Characterisation of the *G. mellonella* cellular innate immune response and a comparison with the human neutrophil

A thesis submitted to the National University of Ireland, Maynooth for the degree of Doctor of Philosophy
by

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Declaration

This Thesis has not been submitted in whole or in part to this or any other university for any degree, and is the original work of the author except where otherwise stated.

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Abbreviations

Abs	Absorbance
AMP	Anti-microbial Peptide
Approx.	Approximately
APS	Ammonium persulphate
ß	beta
bp	Base Pair
BSA	Bovine serum albium
°C	Degrees centigrade
cm	Centimeter
DEPC	Dietyl pyrocarbonate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide 5'-triphosphate
DTT	Dithiothreitol
DPI	Diphenyleneiodonium Chloride
EDTA	Ethylene diamine tetra acetic acid
FACs	Fluorescence Activated Cell sorter
FMLP	N-formyl-methionine- leucine-phenylalanine
g	Grams
hr	Hours
HCL	Hydrochloric acid
HP	High Power
IAA	Iodoacetamide
IEF	Iso-electric focusing
IgG	Immunoglobulin G
kDa	Kilodaltons
L	Litre
M	Molar

MALDI ToF	Matrix-laser desorption/ionization-time of flight
mbar	Millibar
mg	Milligrams
mins.	Minutes
mL	Millilitre
mM	Millimolar
MPO	Myeloperoxidase
mRNA	Messenger ribonucleic acid
µg	Microgram
µL	Microlitre
µM	Micromolar
NADPH	Nicotinamide Adenine Dinucleotide Phosphate (reduced form)
ng	Nanogram
nm	Nanometer
OD	Optical Density
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PMA	Phorbol 12-myristate 13-acetate
PMSF	Phenylmethansulfonyl fluoride
PPO	Prophenoloxidase
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
RT	Reverse transcriptase
SDS	Sodium dodecyl sulfate
sec.	Seconds
SEM	Scanning electron microscopy
SOD	Superoxide dismutase
spp.	Species
TBS	Tris buffered saline

TBST	Tris buffered saline-tween
TCA	Trichloroacetic acid
TEMED	N,N,N',N'-Tetramethlyethlenedimine
temp.	Temperature
TFA	Trifluoroacetic acid
U	Unit
UV	Ultraviolet
V	Volts
v	Volume
v/v	Volume per volume
w/v	Weight per volume
YEPD	Yeast Extract-Peptone-D-Glucose

Abstract

The use of insects as alternative model organisms in areas of research such as pathogen virulence studies, the development of novel therapeutics and the screening of microorganism strains are increasing. Some of the reasons for this are the lower cost of insects, results are often acquired very quickly with insect experiments and there are relatively few ethical issues involved with the use of insects compared to the ethical issues that are involved when using the mammalians.

Larvae of the Lepidopteran Greater Wax Moth, *Galleria mellonella* are used extensively in biological and chemical research. *G. mellonella* larvae possess an immune cell, the haemocyte, which is capable of participating in many of the processes that the human innate immune cell, the neutrophil participates in. The haemocyte and the neutrophil are compared to each other throughout this project in a bid to further elucidate similarities between the human innate immune system and the insect immune system.

Experimental standardizations are studied and optimized in this work in order to characterize ideal conditions for *G. mellonella* larvae to be used in the laboratory and to study the effect that the nutritional status of the insect has on it's ability to fight fungal infections. This was carried out by employing bioassays, proteomic studies and by employing the molecular biology technique, Real Time PCR.

This project also studied a number of similarities between the two immune cells, such as phagocytosis, a crucial component of the immune system's first line of defense. Proteomic similarities between the haemocyte and the neutrophil were studied extensively throughout this work, different techniques were utilised throughout, to identify proteins of interest in the haemocyte. These techniques included SDS-PAGE, mass spectrometry, Western blotting, confocal microscopy and phospho-proteomic staining techniques.

The cytoskeletal properties of the haemocyte were also studied and compared to the neutrophil cytoskeleton due to it's importance in killing processes such as reactive oxygen species production, phagocytosis and pseudopodia production. Work in this Thesis documents the separation of the *G. mellonella* larvae haemocyte population into sub-populations that differ to each other in size and granularity by employing Flow cytometry cell sorting techniques. It is possible

that one of these sub-populations is more similar to the human neutrophil than the others. The presence of granules in the haemocyte was also studied, particularly the presence of MPO-like and lactoferrin-like material.

This work outlines many parallels between the human innate immune system and the immune system of *G. mellonella* larvae and the results demonstrated throughout this Thesis further justify the use of insects as alternative model organisms.

Chapter 1

Introduction

1.1 Insects as Model Organisms

A model organism may be thought of as a species that is widely studied in developmental, genetic or experimental biology in order to understand how particular biological or biochemical processes occur. In most cases the choice of the model organism is dictated by experimental efficiency, cost and the timeline that is involved in a particular project (Hedges, 2002). The idea of using a model organism in an experiment to study human biology is that all organisms on earth most likely share a common ancestor (Medzhitov *et al.,* 2008).

Classically, mammalian models were used, such as rodents, feline and simian models, however due to the costs that are involved, the limits with research project timelines and the ethical challenges involved with mammals more researchers have begun to use insects as their model organism of choice (Heemskerk *et al.,* 2002). In recent years there has been an increase in the use of insects as alternative model organisms to study human pathogens and also to study the toxicity of drugs and compounds (Scully *et al*., 2006). There are many reasons for the increasing use of insects, the advantages include the lower cost involved with insects, the efficiency with experimental timelines, insects can be easily managed in the laboratory and experiments can be carried out in much higher numbers than with mammalian models (Mylonakis and Aballay, 2005). One of the advantages of using larger numbers in the experiment is that it leads to a better statistical outcome. With regard to the ethical concerns involved in experimental research, there are also fewer concerns with using an invertebrate model as opposed to using a mammalian model (Fuchs *et al.,* 2010).

