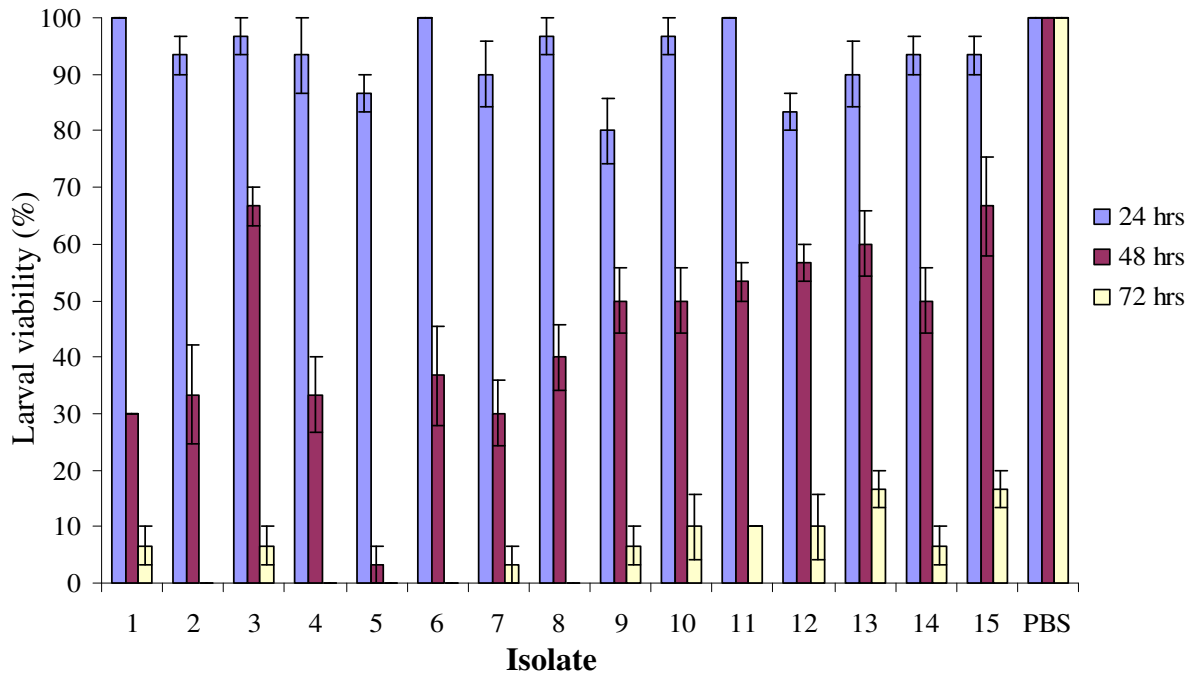
Figure 3.8 Pathogenicity of 28 day old conidia from clinical strains.
Clinical *A. fumigatus* isolates (n = 15) were grown and conidia allowed to mature for 28 days. Conidia were harvested and subsequently injected into *G. mellonella* larvae. Larvae were incubated at 30ºC and viability was recorded 24, 48 and 72 hours post infection.
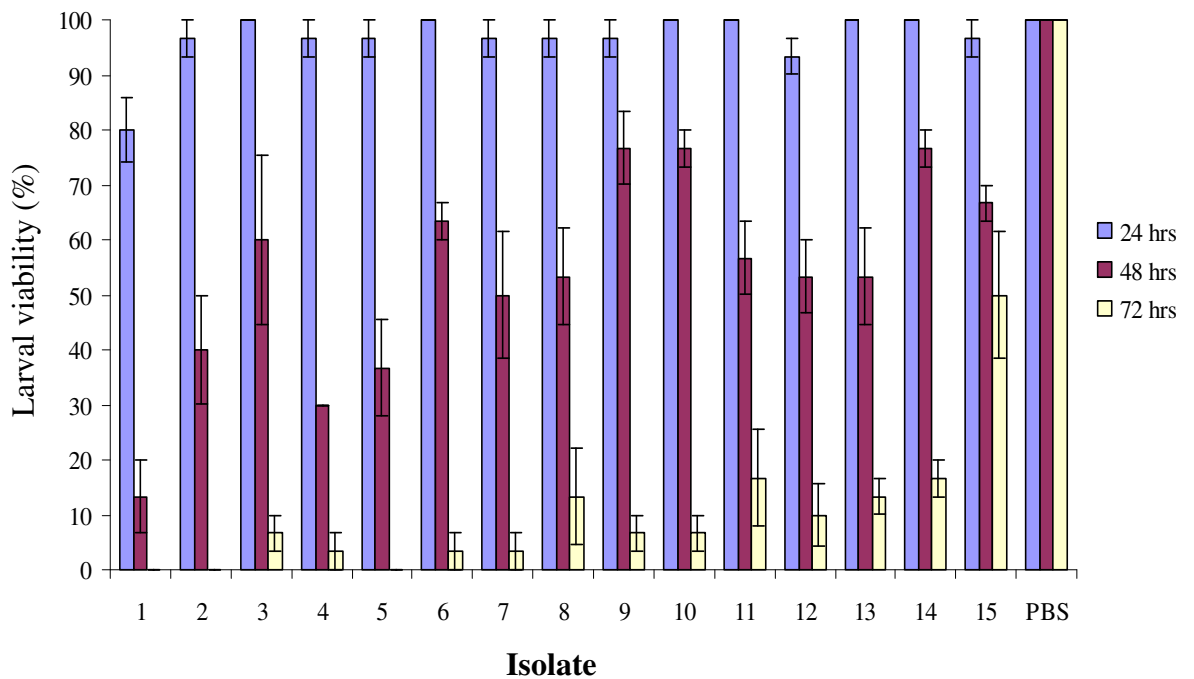
Figure 3.9 Pathogenicity of 28 day old conidia from environmental strains.
Environmental *A. fumigatus* isolates (n = 15) were grown and conidia allowed to mature for 28 days. Conidia were harvested and subsequently injected into *G. mellonella* larvae. Larvae were incubated at 30ºC and viability was recorded 24, 48 and 72 hours post infection.
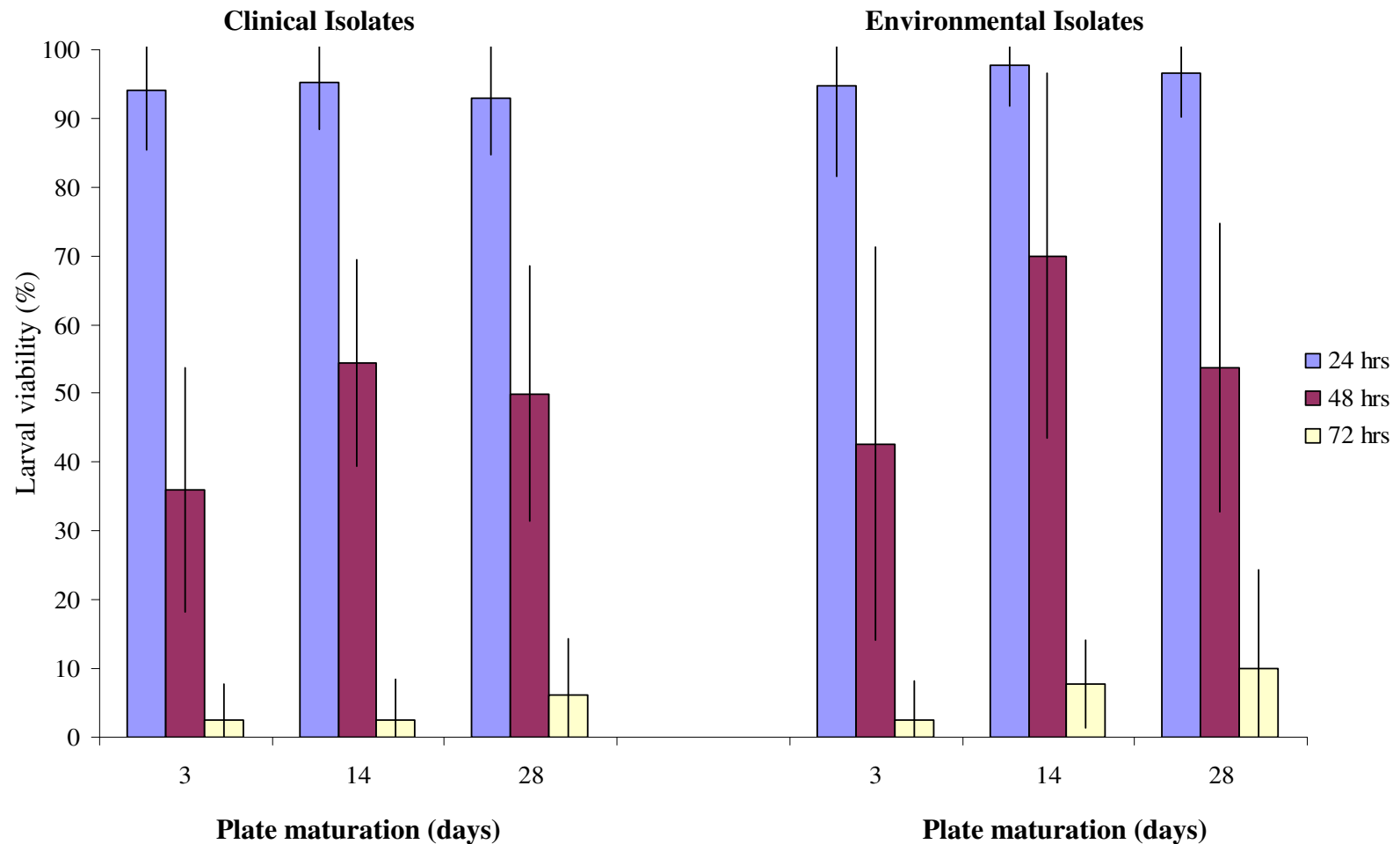
Figure 3.10 Pooled survival data of *G. mellonella* viability following infection with clinical and environmental strains of *A. fumigatus*.
The viability of *G. mellonella* larvae following infection with different aged conidia of clinical and environmental isolates of *A. fumigatus* (n = 15). Bars indicate standard deviation from mean larval survival with the different isolates. The average viability of larvae infected with clinical or environmental isolates varied with the stage of conidial maturation.

significantly more pathogenic than conidia aged for 14 or 28 days, (p ≤ 0.001). Interestingly, 14 day old clinical conidia were significantly less pathogenic 48 hours post-infection (p = 0.001). Analysis of survival 72 hours post-infection revealed that 3 day old conidia remained more pathogenic at this timepoint than 14 or 28 day old conidia (p ≤ 0.001). Comparisons of larval viability when infected with 14 or 28 day old conidia demonstrated that there was no statistically significant difference. The data obtained here show 3 day old clinical conidia is more pathogenic to *G. mellonella* larvae than inoculating with older conidia thus indicating that conidia lose virulence with maturity.

Larvae inoculated with conidia of environmental isolates of *A. fumigatus* also showed variations in survival depending on the level of conidial maturation. Larvae injected with 3 day old environmentally sourced conidia demonstrated significantly less viability 24 hours post-infection than those infected with 14 day old conidia (p = 0.014). Comparisons of larval viability following infection with 28 day old conidia showed no significant difference compared to 3 day old conidia (p = 0.270) but larval survival was significantly higher than larvae infected with 14 day old conidia (p = 0.001). This trend in larval viability was continued 48 hours post-infection. Larvae injected with 3 day old conidia demonstrated significantly reduced viability compared to those injected with 14 day old conidia (p = 0.013). Larvae inoculated with 28 day old conidia did not demonstrate a significant alteration in viability compared to those infected with 3 day old conidia (p = 0.687) but did demonstrate reduced larval survival compared to those larvae infected with 14 day old conidia (p = 0.039). Analysis of 72 hour larval survival following infection with 3 day old conidia revealed that larval viability was significantly reduced compared to that obtained using 14 and 28 day old conidia (p ≤ 0.005). Comparisons of larval survival following infection with 14 and 28 day old conidia showed that there was no statistically significant difference between these two

treatments. This result indicates that "younger" environmentally sourced conidia are more pathogenic in the *G. mellonella* model of infection than older conidia.

The relative mean pathogenicity of environmental and clinical isolates in *G. mellonella* was also compared at the different post-infection time-points. The mean pathogenicity of 3 day old clinical and environmental isolates in *G. mellonella* was not found to be significantly different at 24 or 72 hours ($p = 0.665$ and 1 respectively) but larvae inoculated with clinical isolates did demonstrate reduced viability 48 hours post-infection ($p = 0.041$). An analysis of the relative pathogenicity of 14 day old clinical and environmental conidia revealed that clinical isolates were more pathogenic 24 hours post-infection ($p = 0.044$) but larval viability was similar at 48 and 72 hours ($p = 0.285$ and 1 respectively). Infecting larvae with 28 day old clinical conidia resulted in reduced larval viability 24 and 48 hours post-infection compared to 28 day old environmental conidia ($p = 0.011$ and 0.001 respectively). However at 72 hours larval survival was found not to be significantly different between the two conidial sources ($p = 1$).

3.1.4 Analysis of the pathogenicity of non-*fumigatus Aspergillus* strains in *G. mellonella*.

The pathogenicity of environmental isolates of *A. flavus*, *A. niger*, and *A. terreus* was also assessed in *G. mellonella* with differentially aged conidia (3, 14 or 28 days old). Larvae were injected with 1×10^7 conidia of each strain and incubated at 30ºC. Larval viability was assessed 24, 48 and 72 hours post-infection (Figure 3.11).

Larvae injected with *A. flavus* immediately developed extensive melanisation. Assessment of larval viability 24 hours post-infection with 3 day old conidia showed that all larvae infected with *A. flavus* died quickly and showed extensive melanisation. Larvae injected with *A. niger* demonstrated $93.3 \pm 3.3\%$, $90 \pm 0\%$ and $53.3 \pm 3.3\%$ viability 24, 48 and 72 hours post-infection, respectively. Larvae injected with *A.*
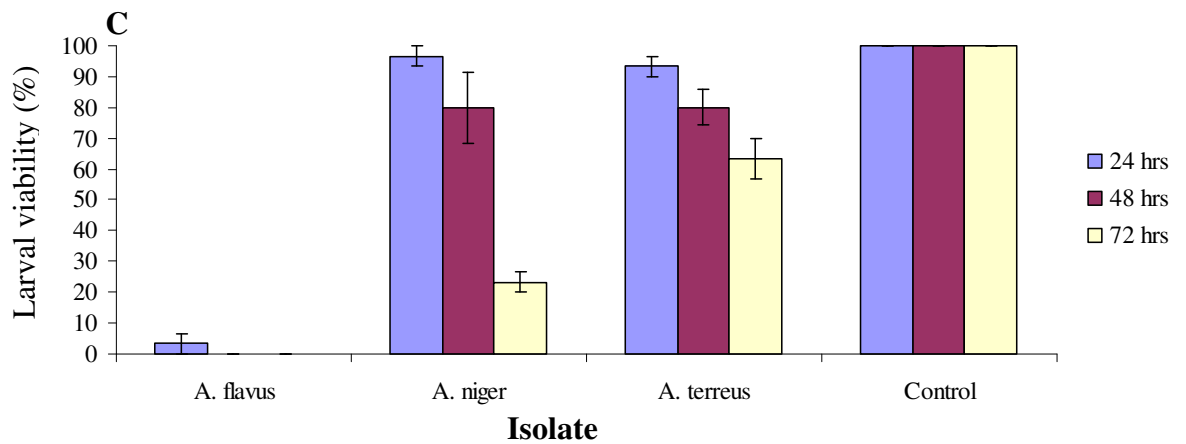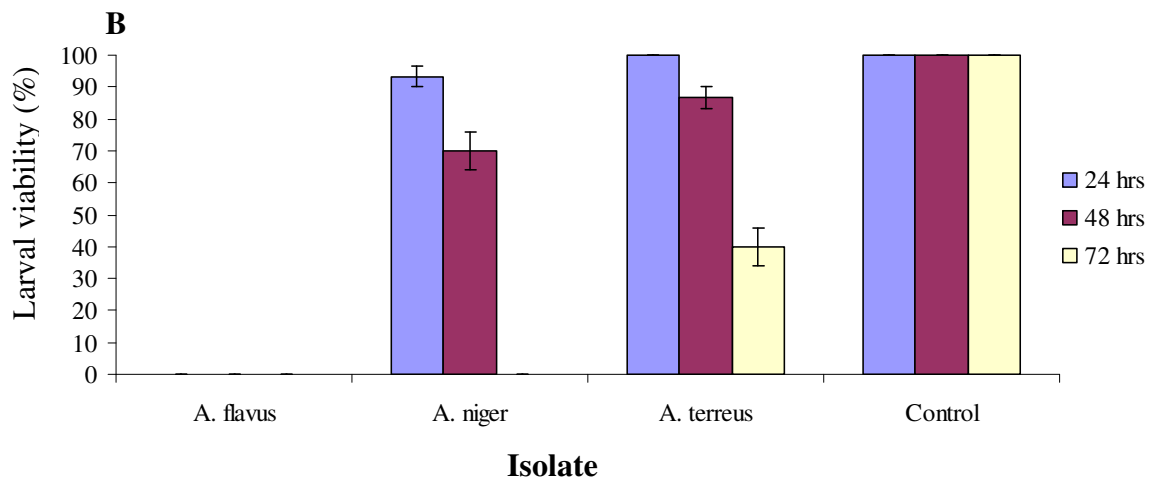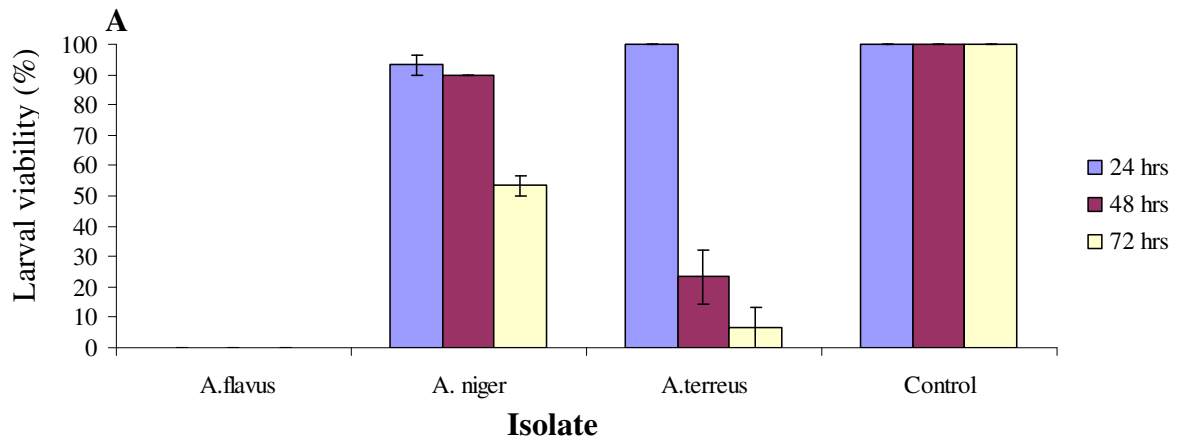
3.11 Viability of larvae following injection with environmental isolates of "non-fumigatus" *Aspergillus* **spp.**

Larvae were injected with conidia harvested from 3 day old, (**A**), 14 day old, (**B**) and 28 day old (**C**) matured plates of environmental isolates of *A. flavus*, *A. niger*, or *A. terreus* conidia. Infection with *A. flavus* resulted in drastically reduced morality at 24 hours. *A. terreus* demonstrated attenuated pathogenicity with increasing conidial maturation. *A. niger* showed variable pathogenicity depending on conidial maturation.
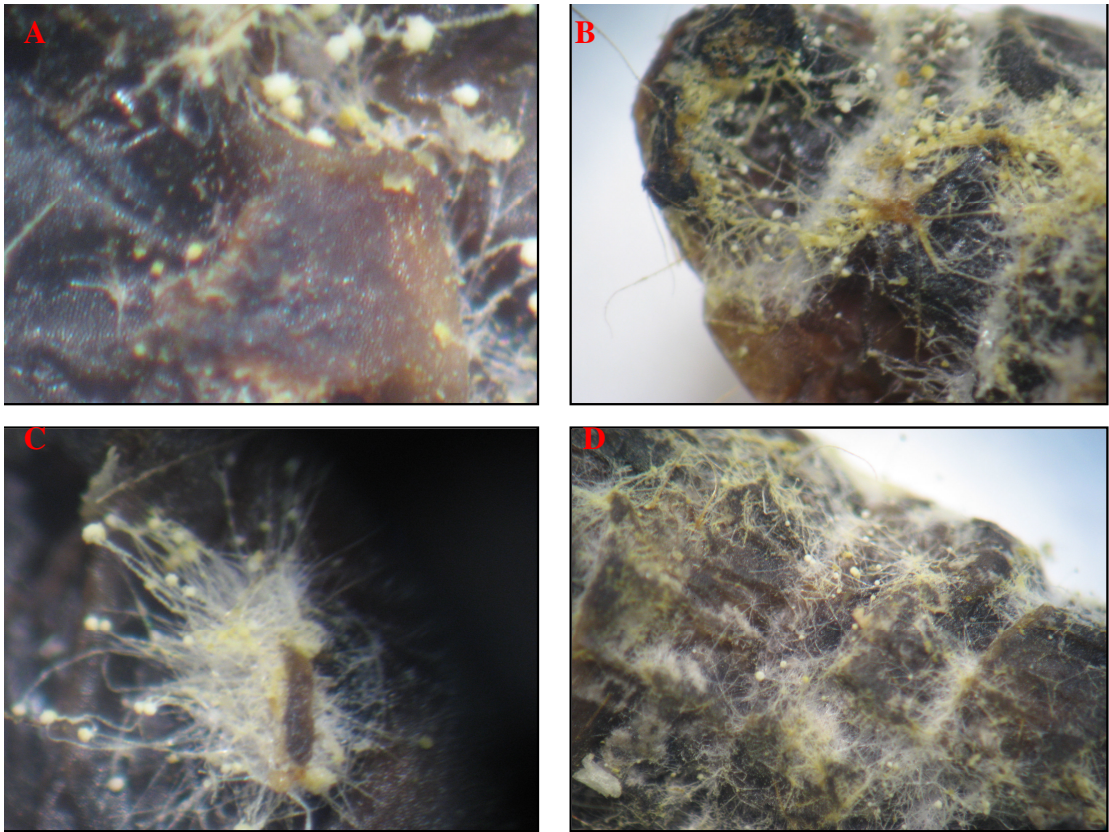
Figure 3.12 Images of *G. mellonella* 72 hours post infection with *A. flavus*.
Larvae were infected with 1 x 10^7 *A. flavus* conidia and were incubated at 30°C. Larvae demonstrated no viability 24 hours post infection. Inspection of larvae under a light microscope demonstrated hyphal growth and conidiophore formation through the insect cuticle (**A** and **D**), anterior region (**B**) and through the pro-legs (**C**).

terreus demonstrated 100%, 23.3 ± 8.8 and 6.6 ± 6.6% viability over the same time period, respectively. Inspection of larvae 48 hours post-infection with *A. flavus* demonstrated the appearance of hyphae growing through the insect cuticle which culminated in the presence of conidiophore development at 72 hours as demonstrated by observing the insect cuticle through a binocular microscope (Figure 3.12).

Analysis of the pathogenicity of 14 day old conidia revealed that larvae infected with *A. flavus* demonstrated no viability 24 hours post-infection and the same outgrowth of hyphae and conidiophores 48 and 72 hours post-infection respectively. Infection of larvae with *A. niger* resulted in 93.3 ± 3.3%, 70 ± 5.7 and 0% viability 24, 48 and 72 hours post-infection. Larvae infected with *A. terreus* demonstrated 100%, 86.7 ± 3.3% and 40 ± 5.8% over the same time period.

Infection of larvae with 28 day old conidia revealed that infection with *A. flavus* did not cause the development of rapid melanisation immediately after infection however 3.3 ± 3.3% of larvae showed viability at 24 hours. Larvae did not develop hyphal outgrowth and until 72 hours post-infection. Larvae infected with *A. niger* demonstrated 96.6 ± 3.3%, 80 ± 11.6% and 23.3 ± 3.3% viability 24, 48 and 72 hours post-infection. *A. terreus* infection resulted in 93.3 ± 3.3%, 80 ± 5.8% and 63.3 ± 6.6% viability at the 24, 48 and 72 hour time-points respectively.

Inspection of data obtained from experiments where larvae were infected with 3, 14 and 28 day old conidia from *A. terreus* showed that larval viability appeared to increase with increasing conidial age. This was evident at the 48 and 72 hour post-infection time-points. Statistical analysis of larval viability 48 hours subsequent to infection with *A. terreus* demonstrated that this increase in viability was significant with 14 or 28 day old conidia compared to 3 day old conidia ($p \leq 0.001$). Comparisons between 14 and 28 day old conidia pathogenicity at this time-point revealed no significant difference ($p = 0.488$). Analysis of 72 hour viability demonstrated that 3 day
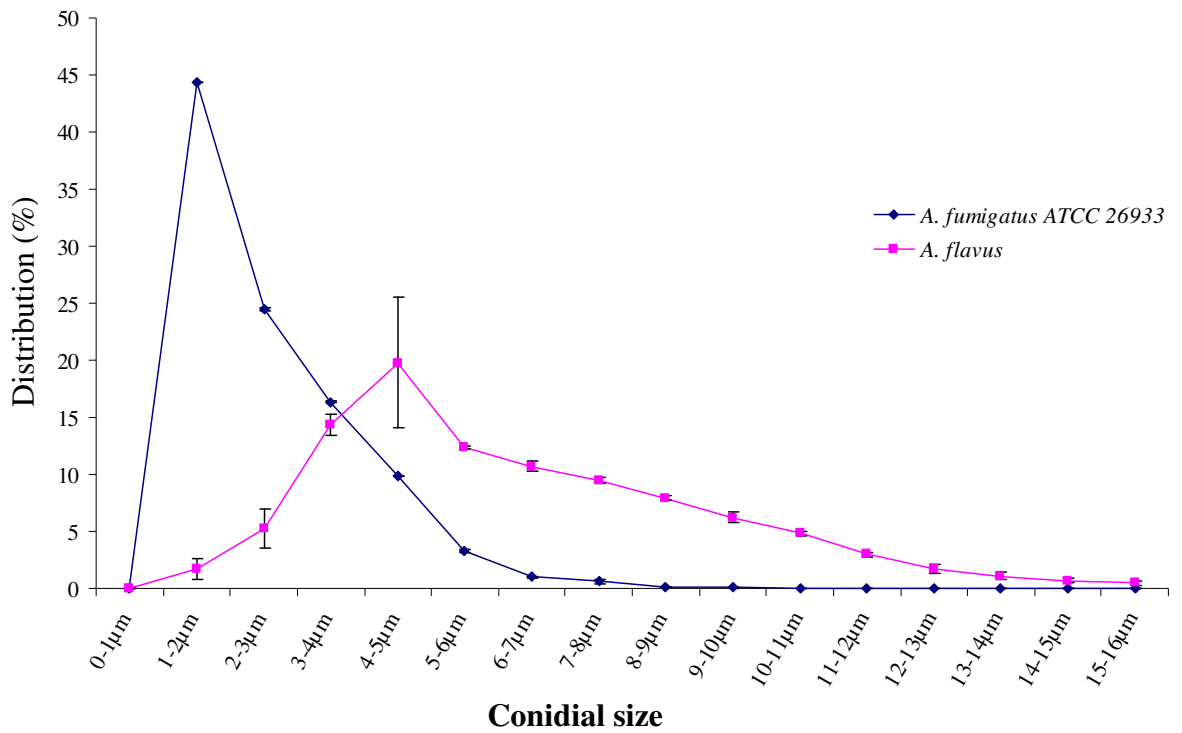
Figure 3.13 Comparative size distributions of *A. fumigatus* and *A. flavus* conidia.
Conidia were matured for 5 days and the size distribution of conidia was determined
using particle sizing counter (PAMAS). The size distribution of *A. flavus* conidia was
greater than that observed for *A. fumigatus* conidia.

old conidia were significantly more pathogenic in larvae than 14 or 28 day old conidia (p = 0.002 and 0.001 respectively). Analysis of statistical significance between 14 and 28 day old conidia revealed a p – value of 0.071. This result shows that *A. terreus* infection in *G. mellonella* larvae becomes attenuated with increasing conidial age. This decrease in pathogenicity appears most prevalent in comparisons between 3 day old conidia and 28 day old conidia indicating a loss in virulence over time.

3.1.5 Determination of conidial size in *A. fumigatus* and *A. flavus*.

A. fumigatus ATCC 26933 and *A. flavus* conidia were matured for 5 days and harvested. The conidial size distribution of both conidial types was ascertained as described (Section 2.5.2). Microscopic analysis of conidial suspensions observed no agglutination of spores. From the data presented (Figure 3.13) it is clear that *A. flavus* conidia show a wider distribution of conidial size relative to *A. fumigatus*. Analysis of *A. fumigatus* conidia showed that the largest size distribution of conidia occurred between 1-2 µm (44.34 ± 0.057%). It was found that that 85% of *A. fumigatus* conidia measured 1-4 µm in diameter with the remaining population measuring slightly larger. *A. flavus* conidia were clearly quite large with 19.78 ± 5.7% of the conidial population measuring 4-5µm. This result indicates the variability in conidial size in *A. flavus*. In contrast to *A. fumigatus* only 1.71 ± 0.9 of conidia were found to measure 1-2 µm in diameter. It was found that 93% of the conidial population demonstrated diameters in excess of 3 µm.

3.1.6 Summary.

The results presented here show that infection of *G. mellonella* with *A. fumigatus* followed by incubation at 37ºC results in larval death at a faster rate than incubation at 30ºC. The inoculation of larvae with 1 x 10^6 conidia and incubating at

37ºC causes a similar reduction in larval death to infection with 1 x 10^7 conidia and incubation at 30ºC. As the immune profile of *G. mellonella* is affected by incubating larvae at 37ºC it was decided to use a temperature of 30ºC for subsequent experiments.

The relative pathogenicity of *A. fumigatus* Af293 which contained deletions for *laeA*, *psoA* or *ftmA* was also assessed. The mutants demonstrated significantly reduced pathogenicity 24 and 48 hours post-infection relative to the "wild-type" Af293 isolate. The Δ*ftmA* and Δ*psoA* demonstrated significantly attenuated pathogenicity at 72 hours, however larvae infected with Δ*laeA* demonstrated an increased, but statistically insignificant, level of viability.

An analysis of different aged clinical and environmental *A. fumigatus* conidia pathogenicity was also performed. There was a large amount of variability in larval viability when infected with conidia of isolates indicating differential pathogenicity of clinical and environmental strains. Larvae infected with 3 day old conidia demonstrated reduced viability compared to older conidia from clinical and environmental isolates.

An analysis of the pathogenicity of environmental isolates of *A. flavus*, *A. niger* and *A. terreus* with 3, 14 or 28 day old conidia was also performed. *A. flavus* demonstrated a high level of pathogenicity in *G. mellonella* larvae culminating in rapid melanisation, loss of larval viability and development of hyphae and conidiophores. Infection of larvae with *A. terreus* resulted in loss of viability. The pathogenicity of *A. terreus* appears to be reduced with increasing conidial age with 3 day old conidia more virulent than 14 or 28 day old conidia. An analysis of conidial size demonstrated that *A. fumigatus* conidia measured 1-4 µm in diameter with *A. flavus* conidia demonstrating a wider distribution and average size.

3.2 Analysis of *A. fumigatus* mycotoxin production *in vivo* and analysis of culture filtrate mediated immunosuppression in *G. mellonella*.

A. *fumigatus* produces a variety of enzymes and secondary metabolites which facilitate the growth and persistence of the fungus within pulmonary tissue (Bennet and Klich, 2003; Ben-Ami *et al*., 2010). As a result an analysis of the production of mycotoxins within *G. mellonella* was necessary to facilitate an improved understanding of *A. fumigatus* pathogenesis *in vivo*. Reeves *et al*., (2004) demonstrated that gliotoxin was produced in significant quantities *in vitro* and reached peak production after 72 hours and it has been speculated that this production may be implicated in fungal pathogenesis. As a result *G. mellonella* larvae were used as an *in vivo* tool to assess the potential immunocompromising effect of clinical and environmental 72 hour culture filtrates.

3.2.1 Determination of the concentration of mycotoxins *in vivo*.

In order to facilitate an understanding of the host-microbe interaction between *G. mellonella* and *A. fumigatus* an analysis of the *in vivo* concentration of gliotoxin and fumagillin in larvae was performed. Larvae were inoculated with *A. fumigatus* ATCC 26933, incubated at 30ºC and mycotoxins were extracted as described 24, 48 and 72 hours post-infection (Section 2.11.1). RP-HPLC analysis of larval extracts (Figure 3.14) revealed that the presence of fumagillin was high 24 hours post-infection (3837 ± 56.4535 ng larval tissue g^{-1}). The presence of fumagillin decreased 48 hours post-infection (1745.2 ± 49.477 ng larval tissue g^{-1}) and was undetectable at 72 hours. In contrast gliotoxin production was detected at concentrations of 1863 ± 34.641, 2480 ± 43.301 and 2312.33 ± 5.025 ng larval tissue g^{-1} at 24, 48 and 72 hours.
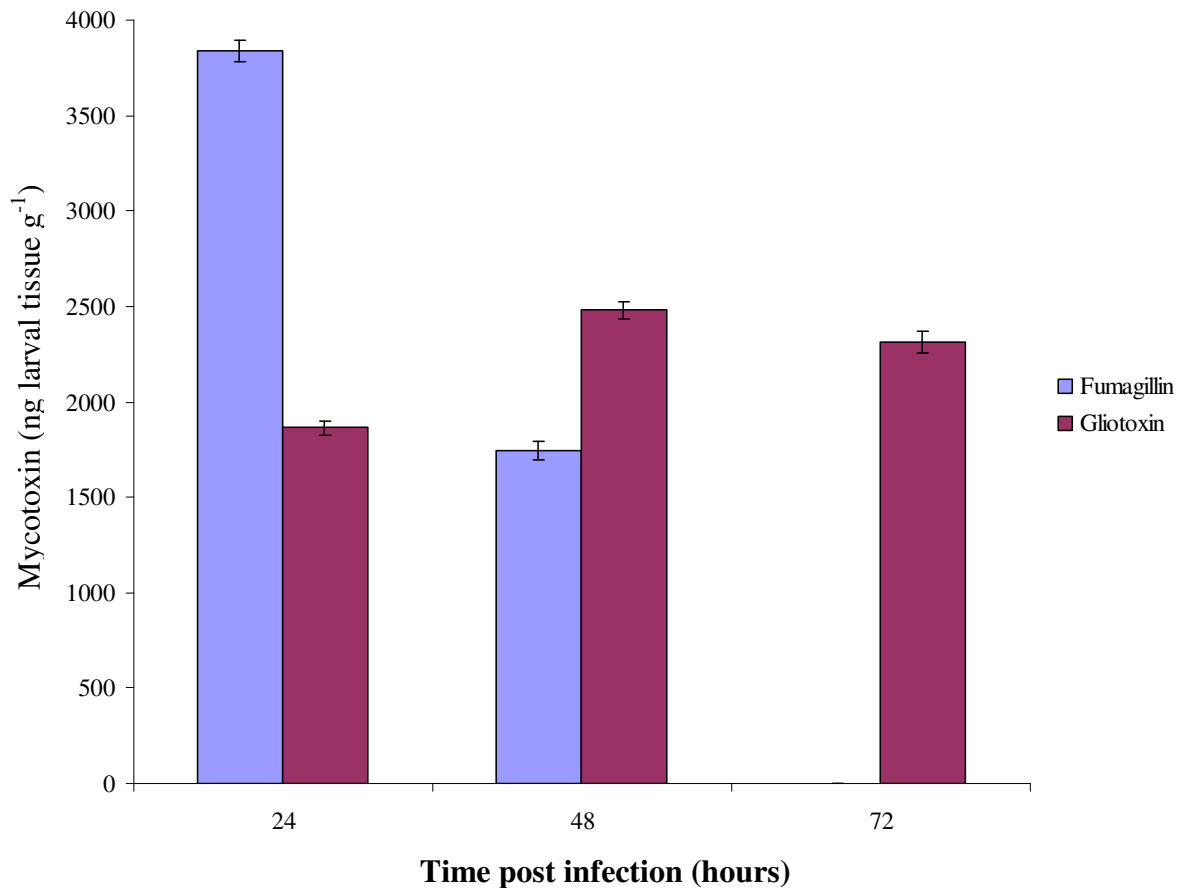
Figure 3.14 *In vivo* **mycotoxin concentrations in** *G. mellonella* **following infection with** *A. fumigatus.*

Larvae were inoculated with *A. fumigatus* ATCC 26933 conidia and incubated at 30ºC. Following incubation for 24, 48 and 72 hours larvae (n = 25) were crushed and organically extracted. Mycotoxin production was quantitatively analysed by RP-HPLC. Gliotoxin was detected at all time-points. Fumagillin was detected at high concentrations 24 hours post infection but declined to undetectable levels after 72 hours.

3.2.2 Determination of the presence of mycotoxins in clinical and environmental *A. fumigatus* culture filtrate.

Clinical and environmental isolates of *A. fumigatus* (CAF 1 – 10 and EAF 1 – 10) were grown in MEM supplemented with 5% (v/v) FCS and were cultured at 37ºC for 72 hours (Section 2.2.11). This was extracted using chloroform as described (Section 2.11.2). RP-HPLC analysis was performed (Section 2.12.3) and the concentration of mycotoxin was calculated by analysing the resulting peak areas and retention times from standards of gliotoxin and fumagillin of known concentration.

Figure 3.15 shows the mycotoxin concentrations calculated from 72 hour culture filtrates. The concentration of gliotoxin and fumagillin in these samples showed great variability. All of the isolates surveyed in this analysis demonstrated fumagillin production. However, two environmental isolates and one clinical isolate did not produce, (or produced undetectable quantities of), gliotoxin. The quantified mycotoxin concentrations from clinical and environmental isolates were pooled in order to ascertain the type of isolate which produced more mycotoxins on average (Figure 3.16). Analysis of mycotoxin production from clinical isolates of *A. fumigatus* demonstrated mean gliotoxin and fumagillin production values of 87 ± 24.9 and 15.5 ± 3.1 ng hyphae mg^{-1} respectively. The environmental isolates in this study demonstrated gliotoxin and fumagillin production of 63.5 ± 27.7 and 22.6 ± 6.6 ng hyphae mg^{-1} respectively. Analysis of these data revealed that production of gliotoxin by clinical and environmental isolates was not statistically different. Fumagillin production in environmental isolates was significantly higher than that from clinical isolates at this timepoint (p = 0.041).
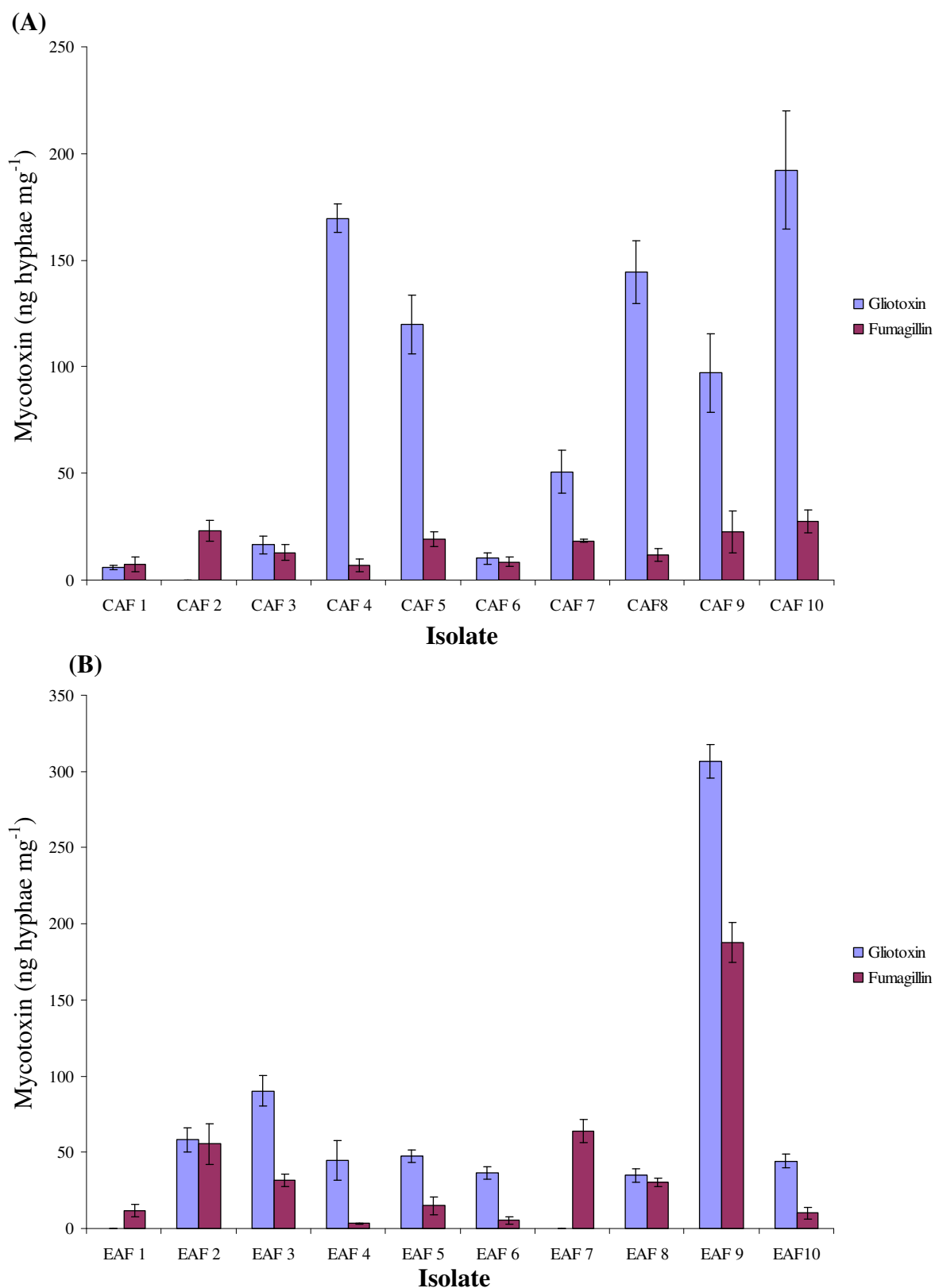
Figure 3.15 RP-HPLC analyses of gliotoxin and fumagillin concentrations in *A. fumigatus* culture filtrates.

Clinical **(A)** and environmental **(B)** isolates of *A. fumigatus* were grown in MEM/5% (v/v) FCS for 72 hours. Following organic extraction of culture filtrates and RP-HPLC analysis the quantity of gliotoxin or fumagillin as a function of fungal biomass was quantified.
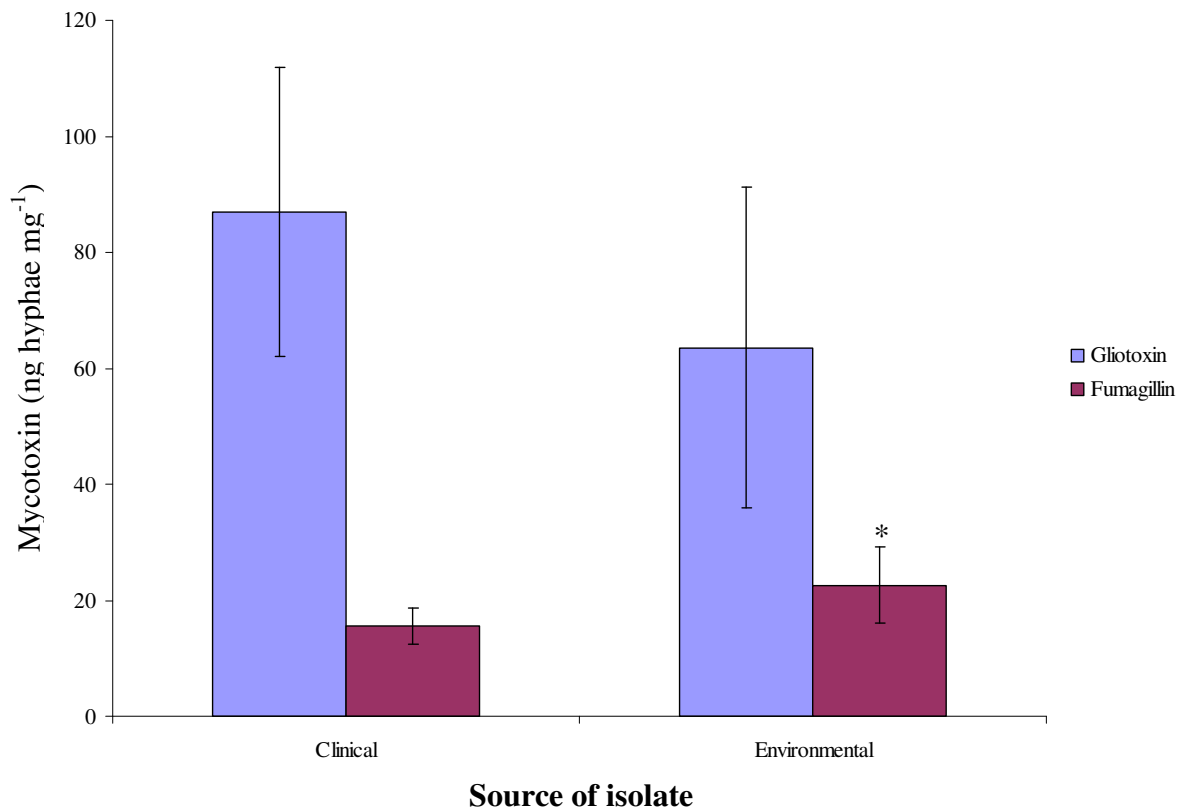
Figure 3.16 Average production of gliotoxin and fumagillin by clinical and environmental isolates of *A. fumigatus*.

The mycotoxin concentration values were grouped according to isolate source. Environmental isolates produced more fumagillin on average (*p = 0.041). Gliotoxin production was highly variable with comparisons between clinical and environmental culture filtrates not demonstrating significant difference.

3.2.3 Determination of the ability of clinical and environmental *A. fumigatus* 72 hour culture filtrates to immunocompromise *G. mellonella* larvae.

Larvae were injected with 20μl of clinical or environmental culture filtrates (filter sterilised) or sterile MEM supplemented with 5% foetal calf serum as a control and incubated at 30ºC for 4 hours after which larvae were inoculated with a follow up dose of 1 x 10^7 *A. fumigatus* ATCC 26933 conidia. Larval viability was assessed 24, 48 and 72 hours post-infection and the results are shown in Figure 3.17. It was noted that injection with culture filtrate without subsequent fungal infection did not result in a loss of larval viability over 72 hours. Larvae injected with MEM/5% (v/v) FCS and subsequently infected with *A. fumigatus* demonstrated 100%, 70 ± 5.8% and 16.6 ± 3.3% viability at 24, 48 and 72 hours, respectively. Larvae injected with clinical and environmental 72 hour culture filtrates prior to infection with 1 x 10^7 conidia clearly demonstrated reduced viability at 24 and 48 hours relative to the control treatment with no viability present at 72 hours (Figure 3.18). Analysis of these data revealed that viabilities were significantly lower than the media control at 24 and 48 hours (p ≤ 0.048). This finding indicates that *A. fumigatus* culture filtrates are immunosuppressive in *G. mellonella* as evidenced by a reduced viability relative to the control.

As it was not feasible to conduct a direct comparison between different isolates due to the number of culture filtrates analysed, viability data from the clinical and environmental culture filtrate pre-treatments were pooled to calculate the mean and standard deviation in order to facilitate a comparison of their immunosuppressive effect (Figure 3.18). Exposing larvae to clinical *A. fumigatus* culture filtrates and subsequent infection with conidia resulted in 67 ± 24.1% and 31.6 ± 21.8% viability after 24 and 48 hours respectively. Larvae injected with environmental *A. fumigatus* culture filtrates prior to conidial inoculation demonstrated 56.6 ± 23.5% and 22.3 ± 18.3% viability after 24 and 48 hours. Statistical analysis of larval survival demonstrated that the
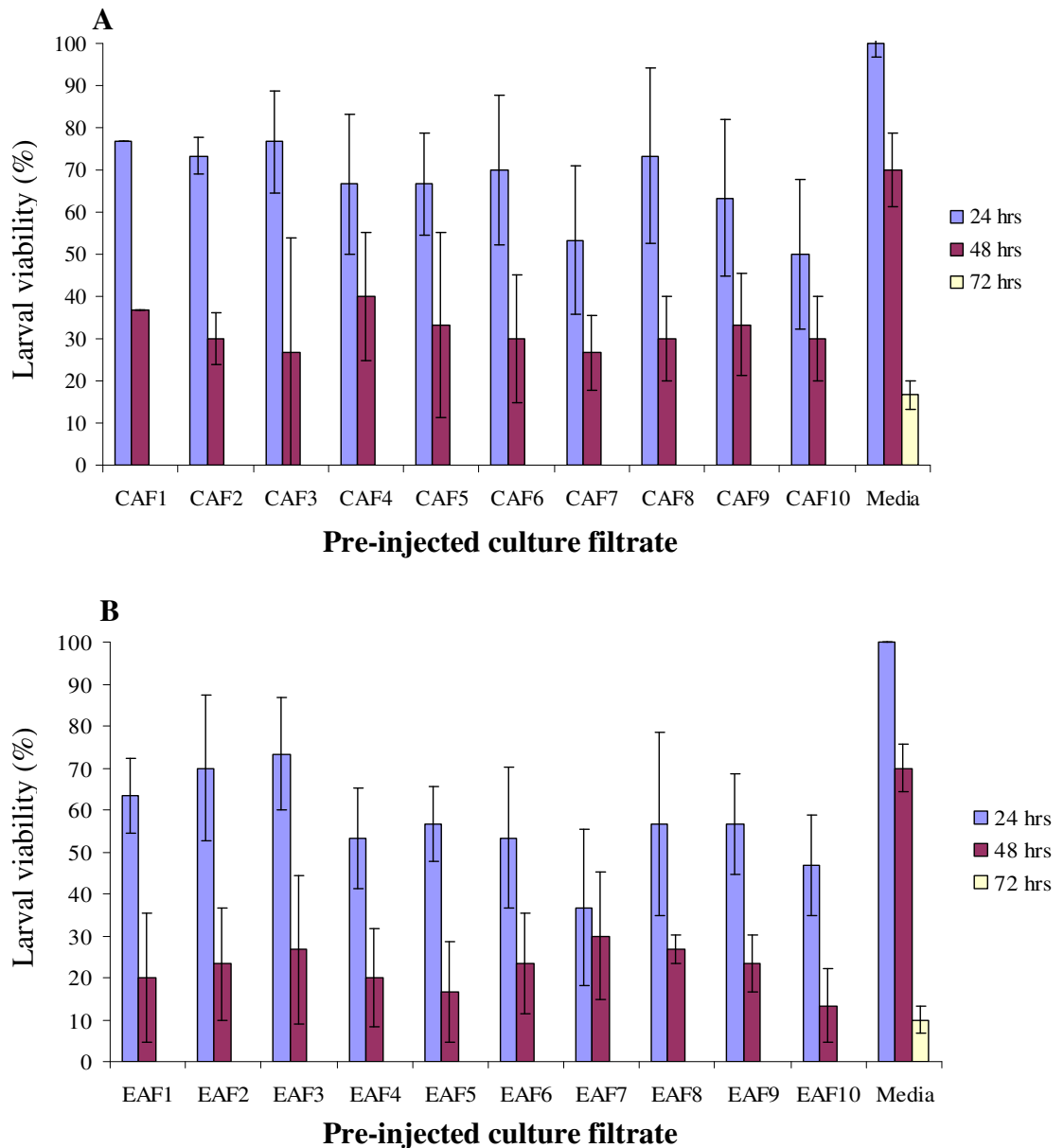
Figure 3.17 Larval viability following pre-exposure to environmental or clinical *A. fumigatus* culture filtrate and subsequent conidial inoculation.
Larvae were inoculated with clinical (**A**) or environmental (**B**) sterilised culture filtrate and incubated at 30ºC for 4 hours. Larvae were infected with 1 x 10^7 *A. fumigatus* ATCC 26933 conidia and incubated at 30ºC. Larval viability was ascertained 24, 48 and 72 hours post infection. Sterile MEM/5% (v/v) FCS was used a pre-treatment control prior to infection with *A. fumigatus*. Larvae administered a prior injection of *A. fumigatus culture* filtrate demonstrated significantly reduced viability at 24 and 48 hours post fungal inoculation (p $\leq$ 0.048).
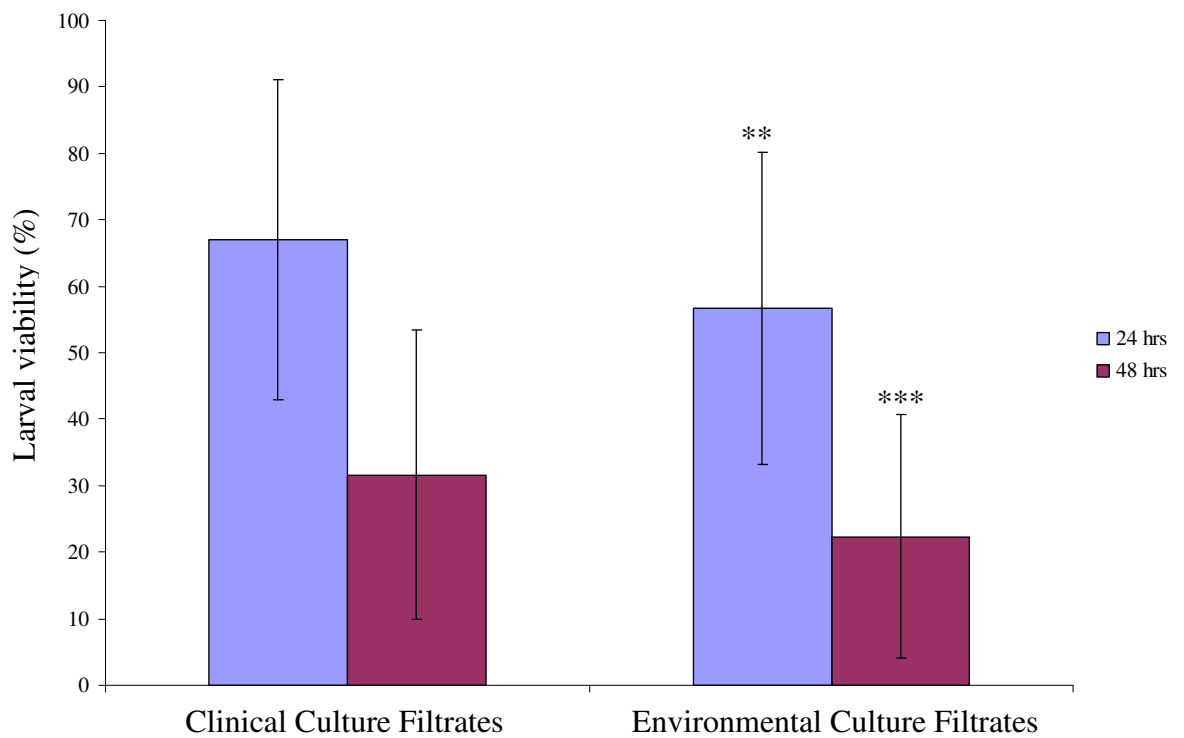
Figure 3.18 Mean larval viability following infection with *A. fumigatus* ATCC 26933 subsequent to exposure to environmental or clinical culture filtrates.
The mean larval viability data from larvae injected with culture filtrate prior to subsequent infection with *A. fumigatus* ATCC 26933 were grouped according to the source of culture filtrate. Prior inoculation with environmental culture filtrate resulted in reduced larval viability at 24 and 48 hours (**p = 0.009, ***p = 0.001).

relative decrease in larval viability caused by inoculating *G. mellonella* with environmental culture filtrates prior to fungal infection was significant in comparison to clinical culture filtrates at 24 and 48 hours (p = 0.009 and 0.001). These data indicate that the environmental *A. fumigatus* culture filtrates employed in this study are significantly more immunosuppressive in *G. mellonella* than clinical culture filtrates.

3.2.4 Summary.

In this section an analysis of *A. fumigatus* ATCC 26933 mycotoxin production in *G. mellonella* was performed. The results demonstrate that fumagillin production is highest 24 hours post-infection. This quantity of fumagillin appears to be reduced 48 hours post-infection with an absence (or undetectable quantity) at 72 hours. In contrast gliotoxin production was detected at all time-points and was significantly high 48 and 72 hours post-infection. This finding indicates production of mycotoxins is variable over time occurring at different stages of fungal growth.

An analysis of the production of mycotoxins from ten clinical and ten environmental isolates of *A. fumigatus* grown in MEM/5% (v/v) FCS was also performed using RP-HPLC analysis of organically extracted culture filtrate. The different strains demonstrated variable mycotoxin production with respect to each other. Combining the data obtained from clinical and environmental isolates of *A. fumigatus* revealed that *in vitro* gliotoxin production was similar between clinical and environmental isolates. The production of fumagillin in environmental isolates appeared to be significantly higher than in clinical isolates.

The culture filtrates were filter sterilised and were used to investigate their ability to weaken the *G. mellonella* response to fungal colonisation using *A. fumigatus* ATCC 26933. The results indicate that all larvae exposed to fungal culture filtrates (irrespective of the source of the isolate) showed reduced viability when subsequently

infected with conidia. Additionally, analysis of mean larval viability when inoculated with environmental culture filtrates prior to infection with conidia was lower than larvae inoculated with clinical isolate culture filtrates. This finding was particularly interesting as it may indicate a differential secretory profile in *A. fumigatus* isolates which results in immunosuppression in *G. mellonella* larvae.

3.3 Determination of the growth rate of clinical and environmental *A. fumigatus* isolates.

A. fumigatus is capable of growing rapidly on a range of carbon sources and in harsh environments which is a major contributing factor to its ubiquitous nature in the environment and facilitates its opportunistic nature as a fungal pathogen (Tekaia and Latgé, 2005). This saprophytic growth and pathogenic virulence of *A. fumigatus* appears to be intrinsically linked to the secretion of enzymes which allow for the acquisition of nutrients from different sources (Gifford *et al*., 2002; Tekaia and Latge, 2005; Robson *et al*., 2005). In this section the growth of clinical and environmental *A. fumigatus* isolates (n = 10) on *Aspegillus* Minimal Medium (AMM) was determined. Furthermore the growth of these *A. fumigatus* isolates on milk agar was investigated as a means of determining their relative growth on complex media due to protease secretion as performed by Richie *et al*., (2009).

3.3.1 Growth of clinical and environmental isolates of *A. fumigatus* on AMM.

A. fumigatus isolates were initially grown on Malt Extract Agar (MEA). As MEA is a very rich nutrient source the rate of growth between different isolates was almost identical. *A. fumigatus* conidia were harvested as described (Section 2.2.10) and the cells were enumerated by haemocytometry. The final concentration was adjusted to 1×10^6 conidia ml^{-1} and a 5 µl drop of this suspension was placed on an AMM agar
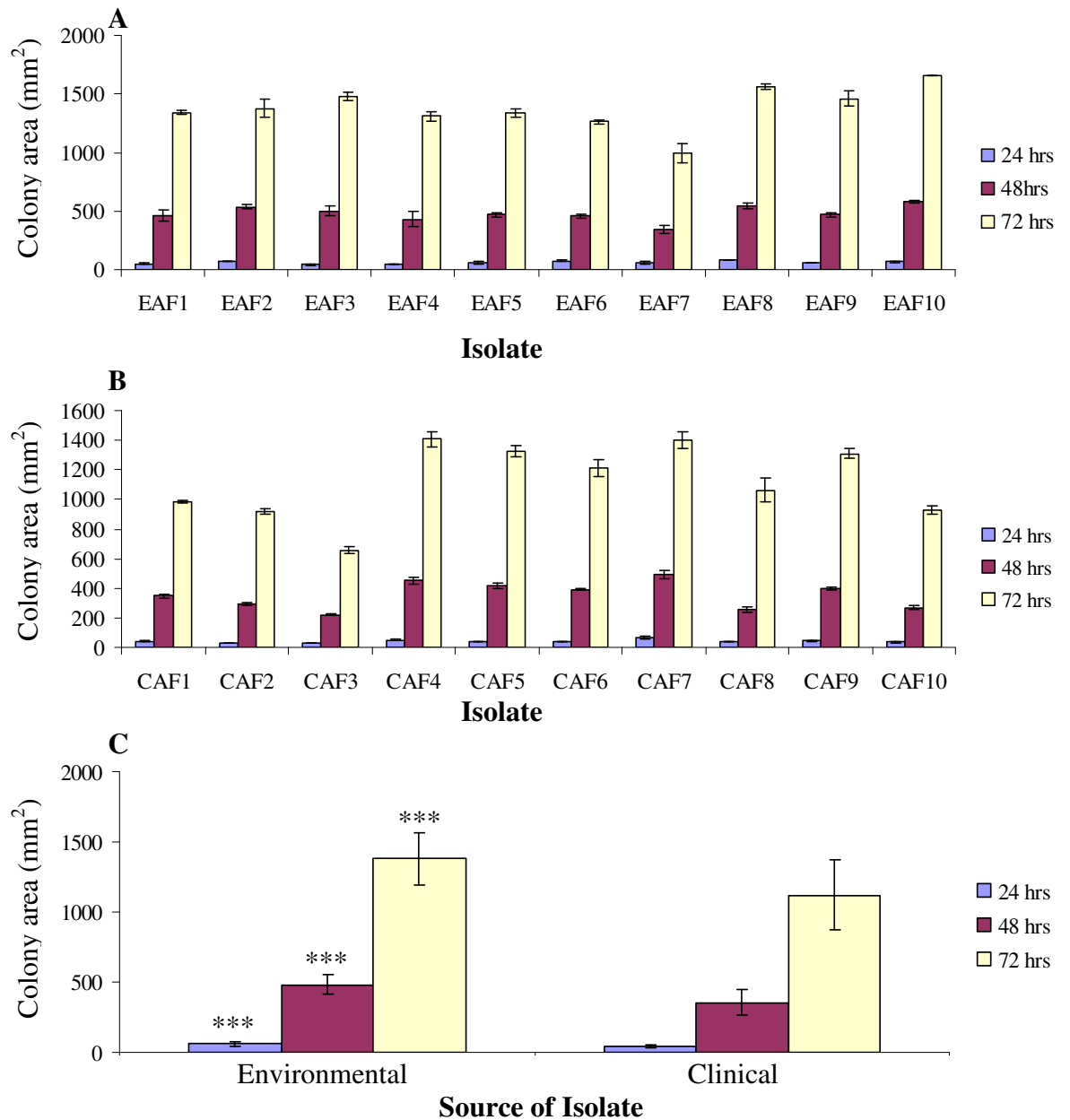
Figure 3.19 Growth of clinical and environmental isolates of *A. fumigatus* on AMM.

Conidia (5×10^3) from environmental (**A**) and clinical (**B**) *A. fumigatus* isolates was spotted on to AMM, incubated at 37ºC and growth was monitored at 24, 48 and 72 hours. Analysis of the average growth of *A. fumigatus* isolates (**C**) indicates that environmental isolates demonstrated improved growth after 24, 48 and 72 hours (***p = 0.001).

plate to give a fixed cell concentration of 5 x 10^3 (Figure 2.5.1). The agar plates were incubated at 37ºC and radial growth of the *A. fumigatus* isolates was measured at 24, 48 and 72 hours and is presented in Figure 3.19 **A** and **B**. Inoculation of plates with environmental and clinical isolates of *A. fumigatus* resulted in variable growth at the different time-points with colony size variability most evident after 72 hours. The data from the environmental and clinical area recordings were grouped to give the mean growth of clinical and environmental *A. fumigatus* isolates at 24, 48 and 72 hours thus facilitating a comparison between the different sources (Figure 3.19 **C**). The colony area of environmental *A. fumigatus* isolates on AMM was calculated as 60.11 ± 14.32 mm^2, 479.89 ± 69.62 mm^2 and 1377.72 ± 188.72 mm^2 at 24, 48 and 72 hours, respectively. Clinical isolates grew to 40.96 ± 11.13 mm^2, 354.17 ± 91.11 mm^2 and 1120.75 ± 249.97 mm^2 over the same period. Statistical analysis of these data showed that environmental isolates demonstrated significantly faster growth than clinical isolates at 24, 48 and 72 hours ($p \leq 0.001$).

3.3.2 Growth of clinical and environmental isolates of *A. fumigatus* on milk agar.

Conidia were harvested as described (Section 2.2.10) and the concentration of conidia was adjusted to give a final concentration of 1 x 10^6 conidia ml^{-1}. A 5 µl droplet was placed on the surface of a milk agar plate (Section 2.2.12) and was allowed to air dry. The plates were incubated at 37ºC and radial growth was measured 24, 48 and 72 hours post inoculation. Analysis of the growth of environmental and clinical isolates (Figure 3.20 **A** and **B**) demonstrated strong growth on milk agar however due to the rapid nature of *A. fumigatus* mycelial growth the expected zone of precipitation ("halo-like" in appearance) was not visible. The pattern of *A. fumigatus* growth did appear to be different from that observed on AMM. For this reason the data obtained from each isolate were pooled into respective clinical and environmental groups and was
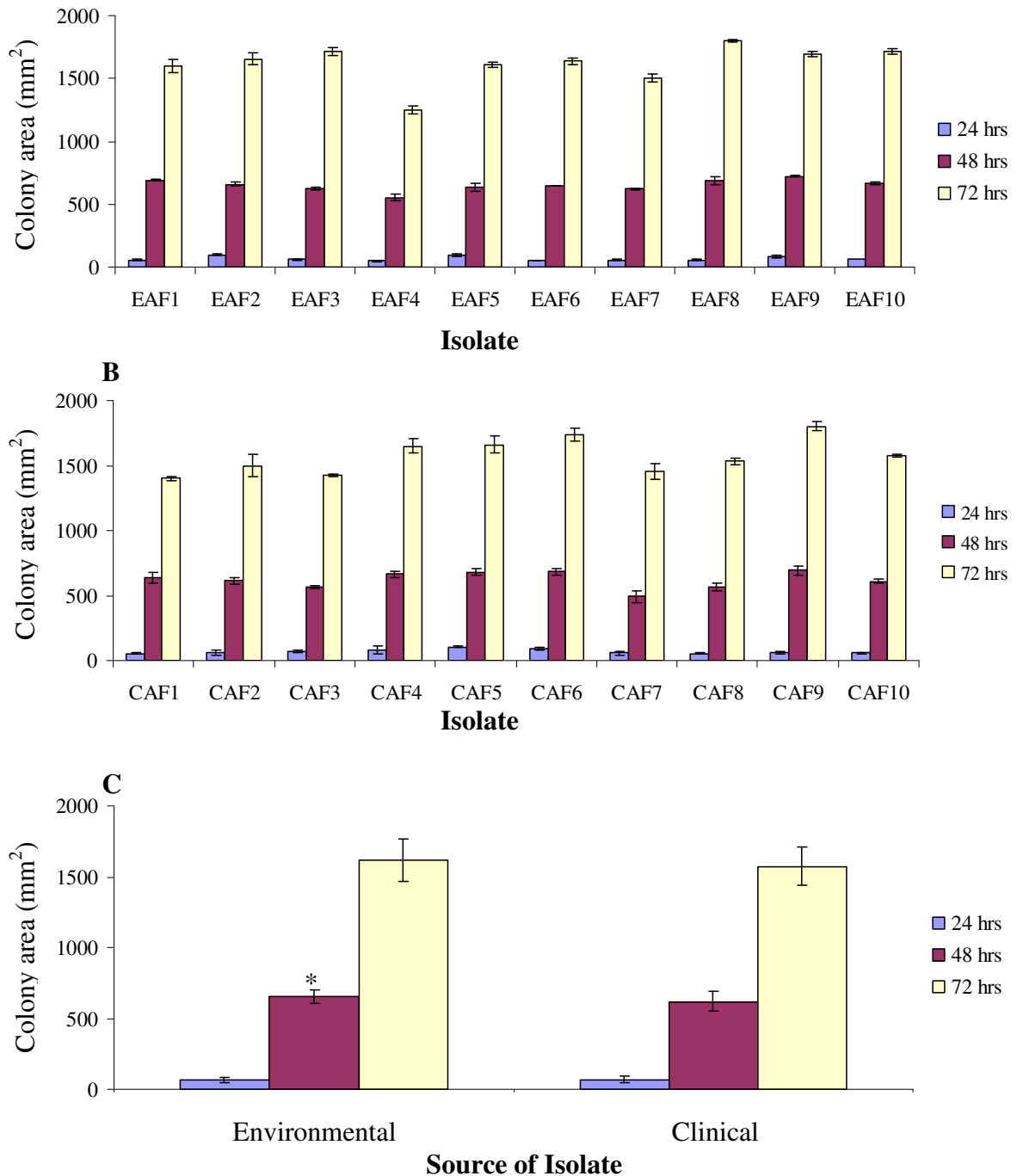
Figure 3.20 Growth of environmental and clinical isolates of *A. fumigatus* on milk agar.

Conidia (5×10^3) from environmental (**A**) and clinical (**B**) *A. fumigatus* isolates were spotted on milk agar plates and incubated at 37°C. The growth of *A. fumigatus* was recorded 24, 48 and 72 hours post inoculation. The average growth of environmental and clinical isolates was grouped and expressed as mean hyphal growth (**C**). Average growth was similar after 24 and 72 hours. Environmental isolates demonstrated improved growth after 48 hours (*$p = 0.035$).

expressed in terms of mean and standard deviation (Figure 3.20 **C**). Environmental isolates grew to 66.52 ± 18.65 mm^2, 650.85 ± 47.92 mm^2 and 1616.28 ± 149.83 mm^2 24, 48 and 72 hours post-inoculation, respectively. Clinical isolates grew to 69.12 ± 21.08 mm^2, 619.86 ± 67.57 mm^2 and 1573.68 ± 136.644 mm^2 at the same times. Comparisons of clinical and environmental growth revealed that the rate of growth was similar at 24 and 72 hours (p = 0.597 and 0.275, respectively). The rate of growth of environmental isolates was higher relative to clinical isolates at 48 hours post inoculation (p = 0.035).

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3.3.4 Summary

In this section the growth of clinical and environmental *A. fumigatus* isolates on AMM and milk agar was compared. Environmental isolates of *A. fumigatus* demonstrated significantly higher growth over clinical isolates at 24, 48 and 72 hours on AMM.

The growth of *A. fumigatus* isolates on milk agar was also ascertained. It was interesting to note that colony growth was similar at 24 and 72 hours with environmental isolates demonstrating faster mean growth 48 post-inoculation.

3.4 Discussion

The use of *G. mellonella* larvae as useful, high throughput and ethical *in vivo* tools to ascertain microbial pathogenicity has increased in the last decade (Cotter *et al*., 2000; Brennan *et al*., 2002; Mahar *et al*., 2004; Mylonakis *et al*., 2005; Jackson *et al*., 2009). The concentration of microbe required in these studies to initiate pathogenicity shows a high level of variation with infection of 2 x 10^6 *C. albicans* cells, 1 x 10^6 *A. fumigatus* conidia and concentrations as low as 20 *Cryptococcus* c.f.u per larvae resulting in larval death. Jackson *et al*., (2009) demonstrated that a 1 x 10^6 inoculum of

A. fumigatus Af293 conidia incubated at 37ºC should be used in *G. mellonella* studies. This contrasted with the work of Reeves *et al*., (2004) and Renwick *et al.,* (2006) where a temperature of 30ºC was used. Mowlds *et al*., (2008) characterised the effect of different temperatures on haemocyte density and the expression of genes encoding antimicrobial peptides in *G. mellonella*. This work demonstrated a 3 - fold increase in haemocyte density in larvae incubated at 37ºC relative to larvae incubated at 30ºC. Furthermore the expression of *gallerimycin* and *transferrin* are significantly increased at 37ºC. Considering that the optimal growing temperature of *A. fumigatus* is 37ºC, the finding that this temperature modulates the immune status of *G. mellonella* is an additional factor which must be considered in such studies. It was found that concentrations of 1×10^4 conidia larvae^{-1} were avirulent a 30ºC or 37ºC. Inoculating larvae with 1×10^5 conidia resulted in no significant change in larval viability but demonstrated some pathogenicity at 37ºC. Infecting larvae with 1×10^6 or 1×10^7 conidia larvae^{-1} demonstrated a distinct change in larval viability depending on the incubation temperature. Infecting larvae with 1×10^6 conidia resulted in a small change to larval viability at 30ºC over 72 hours, however incubating larvae at 37ºC caused a significant loss in viability over the same period of time. The effect of larval incubation at 37ºC is exacerbated further when infected with 1×10^7 conidia with no viability 48 hours post-infection. In contrast infections with the same concentration of conidia and incubating at 30ºC resulted in larval viability being reduced in a manner that was similar to infection with 1×10^6 conidia and incubating at 37ºC. It is therefore clear that studies using *A. fumigatus* may be performed at 30ºC or 37ºC. Mowlds *et al*., (2008), and Wojda and Jacubowicz, (2007) clearly demonstrate that the cellular and humoral immune response of *G. mellonella* is primed at 37ºC. It was decided that for subsequent experimentation a temperature of 30ºC would be used in order to negate the effect of temperature priming of the larval immune response.

The relative pathogenicity of *A. fumigatus* isolates with deletions for genes encoding fumitremorgin (Δ*ftmA*), pseurotin A (Δ*psoA*) and the nuclear associated secondary metabolite regulation protein laeA (Δ*laeA*) from the Af293 parent strain was determined. Larval infection with Af293 resulted in a complete loss of viability 72 hours post-infection. The mutant strains used in this study all demonstrated attenuated virulence over the same time period. The Δ*laeA* strain showed the least attenuation of the three mutant strains. This is interesting as *laeA* has been identified as a global regulator of secondary metabolite biosynthesis thus acting as a significant virulence factor (Bok and Keller, 2004; Bok *et al*., 2005). Perrin *et al*., (2007) showed that *laeA* contributes to the expression of approximately 9.5% of the *A. fumigatus* genome with 13 of 22 metabolite gene clusters adversely affected by *laeA* gene deletion thus confirming the role of this protein in fungal virulence. *PsoA* is a NRPS gene which encodes for the production of pseurotin A. Pseurotin A is a neuritogenic agent and a competitive inhibitor of chitin synthase, (Maiya *et al*., 2007). Recent work by Ishiwika *et al*., (2009) demonstrated that B-cell Ig-E production is suppressed by pseurotin A and its analogues. *FtmA* encodes the brevianamide synthethase which produces brevianamide F, a precursor of fumitremorgins A, B and C which are tremorigenic (Maiya *et al*., 2006). Deletion of a gene encoding for *ftmA* resulted in the most attenuated virulence in larvae thus highlighting the need for improved characterisation of fumitremorgins *in vivo.* Preliminary analysis of Δ*psoA* and Δ*ftmA* virulence in neutropenic mice demonstrated significantly reduced virulence of *psoA* deletants (p < 0.02) but no significant difference was observed in comparisons between Af293 and Δ*ftmA* (Dr. E. Bignell, personal communication). It was perhaps surprising that Δ*laeA* was the least attenuated isolate as its role as a global regulator of secondary metabolite biosynthesis means that mycotoxin production would have been extremely restricted. Furthermore, Bok *et al*., (2005) demonstrated that deletion of *laeA* resulted in increased

phagocytosis of conidia, reduced fungal burden in the murine lung and reduced production of gliotoxin. However the authors found that deletion of *laeA* caused minimal changes to growth rate thus indicating that any changes in virulence are as a result of diminished secondary metabolite production. The finding that deletion of *ftmA* and *psoA* resulted in more attenuated virulence may highlight the requirement for further analysis of *laeA* in fungal pathogenesis. Furthermore it has been previously shown that deletants of *psoA* and *ftmA* do not demonstrate significantly reduced growth (Maiya *et al*., 2006 and 2007). Therefore it would appear likely that the observed attenuation in virulence is as a result of impaired secondary metabolite fuction. The notable discrepancy in virulence of the Δ*ftmA* isolate between *G. mellonella* and neutropenic mouse model is an issue which needs to be addressed through more experimental data.

Comparative analysis of clinical and environmental isolates of *A. fumigatus* has focussed on studies in murine models and relative mycotoxin production with evidence suggesting that a high degree of variability exists between strains (Mondon *et al*., 1996; Aufauvre-Brown *et al*., 1998; Kosalec and Pepeljnjak, 2005). Recent work on characterising the source of these isolates has pointed to azole resistance occurring from the use of fungicides in the environment resulting in a TR/L98H mutations becoming evident in clinical and environmental isolates (Verweij *et al*., 2009). In this Chapter it was hoped to characterise a range of clinical and environmental isolates of *A. fumigatus* at different stages of conidial maturation with regard to their pathogenicity in *G. mellonella*. The results reveal that both clinical and environmental isolates of *A. fumigatus* demonstrated significant variability in larvae with 3 day old conidia showing increased pathogenicity. Infection with 3 day old environmental conidia demonstrated the greatest variation in larval pathogenicity. A comparison between mean larval viability data when larvae were infected with clinical or environmental isolates showed

that virulence is similar for both isolate types. These findings are particularly interesting as a screen of clinical and environmental *A. fumigatus* isolates at different stages of conidial maturation has not been published to date. Significantly the viability data obtained from infecting larvae with environmental isolates would appear to suggest that pathogenicity is variable depending on the stage of conidial maturation. The reasons for this appear unclear although it may be possible that alterations to the quantity of melanin on the conidial surface may play a role in pathogenesis. Jahn *et al*., (1997 and 2000) demonstrated that conidia lacking melanin were less virulent in murine models of infection. In contrast Jackson *et al*., (2009) demonstrated that a loss of melanin on the conidial surface resulted in increased virulence in *G. mellonella*. It may therefore be possible that the structure and quantity of melanin on differentially matured conidia may be a virulence factor which may need to be considered in future studies.

The pathogenicity of environmental "non-fumigatus" *Aspergillus* isolates in *G. mellonella* was also determined. *A. flavus* demonstrated a high level of pathogenicity in larvae with complete mortality present at 24 hours and the observation of condiophores protruding from the larval cuticle at 72 hours. The data obtained from this experiment must be interpreted with a note of caution as it is clear that the infecting dose used in these experiments was excessive. *A. flavus* has been recently described as a major cause of aspergillosis in developing countries (Chakrabarti *et al*., 2010), thus, characterising the pathogenicity of isolates of this fungus may be required on a larger scale. The use of *G. mellonella* to study *A. flavus* pathogenesis has been documented to show host restriction following several propagations through larvae, (Scully and Bidochka, 2005). The large size of *A. flavus* conidia, (Pasqualotto, 2008) may also be contribute to its virulence as Renwick *et al*., (2006) demonstrated that haemocytes were not able to efficiently phagocytose swollen or germinating conidia of *A. fumigatus*. In this Chapter it is clear that *A. flavus* conidia demonstrate a much larger size distribution than *A.*

fumigatus conidia. It may be possible that *A. flavus* conidia are too large to be phagocytosed thus serving as a potential virulence factor in *G. mellonella*. Further clarifiaction regarding the apporapriate level of infection administered to larvae is necessary for future work and may place the occurrence of differential pathogenicity from matured conidia into context. *A. niger* is less documented in fungal pathogenesis studies however it has been extensively studied as a producer of citric acid, (Baker, 2006). Reeves *et al*., (2004) demonstrated that *A. niger* was virulent in *G. mellonella* therefore it is not surprising that pathogenicity was observed in this case. Larval viability following infection with *A. niger* conidia was variable depending on the level of conidial maturation. *A. terreus* has been also been described as a significant emerging pathogen among immunocompromised individuals (Malani and Kauffman, 2007). Larval infection with 3, 14 and 28 day old conidia of *A. terreus* resulted in loss of larval viability. The observed pathogenicity was attenuated with increased conidial maturation. These results coupled to observations of variable pathogenicity of *A. fumigatus* and *A. niger* with different aged conidia further validate the use of *G. mellonella* to screen potentially pathogenic *Aspergilli* however the role of conidial maturation is clearly a factor which must be considered.

The role of *A. fumigatus* mycotoxin production has often focussed on the secretion of gliotoxin as the principle effector of secondary metabolite mediated immunosuppression (Ben-Ami *et al*., 2010). In this work gliotoxin was detected in *G. mellonella* larvae at 24, 48 and 72 hours with the highest amount being evident at 48 hours. This corresponded to the time-point where the greatest variation in larval viability following fungal infection was observed (Figure 3.1). Reeves *et al*., (2004) had previously highlighted the correlation between gliotoxin production and virulence of *Aspergillus* species in larvae. In this study fumagillin was detected at high concentrations *in vivo* 24 hours post-infection. The concentration of fumagillin per

gramme of larval tissue appeared to be reduced at subsequent time-points thus indicating it as a mediator of early fungal pathogenesis. Clearly the production of fumagillin warrants further investigation as it has been shown to be highly bioactive and capable of mediating significant toxicity (Amitani *et al*., 1995; Stevanovic *et al*., 2008).

The mycotoxin concentration in *A. fumigatus* culture filtrates and the potential immunosuppressive effect of these culture filtrates were also assessed. There was variability in the production of gliotoxin and fumagillin in clinical and environmental isolates. The variability in gliotoxin production between different isolates has been previously documented and has been demonstrated to be strongly influenced by conditions which favour mycelial growth (Lewis *et al*., 2005, Kosalec *et al*, 2005) with fumagillin production apparently dependent on the nature of carbon and nitrogen sources, (Yang *et al*., 2003). In this work the average quantity of gliotoxin produced by clinical and environmental isolates of *A. fumigatus* isolates was found to be quite similar. Environmental isolates produced higher concentrations of fumagillin on average thus raising the possibility that its synthesis is based on conferring a competitive advantage against other microbes in nature. The culture filtrates characterised by mycotoxin concentration were also injected into *G. mellonella* larvae prior to infection with *A. fumigatus* ATCC 26933. The data demonstrated the immunosuppressive effect of these culture filtrates as evidenced by reduced larval viability following fungal infection relative to prior inoculation with media alone. Notably the highest producing mycotoxin strains did not correlate entirely with the greatest loss in larval viability when subsequently infected. Therefore it would appear that weakening of the host immune response by secreted factors produced by *A. fumigatus* is not dependent on mycotoxin production alone. It is suggested here that a cumulation of multiple factors (enzymes, mycotoxins) are responsible for the

exacerbation of fungal pathogenesis *in vivo* which mycotoxins play a major, but not exclusive role.

A point of note is the temperatures which were employed in these studies. Genomic sequence analysis performed by Nierman *et al*., (2005) demonstrated that the expression of virulence factor related genes in *A. fumigatus* is highly temperature dependant. In that study it was shown that pathogenicity associated genes demonstrated higher levels of expression at higher tempetures such as 48ºC. Analysis identified 323 genes which showed higher expression at 48ºC with 135 demonstrating higher expression at 37ºC. Interestingly gene expression was markedly increased as temperature increased to 37ºC or 48ºC increased following initial incubation at 30ºC. These findings would therefore suggest that gene expression is likely to be severely reduced when incubated at 30ºC. Therefore it is likely that the observed production of mycotoxins in *vivo* and in *vitro* may not be at optimal levels in these experiments. However, considering the work of Mowlds *et al*., 2008 it is clear that higher temperatures have a priming effect on the immune system of *G. mellonella* larvae therefore strengthening the argument for using a temperature of 30ºC for *A. fumigatus* pathogenicity studies.

The growth of clinical and environmental isolates on defined minimal medium and milk agar was also ascertained in order to characterise the relative growth rates of strains. Environmental isolates demonstrated significantly more growth on AMM than clinical isolates on this media thus indicating a higher capacity to grow on limited nutrients.

The growth of clinical and environmental isolates on milk agar was similar thus indicating conservation of protease activity. Milk agar has been used in bacteriology studies to examine general protease activity evidenced by a zone of precipitation around a growing colony, (DeShazer *et al*., 1999). Applying this method to *A. fumigatus* is

difficult as rapid hyphal growth into assimilated carbon sources is a feature of the saprophytic nature of this fungus. Richie *et al.*, (2009) used this media to characterise the unfolded protein response in *A. fumigatus*. In that particular study Δ*hacA*, a strain deficient in an unfolded protein response transcriptional regulator, demonstrated reduced growth on this medium coupled to a diminution of protease activity. The data presented here would appear to demonstrate that the clinical and environmental isolates used in this study demonstrated equivalent growth on this media therefore it would appear that general protease activity is conserved.

In conclusion this Chapter further validates the use of *G. mellonella* larvae as a novel, reproducible, high throughput tool for the study of systemic *Aspergillus* infections. *A. fumigatus* pathogenicity would appear to be mediated by a combination of *in vivo* mycotoxin production, rapid growth in nutritionally depleted environments coupled to the secretion of protein degrading factors which result in favourable conditions for fungal pathogenesis.

Chapter 4

Investigation of the effect of fumagillin on neutrophil and haemocyte function

4.0 Introduction.

Neutrophils form a primary line of defence against invading microbes by phagocytosing opsonised pathogens, producing superoxide and nitric oxide and the degranulation of azurophilic granules to the phagosome. Degradation of the ingested microbe occurs in the phagocytic vacuole by a combination of oxidative and non-oxidative mechanisms (Babior *et al*., 1973; Curnutte *et al*., 1987; Wheeler *et al*., 1997; Bellochio *et al*., 2004). Central to the oxidative process, the translocation of cytosolic p40^{phox}, p47^{phox}, p67^{phox} and Rac to the membrane is required to form a fully functional NADPH oxidase complex capable of producing superoxide which shows limited bio-accumulation in *vivo* but is rapidly protonated to form hydrogen peroxide (Wientjes *et al*., 1993; Sumimoto *et al*., 1996).

Non-oxidative mechanisms are due to neutral serine proteases, hydrolytic enzymes, and cationic proteins which are packaged in cytoplasmic lysosomes and are released to the phagosome upon cell activation (Spitznagel, 1990). Myeloperoxidase is a key component of these primary granules and catalyses the production of hypochlorous acid upon cell activation and subsequent degranulation, with the additive effect of demonstrating microbicidal activity (Spitznagel, 1990; Standish and Weiser, 2009).

In recent years an increasing amount of evidence has shown that the processes involved in neutrophil function are intricately linked to the formation and subsequent disassembly of F-actin. The work of Zhan *et al*., (2004) demonstrated this principle whereby translocation of p47^{phox} is dependent upon interactions between the PX domain of p47^{phox} and moesin thus acting as the primary mediator for the formation of the NADPH oxidase complex. Additionally, the regulation of F-actin disassembly appears to be intricately linked to this process. Antisense oligonucleotides to cofilin, an actin depolymerising factor, have been demonstrated to reduce the rate of phagocytosis and lower superoxide production (Adachi *et al*., 2002). It follows that tight regulation of the

process of F-actin assembly is essential in the activation process of neutrophils with the effect of eliminating the microbe from the host.

A. fumigatus is capable of producing a variety of toxins and enzymes which facilitate the initial colonisation of lung tissue, exacerbate fungal growth and impair host immune responses (Tekaia and Latgé, 2005). Fumagillin is produced upon hyphal development (Mitchell *et al*., 1997), however the role it plays in the pathogenesis of *A. fumigatus* remains poorly characterised. The most significant evidence to date would appear to show that fumagillin is capable of retarding ciliary beat frequency at a concentration of 10 µg ml^{-1}. Tsunawaki *et al*., (2004) employed concentrations up 128 µg fumagillin ml^{-1} with a cellular exposure time of 7 minutes but failed to see any significant effect on the process of neutrophil degranulation. It would appear however that this concentration is abnormally high as experimental data (presented in Chapter 3) would suggest that the concentration of fumagillin is much lower in *vivo*. Furthermore, it is not clear if the concentration used harms cellular viability therefore normal cellular function could not be possible.

The primary aim of this study was to perform a comprehensive analysis of the effect of fumagillin on neutrophil function. Furthermore, considering the work of Renwick *et al*., (2007), it was deemed necessary to evaluate the effect of equivalent fumagillin concentrations on *G. mellonella* immunocompetence, and haemocyte function in order to clarify if any possible effects viewed in neutrophils are equivalent to those of haemocytes. It is clear that understanding such conservation of cellular responses is crucial to the validation of *G. mellonella* as an accurate in *vivo* model to screen host physiological responses to mycotoxins and other fungal virulence factors, therefore extending the biotechnological utility of this organism.

4.1 Analysis of the effect of fumagillin on the ability of neutrophils to phagocytose and kill a pathogenic target *in vitro*.

In order to understand if fumagillin was capable of inhibiting neutrophil function it was necessary to understand if neutrophil mediated phagocytosis and killing were adversely affected by fumagillin. Considering conflicting data as to the minimum concentration of fumagillin capable of inhibiting function, (Amitani *et al*., 1995, Tsunawaki *et al*. 2004), initial work focussed on identifying a concentration of fumagillin which significantly reduced neutrophil mediated killing of fungal and bacterial human pathogens.

4.1.1 Analysis of the effect of fumagillin on neutrophil mediated killing.

Neutrophils were extracted as described (Section 2.14). A killing assay using *C. albicans* as a target was performed, (Section 2.16.1) and the results are presented in Figure 4.1. The viability of neutrophils did not change significantly throughout the course of the assay, reducing from $98.1 \pm 2.2\%$ before the assay to $94.5\% \pm 1.5\%$ after 60 minutes therefore excluding the possibility that any alteration in *C. albicans* viability resulted from a loss of neutrophil viability.

The viability of *C. albicans* was found to be greatly reduced after 60 minutes when incubated with neutrophils exposed to PBS alone or the methanol control ($5.01 \pm 4.23\%$ and $5.29 \pm 4.31\%$ respectively). Neutrophils incubated with 1μg fumagillin ml^{-1} demonstrated a slight but not statistically significant difference in the ability to kill *C. albicans* recording $11.56 \pm 5.12\%$ viability after 60 minutes ($p = 0.6$). Neutrophils exposed to 2 μg fumagillin ml^{-1} did not show a statistically significant difference in the rate of killing compared to the control up to 20 minutes with *C. albicans* viability at $63.6 \pm 10.98\%$ ($p = 0.247$) however this rate was found to change considerably thereafter. Neutrophils incubated with 2 μg fumagillin ml^{-1} demonstrated a marked
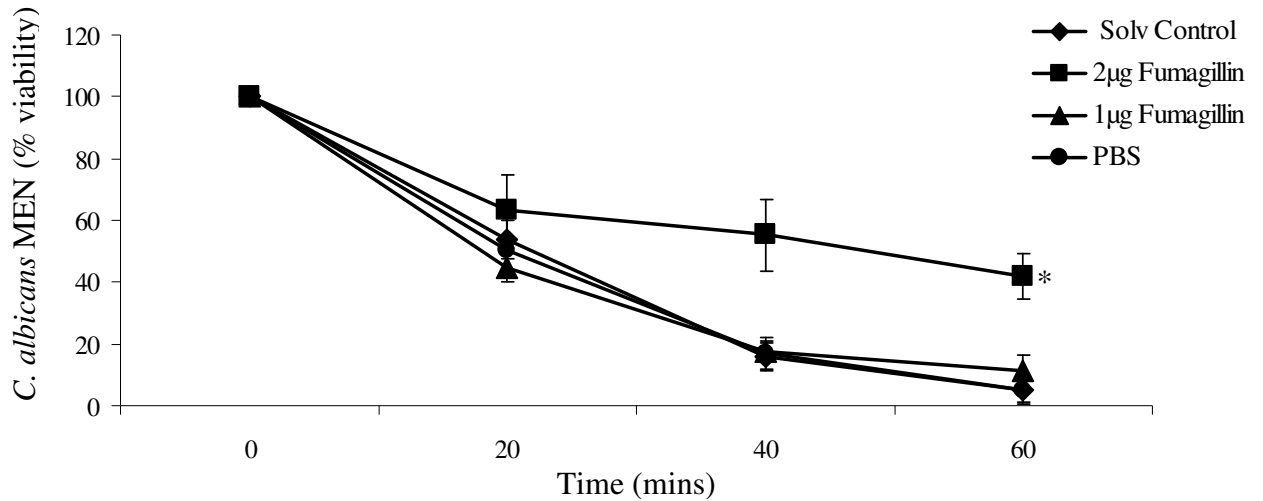
Figure 4.1 Effect of fumagillin on neutrophil mediated *C. albicans* killing.
Neutrophils were isolated and exposed to 1 or 2 µg fumagillin ml^{-1} for 25 minutes at
37ºC and subsequently used in a killing assay using a stirred chamber. Cells exposed to
2 µg fumagillin ml^{-1} demonstrated a reduced capacity to kill *C. albicans* MEN while
neutrophils exposed to the solvent control treatment or 1 µg fumagillin ml^{-1}
demonstrated insignificant changes compared to the PBS control.

* $p \leq 0.02$
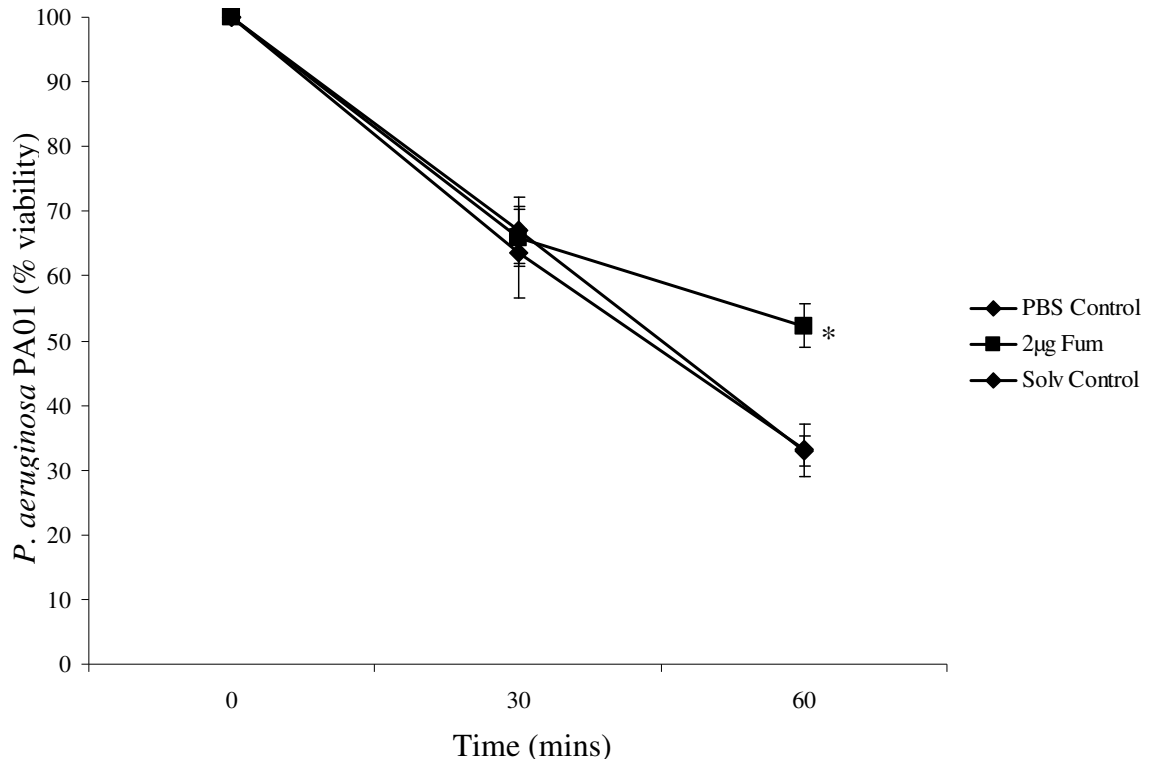
Figure 4.2 Effect of fumagillin on neutrophil mediated killing of *P. aeruginosa*.
Neutrophils were isolated and a killing assay using *P. aeruginosa* as a pathogenic target was performed as described, (Section 2.16.3). Neutrophils exposed to fumagillin (2 µg ml^{-1}) killed significantly less bacterial cells after 60 minutes (*p $\leq$ 0.05) than either positive control treatment.

decreasing in killing activity with yeast viability 58.1 ± 7.4% viability after 60 minutes ($p \leq 0.02$).

Analysis of the fumagillin mediated modulation of neutrophil antimicrobial activity was extended to identifying if the inhibitory effect was applicable to bacteria. Neutrophils were extracted and a killing assay using *P. aeruginosa* was performed as described, (Section 2.16.3). Analysis of the data (Figure 4.2) revealed that there was negligible variability in bacterial viability after 30 minutes. Neutrophils incubated in the presence of PBS or incubated in the presence of 0.002% (v/v) methanol prior to incubation with bacteria killed 67 ± 4.13% and 66.98 ± 2.3% of the *P. aeruginosa* cell population respectively at 60 minutes ($p = 0.991$). This contrasted with the figure observed for neutrophils exposed to 2 µg fumagillin ml^{-1} where 52.26 ± 3.35% of bacteria remained viable. Statistical analysis of the capacity of neutrophils to kill *P. aeruginosa* revealed a significant reduction in cell mediated killing, ($p \leq 0.05$). It was decided for subsequent experimentation that studies on the effect of inhibitory concentrations of fumagillin would focus on the 2 µg ml^{-1} concentration as being the lowest concentration which elicited a physiologically relevant response. As a result this would facilitate a more in depth analysis of the effect of fumagillin on the various cellular parameters involved in neutrophil function.

4.1.2 Analysis of the effect of fumagillin on the ability of neutrophils to phagocytose.

Based on the results in Section 4.1.1 initial work was focussed on investigating the effect of fumagillin on the process of neutrophil mediated phagocytosis as described in Section 2.16.4. Figure 4.3 shows that the rate of phagocytosis increased rapidly in the PBS and methanol control treatments up to 60 minutes with 46.24 ± 1.72% and 44.61 ± 3.11% of cells showing phagocytosis, respectively. However at 60 minutes it is evident
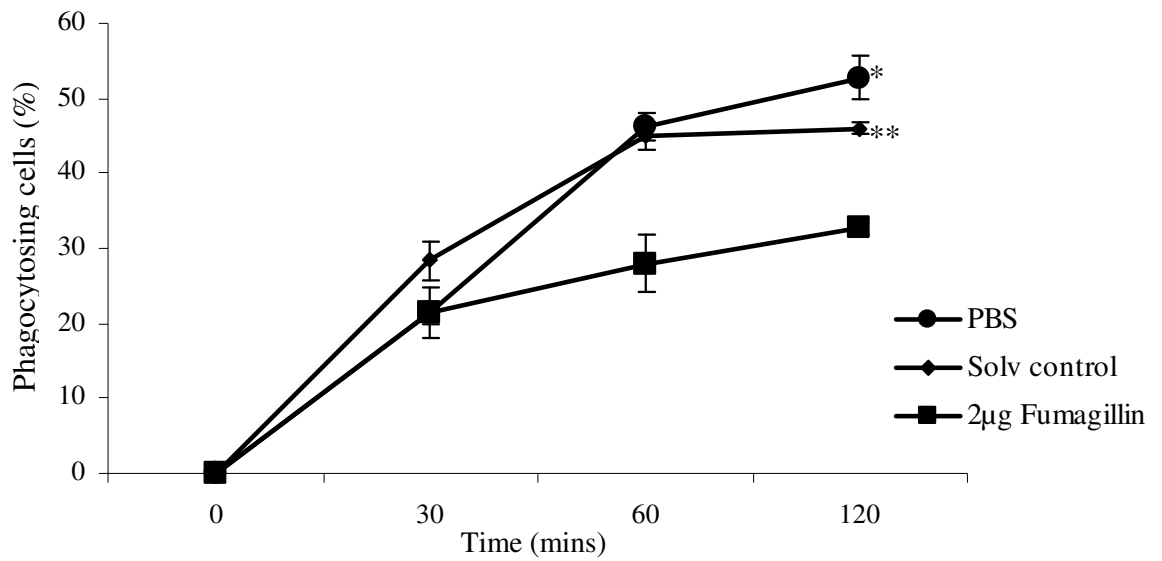
Figure 4.3 Effect of fumagillin on neutrophil mediated phagocytosis.
Neutrophils (1×10^7) were exposed to fumagillin (2 µg ml^{-1}) and were employed in a phagocytosis assay as described, (Section 2.16.4). Neutrophils exposed to fumagillin phagocytosed less conidia than PBS or the 0.002% (v/v) methanol treated control, (p = 0.023* and 0.015* respectively).

that the rate of neutrophil mediated phagocytosis in the 2 µg fumagillin ml^{-1} pre-treatment shows a decrease with 27.94 ± 3.86% of cells demonstrating phagocytosis. This decrease in the rate of phagocytosis was found to be significant compared to PBS, (p = 0.023) and methanol control treated neutrophils, (p = 0.015). The increase in rate of phagocytosis was reduced from 60 to 120 minutes in all treatments compared to the first 60 minutes. At 120 minutes the level of phagocytosis had increased to 52.75 ± 2.82% in the PBS treatment and 46.02 ± 0.736% in the methanol control. This contrasted with the rate of phagocytosis observed in neutrophils exposed to 2 µg fumagillin ml^{-1} where 32.85 ± 1.09% of the cell population had internalised conidia with p – values of 0.013 and 0.01 relative to the PBS and methanol controls respectively.

4.1.3 Summary.

The results presented in this section demonstrates that fumagillin at a concentration of 2 µg ml^{-1} reduces the neutrophil mediated killing of *C. albicans* and *P. aeruginosa*. Lower concentrations of fumagillin were analysed for their effect on neutrophil mediated killing of *C. albicans* however no statistically significant effect was observed. An analysis of the effect of fumagillin on neutrophil mediated phagocytosis showed that exposure of neutrophils to a functionally inhibiting concentration of fumagillin (2 µg ml^{-1}) resulted in reduced internalisation of *A. fumigatus* conidia than the control treatments.

4.2 Analysis of the effect of fumagillin on oxygen consumption and the formation of a functional NADPH oxidase complex.

The work of Tsunawaki *et al*., (2004) demonstrated that the gliotoxin produced by *A. fumigatus* effectively blocks the formation of a functional NADPH oxidase complex by inhibiting the translocation of p47^{phox} and p67^{phox} from the cytosol to the

membrane effectively reducing the association with the flavocytochrome b$_{558}$ complex with the additive effect of a loss of superoxide production. Considering the role of oxygen consumption and the resulting production of superoxide mediated by the formation of this complex it was necessary to undertake an analysis of the effect of inhibitory concentrations of fumagillin on this process. As a result this would potentially demonstrate the effect of fumagillin on a key facet of the antimicrobial activity of neutrophils thus facilitating an understanding of the effect of fumagillin on the balance of the cytosolic and membrane proteome following stimulation.

4.2.1 Effect of fumagillin on oxygen consumption.

Neutrophils were isolated and an assay to determine the effect of fumagillin on the consumption of oxygen was performed as described, (Section 2.18.6). Phorbol-12-mytistate-13-acetate (PMA) at a concentration of 1µg ml^{-1} was used as an artificial stimulus as this has been demonstrated to elicit the activation of neutrophils (Reeves *et al*., 2002). Neutrophils, (1 x 10^7 ml^{-1}) were placed in a stirred chamber and allowed to stabilise for approximately 3 minutes prior to stimulation. The rate of oxygen consumption was monitored as shown in Figure 4.4 over a ten minute period consistent with the methodology described by Bergin *et al*., (2005). Control neutrophils and those exposed to 0.002% (v/v) methanol consumed 55.48 ± 13.12 and 72.63 ± 2.09 nmol ml^{-1} oxygen after 5 minutes respectively. Importantly this value was not deemed as significant, (p = 0.262). It is clear that 5 minutes post stimulation the rate of oxygen depletion by neutrophils pre-treated with 2 µg fumagillin ml^{-1} was significantly reduced relative to the control treatments with 21.48 ± 8.02 nmol ml^{-1} oxygen consumed (p $\leq$ 0.025). This apparent reduction in the ability of neutrophils previously exposed to fumagillin to consume oxygen continued to the ten minute timepoint. Neutrophils previously incubated in the presence of fumagillin consumed 29.03 ± 11.77 nmol
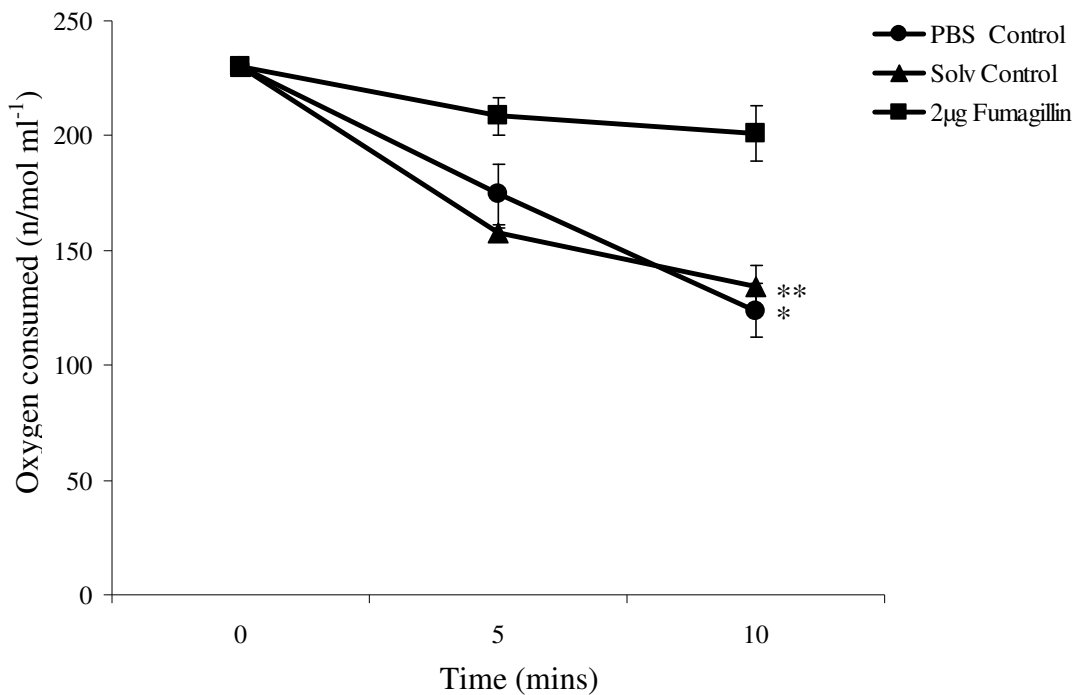
Figure 4.4 Effect of fumagillin on the ability of neutrophils to consume oxygen.
Neutrophils, (1×10^7) were isolated and incubated in the presence of fumagillin. Cells were washed and the rate of oxygen consumption following PMA stimulation ($1 \ \mu g \ ml^{-1}$) was observed (Section 2.18.6). Neutrophils exposed to fumagillin consumed significantly less oxygen than cells incubated in PBS or 0.002% (v/v) methanol, ($p = 0.032^*$ and 0.017^{**} respectively).

oxygen ml^{-1}. This rate of oxygen consumption contrasted with the rate observed in PBS and solvent control treatments where 106.07 ± 11.92 and 95.84 ± 8.97 nmol oxygen ml^{-1} was consumed respectively (p = 0.032 and 0.017). Comparisons between both control treatments demonstrate no statistically significant difference, (p = 0.198) therefore confirming that the reduction in the rate of oxygen consumption is a result of inhibition mediated by fumagillin.

4.2.2 Analysis of the effect of fumagillin on the translocation of p47^{phox}.

As a result of the work presented in Section 4.2.1 an analysis of the formation of a functional NADPH oxidase complex was performed. Neutrophils, (1×10^8 ml^{-1}) were isolated and fractionated as described, (Section 2.18.4). The resulting cytosolic and membrane protein components were subjected to 1–D SDS-PAGE and immunoblotting, (Section 2.7 and 2.9). Rabbit polyclonal antisera raised in rabbits immunised with recombinant p47^{phox} was used at a dilution of 1/2000 in the primary antibody solution. Following washing and probing with secondary anti-rabbit horseradish peroxidase, (HRP) conjugated antibody blots were developed with ECL reagent or DAB staining, (Section 2.9.7). Analysis of p47^{phox} reactivity revealed the identification of a band which displayed size resolution of approximately 47 kDa to be present in the membrane fraction of all treatments, (Figure 4.5 B) confirming the presence of the protein. Importantly, identification of p47^{phox} in membrane fractions demonstrated increased translocation in all treatments where cells received PMA stimulation relative to unstimulated cells. The fluctuation in membrane p47^{phox} was quantified by densitometry and the data resulting from this analysis are presented in Figure 4.5 (C). Cells which were stimulated with PMA and were not pre-incubated in the presence of fumagillin or 0.002% (v/v) methanol demonstrated a 2.59 fold increase in the presence of p47^{phox} in the membrane fraction (p = 0.011). Similarly the solvent control for this experiment
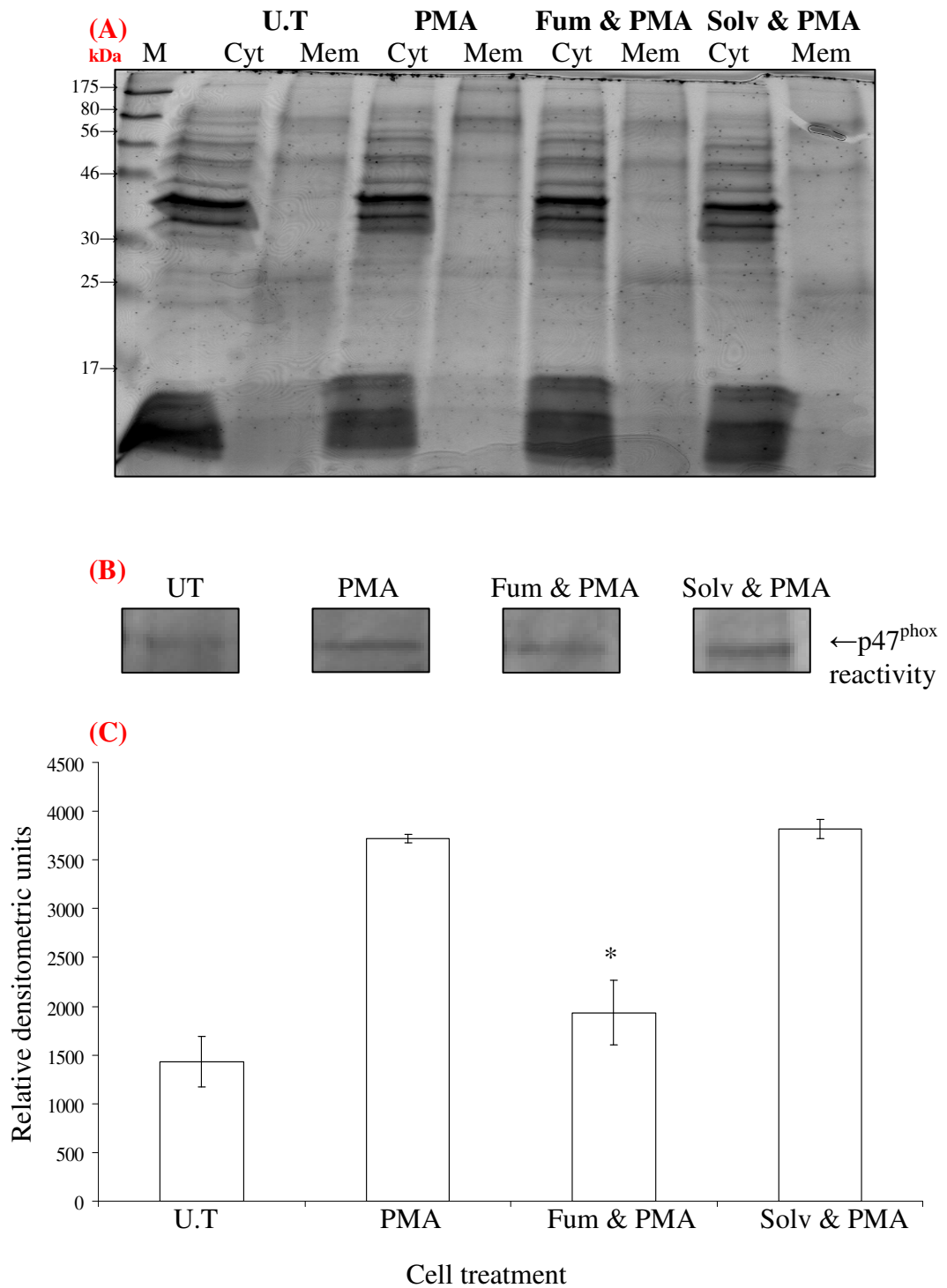
Figure 4.5 Effect of fumagillin on the translocation of p47^{phox} from the cytosol to the membrane.

Neutrophils, (1 x 10^8) were isolated and incubated in the presence of fumagillin (2 µg ml^{-1}). Cells were washed and subsequently fractionated (Section 2.18). Following SDS-PAGE **(A)** and immunoblotting **(B)** the relative translocation of p47^{phox} to the membrane was ascertained by densitometry **(C)**. Neutrophils exposed to fumagillin demonstrated significantly reduced membrane associated p47^{phox} relative to PMA and solvent incubated or PMA stimulated cells (*p ≤ 0.022). Unstimulated neutrophils (U.T) demonstrated some evidence of p47^{phox} in the membrane.

displayed a 2.67 fold increase in the presence of $p47^{phox}$ in the membrane fraction following PMA stimulation, (p = 0.022). Importantly, neutrophils in receipt of PMA stimulation alone and those incubated in the presence of 0.002% (v/v) methanol and then subsequently stimulated with PMA demonstrated almost equivalent levels of $p47^{phox}$ in the membrane fraction, (p = 0.481). This finding indicates that the presence of the solvent used to solubilise fumagillin had a negligible impact on the formation of the NADPH oxidase complex thus supporting the data obtained from the oxygen consumption assay, (Section 4.2.1). Cells previously exposed to fumagillin and subsequently stimulated with PMA showed a marked decrease in the presence of $p47^{phox}$ in the membrane demonstrating 52.08% and 50.65% $p47^{phox}$ translocation compared to PMA and solvent control cells, (p = 0.026 and 0.045 respectively). Cells exposed to fumagillin demonstrated a 1.35 fold increase in membrane $p47^{phox}$ content however it was found that this value was not statistically significant, (p = 0.072). It is clear that exposure of neutrophils to fumagillin prior to PMA stimulation results in the level of membrane bound $p47^{phox}$ to be maintained at near basal levels.

4.2.3 Confocal immunoflouresence microscopy examining the localisation of $p47^{phox}$ following exposure to fumagillin and PMA stimulation.

The translocation event and its inhibition by fumagillin was visualised by immunoflouresence microscopy as described, (Section 2.19). Figure 4.6 clearly shows a wide distribution of $p47^{phox}$ in unstimulated cells demonstrating very few clusters of $p47^{phox}$. Cells which were stimulated with PMA demonstrated localisation of $p47^{phox}$ to the inner periphery of the cell with evidence of localised clustering within the cell which may be a result of association with intracellular membranes. Cells incubated in the presence of 2 µg fumagillin ml^{-1} demonstrate partial translocation to the membrane however the majority of $p47^{phox}$ is retained in the cytosol. This finding further validates
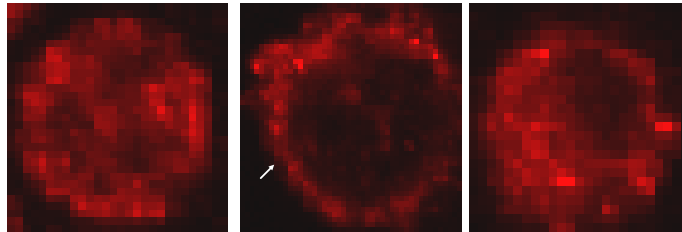
Figure 4.6 Confocal Immunofluoresence imaging of disruption to p47^{phox} translocation mediated by exposure of neutrophils to fumagillin.
Neutrophils were incubated in the presence or absence of fumagillin, (2 µg ml^{-1}) and subsequently stimulated with PMA, (1 µg ml^{-1}) in preparation for immunofluoresence microscopy (Section 2.19.1). Unstimulated neutrophils demonstrate widespread distribution of p47^{phox} throughout. Cells stimulated with PMA demonstrated localised fluorescence to the cell periphery and localised intracellular regions consistent with a translocation event, (highlighted by arrow). Cells previously exposed to fumagillin and subsequently stimulated with PMA show minimal localisation to the cell membrane with the majority of p47^{phox} remaining widely distributed in the cytosol.

previous immunoblot and densitometric analysis, (Section 4.2.2), thus confirming that fumagillin inhibits NADPH oxidase formation by reducing the translocation of p47^{phox} from the cytosol to the membrane.