Probably the most widely studied insect is *Drosophila melanogaster.* Initially *D. melanogaster* was utilised to study Mendelian genetics (Schneider, 2000). Subsequent to these findings, *D. melanogaster* were found to have a homologous signaling transduction pathway to mammals (Imler and Hoffmann, 2005). Mammals possess a number of cellular receptors known as the Toll like receptors; these are homologous to *Drosophila* Toll receptors. In *Drosophila*, the binding of Toll to it's ligand, Spatzle, stimulates the activation of antimicrobial protein expression, through a pathway known as the Toll-Dorsal signaling pathway (Hoffmann *et al.,* 1999).

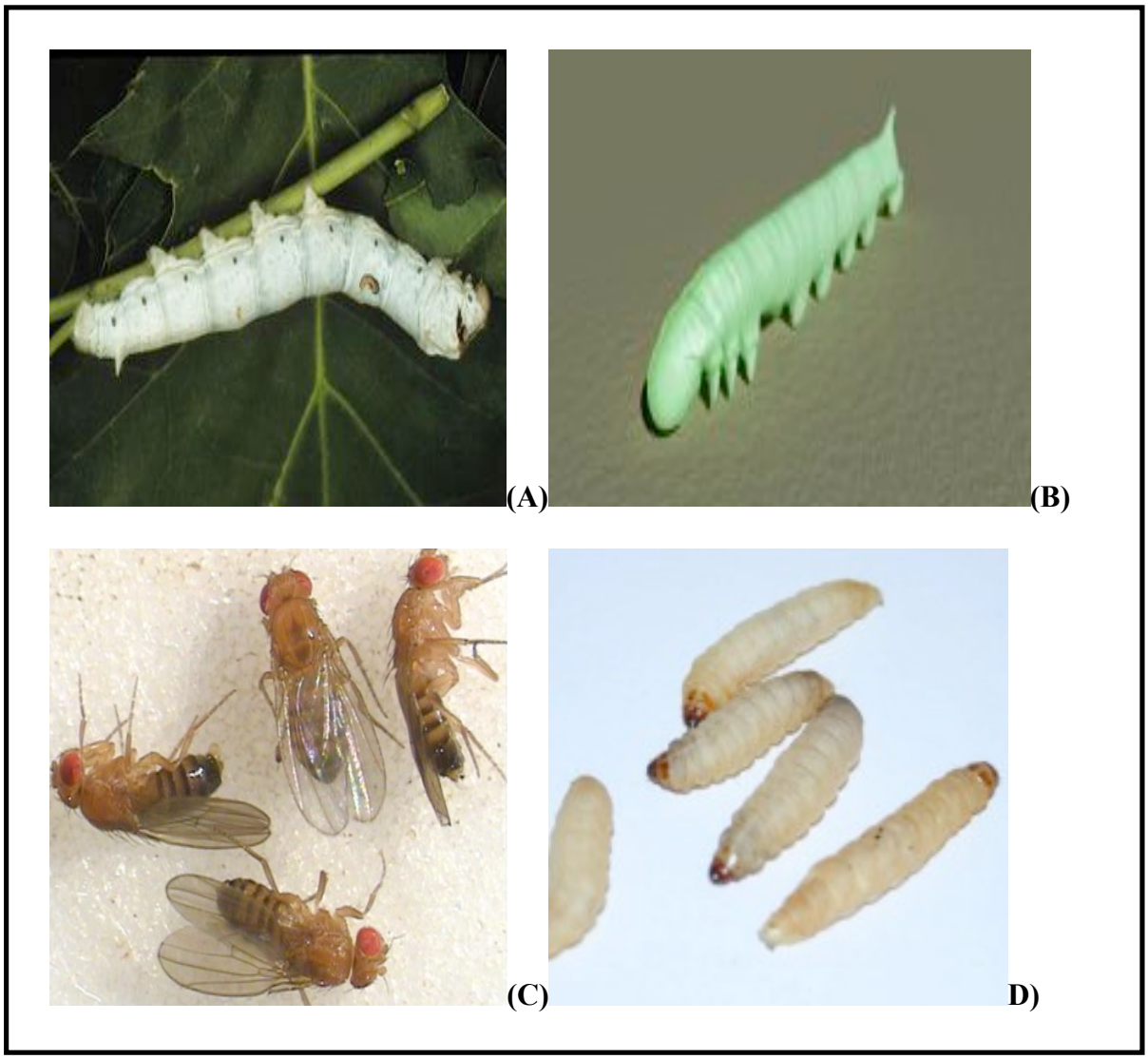


Figure 1.1. Examples of the insects used as model organisms in research (A) The silkworm; *Bombyx mori,* (B) The hornworm; *Manduca sexta,* (C) The fruit fly; *Drosophila melanogaster,* (D) Larvae of the Greater Wax moth; *Galleria mellonella.*

(A) Sourced from http://www.disoverlife.ore

(B) Sourced from http://www.science.nichd.nih.gov

(C) Sourced from http://www.ncbi.nlm.nih.gov

There have been many parallels found between the *Drosophilia* Toll pathway and the mammalian immune signaling cascade that is induced by Interleukin-1 (Hultmark, 1994). Interestingly, the remarkable similarity between human genes and the genes from *D. melanogaster* is known to be so close that many newly studied human genes are now cross-studied against *D. melanogaster*. *D. melanogaster* have also been utilised in biology as model organisms to study areas such as genetics and developmental biology.

Another interesting and widely used insect model is the domesticated silkworm, *Bombyx mori. B. mori* are employed as model organisms for many of the previously highlighted reasons, such as their ease of rearing, low cost, but also due to the importance of *B. mori* in silk production, which is of world-wide economic importance (Li *et al.,* 2009). *B. mori* have been used in fundamental studies of hormones, pheromones, in fact the first known pheromone, bombykol, was characterised from *B. mori* (Sandler *et al.,* 2000).

Manduca sexta is the tobacco hornworm. It is well known in developmental biology for it's large size (can measure up to 5cm) which can facilitate ease in dissection studies to analyse bacterial infections (Silva *et al.,* 2000).