4.2.4 Summary.

In this series of experiments, the effect of fumagillin as a potential inhibitor of neutrophil function mediated by restriction of the assembly of a functional NADPH oxidase resulting in reduced oxygen consumption was examined. The results confirm that fumagillin is inhibitory to neutrophil function and caused a significant reduction in membrane associated p47^{phox}. The downstream effect of this was monitored by assessing the amount of oxygen consumed over a fixed period of time. The results indicate that the restriction of oxygen consumption mediated by fumagillin is possibly as a consequence of reduced p47^{phox} translocation to the membrane therefore reducing the antimicrobial capacity of the cell.

4.3 Analysis of the effect of fumagillin on the process of neutrophil degranulation.

The production of superoxide by PMA-stimulated neutrophils is associated with degranulation and the release of proteolytic enzymes into the phagocytic vacuole and around the cell periphery, thus acting as an essential element of neutrophil antimicrobial response (Spitznagel, 1990; Standish and Weiser, 2009). A comprehensive study of potential mycotoxin mediated inhibition of neutrophil degranulation and activity was warranted to further understand the effect of fumagillin on cellular function.

4.3.1 Analysis of neutrophil myeloperoxidase degranulation.

Neutrophils, (5×10^7), were isolated and a degranulation assay was performed as described, (Section 2.17.1). The degranulated protein extract was concentrated by

acetone precipitation. A 15 µg protein sample from each treatment was subjected to 1–D SDS–PAGE and immunoblotting, (Figure 4.7 A and B). Western blots were initially probed with anti-human myeloperoxidase, (Genway), overnight at a dilution of 1/650. Following washing of the nitrocellulose membrane blots were probed with secondary anti-rabbit HRP conjugated antibody at a dilution of 1/650. The blots were subsequently developed with ECL reagent, (Section 2.9.7). Analysis of the blots identified reactivity against the 59.5 kDa fragment, which was expected as MPO is a heme protein (Reumaux *et al.*, 2003) and electrophoresis was performed under reducing conditions thus clarifying the larger fragment. Visualisation of MPO reactivity showed a variation in MPO reactivity between different treatments and these blots were analysed by densitometry in order to quantify the relative levels of MPO degranulation.

Figure 4.7 C shows that the MPO content in neutrophil degranulation extracts was increased 1.55 and 1.56 fold in the PBS and solvent controls respectively relative to unstimulated cells, ($p = 0.001$). Neutrophils which had been previously exposed to 2µg fumagillin ml^{-1} did demonstrate 1.33 fold increase in MPO degranulation with PMA stimulation compared to untreated cells, ($p = 0.001$) however this was reduced relative to neutrophils stimulated with PBS or with 0.002% (v/v) methanol expressing a 1.47 and 1.48 fold increase in graduation respectively ($p \leq 0.035$).

In order to strengthen this finding the process of degranulation was visualised by employing confocal immunoflouresence microscopy, (Section 2.19). Examination of the localisation of MPO from neutrophils (Figure 4.8) demonstrated that unstimulated cells show a high level of intracellular fluorescence corresponding to concentration of MPO. In contrast cells stimulated with PMA in the absence of prior exposure to 2 µg fumagillin ml^{-1} demonstrated MPO degranulation as evidenced by a loss of intracellular fluorescence and small clusters of fluorescence external to the cell. This finding is interesting as MPO is normally stored within intracellular granules, where it accounts
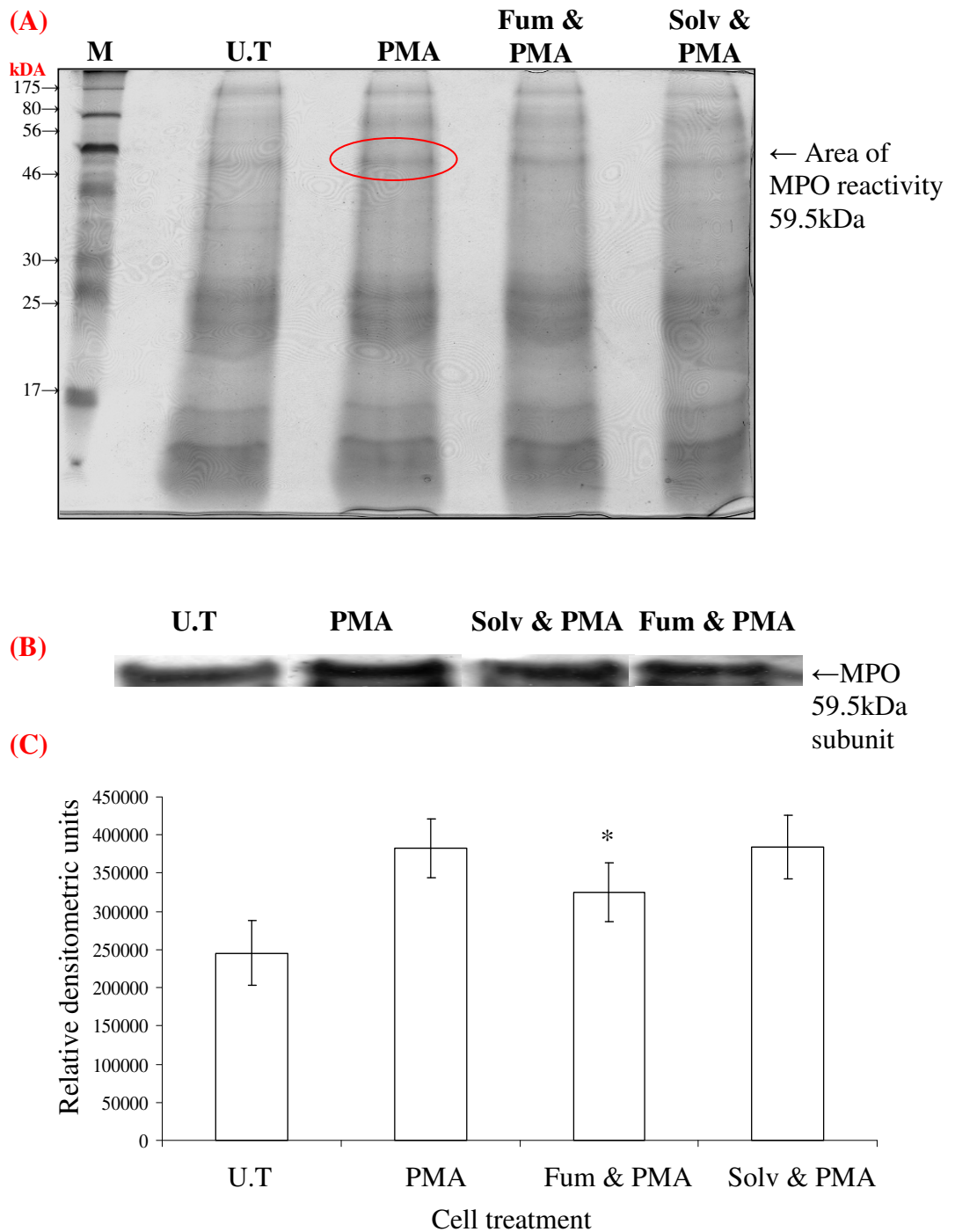
Figure 4.7 Analysis of the effect of fumagillin on neutrophil degranulation.
Neutrophils (5 x 10^7) were incubated in the presence of fumagillin, (2 µg ml^{-1}) for 25 minutes and were stimulated with PMA, (1 µg ml^{-1}) in a thermally controlled stirred chamber at 37ºC, (Section 2.17.1). SDS-PAGE **(A)** and immunoblotting **(B)** against human myeloperoxidase identified a 59.5 kDa subunit of MPO which showed variability in degranulation profile depending on the cell exposure conditions. Densitometric analysis **(C)** demonstrates that the level of MPO degranulation decreased significantly in cells exposed to fumagillin and stimulated with PMA compared to both positive controls, (*p $\leq$ 0.035).

Resting cells

PMA stimulated cells

Fumagillin treated & PMA stimulated cells
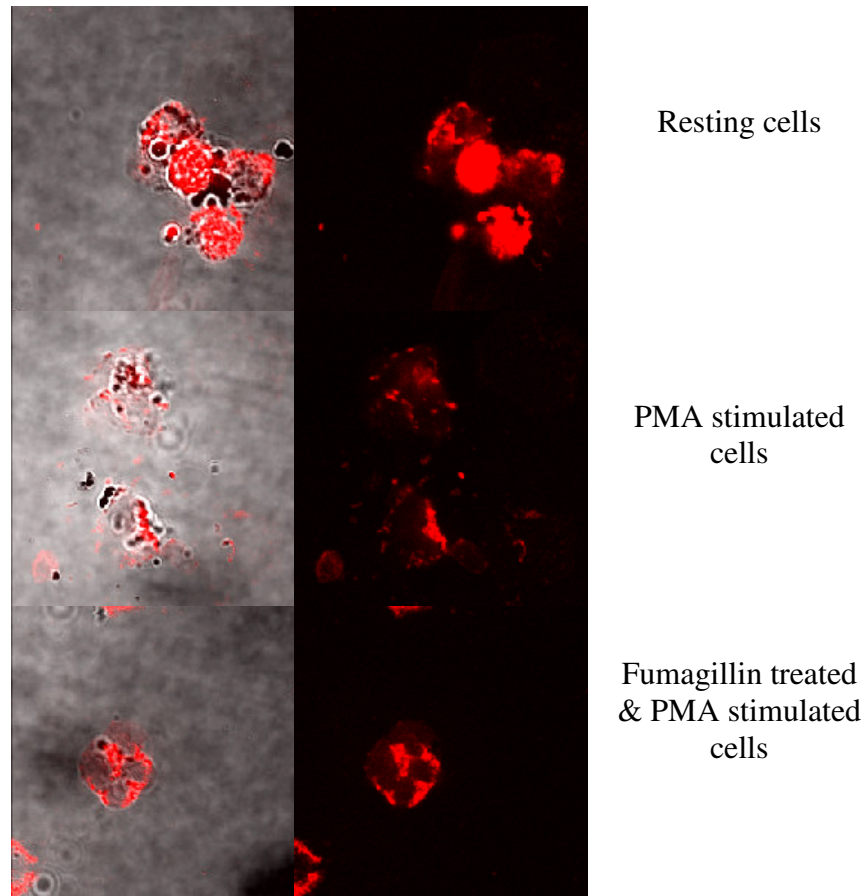
Figure 4.8 Immunoflouresence imaging of inhibition to degranulation of MPO in cells exposed to fumagillin.
Neutrophils were exposed to fumagillin, (2 µg ml^{-1}) and were stimulated with PMA (1 µg ml^{-1}) prior to preparation for immunoflouresence microscopy. Visualisation of the localisation of MPO revealed a high level of intracellular MPO in unstimulated cells with the majority exocytosed extracellulary in distinct granules upon PMA stimulation. Neutrophils exposed to fumagillin and stimulated with PMA demonstrated a partial degranulation of MPO evidenced by some intracellular fluorescence and partial exocytosis of MPO (arrow ←).

for approximately 25% of granule protein and 5% of the total neutrophil proteome, (Segal, 2005). These granules fuse to a phagocytic vacuole or are exocytosed at the site of infection which is demonstrated in this case upon PMA stimulation. The degranulation of MPO in cells previously exposed to 2µg fumagillin ml^{-1} appears to occur in a more restricted manner. There is evidence of partial exocytosis of MPO and the formation of clusters of fluorescence close to the cell periphery however it would appear that this process is frustrated to an extent. Furthermore there is relatively little extracellular granule fluorescence as indicated in Figure 4.8.

In order to gain a comprehensive understanding of the inhibition of neutrophil degranulation mediated by fumagillin it was necessary to quantify the activity of MPO in degranulated cell supernatants following PMA stimulation. The peroxidation activity of MPO was ascertained using a commercially available activity assay kit as described, (Section 2.17.2). Following assay calibration by constructing a standard line using the supplied MPO standards, (Figure 4.9), MPO peroxidation activity was calculated (Figure 4.10). Analysis of the peroxidation activity revealed that unstimulated cells did not show any activity, however extracts from PMA stimulated neutrophils or neutrophils stimulated with PMA subsequent to prior incubation with 0.002% (v/v) methanol did demonstrate peroxidation activity (25.15 ± 1.31 ng MPO ml^{-1} and 25.44 ± 2.08 ng MPO ml^{-1} respectively). This contrasted with experimental data using neutrophils previously incubated with 2 µg fumagillin ml^{-1}. In this case the activity was reduced to 15.38 ± 0.83 ng MPO ml^{-1}. Importantly, this figure was statistically different to both positive controls, ($p \leq 0.019$). In addition comparisons between both positive controls demonstrated that there was no statistically significant difference between cells stimulated with PMA or treated as a solvent control and PMA stimulated, ($p = 0.924$) therefore highlighting fumagillin as an inhibitor.
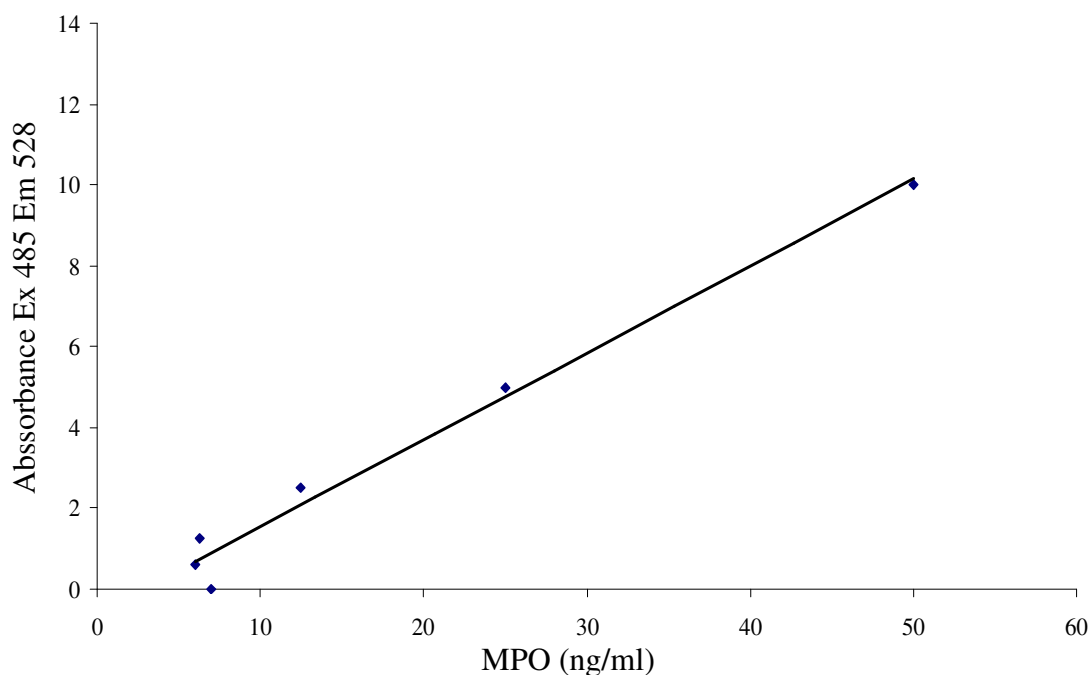
Figure 4.9 MPO peroxidation activity calibration.
MPO standards were prepared according to manufacturer's guidelines and a standard curve was prepared. R^2 = 0.9813, Equation of line: y = 0.2145x – 0.5877.
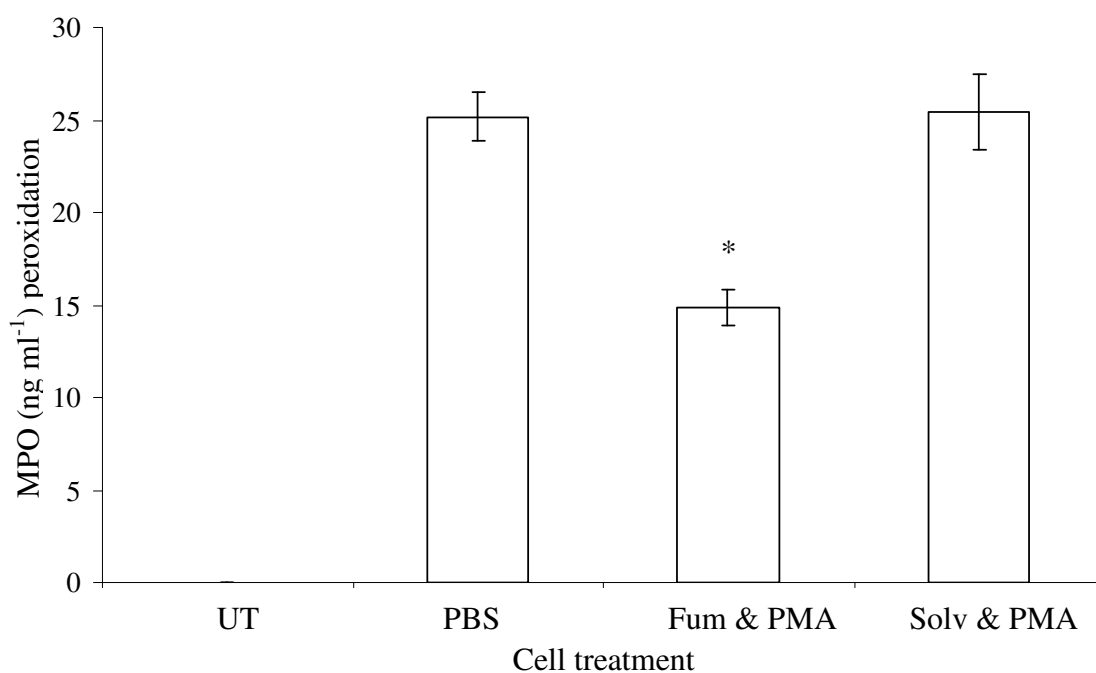
Figure 4.10 Effect of fumagillin on MPO mediated peroxidation activity.
Neutrophils, (5×10^7) were incubated in the presence of fumagillin, (2 µg ml^{-1}) and stimulated with PMA (1 µg ml^{-1}) for 6 minutes. The quantity of protein in the cell free supernatant was standardised for all samples and the peroxidation activity was ascertained according to manufacturers guidelines as described, (Section 2.17.2). Cells exposed to fumagillin demonstrated reduced peroxidation activity relative to both positive controls, (*p $\leq$ 0.019).

4.3.2 Summary

It is clear that a concentration of fumagillin which is inhibitory to neutrophil function, (2 µg ml^{-1}), the process of degranulation appears to be reduced significantly as evidenced by reduced detection of MPO from the supernatant of PMA stimulated neutrophils previously exposed to 2 µg fumagillin ml^{-1}. The immunoblot and densitometric analysis is strengthened by the confocal immunoflouresence microscopy images presented in Figure 4.8. Cells stimulated with PMA show a far higher level of MPO degranulation as demonstrated by a loss of intracellular fluorescence and appearance of external granules. The increase in the detection of peroxidation activity further supports this work as neutrophils exposed to fumagillin show almost 40% less activity than that of both positive controls. Neutrophils previously incubated with 2 µg fumagillin ml^{-1} showed evidence of significantly more degranulation than unstimulated cells therefore fumagillin does not entirely block neutrophil function but suppresses it significantly compared to uninhibited cells.

4.4 Analysis of the effect of fumagillin on the assembly of F-actin.

Actin in unstimulated neutrophils exists in a globular, (G) form however upon stimulation it is converted to filamentous (F) actin in order to mediate cellular processes such as phagocytosis, protein translocation and degranulation, (Zhan *et al*., 2004; Comera *et al*., 2007). The work of Zhan *et al*., (2004) has particular relevance to this study as it demonstrates the p47^{phox} PX domain associates directly with moesin and it is this association which allows the translocation of p47^{phox} from the cytosol to the membrane thus initiating the assembly with flavocytochrome b$_{558}$. In this section the effect of fumagillin on the process of F-actin assembly which plays a key role in this process is examined using immunoblot analysis and confocal immunoflouresence microscopy imaging.

4.4.1 Immunoblot detection of F-actin assembly in stimulated neutrophils.

Immunoblot detection of F-actin assembly was performed as described, (Section 2.18.7). Following the identification of the reactive band (Figure 4.11) it was clear that the level of reactivity increased following 1 µg PMA ml^{-1} stimulation. This finding is important as it validated the hypothesis that F-actin band reactivity would increase with cellular activation mediated by PMA. Neutrophils stimulated with PMA and cells exposed to 0.002% (v/v) methanol prior to PMA stimulation demonstrated a 1.4 and 1.48 fold increase in the formation of F-actin relative to unstimulated cells, ($p \leq 0.05$). Consistent with previous experimentation densitometric values for both positive controls did not record any statistical difference, ($p = 0.221$). Additionally, neutrophils exposed to 2 µg fumagillin ml^{-1} did show a statistically significant increase in F-actin assembly over unstimulated cells further strengthening the hypothesis that the effective concentration of fumagillin employed in this study significantly inhibited but did not completely diminish cellular processes. Neutrophils exposed to 2 µg fumagillin ml^{-1} demonstrated approximately 15% less F-actin formation that that observed for the positive controls, ($p \leq 0.05$).

4.4.2 Rhodamine coupled phalloidin staining of F-actin assembly.

In order to strengthen the hypothesis that neutrophil function was being inhibited by fumagillin it was necessary to assess the formation of F-actin using confocal immunoflouresence microscopy with rhodamine coupled phalloidin as described, (Section 2.19.2). Comera *et al*., (2007) and Misu *et al*., (2009), demonstrated the use of rhodamine coupled phalloidin in studies of F-actin assembly as it has affinity for F-actin filaments. Figure 4.12 demonstrates variable localised fluorescence intensity across different treatments. Neutrophils stimulated with PMA clearly demonstrate increased fluorescence compared to unstimulated cells consistent with the formation of F-actin.
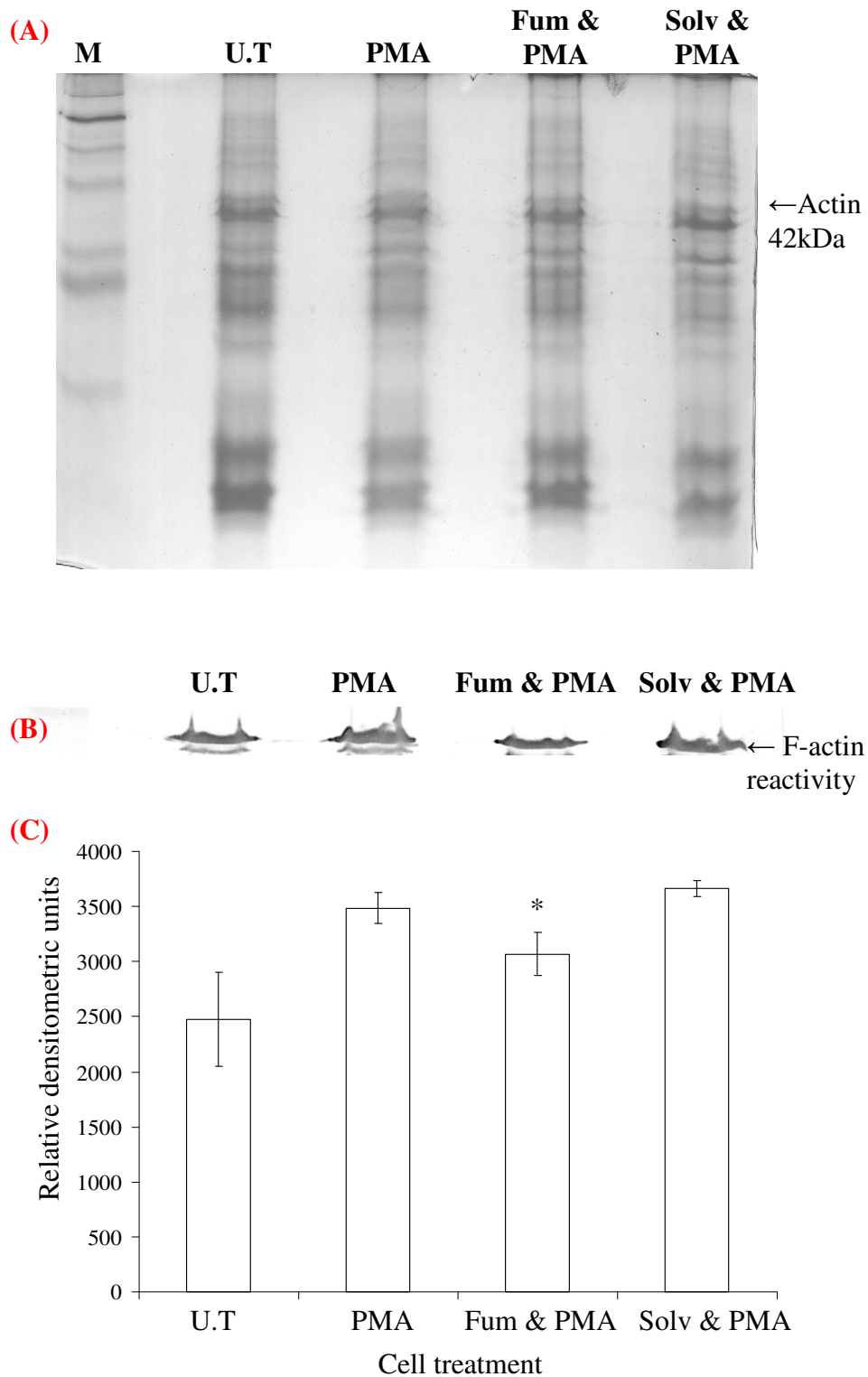
Figure 4.11 Effect of fumagillin on the formation of neutrophil F-actin.
Neutrophils (5×10^7) were incubated in the presence of fumagillin, ($2 \ \mu g \ ml^{-1}$), and were subsequently stimulated with PMA ($1 \ \mu g \ ml^{-1}$) prior to whole cell protein extraction as described, (Section 2.18.7). SDS-PAGE **(A)** and immunoblotting **(B)** coupled to densitometric analysis **(C)** demonstrates reduction of F-actin assembly in neutrophils exposed to fumagillin when subsequently stimulated with PMA compared to both positive control treatments, ($p \leq 0.05$).

Untreated	PMA stimulated	Fum & PMA stimulated

Untreated	PMA stimulated	Fum & PMA stimulated
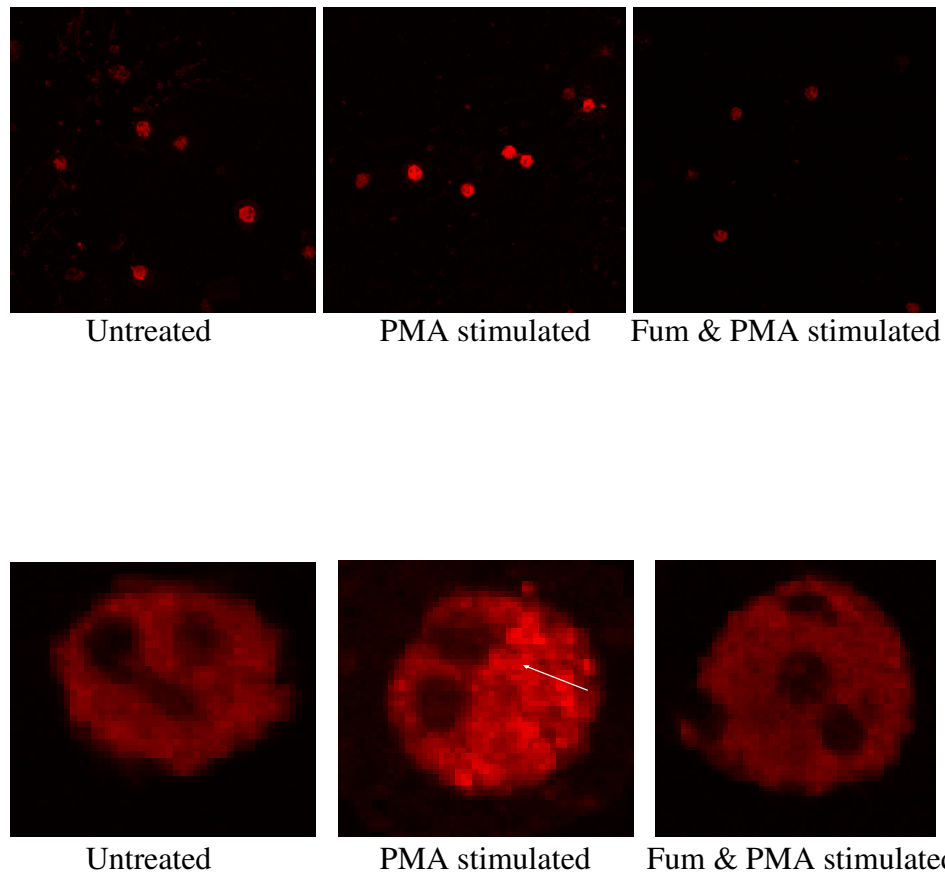
Figure 4.12 Fluorescent visualisation of rhodamine coupled phalloidin stained F-actin assembly in neutrophils following PMA stimulation.
Cells were incubated in the presence or absence of fumagillin, (2 µg ml^{-1}) and were subsequently stimulated with PMA (1 µg ml^{-1}) or left untreated. Neutrophils were then prepared for rhodamine coupled phalloidin staining as described, (Section 2.19.2). Unstimulated neutrophils showed a low level of fluorescence consistent with basal levels of F-actin. This contrasted with the level of fluorescence present in PMA stimulated cells, (indicated by arrow) with localised F-actin staining occurring throughout. Neutrophils exposed to fumagillin and subsequently stimulated with PMA demonstrate some reactivity but this is clearly reduced relative to the positive control

Neutrophils previously exposed to 2 µg fumagillin ml^{-1} and subsequently stimulated with PMA demonstrated only moderate rhodamine coupled phalloidin F-actin staining compared to the positive control. This finding further validates the immunoblot analysis presented in this section where disruption to F-actin assembly is caused by exposure of neutrophils to fumagillin.

4.4.3 Summary.

The data presented in this section demonstrated that the minimum inhibitory concentration of fumagillin (2 µg ml^{-1}) causes disruption to the formation of F-actin in neutrophils subsequently stimulated with 1 µg PMA ml^{-1}. This is evidenced by immunoblot detection of neutrophil F-actin assembly. In addition rhodamine coupled phalloidin staining of neutrophils demonstrated reduced F-actin assembly where neutrophils were previously exposed to fumagillin.

4.5 Analysis of the effect of fumagillin on *G. mellonella* viability and haemocyte function.

In recent years *G. mellonella* larvae have been validated as useful models for *in vivo* studies of immunological pathways common to both vertebrates and invertebrates, (Kavanagh and Reeves, 2004; Fuchs and Myolonakis, 2006). Central to their utility as model organisms, there exists a high level of similiarity between insect haemocytes and human neutrophils. Bergin *et al*., (2005) highlighted that *G. mellonella* haemocytes are able to consume oxygen and produce superoxide mediated by a NADPH oxidase complex that contains proteins homologous to p40^{phox}, p47^{phox}, p67^{phox} and gp91^{phox} of human neutrophils. In addition, Renwick *et al*., (2007) demonstrated that haemocyte p40, p47 and p67 proteins translocate from the cytosol to the membrane in a comparable manner to neutrophils. Furthermore, it was found that gliotoxin disrupted this

translocation process in haemocytes with the effect of lowering superoxide production thus limiting pathogen targeted killing. Therefore the effect of fumagillin on the cellular immune response of *G. mellonella* was investigated, as characterising any potential modulatory effect on immune function would serve to further validate *G. mellonella* as an alternative model to assess mycotoxin/immune system interactions. In addition, the same analysis may lead to identification of previously unknown effects of fumagillin not seen in human neutrophils.

4.5.1 Analysis of the modulation of larval viability by fumagillin.

Considering the data presented elsewhere in this Chapter an initial study to assess if fumagillin was capable of immunosuppressing larvae to *A. fumigatus* infection was employed. As the lowest inhibitory concentration of fumagillin identified as capable of restricting cellular function in human neutrophils was 2 µg ml^{-1} it was decided that an initial study assessing any immuno-modulatory effect would use this mycotoxin concentration. Larvae were injected (Section 2.1.5) with 2, 1, and 0.1 µg fumagillin 20 µl^{-1}. As a control larvae were injected with a concentration of methanol corresponding to the highest concentration of solvent in the fumagillin treatment. Larvae were incubated for four hours at 30ºC and were subsequently injected with 1 x 10^7 *A. fumigatus* ATCC 26933 conidia. As an additional control, larvae which were initially left untreated but incubated for 4 hours at 30ºC were injected with 1 x 10^7 *A. fumigatus*. Larvae were incubated at 30ºC and larval viability was recorded at 24, 48 and 72 hours (Figure 4.13).

Examination of larvae at 24 hours showed no adverse effect on viability. However inspection of larvae 48 hours post *A. fumigatus* infection identified the first significant change in viability. Larvae previously injected with 2µg 20 µl^{-1} fumagillin demonstrated 60 ± 10% viability. This contrasted with viability data observed for larvae
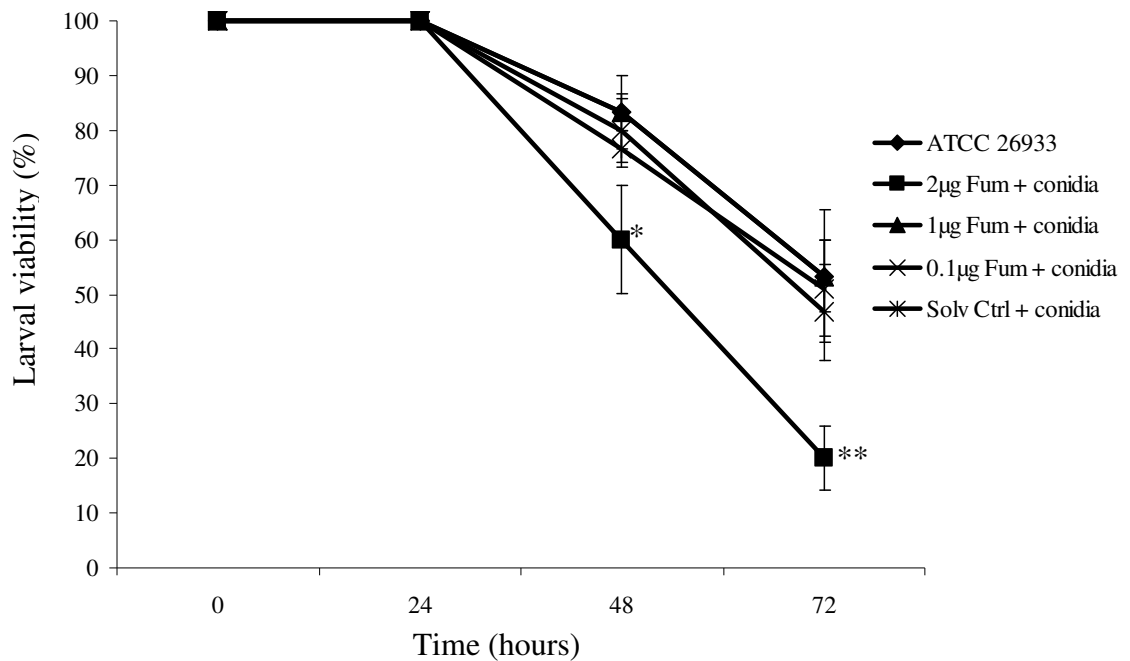
Figure 4.13 Analysis of the effect of fumagillin on *G. mellonella* susceptibility *to A. fumigatus* infection.

Larvae were injected with different concentrations of fumagillin, (0.1, 1 or 2 µg 20 µl^{-1}) or a methanol control and were incubated at 30ºC for 4 hours when they were administered a follow up inoculum of 1 x 10^{7} *A. fumigatus* ATCC 26933 conidia. Larval viability was monitored at 24, 48 and 72 hours post conidial infection. Larvae previously injected with 2 µg fumagillin demonstrated reduced viability at 48 and 72 hours compared to larvae injected with conidia alone (p = 0.045* and 0.015**, respectively).

injected with *A. fumigatus* alone, (83.3 ± 6.6%, p = 0.045). It was noted that viability of larvae injected with fumagillin (1 and 0.1 µg fumagillin 20 µl^{-1}) and the solvent control and subsequently followed up with 1 x 10^7 conidia displayed viabilities of 83.3 ± 3.3%, 80 ± 5.7% and 76.6 ± 3.3%. Statistical analysis revealed that none of these values were significantly different to the *A. fumigatus* infected control, (p ≥ 0.519), thus the inoculation of 2 µg fumagillin per larva demonstrated the first statistically significant alteration to viability when subsequently challenged with conidia at 48 hours.

Larvae injected with 2 µg fumagillin and inoculated with 1 x 10^7 conidia demonstrated significantly reduced viability at 72 hours (20 ± 5.7%). This contrasted sharply with data recorded for larvae injected with only *A. fumigatus,* where 53.3 ± 12% of larvae remained viable, (p = 0.007). In comparison larvae administered an equivalent concentration of solvent and subsequently infected with 1 x 10^7 conidia demonstrated 51.1 ± 8.9% viability. Larvae inoculated with *A. fumigatus* alone did not demonstrate significantly altered viability compared to the solvent control, (p = 0.796), however comparisons between the solvent control and larvae immunologically challenged with 2 µg fumagillin demonstrated a p – value of 0.015. These findings exclude the possibility that the alteration in viability was mediated by the solvent used to solubilise fumagillin. Larvae injected with 1 or 0.1 µg fumagillin larvae^{-1} prior to fungal challenge demonstrated 53.3 ± 6.6 and 46.6 ± 8.8% at 72 hours respectively. These treatments were not statistically different, (p ≥ 0.606) compared to both positive controls. However in comparisons with the 2µg fumagillin larvae^{-1} pre-treatment a p – value of 0.007 and 0.028 was recorded for 1 and 0.1 µg fumagillin larvae^{-1} respectively. This finding confirmed that larvae exposed to 2µg fumagillin are more susceptible to subsequent fungal infection.

4.5.2 Analysis of *in vivo* haemocyte densities of larvae injected with fumagillin.

G. mellonella larvae were injected with PBS, 2µg fumagillin or 10% (v/v) methanol in a 20µl volume and incubated at 37ºC for 4 or 24 hours. Larvae were bled and haemocyte density μl^{-1} of haemolymph was ascertained, (Section 2.15.3). Larvae injected with PBS, 10% methanol or 2µg fumagillin demonstrated $2.44 \pm 0.244 \times 10^3$, $2.86 \pm 0.48 \times 10^3$, and $2.41 \pm 0.19 \times 10^3$ haemocytes μl^{-1} 24 hours post-inoculation respectively (Figure 4.14). Statistical analysis of this data revealed that there were no significant differences between any of these treatments, ($p \geq 0.132$). This finding was interesting as it eliminated the possibility that the immunosuppressive effect mediated by pre-exposing larvae to 2 µg fumagillin was mediated by cytotoxicity to the larval haemocyte population.

4.5.3 Analysis of the effect of fumagillin on haemocyte mediated pathogen killing.

Considering the observation that 2 µg fumagillin ml^{-1} mediated an immunosuppressive effect with *in vitro* neutrophil experimentation coupled to the immunosuppressive effect mediated by the same concentration of fumagillin using *G. mellonella*, it was hypothesised that the cellular function of larval haemocytes could be diminished. To test this hypothesis a killing assay using *C. albicans* MEN as a target was performed, (Section 2.16.2). Haemocytes previously exposed to 2 µg fumagillin ml^{-1} for 25 minutes killed $66.44 \pm 4.36\%$ of *C. albicans* MEN cells after 60 minutes (Figure 4.15). This figure contrasted with 85.11 ± 2.81 % and $88.41 \pm 1.06\%$ *C. albicans* MEN viability where yeast cells were incubated with the PBS and 0.002% (v/v) methanol treated haemocytes respectively, ($p \leq 0.048$). Crucially, there was no statistical significance recorded between the two positive controls therefore the inhibitory effect was mediated by fumagillin.
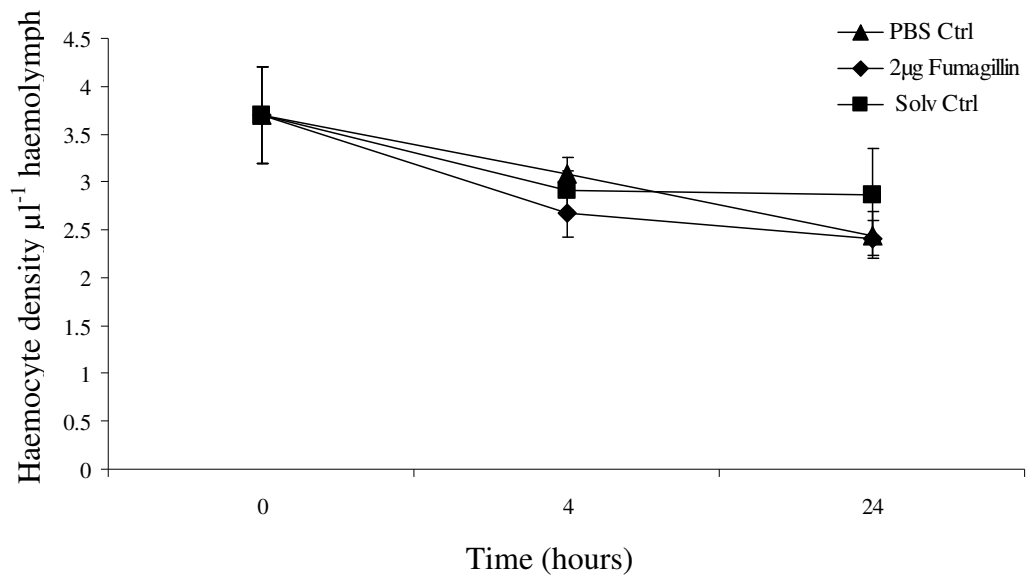
Figure 4.14 Effect of fumagillin on *in vivo* haemocyte density of *G. mellonella* larvae.

Larvae were injected with fumagillin (2 µg), PBS, or 10% methanol as an equivalent solvent control for the injection of fumagillin. Haemocyte density μl^{-1} haemolymph was ascertained as described, (Section 2.15.3). Haemocyte density was found to be reduced slightly in all treatments however there was no statistically significant difference between the different treatments.
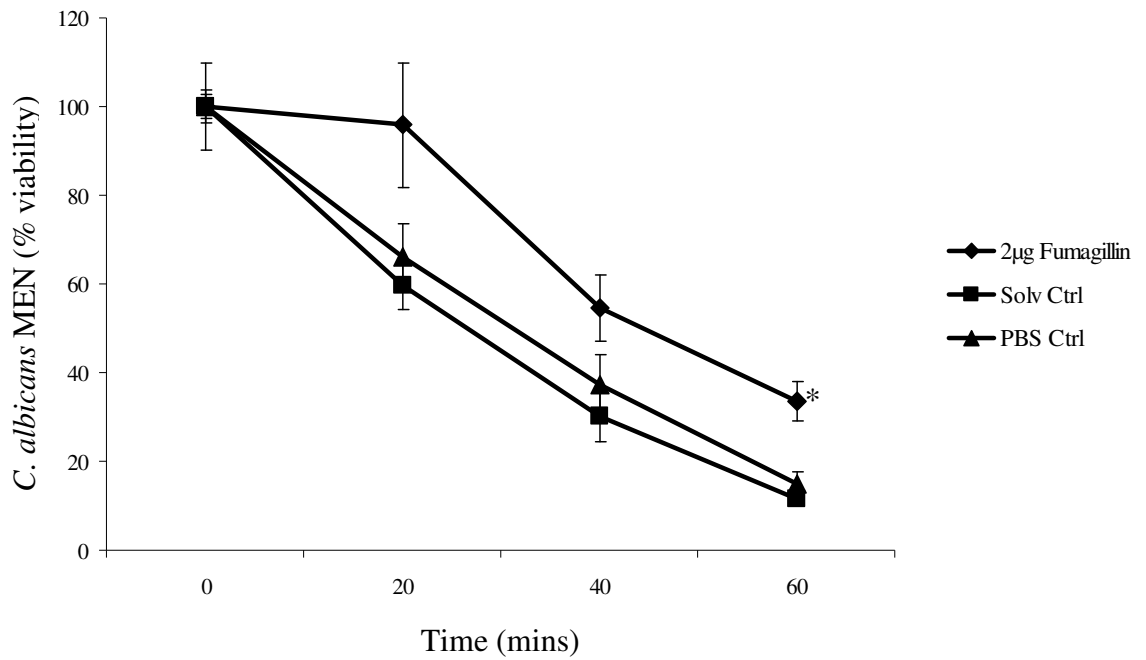
Figure 4.15 Effect of fumagillin on the ability of haemocytes to kill *C. albicans*.
Haemocytes (5×10^6) were incubated in the presence or absence of fumagillin, ($2 \ \mu g$ ml^{-1}) and were prepared for a killing assay using *C. albicans* MEN as described, (Section 2.16.2). Cells exposed to fumagillin exhibited a significantly reduced ability to kill *C. albicans* relative to both positive controls, (*$p \leq 0.048$).

4.5.4 Analysis of the effect of fumagillin on haemocyte phagocytosis.

In section 4.1.2 it was demonstrated that neutrophils previously exposed to 2 µg fumagillin ml^{-1} were significantly less capable of phagocytosing conidia thus inhibiting the initial neutrophil killing process. Considering the reduction in haemocyte mediated pathogen killing, (Section 4.5.3), an analysis of the effect of fumagillin on haemocyte phagocytosis was performed.

Analysis of phagocytosis data, (Figure 4.16) revealed that the rate of phagocytosis occurred in a manner that was comparable to the same process in human neutrophils (Figure 4.3). The rate of phagocytosis increased sharply in the first 60 minutes however the number of haemocytes identified to have engulfed conidia appeared to remain relatively stable up to 120 minutes. Statistical analysis of the rate of phagocytosis revealed that there was no significant increase in the second 60 minutes of the assay, ($p \geq 0.376$), for all three treatments. After 30 minutes the rate of phagocytosis between the different haemocyte treatments revealed only very slight alterations in the number of engulfed conidia. However, after 60 minutes $50.93 \pm 2.78\%$, $54.95 \pm 2.12\%$ and $41.45 \pm 1.48\%$ phagocytosis was observed in haemocytes in receipt of the PBS, 0.002% (v/v) methanol or 2 µg fumagillin ml^{-1} treatments. Comparative analysis of these rates demonstrated that haemocytes exposed to fumagillin demonstrated significantly reduced phagocytosis, ($p \leq 0.05$), compared to the controls. However the relatively small difference between these treatments would suggest that the inhibitory process observed in this case is not the main feature of how fumagillin functions. Therefore a more detailed analysis of the biochemical pathways affected by exposing haemocytes to 2 µg fumagillin ml^{-1} was required.
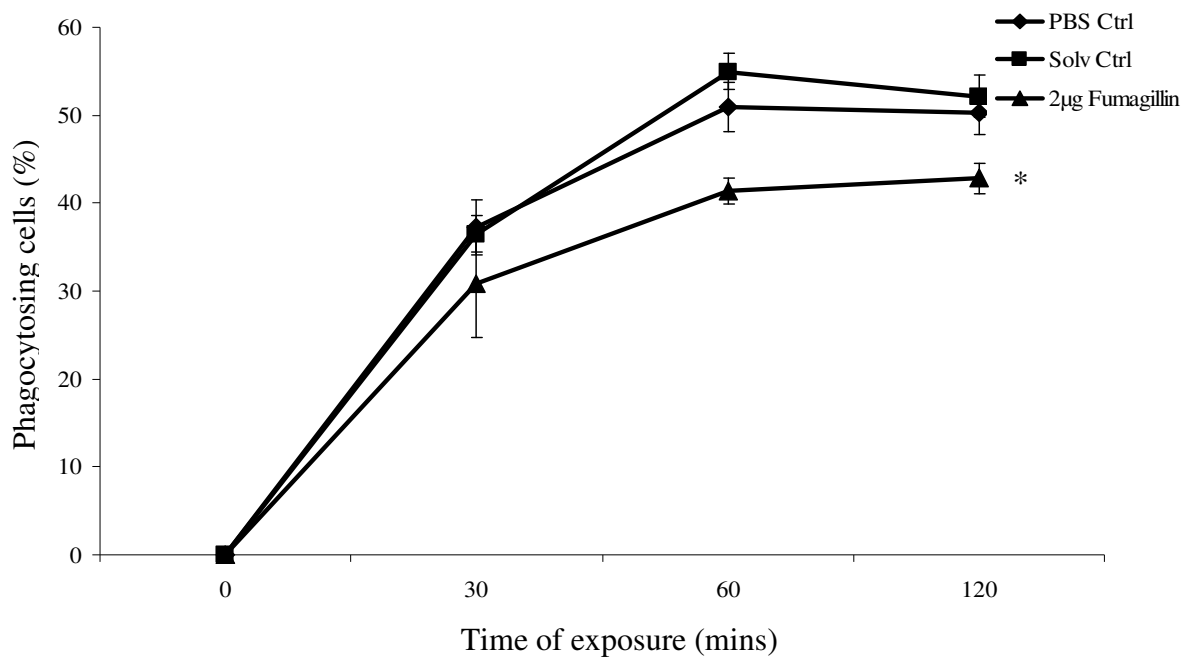
Figure 4.16 Effect of fumagillin on haemocyte phagocytosis.
Haemocytes, (5 x 10^6) were isolated and a phagocytosis assay using *A. fumigatus* conidia as a target was performed. Haemocytes incubated with fumagillin, (2 µg ml^{-1}) demonstrated a reduced capacity to engulf conidia, (*p $\leq$ 0.05)

4.5.5 Summary.

The possibility that *G. mellonella* larvae could be immunocompromised by fumagillin and the killing function of haemocytes could be reduced in a similar manner to neutrophils was investigated. The results demonstrate that inoculating larvae with 2μg fumagillin larva^{-1} reduced immunocompetence *in vivo* thus increasing their susceptibility to fungal colonisation. This was not mediated by a significant reduction in circulating haemocyte density *in vivo* but as a result of a reduction in haemocyte function as indicated by decreased ability to kill pathogens and reduced levels of phagocytosis.

4.6 Analysis of the effect of fumagillin on NADPH oxidase complex-like formation and resulting oxygen consumption.

Bergin *et al*., (2005) and Renwick *et al*., (2007) identified the presence of p47, p67 and p40 homologues in *G. mellonella* haemocytes. Furthermore the production of superoxide coincided with the translocation of these proteins from the cytosol to the membrane. Inhibition of this process mediated by neutrophil inhibitors, (DPI, gliotoxin) points to a conservation of functional pathways between both cell types. As a result of these findings and the inhibition of NADPH oxidase formation resulting in a loss of oxygen consumption observed by exposing human neutrophils to 2 μg fumagillin ml^{-1} (Section 4.2) an analysis of the same cellular pathway in haemocytes was performed.

4.6.1 Analysis of the effect of fumagillin on the ability of *G. mellonella* haemocytes to consume oxygen.

The rate of oxygen consumption of haemocytes following PMA stimulation was investigated as described, (Section 2.18.6). Analysis of the rate of oxygen consumption, (Figure 4.17), 5 minutes post PMA stimulation revealed that there was a slight but not
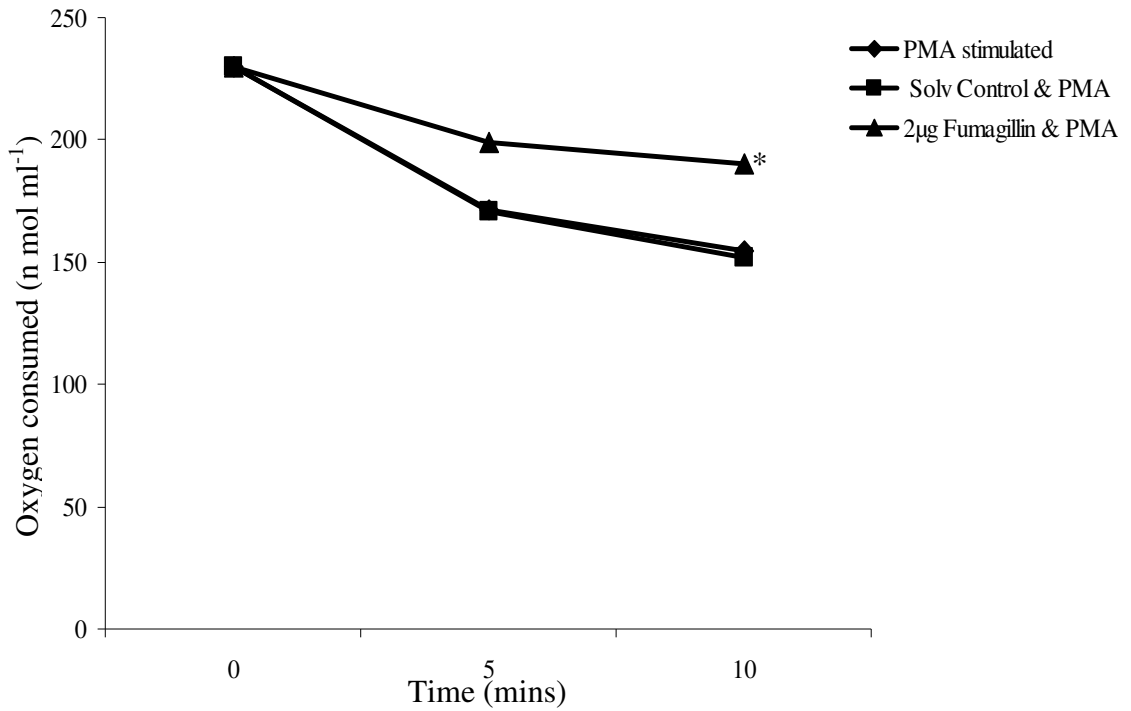
Figure 4.17 Effect of fumagillin on haemocyte mediated oxygen consumption.
Haemocytes (5×10^7) were extracted and incubated in the presence or absence of fumagillin (2 µg ml^{-1}) and were PMA stimulated (1 µg ml^{-1}) in a Clark type oxygen electrode as outlined, (Section 2.18.6). Haemocytes that were exposed to fumagillin demonstrated reduced oxygen consumption over a ten minute time period relative to controls (*$p \leq 0.041$).

statistically significant change in the rate of oxygen consumption, (p ≥ 0.098), however there is a clear separation in the rate of oxygen consumption at this point. Haemocytes previously exposed to 2 µg fumagillin ml^{-1} consumed 39.9 ± 3.99 nmol oxygen ml^{-1} ten minutes post PMA stimulation. This figure contrasted with the rate observed for the PBS and 0.002% (v/v) methanol treatments where haemocytes consumed 75.59 ± 3.49 and 78.38 ± 6.05 nmol oxygen ml^{-1} respectively, (p ≤ 0.041). The positive controls for this experiment both yielded rates of oxygen consumption that were not regarded as significantly different, (p = 0.797).

4.6.2 Analysis of the effect of fumagillin on haemocyte p47 translocation.

Haemocytes were incubated in the presence or absence of 2 µg fumagillin ml^{-1}, stimulated with 1µg PMA ml^{-1} for 6 minutes and were fractionated as described (Section 2.18.5). Following acetone precipitation, solubilisation of concentrated protein and SDS - PAGE of cytosolic and membrane fractions, the resulting gels were subjected to immunoblotting, (Figure 4.18). Rabbit polyclonal antisera raised in rabbits immunised with recombinant p47^{phox} was used at a dilution of 1/2000 in the primary antibody solution overnight. Following membrane washing the blots were exposed to secondary anti-rabbit HRP conjugated antisera used at a dilution of 1/2000 and were subsequently developed using DAB, (Section 2.9.7).

Analysis of membrane development identified a protein of 47 kDa which showed variability in membrane and cytosolic fractions consistent with the presence or absence of PMA stimulation, (Figure 4.18 B). Following densitometric analysis, (Figure 4.18 C) the relative p47 content in the cytosolic and membrane fractions for all the treatments was ascertained by densitometry and was expressed as a percentage of the total p47 content between the treatments. Analysis of untreated haemocyte fractions revealed that 84.11 ± 6.62% of p47 protein remained in the cytosol with only a very
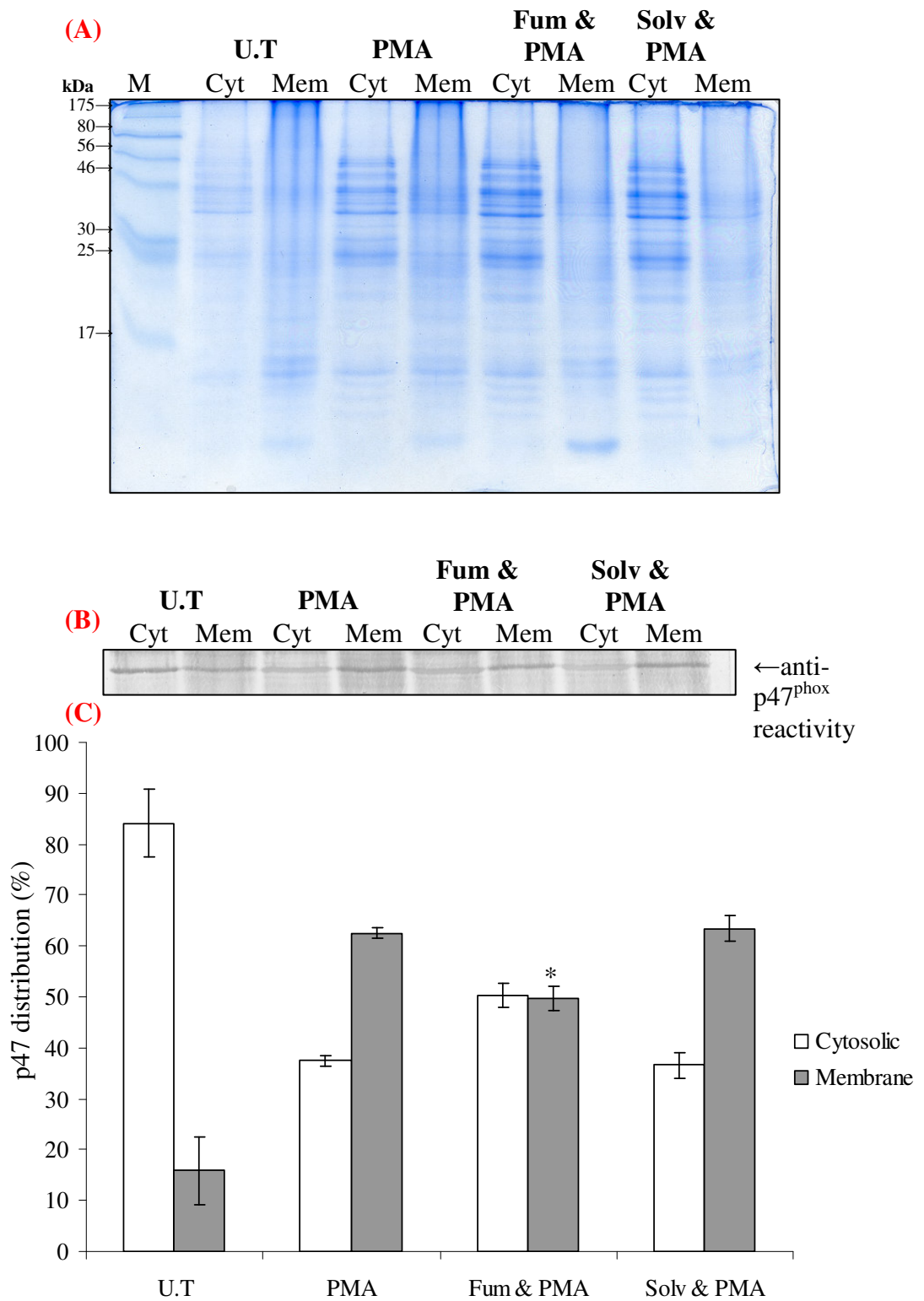
Figure 4.18 Effect of fumagillin on haemocyte p47 translocation.
Haemocytes (1×10^8) were extracted and incubated in the presence or absence of fumagillin, $(2 \ \mu g \ ml^{-1})$. Following stimulation and fractionation, (Section 2.18.5) the cytosolic and membrane components were electrophoresed **(A)** and probed for the presence of p47 **(B)**. Densitometric analysis **(C)** revealed that the percentage of p47 bound to the membrane was decreased relative to both positive controls $(p \leq 0.034)$.

small amount of protein residing in the membrane fraction. This value contrasted sharply with data obtained from haemocytes stimulated with 1 µg PMA ml^{-1} whereby 62.51 ± 1.09% p47 had translocated to the membrane (p = 0.014). This trend in the localisation of p47 is mirrored in haemocytes previously exposed to 0.002% (v/v) methanol as a control where 63.34 ± 2.52% of p47 was in the membrane, (p = 0.717). In contrast haemocytes exposed to 2µg ml^{-1} fumagillin and subsequently stimulated with 1 µg ml^{-1} PMA demonstrated 49.74 ± 2.52% of p47 in the membrane. This reduction of membrane p47 was significantly less than that of the positive controls, (p ≤ 0.034), but was significantly greater than the value observed for unstimulated haemocytes, (p = 0.016).

4.6.3 Summary.

The effect of fumagillin (2 µg ml^{-1}) on the process of haemocyte mediated oxygen consumption was investigated. In addition an analysis of the translocation of a protein homologous to neutrophil p47^{phox} was performed. The process of oxygen consumption in haemocytes was inhibited by exposing haemocytes to fumagillin. Immunoblot and densitometric analysis of haemocyte p47 translocation demonstrated that this process was significantly reduced by fumagillin compared to PBS or 0.002% (v/v) methanol treated haemocytes.

4.7 Analysis of the effect of fumagillin on haemocyte degranulation and F-actin assembly.

Considering homology between neutrophil and haemocyte responses to fumagillin presented elsewhere in this Chapter it was deemed necessary to expand the study to analyse any possible effects on haemocyte degranulation. It was hypothesised that degranulated haemocyte extract would identify a protein with immunoreactivity to

human myeloperoxidase antisera, and could be identified using LC/MS analysis. Furthermore, a study of other degranulated proteins identified as being differentially released was deemed necessary to acquire a more comprehensive understanding of PMA stimulated degranulation and inhibition of this process in haemocytes.

4.7.1 Analysis of the effect of fumagillin on a protein with anti-MPO reactivity and identification using LC/MS analysis.

Haemocytes, (5 x 10^7), were isolated and a degranulation assay was performed as described, (Section 2.17.1). The degranulated protein extract was concentrated by acetone precipitation and a 15 µg protein sample from each treatment was subjected to 1–D SDS–PAGE and immunoblotting, (Figure 4.19 A and B). Western Blots were initially probed with anti-human myeloperoxidase, overnight at a dilution of 1/650. Following washing of the nitrocellulose membrane blots were probed with secondary anti-rabbit HRP conjugated antibody at a dilution of 1/650. The blots were subsequently developed with ECL reagent, (Section 2.9.7) and differences between resulting reactivity were quantified by densitometry. As the identity of the reactive proteins were unknown the corresponding proteins were excised and subjected to tryptic digestion followed my LC/MS analysis, (Section 2.13.3). The upper protein band of interest (59 kDa) was identified as prophenoloxidase subunit 2 (Table 4.1). As band immunoreactivity revealed the detection of two bands the entire reactive region from a corresponsding SDS-PAGE gel was analysed and was identified as prophenoloxidase (Band 1* from Table 4.1).

Densitometric analysis of prophenoloxidase subunit 2 immunoreactivity (Figure 4.1 C) revealed clear differences in the relative level of degranulation. Haemocytes stimulated with 1 µg PMA ml^{-1} demonstrated a 1.75 fold increase in reactivity relative to unstimulated cells, (p = 0.005). This increase was also evident in the solvent control
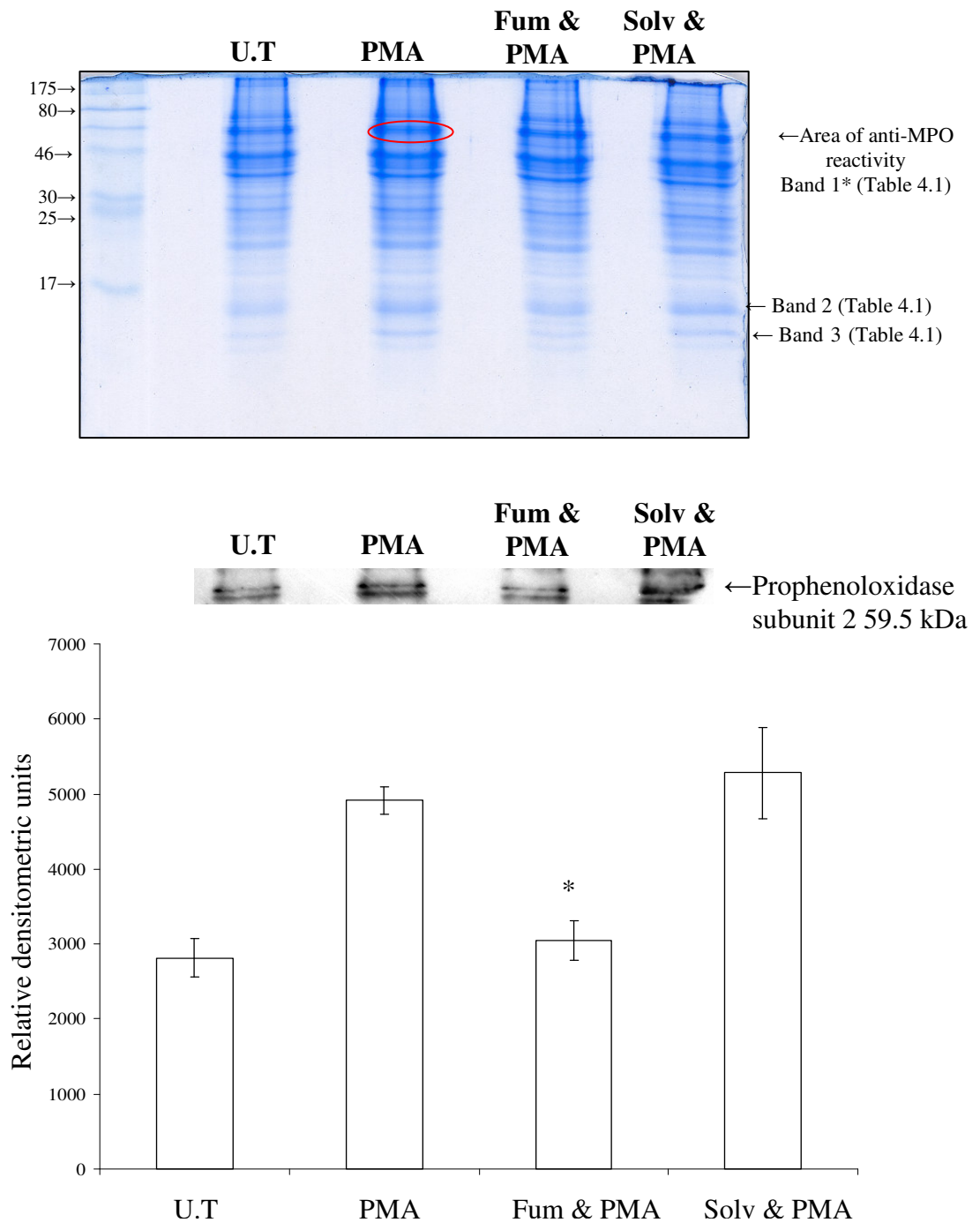
Figure 4.19 Analysis of the effect of fumagillin against a protein with MPO-like immunoreactivity.
Haemocytes (5 x 10^7) were isolated and were incubated in the presence or absence of fumagillin (2 µg ml^{-1}). The cells were washed and a degranulation procedure was performed as outlined, (Section 2.17.1). SDS-PAGE (A) followed by immunoblotting with human MPO antisera identified reactivity at 59.5 kDa (B). The band of interest was excised from a stained gel and was identified by LC/MS, (Table 4.1) as prophenoloxidase subunit 2. Densitometric analysis (C) revealed that the degranulation of this protein was significantly reduced compared to other PMA stimulated treatments, (*p ≤ 0.049). Bands 2 and 3 were also analysed by densitometry and identified by LC/MS and presented in Figure 4.21.

```
CLUSTAL W (1.83) multiple sequence alignment


4557759      MGVPFFSSLRCMVDLGPCWAGGLTAEMKLLLALAGLLAILATPQPSEGAAPAVLGEVDTS 60
14517795     ---------------------MSDSKSRLLLFFDRPSEPCFLQKGDDNVAFEVPDHYYS 38
                                   ::. . . ** : . :  . * .:. .. : :   *

4557759      LVLSSMEEAKQLVDKAYKERRESIKQRLRSGSASPMELLSYFKQPVAATRTAVRAADYLH 120
14517795     DKYKSLTNTLANRFGSGEVRTIPVKNIALPDLSLPLQLPYNDQFSLFVAKHRRMAGKLID 98
               .*: ::       : : *  .:*:    ..: *:::*    : .: .::    *.. :.

4557759      VALDLLER----KLRSLWRRPFNVTDVLTPAQLNVLSKSSGCAYQDVGVTCPEQDKYRTI 176
14517795     IFINMRDVDDLISLCSYCQMRVNPYMFNYCLSVAILHRPDTKGLQVPPVVETFPDKFMDP 158
             : ::: :    .* * : .*    .: :* :.. . *   *. .  **:

4557759      TGMCNNRRSPTLGASNRAFVRWLPAEYEDGFSLPYGWTPGVKRNGFPVALARAVSNEIVR 236
14517795     KVFRKARETTTVNASGNRMPITIPTNYTASNSEPE-QRVAYFREDIGINLHHWHWHLVYP 217
             . : : *.:.*:.**.. :    :*::*  . * *    .  *:.: * * :   : :

4557759      FPTDQLTPDQERSLMFMQWGQLLDHDLDFTPEPAARASFVTGVNCETSCVQQPPCFPLKI 296
14517795     FEAAREIVKKDR------RGELFYYMHQQIIARYNAERLCNGLGRVTRYSDFRAPIGEGY 271
             * : :   .::*       *:*: : :        : .*. *   : . :

4557759      PPNDPRIKNQADCIPFFRSCPACPGSNITIRNQINALTSFVDASMVYGSEEPLARNLRNM 356
14517795     FP---KLDSQVASRSWPPRFANTVIRDIDRPVNEIKIDVFQLETWRDRFLQAIDSNAINM 328
             *     ::..*. . .:   .     :*    :   :   *   :      :.:  *  ** 

4557759      SNQLGLLAVNQRFQDNGRALLPFDNLHDDPCLLTNRSARIPCFLAG--DTRSSEMPELTS 414
14517795     PNGRKVPLNEETGIDELGNLMESSILSLNRGYYGDLHNMGHVFIAYSHDPDHRHLEEYGV 388
             .*   :   ::   *:   *:   * : .  *   :        *:*  *.  .: *

4557759      MHTLLLREHNRLATELKSLNPRWDGERLYQEARKIVG-AMVQIITYRDYLPLVLGPTAMR 473
14517795     MGDSATAMRDPVFYRWHAYIDDIFN--LYKSKLTPYGDSQLDYPGIRVSSISVEGPAGAN 446
             *       :: :  . .::       . **:. . * : ::     *     * **:. . 

4557759      KYLPTYRSYNDSVDPRIANVFTNAFRYGHTLIQPFMFRLDNRYQPMEPNPRVPLSRVFFA 533
14517795     RFATQWQQSLVELSQGLDFTPRGSVLAKFTHLQHEEFTYVIEVNNTSGQSKMGTFRVFMA 506
             ::. . ::.   .:. :   .  .:.   .* :*   *   .: . :.:: ***:*

4557759      SWRVVLEGGIDP-ILRGLMATPAKLNRQNQIAVDEIRERLFEQVMRIGLDLPALNMQRSR 592
14517795     PKTDERGQPLAFEDQRRLMIELDKFTRGLKPGNNTIRQRSLDSSVTIPFERTFRNQANRP 566
             .       :      * **   *:.*  : . : **:* ::. : * :: .  *  .

4557759      DHGLPGYNAWRRFCGLPQPETVGQLGTVLRNLKLARKLMEQYGTPNNIDIWMGGVSEPLK 652
14517795     GDPGSATAAEFDFCGCGWP----------HHMLIPKGTEQGY--PVVLYVMVSDWNADKI 614
             ..  .. *   *** *        :::  :.:  : *  *  : :  :.. .

4557759      RKGRVGPLLACIIGTQFRKLRDGDRFWWENEGVFSMQQRQALAQISLPRIICDNTGITTV 712
14517795     EQDTVG---ACNDAASYCGLRD-RKYPDKRHMGFPFDRR------------------SEA 652
             .:. **   **  .:.:  *** ::  :..  *.:::*                   : .

4557759      SKNNIFMSNSYPRDFVNCSTLPALNLASWREAS 745
14517795     RNLTDFLKPNMATRDCTIKFTDAIREGTQRQ-- 683
               : .  *:. .   . ..    *:. .: *:
```

Figure 4.20 CLUSTAL sequence alignment of Human MPO and *G. mellonella* PPO.

The sequences of human MPO and *G. mellonella* PPO were aligned using CLUSTAL W to identify regions of conservation. The points of similiarity are highlighted (*)- identical, (:) conserved amino acid substitution and (.) semi-conserved substitution thus identifying potential regions to explain cross-reactivity between PPO and human MPO.

<u>**Residues**</u>

Red: Small hydrophobic amino acids
Blue: Acidic amino acids
Magenta: Basic amino acids
Green: Hydroxyl amine basic amino acids

gi 4557759 : Myeloperoxidase (*Homo sapiens*)
gi 14517795: Prophenoloxidase (*Galleria mellonella*)

Band No.	Band I.D	gi	Score	Peptides matched	Coverage
1	Prophenoloxidase subunit 2	34556399	501	10	24%
1*	Prophenoloxidase	14517795	309	5	13%
2	Cellular retinoic acid binding protein	112983600	212	8	25%
3	Cofilin/ Actin depolymerisation factor	110751158	182	10	20%

Table 4.1 Proteins identified by LC/MS analysis demonstrating alternative abundances from degranulated haemocytes.
Numbered bands of interest which showed notable changes from SDS-PAGE and immunoblot analysis in Figure 4.19 were excised and subjected to trypsin digestion. The identified bands are listed along with identification of two low molecular weight proteins, (cellular retinoic acid binding and actin depolymerisation factor homologue/cofilin) which show differential levels of degranulation from haemocytes upon PMA stimulation.
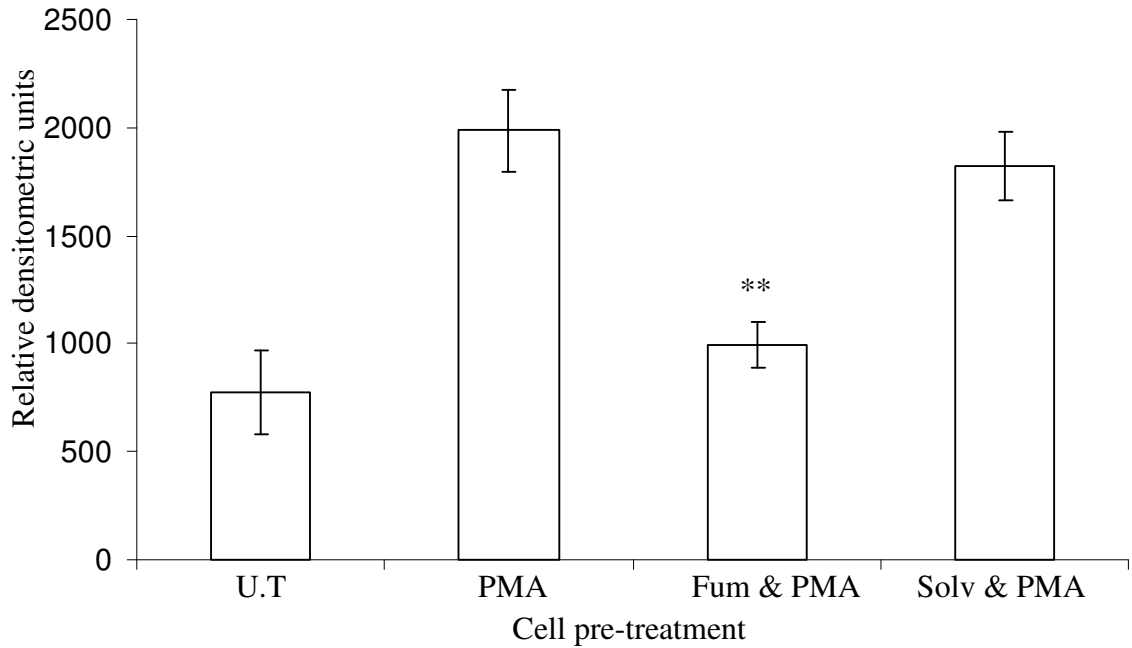
Figure 4.21 Densitometric analysis of degranulation of cellular retinoic acid binding protein from *G. mellonella* haemocytes (Band 2 from Figure 4.19 A).
Identification of CRABP, (Figure 4.19 **A** Table 4.1) coupled to densitometric analysis demonstrates CRABP degranulation is significantly reduced in haemocytes previously exposed to fumagillin compared to both positive controls, (**p ≤ 0.006).
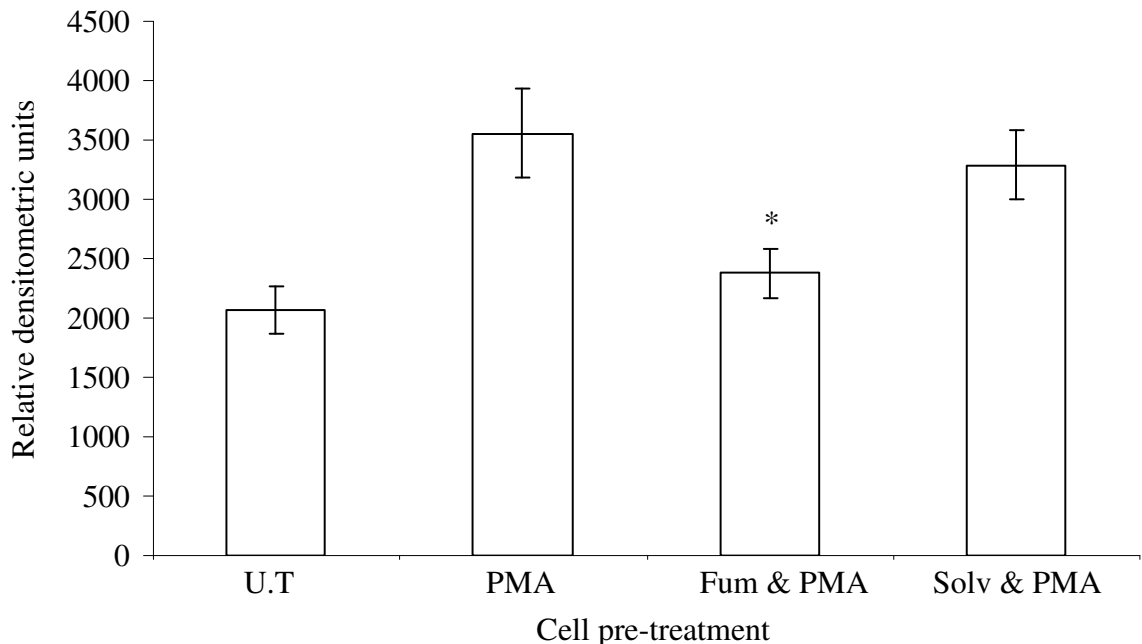
Figure 4.22 Densitometric analysis of degranulation of cofilin from *G. mellonella* haemocytes (Band 3 from Figure 4.19 A).
Densitometric analysis of cofilin degranulation (Figure 4.19 **A** and Table 4.1) from haemocytes exposed to fumagillin (2 µg ml^{-1}) and stimulated with PMA. Haemocytes exposed to fumagillin demonstrate reduced exocytosis of cofilin relative to both positive controls, (*p ≤ 0.028).

treatment where a 1.87 fold increase in the level of PPO subunit 2 reactivity compared to the negative control, (p = 0.039). There was no significant difference between PBS and 0.002% (v/v) methanol controls, (p = 0.920). Haemocytes previously exposed to fumagillin (2 µg ml^{-1}) and subsequently stimulated with PMA demonstrated a 1.08 fold increase in the PPO subunit 2 reactivity compared to the negative control however this was not deemed significant, (p = 0.506). In contrast haemocytes exposed to fumagillin and stimulated with PMA and both positive controls showed that the reduction in PPO subunit 2 attributed to incubating cells with fumagillin was statistically significant, (p ≤ 0.049). Figure 4.20 demonstrates sequence alignment between the full sequence of PPO and MPO using ClustalW software. It was observed that there are localised regions of sequence similiarity between both sequences, with evidence of conserved and semi-conserved substitutions along the sequence alignment.

4.7.2 Identification and densitometric analysis of low molecular weight haemocyte proteins with altered degranulation profiles following exposure of haemocytes to fumagillin.

SDS–PAGE analysis of degranulated haemocyte extracts identified low molecular weight bands which show alterations in abundance between different treatments. These bands were excised and subjected to tryptic digestion prior to LC/MS analysis as described, (Section 2.13.3) and are presented in Table 4.1. Following identification, densitometric analysis was performed on these bands and is presented in Figure 4.21 and 4.22.

A protein of 14.9 kDa was identified as homologous to haemocyte cellular retinoic acid binding protein, (CRABP) from *Bombyx mori*. Densitometric analysis of CRABP identified variable levels of degranulation following PMA stimulation (Figure 4.21). Larvae stimulated with 1 µg PMA ml^{-1} showed a 2.38 fold increase in CRABP

degranulation. This figure compared favourably with a 2.15 fold increase observed in the solvent control following PMA stimulation, (p = 0.2). Analysis of degranulated CRABP from stimulated cells previously exposed to 2 µg fumagillin ml^{-1} identified a 1.19 fold increase in CRABP degranulation however this value was not deemed to be significant compared to unstimulated cells, (p = 0.211). In contrast cells stimulated with PMA or methanol treated cells which were subsequently stimulated with PMA showed significantly increased CRABP degranulation, (p ≤ 0.006).

The second protein of interest to be identified was a 17.05 kDa protein identified as having homology with cofilin/actin-depolymerising factor homologue from *Apis mellifera*. Bioinfomatic analysis of this protein using BLAST identified this protein as a member of the ADF superfamily with a high level of similiarity to other actin depolymerising factors in *B. mori* and *Drosophila* species. Densitometric analysis of degranulated actin depolymerising factor/cofilin (Figure 4.22) demonstrated a 1.72 and 1.59 fold increase in the release of this protein in PMA stimulated cells and solvent control cells relative to unstimulated haemocytes, (p ≤ 0.021). Cells previously exposed to 2 µg fumagillin ml^{-1} and subsequently exposed to 1 µg PMA ml^{-1} demonstrated only a 1.14 fold increase in the degranulation of cofilin relative to unstimulated cells, (p = 0.186) however the reduced degranulation of this protein compared to both positive control treatments was considered significant, (p ≤ 0.028).

4.7.3 Analysis of F-actin immunoreactivity following haemocyte exposure to fumagillin.

Immunoblot detection of haemocyte F-actin assembly was performed as described, (Section 2.18.7). SDS-PAGE and immunoblot detection of F-actin antisera raised against the human form of the protein revealed reactivity (Figure 4.23). Analysis of this blot demonstrated insignificant variation in F-actin immunoreactivity following

(A)

U.T   PMA   **Fum & PMA**   **Solv & PMA**

←Actin 42kDa

(B)

U.T   PMA   **Fum & PMA**   **Solv & PMA**

←F-actin reactivity
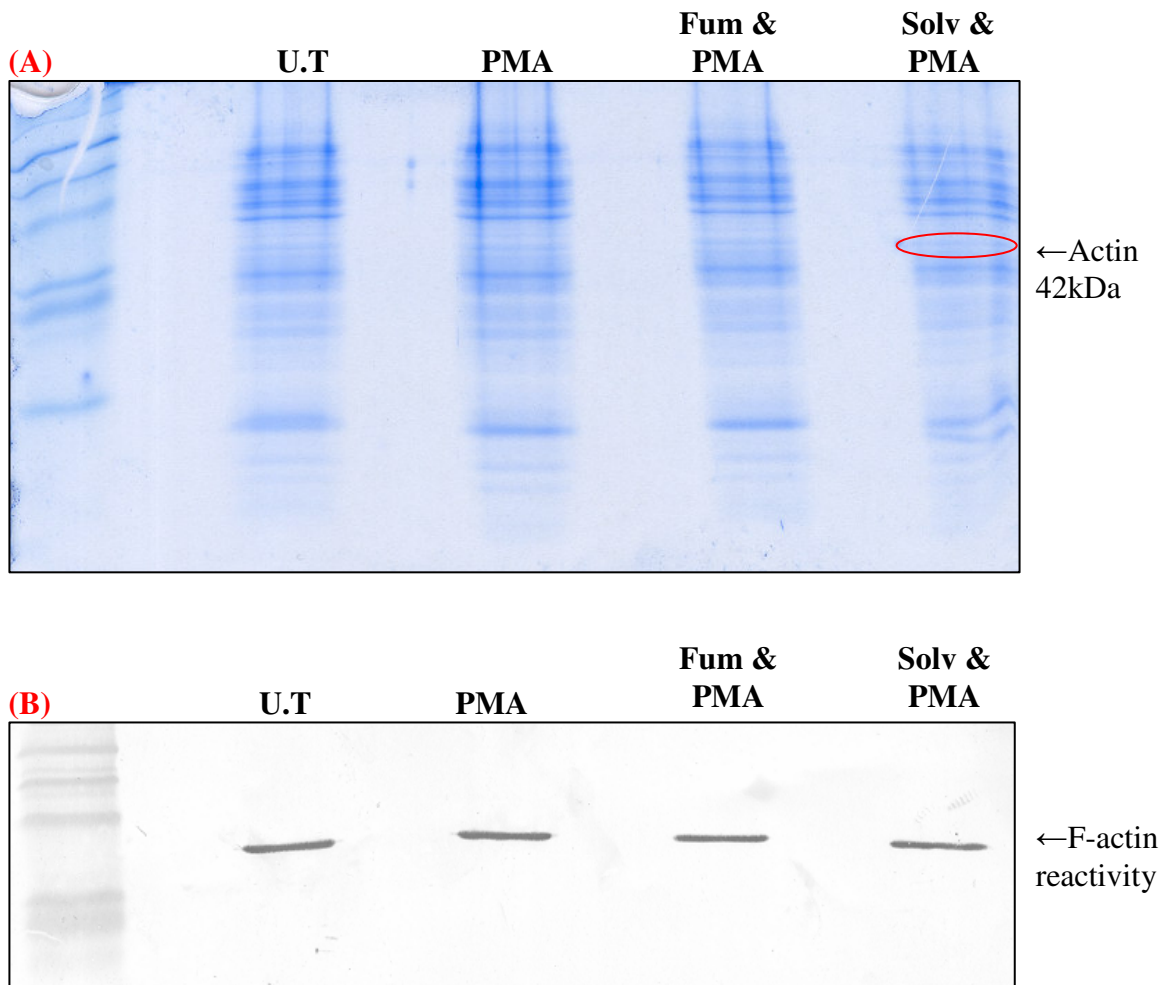
(C)   **LC/MS Protein identification**
Actin – *Aedes aegypti* (Yellow fever mosquito)
gi: 67782283
Score 510
Peptides matched: 10
Coverage: 40%

Figure 4.23 Analysis of F-actin in haemocytes following exposure to fumagillin.
Haemocytes (5×10^{7}) were isolated and were incubated in the presence or absence of fumagillin ($2~\mu g~ml^{-1}$). Following PMA stimulation ($1~\mu g~ml^{-1}$) immunoblot detection of F-actin assembly was performed. Immunoblot detection **(B)** demonstrated no significant variation in F-actin assembly. Excision of corresponding stained band from SDS-PAGE gel **(A)** revealed identification of invertebrate actin **(C)**.

PMA stimulation or incubation of cells with fumagillin (2 µg ml^{-1}). The corresponding protein band from a stained SDS-PAGE gel of the same extract was excised and subjected to trypsin digestion and LC/MS analysis. The immunoreactive band was identified as actin from *Aedes aegypti* (Yellowfever mosquito).

4.7.4 Summary.

The effect of fumagillin (2 µg ml^{-1}) on haemocyte degranulation and F-actin assembly was investigated. Analysis of degranulated extract immunoreactivity with antisera raised against human MPO demonstrated reactivity at 59 kDa. LC/MS analysis of this band revealed the identification of prophenoloxidase subunit 2. Haemocytes previously exposed to fumagillin and subsequently stimulated with PMA demonstrated a significant reduction in PPO degranulation relative to haemocytes stimulated with PMA alone or stimulated with PMA subsequent to incubation with 0.002% (v/v) methanol. Furthermore LC/MS analysis of two low molecular weight bands from the degranulated samples identified the presence of CRABP and actin depolymerising factor/cofilin which demonstrated reduced degranulation in cells previously incubated in the presence of fumagillin. Immunoblot detection of haemocyte F-actin assembly demonstrated no alteration in the formation of F-actin. LC/MS analysis of the immunoreactive band from a corresponding SDS-PAGE gel demonstrated the identification of actin from *Aedes aegyptii*.

4.8 Discussion.

Mycotoxin production by *A. fumigatus* is a key factor in the development and persistence of aspergillosis *in vivo*. This was highlighted by Bok *et al*., (2005) with the finding that isolates containing deletions of the *lae*A gene demonstrated reduced virulence in murine models characterised by low levels of fungal invasion in murine

host tissue. Moreover Δ*laeA* strains were less capable of initiating cytotoxicity in neutrophil cell lines. One of the toxins under the control of *lae*A, fumagillin, was demonstrated to be produced at high amounts using *G. mellonella* larvae (Chapter 3). It was hypothesised that detection of fumagillin 24 hours post infection with *A. fumigatus* ATCC 26933 may indicate a role for fumagillin in the earlier phases of invasive infection. Fumagillin has previously been demonstrated as cilio-inhibitory at concentrations of 10 µg ml^{-1} (Amitani *et al*., 1995). Tsunawaki *et al*., (2004) examined the interaction of fumagillin with neutrophils using concentrations up to 125 µg ml^{-1}. No statistically significant changes in superoxide production were observed at concentrations of 64 µg fumagillin ml^{-1}. This article used incubation times of seven minutes with the mycotoxin prior to conducting any of the assays. Amitani *et al*., (1995) demonstrated ciliostasis mediated by fumagillin when cells were incubated for between 1-6 hours. In this work the duration of incubation was extended to 25 minutes to investigate the effect of fumagillin on neutrophil function. In addition to this an analysis of the effect of fumagillin was performed using haemocytes isolated from larvae of *G. mellonella*. Bergin *et al*., (2005) and Renwick *et al*., (2007) identified that *G. mellonella* haemocytes demonstrated superoxide production coinciding with the consumption of oxygen in response to PMA stimulation which was inhibited by the presence of gliotoxin and DPI. In addition an investigation of the cytosolic proteome of *G. mellonella* demonstrated the presence of conserved immunoreactivity and sequence homology with neutrophil p47^{phox} and p67^{phox}. Considering these previous findings, an analysis of the effect of fumagillin on haemocyte function was warranted.

The work presented in this Chapter indicates that incubation of neutrophils with fumagillin (2 µg ml^{-1}) inhibits cellular function. This finding was conserved, albeit at different levels of inhibition, when *C. albicans* MEN and *P. aeruginosa* were used as pathogenic targets in the phagocyte killing assay. Furthermore due to the prevalence of

A. fumigatus in the sputum of patients with cystic fibrosis (Pihet *et al*., 2008) the finding that fumagillin is capable of inhibiting neutrophil mediated killing is of particular importance due to the diverse array of bacterial and fungal pathogens which commonly populate the airway mucosa (Festini *et al*., 2006). *P. aeruginosa* is the most common respiratory pathogen in the cystic fibrosis airway mucosa resulting in debilitating illness and significant mortality (Davies, 2002). The finding that fumagillin inhibits neutrophil mediated killing of this pathogen is of particular interest given the importance of a competent local immune response in cystic fibrosis patients.

The process of phagocytosis is the initial phase of the neutrophil mediated killing and is initiated through the recognition of non-self entities through the binding of antibody by Fc receptors or through the recognition of complement on the microbial surface (Aderem and Underhill, 1999). The work presented here demonstrates that fumagillin is capable of inhibiting phagocytosis evidenced by the reduced internalisation of conidia within neutrophils. The mycotoxin mediated inhibition of phagocytosis has been previously demonstrated by Comera *et al*., (2007) with the finding that gliotoxin is capable of reducing phagocytosis through the collapse of F-actin filaments. The results presented in this Chapter suggest that the successful formation of F-actin is an integral element in this process and the inhibition of its formation in neutrophils exposed to a functionally inhibiting concentration of fumagillin suggest that the decrease in phagocytosis may be mediated by actin disruption.

The consumption of oxygen coinciding with superoxide production mediated by the formation of a functional NADPH oxidase complex is an integral element in neutrophil function. The formation of the NADPH oxidase complex in neutrophils is dependent on the translocation of $p40^{phox}$, $p47^{phox}$ and $p67^{phox}$ from the cytosol to the membrane bound flavocytochrome b_{558} (Segal, 2005). Dang *et al*., (2002), identified that the phosphorylation of $p47^{phox}$ enhanced the binding of $p40^{phox}$ to the cytochrome

complex. The association of both proteins effectively enhanced the binding of p67^{phox} by acting as an adapter. The work of Mizuki *et al*., (2005) demonstrated that in stimulated cells p47^{phox} interacts with p67^{phox} via simultaneous binding of the p67^{phox} C-terminal SH3 domain to both the proline rich region of amino-acid residues 360-369 and the C-terminally flanking region of p47^{phox}.This complex then translocates as a unit to membrane flavocytochrome b$_{558}$ where the activate NADPH oxidase complex is formed. Therefore, the translocation of p47^{phox} plays an essential role in the formation of a functional NADPH oxidase complex. In this Chapter, exposure of neutrophils to fumagillin (2 µg ml^{-1}) caused reduced oxygen consumption. Furthermore immunoblot detection of p47^{phox} in the cytosolic and membrane components of fractionated neutrophils following PMA stimulation revealed that cells exposed to fumagillin demonstrated evidence of reduced translocation to the membrane. This finding is further supported by immunoflouresence microscopy. Unstimulated neutrophils demonstrate a wide dispersal of p47^{phox} immunoflouresence in the cytosol with PMA stimulated cells demonstrating a significant level of localisation with the cell periphery. Neutrophils exposed to fumagillin (2 µg ml^{-1}) demonstrated limited association with the cell membrane consistent with the immunoblot data with the majority of immunoflouresence maintained in the cytosol. This restriction in translocation would explain the reduction in oxygen consumption mediated by incubating neutrophils with fumagillin. Considering the restriction in F-actin assembly mediated by fumagillin the reduced translocation of p47^{phox} is particularly interesting. This finding is significant considering the work of Zhan *et al*., (2002) as it was demonstrated that p47^{phox} PX domain lipid binding domain mutants were able to translocate to the membrane independent of phosphoinositide binding therefore limiting the argument that association of p47^{phox} with the membrane is a result of lipid affinity. Zhan *et al*., (2004) demonstrated that the p47^{phox} PX domain was responsible for protein translocation *via*

moesin mediated associations with F-actin. In this process it was shown that F-actin polymerisation on membranes accompanied by p47^{phox} interaction with moesin serves to initiate this translocation process. Therefore disruption to F-actin assembly would diminish any potential translocation event due to a lack of membrane association. Considering the work of Zhan *et al*., (2004), in parallel with the data presented here, it would appear that disruption to F-actin assembly reduces translocation in addition to alterations to cell structure and phagocytic capacity as demonstrated by Yürüker and Niggli, (1992).

The process of degranulation plays an important role in the micro-biocidal activity of neutrophils, (Segal, 2005). Segal *et al*., (1980) identified that degranulation coincides with NADPH oxidase activity with an estimated lag-phase of approximately twenty seconds. Additionally, MPO has been identified as an important mediator of this process by catalysing the formation of hypochlorous acid from hydrogen peroxide, a product of NADPH oxidase mediated superoxide formation, (Kettle and Winterbourn, 2001). A review by Nauseef, (2007) identified that MPO-hydrogen peroxide system oxidises Br$^-$ and SCN$^-$ thus leading to the formation of HOBr and HOSCN respectively which in turn have toxic effects *in vivo* depending on pH. A recent study by Allen and Stephens, (2010) identified that MPO is capable of selectively binding to *E. coli, P. aeruginosa* and *S. aureus* and furthermore mediating selective killing on these microbes, therefore the degranulation of MPO in the phagosome and extracellularly may be a method by which neutrophils kill pathogenic bacteria. Considering the experimental results discussed previously, (Section 4.2), it was therefore necessary to employ a comprehensive analysis of the process of degranulation and resulting MPO activity from degranulated cell extracts. The data in this Chapter demonstrate that the degranulation of MPO is reduced in neutrophils exposed to fumagillin as evidenced by immunoblot analysis and immunoflouresence imaging of neutrophils following PMA

stimulation. In addition to this the peroxidation activity in cell free supernatants is strongly reduced where neutrophils were exposed to fumagillin relative to both positive control treatments. Considering the synergy between NADPH oxidase formation and myeloperoxidase degranulation and activity, the finding that both of these factors are inhibited by fumagillin is an interesting development in the interplay between the innate immune system and fungal mycotoxins. Furthermore it may be possible that the restriction in neutrophil mediated pathogen killing is a result of the additive effect of restrictions to phagocytosis, NADPH oxidase formation and degranulation. The finding that PMA induced F-actin assembly in neutrophils is restricted as a result of exposure to fumagillin may also point to an underlying cause for the restriction of cell function.

The effect of fumagillin on increasing the susceptibility of *G. mellonella* to infection was investigated. Larvae injected with 2 µg fumagillin 20µl^{-1} prior to infection with *A. fumigatus* conidia demonstrated reduced viability relative to larvae injected with *A. fumigatus* alone, or following pre-injection with 1µg fumagillin or the solvent control prior to fungal infection. This finding indicates a systemic weakening of the larval immune response following injection with fumagillin. From an entomological point of view it is significant as fumagillin has been routinely used as an anti-microsporidial agent in the treatment of nosemosis in honeybees however it is no longer licensed for use in the EU (Porrini *et al*., 2010).

Larvae injected with fumagillin demonstrated an insignificant change in haemocyte density relative to other treatments therefore eliminating the possibility that the alteration in susceptibility was a direct result of reduced haemocyte circulation. Considering the finding that fumagillin (2 µg) was capable of increasing the susceptibility of *G. mellonella* larvae to infection and in light of the finding that neutrophil function was impaired following exposure to fumagillin (2 µg ml^{-1}) an

analysis of the effect of fumagillin at this concentration on haemocyte function was performed.

Incubation of haemocytes with 2 µg fumagillin ml^{-1} caused a significant reduction in haemocyte mediated killing of *C. albicans*. As *A. fumigatus* conidia are visible once phagocytosed under light microscopy an analysis of the effect of fumagillin on haemocyte phagocytosis was performed. Haemocytes incubated with fumagillin (2 µg ml^{-1}) demonstrated reduced phagocytosis of *A. fumigatus* conidia. The restriction in haemocyte killing and phagocytosis showed a high level of similarity with the data obtained from neutrophils. The rate of killing and phagocytosis, while occurring at slightly different rates to neutrophils demonstrated a similar level of statistical significance at the endpoint of the experiment. The level of phagocytosis appears to stabilise between 60 and 120 minutes for both cell types with cells exposed to fumagillin demonstrating significantly reduced phagocytosis relative to the control treatments. The effect of inhibition of *G. mellonella* phagocytosis has been documented with mycotoxins produced by the entamopathogen *Metarhizum anisopliae* capable of inhibiting phagocytosis through the secretion of destruxins which cause disruption to the actin cytoskeleton (Vilcinskas *et al*., 1997). The finding that fumagillin inhibits haemocyte phagocytosis is a novel finding and represents a detrimental effect on the first phase of insect phagocyte activity.

Bergin *et al*., (2005) identified that *G. mellonella* haemocytes were capable of producing superoxide following PMA stimulation (1 µg ml^{-1}) and identified the presence of proteins which demonstrated homology to neutrophil p47^{phox} and p67^{phox}. The production of superoxide was inversely related to the consumption of oxygen by haemocytes. Renwick *et al*., (2007) demonstrated that the p47 and p67 homologue translocation was inhibited relative to control treatments by incubating haemocytes in the presence of gliotoxin prior to PMA stimulation. In this work fumagillin (2 µg ml^{-1})

reduced the consumption of oxygen by *G. mellonella*. The experimental data resemble that observed in neutrophils. Furthermore densitometric analysis of p47 translocation following PMA stimulation subsequent to fumagillin exposure revealed that approximately 50% of the haemocyte p47 content was translocated to the membrane with approximately 60% translocation occurring in the PMA, and 0.002% (v/v) methanol plus PMA treatments. This reduction in translocation was significant relative to these control treatments but was notably higher than that observed for unstimulated haemocytes. These data indicate that fumagillin, ($2\mu g\ ml^{-1}$) causes a reduction in the level of oxygen consumption. This is mediated by diminished translocation of a protein homologous to p47^{phox} and is similar to comparative neutrophil/haemocyte data presented by Renwick *et al.*, (2007) although the concentration of gliotoxin required to elicit an inhibitory response is much less than that of fumagillin. Nevertheless, the data adds another parallel where an inhibitory response mediated by mycotoxins is similar in neutrophils and haemocytes.

The process of degranulation is poorly understood in *G. mellonella* haemocytes. Some work on haemocyte degranulation has been undertaken with other invertebrate species (Kurata *et al.*, 2006) however the issue of proteins homologous to the invertebrate immune response has not been addressed. In this study we attempted to assess the effect of fumagillin on *G. mellonella* haemocyte degranulation and to employ a proteomic approach in conjunction with immunoblot and densitometric analysis to identify proteins of significance in this process. Haemocytes exposed to fumagillin (2 $\mu g\ ml^{-1}$) demonstrated reduced degranulation of a protein of approximately 59 kDa following PMA stimulation relative to the positive and solvent control using an antibody raised against human MPO. LC/MS analysis of the immunoreactive protein from a corresponding SDS-PAGE gel revealed the identification of prophenoloxidase subunit 2. Prophenoloxidase (PPO) is the native zymogen of the phenoloxidase (PO)

pathway and its role in insect immunity has been discussed in Section 1.5.2. The degranulation of components of the PPO activating system has been previously discussed in other invertebrates (Johansson and Söderhall, 1985) however the degranulation of PPO has not been described. The data presented here indicate that not only is PPO released upon haemocyte activation but mycotoxins such as fumagillin can inhibit its release. In addition ClustalW linear alignment of PPO and MPO amino acid sequences identified regions of similarity between both protein sequences with conserved and semi-conserved regions also present however an analysis of conserved epitope binding would be necessary to understand how cross-reactivity occurs.

Densitometry coupled to LC/MS analysis identified fluctuating degranulation of two low molecular weight proteins, CRABP and cofilin following PMA stimulation subsequent to the presence or absence of fumagillin. Retinoic acid (RA) derived from dietary vitamin A plays a key role in the regulation of embryo development and cell morphogenesis, and CRABP plays a key role in the transportation of RA (Mansfield *et al*., 1998). Nakamura *et al*., (2007) demonstrated that injection of retinoic acid into larvae of *Rhodnius prolixus* reduced phenoloxidase activity thus potentially acting as a regulator of the humoral immune response. This hypothesis was supported by Wang *et al*., (2007) where it was shown that CRABP from *B. mori* reduced the physiological activity of R.A. thus furthering the concept that this protein modulates immune function. In this study the finding that CRABP is degranulated may point to a regulatory mechanism employed by larvae to control the level of phenoloxidase activity in haemolymph however the precise mechanism of how this may occur remains to be elucidated.

The increased degranulation of cofilin following PMA stimulation is a novel development in the understanding of *G. mellonella* haemocyte function. Haemocytes exposed to fumagillin demonstrated decreased cofilin degranulation following PMA

stimulation. In *B. mori* actin depolymerising factor homologues are identified as being crucial in the recycling and regulation of F-actin and contribute to the regulation of cellular function, (Zhang *et al*., 2006). Considering the reduction in intracellular cofilin it is possible that degranulation of a protein involved in the depolymerisation of F-actin may play a role in the regulation of assembly. Adachi *et al*., (2002) demonstrated disruption to cofilin expression which resulted in increased phagocytic cell respiratory burst and pathogen internalisation thus cell stimulation may cause a reduction in the degranulation of this protein. The reduction in oxygen consumption caused by disruption to the translocation of p47 may be influenced by reduced depolymerisation of F-actin mediated by cofilin. The significantly reduced cofilin titres from stimulated haemocytes previously exposed to 2 µg ml^{-1} fumagillin points to its intracellular sequesterisation resulting in F-actin depolymerisation and subsequent limiting of phagocytosis and NADPH oxidase complex formation.

The identification of cross-reactivity between an antibody raised against human F-actin and an invertebrate actin is not surprising as actin is a highly conserved protein in nature (Mullins *et al*., 1997). Surprisingly, considering the previous findings with the alterations in cofilin degranulation there was no statistically significant alteration observed in F-actin assembly. The reasons for this remain unclear and conflict with previous parallels in the haemocyte function in response to PMA stimulation following exposure to mycotoxins. It may be possible that the different antibody specificity may account for some loss of sensitivity however this is an area which requires further investigation or a different methodological approach to fully understand the interplay between F-actin formation and subsequent depolymerisation on haemocyte function.

This Chapter clearly demonstrates that fumagillin at a concentration of 2 µg ml^{-1} causes a reduction in neutrophil and haemocyte function through decreased killing of a pathogenic target and reduced phagocytosis. The translocation neutrophil p47^{phox} and its

haemocyte homologue is disrupted following cellular exposure to fumagillin and results in similar levels of reduced oxygen consumption. The process of degranulation is inhibited in both cell types with MPO degranulation and activity reduced in neutrophil samples following exposure to fumagillin. A parallel study in haemocytes revealed cross reactivity of MPO anti-sera with *G. mellonella* prophenoloxidase with haemocytes exposed to fumagillin demonstrating significantly reduced PPO degranulation following PMA stimulation. In addition the decreased degranulation of an actin depolymerising factor (cofilin) following haemocyte exposure to fumagillin may explain a reduction in F-actin assembly. It is postulated that this inhibition may be the underlying reason for the observed loss of neutrophil function following exposure to fumagillin. However, immunoblot analysis of F-actin assembly in haemocytes revealed no significant changes in any of the treatments applied to haemocytes. It remains to be understood if the parallels between neutrophil and haemocyte function following exposure to fumagillin are also extended to the process of F-actin assembly. Nevertheless the work presented here clearly illustrates that fumagillin is capable of inhibiting neutrophil and haemocyte activity following PMA stimulation and it is speculated that a diminution of F-actin assembly may be the underlying cause.

Chapter 5

Analysis of alterations to *Galleria mellonella* haemolymph proteome and serum protein activity in response to fumagillin

5.0 Introduction

In order to comprehensively understand the immune response to infection it is necessary to complement data obtained from cellular studies with studies concerning humoral immunity. The recognition of pathogens is a key process in the initiation of an effective immune response leading to successful eradication of microbes from host tissue, and is reliant on the detection of pathogen associated molecular patterns (Lavine and Strand, 2001; Janeway and Medzhitov, 2002). In mammals, studies concerning humoral defences against invading microbes tend to focus on the levels of pathogen specific antibodies initially secreted by memory B-cells and subsequently by terminally differentiated plasma cells, (Abney *et al*., 1978; Ahmed and Gray, 1996). In insects a direct comparison is not possible due to the absence of an adaptive immune response however various elements of the innate immune system are conserved between mammals and insects, (Vilmos and Kurucz, 1998; Salzet, 2001). In recent years the application of 2-Dimensional electrophoresis coupled to mass spectrometry analysis of proteomic profile changes in *G. mellonella* larvae has provided novel insights into alterations to the larval humoral proteome following microbial, microbial subunit or physical stimuli, (Bergin *et al*., 2006; Mowlds *et al*., 2008; Mowlds *et al*., 2010). As a result an analysis of the effect of mycotoxins *in vivo* is required to further validate the *G. mellonella* model.

The effect of gliotoxin on the ability of *G. mellonella* to combat fungal infection has been discussed by Reeves *et al*., (2004) and provides an interesting insight into the effect of mycotoxins *in vivo*. In the work presented here a profile of proteins capable of binding to *A. fumigatus* conidia was analysed. Furthermore, the effect of fumagillin on the ability of haemolymph proteins to bind *A. fumigatus* conidia is discussed thus offering an insight into the *in vivo* effects of fumagillin. An analysis of pooled human

serum binding to conidia and the effects of fumagillin on this binding was performed thus facilitating a comparative analysis of serum/haemolymph pathogen recognition.

5.1 Proteomic and enzymatic analysis of changes in *G. mellonella* cell free haemolymph in response to fumagillin.

In this section a proteomic approach was employed to further understand the effect of an immunosuppressive concentration of fumagillin on *G. mellonella*. The aim of this work was to identify alterations in the expression of proteins/peptides in larval haemolymph thus allowing a comprehensive understanding of the physiological and immunological impact of exposing larvae to this toxin

5.1.1 2-Dimensional electrophoresis of haemolymph and LC/MS analysis of spots showing altered expression in *G. mellonella* larvae.

Larvae were injected with PBS, 2 µg fumagillin or 10% (v/v) methanol. As an additional control larvae were left un-injected. Larvae were incubated at 30ºC for 24h prior to haemolymph extraction. Larval haemolymph was subjected to isoelectric focussing and 12.5% SDS-PAGE (Figure 5.1) as described, (Section 2.7.2) followed by Colloidal Coomassie staining, (Section 2.8.3) and Progenesis SameSpot™ analysis. The protein profile of haemolymph from larvae that were left untreated and incubated at 30ºC was used as a reference point for comparisons with other treatments, (Figure 5.2).

Following Progenesis SameSpot analysis protein spots of interest were excised and subjected to trypsin digestion prior to LC/MS analysis, (Section 2.13). Thirteen spots were identified by LC/MS analysis with eleven, (Figure 5.3 A) showing a statistically significant alteration in expression in fumagillin injected larvae, (p $\leq$ 0.05). Identified proteins are presented in Table 5.1.
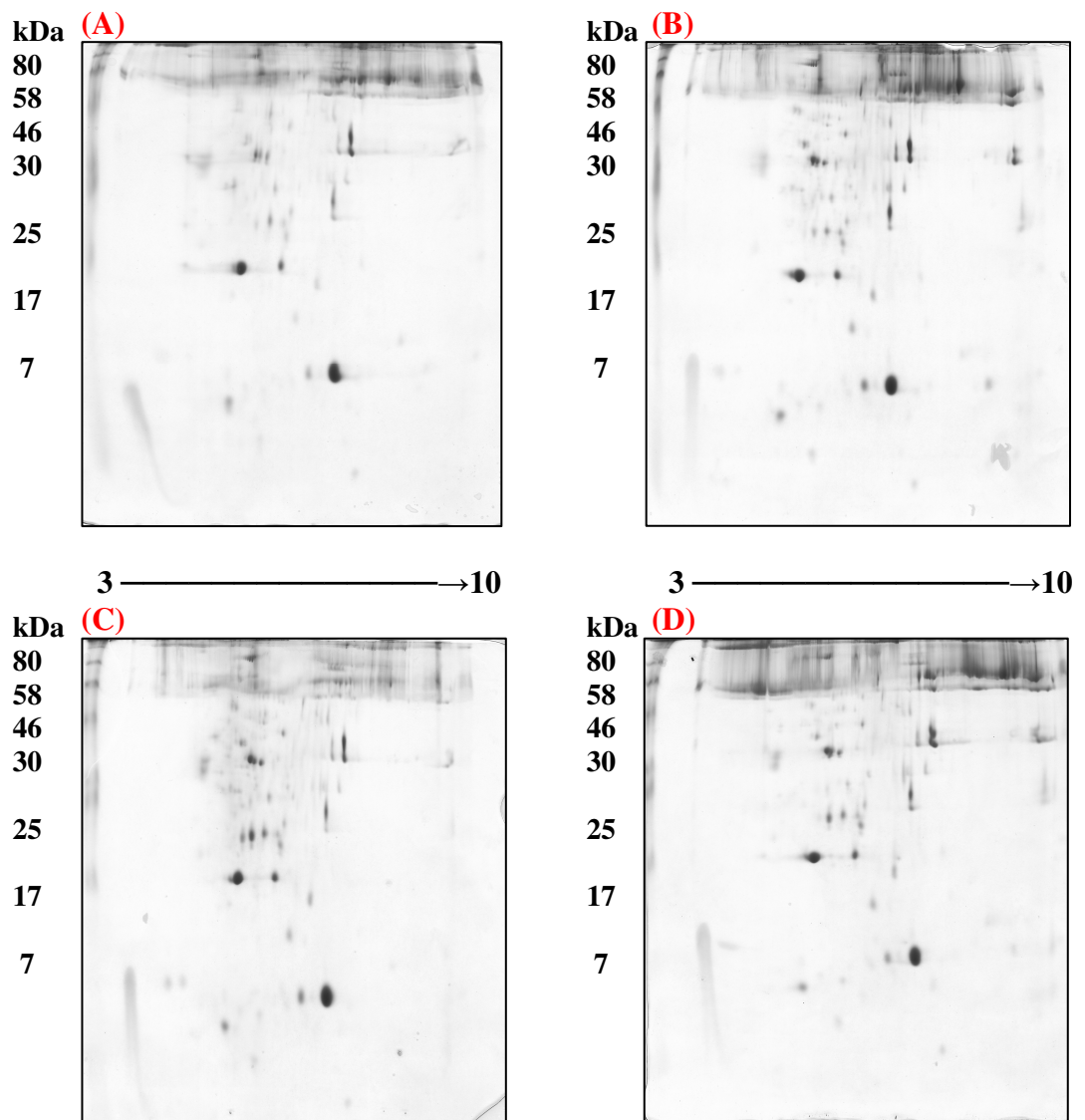
Figure 5.1 Larval haemolymph proteomic profile following fumagillin injection.
Larvae were left unstimulated **(A)**, injected with PBS **(B)**, injected with 2 µg fumagillin **(C)** or injected with 10% methanol as a solvent control **(D)**. Following 2-dimensional electrophoresis of cell free haemolymph protein spots were visualised using Colloidal Coomassie staining prior to Progenesis™ same spot analysis.