The validity of using insects as models in biological research is further substantiated by parallels that have been outlined between the immune response of insects and the innate immune response of humans (Kavanagh and Reeves, 2004; Zhao and Kanost, 1996). There exists many anatomical similarities such as the insect's cuticle, which serves as a primary physical barrier and this is analogous to the skin (Maeda, 1996). The circulating haemolymph that is contained within the body cavity of the insect is functionally similar to human blood. This transports nutrients, amino acids, vitamins and the cellular immune components (Matha and Mracek, 1984). However, insects do not possess a vasculatory system. The insect midgut contains microvilli that are analogous to intestinal microvilli in humans, the microvilli are where toxins produced from pathogens can bind receptors and an immune response is initiated (Scully and Bidochka, 1996). Another similarity with human innate immunity is the insect's immune cell, the haemocyte, which has the ability to recognize and phagocytose microbes, and this cell is similar to the human neutrophil (García-García *et al*., 2009). The above points underline the authenticity of the use of insects as model organisms and the advantages of the

model as opposed to the traditional mammalian model. Some examples of insect model organisms can be seen in Figure 1.1.

1.1.1 *Galleria mellonella*

The larvae of *G. mellonella*, the greater wax moth or honeycomb moth, is another insect that has recently emerged as an alternative model organism in research and development. *G. mellonella* larvae are a pest of bees hives and they feed on the honeycomb that is produced inside the hives. In some cases they have also been found in wasp nests (Freitak *et al*., 2007). Classically *G. mellonella* were known for their use as fishing bait but have recently been used in biological research as models, much like the insect models mentioned previously. *G. mellonella* larvae provides a simple, yet effective tool for studying many pathogens, such as bacteria and fungi, but they are also useful in the study of the development of novel therapeutic agents (Rowan *et al*., 2009).

G. mellonella larvae are useful as model organisms due to their low cost, their availability from suppliers and *G. mellonella* larvae can survive at mammalian temperatures (37°C) and at room temperature (Cotter *et al.,* 2000). They are easily inoculated by hand with a small syringe and do not need to be propagated in the lab. *G. mellonella* larvae may also be inoculated topically or by oral inoculation. Another advantage of using *G. mellonella* larvae is that because of their natural habitat, which is beehives, they encounter many microbes and this has led to the evolution of a sophisticated immune response (Fuchs *et al*., 2010).

The experimental findings with studies of *G. mellonella* larvae have also yielded extraordinarily similar results to those found in parallel studies with mammalian models (Brennan *et al*., 2002; Renwick *et al*., 2007). Pathogen virulence in *G. mellonella* can be easily measured by observing larval survival over a known time period. *G. mellonella* larvae's amenability to infection combined with it's evolved immune response makes it a highly attractive biological research model organism (Mukherjee *et al.*, 2010). Inoculation of *G. mellonella* larvae can be seen in Figure 1.2.

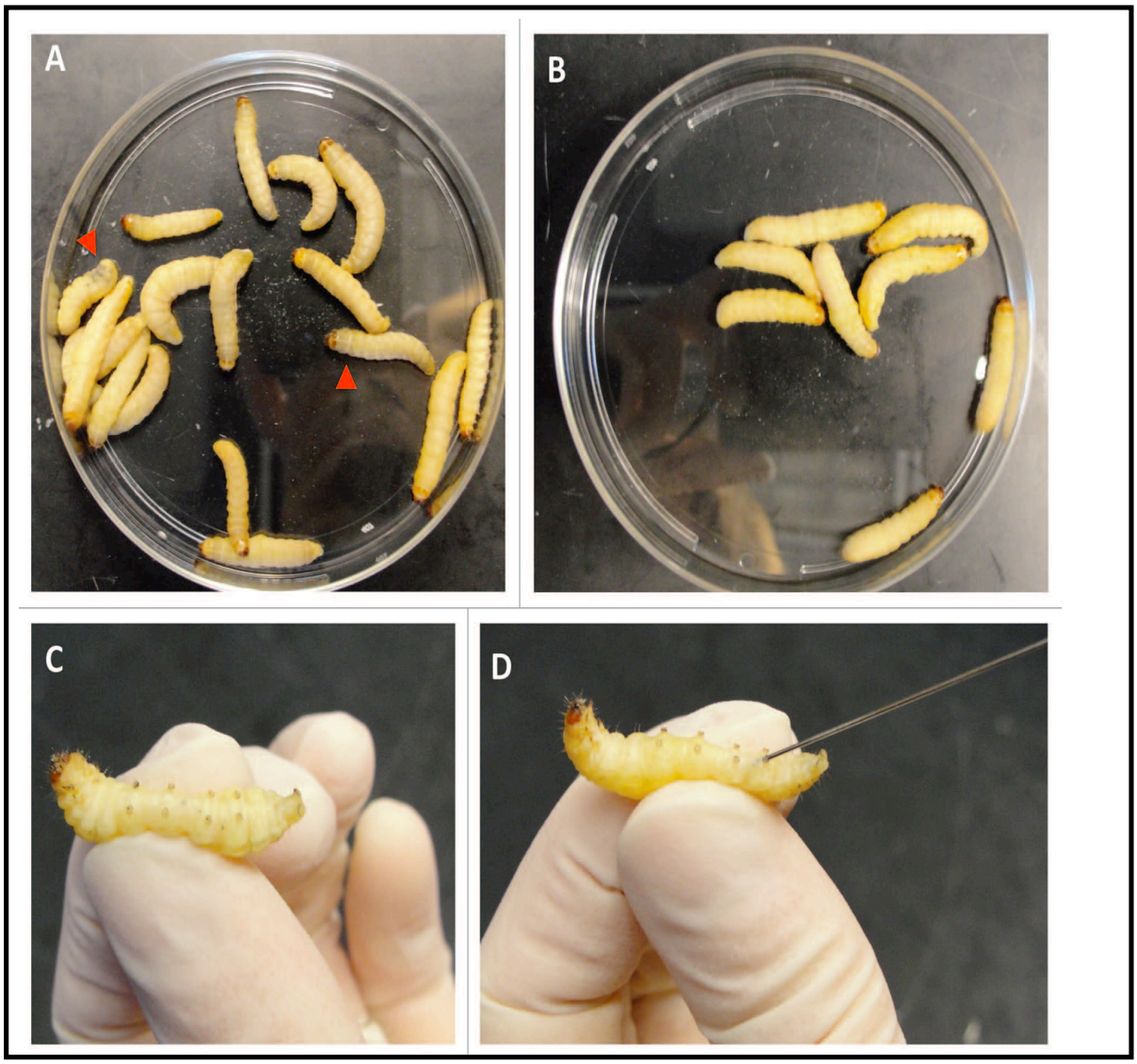


Figure 1.2 *G. mellonella* larvae acquired from suppliers are various weights and may show different health status (A), The larvae that are chosen for experiments should show no signs of cuticle melanisation (no visible dark spots or pigmentation on cuticle) as this could be a sign of an underlying infection (B), Larvae can be easily injected with a myco-syringe through the last left pro-leg (C) + (D).