Isoelectric point (p.I)

3 —————————————————→10
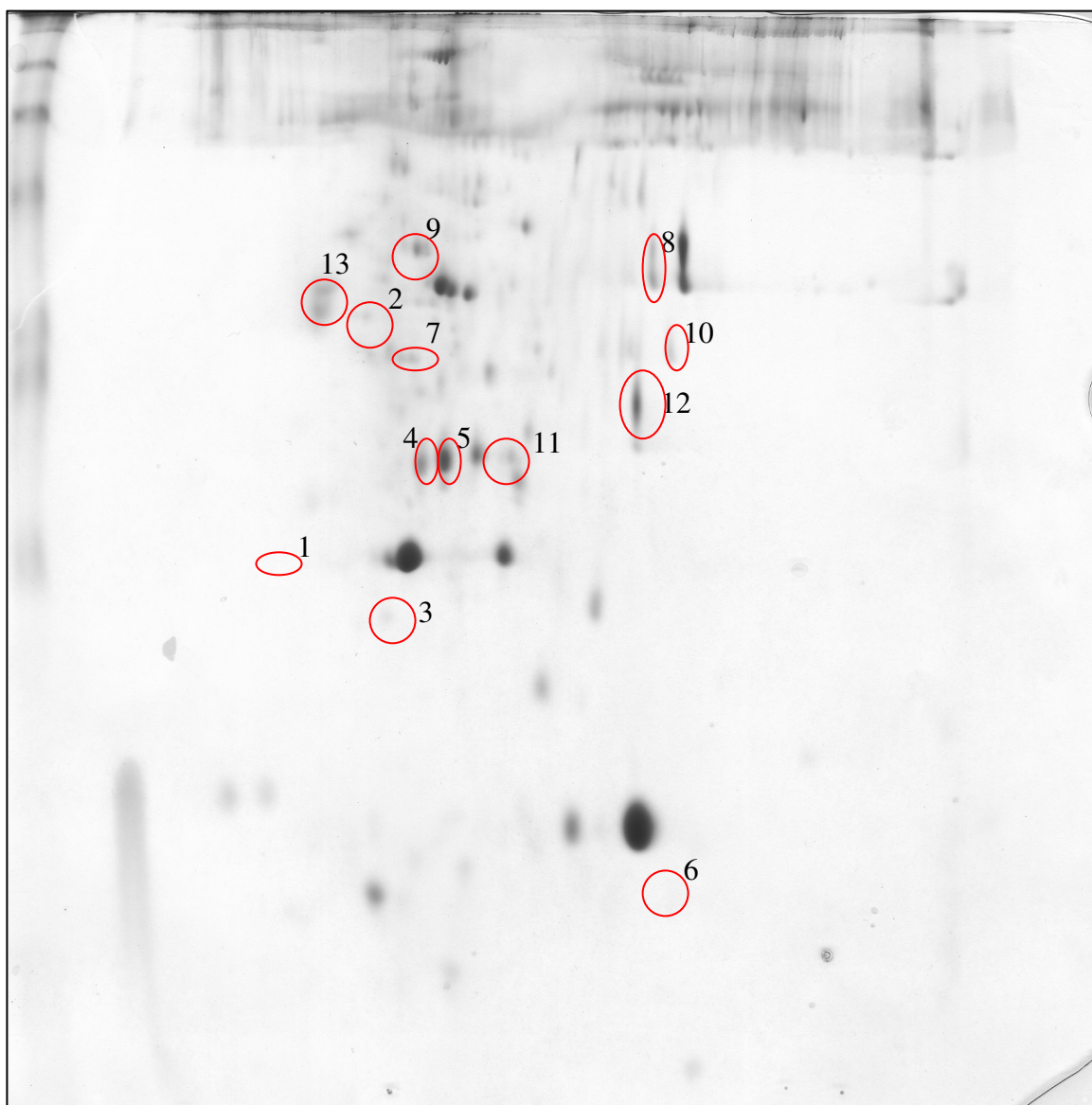
Figure 5.2 Spots excised for fold change analysis from *G. mellonella* haemolymph profiles.

An unstimulated larval treatment was used to excise spots which showed alterations in expression as determined using Progenesis™ same spot analysis. Highlighted spots are comparatively analysed in Figure 5.3

Spot 1 was identified as 27 kDa haemolymph protein (P27K) from *G. mellonella* and demonstrated a 7.6 and 4.24 fold increase in expression in larvae injected with PBS and 10% (v/v) methanol, respectively. Larvae injected with fumagillin (2 µg) expressed a 1.47 fold increase in P27K expression compared to untreated larvae however this was significantly less than that observed for both positive controls. Uniprot analysis reveals that P27K is a member of the UPF0408 family which consists of two other 27 kDa glycoproteins from *Manduca sexta* and *Bombyx mori* however the functions of these proteins remain unknown.

Spot 2 demonstrated homology with Glyceraldehyde-3-phosphate dehydrogenase, (GAPDH) from *Staphylococcus sciuri*. Larvae demonstrated a 3.76, 4.97 and 2.14 fold change in GAPDH expression relative to untreated larvae following injection with PBS, 2 µg fumagillin or 10% (v/v) methanol injected larvae. GAPDH catalyses NAD^+ dependent oxidative phosphorylation of glyceraldehyde-3-phosphate to 1,3-biphosphoglycerate and has been demonstrated to play a role in DNA repair and interaction with cellular components such as RNA and nitric oxide (Sirover, 1996 and 1999). Expression of GAPDH has been demonstrated to be increased in the presence of reactive oxygen species, (Ito *et al*., 1996).

Spots 3 and 4 showed homology to alkyl hydroperoxide reductase (AHP) C22 subunit and peroxiredoxin from *Acinetobacter johnsonii* SHO46 and *Acinetobacter calcoceticus* respectively which are annotated as functionally related proteins. Larvae injected with fumagillin demonstrated a 4.9 fold increase in the expression of AHP relative to untreated larvae. In comparison, PBS and methanol control injected larvae showed 3.11 and 3.32 fold increases in AHP expression respectively compared to untreated larvae. AHP has been demonstrated to protect host organisms from oxidative stress by reducing hydrogen peroxide, peroxynitrite and organic hydroperoxides, (Jacobson *et al*., 1989; Storz *et al*., 1989). Larvae injected with fumagillin demonstrated

Protein	Spot I.D corresponding to treatment				Fold expression			
	Untreated	PBS	2µg Fum	Solv Ctrl	U.T	PBS	2µg Fum	Solv Ctrl
1. 27 kDa haemolymph protein					1	7.6	1.47	4.24
2. Glyceraldehyde 3 phosphate dehydrogenase					1	3.76	4.97	2.14
3. Alkyl hydroperoxide dehydrogenase					1	3.11	4.9	3.32
4. Peroxiredoxin					1	1.38	4.92	2.05
5. Eukaryotic Initiation factor					1	1.26	3.85	2.41
6. Cellular retinoic acid binding protein					1	1.12	0.51	1.52
7. Arylphorin					1	1.68	0.67	1.82
8. Hexamerin					1	1.51	0.82	1.24
9. Membrane ATP synthase					1	1.32	1.76	1.31
10. GI20232 (*Drosophila mojavensis*)					1	1.22	1.7	1.03
11. 32 kDa ferritin subunit					1	1.43	1.19	1.62

Figure 5.3 (A)

Figure 5.3 (A) Identification of proteins with significantly altered expression following larval injection with fumagillin.
Proteins demonstrating significant alterations in expression, (p ≤ 0.05) identified using Progenesis™ same spots software are presented with the fold change expressed relative to unstimulated larvae.
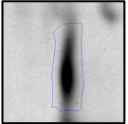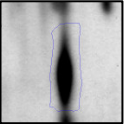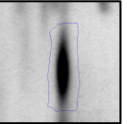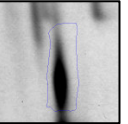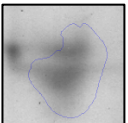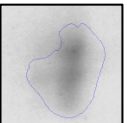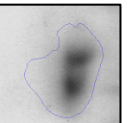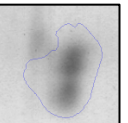
Protein	Spot I.D corresponding to treatment				Fold expression			
	Untreated	PBS	2µg Fum	Solv Ctrl	U.T	PBS	2µg Fum	Solv Ctrl
12. Juvenile hormone binding protein					1	1.2	0.81	1.03
13. Translation elongation factor					1	0.72	0.98	0.735

Figure 5.3 (B) Individual spot analyses of proteins which did not show significantly changed expression following injection with fumagillin.
Proteins identified by LC/MS analysis subsequent to Progenesis™ same spots identification. The expression of spots identified as JHBP and translation elongation factor did not vary significantly with different stimuli.

Spot I.D	gi	Species	Score	Peptides Matched	Coverage
27 kDa haemolymph protein	46396271	*Galleria mellonella*	333	6	41%
Glyceraldehyde 3 phosphate dehydrogenase	20799626	*Stapylococcus sciuri*	368	7	20%
Alkyl hydroperoxide dehydrogenase C22 subunit	262369692	*Acinetobacter johnsonii SHO46*	415	8	51%
Peroxiredoxin	262279671	*Acinetobacter calcoceticus*	206	3	20%
Eukaryotic Initiation factor	159482685	*Chlamyldomonas reinhardtii*	69	1	3%
Cellular retinoic acid binding protein	112983600	*Bombyx mori*	96	2	15%
Arylphorin	159078	*Galleria mellonella*	490	9	11%
Hexamerin	347090	*Galleria mellonella*	170	3	4%
Membrane bound ATP synthase	262371347	*Acinetobacter johnsonii SHO46*	673	11	41%
GI20232	195120978	*Drosophila mojavensis*	91	2	6%
32 kDa ferritin subunit	17901818	*Galleria mellonella*	69	2	9%
Juvenile hormone binding protein	158515746	*Galleria mellonella*	372	11	31%
Translation elongation factor	59806076	*Stapylococcus sciuri*	89	1	12%

Table 5.1 Summary of proteins identified by LC/MS analysis.
MASCOT identification of spots presented in Figure 5.3 (A) and (B). The proteins demonstrating the greatest fold change relative to untreated larvae ($p \leq 0.05$) down to the lowest (non-significant) changes are presented in order. For identification a MASCOT score lower limit of 62 was used.

a 4.92 fold increase in peroxiredoxin (PRX) expression. This contrasts with the 1.38 and 2.05 fold increases in expression demonstrated by larvae injected with PBS or solvent control. Peroxiredoxins are a ubiquitous family of proteins of ancient origin which play a key role in the protection of host tissue to damaging oxidative stresses whereby hydroperoxides are reduced and detoxified, (Wood *et al.*, 2003; Knoops *et al.*, 2007). In mammalian cells Prx's may comprise of 0.1-0.8% of soluble protein where, in the presence of thioredoxin, they play an integral role in the regulation of oxidative stress responses in host tissue, (Chae *et al.*, 1999). Furthermore, studies using other lepidopteran species have demonstrated that alterations in the expression and synthesis of Prxs are intricately linked to the need to detoxify host tissue and other aberrant stimuli, (Wang *et al.*, 2008; Fang *et al.*, 2010)

Spot 5 was identified as a protein with homology with eukaryotic initiation factor from *Chlamydomonas reinhardtii*. Larvae injected with fumagillin demonstrated a 3.85 fold increase in eukaryotic initiation factor relative to untreated larvae. Eukaryotic initiation factors function in the initiation of protein translation in eukaryotic species, (Pestova and Hellen, 2000). In addition the synthesis of eukaryotic initiation factors increases at the onset of metamorphosis in some insect species indicate that altered expression of this protein is developmentally regulated, (Dong *et al.*, 2009).

Spot 6 was identified as cellular retinoic acid binding protein, (CRABP). Interestingly, larvae injected with fumagillin expressed CRABP 0.51 fold relative to untreated larvae. Larvae injected with PBS or the methanol control demonstrated a 1.12 and 1.52 increases in the expression of CRABP respectively. Considering the reduction in haemolymph CRABP and the results presented in Section 4.7.2 it is apparent that fumagillin, (2 µg) is capable of reducing the presence of this protein *in vivo* as well as the limiting degranulation of CRABP from haemocytes. Mehta *et al.*, (1994) demonstrated that CRABP reduced the expression of TNF-α and nitric oxide production

in macrophage cell lines however it remains to be understood if a similar process takes place in haemocytes.

Spots 7 and 8 were identified as arylphorin and hexamerin respectively. Larvae injected with PBS or 10% (v/v) methanol demonstrated a 1.68 and 1.82 fold increase in arylphorin expression relative to unstimulated larvae. This contrasted with the reduced levels of arylphorin present in the haemolymph of larvae injected with fumagillin where it was expressed 0.67 fold relative to untreated larvae. The expression of hexamerin in larvae injected with fumagillin was 0.82 fold of untreated larvae. Larvae injected with PBS or 10% (v/v) methanol demonstrated increased hexamerin synthesis, (1.51 and 1.24 fold, respectively). Arylphorin and hexamerin are annotated as members of the haemocyanin superfamily, and primarily function to store macromolecules and in oxygen transportation.

Spot 9 was identified as a membrane bound ATP synthase. Larvae injected with PBS, 10% (v/v) methanol or 2 µg fumagillin demonstrated a 1.32, 1.31 and 1.76 fold increase in the expression of ATP synthase relative to untreated larvae, respectively. ATP synthases are ubiquitous and function to convert ADP and inorganic phosphates to form ATP, (Mitchell, 1961; Boyer, 1997). Due to the membrane localisation of ATP synthases, drastic increases in expression are associated with disruption to cell structure and cell lysis, (Lian *et al*., 2008).

Spot 10 showed sequence homology with GI20232 in *D. mojavensis*. Annotation of this protein identified it as an aldo-keto reductase (AKR). Larvae injected with fumagillin demonstrate a 1.7 fold increase in the expression of GI20232 compared to untreated larvae. AKRs are a well characterised family of proteins which comprise over 150 members, and are linked to the metabolism of molecules with aldehyde or ketone groups, drugs or carcinogens, (Jez *et al*., 1997, Masser, 2004).

Spot 11 was the last protein which demonstrated a significant variation in the level of expression in larvae injected with fumagillin compared to other treatments and was identified as 32 kDa ferritin subunit from *G. mellonella*. Larvae injected with PBS or 10% (v/v) methanol demonstrated a 1.43 and 1.62 fold increase in the expression of 32 kDa ferritin subunit. In contrast, larvae injected with fumagillin demonstrated a 1.19 fold increase in the level of 32 kDa ferritin subunit expression which was found to be significantly less than both positive controls. Ferritin plays an important role in the regulation of iron homeostasis as aberrant iron levels in host tissue contribute to the formation of free radicals, (You and Wang, 2005). Ferritin is a highly conserved protein which has been isolated from plant, vertebrate and invertebrate sources and is intrinsically linked to the acute phase response in infected tissues thus serving as potential biomarker of host immunological status, (Beck *et al*., 2002).

Spots 12 and 13 did not demonstrate significant alterations in expression and are presented in Figure 5.3 (B). Spot 12 was identified as juvenile hormone binding protein, (JHBP). JHBP acts as a carrier protein for juvenile hormone which in turn acts to regulate development and metamorphosis in insects, (Truman and Riddiford, 1999; Gilbert *et al*., 2000). The work of Zalewska *et al.*, (2009), identified that the exocytosis of JHBP from fat body cells to the haemolymph is mediated by interactions with ATP synthase. Larvae injected with PBS, fumagillin or solvent control demonstrated 1.2, 0.81 and 1.03 fold expression values compared to unstimulated larvae.

Spot 13 was identified as a translation elongation factor. In eukaryotes translation elongation factors function in polypeptide chain synthesis through interactions with t-RNA and m-RNA in the ribosome (Lamberti *et al*., 2004). Larvae injected with PBS, fumagillin or 10% (v/v) methanol demonstrated diminished levels of translation elongation factor expressing 0.72, 0.98 and 0.73 fold of untreated larvae

respectively compared to unstimulated larvae, however these reductions were calculated as insignificant, ($p \geq 0.12$).

5.1.2 Analysis of the effect of fumagillin on phenoloxidase activity in larval haemolymph.

In order to ascertain if fumagillin affected the activation of the phenoloxidase (PO) pathway in larval haemolymph it was necessary to establish a means of activating the pathway in the absence of infection. Mowlds and Kavanagh (2008) demonstrated that humoral immunity in *G. mellonella* larvae may be affected by increases in temperature as evidenced by increases in antimicrobial peptide production and changes to the proteomic profile. Therefore the effect of increasing temperature on PO activity was assessed. Larvae were incubated at 30ºC or 37ºC for 24 hours and a PO activity assay was performed as described, (Section 2.20.2). Figure 5.4 demonstrates that larvae incubated at 37ºC show a 1.77 fold increase in PO activity compared to larvae incubated at 30ºC, ($p \leq 0.001$). This finding was interesting as it indicated that the phenoloxidase cascade may be affected by non-microbial stimuli. As a non-infective means to affect PO activation was established the effect of an immunosuppressive concentration of fumagillin on the activity of PO *in vivo* was assessed. Larvae were left untreated or injected with PBS, 2 µg fumagillin or 10% (v/v) methanol and incubated at 37ºC prior to performing PO activity analysis. Haemolymph from untreated larvae demonstrated approximately the same level of absorbance at 490nm as the 37ºC treatment described in Figure 5.4, (0.124 ± 0.005). This figure contrasted with the absorbance observed for haemolymph from larvae injected with PBS or 0.002% (v/v) methanol where figures of 0.158 ± 0.006 and 0.157 ± 0.005 were recorded, ($p \leq 0.007$). Haemolymph from larvae injected with PBS or 10% (v/v) methanol did not demonstrate statistically significant absorbance values from each other, ($p = 0.15$). Haemolymph extracted from larvae
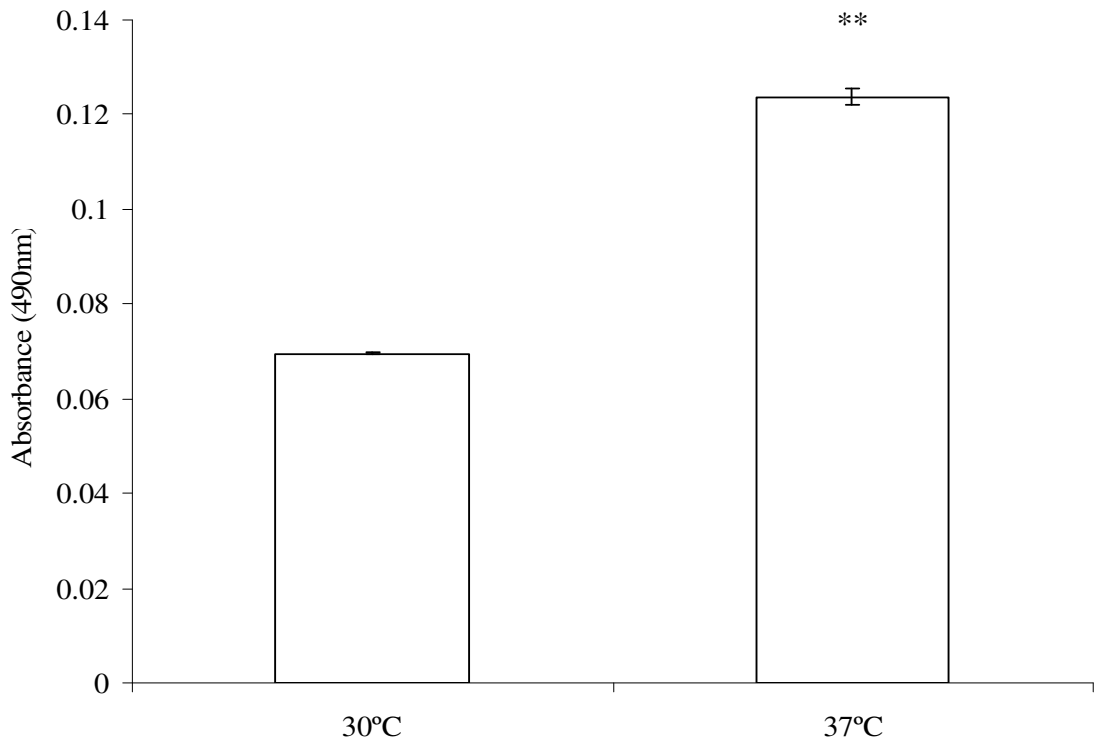
Figure 5.4 Phenoloxidase activity of *G. mellonella* haemolymph following incubation at 30ºC or 37ºC.
Larvae were incubated at 30ºC or 37ºC for 24 hours and phenoloxidase activity was ascertained at 490 nm. Larvae incubated at 37ºC demonstrated significantly increased phenoloxidase activity, (p = 0.001).
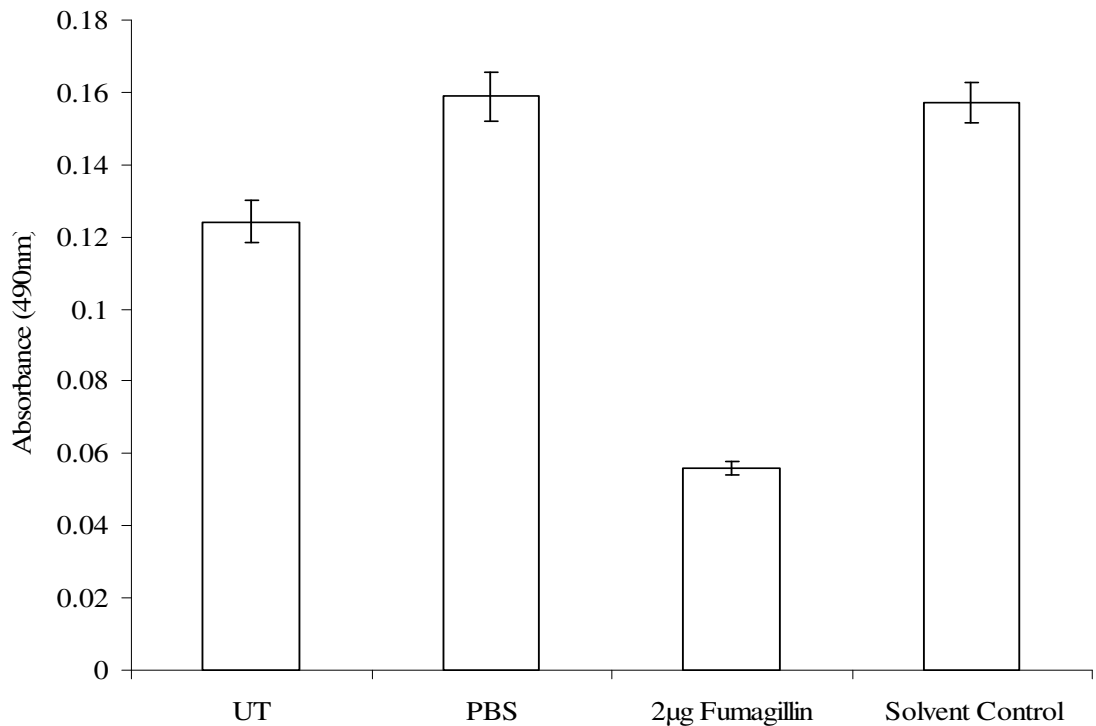
Figure 5.5 Effect of fumagillin on *G. mellonella* haemolymph phenoloxidase activity.
Larvae were injected with PBS, 2 μg fumagillin or 10% methanol or were left untreated and incubated at 37ºC for 24 hours. Larvae injected with fumagillin demonstrated reduced phenoloxidase activity compared to all other treatments, (p ≤ 0.001).

injected with fumagillin (2 µg) demonstrated greatly reduced PO activity compared to all other treatments with absorbance at 0.055 ± 0.001. The reduction in absorbance was significant compared to all other treatments, ($p \leq 0.001$).

5.1.3 Summary.

In this section the effects of fumagillin on the haemolymph profile and the phenoloxidase cascade of *G. mellonella* larvae was assessed. Larvae injected with an immunosuppressive concentration of fumagillin (2µg), demonstrated significant increases in expression of enzymes that catalyse the glycolytic pathway, peroxide breakdown, initiation of protein translation, ATP synthesis, and an aldo-keto reductase. These increases are coupled to the reduced expression of CRABP, haemocyanins and ferritin. In addition the effect of fumagillin on the activation of the phenoloxidase cascade was determined. Phenoloxidase activity is significantly reduced in larvae injected with fumagillin. These results indicate that larval exposure to fumagillin appears to reduce PO activity and leads to the induction of oxidative stress *in vivo* as evidenced by the increased expression of detoxification enzymes.

5.2 Analysis of the binding profiles of haemolymph proteins with affinity for *A. fumigatus* conidia.

The insect humoral immune system has been demonstrated to participate in the recognition of pathogens and the cellular neutralisation of invading microbes, (Dunphy *et al*., 1986; da Silva *et al*., 2000). In order to develop a more comprehensive understanding of the interaction between *A. fumigatus* and *G. mellonella* larvae at the point of infection a study to assess the interaction of the humoral response with *A. fumigatus* conidia was performed.

5.2.1 SDS-PAGE and LC/MS identification of larval haemolymph proteins binding to *A. fumigatus* conidia *in vitro*.

Larvae (10) were bled and a haemolymph binding assay was performed as described, (Section 2.20). Following protein solubilisation and SDS-PAGE analysis, (Figure 5.6) all visible bands were excised and subjected to trypsin digestion prior to LC/MS analysis. Four bands of interest were identified and are presented in Table 5.2.

Band 1 was identified as apolipophorin, (apo 1). Apo 1 primarily functions in lipid transport and lipoprotein metabolism, (Weers and Ryan, 2006) however the work of Ma *et al*., (2006) identified a role for this protein in oligomeric interactions with LPS thus initiating particle neutralisation. This process is further complexed by interactions with lipids, glycolipids and hemolin leading to a "cage-like" sequesterisation of LPS moieties within the haemocoel, (Schmidt *et al*., 2010).

Band 2 was identified as arylphorin. Arylphorin has been discussed previously in this Chapter as an amino acid storage and transport protein, (Telfer, 1991). However the work of Beresford *et al*., (1997) indicated an additional role for arylphorin as an antimicrobial. The N-terminus of gallysin-1, a potent elicitor of the antimicrobial response of *G. mellonella* haemolytically active material, (HAM) is identical to the first 20 amino acids of the arylphorin sequence.

Band 3 was identified as prophenoloxidase subunit 2. PPO is a key component of the melanisation pathway which catalyses *o*-hydroxylation of monophenols and oxidation of phenols to quinines which polymerise to form melanin, (Soderhall and Cerenius, 1998). The activation of PO is mediated by the prophenoloxidase activating system, (ProPO-AS) consisting of C-type lectins which bind LPS, peptidoglycan and β-1,3 glucan and thus initiate the controlled activation of PO, (Kavanagh and Reeves, 2004).
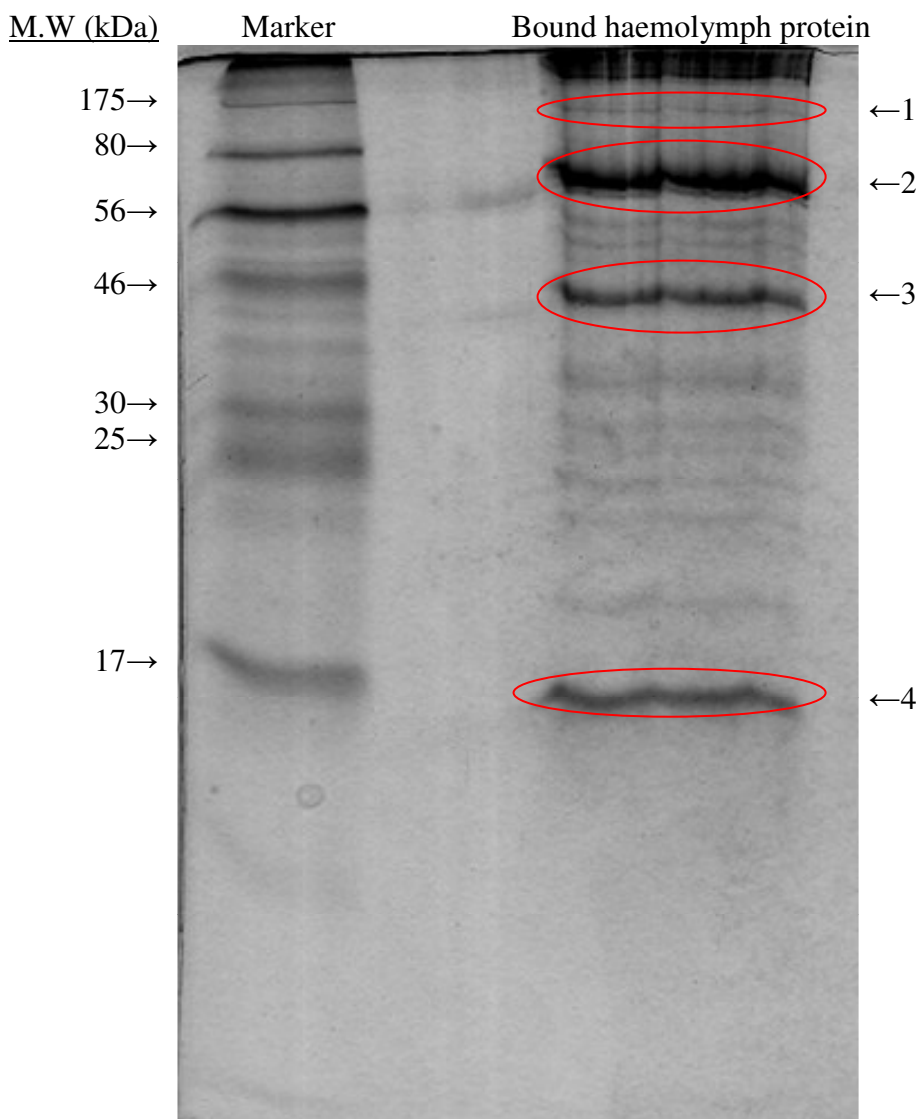
Figure 5.6 SDS-PAGE analysis of proteins bound to conidia.
The haemolymph binding assay was performed as described (Section 2.20). Following protein extraction and 12.5% SDS-PAGE bands of interest were excised and trypsin digested prior to subsequent identification by LC/MS analysis.

Band No.	Identified	gi	Score	Peptides matched	Coverage
1	Apolipophorin	50404098	151	3	4%
2	Arylphorin	159078	271	6	10%
3	Prophenoloxidase subunit 2	34556399	167	3	4%
4	Apolipophorin III	5915688	170	4	29%

Table 5.2 Identified proteins from *G. mellonella* haemolymph interaction study.
Bands of interest (Figure 5.6) were excised and enzymatically digested using trypsin. Following peptide extraction LC/MS analysis yielded identification of four major proteins with binding affinity for *A. fumigatus* conidia.

Band 4 was identified as apolipophorin III, (apoLp III). ApoLp III mediates binding to lipotechtoic acid and the detoxification of LPS, (Kato *et al*., 1994; Dunphy and Halwani, 1997). In addition apoLp III enhances haemolymph antibacterial activity by promoting phagocytosis and haemocyte mediated superoxide production, (Wiesener *et al*., 1997). ApoLp III activates the PPO cascade and demonstrates functional and structural homology with mammalian apolipoprotein E, (Cole *et al*., 1987). At normal physiological levels apoLp III is involved in lipid transport and provides diacylglycerol (DAG) to flight muscles, (van der Horst *et al*., 2002; van der Horst 2003).

5.2.2 Comparative analysis of the effect of fumagillin on haemolymph protein binding to conidia *in vitro*.

The haemolymph binding assay was performed as described, (Section 2.20.3) however haemolymph was spiked with fumagillin (1 or 2 µg ml^{-1}) for 25 minutes or methanol (0.002% v/v). From there the assay was performed as normal with the concentration of conidia in each extraction sample checked for equal loading. The protein lysates were separated and visualised by 1-D SDS-PAGE and Colloidal Coomassie staining, (Figure 5.7). The profiles of identified bands were analysed by densitometry to ascertain if differential binding took place in the presence or absence of immunomodulatory concentrations of fumagillin. Densitometric analysis of larval haemolymph proteins revealed that bands identified as arylphorin, prophenoloxidase subunit 2 and apolipophorin IIII demonstrated altered levels of binding to conidia following co-incubation with fumagillin.

In Figure 5.8 densitometric quantification of differential arylphorin binding to conidia is presented. Arylphorin binding was reduced 0.57 and 0.66 fold where haemolymph was pre-incubated with 2 or 1 µg fumagillin ml^{-1} respectively compared to
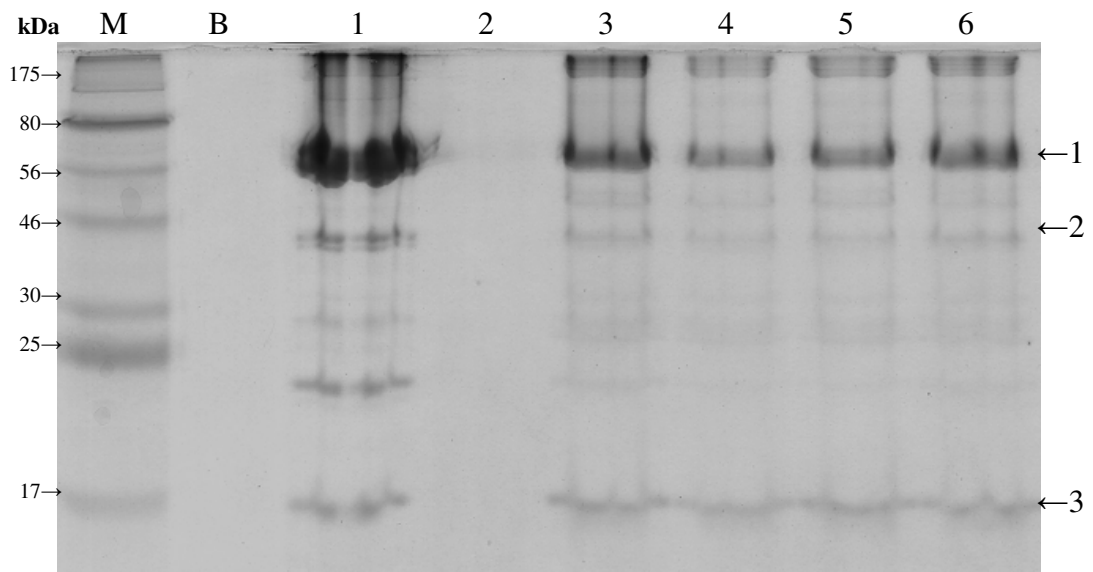
Figure 5.7 Haemolymph binding profiles of proteins bound to conidia following incubation of haemolymph with fumagillin.
Larvae were bled and resulting cell free haemolymph was incubated with 2 or 1 µg fumagillin ml^{-1} for 25 minutes or incubated in the presence of 0.002% (v/v) methanol as a solvent control. Resulting proteins were visualised by SDS-PAGE and Colloidal Comassie staining.

Lanes
1. Full Haemolymph profile (20 µg protein)
2. Conidia surface extraction
3. Control haemolymph interaction
4. 2 µg fumagillin ml^{-1} haemolymph interaction
5. 1 µg fumagillin ml^{-1} haemolymph interaction
6. Solvent control (0.002% v/v methanol) haemolymph interaction

Bands marked 1 (arylphorin), 2 (PPO subunit 2) and 3 (apolipophorin III) were identified and relative binding was ascertained by densitometry
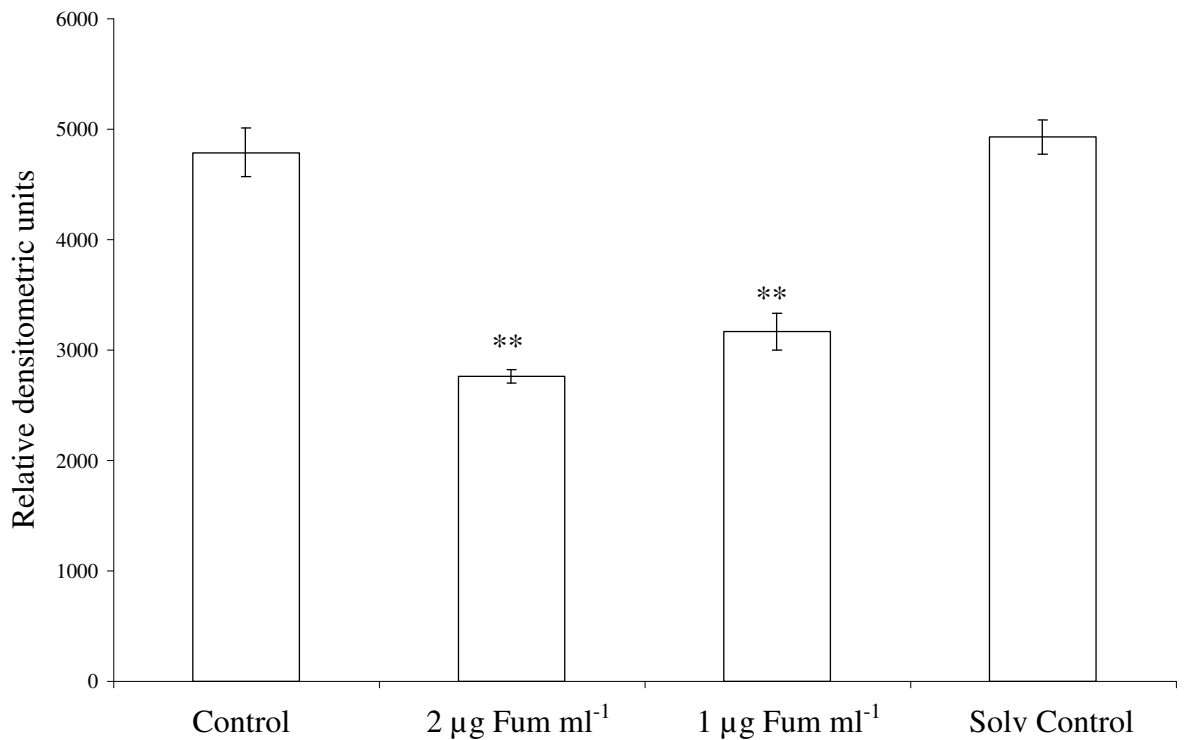
Figure 5.8 Densitometric analysis of arylphorin binding to *A. fumigatus* conidia.
The conidial binding of arylphorin from larval haemolymph pre-treated with fumagillin, (2 or 1 µg ml^{-1}) is presented. Haemolymph pre-treated with fumagillin demonstrated reduced binding to conidia, (**p ≤ 0.006) relative to control haemolymph.
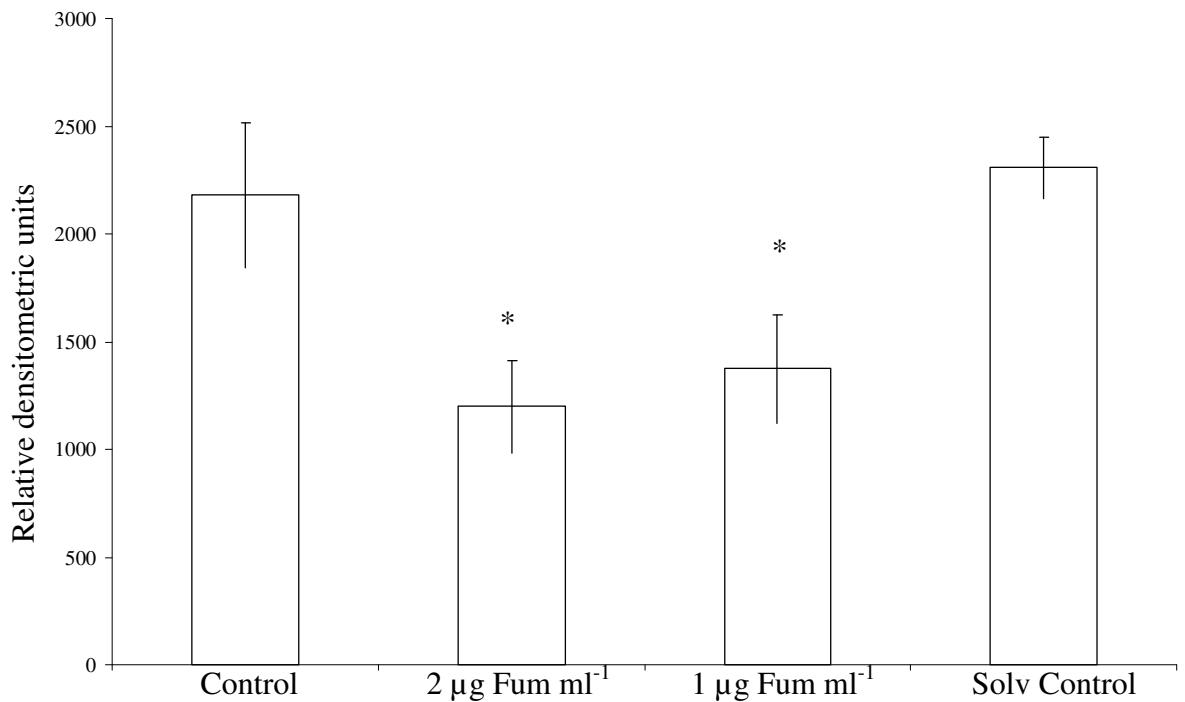
Figure 5.9 Densitometric analysis of prophenoloxidase subunit 2 binding to *A. fumigatus* conidia.
The conidial binding of prophenoloxidase subunit 2 from larval haemolymph pre-treated with fumagillin, (2 or 1 µg ml^{-1}) is presented. Haemolymph pre-treated with fumagillin demonstrated reduced binding to conidia, (p ≤ 0.014) relative to control haemolymph.
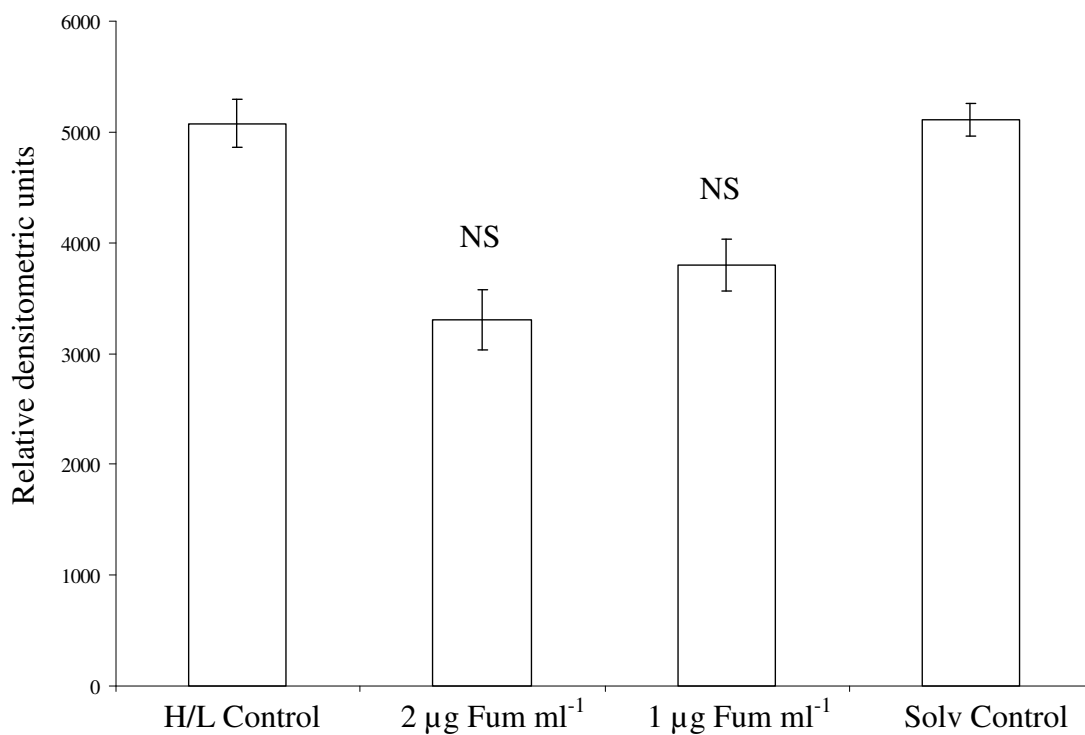
Figure 5.10 Densitometric analysis of Apolipophorin III binding to *A. fumigatus* conidia.

The conidial binding of apolipophorin III from larval haemolymph pre-treated with fumagillin, (2 or 1 μg ml^{-1}) is presented. There was a reduction in the level of apolipophorin III binding however the alteration was not considered statistically significant, (p $\geq$ 0.064).

untreated haemolymph ($p \leq 0.006$). Haemolymph pre-treated with 0.002% (v/v) methanol did not demonstrate a notable change in arylphorin binding ($p = 0.593$).

Exposure of haemolymph to fumagillin caused significant alterations in the binding of prophenoloxidase subunit 2 to conidia, (Figure 5.9). Haemolymph incubated with fumagillin (1 or 2 µg ml^{-1}) demonstrated a 0.55 and 0.63 fold decrease in prophenoloxidase subunit 2 binding respectively compared to control haemolymph, ($p \leq 0.014$). Haemolymph incubated in the presence of 0.002% (v/v) methanol did not demonstrate a significant change in prophenoloxidase binding ($p = 0.754$).

The binding of apoLp III was altered by incubating haemolymph with fumagillin (Figure 5.10). Haemolymph incubated with fumagillin (1 or 2 µg ml^{-1}) demonstrated a 0.65 and 0.74 fold decrease in apoLp III binding compared to control haemolymph however the reduction in binding was not considered to be significant, ($p \geq 0.064$). This finding indicates that while alterations in protein binding occurs following exposure of haemolymph to fumagillin the relative change is not universally significant thus indicating specific inhibition to pathogen recognition. This is supported by the finding that apolipophorin 1 did not demonstrate altered binding to *A. fumigatus* conidia when previously incubated in the presence of fumagillin.

5.2.3 SDS-PAGE and LC/MS identification of human serum proteins with binding activity to *A. fumigatus* conidia *in vitro*.

In order to perform a comparison with the human innate immune system cell free serum pooled from multiple donors was used in an identical manner to that of haemolymph (Section 2.20.3). The resulting binding profile is presented in Figure 5.11 and a list of analysed proteins is presented in Table 5.3.

Band number 1 was identified as inter-alpha-trypsin inhibitor family: heavy chain related protein (ITIH4). ITIH4 is highly conserved in pigs, humans and cattle and

is regarded as a biomarker for the acute phase reactions to aberrant stress and may be regulated by IL-6, (Pineiro *et al*., 1996; Pineiro *et al*, 2004). It has been demonstrated that ITIH4 may play a role in the restriction of actin polymerisation and phagocytosis in neutrophils, (Choi-Mura *et al*., 2000).

Band number 2 was identified as a gelsolin isoform. Intracellular gelsolin is involved in the reorganisation of actin filaments thus modulating cell shape and motility, (Yin *et al*., 1979; Kwiatkowski, 1989). Recent work performed by Bucki *et al*., 2008 demonstrated a role for this protein in the binding of liotechtoic acid, (LTA) and LPS. The interaction of gelsolin with LPS and LTA causes a reduction in aggregate size of endotoxins resulting in restrictions to F-actin depolymerisation activity leading to the formation of an immune response *in vivo*.

Band number 3 was identified as angiotensinogen. Angiotensinogen is the parent molecule of angiotensin which functions in blood pressure regulation, renal haemodynamics, and electrolyte homeostaisis, (Chen *et al*., 2004). The work of Naim *et al*., (2004) demonstrated that Angiotensin II, (the main effector peptide of the rennin angiotensin system) is capable of inducing rapid neutrophil recruitment mediated by the CXC chemokines CINC/KC and MIP-2 and IL-8 in humans. The role of Angiotensin II in the proinflammatory reaction is supported by work of Ito *et al*., (2001) where it was demonstrated that neutrophils possessed Angiotensin II receptor.

Band number 4 was identified as alpha-1 antitrypsin: A canonical template for active serpins, (AAT). AAT is annotated as a member of the serpin superfamily and primarily functions as a scavenger of neutrophil elastase. Abnormal variants of this protein cause affected individuals to lose lung elasticity, (Carrell *et al*., 1982). Apart from eliciting an antiprotease function AAT can inhibit LPS stimulated synthesis of TNF-α and IL-1β but can enhance IL-10, (Janciauskiene *et al*., 2004). Luisetti *et al*.,
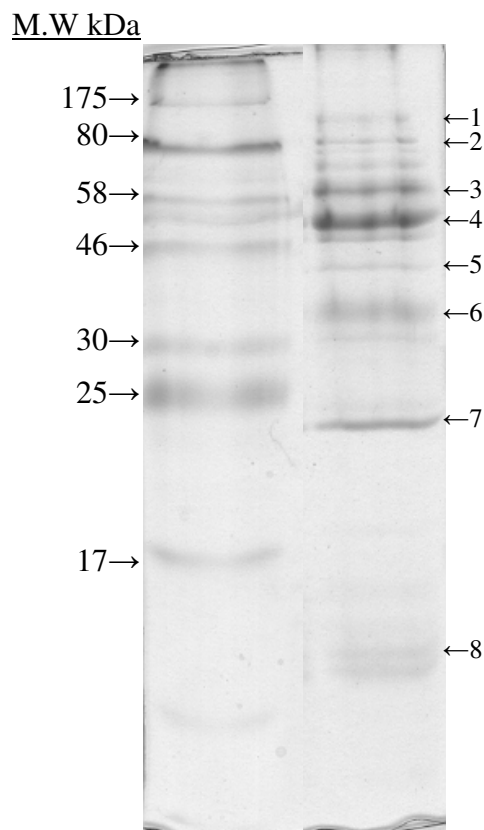
Figure 5.11 1-D SDS-PAGE profile of serum proteins with affinity for *A. fumigatus* conidia.
Serum proteins bound to conidia were analysed by 1-D SDS-PAGE and visualised with Colloidal Comassie staining. Resulting bands were excised and trypsin digested prior to LC/MS identifiaction

Band No.	Identified	gi	Score	Peptides matched	Coverage
1	inter-alpha-trypsin inhibitor family: heavy chain related protein (HRP)	1483187	1018	21	27%
2	gelsolin isoform	4504165	243	7	8%
3	Angiotensinogen	532198	154	6	13%
4	Alpha-1antitrypsin: A canonical tamplate for active serpins	6137432	828	16	44%
5	Apolipoprotein A-IV precursor	71773110	823	18	49%
6	Apolipoprotein E	178849	162	10	10%
7	Immunoglobulin kappa - light chain	3169770	105	8	24%
8	Apolipoprotein C III	521205	144	2	27%

Table 5.3 Identification of serum proteins with binding affinity to *A. fumigatus* conidia.
Bands of interest from Figure 5.11 were excised and subjected to trypsin digestion and LC/MS analysis.

(1991), did demonstrated that sepharose a protease produced by *A. mellus* was not inhibited by AAT.

Band number 5 was identified as apolipoprotein A-IV precursor, (apoA-IV). ApoA-IV is a 46 kDa serum protein synthesised in the enterocytes of the small intestine. ApoA-IV is believed to participate in lipid absorption and cholesterol transport in addition to anti-oxidant properties, and may play a role in the immune response by inhibiting LPS-induced stimulation of monocytes, (Stan *et al*., 2003; Recalde *et al*., 2004). Khovidhunkit *et al*., (2004) identified that the expression of apo A-IV is substantially increased in mice injected with LPS further highlighting the role of apo A-IV *in vivo*.

Band number 6 was identified as apolipoprotein E (apoE). ApoE plays a key role in the activation of the immune response by reducing endotoxic shock mediated by LPS, promoting phagocytosis and nitric oxide release, (Feingold *et al*., 1995; de Bont *et al*., 2000; Carvalho *et al*., 2000; Riddell *et al*., 1997). Vonk *et al*., (2004) indicated that apoE deficient mice demonstrated increased candidemia mediated by higher lipid availability and the neutralisation of candidacidal factors by very low density lipoproteins, (VLDL).

Band number 7 was identified as immunoglobulin kappa-light chain. Immunoglobulin kappa-light chain is a component of the variable domain of the light chain of immunoglobulin and shows a great level of variation through conservation of amino acid replacements at multiple positions thus conferring moiety specificity, and pathogen recognition (Hill *et al*., 1966; Das *et al*., 2008).

Band number 8 was identified as apolipoprotein CIII (Apo CIII). Apo CIII is a very low density lipoprotein (VLDL) and may serve as a ligand for TLR-2 thus mediating inflammatory activation of endothelial cells, (Kawakami *et al*., 2008).

5.2.4 An analysis of the effect of fumagillin on binding of serum proteins to conidia *in vitro*.

The serum binding assay was performed as described, (Section 2.20.3) however serum was spiked with fumagillin (1 or 2 µg ml^{-1}) for 25 minutes or 0.002% (v/v) methanol. From there the assay was performed as previously described with the concentration of conidia in each extraction sample checked for equal loading. The protein lysates were separated and visualised by 1-D SDS-PAGE and Colloidal Coomassie staining, (Figure 5.12). The profiles of identified bands were then analysed by densitometry to ascertain if differential binding took place in the presence of fumagillin. From the results only 2 identified bands showed differential binding in response to the toxin, however due to the homology that exists between apoLp III and apoE (Cole *et al*., 1987) and the identification of apoLp III binding to conidia (Sections 5.2.1 and 5.2.2) it was deemed necessary to apply densitometric analysis to the binding profile of apoE.

In Figure 5.13 serum pre-treated with fumagillin (2 or 1 µg ml^{-1}) demonstrated 0.44 and 0.83 fold decrease in angiotensinogen binding to conidia relative to control serum, ($p \leq 0.007$). Serum incubated in the presence of 0.002% (v/v) methanol did not demonstrate any significant differences compared to control haemolymph ($p = 0.874$) but did demonstrate significantly increased angiotensinogen binding compared to fumagillin treated serum, ($p \leq 0.026$).

Immunoglobulin kappa binding to conidia was also altered (Figure 5.14). Serum pre-treated with fumagillin (1 or 2 µg ml^{-1}) demonstrated 0.84 and 0.85 fold reduced binding of immunoglobulin to conidial surfaces, ($p \leq 0.009$) relative to control serum. Serum incubated in the presence of 0.002% (v/v) methanol demonstrated no significant difference compared to the control ($p = 0.3$) but did show a higher level of binding compared to the fumagillin treatments ($p = 0.014$).
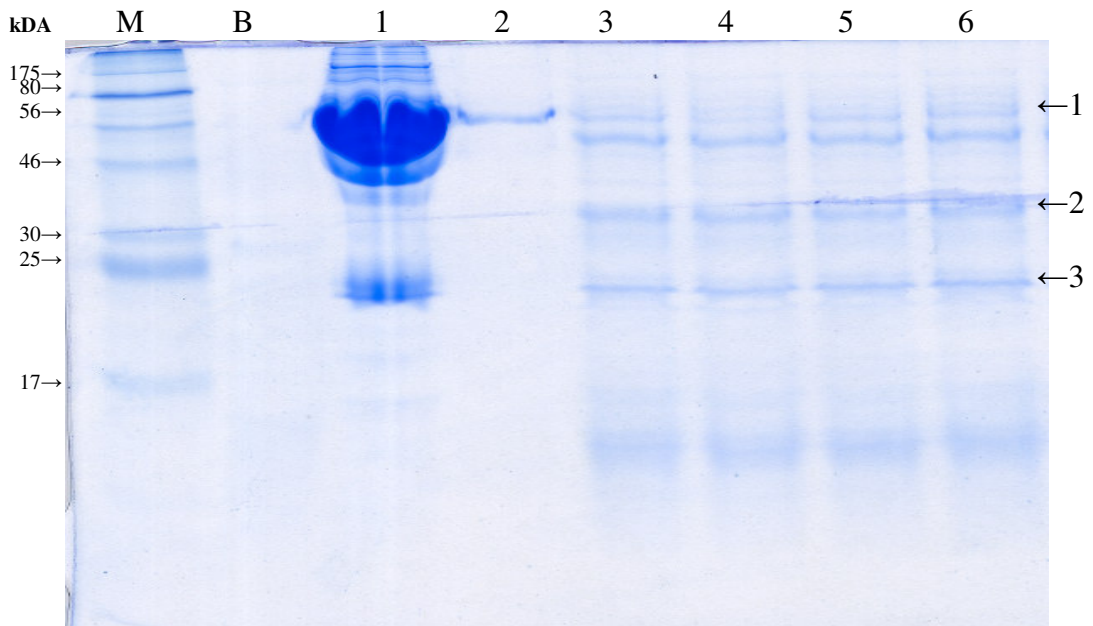
Figure 5.12 Binding profiles of proteins bound to conidia following incubation of human serum with fumagillin.

Serum was pooled from a variety of donors (n = 10) was diluted 1/10 (approx 7.5 mg protein ml^{-1}). Serum was incubated with 2 or 1 µg fumagillin ml^{-1} for 25 minutes or incubated in the presence of 0.002% v/v methanol as a solvent control. Resulting proteins were visualised by SDS-PAGE and Colloidal Coomassie staining.