Image sourced from Fuchs *et al.,* 2010.

1.1.2 Studies using *G. mellonella* larvae as model organisms

G. mellonella larvae have been used as reliable models to study many pathogens and also to test the toxicity of novel therapeutic compounds. Many of the aspects of the immune response of *G. mellonella* larvae are extremely similar to those in mammalian models and for this reason the use of *G. mellonella* larvae is growing, along with the cost and efficiency advantages that were outlined in the previous sections. *G. mellonella* larvae have been utilised in virulence studies of bacteria and fungi, where it has been shown that the virulence mechanisms and mutant strain pathogenicity of the microbes in *G. mellonella* larvae parallels the results observed in studies using vertebrate models (Brennan *et al*., 2002; Seed and Dennis, 2008).

Listeria monocytogenes is an extremely virulent food borne pathogen and can lead to advanced septicemia in immunocompromised individuals and to abortions in pregnant women. Mukherjee *et al.,* (2010) demonstrated that *G. mellonella* larvae could be utilised as a model to study the virulence of *L. monocytogenes* and various mutants of the strain. The experimental results that were obtained paralleled those with mice. The ease of larval inoculation and the ability to use large numbers of larvae in the studies (ultimately allowing accurate statistical analysis) were also noted.

Burkholderia cepacia and *Pseudomonas aeruginosa* are opportunistic pathogens that are the primary infectious agents in patients with cystic fibrosis (CF), (Seed and Dennis, 2008). Seed and Dennis, (2009) further utilised *G. mellonella* larvae as a model in the study of alternative therapies for *B. cepacia* infections. It was found that bacteriophage therapy was a successful replacement to antibiotic therapy for *B. cepacia* infections in the larvae. This study provided evidence for the replacement of antibiotic therapies with bacteriophage therapies in patients with a *B. cepacia* infection. Ryan *et al.,* (2009) studied intraspecies signaling in *B. cenocepacia* (closely related to *B. cepacia*) by using *G. mellonella* larvae as the infection model. Lander *et al*., (2000) demonstrated correlating results between the pathogenesis of clinical isolates of *P. aeruginosa* in murine models and in *G. mellonella* larvae. Ryan *et al.,* (2009) also effectively characterised the virulence of *P. aeruginosa* mutants in *G. mellonella* larvae.

Similarities have also been identified between the virulence of *Candida albicans* mutants in *G. mellonella* larvae and the virulence of these mutants in BalbC mice (Brennan *et al.,* 2002). Other human pathogens that have been studied and

assessed using *G. mellonella* larvae are *Aspergillus fumigatus* and *Aspergillus flavus* (Reeves *et al*., 2004; Scully and Bidochka, 2005). It has been found that the pathogenicity of the *Aspergillus* spp. in mammalian models matches the pathogenicity in *G. mellonella* larvae.

Francisella tularensis is one of the most infectious bacterial pathogens known and is the causative agent of tularemia. Tularemia has an 80% fatality rate and is a Class A biothreat. *G. mellonella* larvae have effectively been used as an invertebrate host system to study the bacterial pathogenesis of *F. tularensis*, and to study the efficacy of anti-bacterial compounds (such as ampicillin and streptomycin) on the pathogen (Aperis *et al*., 2007).

G. mellonella larvae have been employed to study the efficacy of novel therapeutics compounds such as silver biometal complexes (Rowan *et al.,* 2009). Cowen *et al*., (2009) used *G. mellonella* larvae successfully as a model to study Hsp90 inhibitors as novel therapeutic agents against fungal pathogens such as *C. albicans*. *Staphylococus aureus* and methicillin resistant *S. aureus* can be lethal in individuals, especially those who are immunocompromised. Desbois and Coote, (2011) utilised *G. mellonella* larvae as an alternative *in vivo* model for testing the efficacy of anti-staphyloccal antibiotics on the bacteria, such as daptomycin and vancomycin.

Many bacteria have the ability to grow and disperse by the production of biofilms, Brackman *et al*., (2011) synchronized quorum sensing (QS) inhibitors (quorum sensing is a strategy involved in the production of biofilms) to antibiotics in order to analyse the affect on the growth of bacteria such as *P. aeruginosa*. The *in vivo* model that the group effectively utilised was *G. mellonella* larvae. It was found that a significantly higher number of *G. mellonella* larvae survived bacterial infection with this synchronized treatment of conventional antibiotics and QS inhibitors.

The increasing volume of studies using *G. mellonella* larvae as a prime model for analysing a number of infectious and opportunistic pathogens virulence mechanisms underlines the insects importance in current biological research.

G. mellonella larvae's ability to be used successfully and efficiently as an alternative research model both biologically and chemically further supports future research into the similarities that exist between *G. mellonella* larvae's immune response and the immune response of humans. *G. mellonella* larvae provide a means

of studying mammalian pathogens at mammalian temperatures and the larvae provide a facile approach to evaluating the effect of novel therapeutics on various pathogens.

1.1.3 The immune response of *G. mellonella* larvae

The *G. mellonella* immune response consists of both humoral and cellular components. The humoral defense mechanism is composed of soluble molecules such as antimicrobial peptides (AMPs) and proteins that are similar to the mammalian complement proteins (Vogel *et al*., 2011). The humoral defense mechanism also encompasses signaling cascades that lead to processes such as melanisation and clotting (Gillespie *et al.*, 1997). The cellular immune response leads to phagocytosis and the production of reactive oxygen species.

Antimicrobial peptides have been found to be highly conserved through species (Hoffmann *et al.*, 1999) and act on pathogens in two ways, they are either immunomodulatory and/or are bacteriocidal agents. The antimicrobial peptides are usually produced in the larval fat body, found in the mid-gut of *G. mellonella*, and circulate in the haemolymph (Gillespie *et al.,* 1997) . These include lysozyme; which breaks down bacterial cell walls, cecrophins; which have been found to also attack bacterial cell walls, defensins; these peptides attack Gram positive bacteria and apolipophorin 3, which has been identified to bind components of bacterial cell walls such as lipopolysaccarides (LPS) (Ratcliff, 1985; Kavanagh and Reeves, 2004).

AMPs have also been demonstrated to be produced and stored in the immune cell of *G. mellonella* larvae, the haemocyte (Strand, 2008). Apolipophorin 3 has been found to act in many different ways in the immune response of *G. mellonella* larvae (Zdybicka-Barabas & Cytryńska, 2010). It can bind to and inhibit many of the components of bacterial and fungal cells walls (such as LPS and glucan). It has also been found to stimulate phagocytosis in the plasmatocytes of *G. mellonella* larvae, it has been identified to induce haemocyte adhesion during encapsulation and to activate the prophenoloxidase cascade (Wiesner *et al.,* 1997; Whitten *et al*., 2004).

The melanisation of a wounded insect is a result of the activation of the prophenoloxidase cascade, prophenoloxidase is in turn activated by serine proteases (Gillespie *et al.*, 1997). This process leads to the production of compounds such as quinones, which indirectly contribute to the production of ROS, this environment is

cytotoxic for pathogens (Nappi *et al.,* 1999). Prophenoloxidase is present in larval haemocytes, haemolymph and the cuticle and it is important in the immune response of the insect. Melanisation is an important mechanism in processes such as wound healing and in pathogen sequestration. It functions to harden wound-healing clots in the epidermis (Theobald *et al*., 2004).

1.1.4 The cellular component of *G. mellonella* larvae's immune response; the haemocyte

The cellular defence mechanism of *G. mellonella* larvae is comprised of different subpopulations of haemocytes. Haemocytes have the ability to recognize a variety of non-self particles through complex signaling cascades (Sideri *et al*., 2007). Changes in haemocyte concentrations and cellular morphology can determine the effect that an infection is having on larvae (Mowlds *et al.,* 2010). The recognition of non-self material is made through either direct interaction of surface receptors on the haemocyte with molecules on the invading particle (such as LPS and zymogen). Haemocytes may also recognize non-self material by the indirect interaction of the humoral immune response to that particle, the humoral immune response can opsonise the material by the binding of circulating antimicrobial peptides to the material (Hoffmann, 1994). It has been known for many years that there exists more than one type of haemocyte in *G. mellonella* larvae. These different subpopulations most likely perform different tasks in the haemolymph, such as phagocytosis, encapsulation and nodulation (Lackie, 1988).

The process of phagocytosis in insects is known to be lectin mediated and is similar to what occurs in the human neutrophil (Li and Christensen, 1992). Phagocytosis is an extremely effective defense mechanism against invading material. It is also highly conserved between species. It is thought that invertebrate phagocytosis is induced by substances such as bacterial cell wall components (Rohloff *et al.,* 1994). Phagcoytosis has been visualised in haemocytes of *G. mellonella* larvae microscopically, it involves the engulfment of non-self particles by the extension of pseudopodia and the formation of vacuoles, in a manner that is extremely similar to phagocytosis by mammalian neutrophils (Wiesner, 1996). In order for the haemocyte to allow phagocytosis to occur, there must be a number of cellular processes that occur, such as cellular attachment, recognition, activation of

signaling cascades, actin polymerization and ultimately particle engulfment (Strand, 2008).

Encapsulation involves the binding of haemocytes to non-self particles such as microbes and forming layers of haemocytes around these non-self particles. The particles are then destroyed by the release of toxic compounds from the haemocytes, such as reactive oxygen species (Lavine and Strand, 2003). Recently, the antimicrobial peptide apolipophorin 3 has been identified to stimulate the encapsulation process in *G. mellonella* larvae (Whitten *et al.*, 2011). A related process to encapsulation is the formation of nodules around non-self material. Nodules are large aggregates of haemocytes that form a bundle of extracellular material around non-self particles, it is thought that the LPS produced from bacteria stimulate the formation of nodules. Subsequent to nodule formation, the aggregates are then encapsulated and broken down (Whitten *et al.*, 2004). There is likely to be a chemical signaling relationship between the processes of encapsulation and nodule formation in the insect's haemolymph, they are also tightly linked to the humoral response in *G. mellonella* larvae (Anngrgraeni and Ratcliff, 1991).

In the larval stage of Lepidoptera it is likely that there are four morphologically distinct subpopulations of haemocytes (Lavine and Strand, 2002). The total haemocyte population is thought to be composed of granular cells (granulocytes), plasmatocytes, oenocytoids and spherulocytes (Ribeiro and Brehelin, 2006). The maturation of these different sub-populations of haemocytes occurs in a process known as haematopoiesis (Nardi, 2004).

Lepidopteran haemopoietic organs are found in the meso and metathorax of the insect and haematopoiesis occurs during insect embryogenesis. These organs are the primary sources of prohaemocytes and plasmatocytes (Ling *et al.,* 2005). Circulating granulocytes, oenocytes and the spherulocytes most likely derive from the prohaemocytes that are already in circulation in the haemolymph (Nardi *et al.,* 2003). There have been similarities identified between the development of haemocytes in Lepidopteran insects and those that are known to occur in *D. melanogaster,* which essentially suggests that there exists a conserved mechanism of haematopoiesis (Strand, 2008). However there are still areas of molecular and signaling cascades in Lepidopteran haematopoiesis development that are unknown (Lavine and Strand, 2001).