Lanes
1. Full Haemolymph profile (20µg protein)
2. Conidia surface extraction
3. Control haemolymph interaction
4. 2 µg fumagillin ml^{-1} serum interaction
5. 1 µg fumagillin ml^{-1} serum interaction
6. Solvent control (0.002% v/v methanol) haemolymph interaction

Bands marked 1 (angiotensinogen), 2 (apolipoprotein E) and 3 (immunoglobulin kappa-light chain) were identified and relative binding was ascertained by densitometry (Figures 5.13, 5.14 and 5.15).
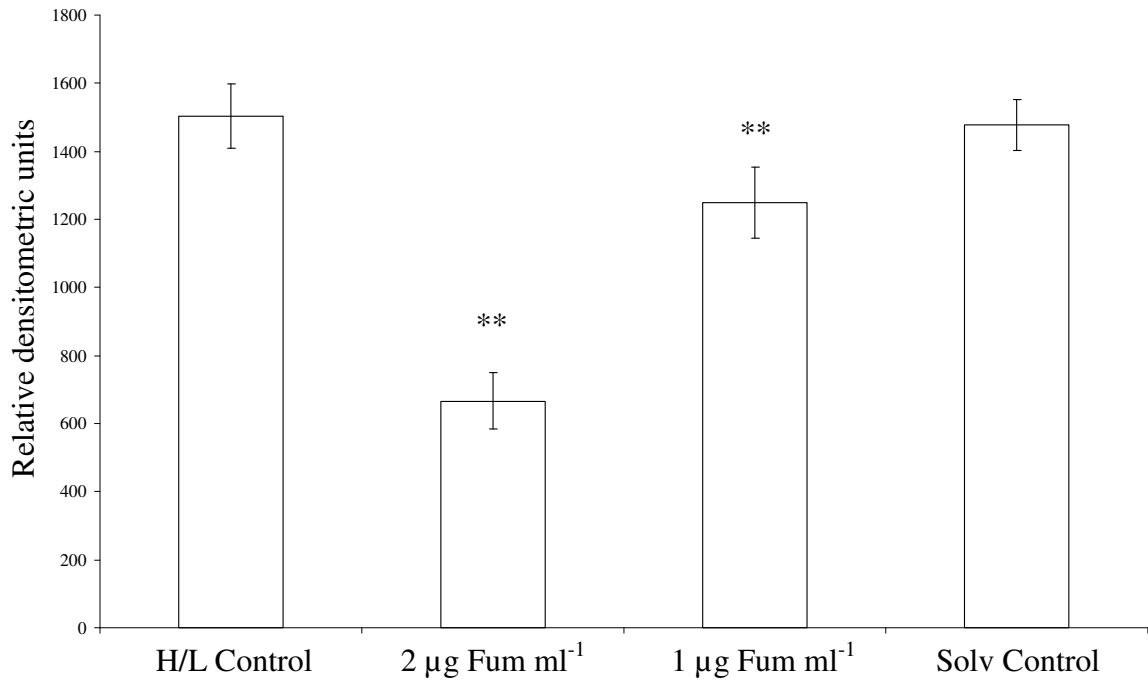
Figure 5.13 Densitometric analysis of angiotensinogen binding to *A. fumigatus* conidia.
The conidial binding of angiotensinogen from human serum pre-treated with fumagillin, (2 or 1 µg ml^{-1}) is presented. Serum pre-treated with fumagillin (2 or 1 µg ml^{-1}) demonstrated significantly reduced angiotensinogen binding relative to the control, (**p ≤ 0.007).
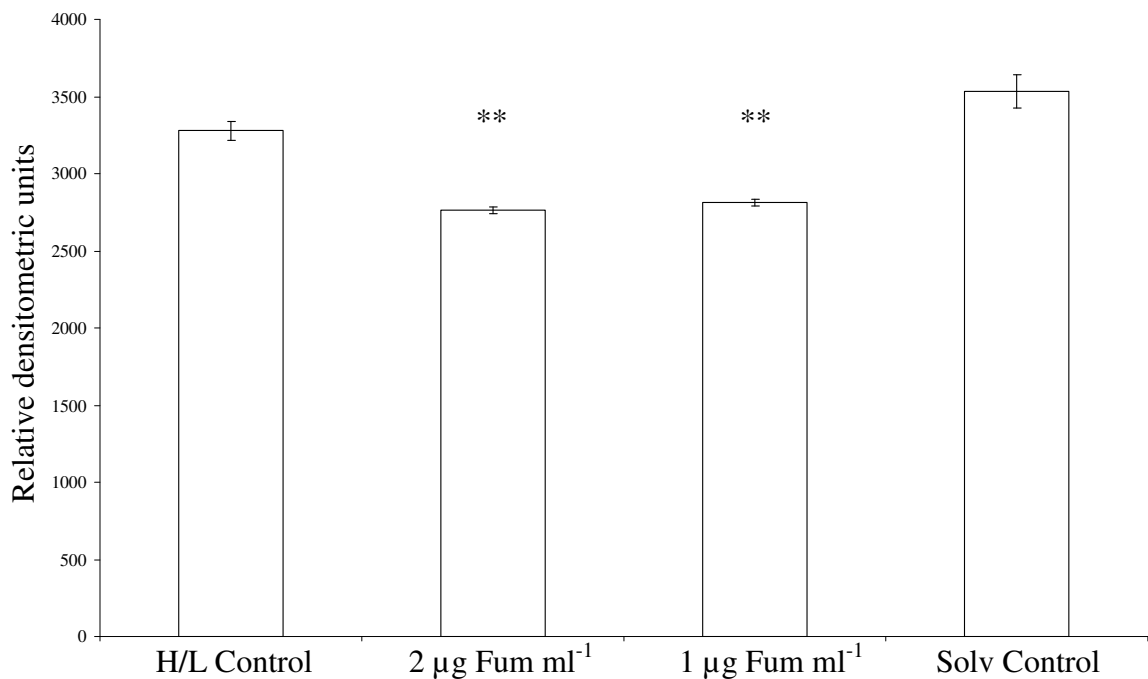
Figure 5.14 Densitometric analysis of immunoglobulin kappa binding to *A. fumigatus* conidia.
The conidial binding of Ig-kappa from human serum pre-treated with fumagillin, (2 or 1µg ml^{-1}) is presented. Serum pre-treated with fumagillin (2 or 1 µg ml^{-1}) demonstrated significantly reduced Ig-kappa binding relative to the control, (**p ≤ 0.009).
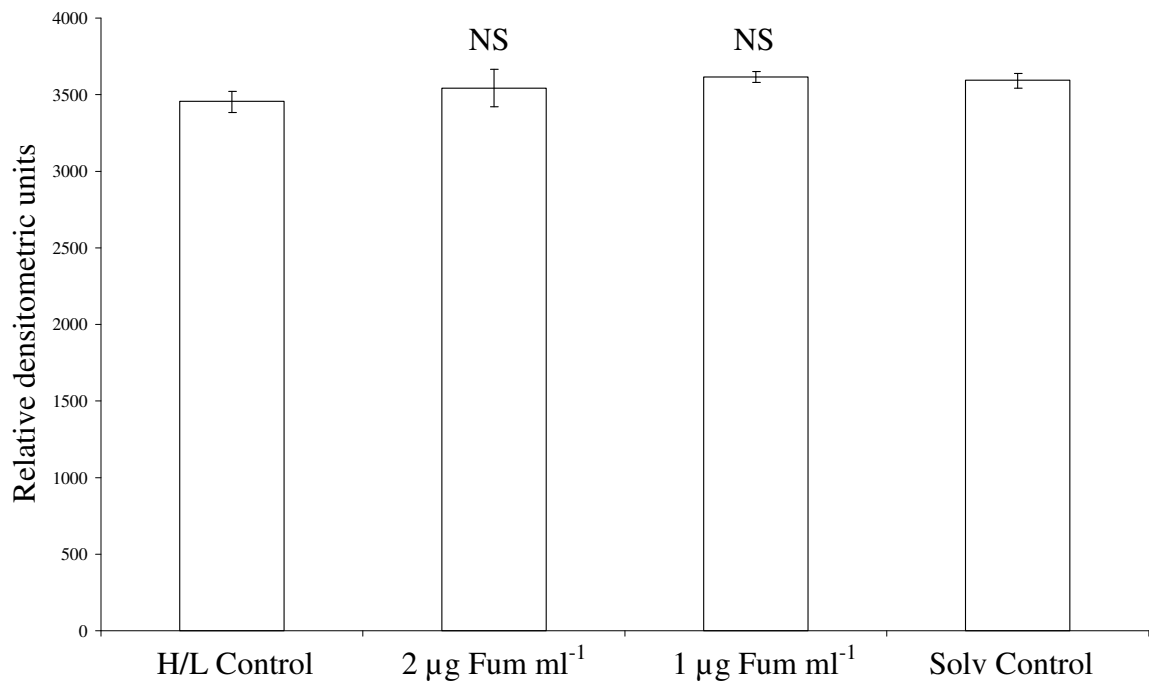
Figure 5.15 Densitometric analysis of apolipoprotein E binding to *A. fumigatus* conidia.

The binding of apolipoprotein E from human serum following exposure to fumagillin is presented. Serum pre-treated with fumagillin did not demonstrate any significant variability in apoE binding (NS).

There was a minimal change in the level of apolipoprotein E binding to conidia following incubation of serum with fumagillin, (Figure 5.15). Comparisons between control and serum control treatments revealed no significant alterations to binding, (p = 0.45). Furthermore, comparisons with both control treatments and serum exposed to fumagillin (1 or 2 μg ml^{-1}) show that apolipoprotein E binding was not affected (p $\geq$ 0.1).

5.2.5 Summary.

An analysis of the binding of proteins from *G. mellonella* larval haemolymph identified by LC/MS analysis to conidia was performed to further understand the interactions between the larval immune system and *A. fumigatus* conidia. This analysis identified four proteins which demonstrated binding to conidia using an *in vitro* assay. The four proteins, (apolipophorin, arylphorin, prophenoloxidase subunit 2 and apolipophorin III) have been previously implicated in immune reactions in *G. mellonella* larvae. A parallel study with pooled human serum identified 8 proteins binding to conidia (Figure 5.11 and Table 5.3). The proteins have been previously identified as components of the inflammatory response, pathogen recognition, mediators of leukocyte pathogen recognition and as biomarkers for the acute phase response to infection. An analysis of the effect of fumagillin on serum binding to conidia revealed that only two proteins varied in binding to conidia, (angiotensinogen and immunoglobulin kappa light chain). Due to the structural and functional similiarity that human apoE shares with invertebrate apoLp III, a densitometric analysis of apoE binding was performed. Densitometric analysis of both protein profiles revealed that variations in the binding of to conidia were present but not statistically significant.

5.3 Discussion.

The ability of *A. fumigatus* to evade the host immune response has received considerable attention throughout the course of research on this pathogen. The production of a variety of enzymes and toxins which impair immune function and alter the physiology of host tissue create a favourable environment for fungal growth and further microbial colonisation (Kolattukudy *et al*., 1993; Sutton *et al*., 1996; Bertout *et al*., 2002; Ben-Ami *et al*., 2010). The work presented in this Chapter serves to complement the cellular data presented in Chapter four where the effect of fumagillin on neutrophils and haemocytes was characterised.

The effect of an immunosuppressive concentration of fumagillin on the proteome of larval haemolymph was described, (Figures 5.1, 5.2 and 5.3). It was hypothesised that analysis of the humoral proteomic profile of *G. mellonella* may identify other effects of fumagillin which were not directly related to an immune response. The significant increase in expression of proteins demonstrating homology with glyceraldehyde-3-phosphate dehydrogenase, alkyl hydroperoxide dehydrogenase and peroxiredoxin in larvae injected with fumagillin (2 µg) may be indicative of the development of mycotoxin mediated oxidative stress *in vivo*. Schütze *et al*., (2010) provided a recent insight into this process. Using Balb/c mice, exposure to gliotoxin and patulin induced lipid peroxidation which was corrected by the administration of N-acetylcysteine or glutathione ethyl ester. In addition studies concerning the effect of TNP-470 (a synthetic derivative of fumagillin and inhibitor of angiogenesis), demonstrated induction of cell death through the generation of reactive oxygen species thus mediating toxicity in murine endothelium cell lines. In this case the addition of N-acetylcysteine caused a reduction in cell death mediated by TNP-470 (Okroj *et al*., 2005). Considering the inhibition of phagocytic cell oxidative responses mediated by mycotoxins *in vitro*, (Tsunawaki *et al*., 2004; Renwick *et al*., 2007), it is interesting that

fumagillin at an immunosuppressive concentration mediates an oxidative stress in host tissue. The increased expression of GAPDH, peroxiredoxin and alkyl hydroperoxide dehydrogenase may be indicative of such an oxidative stress mediated by fumagillin. The increase in expression of an aldo-keto reductase is also indicative of the presence of an oxidative stress mediated by fumagillin possibly as a result of lipid peroxidation. A human aldo-keto reductase (AKR1C1) mediates reduction of α,β-unsaturated aldehydes produced during lipid peroxidation caused by the presence of reactive oxygen species thus indicating a role for AKRs in oxidative stress response *in vivo* (Burczynski *et al*., 2001).

The increased expression of a protein showing homology with eukaryotic initiation factor in response to fumagillin is interesting. Datta *et al*., (2004) revealed that fumagillin inhibited the turnover of eukaryotic initiation factor 2-associated glycoprotein p67 in murine C3C12 myoblasts. The biochemical implication of this interaction is that the stabilisation of p67 leads to reduced phosphorylation of eukaryotic elongation factor 2. To date there is no record of such a process in *G. mellonella* however given the conservation of cellular pathways between vertebrates and invertebrates such an occurrence may occur.

Haemocyanins, (arylphorin, hexamerin) function as oxygen transporters and amino acid storage molecules (Beintema *et al*., 1994). The decrease in the expression of two haemocyanin proteins following larval exposure to fumagillin may be a result of reduced oxygen transportation and alterations to physiological processes *in vivo.*

The reduction of ferritin expression in fumagillin treated larvae may contribute to the weakening of the immune response prior to a microbial insult. Ferritin plays a key role in iron homeostasis and in restricting the availability of iron to pathogenic micro-organisms (Haas *et al*., 2008; Schrettl *et al*., 2004). It is evident that the inability of

larvae to sequester iron may have a negative effect on larval fitness prior to pathogenic stimuli.

The activation of the phenoloxidase cascade plays a key role in protection against infection in invertebrates and is activated upon physical and microbial stress, (Soderhall and Cerenius, 1998; Bogus *et al*., 2007). Temperature mediated alterations to phenoloxidase activity have been previously discussed for other invertebrates, (Sung *et al*., 1998). Increasing the incubation temperature of *G. mellonella* resulted in a significant increase in phenoloxidase activity therefore establishing a non-infective method for inducing activity. Larvae injected with fumagillin demonstrated extremely low levels of activity. The finding that phenoloxidase activity may be reduced in insects following mycotoxin exposure has been previously demonstrated by Huxham *et al*., (1989), where exposure of *Periplaneta americana* and *Schistocerca gregaria* to destruxin produced by *Metarhizium anisopliae* caused a reduction in phenoloxidase activity. The result presented here potentially implies that fumagillin may mediate reductions in the activity of this enzyme thus leaving larvae incapable of effective clotting and melanin production against microbial stimuli. The identification of four larval haemolymph proteins with binding affinity for *A. fumigatus* conidia offers a further insight into the recognition of fungal pathogens by the larval immune system. Apolipophorin binding to LPS has been demonstrated by Ma *et al*., (2006) and reviewed in Schmidt *et al*., (2010) with the latter suggesting the formation of a "cage-like" structure resulting in the neutralisation of LPS. The finding that apolipophorin is capable of binding to conidia may highlight that a related mechanism may take place with the effect of sequestering conidia upstream of other humoral or cellular processes. The binding of this protein to *A. fumigatus* was not significantly affected by haemolymph exposed to fumagillin. The binding of prophenoloxidase subunit 2 and arylphorin is a novel development on the interaction of the insect humoral immune

response to *A. fumigatus*. Beresford *et al*., (1997) demonstrated that the N-terminus of gallysin-1 a potent elicitor of the antimicrobial response of *G. mellonella* haemolytically active material, (HAM) is identical to the first 20 amino acids of the arylphorin sequence. This work was preceded by Phipps *et al*., (1994), where it was demonstrated that gallysin-1 was capable of causing cell damage but not cell death but could elicit cytotoxicity in the presence of lysozyme. It may be possible that the binding of arylphorin to conidia may be linked to this process by functioning as an adaptor protein for other effector protein(s). The binding of a subunit of phenoloxidase may offer an additional insight to the already well characterised mechanism of phenoloxidase cascade activation. The activation of the phenoloxidase cascade has been shown to be dependent on the Ca^{2+} dependent serine protease cascade which results in the activation of a prophenoloxidase activating enzyme which catalyses the formation quinones. The subsequent phenoloxidase mediated oxidation of phenols to form cytotoxic quinones can be toxic to the host if not regulated and localised to the site of infection, (Ashida, 1990). Thus the association of prophenoloxidase with conidia may result in the localisation of this response to the pathogen of interest with beneficial effects to the host *in vivo.* Furthermore the fumagillin mediated inhibition of binding, (Figure 5.9) may offer support to the hypothesis that mycotoxins initiate toxic effects to host tissue by dysregulation of immunological cascades. The identification of apoLp III binding to *A. fumigatus* conidia is not surprising as Whitten *et al*., (2004) identified a role for this protein in the binding to β1,3-glucan thus positively affecting phagocytosis and eradication of conidia from the haemocoel. Therefore it is possible that apoLp III binding to *A. fumigatus* initiates a similar process. Exposure of haemolymph to fumagillin resulted in non-significant alterations in apolipophorin III binding to conidia. The reason for this occurrence is not clear however it does demonstrate that the identified haemolymph proteins bound to conidia display variable levels of pathogen

affinity in the presence of fumagillin therefore suggesting the observed inhibitions were protein specific.

A comparative analysis of protein binding from human serum yielded identification of eight proteins. The functions of these proteins vary from regulation of F-actin assembly, neutrophil recruitment, inflammation, phagocytosis, endotoxin neutralisation, pathogen recognition, digestion of neutrophil elastase and as biomarkers for the acute phase response to infection. Of the eight proteins identified only two demonstrated significantly altered binding when incubated in the presence of fumagillin, (Angiotensinogen and immunoglobulin kappa light chain). It is of particular interest to note that both immunoglobulins and angiotensin II (the digested product of angiotensinogen) have receptors on the surface of neutrophils, (Ito *et al*., 2001; Cutler *et al*., 1991; Gordon *et al*., 1986). It is therefore possible that a loss of protein binding may result in reduced opsonisation of target conidia. The finding that apoLp III and apoE binding to conidia was not significantly affected by fumagillin is of particular interest as it has been commented that both proteins share functional and structural homology, (Cole *et al* 1987; Whitten *et al*., 2004). The work presented here describes how both lipoproteins can bind to conidia and may play a role in the phagocyte mediated eradication of the pathogen from the host. Similarly, both proteins are not significantly affected by previous exposure to fumagillin, (Figures 5.10 and 5.15).

In conclusion this Chapter demonstrates that exposure of *G. mellonella* larvae to fumagillin results in the expression of proteins indicative of oxidative stress *in vivo* and alterations to proteins involved in normal larval physiology. Furthermore the temperature induced activation of the phenoloxidase pathway is significantly reduced by *in* vivo exposure to fumagillin thus limiting the ability of *G. mellonella* to form melanin. These findings are complemented by the identification of four larval haemolymph and eight human serum proteins which exhibit binding affinity to *A.*

fumigatus conidia. Some of these have not been previously implicated in the clearance of pathogenic fungi from host tissue. The inhibition of conidia binding by two haemolymph and serum proteins when previously exposed to fumagillin demonstrates that inhibition to binding is protein specific and may imply that restrictions to pathogen recognition may confer immunosuppressive effects. Additionally, two homologous lipoproteins from haemolymph and serum (ApoLp III and apoE) also exhibit conidial binding but are not affected by the presence of fumagillin *in vitro*.

Chapter 6

Exposure to sub-lethal concentrations of *Aspergillus fumigatus* conidia primes the *Galleria mellonella* larval immune response to subsequent fungal infection

6.0 Introduction.

Invertebrates, unlike vertebrates, rely solely on an effective innate immune system in response to infection. In recent years it has been demonstrated that not only is this response directed to the infecting micro-organism but it can also be influenced by pathogen subunit motifs (Mowlds *et al*., 2010). The increased density of circulating haemocytes, elevated synthesis of immunologically relevant proteins, activation of enzymatic cascades and altered expression of antimicrobial peptides all contribute to a diverse repertoire of responses which contribute to the localisation and eradication of pathogens within the host (Kavanagh and Reeves, 2004). However the interplay between cellular and humoral immune responses remains an area which needs to be comprehensively understood in order to utilise invertebrate models as an appropriate means of understanding the interaction of the innate immune response of *G. mellonella* larvae to opportunistic pathogens such as *A. fumigatus*.

Immunological priming of insects prior to a harmful inoculum of a pathogenic micro-organism has provided a useful understanding of the potential to manipulate innate defence mechanisms against pathogens (Mowlds and Kavanagh, 2008). In order to understand the alterations in viability it is therefore necessary to investigate if increasing the larval capacity to combat a microbial stress results in a cost to other larval fitness traits as has been demonstrated in other invertebrate species (Jacot *et al*., 2005).

6.1 Assessment of larval viability and haemocyte density resulting from non-lethal *A. fumigatus* priming.

In Chapter 3 the pathogenicity of *A. fumigatus* ATCC 26933 in larvae was determined. It was demonstrated that infection of larvae with 1 x 10^7 conidia and incubation at 30ºC resulted in larval death. However concentrations from 1 x 10^6 conidia larvae^{-1} and below did not result in larval death. In this section the effect of priming larvae with sub-lethal concentrations of conidia on larval viability prior to infection with a lethal inoculum of conidia was ascertained. The duration of priming with conidia was varied in order to determine if any protective effect was time dependent. In addition an analysis of alterations to haemocyte density was also performed.

6.1.1 Determination of alterations in larval viability after 4, 24 and 48 hours immune priming.

Larvae were injected with 1 x 10^4, 1 x 10^5 or 1 x 10^6 *A. fumigatus* conidia ATCC 26933 20 μl^{-1}, incubated at 30ºC and subsequently administered with a lethal concentration of conidia, (1 x 10^7), 4, 24, or 48 hours post immune priming. The viability of larvae was assessed at 24, 48, and 72 hours following the injection of the 1 x 10^7 inoculum (Figure 6.1). As a control larvae were injected with sterile PBS prior to infection with 1 x 10^7 conidia. It was observed that all larvae primed with *A. fumigatus* and PBS remained viable and showed no evidence of declining fitness therefore any loss of larval viability was due to the infection of larvae with 1 x 10^7 conidia.

Larvae primed with non-lethal concentrations of *A. fumigatus* 4 hours prior to receiving a 1 x 10^7 dose showed no loss in viability 24 hours post lethal infection at all pre-treatments. At 48 hours there were slight but not statistically significant variations in larval viability, ($p \geq 0.129$). Analysis of larvae 72 hours post lethal infection revealed
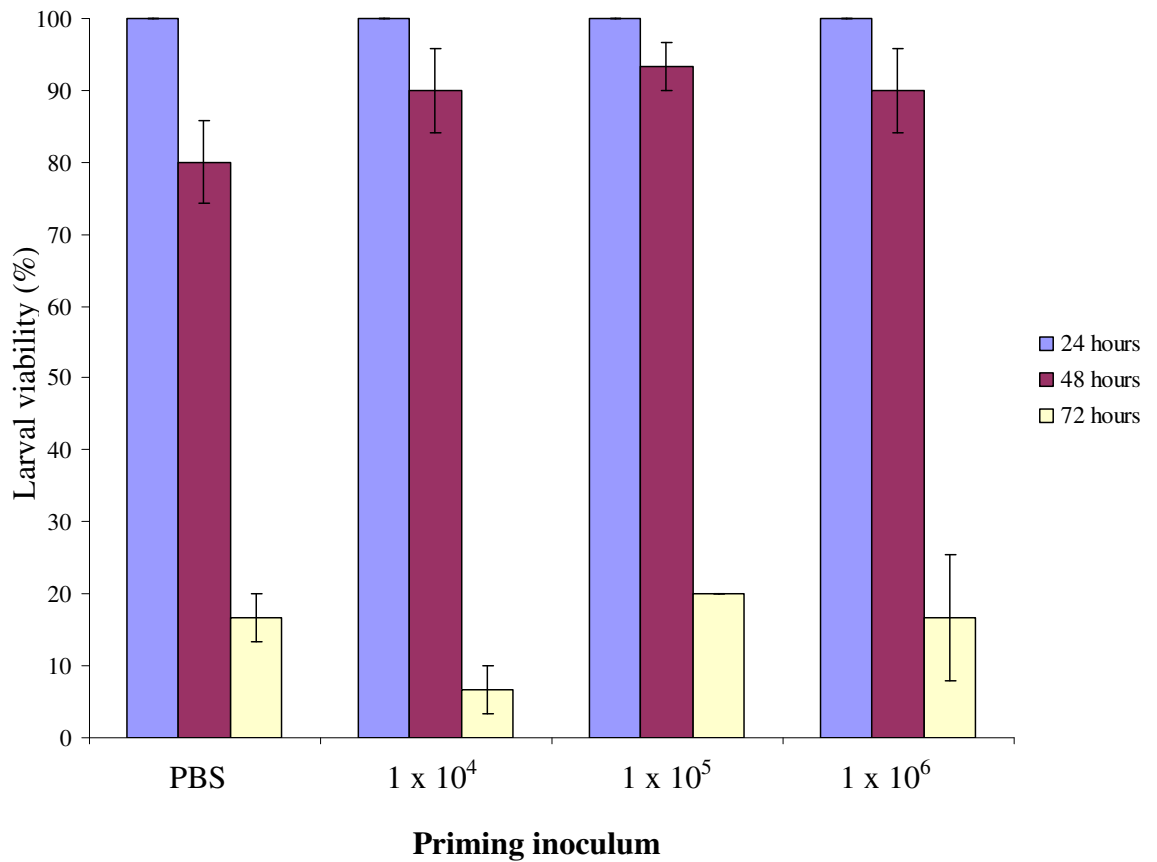
Figure 6.1 Determination of immuno-protection after 4 hours priming with *A. fumigatus* conidia.
Larvae were inoculated with PBS, 1 x 10^4, 1 x 10^5 or 1 x 10^6 *A. fumigatus* ATCC 26933 conidia. Following 4 hours incubation at 30ºC larvae were injected with a lethal (1 x 10^7) concentration of conidia and viability was recorded at 24, 48, and 72 hours.

No statistically significant change in viability was recorded.
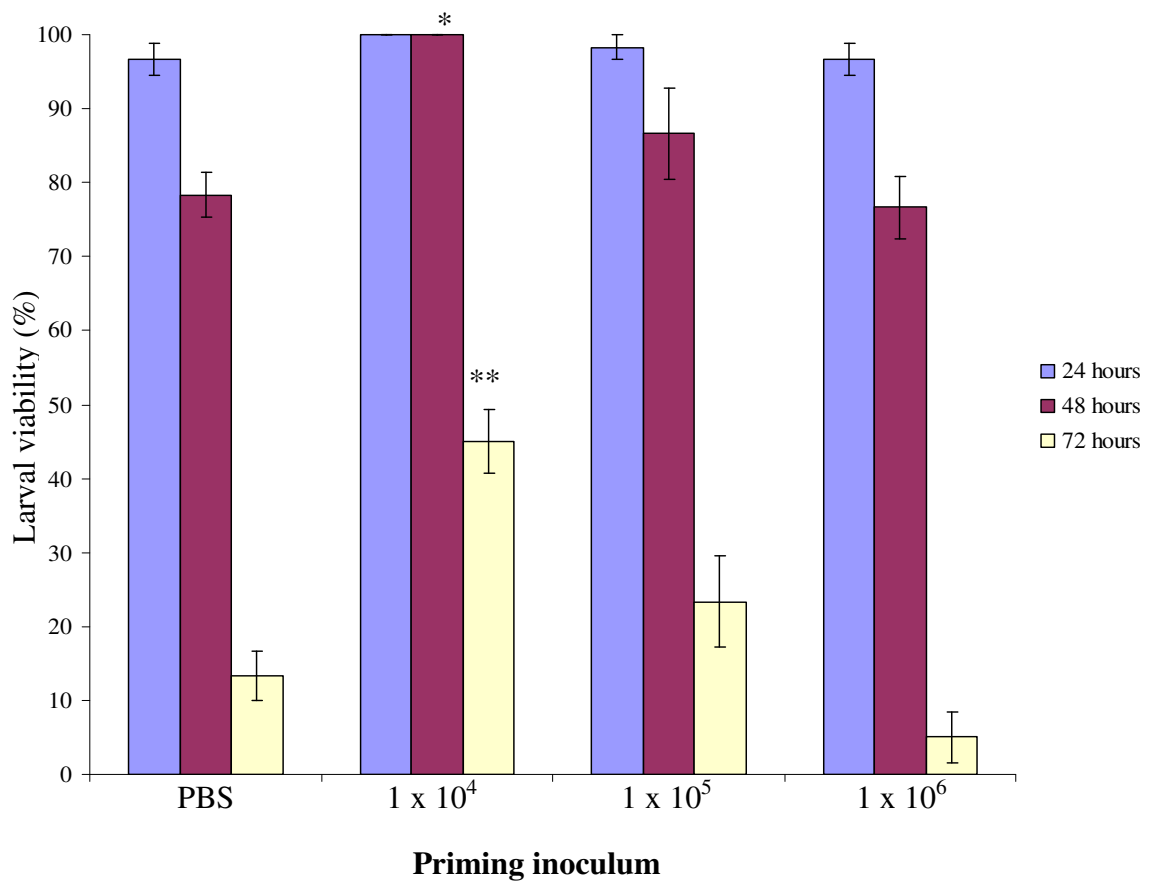
Figure 6.2 Determination of immuno-protection after 24 hours priming with *A. fumigatus* **conidia.**

Larvae were inoculated with PBS, 1×10^4, 1×10^5 or 1×10^6 *A. fumigatus* ATCC 26933 conidia. Following 24 hours incubation at 30ºC larvae were injected with a lethal (1×10^7) concentration of conidia and viability was recorded at 24, 48, and 72 hours.

$*p < 0.05$ relative to PBS treatment
$**p < 0.012$ relative to PBS treatment
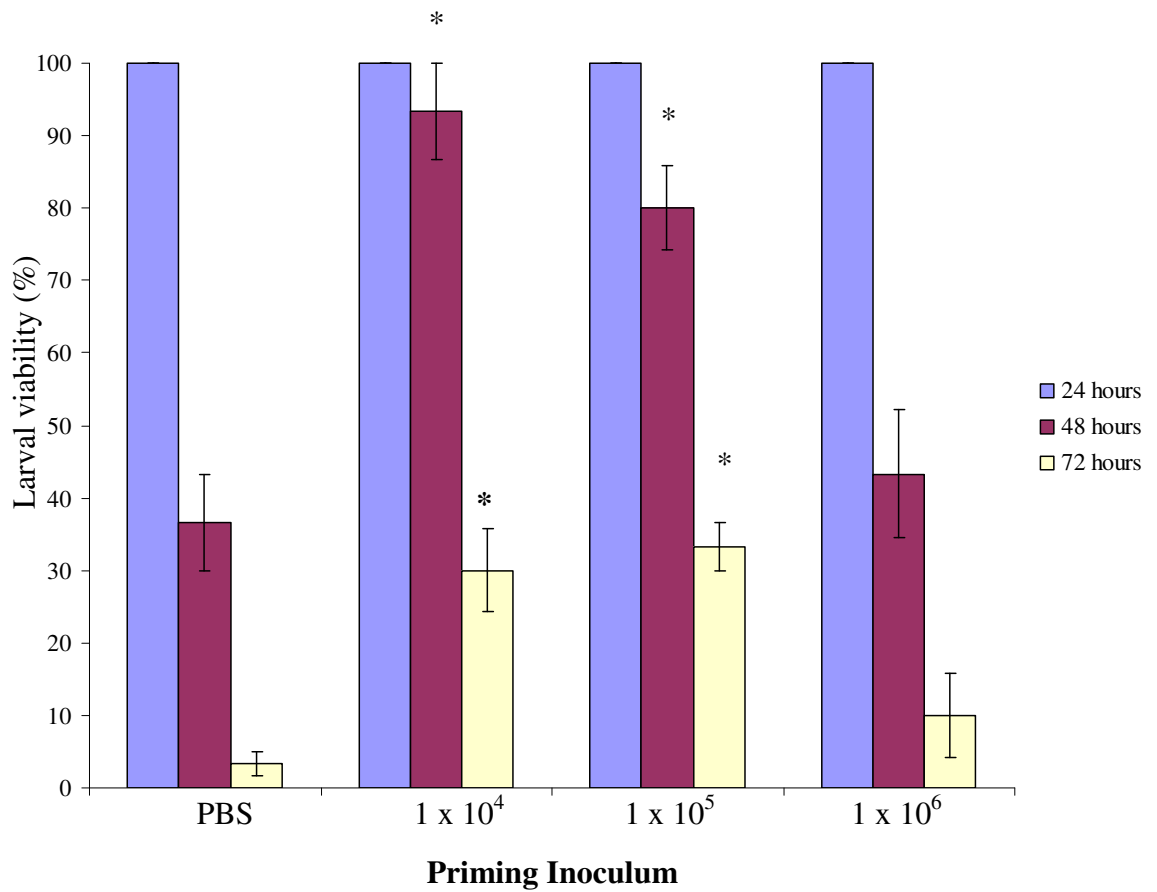
Figure 6.3 Determination of immuno-protection after 48 hours priming with *A. fumigatus* conidia.
Larvae were inoculated with PBS, 1 x 10^4, 1 x 10^5, and 1 x 10^6 *A. fumigatus* ATCC 26933 conidia. Following 48 hours incubation at 30ºC larvae were injected with a lethal (1 x 10^7) concentration of conidia and viability was recorded at 24, 48, and 72 hours.

* p ≤ 0.02 relative to PBS treatment

non-significant changes in viability, ($p \geq 0.129$) compared to control larvae. In addition the variations in larval viability between *A. fumigatus* priming treatments did not vary with all comparisons between treatments ($p \geq 0.228$).

Due to negligible effect of non-lethal doses of *A. fumigatus* over a period of 4 hours it was necessary to evaluate if extending the duration of incubation was necessary. Larvae were pre-treated as before but were incubated for 24 hours prior to injection with 1×10^7 *A. fumigatus* conidia (Figure 6.2)

Larval viability did not change 24 hours post-infection. At 48 hours there was a notable change in survival with larval viability maintained at 100% when larvae were primed with a 1×10^4 conidia pre-injection compared to a $21.7 \pm 3.1\%$ reduction in viability, ($p < 0.05$) where larvae were pre-treated with PBS. Larvae primed with 1×10^5 and 1×10^6 conidia decreased in viability by $13.3 \pm 6.1\%$, ($p = 0.230$), and $23.3 \pm 4.2\%$ respectively relative to the control treatment however this value was not considered significant.

Larvae inspected at 72 hours showed a marked alteration in viability depending on the pre-treatment. Larvae primed with PBS, 1×10^4, 1×10^5 and 1×10^6 conidia recorded viabilities of $13.3 \pm 3.3\%$, $45 \pm 4.3\%$, $23.3 \pm 6.1\%$, and $5 \pm 3.4\%$ respectively. Larvae pre-treated with 1×10^4 conidia showed a significant increase in viability compared to PBS, 1×10^5 and 1×10^6 inoculated larvae recording p - values of ≤ 0.012. However 1×10^5 and 1×10^6 injected larvae did not show a statistically significant change in viability compared to PBS injected larvae, ($p \leq 0.114$). Taken together the data presented here show that larvae primed with 1×10^4 conidia prior to inoculation with 1×10^7 conidia 24 hours later demonstrate a significant increase in survival at 48 hours which becomes even more pronounced at 72 hours.

As a result of evident immune priming event that takes place 24 hours post-infection with 1×10^4 conidia it became necessary to investigate if immunological

priming is maintained after 48 hours incubation (Figure 6.3). Larvae were inoculated with sub-lethal concentrations of conidia and incubated at 30ºC for 48 hours prior to administration with 1×10^7 conidia. Larval viability remained at 100% 24 hours post-lethal infection.

At the 48 hour timepoint larval viability was $36.6 \pm 6.6\%$, $93.3 \pm 6.6\%$, $80 \pm 5.7\%$, and $43.3 \pm 8.8\%$ for larvae injected with PBS, 1×10^4, 1×10^5 and 1×10^6 conidia respectively. Statistically, the 1×10^4 and 1×10^5 conidial treatments were the most significant compared to the PBS control, ($p = 0$ and 0.001 respectively), however larvae primed with 1×10^6 conidia did not display significant changes in survival, ($p = 0.598$). This marked decrease in larval survival was notable compared to the 1×10^4 and 1×10^5 treated larvae, ($p \leq 0.003$). Larval viability was recorded 72 hours post-infection as $6.7 \pm 3.3\%$, $30 \pm 5.7\%$, $33.3 \pm 3.3\%$, and $10 \pm 5.7\%$ where larvae were primed with PBS, 1×10^4, 1×10^5 and 1×10^6 conidia prior to the lethal inoculum. Statistical analysis of these data revealed $p \leq 0.02$ at the 1×10^4 and 1×10^5 conidia priming treatments relative to the PBS control. Larvae primed with 1×10^6 conidia did not show any significant change in viability relative to the PBS control ($p = 0.64$).

6.1.2 Effect of immune priming concentrations of *A. fumigatus* on haemocyte density.

G. mellonella larvae were injected with 1×10^4, 1×10^5 or 1×10^6 *A. fumigatus* conidia ATCC 26933 or were injected with sterile PBS as a control and incubated for 4, 24 or 48 hours at 30ºC (Figure 6.4). Larvae were bled and haemocyte density was recorded by light microscopy.

Analysis of haemocyte density after four hours demonstrated a decrease in cell number in *A. fumigatus* conidia injected larvae relative to the PBS control falling from $5.1 \pm 0.35 \times 10^3$ cells μl^{-1} to $3.7 \pm 0.75 \times 10^3$ cells μl^{-1}, $3.35 \pm 0.08 \times 10^3$ cells μl^{-1} and

4.6 ± 0.46 x 10^3 cells μl^{-1} in larvae injected with 1 x 10^4, 1 x 10^5 or 1 x 10^6 conidia, respectively. Significantly the reductions in haemocyte density recorded p – values ≤ 0.041 relative to PBS injected larvae indicating a possible weakening of the host cellular immune response in the initial phase of self–protection against the fungus. It was found that the difference in haemocyte density between 1 x 10^5 and 1 x 10^6 conidia injected larvae was also significant at p = 0.045. This was not found to be the case in comparisons between 1 x 10^4 and 1 x10^5 injected larvae or 1 x 10^4 and 1 x 10^6 injected larvae (p ≥ 0.05).

At 24 hours the changes in haemocyte densities were far more pronounced and showed a significant increase depending on the size of the initial fungal inoculum increasing from 3.94 ± 0.63 x 10^3 cells μl^{-1} in PBS injected larvae to 7.06 ± 1.27 x 10^3 cells μl^{-1}, 5.9 ± 0.55 x 10^3 cells μl^{-1} and 6.74 ± 0.74 x 10^3 cells μl^{-1} for 1 x 10^4, 1 x 10^5 and 1 x 10^6 conidia injected larvae, respectively, (p ≤ 0.028). Analysis of haemocyte densities in larvae inoculated with priming concentrations of conidia demonstrated that no statistical significance in comparisons made between priming treatments existed (p ≥ 0.05).

Larval haemocyte density was ascertained at 48 hours in order to understand if the impact of non-lethal fungal inoculation varied after 24 hours. Analysis of observed haemocyte densities revealed a decrease in the number of haemocytes to 3.62 ± 0.56 x 10^3 cells μl^{-1}, 3 ± 0.48 x 10^3 cells μl^{-1}, 4.92 ± 0.81 x 10^3 cells μl^{-1} and 3.92 ± 0.31 x 10^3 cells μl^{-1} for PBS, 1 x 10^4 , 1 x 10^5 and 1 x 10^6 primed larvae, respectively. Priming inoculations of conidia did not confer a statistically significant change in haemocyte density relative to the PBS control (p = 0.220). Larval haemocyte densities following injection with conidia did not vary significantly in comparisons made between infected treatments at this timepoint (p ≥ 0.063). Haemocyte densities decreased significantly in larvae that received 1 x 10^4 and 1 x 10^6 conidia from 24 to 48 hours (p = 0.036 and
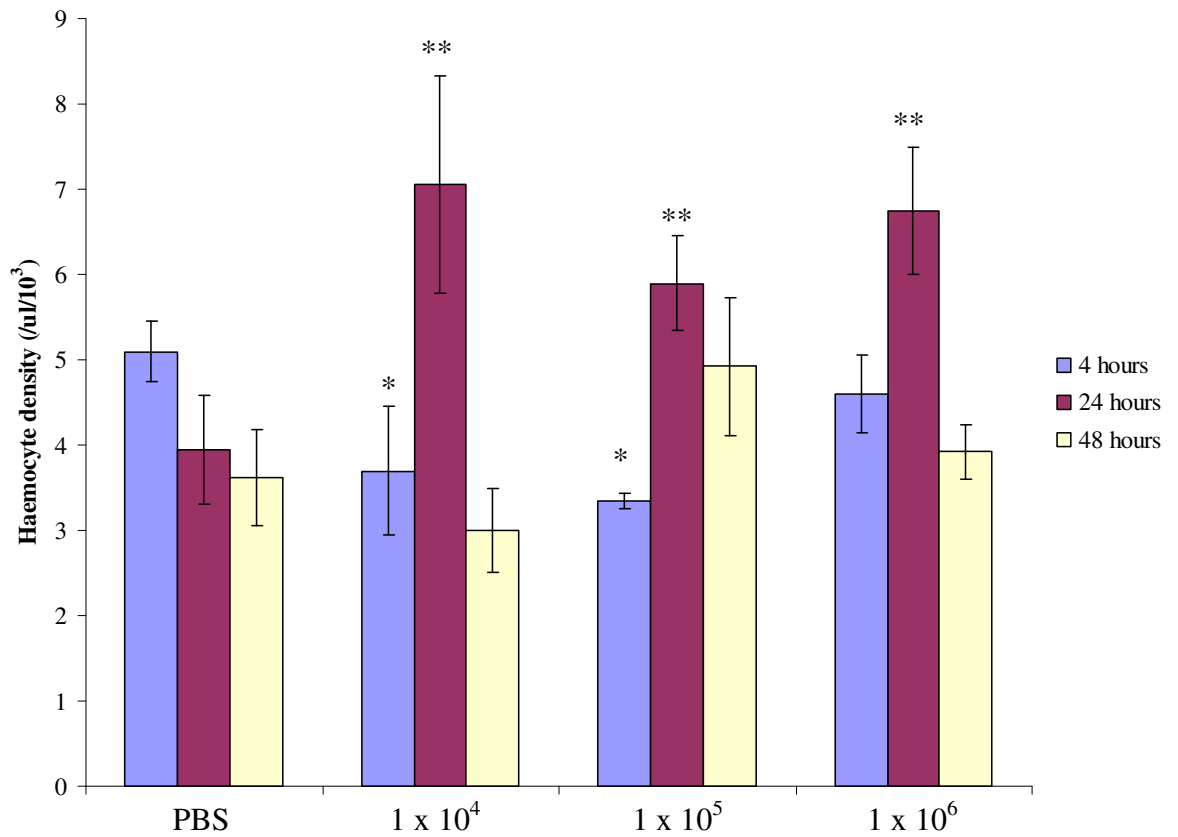
Figure 6.4 Haemocyte densities in response to *A. fumigatus*.
Larvae were injected with PBS or non-lethal concentrations of *A. fumigatus* conidia. Larvae were subsequently bled at 4, 24 or 48 hours and haemocyte densities were ascertained.

 * p $\leq$ 0.05 relative to PBS
** p $\leq$ 0.028 relative to PBS

0.028 respectively) however this reduction was not significant in larvae injected with 1 x 10^5 conidia (p = 0.445).

6.1.3 Summary.

Examination of larval viability data demonstrated significant changes in larvae in receipt of non-lethal concentrations of *A. fumigatus*. The protective effect does not appear to occur in the early phase of immune priming (after four hours). Priming appears to peak at 24 hours with significant increases in viability in 1 x 10^4 conidia primed larvae. Surprisingly this priming event does not occur at higher non-lethal concentrations. Immunological priming of larvae for 48 hours with 1 x 10^4 conidia induces a significant increase in larval viability when subsequently infected with 1 x 10^7 conidia. Larvae primed with 1 x 10^5 conidia for 48 hours demonstrated a statistically significant increase in viability at the 72 hour time-point. The priming responses appear to be dose-specific but do not increase correlating to the fungal burden to which the larvae are exposed. In addition the 48 hour priming data suggest that the maximum protective effect of inoculating larvae with sub-lethal doses of conidia may be temporary (approximately 24 hours) with the onset of a return to increased susceptibility to fungal infection. The population of circulating haemocytes in *G. mellonella* haemolymph shows significant variations depending on concentration of conidial pre-treatment and the duration of time that the larvae were incubated. It was found that inoculation with 1 x 10^4 *A. fumigatus* conidia and incubation for 24 hours appeared to leave larvae highly primed at a cellular level as evidenced by the presence of the greatest number of circulating haemocytes at this time-point.

6.2 Detection of prophenoloxidase and apolipophorin III binding analysis.

Prophenoloxidase (PPO) and apolipophorin III (apoLp III) have been previously identified as major components of the humoral immune response in *G. mellonella*, (Whitten *et al*., 2004; Park *et al*., 2005). Whitten *et al*., (2004) identified a role for apoLp III as a pathogen recognition receptor of β1,3-glucan, a key component of the fungal cell wall. The immunological relevance of PPO has been well characterised in invertebrates as an effector protein in the limitation of microbial spread and synthesis of melanin (Schmidt *et al*., 2010). In this study an analysis of apoLp III binding to conidia from haemolymph isolated from larvae primed with conidia was performed. In addition, following from the results of Chapter 4 where it was found that human myeloperoxidase antisera demonstrated immunoreactivity for larval PPO an immunoblot analysis of whole haemolymph was necessary. It was hoped that this might lead to a quantitative assessment of PPO in whole haemolymph thus indicating the potential melanisation in insect haemolymph.

6.2.1 Prophenoloxidase detection in larvae.

G. mellonella larvae were injected with 1 x 10^4, 1 x 10^5 or 1 x 10^6 *A. fumigatus* ATCC 26933 conidia and were incubated at 30ºC for 4, 24 or 48 hours. Cell-free haemolymph was extracted following centrifugation and solubilised in 5X solubilisation buffer prior to being subjected to 1–D SDS–PAGE and immunoblotting with human MPO antisera which was demonstrated in Chapter 4 to be cross-reactive with *G. mellonella* prophenoloxidase. Upon development of Western blots (Figure 6.5) band reactivity (approximately 79 kDa) was analysed by densitometry (Figure 6.6). The band of interest was excised from the corresponding SDS-PAGE gel and subjected to trypsin digestion prior to LC/MS analysis and was identified as *G. mellonella*

(A)

U.T   PBS 1×10^4 1×10^5 1×10^6

←79 kDa

(B)

U.T   PBS 1×10^4 1×10^5 1×10^6

←79 kDa

(C)

U.T   PBS 1×10^4 1×10^5 1×10^6

←79 kDa
←58 kDa
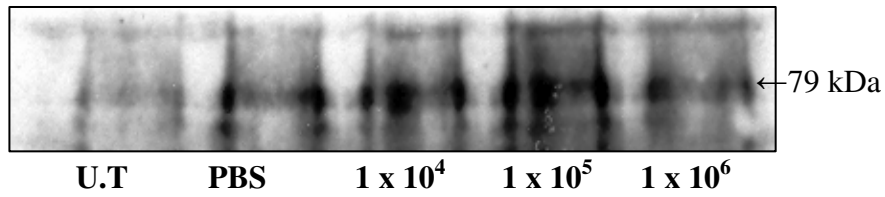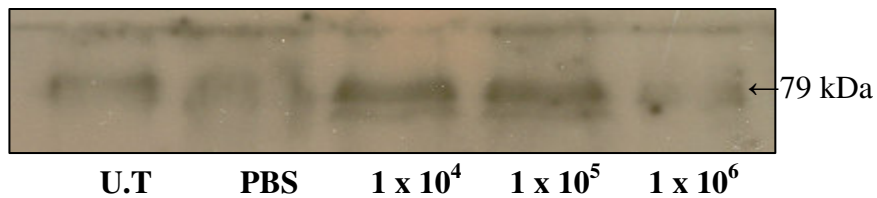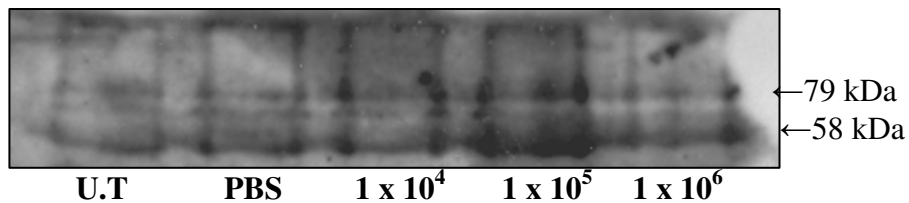
(D)

<u>**LC/MS analysis**</u>

Prophenoloxidase (*G. mellonella*)
gi:14517795
Mass: 79076
Score: 259
Matches: 4
Coverage: 6%

Figure 6.5 Immunoblot detection and LC/MS verification of haemolymph PPO.
Western Blot detection of PPO at 4, 24 and 48 hours following conidial infection (**A**, **B**, and **C**) and Mascot verification of band following tryptic digestion coupled to LC/MS analysis. Analysis of 4 and 24 hours show detection of PPO band at various levels at 4 and 24 hours. Analysis of 48 hours Western blots reveal loss of immunoreactivity at 79 kDa but detection of possible breakdown products at approx. 58 kDa.
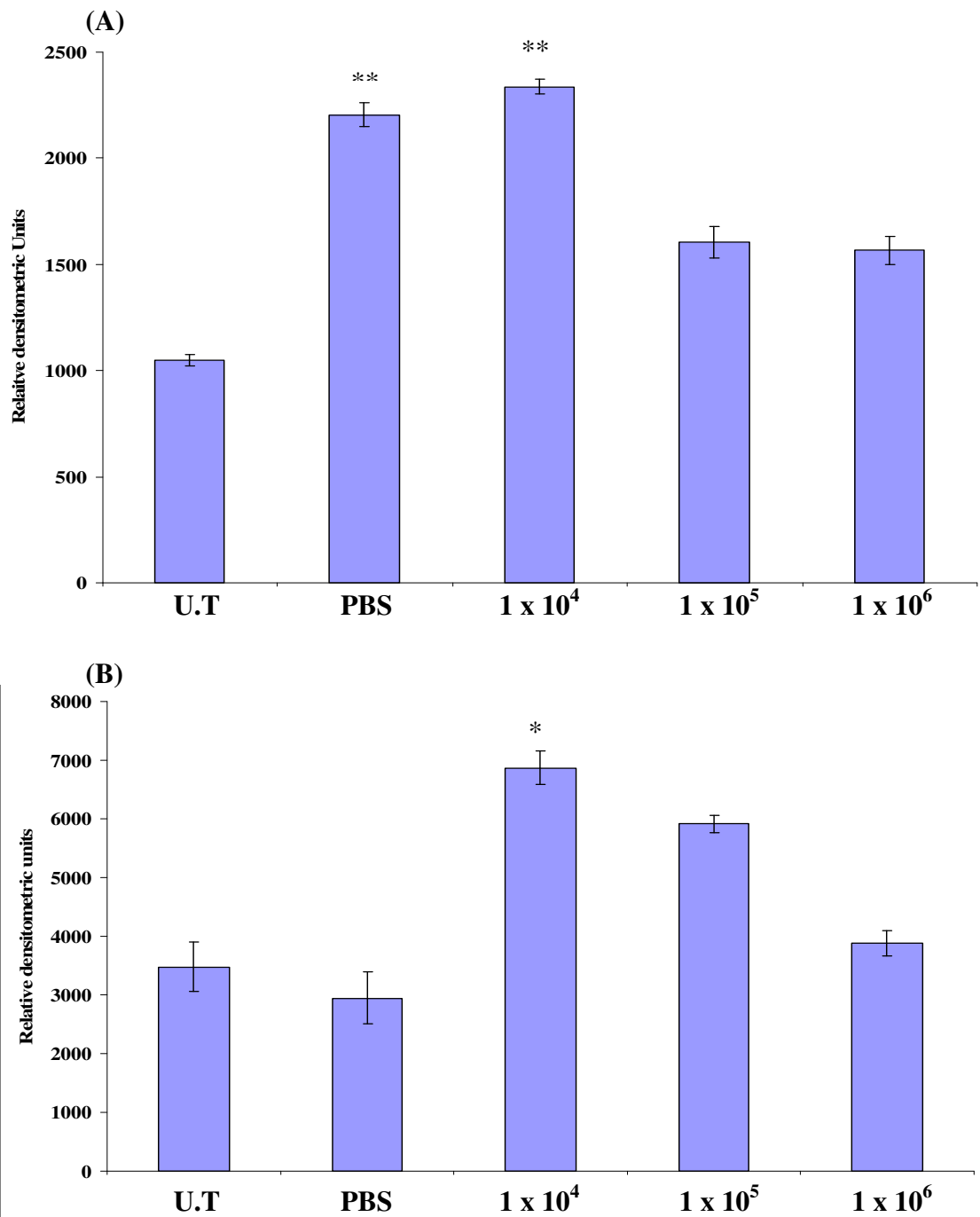
Figure 6.6 Densitometric analysis of PPO reactivity at 4 and 24 hours.
Anti-PPO (79 kDa immunoreactive zymogen) profiles at 4 hours **(A)** and 24 hours **(B)** from Figure 6.5 **(A)** and **(B)** were analysed by densitometry. Larvae primed with PBS and 1×10^4 conidia for 4 hours expressed a significant increase in humoral PPO ($p \leq 0.014$). Larvae incubated with 1×10^4 conidia for 24 hours demonstrated increased PPO reactivity ($p = 0.026$) and those incubated with 1×10^6 conidia over the same incubation conditions ($p = 0.014$).

$** \ p \leq 0.005$
$* \ p \leq 0.026$

prophenoloxidase; (gi: 14517795, Mass: 79,076 Da, Score: 259, Matches: 4, Coverage: 6%).

At 4 hours there is a significant increase in the presence of PPO relative to untreated larvae (Figure 6.6 **A**). Larvae injected with PBS showed a significant increase in the presence of PPO at this timepoint increasing 2.1 fold, (p = 0.005). Larvae injected with non-lethal inocula of *A. fumigatus* conidia showed an increased presence of PPO (p $\leq$ 0.014) with larvae injected with 1 x 10^4 conidia expressing the most significant increase (2.26 fold) relative to untreated larvae (p = 0.001). The observed PPO levels in larvae inoculated with 1 x 10^4 conidia after 4 hours at 30ºC relative to 1 x 10^5 and 1 x 10^6 inoculations is notable as there is a 1.45 and 1.49 fold increase, respectively (p $\leq$ 0.004). In addition larvae injected with PBS demonstrated significantly more PPO reactivity than larvae inoculated with 1 x 10^5 (p = 0.021) and 1 x 10^6 (p = 0.016) conidia. The data suggest that in the early phase of immune priming *G. mellonella* larvae synthesise or degranulate significantly more PPO in response to a pathogen or injury with a sterile stimulus such as PBS. Importantly at this time-point this arm of the innate immune system does not appear to be directed towards a specific dose of pathogen however the reduction in PPO levels in 1 x 10^5 and 1 x 10^6 conidia injected larvae would suggest a degradation of the native enzyme as part a function of the phenoloxidase (PO) pathway.

At 24 hours (Figure 6.6 **B**) the relative levels of PPO show a notable variation to that observed at 4 hours. There is a noteworthy decrease in the reactivity of the PPO band in PBS injected larvae (p = 0.027) falling to 84.83% of that observed in untreated larvae. Surprisingly, only larvae injected with 1 x 10^4 conidia show a statistically significant increase in PPO reactivity increasing 1.97 fold relative to the control (p = 0.026). Larvae injected with 1 x 10^5 and 1 x 10^6 conidia present 1.7 (p = 0.071) and 1.1 fold (p = 0.290) increases respectively relative to the control. In comparing the relative

presence of PPO reactivity of 1×10^4 conidia injected larvae to those administered with 1×10^5 conidia there is a 13.8% reduction which is not regarded as significant (p = 0.090) however larvae which received the 1×10^6 inoculum demonstrated a 43% reduction relative to larvae injected with 1×10^4 conidia (p = 0.014). Increases in PPO reactivity appear to correlate with immune sensing of conidia however this does not increase in a dose specific manner and seems to occur inversely to the level of infection.

Prophenoloxidase levels in larvae which had been incubated for 48 hours (Figure 6.5 **C**) demonstrate an almost complete loss of band reactivity at approximately 79 kDa. As a result it was not possible to perform comparative densitometric analysis of this band. There was a low detection of a 56-58 kDa band in untreated larvae. This was accompanied by the detection of lower molecular weight bands which were particularly prevalent in larvae that received the 1×10^5 conidial inoculum; however its presence appears greatly reduced in all other treatments.

6.2.2 Apolipophorin III binding to conidia following 24 hours conidial priming.

Larvae were injected with PBS, 1×10^4, 1×10^5 or 1×10^6 conidia or remained untreated and were incubated at 30ºC for 24 hours. Larvae were bled and cell free haemolymph was extracted. The binding assay was performed as per Section 2.20.3. A 10 µl aliquot of this extract was removed and subjected to 1-D SDS-PAGE and Colloidal Coomassie staining (Figure 6.7 **A**). Analysis of resulting gels showed variations in the level of protein which was solubilised from the conidial surface, (particularly at 20 kDa). The 20 kDa band was excised and subjected to tryptic digestion prior to LC/MS analysis as outlined in Section 2.13.3. The resulting analysis (Figure 6.7 **C**) confirmed the band identity of apoLp III (Mass: 20.499 kDa, Score: 704, Sequences matched: 15, Coverage 71%). Densitometric analysis of the identified band (Figure 6.7 **B**) revealed haemolymph of larvae in receipt of *A. fumigatus* inoculations displayed a

A

U.T   PBS 1×10^4 1×10^5 1×10^6

LC/MS analysis
Apolipophorin III
gi: 5915688
Mass: 20.499 kDa
Score: 704
Matches: 15
Coverage: 71%

B

C
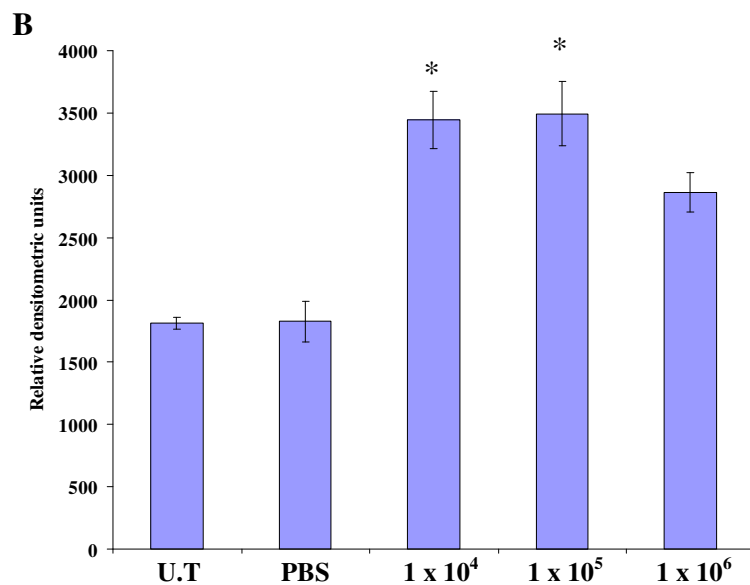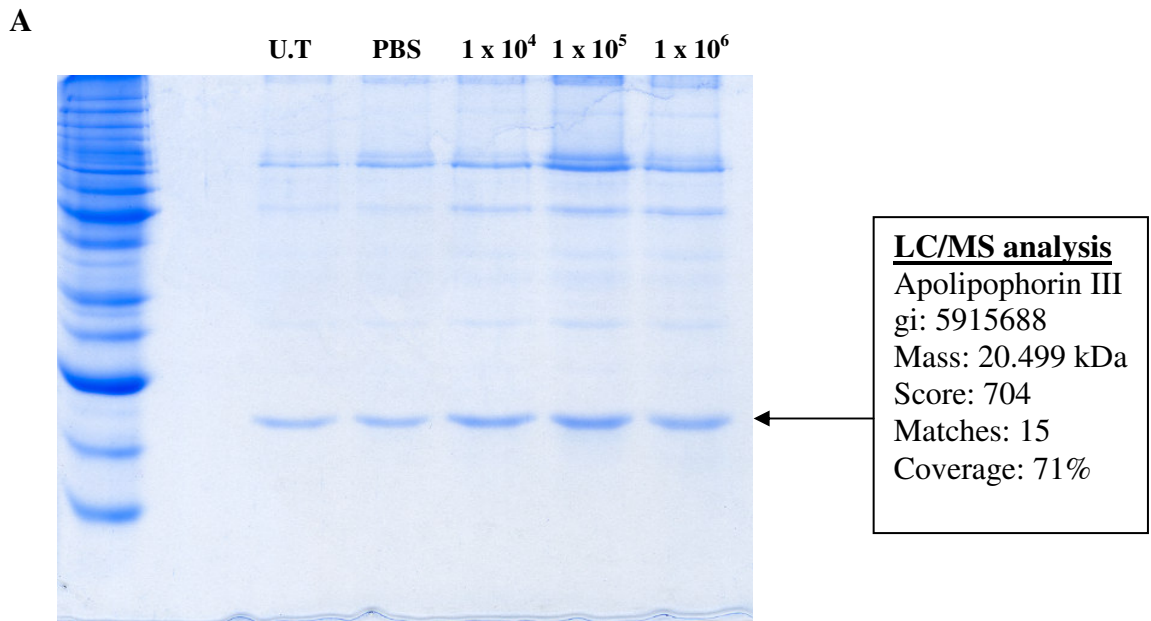
```
  1 MAAKYVFVVA ACSALAQAGI VRRDASTPLQ DLEKHAAEFQ KTFSEQLNAF
 51 TNSKDTKEFN TALKEGSDSV LQQLNALASS LQKALNDANG KAKEALEQTR
101 TNLERTAEEL RRAHPDVERQ AGALRDRLQT AVQATVQETQ KLAKTVGANL
151 EETNKKLAPQ IKSAYDDFVK QAQEVQKKLH EAASKQ
```

Figure 6.7 Haemolymph protein binding profile from larvae primed with *A. fumigatus* conidia.
SDS – PAGE and LC/MS analysis (A), densitometric analysis (B) of apoLp III binding to conidia after 24 hours immune priming in larvae. Amino acid sequence coverage (highlighted) obtained from LC/MS data confirming identity of apoLp III (C).