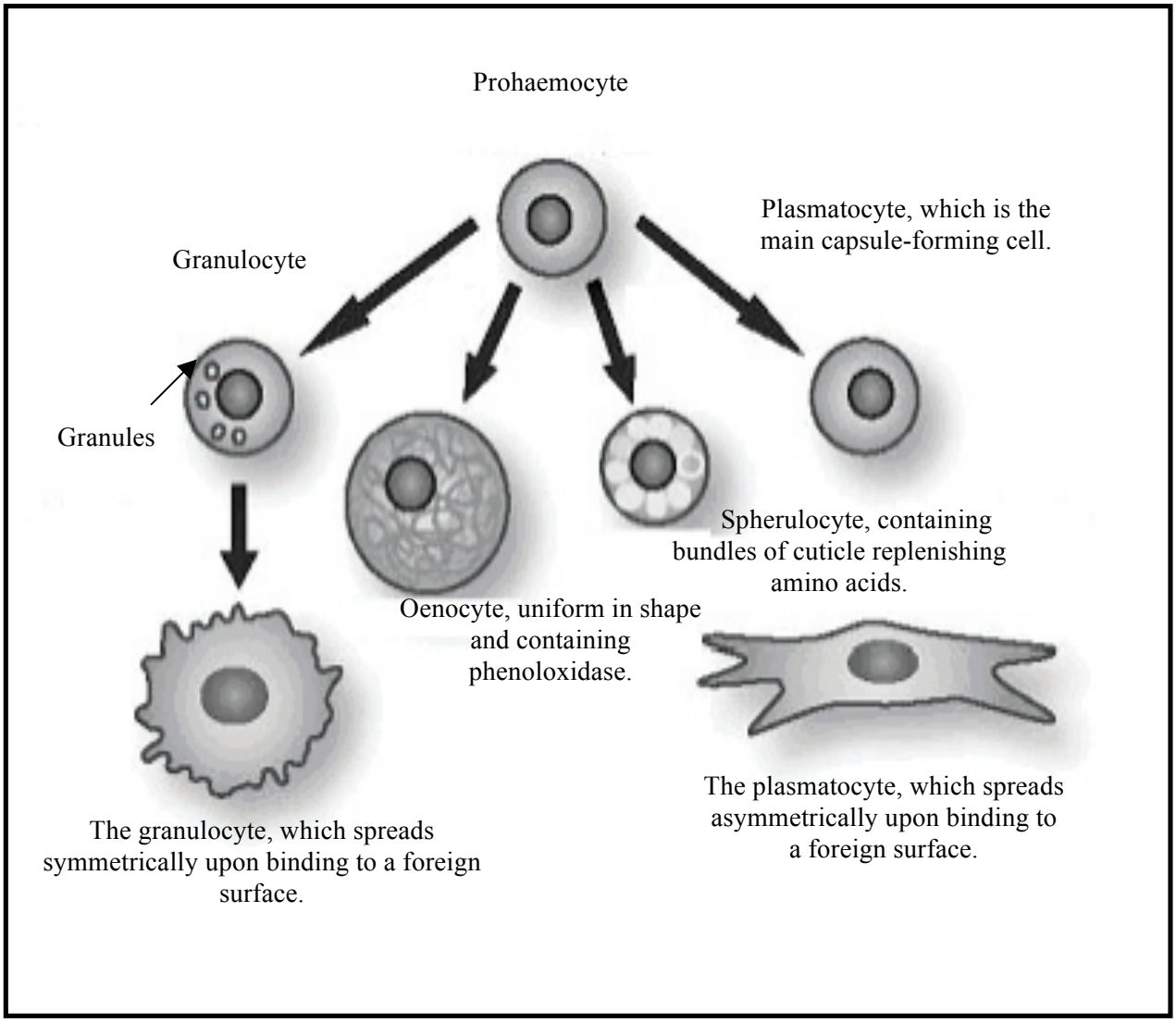


Figure 1.3 Hypothesised model for the process of haemocyte sub-population development in *G. mellonella* larvae.

Adapted from Strand, 2008.

1.1.5 The different functions of the haemocyte subpopulations

The granular haemocytes of *G. mellonella* larvae are the most abundant in the haemolymph. They are known to be strongly adhesive to surfaces and are the predominant phagocytic cells in *G. mellonella* larvae. Morphologically granulocytes are circular in shape. Besides their ability to phagocytose, the granulocytes have also been identified to play a role in the encapsulation process, they appear to be randomly distributed with the plasmatocytes (Strand *et al.,* 2006). Granulocytes of *G. mellonella* larvae have been identified forming the outer layer of the haemolymph clots that leads to extracellular wound healing (Theobald *et al.,* 2004).

The important process of encapsulation in Lepidoptera is mediated by the plasmatocytes. Plasmatocytes are shaped similarly to the granulocyte, the cell is rounded and plasmotocytes have been demonstrated to spread asymmetrically on surfaces. Plasmatocytes attach to the foreign particles in large numbers and there are indications that there is a complex signaling relationship between granulocytes and plasmatocytes in order to recruit the plasmatocytes to areas of infection, or perhaps to a particle that is too large for the granulocyte to phagocytose (Liu *et al.,* 1998). The plasmatocytes are the primary cell involved in encapsulation. The oenocytoids and spherulocytes are the cellular components that participate in events such as the prophenoloxidase cascade and they are also suggested to be the important amino acid storage cells in the haemolymph, containing cuticle replenishing components (Lavine *et al.,* 2005). These cells are non-adhesive and have also been shown to contain lysozyme, an important defense mechanism enzyme that can break down bacterial cell walls. The oenocytoid is the principal cell containing phenoloxidase, phenoloxidase functions in cuticle repair and in the melanisation process (Castillo *et al.,* 2006). It is well documented that the number of haemocytes in circulation will change rapidly once infection/stress occurs (Kavanagh and Reeves, 2007; Bergin *et al.,* 2005), this is a defined marker of stress in the insect and is utilised in studies of pathogen virulence and drug toxicity screening (Mowlds *et al.,* 2010; Fuchs *et al.*, 2010).

Recognition of the pathogen by the haemocyte in Lepidopteran insects is dependent upon signal recognition responses to molecules such as LPS, bacterial glucans and Gram-negative bacteria recognition proteins. These bind to receptors such as LPS-binding protein and Gram-negative bacteria recognition proteins (Strand, 2008; Dong *et al.,* 2006). The activation of these receptors initiates processes such as

phagocytosis and engulfment (Chen *et al.,* 2005). It is very likely that the activation of these cascades results in fluctuations in the sub-populations of haemocytes of *G. mellonella* larvae. Lavine *et al.,* (2005) demonstrated that the production of AMPs in different haemocyte populations of the moth *Pseudoplusia includens* was varied. The proposed model for haemocyte development can be seen in Figure 1.3.

1.1.6 Methods for the purification of insect haemocyte subpopulations

Classically, the purification of the sub-populations of insect haemocytes was based on methods such as percoll gradient separation techniques, morphological differences based on microscopical, density centrifugation methods and lectin staining techniques (Mead *et al.,* 1996; McKenzie and Preston, 1992). Using morphological variancations to distinguish groups of haemocyte subtypes is difficult and the results are often different between research groups. For example, lectin staining can often recognise unspecific carbohydrate structures present on tissues and therefore this method is very limiting (Rizki and Rizki, 1986). The other drawbacks with these methods is that they have been found to work more effectively with cells that have significant physical differences such as in the separation of the human blood cells; the leukocytes and the erythrocytes (red blood cells). Due to the lack of a nucleus in the red blood cells, simple centrifugation separation can be utilised to sort white blood cells from red blood cells (English and Anderson, 1974). For these reasons there are varying subjective views on haemocyte sub-population classification methods in the literature and this has led to a certain degree of confusion in haemocyte nomenclature (Strand, 2008).

Flow cytometry analysis of single cells dates back to the 1940s where it was first designed to study dust particles (Gucker *et al*., 1947). The instrument that is based on flow cytometry is known as a fluorescence activated cell sorter (FACS). Today it is widely used in the field of cell sorting. The advantages of flow cytometry include multiparameter data-aquisition, multi-variate data analysis and the high-speed ability to sort cells into different subpopulations. Recent advances in the field of flow cytometry have allowed insect immunologists to develop an alternative and more accurate means of purifying the different haemocyte subpopulations (Davey and Kell, 1996). Flow cytometry has the ability to identify different populations of cells by measuring the light that different cells scatter and the omission of fluorescence from

the different cells as they pass through a laser beam (Parks *et al.,* 1986). The laser scatter properties obtained from flow cytometry allows detailed morphometric analysis of the subpopulations and can accurately distinguish groups of haemocytes.

Flow cytometric analysis has been used to characterise haemocyte subpopulations of the silkworm *B.mori,* where the four subpopulations were sorted into granulocytes, plasmatocytes, oenocytoids and spherulocytes (Nakahara *et al.,* 2009). Flow cytometry has also been utilised in the analysis of the haemocyte populations in *M. sexta* (Nardi *et al.,* 2003). While the ability of the haemocyte of *G. mellonella* to phagocytose particles has been analysed using flow cytometry (Mowlds *et al.,* 2010; Fuchs *et al.*, 2010), the fundamental aspect of sorting the haemocytes into the subpopulations has not yet been studied.

1.2 The Innate Immune response of Humans

Innate immunity refers to the host's first line of defense against infection. Ultimately the innate immune system serves to eliminate the infection in the early hours of pathogen invasion. The human immune system protects against infection by maintaining a complex relationship between the innate immune system and the adaptive immune system, however it has recently been argued that the innate immune system is the more important (Hoffmann *et al*., 1999).

Innate immunity is comprised of many factors including the physical barriers to prevent infection, i.e. the skin, the peristalsis movement in the gastrointestinal tract, the movement of the cilia in the respiratory tract, the production of saliva and mucus in the nasopharynx and the flushing action of tears from eyes (Ganz, 2002). Innate immunity in humans also involves inflammation, the complement system and cellular aspects such as the neutrophil. Inflammation is stimulated by chemical factors that are released from cells that are present at the sites of infection; such as dendritic cells (Parks *et al*., 2004). The release of cytokines (e.g. interleukins) in response to pathogen associated molecular patterns (PAMPs), e.g. bacterial LPS can further recruit immune cells to the site of infection and induce the clinical signs of inflammation, such as vascular injury, tissue damage and a temperature increase in the body. This also leads to the activation of the Toll signaling pathway (Akira *et al*., 2001).

The complement system of innate immunity consists of circulating glycoproteins that can become activated by bacterial products to further enhance processes such as inflammation and the recruitment of the cells involved in the adaptive immune response (Goldman and Prabhakar, 1996).

The human innate immune system is also composed of cellular components. These cells are from the bone marrow derived-myeloid lineage. The myeloid lineages of immune cells are highly phagocytic, extremely motile and have the ability to provide a first line of defence against most infectious pathogens. They include the neutrophils, eosinophils, basinophils and monocytes/macrophages (Greenberger *et al.,* 1983).

Neutrophils are a dominant cell in the first line of denfence; they have a short half life span and can rapidly ingest, kill and digest microorganisms that have managed to breach the physical, anatomical barriers of the innate immune response. Another myeloid cell is the eosinophil which serves in the destruction of parasites and inducement of inflammatory responses. The basophils are cells that rapidly produce cytokines and contain large cytoplasmic granules where chemicals such as histamine reside. Monocytes and macrophages are also myeloid derived cells which are extremely important in terms of recruiting T lymphocytes, which are components of the body's adaptive immune response (Goldman and Prabhakar, 1996).

The human innate immune system is essential for survival and it is clear that innate immunity is highly complex and there also exists strong relationships between innate immunity and adaptive immunity. Interestingly, innate immunity has only recently been considered as important as the adaptive immune response in the hierarchy of immunity. The relatively recent interest in the innate immune response has been greatly underlined by the genetic and proteomic research that is conducted in model organisms (Dionne and Scheider, 2008).

1.2.1 The Neutrophil

The neutrophil (also known as polymorphonuclear leukocyes due to the presence of a lobed nucleus) is an essential component of the human innate immune system. Elie Metchnikoff first discovered neutrophils and he demonstrated these cells to be phagocytic, motile and to contain granules. Subsequent to Metchnikoff's findings the granule content of the neutrophil was characterised and it was found that the protein content of the granules have a microbicidal function (Segal, 2005). Studies investigating the content of these granules identified the respiratory burst that occurs with neutrophil phagocytosis. It was soon after this discovery that chronic granulomatous disease (CGD) was identified and it was discovered that this disease is a result of a defective neutrophil respiratory burst in patients (Thrasher *et al*., 1994). Neutrophils in CGD patients are unable to produce free radicals such as superoxide anion, ultimately leading to a compromised immune response. Studies of CGD has lead to many findings with regard to the killing ability of the neutrophil (Segal *et al.,* 1982).