* $p = 0.048$ relative to untreated larvae

higher binding activity towards conidia than untreated or PBS injected larvae, (p ≤ 0.028 and 0.048 respectively). Unlike analysis of the presence of PPO there was no significant change in apoLp III in comparisons between untreated and PBS injected larvae after 24 hours thus clarifying the observed changes were as a result of the initial microbial challenge and not as a result of physical insult. Larvae infected with 1 x 10^4, 1 x 10^5 and 1 x 10^6 conidia demonstrated 1.89, 1.92 and 1.57 fold increases in the level of apoLp III binding respectively compared to untreated larvae however only larvae injected with 1 x 10^4 and 1 x 10^5 conidia demonstrated a statistically significant increase in apoLp III (p ≤ 0.048).

6.2.3 Summary.

Larvae injected with conidia demonstrated increased levels of PPO in cell free haemolymph 4 and 24 hours post-immune priming with 1 x 10^4 conidia. The detection of the 79 kDa PPO native zymogen appeared to be lost 48 hours post infection with the appearance of lower molecular weight reactivity. A conidial binding assay using haemolymph from larvae primed for 24 hours with 1 x 10^4 and 1 x 10^5 conidia demonstrated higher apoLp III binding to the conidia than other treatments.

6.3 Proteomic analysis of *G. mellonella* cell free haemolymph following larval priming with *A. fumigatus* conidia.

Cytrnska *et al*., (2007) and Mak *et al*., (2010) demonstrated the role of a diverse repertoire of low molecular weight proteins and peptides in response to fungal infection by utilising RP-HPLC and SDS–PAGE isolation and mass spectroscopy analysis to aid identification of factors demonstrating differential expression upon fungal or bacterial challenge. In this section it was sought to establish which peptides/low molecular

weight proteins demonstrated differential expression following sub-lethal infection with *A. fumigatus* conidia.

In addition to this work a 2-D SDS-PAGE coupled to LC/MS approach was employed to identify proteins with altered expression following immune priming with *A. fumigatus*.

6.3.1 RP-HPLC and SDS-PAGE of *G. mellonella* peptide extracts.

Untreated larvae (10) were bled and haemolymph peptides were extracted as per Section 2.10. Extracted peptides were analysed at 220 nm, 254 nm and 280 nm, for the detection of peptide bonds, disulphide bonding, or aromatic groups, (Figure 6.8 A). Overlaying the peaks demonstrated conservation of detection at certain retention times (e.g 11.388 mins) indicating the presence of all three components however other peaks (e.g 18.216 mins) indicated the absence of usually detectable residues such as tryptophan, or the absence of disulphide bonding adding to the complexity of the peptide/protein profile attained. It was clear from the analysis that at least 4 distinct peptide peaks are present and these were isolated by fraction collection and digested with trypsin for identification.

The lyophilised peptide extract was subjected to 1–D SDS–PAGE under denaturing conditions on a 12.5 or 15% (w/v) acrylamide gel (Figure 6.8 B). The purpose of this step was to clarify the absence of higher molecular weigh proteins (> 30 kDa). SDS-PAGE analysis demonstrated the loss of protein content above 30 kDa. The quality of band resolution increases with a higher percentage of acrylamide. It was subsequently decided to subject all peptide extractions to 15 % (w/v) acrylamide 1–D SDS–PAGE to aid improved resolution.

Figure 6.8 (A) Multiple wavelength analysis of peptide RP-HPLC profiles.
RP- HPLC haemolymph peptide analysis from untreated larvae was performed initially using multiple wavelength DAD at 220nm **(A)** 254nm **(B)** and 280nm **(C)**. Inspection of peptide bonds **(A)** indicated some peptides lacked disulphide bonding **(B)** and the presence of aromatic groups **(C)** thus indicating differential properties of peptides present in larval haemolymph.

12.5% Acrylamide   **15% Acrylamide**

← 30 kDa →
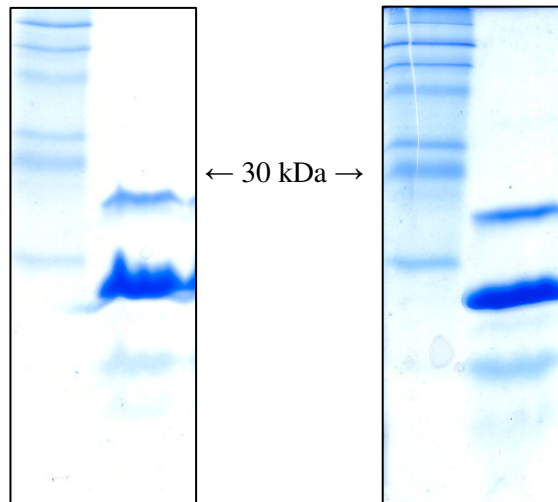
Figure 6.8 (B). Visualisation of peptide extraction by SDS-PAGE.
Lyophilised peptide extracts were subjected to 1–D SDS–PAGE under increasing (w/v) concentrations of acrylamide. The purpose of this step was to confirm the absence of high molecular weight proteins and peptides and assess the purity of the sample. At 12.5 % acrylamide the absence of protein above 30 kDa is verified however it was found that improved visualisation was possible at higher concentrations of acrylamide.

6.3.2 RP–HPLC fractionation and LC/MS analysis of haemolymph peptides.

Peptides were extracted from larvae (Section 2.10) and a 50 µl sample was loaded on an Agilent C-18 column and fractionated based on peak retention time over an Acetonitrile gradient (Figure 6.9 and Table 6.1). Following lyophilisation and tryptic digestion all samples were analysed by LC/MS. As the first 5 minutes of the run uses an isocratic flow-through, all meaningful analysis takes place from the commencement of gradient elution. Major peaks at 11.089, 12.597, and 17.989 minutes and minor peaks at 13.736 and 16.859 minutes were identified. These are presented in Figure 6.10 and Table 6.2.

Peak number one was identified as *G. mellonella* larval haemolymph protein. UNIPROT and BLAST analysis of the peptide sequence revealed larval haemolymph protein (lhp-1) to be a member of the invertebrate haemocyanin family which contains other methionine rich proteins with an affinity for copper binding. Analysis of this protein reveals a di-copper binding centre which is unlike most other insect haemocyanins which do not possess such a molecule (Jiravanichpaisal *et al*., 2006). Analysis of this family of proteins demonstrate that haemocyanins function as oxygen transporters in a manner analogous to haemoglobin, (Burmester, 2001) however unlike haemoglobin they are not associated with a particular type of cell but function unbound to transport oxygen around the haemocoel.

Peak number 2 was identified as anionic antimicrobial peptide 2, a 60 amino acid antimicrobial peptide which is secreted in response to microbial infection (Cytrynska *et al*., 2006). Recent work has demonstrated this peptide to be highly abundant in haemolymph however little change in abundance was recorded following infection with *C. albicans* or *Fusarium oxysporum,* (Mak *et al*., 2010). Cytrnska *et al*., (2007) noted that anionic antimicrobial peptide 2 appears lack to antifungal activity
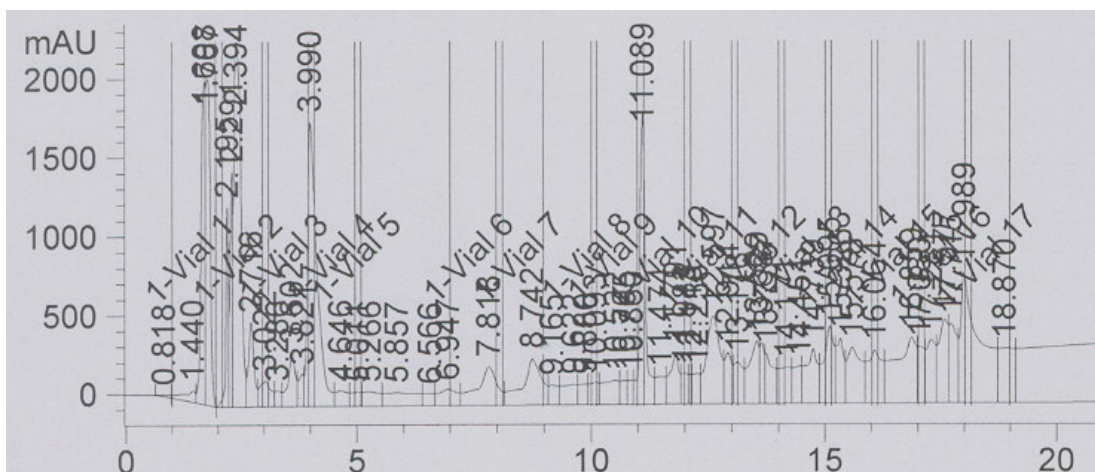
Figure 6.9 Peak fractionation by RP-HPLC.
Peptides from untreated larvae were extracted and individual peaks were isolated and collected using RP-HPLC fractionation.

```
Fraction collection using a timetable
==================================================================
Frac   Well   Location    Volume     BeginTime   EndTime     Reason
 #      #                  [µl]       [min]       [min]
-----|-----|----------|----------|----------|----------|-------
  1     1    1-Vial 1     967.92      0.9998      1.9528   DAD1
  2     1    1-Vial 2     889.58      2.0848      2.9594   DAD1
  3     1    1-Vial 3     884.17      3.0905      3.9596   DAD1
  4     1    1-Vial 4     886.04      4.0886      4.9596   DAD1
  5     1    1-Vial 5       0.83      5.0905      5.0905   DAD1
  6     1    1-Vial 6     994.58      6.9998      7.9794   DAD1
  7     1    1-Vial 7     888.12      8.1119      8.9850   DAD1
  8     1    1-Vial 8       1.46      9.9998      9.9998   DAD1
  9     1    1-Vial 9     874.38     10.1269     10.9863   DAD1
 10     1    1-Vial 10    877.92     11.1298     11.9927   DAD1
 11     1    1-Vial 11    882.92     12.1315     12.9994   DAD1
 12     1    1-Vial 12    893.54     13.1275     14.0061   DAD1
 13     1    1-Vial 13    887.92     14.1334     15.0063   DAD1
 14     1    1-Vial 14    887.29     15.1340     16.0063   DAD1
 15     1    1-Vial 15    892.71     16.1350     17.0128   DAD1
 16     1    1-Vial 16    889.17     17.1452     18.0194   DAD1
 17     1    1-Vial 17    847.50     18.1525     18.9850   DAD1
```

Table 6.1 List collections from RP-HPLC analysis of *G. mellonella* peptides following faction collection.
Fractions of interest from RP-HPLC chromatogram analysis (Figure 6.9) were collected in glass vials using a fraction collector. Following lyophilisation these fractions were subjected to tryptic digestion and LC/MS analysis.
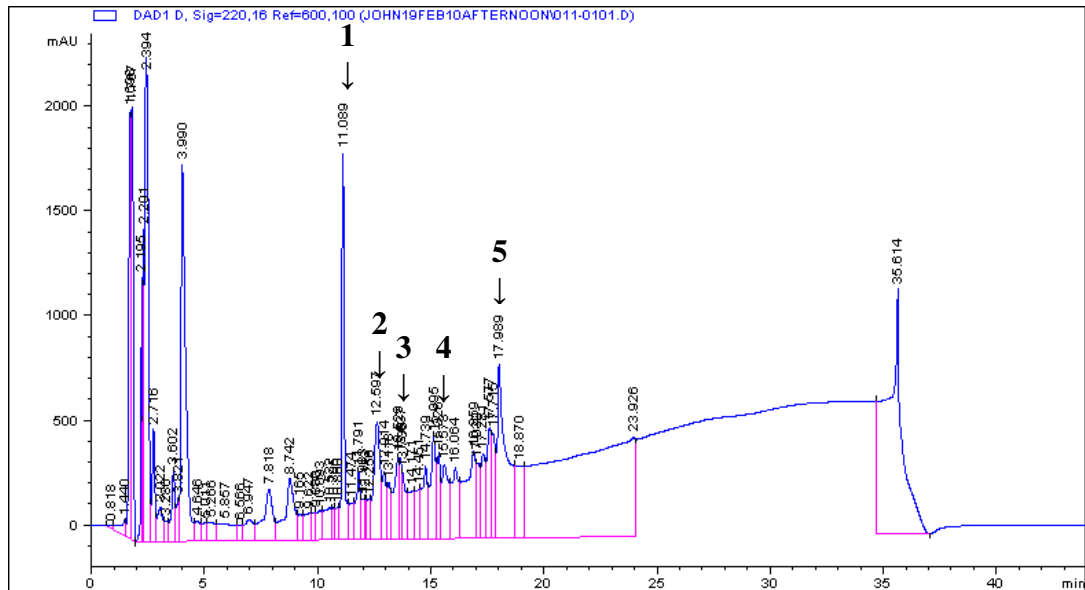
Figure 6.10 Labelling of identified peaks from RP-HPLC.
The C-18 HPLC column was loaded with 50 µl of sample and fractionated by peak.
Peaks which were subsequently identified by LC/MS analysis (Table 6.2) are
highlighted by numbered arrows (↓).

Peak Number	Identified	gi	M.W (Da)	Mascot Score	Peptides I.D	Coverage (%)
1	Larval haemolymph protein	110812735	13783	154	3	31
2	Anionic antimicrobial peptide 2	156630460	6975	265	4	78
3	Apolipophorin III	5915688	20499	286	6	26
4	Apolipophorin III	5915688	20499	397	9	45
5	Apolipophorin III	5915688	2499	479	16	48

Table 6.2 Identification of fractionated peaks of interest by LC/MS analysis.
Peaks highlighted in Figure 6.10 as being identified by LC/MS are presented according
to a corresponding number. Peaks 1 and 2 were identified as lhp-1 and anionic
antimicrobial peptide 2, respectively. It was found that peaks from 13.2 – 18 minutes
identified as apoLp III however a significant apoLp III peak occurred at approx 17.989
minutes which was present in all samples subjected to HPLC analysis.

except against *P. pastoris* and *P. stipidis*, and appeared to be induced by Gram-positive bacterial infection.

Peaks three, four and five were all identified as apoLp III. Peak 5 (approx 18 minutes) was identified as the main apoLp III peak present in the sample. The abundance of apoLp III is of interest as it plays a key role in mediating immune responses in *G. mellonella* where it has been shown to act as a pattern recognition receptor as a support to the cellular immune response as well as an activator of the phenoloxidase cascade *in vitro*, (Whitten *et al*., 2004, Park *et al*., 2005). The work of Mak *et al*., (2010) demonstrated a small increase in the abundance of an apoLp III derived peptide in response to fungal infection with *C.albicans* or *F. oxysporum.*

6.3.3 Removal of lipids by n-hexane.

Cytrynska *et al*., (2006) performed lipid removal of initial larval haemolymph extracts prior to RP-HPLC analysis. Given the lipid rich nature of larval haemolymph it was necessary to investigate the effect of n-hexane removal on the extraction procedure and furthermore to understand if such removal could potentially remove peaks of interest from chromatogram analysis. Analysis of RP-HPLC profiles showed that the 3 major peaks remain present after lipid removal however the size of these peaks changed (Figure 6.11 **A**). Larval haemolymph protein, anionic antimicrobial peptide 2 and apolipophorin III peak area values were reduced 2.1, 2.12, and 1.47 fold respectively. This raised the question of the requirement for lipid removal and more importantly the risk of error in preparing the samples. For subsequent analysis the extraction procedure was performed without lipid removal (as in Figure 6.11 **B**) to give a more precise reflection of the relative abundance of these peptides in response to larval infection.

(A)

(B)
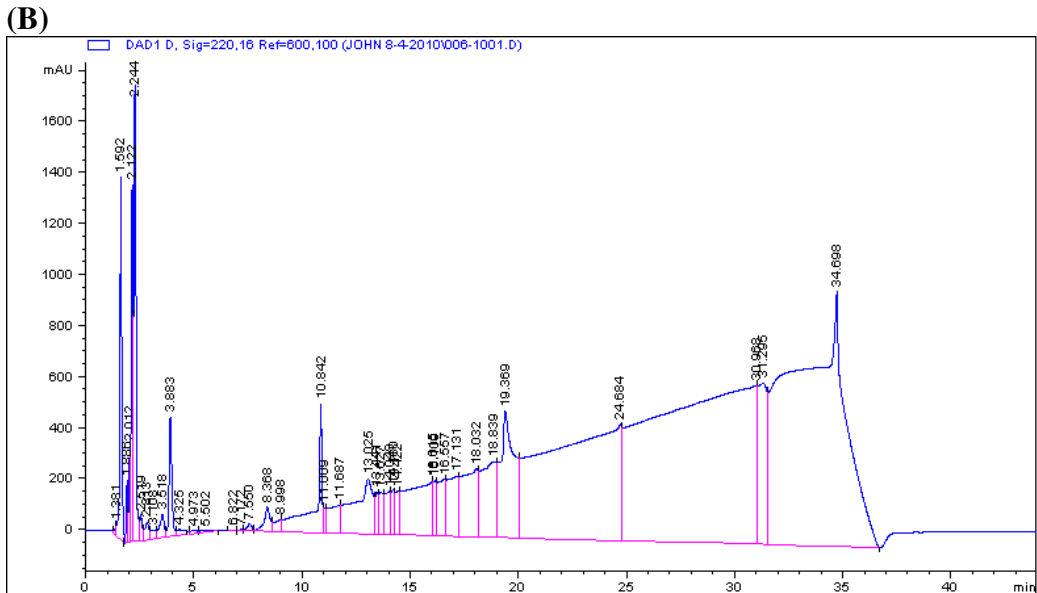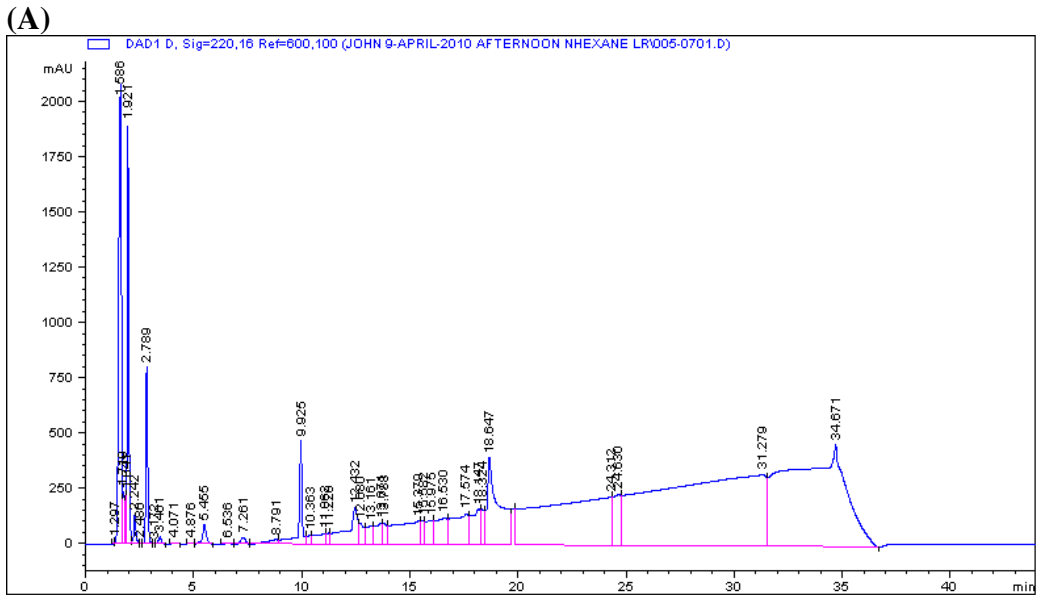
Figure 6.11 Changes to HPLC profiling following lipid removal.
Larval haemolymph peptide extracts from untreated larvae were lipid removed using n-hexane and ethyl acetate **(A)**. Chromatogram **(B)** shows the same extraction method with the exception of lipid removal demonstrating few notable differences in HPLC profiling.

(A)

(B)

(C)

(D)
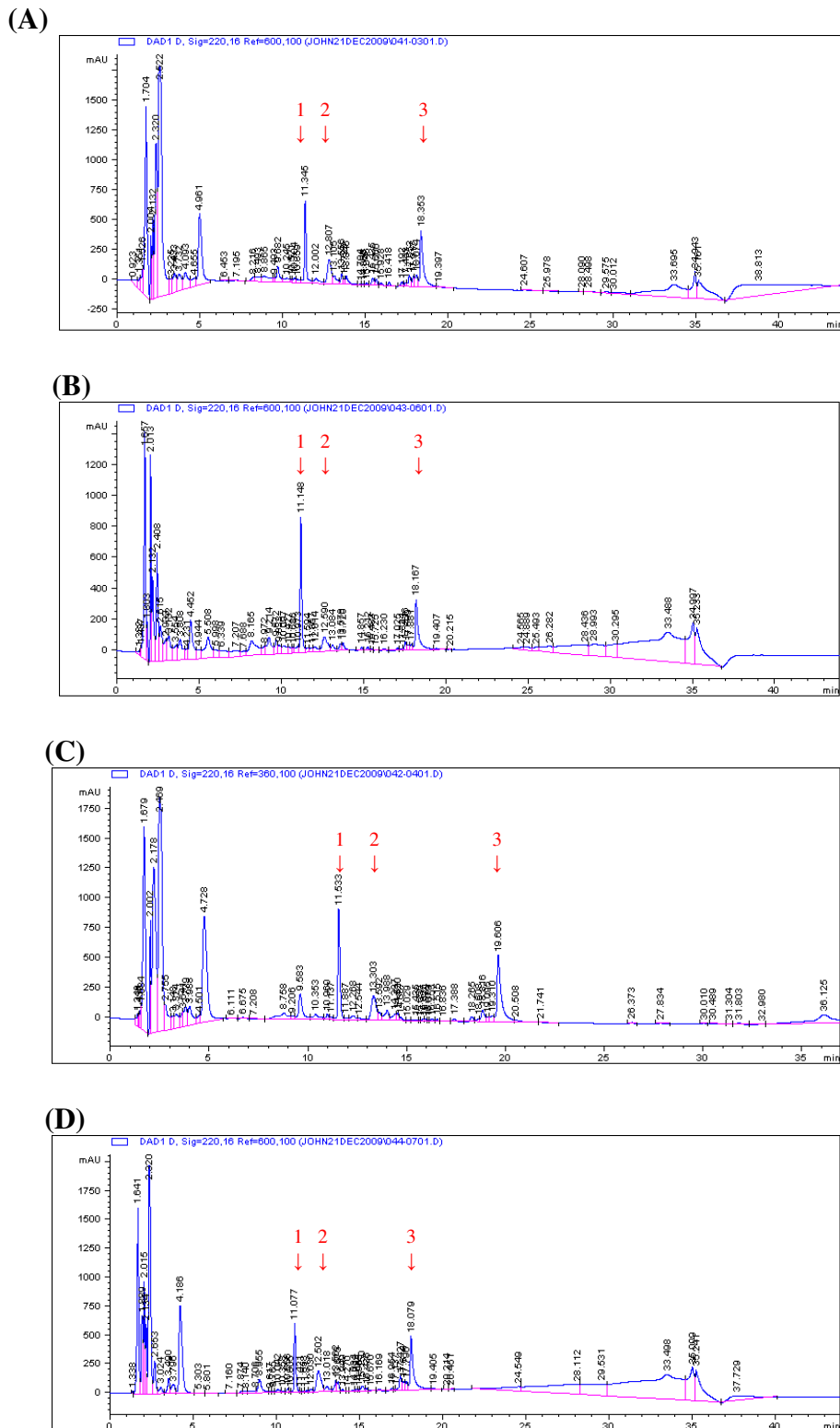
Figure 6.12 HPLC profiles of untreated larvae and larvae inoculated with 1 x 10^7 conidia at 4 and 24 hours.

RP-HPLC larval peptide analysis of larval peptides demonstrate changes to peptide profiles with *A. fumigatus* infection, [U.T 4 hrs **(A)** 1 x 10^7 4 hrs **(B)**, U.T 24 hrs **(C)** and 1 x 10^7 24 hours **(D)**]. Peak identification was; **1**. Lhp-1, **2**. Anionic antimicrobial peptide 2, **3**. ApoLp III. This result verified that intensity of the previously identified peaks of interest changed following infection with *A. fumigatus*.

6.3.4 Changes in peptide peak area as a result of lethal fungal inocula.

Larvae were injected with 1×10^7 *A. fumigatus* ATCC 26933 conidia or with PBS, incubated for 4 or 24 hours at 30ºC and peptides were extracted for HPLC analysis as described (Section 2.10.2). Analysis of peaks of interest showed that at 4 hours larval haemolymph protein, anionic antimicrobial peptide 2, and apoLp III showed a 1.3, 1.01, and 1.29 fold increases in peak area in larvae infected with 1×10^7 conidia respectively (Figure 6.12). Such changes are minor but may point to an increase in initial proteomic responses following infection. In contrast the HPLC profiles obtained at 24 hours demonstrated larval haemolymph protein expression reduced 1.5 fold, anionic antimicrobial peptide 2 concentration did not change with apoLp III peak area increased 1.5 fold. Such an alteration points to a possible variation of the immune response following fungal challenge and supports the initial finding that larval immune responses are most evident 24 hours post-infection. As a result subsequent proteomic analysis of priming concentrations of *A. fumigatus* conidia were focussed at 24 hours post-infection.

6.3.5 Non-lethal infection with *A. fumigatus* results in increased presence of peptides and proteins of interest at 24 hours.

Larvae were injected with PBS or 1×10^4, 1×10^5 or 1×10^6 *A. fumigatus* conidia incubated at 30ºC for 24 hours and peptides were extracted and subjected to RP-HPLC and analysed at 220 nm for the relative abundance of proteins and peptides identified previously (Figure 6.13 and Table 6.3). Significantly, peak number 1 identified as larval haemolymph protein demonstrated a 2 and 2.1 fold increase ($p \leq 0.01$) in abundance following priming with *A. fumigatus* ATCC 26933. Larvae injected with 1×10^6 conidia showed a decrease in this peak area relative to other priming doses. Considering the significant increases in viability conferred by 1×10^4 and 1×10^5 conidial inoculations
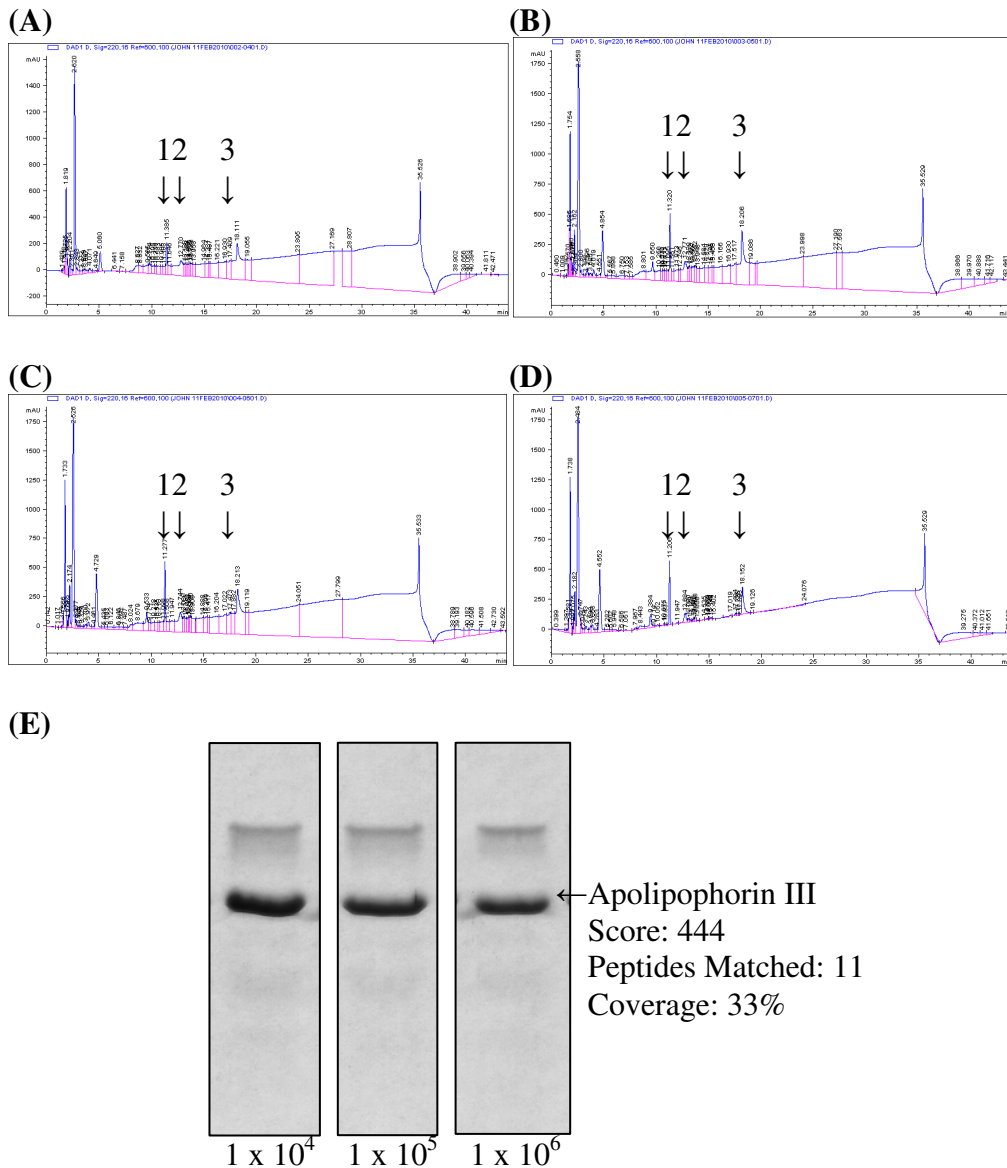
Figure 6.13 Changes to peptide peak area in response to priming doses of conidia and evidence of sample integrity.

HPLC profiles of haemolymph peptide analysis from larvae injected with PBS (**A**), 1 x 10^4 (**B**), 1 x 10^5 (**C**) and 1 x 10^6 (**D**) conidia. SDS-PAGE (15%) clarification of sample integrity (**E**). Larvae injected with conidia did not demonstrate breakdown of apolipophorin III as verified by in gel digestion and LC/MS analysis. A list of identified peaks and their fold expression to untreated control larvae is presented in Table 6.3.

Peak No	Peptide I.D	Peak area fold change			
		Control	1×10^4	1×10^5	1×10^6
1	Larval haemolymph protein	1	2	2.1	1.58
2	Anionic antimicrobial peptide 2	1	0.89	0.9	0.223
3	Apolipophorin III	1	1.27	1.1	0.243

Table 6.3 Fold change of identified larval peptides following infection with non-lethal *A. fumigatus* inocula.

Identified peak areas were expressed as a fold change relative to larvae injected with PBS from the HPLC analysis presented in Figure 6.13. Larvae that received doses of 1×10^4 and 1×10^5 conidia demonstrated a significantly higher presence of larval haemolymph protein ($p \leq 0.01$) but did not vary in the amount of anionic antimicrobial peptide 2 or apoLp III, ($p \geq 0.055$ and 0.065). Priming with 1×10^6 conidia resulted in a significant decline in the presence of anionic antimicrobial peptide 2 and apoLp III, (0.002 and 0.0032 respectively).

to *G. mellonella* larvae after 24 hours and the association of these doses with the increased expression of larval haemolymph protein it may be possible that synthesis of this protein is increased in healthy larvae as a means of supporting the cellular immune response.

Anionic antimicrobial peptide 2 peaks decreased approximately 10% with fungal inoculation of 1 x 10^4 or 1 x 10^5 conidia (p $\geq$ 0.055) and by 77% when larvae were injected with 1 x 10^6 conidia (p = 0.002). This finding is particularly interesting as the work of Mak *et al*., (2010) and Cytrnska *et al*., (2007) demonstrated that induction of this protein in *G. mellonella* larvae took place upon bacterial challenge. It is therefore interesting that the decrease in abundance of this peptide in larvae challenged with *A. fumigatus* may be part of a specialisation of the host immune response in terms of targeting the infecting microbe. Alternatively this may be a result of proteolytic cleavage of host proteins by secreted extracellular proteases produced by *A. fumigatus*.

Peak number 3 identified as apoLp III was present in infected and non-infected larvae. The relative increase of apoLp III in larvae inoculated with 1 x 10^4 (1.27 fold, p = 0.065) and 1 x 10^5 (1.1 fold, p = 0.12) and subsequent decrease with 1 x 10^6 conidia (0.243 fold, p = 0.0032) was interesting as it pointed to a potential degradation of this protein over time. Such an occurrence has been observed previously in studies concerning apoLp III. Andreijko *et al*., (2008) demonstrated degradation of apoLp III in cell free haemolymph 24 hours post-infection with *P. aeruginosa* by the appearance of immunoreactive polypeptides at 15, 13.3, 12 and 9.5 kDa which was speculated to be the result of the production of a protease IV by *P. aerunginosa* for which apoLp III is a substrate, (Andrejko *et al*., 2005). In Figure 6.13 E there did not appear to be any significant breakdown associated with infection with 1 x 10^6 conidia larvae^{-1} therefore the reasons for reduction at this concentration may be linked to a subsequent decline in larval immunocompetence prior to further infection (Figure 6.2).

6.3.6 Identification of proteins with altered expression following immune priming, by 2-D electrophoresis and LC/MS analysis.

The aim of this section was to analyse the changes in the humoral proteomic profile of *G. mellonella* larvae that were expressed in response to a non-lethal conidial inoculum.

Larvae were left untouched or injected with PBS, 1×10^4 or 1×10^5 *A. fumigatus* ATCC 26933 conidia and incubated for 24 hours at 30ºC. Cell free hemolymph was extracted, subjected to 2-dimensional electrophoresis (Figure 6.14) and analysed using Progenesis software using a haemolymph profile from untreated larvae as a reference map (Figure 6.15). Following Progenesis analysis spots were excised and subjected to tryptic digestion and LC/MS analysis with database interrogation using the MASCOT search engine (Section 2.13). Initially all peptides of interest were searched for larval homology (Figure 6.16 and Table 6.4). Any spots which failed to yield identity were re-searched for homology against all invertebrates (Figure 6.17 and Table 6.5) with an ion score cut-off of 48. Identified proteins are expressed relative to untreated larvae (U.T).

Spot number 1, identified as having the highest level of change in response to non-lethal fungal stimuli was identified as hexamerin an 81.8 kDa protein which was shown to increase in expression 4.38 fold in larvae injected with 1×10^5 conidia. Hexamerin biosynthesis takes place in the fat body and is subsequently released into the haemolymph where it is considered to function as a source of amino acids, (Kanost *et al*., 1990; Burmester and Scheller, 1999). It has been demonstrated that hexamerins are endocytosed by fat body cells and act as storage proteins during larval feeding prior to metamorphosis, (Gilbert *et al*., 2000).

Given the increase in hexamerin it is not surprising that a 7.3-fold increase in a homologous hexamerin receptor identified in *Corcyra cephalonica* is observed in larvae injected with 1×10^5 conidia (Spot number 2). The hexamerin receptor has been

(A)

(B)

3 ————————→10
pH

3 ————————→10
pH

(C)

kDa
80
58

46
30

25

17

7

(D)

kDa
80
58

46
30

25

17

7
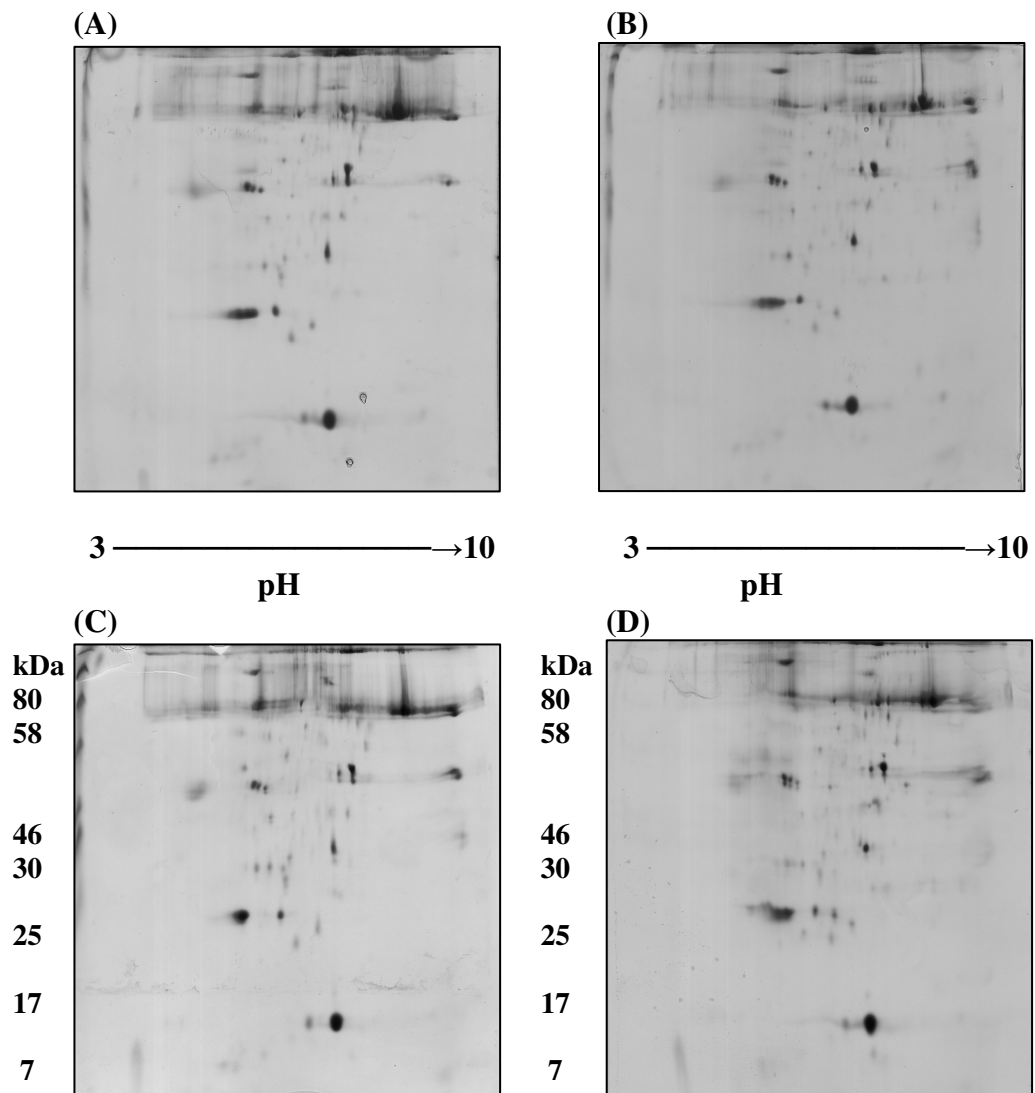
Figure 6.14 Larval haemolymph proteomic profile with non-lethal *A. fumigatus* treatments.
Larvae were left untreated **(A)** or injected with PBS **(B)**, 1 x 10^4 **(C)** or 1 x 10^5 **(D)** *A. fumigatus* ATCC 26933 conidia and incubated at 30ºC for 24 hours. Protein was extracted and the proteomic profile was examined using 2-dimensional electrophoresis. All subsequent changes were compared to larvae that were incubated at 30ºC but were not injected with conidia.
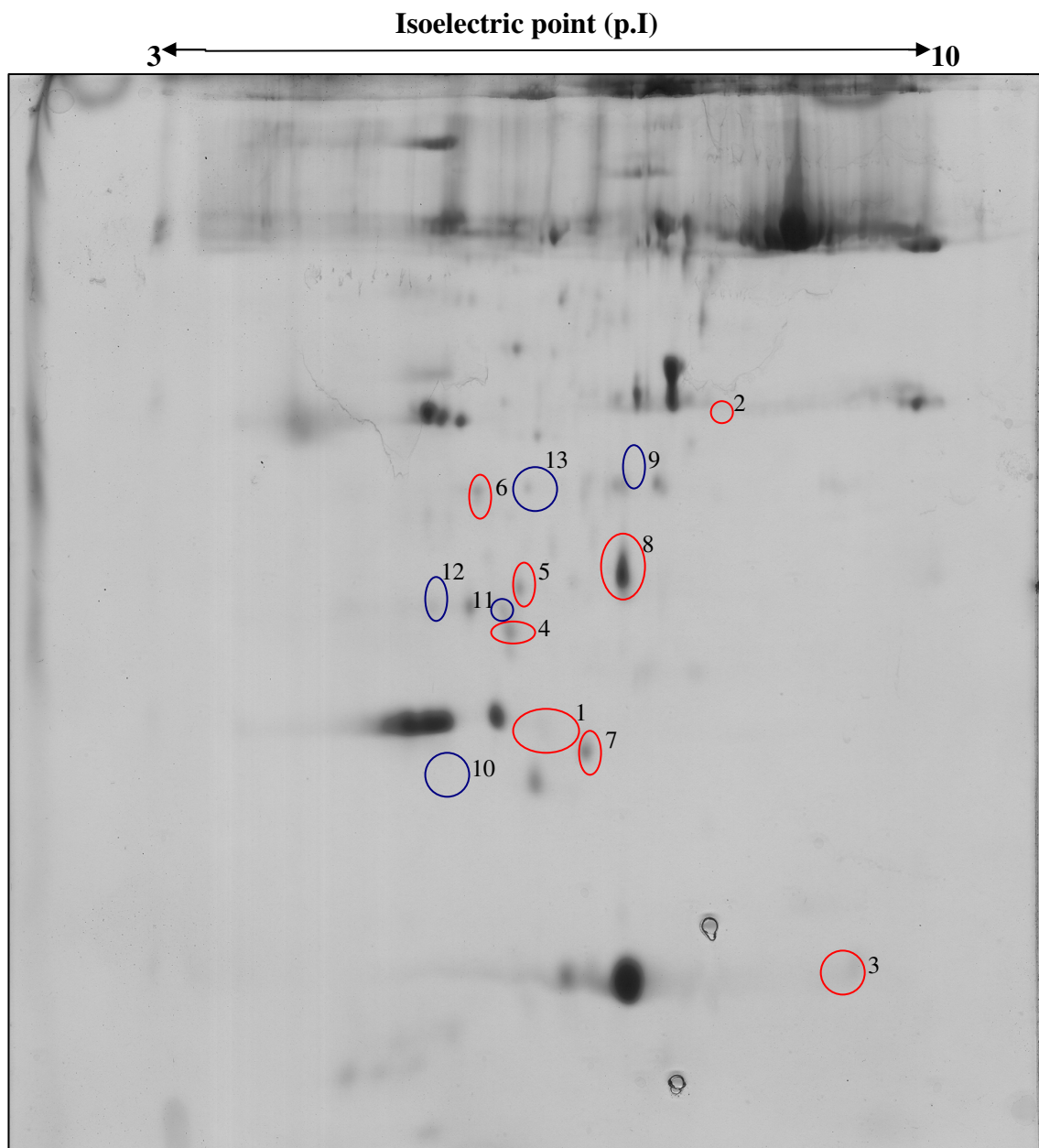
Figure 6.15 Spots excised for fold change analysis from *G. mellonella* haemolymph profiles following immune priming.
An unstimulated larval treatment was used to excise spots which showed alterations in expression as determined by Progenesis™ same spot analysis. Spots circled red (1-8) were identified in *G. mellonella* or other moth larvae. Spots circled blue (9-13) were identified to homologous proteins from other invertebrate species.
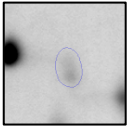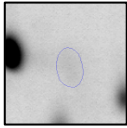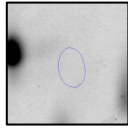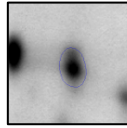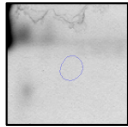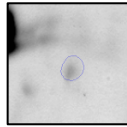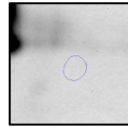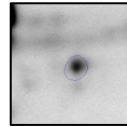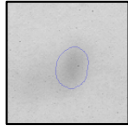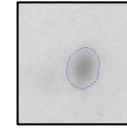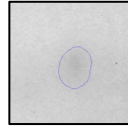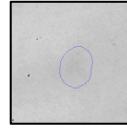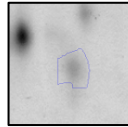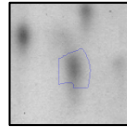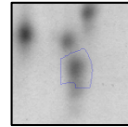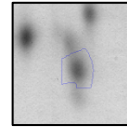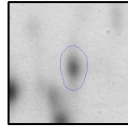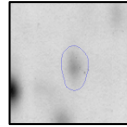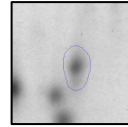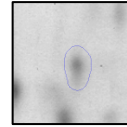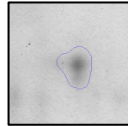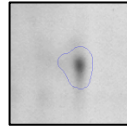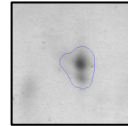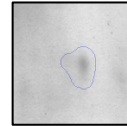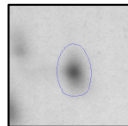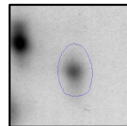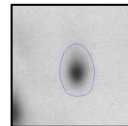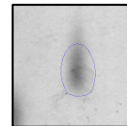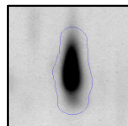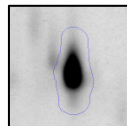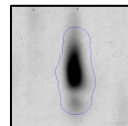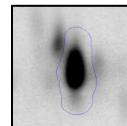
Protein	Spot I.D corresponding to treatment				Fold expression			
	Untreated	PBS	1×10^4	1×10^5	U.T	PBS	1×10^4	1×10^5
1. Hexamerin					1	1.19	0.293	4.38
2. Hexamerin Receptor					1	2.65	0.79	7.3
3. ApoLp III					1	1.44	0.67	0.64
4. 32 kDa Ferritin subunit					1	1.28	2.08	1.96
5. Arylphorin					1	0.62	1.16	0.69
6. Apolipophorin					1	1.44	1.77	0.98
7. Gallerin					1	1.03	1.21	1.33
8. Juvenile hormone binding protein					1	0.93	0.92	1.06

Figure 6.16 Individual larval haemolymph spot analyses of proteins which show differential abundance with immune priming.
Gels were analysed using Progenesis™ "Same spot" software. Spots which showed differential expression ($p \leq 0.05$) were excised and identified using LC/MS analysis (except juvenile hormone binding protein which did not change significantly ($p \geq 0.1$)). The fold expression relative to untreated larvae (U.T) is also displayed.
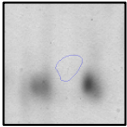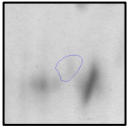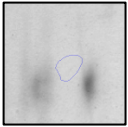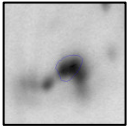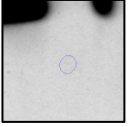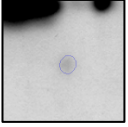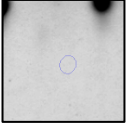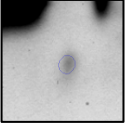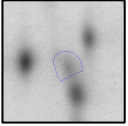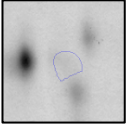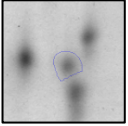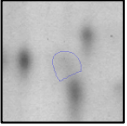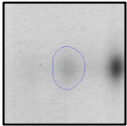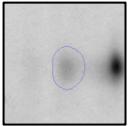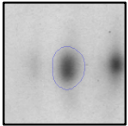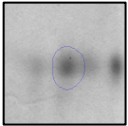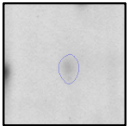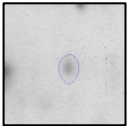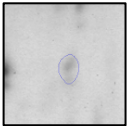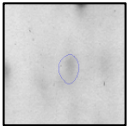
Protein	Spot I.D corresponding to treatment				Fold expression			
	Untreated	PBS	1×10^4	1×10^5	U.T	PBS	1×10^4	1×10^5
9. GJ23138 *Drosophila virilis*					1	0.92	0.726	6.99
10. GF17786 *Drosophila ananassae*					1	2.21	0.81	4.1
11. GJ14984 *Drosophila virilis*					1	0.48	1.53	0.68
12. GM22874 *Drosophila sechella*					1	1.0	2.76	1.8
13. Arginine Kinase *Homarus gammarus*					1	1.35	1.74	0.73

Figure 6.17 Individual spot analysis identified in other species.
In some cases it was not possible to attain spot identity in *G. mellonella*. Other invertebrate databases were searched for protein homologues to aid identification of spots of interest. The fold change in expression relative to untreated larvae (U.T) is also presented.

Spot I.D	gi	Score	Peptides matched	Coverage
Hexamerin	347090	183	5	8%
Hexamerin receptor	282722536	96	2	2%
Apolipophorin III	5915688	241	6	33%
32 kDa ferritin subunit	17901818	270	7	25%
Arylphorin	384243	340	7	14%
Apolipophorin	50404098	132	3	28%
Gallerin	1146408	169	2	12%
Juvenile hormone binding protein	158515746	165	8	17%

Table 6.4 Identified larval proteins data.
MASCOT identification data from excised spots presented in Figure 6.16. All spots were associated with *G. mellonella* larvae except hexamerin receptor which was identified in *Corcyra cephalonica* (rice moth). A MASCOT score cut-off of 62 was used for positive identification of proteins.

Spot I.D	Organism	gi	Score	Peptides matched	Coverage
GJ23138	*D. virilis*	195390311	64	2	3%
GF17786	*D. ananassae*	190626532	54	1	6%
GJ14984	*D. virilis*	195402607	52	1	1%
GM22874	*D. sechella*	195345353	59	3	1%
Arginine Kinase	*H. gammarus*	585342	151	3	12%

Table 6.5 Data of spots identified in other invertebrate species.
MASCOT identification of spots presented in Figure 6.17. Peptide ions which were not identified in larvae were identified in *Drosophila* spp. and *Homarus gammarus*, (European lobster). A MASCOT score cut-off of 62 was used for positive identification of proteins.

identified as a member of the low density lipoprotein receptor superfamily, (Burmester and Scheller 1999) and functions as a binding site for hexamerin in fat body cells, (Zhou *et al*., 2006).

Spot number 3, was identified as apoLp III which decreased in expression 0.67 and 0.64 fold in larvae that received 1 x 10^4 and 1 x 10^5 conidia respectively but demonstrated a 1.44 fold increase in PBS injected larvae. ApoLp III has been shown to bind β1,3-glucan (Whitten *et al*., 2004), and lipotechtoic acid in Gram positive bacteria, (Halwani *et al*., 2000). ApoLp III has been previously shown in this Chapter to be particularly abundant in larval haemolymph, while the identified spot in this section is quite low in intensity. ApoLp III is an insect low density lipoprotein (LDL) which holds critical functions in terms of reversible lipid binding and transport characterised by amphipathic α-helices which contribute to the presence of lipid bound and lipid free forms of apoLp III (Segrest *et al*., 1992; Weers and Ryan, 2006). It may be possible that the lipid binding nature of this protein caused a change in the surface charge of the protein thus causing a shift in isoelectric focussing of the spot.

The fourth spot of interest was identified as a 32 kDa ferritin subunit. Cloning and expression studies of this protein initially demonstrated a high level of expression of this protein following iron feeding (Kim *et al*., 2002), as iron is toxic to host tissue due to its role in the formation of free radicals (You and Wang, 2005). Ferritin contributes to cellular iron ion homeostasis by sequestering free iron and has also been demonstrated to be increased in expression following bacterial infection in *Drosophila melanogaster*, (Vierstraete *et al*., 2004). Interestingly ferritin is also present in mammals where sequesterisation of iron is critical to limiting the spread of infection due to the necessity of microbes to access free iron (Haas *et al*., 2008; Schrettl *et al*., 2004). The role of ferritin is of particular importance in this case as inoculations with sub-lethal concentrations of *A. fumigatus* conidia results in increased expression of the 32 kDa

ferritin subunit. Larvae injected with 1 x 10^4 and 1 x 10^5 conidia exhibited 2.08 and 1.96 fold increases in the expression of ferritin respectively. This finding strengthens the link between the control of the spread of fungal infection and the limitation of free iron accessible to the invading microbe.

Spot number 5 was identified as arylphorin. Arylphorin is a member of the insect haemocyanin family and BLAST analysis of this protein indicates a high level of homology with other arylphorins and hexamerins from insect species (data not presented). Larvae injected with 1 x 10^4 conidia showed a significant increase in the expression of arylphorin 24 hours post infection compared to PBS and 1 x 10^5 injected larvae which showed a decrease in expression. At present there is no evidence to suggest a direct antifungal role for arylphorin in insect species rather its annotated role is as a storage protein and oxygen transporter activity. A proteomic approach to the effect of *E. coli* and *M. luteus* bacterial feeding in larvae of *Trichoplusia ni* demonstrated a significant increase in the presence of arylphorin as one of a number of key proteomic changes associated with resistance to further infection, (Freitak *et al.*, 2007). It may be possible that the lowest concentration of conidia injected into larvae stimulates a response similar to controlled low doses that would be possible with a feeding event however considering the relative change in expression relative to uninfected larvae it is likely that alterations in arylphorin expression is related to an antibacterial response.

Spot number 6 was identified as apolipophorin (apo 1). Apo 1 was expressed 1.44, 1.77 and 0.98 fold when injected with PBS or 1 x 10^4 and 1 x 10^5 conidia, respectively, compared to untreated larvae. While apo 1 is classed among the same family of lipophorin based apolipoproteins as apoLp III there appears to be no previous evidence to suggest that apo 1 is directly involved in the antifungal response of insects. Apo 1 has been demonstrated to display conserved functions with other proteins within

this family of lipid transport and lipoprotein metabolism, (Weers and Ryan, 2006). Apo 1 does display functionality in response to bacterial challenge where coagulation of apo 1 takes place to form a globular structure resulting from oligomeric interactions with LPS thus neutralising LPS from the surrounding environment (Ma *et al*., 2006).

Spot number 7 was identified as gallerin. Larvae inoculated with 1 x 10^4 and 1 x 10^5 *A. fumigatus* conidia display a 1.21 and 1.33 fold increase in gallerin expression relative to untreated larvae. Gallerin is annotated as a member of the calycin family of immunologically relevant proteins. Other members of this family include alpha-1-acid glycoprotein 1 which functions in the acute phase response to infection and regulates the immune system processes. This process is mediated in neutrophils by cytosolic Ca^{2+} through the binding of immunoglobulin-like lectins with the effect of inhibiting superoxide production (Laine *et al*., 1990; Gunnarson *et al*., 2007). The presence of a functionally similar protein in *G. mellonella* larvae is of particular interest as the precise role of gallerin in fungal infections is unknown. The expression of this protein increases with the level of fungal infection therefore pointing to a role in the regulation of the cellular immune response to pathogenic fungi.

Spot number 8 was identified as juvenile hormone binding protein (JHBP) which did not show a statistically significant change in expression following fungal infection. JHBP is synthesised in fat body cells and serves a carrier protein for juvenile hormone (JH) which plays a key role in regulation of metamorphosis and development in insects, (Gilbert *et al*., 2000; Zalewska et al., 2009). Flatt *et al*., (2008), identified that juvenile hormone antagonistically regulates the production of antimicrobial peptides in *D. melanogaster,* however it remains unclear whether such a process is evident in *G. mellonella*.

Certain spots which demonstrated significant alterations in expression were not identified in larvae but did show sequence homology with proteins in other

invertebrates (Figure 6.16 and marked as blue circles in Figure 6.15). There was a 6.9 fold increase in the presence a protein homologous to DNA binding protein GJ23138 identified from *Drosophila virilis* (Spot number 9) in 1 x 10^5 injected larvae. BLAST analysis of this protein identifies a GJ23138 to have a motif present in the pipsqueak protein of *Drosophila* which is a helix turn helix protein that binds to GAGA sequences (Lehmann *et al*., 1998).

There was a 4.1 fold increase in an isochorismatase-like protein annotated as GF17786 from *Drosophila ananassae* (Spot number 10). An isochorismatase homologue occurs in *E. coli* and is known to be involved in the hydrolyzation reactions, (Hamano *et al*., 2006).

A 1.53 fold increase in the presence of protein homologous to GJ14984 from *D. virilis* (Spot number 11) was observed when larvae were injected with 1 x 10^4 conidia. BLAST analysis of GJ14984 reveals 95% coverage with an organic anion transporting polypeptide from *D. melanogaster* which functions in the transport of macromolecules.

A 2.76 and 1.8 fold increase in the expression of a protein homologous to GM22874 from *Drosophila sechella* was observed in 1 x 10^4 conidia injected larvae (Spot number 12). BLAST analysis of this protein displays 99% sequence homology with the dynein heavy chain at 16F protein from *D. melanogaster*. Dynein is a microtubule motor protein. Annotations for this protein indicate ATP binding and ATPase activity as critical functions of this protein (Asai and Wilkes, 2004).

Spot number 13 was identified as a protein showing sequence homology with Arginine kinase (AK) from *Homarus gammarus* (European Lobster). There was a 1.74 fold increase in the expression of AK in larvae primed with 1 x 10^4 conidia relative to untreated larvae with larvae inoculated with PBS and 1 x 10^5 conidia demonstrating 1.35 and 0.73 fold alterations in expression respectively. The identification and analysis of the expression of arginine kinase is particularly interesting as AK is the most

abundant phosphagen kinase in invertebrates, with the function of catalysing the transfer of a phosphoryl group to ADP thus forming ATP (Kotlyar *et al*., 2000).

6.3.7 Summary.

In this section a comprehensive approach to elucidate changes in proteomic profile following infection with non-lethal conidial inocula was employed. RP-HPLC coupled to LC/MS analysis of *G. mellonella* peptides demonstrated the increase in expression of *G. mellonella* larval haemolymph protein which functions in oxygen transportation. This coincided with reduced expression of anionic antimicrobial peptide 2 which functions in the bacterial response. Interestingly the expression of apoLp III did not appear to be greatly influenced by non-lethal conidial infection. Analysis of differential expression of haemolymph proteins by 2-dimensional electrophoresis coupled to LC/MS revealed the increased expression of proteins involved in macromolecule storage, iron sequesterisation, clotting, acute phase response and ADP phosphorylation following priming with 1×10^{4} *A. fumigatus* conidia.

6.4 RT-PCR analysis of *G. mellonella* AMP gene expression.

The expression and synthesis of antimicrobial peptides (AMPs) is a key component of the innate immune system of insects, (Bergin *et al*., 2006; Brown *et al*., 2008). Recent work by Mowlds *et al*., (2009) demonstrated that the innate immune system of *G. mellonella* larvae could be effectively protected against *C. albicans* infection by utilising the priming response initiated by injecting larvae with β-glucan. Such a response is interesting as it points to a short-term increase in the immune status of the host in advance of subsequent infection. Therefore, a study investigating whether non-lethal concentrations of *A. fumigatus* resulted in a significant increase in AMP gene expression was warranted.

6.4.1 RT-PCR analysis of larval AMP genes following inoculation with non-lethal conidial inocula.

Larvae were inoculated with PBS or 1×10^4, 1×10^5 or 1×10^6 conidia and incubated at 30ºC for 4, 24 and 48 hours. As an additional control larvae were left untreated. RNA extraction, cDNA synthesis and PCR amplification of transcribed genes of interest was performed as described (Section 2.21). Resulting PCR products were quantified by densitometry using ImageQuant software. All values were normalised by β-actin and were expressed as a fold change relative to untreated larvae.

Analysis of gene expression demonstrated only slight alterations in PCR product band intensity after 4 hours priming at 30ºC (Figure 6.18). There was no statistically significant change to the expression of *galiomycin*, *transferrin* or *impi* at this timepoint. Larvae injected with 1×10^5 conidia displayed a 12% increase in the expression of *gallerimycin* (p = 0.002) 4 hours post infection. This result was not surprising as low doses of *A. fumigatus* have been demonstrated to have a limited effect on larval viability when larvae are subsequently injected with lethal concentrations of *A. fumigatus*.

At 24 hours there were clear differences in relative gene expression (Figure 6.19). Larvae injected with 1×10^4, 1×10^5 and 1×10^6 conidia demonstrated 2.29 ± 0.096, 2.66 ± 0.11 and 3.03 ± 0.17 fold increases in *gallerimycin* expression respectively (p = 0.001). *Galiomycin* expression was not altered significantly at this timepoint with p ≥ 0.128 in all comparisons. The expression of *transferrin* was also significantly increased following larval infection showing 1.11 ± 0.04, 1.23 ± 0.04 and 1.24 ± 0.04 fold (p ≤ 0.007) fold increases in expression following 1×10^4, 1×10^5 and 1×10^6 conidial infection. In addition *impi* expression increased 1.11 ± 0.05 1.16 ± 0.05 and 1.19 ± 0.05 fold (p ≤ 0.009) following the same rate of infection.

At 48 hours, (Figure 6.20) *gallerimycin* expression was demonstrated to be significantly increased in larvae injected with 1×10^5 and 1×10^6 conidia relative to
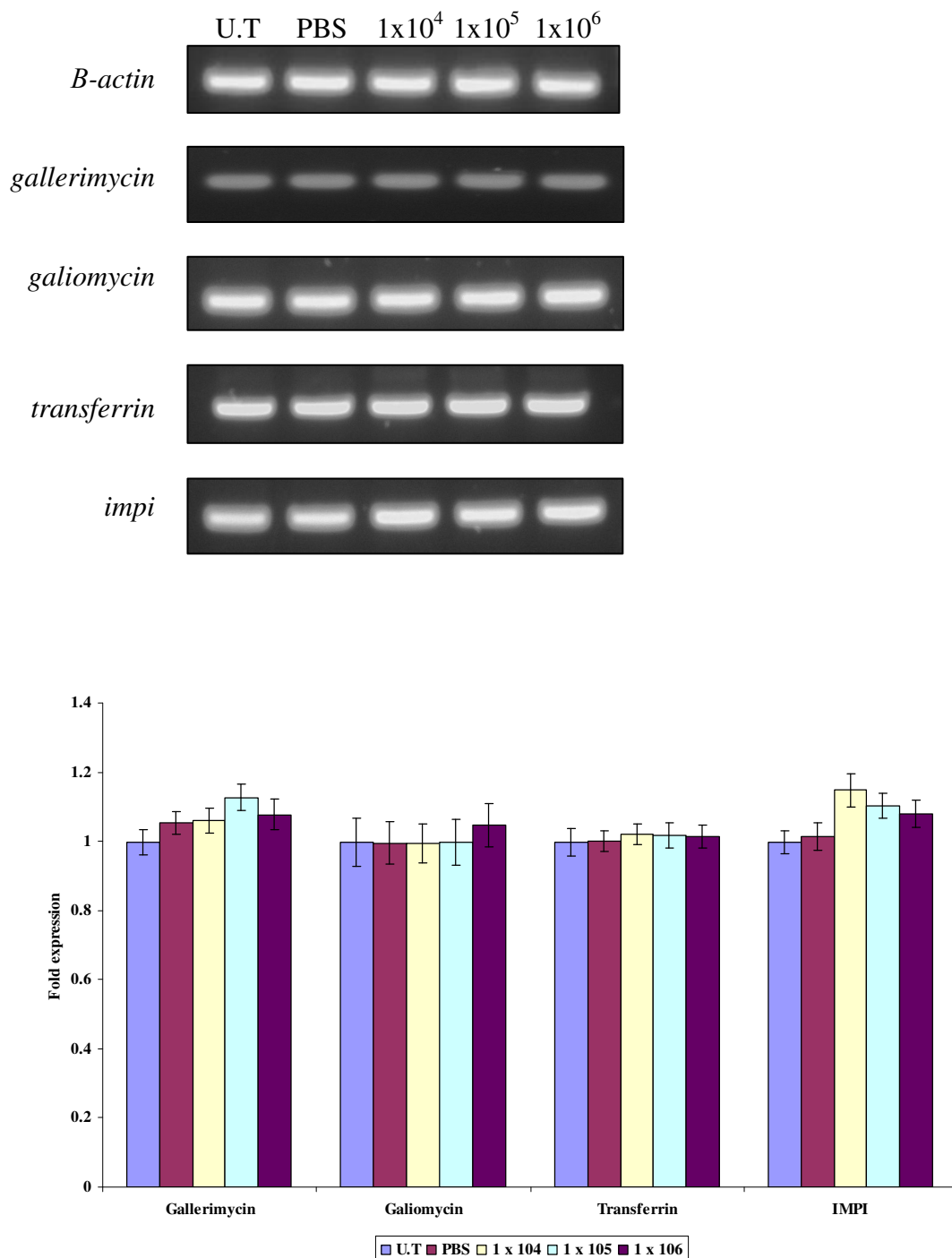
Figure 6.18 RT-PCR analysis of *G. mellonella* cDNA extracted from whole larvae after 4 hours and separated on a 1% agarose gel.
Larvae were injected with PBS or inoculated with 1×10^4, 1×10^5 or 1×10^6 *A. fumigatus* ATCC 26933 conidia or left untreated (U.T). Larvae were incubated for 4 hours, RNA was extracted and cDNA was synthesised. PCR was performed using primers for *β-actin*, *gallerimycin*, *galiomycin*, *transferrin* and *impi*. Resulting PCR products were visualised on a 1% agarose gel and bands were quantified using densitometry. *β-actin* was used as a control which was demonstrated to show no change in expression and gene expression was calculated as a fold change from untouched larvae. Quantification was achieved by densitometric analysis of four independent PCRs for each gene.
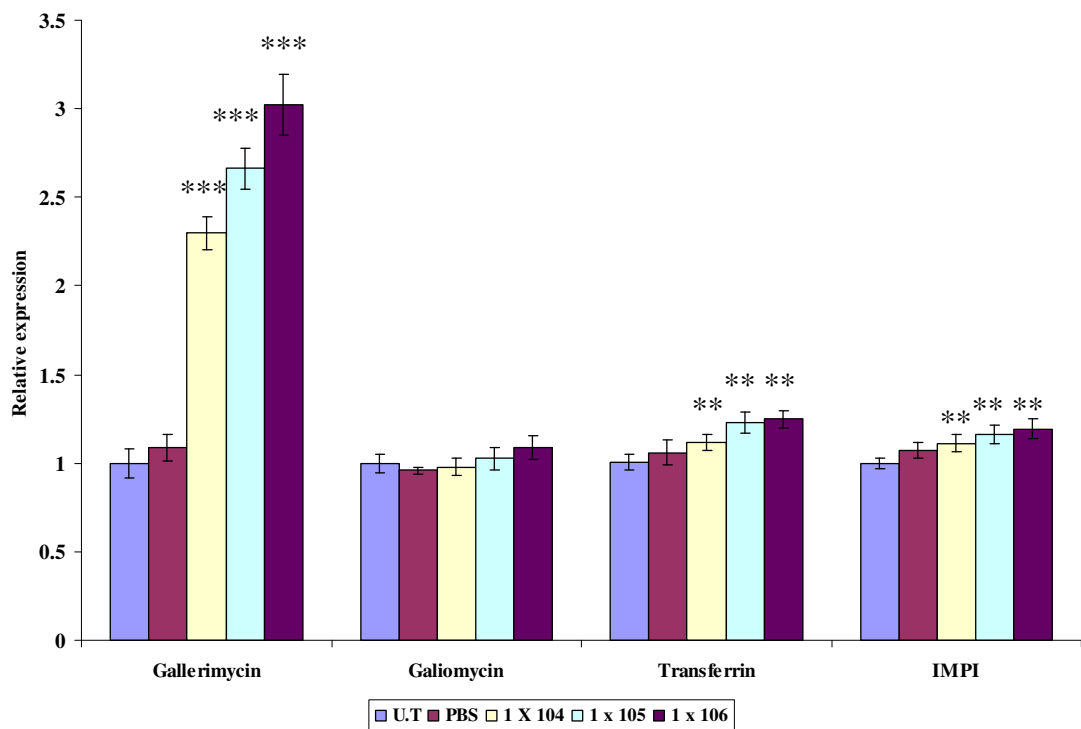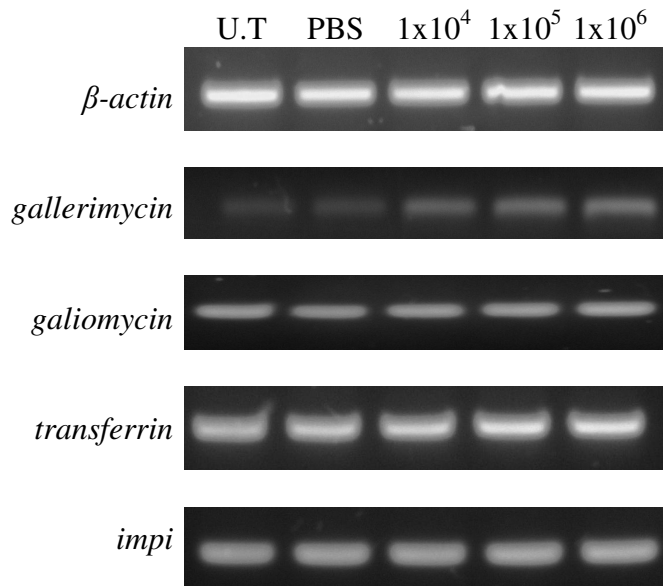
Figure 6.19 RT-PCR analysis of *G. mellonella* cDNA extracted from whole larvae after 24 hours and separated on a 1% agarose gel.
Larvae were injected with PBS or inoculated with 1×10^4, 1×10^5 or 1×10^6 *A. fumigatus* ATCC 26933 conidia or left untreated (U.T). Larvae were incubated for 24 hours, RNA was extracted and cDNA was synthesised. PCR was performed using primers for *β-actin*, *gallerimycin*, *galiomycin*, *transferrin* and *impi*. Resulting PCR products were visualised on a 1% agarose gel and bands were quantified using densitometry. *β-actin* was used as a control which was demonstrated to show no change in expression and gene expression was calculated as a fold change from untouched larvae. Quantification was achieved by densitometric analysis of four independent PCRs for each gene.
*** p = 0.001
**   p < 0.01
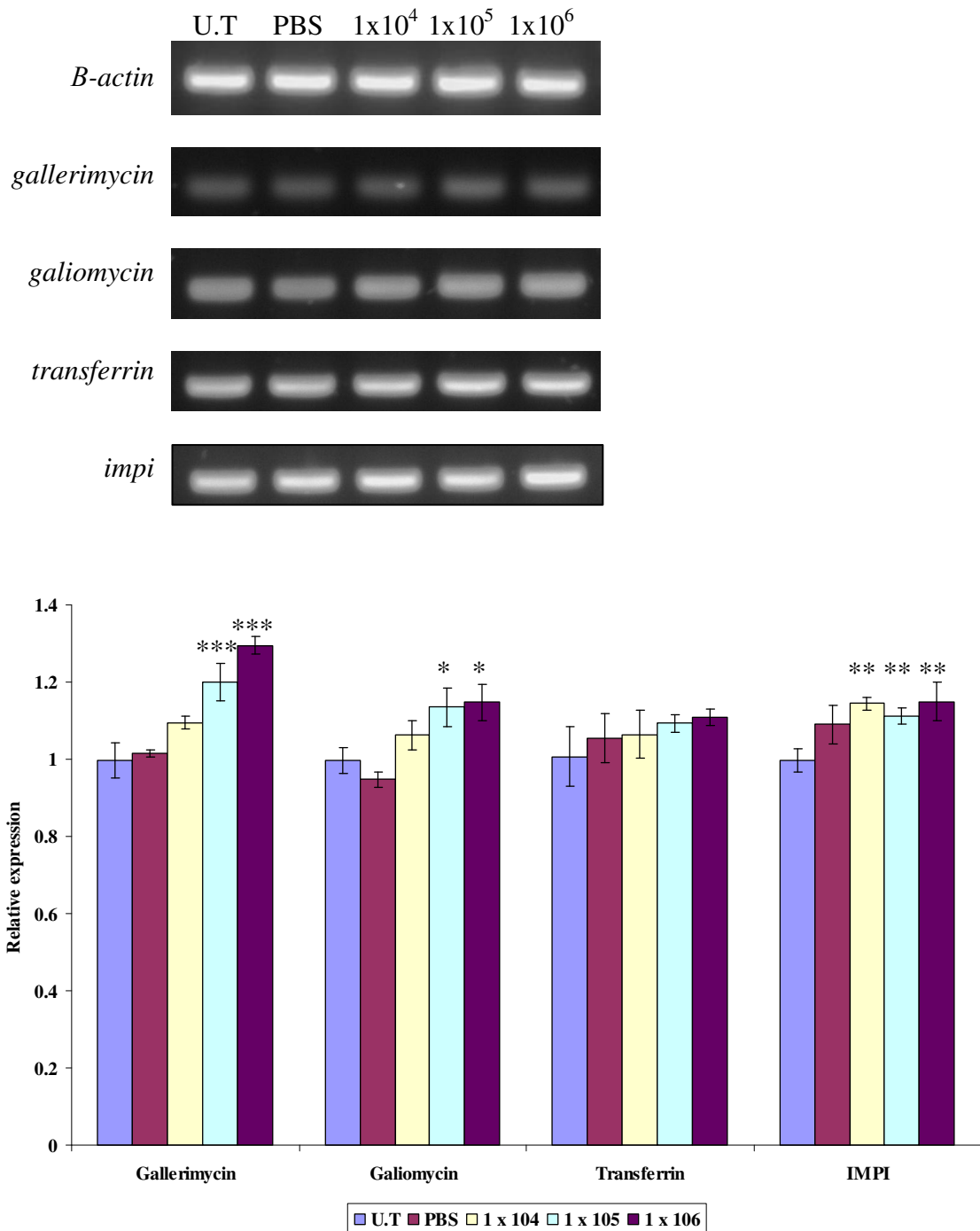
Figure 6.20 RT-PCR analysis of *G. mellonella* cDNA extracted from whole larvae after 48 hours and separated on a 1% agarose gel.

Larvae were injected with PBS or inoculated with 1×10^4, 1×10^5 or 1×10^6 *A. fumigatus* ATCC 26933 conidia or left untreated (U.T). Larvae were incubated for 48 hours, RNA was extracted and cDNA was synthesised. PCR was performed using primers for *β-actin*, *gallerimycin*, *galiomycin*, *transferrin* and *impi*. Resulting PCR products were visualised on a 1% agarose gel and bands were quantified using densitometry. *β-actin* was used as a control which demonstrated to show no change in expression and gene expression was calculated as a fold change from untreated larvae. Quantification was achieved by densitometric analysis of four independent PCRs for each gene.

*** $p \leq 0.003$ * $p \leq 0.009$

** $p \leq 0.006$

untreated larvae demonstrating relative increases of 1.2 ± 0.09 and 1.29 ± 0.02 fold (p ≤ 0.003) however these values had dropped significantly compared to relative fold changes at 24 hours (Figure 6.19). Larvae injected with PBS or 1 x 10^4 conidia did not demonstrate any significant increase in expression at this time. *Galiomycin* expression was increased 1.13 ± 0.05 and 1.27 ± 0.05 fold at this timepoint in larvae injected with 1 x 10^5 and 1 x 10^6 conidia respectively (p ≤ 0.009). *Transferrin* expression was not altered significantly after 48h displaying a return to baseline levels of expression at this timepoint. *IMPI* expression was significantly increased at 48 hours displaying a 1.14 ± 0.02, 1.11 ± 0.02 and 1.15 ± 0.05 (p ≤ 0.006) fold increase over untreated larvae when injected with 1 x 10^4, 1 x 10^5 and 1 x 10^6 conidia, respectively.

6.4.2 Summary.

The effect of infecting *G. mellonella* larvae with sub-lethal concentrations of *A. fumigatus* on AMP gene expression was investigated by RT-PCR in combination with densitometric analysis of resulting gel products. Analysis of *galiomycin*, *gallerimycin*, *transferrin* and *impi* expression at 4 hours post infection with 1 x 10^4, 1 x 10^5 and 1 x 10^6 conidia demonstrated no significant change in expression. At 24 hours post infection there were highly significant increases in *gallerimycin* expression following immune priming (p ≤ 0.001). In addition there were also significant increases in *transferrin* and *impi* expression following conidial priming (p ≤ 0.007 and 0.009 respectively). At 48 hours the levels of expression did not vary as much however there were significant increases in *gallerimycin*, *galiomycin* and *impi* expression (p ≤ 0.003, 0.009 and 0.006 respectively).

6.7 Discussion.

The adaptability of the innate immune response of *G. mellonella* has been identified in recent years as being far more advanced than previously thought. Central to this are fluctuations in haemocyte density, synthesis of antimicrobial peptides and notable shifts in the proteome of the humoral immune response which effectively deal with pathogens, (Whitten *et al*., 2004; Bergin *et al*., 2006; Andrejko *et al*., 2009).

The results presented here showed that non-lethal inocula with conidia conferred a significant protective response in larvae injected with 1×10^4 conidia but larvae injected with 1×10^5 or 1×10^6 conidia did not record statistically significant increases in viability when administered a follow-up injection of 1×10^7 conidia. This immune priming event is continued at 48 hours post infection with larvae inoculated with 1×10^4 and 1×10^5 conidia and subsequently injected with 1×10^7 conidia demonstrating increased viability at the endpoint of this study ($p \leq 0.02$). The 1×10^6 conidial inoculum did not prime larvae against subsequent fungal infection however this may be a result of a cumulative fungal burden over time. It is worth noting that the survival of larvae injected with PBS prior to subsequent infection with *A. fumigatus* was reduced in a stepwise manner 4, 24 and 48 hours. This is a key factor as it contributed to the significance of the data obtained. This a suprising result however it would appear to be correlated with a reduction in haemocyte density at the same timepoints as observed in Figure 6.4. The reasons for this occurrence are as yet unclear.

To fully understand the key changes in the balance of the immune status corresponding to priming events and the fluctuations that take place over time, a study identifying changes to different parameters of the immune response was employed. Larvae injected with conidia demonstrated a statistically significant increase in the number of circulating haemocytes μl^{-1} of haemolymph increasing 1.79, 1.49, and 1.71 fold 24 hours post-infection with 1×10^4, 1×10^5 and 1×10^6 conidia respectively ($p \leq$

0.028). It was particularly interesting to note that the number of circulating haemocytes had declined rapidly by 48 hours. Such a reduction in the number of haemocytes is indicative of a possible return to a normalised immune state. It has been previously demonstrated that a priming event initiated by physical stress results in haemocyte densities approximately 2.3 fold greater than control larvae after 48 hours (Mowlds *et al*., 2008). Initial investigations of the importance of an adequate number of circulating haemocytes and general larval fitness in *G. mellonella* demonstrated that highly pathogenic yeast isolates cause a reduction in the number of haemocytes thus assisting in the proliferation of the microbe throughout the insect (Bergin *et al*., 2003) due to the fact that haemocytes bind to yeast cells. Considering the improved viability of *G. mellonella* primed with 1 x 10^4 conidia for 24 hours (Figure 6.2) and the observation that haemocyte density of larvae peaks 24 hours following inoculation with the same dose (Figure 6.4) it is not unreasonable to suggest that there is a strong link between the two. Dean *et al*., (2004) demonstrated the appearance of a class of haemocyte in *M. sexta* with extreme spreading ability with the additive effect of forming nodules which limit the proliferation and spread of an invasive pathogen. Given the functional similarities demonstrated between the immune system of both of *G. mellonella* and *M. sexta* it would be interesting to investigate if such process is present in *G. mellonella*. Furthermore a study of such a fluctuation in response to natural infection and targeted injection of non-lethal doses is warranted.

The work of Whitten *et al*., (2004), demonstrated a role for the lipoprotein apoLp III in protection against fungal infection with lipid associated form of the protein thus acting as a pattern recognition receptor (PRR) within the haemocoel. The additive effect of apoLp III binding to conidia is enhanced cell attachment and spreading. From the data presented here there appears to be small alterations in levels of apoLp III in larvae as detected by RP-HPLC following infection with 1 x 10^4 and 1 x 10^5 inocula of

conidia at 24 hours, (Figure 6.13 and Table 6.1), however the presence of apoLp III in 1 x 10^6 conidia injected larvae appears drastically reduced. It may be possible that apoLp III levels are intrinsically linked to larval immunological health in response to fungal pathogens. Critically, haemolymph of larvae primed for 24 hours with *A. fumigatus* conidia demonstrate a higher affinity for conidia as presented in Figure 6.5. Haemolymph from larvae primed with 1 x 10^4 and 1 x 10^5 conidia showed significant increases in apoLp III binding at this timepoint ($p \leq 0.048$). These findings along with the work of others (Zakarian *et al*., 2002) strengthens the assertion that apoLp III plays a critical role in linking the cellular and humoral immune responses with the additive effect of improving larval resistance to invading fungal pathogens. However, further work is necessary to understand if apoLp III binding is altered when larvae are incubated at 4 or 48 hours as this would place this occurrence of the immune response into context.

Prophenoloxidase (PPO), a 79 kDa zymogen (in its native form) plays a critical role in initiating melanisation and limitation of microbial proliferation upon infection, (Kavanagh and Reeves, 2004). The activation of this response is mediated by controlled serine protease cleaving of PPO with the combined effect of forming melanin within the haemocoel which is toxic to the microbe, and to the host at high concentrations. Figures 6.6 and 6.7 demonstrate that not only is the presence of PPO increased upon fungal stimulation at 4 and 24 hours post-infection but there is a significant (1.97 fold) increase in the relative expression of PPO at 24 hours in larvae infected with 1 x 10^4 conidia ($p = 0.026$). At 48 hours there was evidence of a complete breakdown of native PPO with the detection of lower molecular weight bands indicating cleavage of the native protein therefore any protective effect native PPO could play in the eradication of subsequent infection is diminished. This is further supported by the non-specific cleavage of the enzyme as is evidenced by the breakdown products in haemolymph from untreated and

PBS injected larvae when probed for PPO. The work presented here emphasises the role of an abundance of PPO in haemolymph as being a critical factor in the priming of the immune state thus contradicting the previous assertion made by Hung and Boucias, (1996) that strong activation of the PO cascade was critical to immunoprotection.

Analysis of the RP-HPLC profile coupled to LC/MS identification of peaks also identified a key factor in the reduction in peak area associated with anionic antimicrobial peptide 2. At 24 hours following injections with 1×10^4, 1×10^5 and 1×10^6 conidia there was a marked decrease in the abundance of this peptide in the larval peptide profile. This finding is particularly interesting as anionic antimicrobial peptide 2 does not display any activity against *Aspergilli*, (Cytrnska *et al*., 2007). The observed reduction in the abundance of this peptide point to a specialisation of the immune response against a fungal pathogen following infection. However it remains to be fully understood if this process is as a result of differential signalling through a Toll receptor pathway (Lemaitre *et al*., 1996).

The increased expression of larval haemolymph protein (lhp-1) in response to immunoprotective fungal priming and the association between high titres of the peptide and larval survival is of great interest. Electronic annotation of this peptide points to a role in oxygen transport due to the presence of a haemocyanin di-copper binding domain. Such a role for a low molecular weight protein is novel as the impact of microbial infection and oxygen transport within larvae receives little attention. It remains to be understood what transcriptional regulation takes place in response to fungal microbe sensing in order to cause enhanced synthesis of this product however the abundance of this peptide appears to be intrinsically linked to larval fitness in advance of a subsequent infection. Another role for enhanced oxygen transport could be to support the cellular immune response which it has been demonstrated to form a functional NADPH oxidase-like complex, with the resulting formation of superoxide

(Renwick *et al*., 2007). It is worth noting that the decrease in lhp-1 and anionic antimicrobial peptide 2 in larvae injected with 1 x 10^6 conidia relative to larvae injected with 1 x 10^4 or 1 x 10^5 conidia may be as a direct result of injection proteolytic cleavage cuased by secreted extracellular proteases produced by *A. fumigatus*. Extracellular proteases produced by *A. fumigatus* are important virulence factors (Alp and Arikan, 2008, Behnsen *et al*., 2010 and Birch *et al*., 2004) therefore it is possible that the heavier fungal burden may have resulted in the accumulation of such an effect in larvae.

The observed cellular and metabolic shifts observed in the data presented here are further supported by LC/MS data. Spots identified as hexamerin, hexamerin receptor, 32 kDa ferritin subunit, apolipophorin and gallerin and arginine kinase show marked increases in expression 24 hours post infection with 1 x 10^5 conidia. Considering the role of hexamerin as a storage protein which is endocytosed by fat body cells (Gilbert *et al*., 2000) it is perhaps surprising that such an emphasis on the storage of amino acids and nutrients is evidenced at the proteomic level. As a result of the fold increase in hexamerin synthesis following fungal inoculation it may be possible that as well as functioning as a storage protein particularly high expression of hexamerin may be a biomarker for a respiratory shift at the onset of more acute larval infection but not necessarily efficient priming of the immune response. It is noted that both the expression of hexamerin and hexamerin receptor are greatly reduced in larva injected with 1 x 10^4 conidia relative to infection with 1 x 10^5 conidia. This finding is perhaps surprising considering the impact of higher fungal inocula. However the "trade off" between larval fitness and effective immune priming has been documented by Freitak *et al*., (2007) in a study of priming in *Trichoplusia ni* larvae. Effective priming of larvae resulted in a negative impact on "fitness-traits" such as mass and pupal development which is interesting considering the work of Zalewska *et al*., (2009) where it was

demonstrated that hexamerin binds juvenile hormone and thus plays a role in the development of larvae form the larval to the pupal stage. Therefore the effective priming of larvae induced by 1 x 10^4 conidia viewed elsewhere in this Chapter may have a side-effect of reducing conventional larval fitness with the benefit of a more efficient immune system aimed at certain pathogens.

The approximate two-fold increase in expression of the 32 kDa ferritin subunit is an interesting development in understanding the balance between priming larvae appropriately for fungal infection and maintaining physiologically sustainable levels of available iron within the haemocoel. This balancing of iron levels has been demonstrated by others to play a key role in the immune system/fungal iron seqesterisation balance (Vierstraete *et al*., 2004: Schrettl *et al*., 2004). From the data presented in Figure 6.16 it is proposed that enhanced synthesis of ferritin as a means of sequestering any free iron from *Aspergillus* siderophores plays a key role in protecting the host against the higher fungal inoculum by effectively depleting the fungus of its iron requirement.

The increased expression of apolipophorin (apo 1) is interesting in the light of work presented by Ma *et al*., (2006) as it may suggest a role for apo 1 in the neutralisation and localisation of *A. fumigatus* within the haemocoel. The process of apo 1 based neutralisation of LPS is a means of immunoprotection that has yet to be studied for fungi however the increased expression of this protein in response to an immunoprotective concentration of conidia may warrant further research. The increase in the expression of gallerin is an interesting finding considering that this protein has been annotated a functional role in the acute phase response of fungal infections, a trait that is conserved with human alpha-1-acid glycoprotein 1. Laine *et al*., (1990) identified that alpha-1-acid glycoprotein 1 plays a role in inhibiting superoxide production. This observation, considering the increase in gallerin expression 24 hours post-infection with

a sub-lethal fungal inoculum may point to a functionally conserved role between vertebrates and invertebrates as superoxide production at unchecked levels is damaging to host tissue.

Arginine kinase (AK) plays a highly conserved role in the phosphorylation of ADP (Kotlyar *et al*., 2000), and the 1.74 fold increase in expression observed following immunoprotective stimulation (1 x 10^4 conidial inoculum) points to a significant change in the metabolic profile of larvae in an immune priming event. It is proposed that enhanced synthesis of ATP as evidenced by the change in AK expression is indicative of an altered rate of respiration with the effect of serving a protective role to larvae, in parallel with other factors such as reduction in hexamerin expression and the energy requirement posed by an increase in circulating haemocytes (Figure 6.4).

The observed changes in *gallerimycin* expression 24 hours post infection with non-lethal *A. fumigatus* inocula are particularly interesting as it points to the synthesis of an antimicrobial peptide immune response which serves as an addition to changes in proteomic profile, protein activity, and cellular processes commented upon in this Chapter. In light of the work of Schuhmann *et al*., (2003) it is clear that expression of *gallerimycin* (Figure 6.19) and related peptide genes in other invertebrates is influenced by the detection of a filamentous fungus, to which it is a potent fungicidal agent. This finding is particularly interesting as the work of Schuhmann *et al*., (2003) demonstrated that the *G. mellonella* gallerimycin peptide which shows sequence similiarity with drosomycin and heliomycin showed activity against the filamentous fungi *Metarhizum anisopliae* but demonstrated negligible activity against yeast or bacteria. The authors of this article and Mowlds *et al*., (2010) demonstrate the use of priming larvae with LPS or β-glucan as a means of understanding upregulation of the immune response corresponding to infection with a pathogen subunit. These findings while particularly valuable to advancing the understanding of larval innate immune responses do not

distinguish between different classes of microorganisms and the specifically tailored immune responses required for the effective eradication of the infecting microbe as is demonstrated in the work of Bergin *et al*., (2006). It is therefore postulated that upon infection with *A. fumigatus* conidia a significant increase in the expression of *gallerimycin* selectively through activation of the Toll pathway targets the immune response to the pathogen of interest. Given the immunoprotective effect which these non-lethal doses confer to larvae to subsequent infection and the timing of this response it is clear that a specialisation of the immune response takes place which is not accounted for by injecting with pathogen subunits alone. In interpreting the changes in expression for other genes a note of caution must be sounded. While the changes in expression were found to be statistically significant it may not hold significance at a biological level. For future work it would be necessary to analyse the changes in expression using quantitative PCR (q-PCR).

The data presented in this Chapter demonstrate that larval immune priming responses against *A. fumigatus* appear to peak 24 hours following non-lethal infection. This immune priming effect however appears to reduce slightly at 48 hours. In summary it is proposed that sensing of specific low doses of pathogen mediate protective responses but the scale of this response is not necessarily dose dependent. Injections with 1 x 10^4 conidia result in an increase in the number of circulating haemocytes. As a result increase PPO levels within the haemolymph as PPO is exocytosed during the cellular response (Kavanagh and Reeves, 2004). This process is supported by the enhanced binding activity of apoLp III to conidia and presence of larval haemolymph protein (lhp-1) which functions as an oxygen transporter for the humoral response. As the antifungal priming event takes place anti-bacterial activity of haemolymph is reduced as evidenced by a lowering of anionic antimicrobial peptide 2. There is a notable metabolic shift as demonstrated by increased arginine kinase

expression but a significant reduction in hexamerin and hexamerin receptor which in turn may affect conventional fitness of larvae. However the protective effect further mediated by sequesterisation of iron and increased expression of specific antifungal peptides point to a specific immune response to the pathogen of interest.

Chapter 7

General Discussion

7.1 Discussion

Aspergillus fumigatus is a significant pathogen among the immunocompromised, organ transplant recipients, asthmatics and cystic fibrosis patients (Tekaia and Latgé, 2005). The treatment of ABPA, aspergilloma or invasive aspergillosis has many drawbacks such as the economic cost of chemotherapy, the toxic side-effects of antifungals, the invasive nature of fungal ball removal and the time limit imposed by the rapid growing and debilitating nature of fungal pathogenesis. Additionally the use of corticosteroids in the treatment of ABPA may leave individuals more susceptible to subsequent microbial infection. In recent years the overuse of antifungal chemotherapy coupled to the emergence of azole resistance originally as a result of mutations in the *cyp51A* gene however it is now believed that other genes may be responsible for this (Verweij *et al*., 2009; Bueid *et al*., 2010).

The testing of pathogenic microbes has previously centred on the use of murine models of infection however in recent years larvae of the greater wax moth *G. mellonella* have been demonstrated as an efficient high throughput model of invasive aspergillosis with *A. fumigatus* isolates of attenuated virulence (Slater *et al*., 2010). Such findings support a growing body of work in this area which demonstrated the use of *G. mellonella* as an alternative high throughput model for examining the relative virulence of *C. albicans, Burholderia cepacia* and *Yersinia pseudotuberculosis* (Brennan *et al*., 2002; Seed and Dennis 2008; Champion *et al*., 2009). In Chapter 3 the use of *G. mellonella* as an alternative model to study the pathogenicity *A. fumigatus* isolates was assessed. Incubation of larvae at 37ºC results in lower rates of larval survival than at 30ºC. Mowlds and Kavanagh, (2008) comprehensively demonstrated that incubation of larvae at 37ºC primed the immune response of larvae against subsequent infection relative to incubation at 30ºC. Considering this and the similar survival rates obtained when infecting larvae with 1×10^6 conidia and incubating at

37ºC or infecting with 1 x 10^7 conidia and incubating at 30ºC the latter infection conditions were employed. Screening of *A. fumigatus* AF293 and the gene deletant strains Δ*ftmA*, Δ*psoA* and Δ*laeA* in larvae demonstrated attenuated virulence in all mutant strains. Considering the work of Bok *et al*., (2005) the scale of attenuation of Δ*laeA* was perhaps quite low considering that *laeA* is a global regulator of secondary metabolite biosynthesis and murine data suggested significantly reduced virulence. Infection of larvae with differentially matured conidia of clinical and environmental isolates of *A. fumigatus* demonstrated that larval viability was variable depending on the source of the isolate and the level of conidial maturation. Three day old conidia demonstrated higher virulence than 14 and 28 day old conidia in clinical and environmental isolates. However there was some evidence to suggest that larvae injected with 14 or 28 day old clinical *A. fumigatus* conidia demonstrated slightly reduced viability on average relative to environmental isolates. An analysis of the pathogenicity of *A. flavus*, *A. terreus* and *A. niger* was also performed. *A. flavus* was highly pathogenic in *G. mellonella* larvae and appeared to grow aggressively through the larval cuticle with clear evidence of conidiophore formation. *A. flavus* has been described as an entamopathogen of variable pathogenic potential (Lage de Morales *et al*., 2001). In this study *A. flavus* is demonstrated as severely pathogenic in *G. mellonella*. The reasons for this are not entirely clear however Renwick *et al*., (2006) did demonstrate that conidial size is inversely correlated with phagocytosis. *A. flavus* does demonstrate pathogenicity in humans however it is normally associated with complications of the upper respiratory tract (Pasqualotto, 2009). The validation of *G. mellonella* as an *in vivo* tool to assess the pathogenicity of *A. flavus* complements the work of Scully and Bidochka, (2005) where it was shown that *A. flavus* demonstrated host specialisation in larvae following serially repeated infection. *A. terreus* and *A.*

niger also demonstrated virulence in larvae with *A. terreus* significantly attenuated with increased conidial maturation.

In this study mycotoxin production in 72 hour culture filtrates was assessed in clinical and environmental *A. fumigatus* isolates and revealed a high level of variability among different strains with fumagillin production being higher on average in environmental isolates. Gliotoxin production, while variable between different strains, was statistically similar in clinical and environmental *A. fumigatus* isolates. Watanabe *et al.*, (2003) demonstrated that *A. fumigatus* culture filtrate did not directly result in murine mortality however exposure to culture filtrate and conidia caused increased larval mortality. The results presented in this study demonstrate that injection of culture filtrates from clinical and environmental *A. fumigatus* isolates rendered larvae susceptible to subsequent infection however it is unclear if specific mycotoxins are directly responsible for this. It would appear that culture filtrate mediated immunosuppression is multi-faceted and future work will require a comprehensive analysis of the synergistic effects of *A. fumigatus* virulence factors *in vivo*. *In vitro* growth of *A. fumigatus* has been correlated to *in vivo* virulence (Paisley *et al.*, 2005). In this study clinical and environmental isolates of *A. fumigatus* demonstrated variable growth on AMM and milk agar. Environmental *A. fumigatus* isolates demonstrated better average growth on AMM relative to clinical isolates indicating improved growth on minimal nutrient requirements which may be attributable as a virulence factor. Growth on milk agar while showing isolate variability did not distinguish isolates in terms of protease capacity. It must be cautioned however that this is a relatively small study of the growth of *A. fumigatus* on different media. It is possible that a wider study utilising a greater number of isolates may demonstrate a greater difference between both isolate sources.

Reeves *et al*., (2004) demonstrated that *in vivo* gliotoxin production reached levels of approximately 2400 ng larval tissue g^{-1}. In this study it was demonstrated that gliotoxin production reached similar levels 48 hours post infection. An analysis of *in vivo* fumagillin production demonstrated high levels of mycotoxin production 24 hours post-infection with none detected at 72 hours. This finding was particularly interesting as the production of fumagillin *in vivo* during *A. fumigatus* pathogenesis has not been addressed in previous literature and may be representative of the early phases of invasive fungal pathogenesis.

The significant production of fumagillin after 24 hours prompted a study to investigate the effect of fumagillin on neutrophil function. An investigation of the immunosuppressive effect of fumagillin in *G. mellonella* larvae demonstrated that injection with 2 µg fumagillin 20 µl^{-1} increased larval susceptibility to *A. fumigatus* infection. Fumagillin (2 µg ml^{-1}) significantly reduced the killing ability of neutrophils and haemocytes *in vitro* without inducing necrotic effects. It was notable that killing was not adversely affected by pre-exposing neutrophils or haemocytes to fumagillin however it was evident that the inhibitory effect was not observed at all time-points but was significant 60 minutes post-incubation. Incubation of larvae with fumagillin caused a reduction in phagocytosis coupled to a decrease in oxygen consumption in PMA stimulated neutrophils and haemocytes. Bergin *et a*l., (2005) and Renwick *et al*., (2006) demonstrated sequence and functional similarities between the cytosolic components of neutrophil NADPH oxidase complex and *G. mellonella* haemocytes. The proteomic conservation of the NADPH oxidase complex between the two cell types aided the identification of reduced p47 translocation in haemocytes from the cytosol to the membrane. This complements the findings of Renwick *et al*., (2007) as it would appear that mycotoxin mediated inhibition of NADPH oxidase formation is conserved between both cell types thus extending the similarities between both immune responses. It must

be noted that the data presented here conflict with the findings of Tsunawaki *et al*., (2004). In their work a concentration of 128 µg fumagillin ml^{-1} was required to inhibit superoxide production. It is possible that the methodology used in that paper may have been lacking in sensitivity as 2 µg gliotoxin ml^{-1} was required to inhibit superoxide production. Subsequent work by Renwick *et al*., (2007) demonstrated reduced neutrophil p47^{phox} translocation after exposure to 0.25 µg gliotoxin ml^{-1}. This 8-fold difference coupled to the fact that neutrophils were exposed to mycotoxin for only 7 minutes as opposed to the 25 minute mycotoxin-phagocyte incubation time used in the results presented here may partially explain the results obtained by Tsunawaki *et al*., (2004).

NADPH oxidase formation co-incides with degranulation, with MPO forming a major component of this process. Neutrophils exposed to fumagillin demonstrated reduced extracellular myeloperoxidase following PMA stimulation relative to positive controls. This finding was further supported by the decreased myeloperoxidase mediated peroxidation activity from degranulated extracts using the same protocols. In contrast the process of degranulation in *G. mellonella* haemocytes is poorly understood. Haemocytes incubated with fumagillin demonstrated reduced exocytosis of PPO which was cross reactive with MPO antisera. ClustalW analysis of MPO and PPO revealed amino acid matching, conservation and semi-conservation in peptide sequence alignments however it is unknown if these proteins are related. This may explain antibody cross-reactivity between both proteins however a study of conserved epitope binding would be required to fully understand the reasons for this. Zhan *et al*., (2004) demonstrated that F-actin assembly coupled to the interaction p47^{phox} with moesin initiated the early events of translocation leading to the formation of a fully functional NADPH oxidase complex. The data presented here demonstrate that F-actin assembly was reduced in fumagillin treated neutrophils following PMA stimulation. This in turn

may explain the observed inhibition of translocation as well as the failure of neutrophils to engage in effective phagocytosis. The application of the same method in haemocytes did not demonstrate any change in F-actin assembly irrespective of PMA stimulation of pre-treatment with fumagillin. LC/MS analysis did reveal the increased degranulation of cofilin in PMA stimulated cells however the level of cofilin was significantly reduced in fumagillin treated cells. Adachi *et al*., (2002) demonstrated that reduction of cofilin expression *in vitro* resulted in increased phagocytosis and NADPH oxidase formation thus indicating an antagonistic effect on F-actin formation. It is proposed that increased turnover of F-actin resulting from sequesterisation of cofilin may result in decreased p47 translocation and phagocytosis in haemocytes.

The humoral immune response of insects represents a complex mixture of cell free responses which include clotting, melanisation, production of antimicrobial peptides and proteins which confer host protection either through acting as pattern recognition receptors in support of the cellular response or in detoxification (Kavanagh and Reeves, 2004). In Chapter 5 the effect of fumagillin on the humoral component of *G. mellonella* immune response was assessed. A 2-dimensional electrophoresis coupled to LC/MS approach identified a number of changes in the proteome of larval haemolymph which are indicative of the presence of oxidative stress. Wang *et al*., (2008) and Fang *et al*., (2010) demonstrated the increased expression of peroxiredoxins following oxidative stress *in vivo*. Ito *et al*., (1996) also demonstrated the increased expression of GAPDH in the presence of oxidative stress. The increased expression of GAPDH and the functionally related AHP and PRX proteins in haemolymph obtained from larvae injected with fumagillin is indicative of the presence of such oxidative stresses. Mycotoxin mediated lipid peroxidation has been demonstrated for gliotoxin (Schütze *et al*., 2010) and TNP-470, a synthetic analogue of fumagillin, has also been demonstrated to initiate lipid peroxidation (Okroj *et al*., 2005). The increased

expression of an aldo-keto reductase in fumagillin injected larvae further suggests that oxidative stress as a result of lipid peroxidation is the cause of the increased expression of these proteins. It is possible that the occurrence of lipid peroxidation may cause damage to tissues in larvae which, while not fatal, may weaken host responses through damage to cell membranes. Such a finding has been previously identified in *G. mellonella* larvae following endotoxin exposure *in vivo* (Dubovskiy *et al*., 2008) and demonstrates another element of the harmful effects of fumagillin.

The decreased expression of ferritin in larvae injected with fumagillin may also account for the substantial loss of viability in larvae injected with fumagillin and subsequently challenged with *A. fumigatus* ATCC 26933. Ferritin plays a key role in the sequesterisation of iron *in vivo* and the limitation of free iron to microbes is an important element in reducing growth (Schrettl *et al*., 2004). In Chapter 6 priming of *G. mellonella* larvae with *A. fumigatus* conidia resulted in increased ferritin expression. These two studies clearly demonstrate that ferritin levels are correlated with susceptibility to subsequent fungal infection. This finding is an interesting development in understanding the larval immune response and may have the potential to be viewed as a biomarker of insect immunological status.

The temperature mediated activation of the phenoloxidase pathway independent of microbial infection is an interesting finding. Cerenius and Soderhall, (2004) previously identified that exposure to peptidoglycan, lipopolysaccharide or glucan is required to activate the phenoloxidase cascade. In this study phenoloxidase activity was initiated through larval incubation at 37ºC. In light of the data presented by Mowlds and Kavanagh, (2008) where it was shown that such incubation primed larvae against subsequent infection this finding further exemplifies the issues of incubating larvae at 37ºC thus leading to temperature mediated effects which may lead to erroneous data. The reduced phenoloxidase activity in larval haemolymph following injection with

fumagillin demonstrated another damaging effect mediated by fumagillin. The implication of this is reduced pathogen recognition and exacerbation of fungal colonisation *in vivo*.

A novel approach to understanding the interaction between the larval immune response and *A. fumigatus* conidia was performed through the haemolymph binding assay. The results show the binding of four proteins (apo 1, arylphorin, PPO and apoLp III) of immunological relevance which may explain the initiation of the humoral immune response. Ma *et al*., (2006) and Schmidt *et al*., (2010) demonstrated that apo1 functions in the neutralisation of LPS in insects. In Chapter 6 immunoprotective priming of larvae with *A. fumigatus* conidia resulted in the increased expression of apo 1. The findings from both of these studies are interesting as they indicate that apo 1 may play a role in the limitation of fungal dissemination *in vivo* and that the expression of this protein may be induced through fungal priming thus conferring a protective effect to the host from subsequent infection. The observed interaction between prophenoloxidase subunit 2 and conidia is particularly interesting given the important function of the PO pathway *in vivo*. The activation of the phenoloxidase cascade is a tightly regulated process as quinines are toxic to host tissue (Ashida, 1990). The observed association of prophenoloxidase with *A. fumigatus* conidia may represent another mechanism where the humoral response is localised to specific sites of infection therefore directing melanin production to specific sites of infection. Inhibition of this binding activity in haemolymph exposed to fumagillin may cause dysregulation of immunological cascades therefore leading to impaired responses to infection and the induction of oxidative stress.

ApoLp III and its mammalian homologue apo E have been demonstrated as having conserved functions in innate immune defences to fungal infection. Furthermore Whitten *et al*., (2004) identified that apoLp III was capable of acting as a pattern

recognition receptor to β1,3-glucan. The finding that both apo E and apoLp III bind to *A. fumigatus* conidia complements previous research and may have implications for other immune responses such as cell adhesion, encapsulation, phagocytosis, reactive oxygen intermediate production and prophenoloxidase activation. Incubation of serum or haemolymph with fumagillin resulted in negligible effects on apoLp III and apo E binding however larval priming with immunoprotective concentrations of *A. fumigatus* conidia resulted in increased apoLp III binding activity 24 hours post infection which was directly correlated with increased larval survival. Vonk *et al.*, (2004) demonstrated that apo E deficient mice were more susceptible to *C. albicans* infection. The results presented in Chapters 5 and 6 indicate that the functionally related lipoproteins may play a role in protection against fungal colonisation through their binding efficacy and stimulation of cellular and humoral immune responses in vertebrates and invertebrates.

The application of the same binding technique to serum as was performed with larval haemolymph identified several proteins of interest, two of which (angiotensinogen and immunoglobulin kappa light chain) demonstrated reduced binding following exposure to fumagillin. As both proteins are recognised by neutrophils through the presence of receptors on the cell surface (Gordon *et al.*, 1986; Cutler *et al.*, 1991; Ito *et al.*, 2001) the inhibition of binding in serum exposed to fumagillin implies that neutrophil recognition of a pathogenic target may be reduced by this mycotoxin. The observed binding of apolipoprotein C-III and A-IV also demonstrated the possible roles of apolipoproteins in inflammation and acute phase responses to infections as has been previously described (Khovidhunkit *et al.*, 2004; Kawakami *et al.*, 2008). Future work may need to focus on murine models of infection deficient in these proteins to understand their role in the pathogen pathogenesis of *A. fumigatus*.

The experimental design in Chapter 6 was based on recent developments in the area of immunological priming (Mowlds and Kavanagh 2008; Mowlds *et al.*, 2008;

Mowlds *et al*., 2010). In order to fully exploit the *G. mellonella* model to it is clear that an understanding on the immune response to specific microbes is warranted. The work of Mowlds *et al*., (2010) while invaluable in understanding larval responses to fungal challenge does not discern the specific microbe of interest as glucan is an ubiquitous element of fungal microflora. Infection of larvae with non-lethal concentrations of *A. fumigatus* conidia resulted in protection in a manner that did not increase in a dose responsive manner. Larval priming for 24 hours appeared to elicit the strongest priming relative to priming for 4 hours and 48 hours. The time dependent increase in immune priming is evidenced by increased haemocyte density 24 hours post-infection which decreased at the 48 hour timepoint. Bergin *et al*., (2003) showed that the higher haemocyte densities were indicative of improved larval health. Furthermore infection with pathogenic yeast isolates are directly correlated with decreased haemocyte density. The decrease in circulating haemocytes may be a result of efficient cellular attachment to microbes leaving larvae more susceptible to subsequent infection. This has been previously demonstrated by Bogús *et al*., (2007) as *G. mellonella* larvae were shown to effectively engage in cellular attachment and encapsulation of entamopathogenic fungi. It is proposed that the immunoprotective increases in haemocyte density are initiated by increased cellular detachment from the haemocoel coupled to lower fungal loading therefore resulting in improved competence to subsequent infection. The increase in PPO as quantified by immunoblot and densitometric analysis may also be linked to this process. PPO binding to *A. fumigatus* conidia was demonstrated in Chapter 5. The reduced levels of PPO detected by Western blotting following infection with 1×10^6 *A. fumigatus* conidia may be indicative of increased binding to *A. fumigatus* conidia. Furthermore the breakdown of PPO at 48 hours may be as a result of the same temperature mediated activation of the PO pathway observed in Chapter 5 as the normal storage temperature of *G. mellonella* is 15ºC.

RP-HPLC and 2-dimensional electrophoresis of larval haemolymph coupled to LC/MS analysis identified several alterations in the metabolic and immunological status of *G. mellonella* larvae following conidial priming. The increased expression of larval haemolymph protein (electronically annotated as an oxygen transporter) in larvae injected with 1×10^4 conidia is indicative of the increased demand for oxygen in haemocyte mediated superoxide production in response to haemocyte activation as demonstrated by Renwick *et al*., (2007). Anionic antimicrobial peptide 2 has been described as having antibacterial activity (Cytrnska *et al*., 2007). The decreased expression of this peptide in larvae challenged with conidia indicates a reduction in immunocompetence to bacterial infection possibly through differential signalling through the Toll pathway (Lemaitre *et al*., 1996). This tailoring of the immune response is further evidenced by the previously discussed increases in ferritin and apo 1 expression thus leading to effective protection against fungal infection. A change in the metabolic profile of *G. mellonella* larvae is evidenced by the increased expression of arginine kinase (AK). As AK functions in ADP phosphorylation in invertebrates (Kotlyar *et al*., 2000) the discovery of a homologous protein in *G. mellonella* haemolymph may indicate an increase in ATP output in response to immune priming thus supporting the cellular and humoral responses to infection. RT-PCR analysis of larvae challenged with *A. fumigatus* conidia demonstrated a significant dose responsive increase in *gallerimycin* expression following conidial challenge. Schuhmann *et al*., (2003) identified that the gallerimycin peptide is specifically active against filamentous fungi. In *Drosophila* a homologous protein drosomycin is expressed through activation of the Toll pathway in response to fungal infection (Lemaitre *et al*., 1996).

The results presented in this thesis clearly demonstrate the benefits of using the *G. mellonella* model as a high throughput model to assess the pathogenicity of *Aspergillus* isolates, the production of mycotoxins *in vivo* and the immunosuppressive

effects of culture filtrates. This study has demonstrated a possible role for fumagillin in the pathogenesis of *A. fumigatus* as a mycotoxin that is produced in the early stages of invasive fungal colonisation. Furthermore it was found that fumagillin inhibited the function of neutrophils and haemocyte thus extending the parallels between both cell types. Fumagillin elicited inhibitory effects on cellular killing through restrictions to phagocytosis, NADPH oxidase complex formation and degranulation. It is speculated that these effects may be as a result of a diminution in F-actin formation. In support of this a comparative study of cell-free haemolymph and human serum demonstrated the decreased expression and activity of elements in the humoral immune response following exposure to fumagillin. Previous mycotoxin research in *A. fumigatus* has focused on the *in vivo* production of gliotoxin. As gliotoxin is a highly potent molecule this approach is merited however it is evident that a wider approach to mycotoxin production in the study of *A. fumigatus* pathogenesis is warranted. Furthermore a re-evaluation of the functions of serum and haemolymph proteins may be required.

The immune response of *G. mellonella* larvae to fungal infection was also addressed. Larvae were effectively primed against subsequent infection following infection with low concentrations of *A. fumigatus*. The application of proteomic methods demonstrated a role for several proteins involved in pathogen recognition, immunological cascades and macromolecule storage culminating in the specific tailoring of the larval immune response. These findings are a valuable addition to a growing body of information validating the use of *G. mellonella* as an alternative model in the study of human pathogens.

7.2 Future work.

The work presented in this thesis has revealed future area of work which needs to be addressed. The use of the *G. mellonella* model of infection is clearly a valuable

tool in the assessment of the pathogenicity of *A. fumigatus*. Although there is a growing body of work correlating murine pathogenicity with that of larvae more work needs to be done to describe the development of genetic mutants of *A. fumigatus* in larvae. Also there is a requirement to develop an alternative infection method with *A. fumigatus* conidia. *A. fumigatus* infections are not initiated at an invasive level therefore a natural method of infection possibly through feeding conidia to larvae needs to be developed. This thesis demonstrates the application of organic extraction coupled to RP-HPLC analysis to the identification of the presence of mycotoxins *in vivo*. The multi-faceted nature of virulence factors of *A. fumigatus* and gene deletant strains of the fungus may be assessed for production *in vivo* using *G. mellonella* therefore offering further insights into the pathobiology of the fungus.

The identification of fumagillin as an immunosuppressant is an area which requires further research. To date there has been a surprising lack of literature outlining a pathway for the biosynthesis of fumagillin aside from the finding that laeA functions as a regulator of its production. It is suggested that future work should be directed towards the identification of such a pathway in the manner that has been applied for gliotoxin. In addition the production of fumagillin at the early stages of invasive aspergillosis should also be addressed in light of the findings of high levels of fumagillin 24 hours post-infection in *G. mellonella*. It may be possible that fumagillin may also be produced in the early stages of invasive aspergillosis in mammals and the application of murine infection coupled to biochemical analysis of its prevalence in vivo may offer an additional insight to the understanding of interaction between mycotoxins and host tissue. It is also suggested that the application of neutropenic or chemically immunosuppressed mice may be of worth in understanding these interactions. To date the entry of fumagillin into the cell is an area which has not been addressed. While such

mechanisms have been identified in gliotoxin uptake the identification of the cellular target of fumagillin would offer further insights into this molecule.

This thesis has identified several proteins or peptides which are expressed differentially depending on the exposure of larvae to fumagillin or low levels of fungal infection. Currently, the genome sequence of *G. mellonella* has not been made publicly available. Considering the substantial use of *G. mellonella* in studies of microbial pathogenesis and the potential offered by applying modern molecular and proteomic techniques there is an increasing need for such a database. The identification of protein homologues with other species in this thesis further validates this urgent requirement in the understanding of *G. mellonella* and would substantially increase scientific output in this area of research.

Chapter 8

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