The killing ability of the neutrophil is an extremely complex multilayered process, consisting of the formation of a functional nicotinamide adenine dinucleotide phosphate reduced form (NADPH) oxidase, generation of ROS, the neutrophil's ability to degranulate many different proteolytic enzymes and the conditions that exist in the phagocytic vacuole (Fuchs *et al.,* 2010).

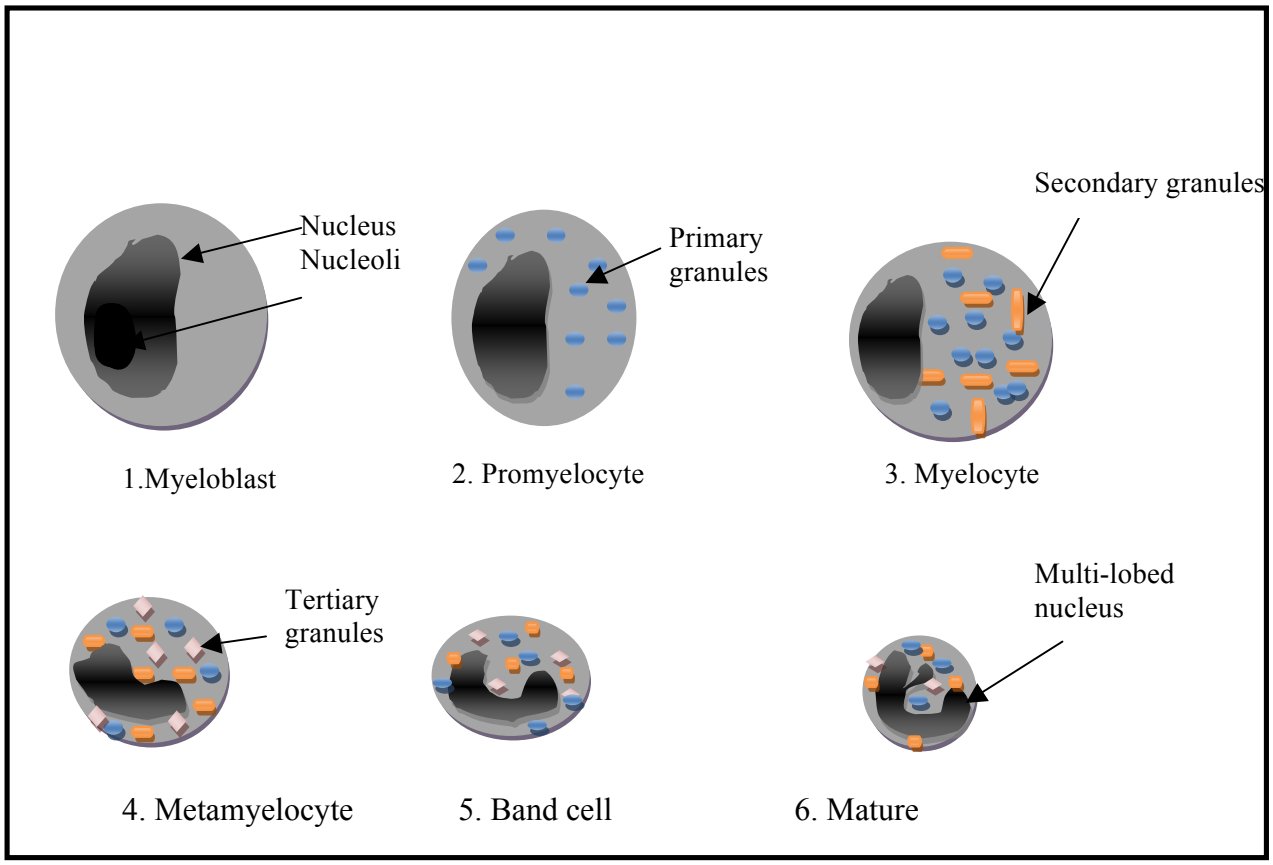


Figure 1.4 Neutrophil developments in humans, the cells are formed in the process known as haematopoiesis, the myeloblast, promyelocyte, myelocyte, metamyelocyte and band cell are located in the bone marrow and the mature neutrophil is found in circulating blood.

Adapted from Bainton *et al.,* 1971.

The NADPH oxidase is a multi-subunit enzyme that is integrated in the cell membrane and is also located in the cell cytosol in the neutrophil. The membrane components of the oxidase are $p22^{phox}$ and $gp91^{phox}$ (these two proteins form flavocytochrome b_{558}) while $p40^{phox}$, $p47^{phox}$, $p67^{phox}$ and small GTPase Rac all reside in the cytosol in the resting neutrophil (García-García *et al.,* 2009). Upon activation of the neutrophil, which occurs with the binding of immune signaling mediators to the neutrophil cellular receptors, the cytosolic components of the NADPH oxidase translocate to the membrane and bind to the flavocytochrome complex. This leads to the catalysis of the reactive oxygen species that aid in microbe destruction and pathogen killing. This NADPH oxidase formation also triggers a phenonomen known as degranulation (Xu *et al*., 2009).

The neutrophil contains four subtypes of granules all of which have different functions and these granules release their contents in the process known as degranulation. Neutrophil development can be visualised in Figure 1.4, where the development of the lobed nucleus and distinct granules is visualised. Degranulation is a sequestered event that is regulated and can be triggered by microbial molecules detected by the neutrophil. The primary/azurophilic granules contain enzymes such as myeloperoxidase, which forms the toxic compound hypochlorous acid, cathepsin G and elastase (Segal, 2005). The secondary/specific granules contain lactoferrin, which aids in sequesterisation of iron and copper from microorganisms. Approximately two thirds of neutrophil lysozyme concentration is contained in the specific granules (Stie & Jesaitis, 2007). Tertiary/gelatinase granules contain gelatinase while secretory/storage granules contain many storage amino acids such as serum albumin and aid in the replacement of the plasma membrane upon phagocytosis and engulfment (Wong *et al.,* 2009). These granules enclose their enzymes and proteins in an acidic pH environment which maintains the enzymes in an inactive state until the pH of the granule is altered, which occurs upon activation of the NADPH oxidase and this leads to a change in the membrane potential of the neutrophil (Styrt and Klempner, 1982).

The other killing process of the neutrophil is the ability of the cell to produce pseudopodia and engulf an opsonised particle. Within a few minutes of arriving at the site of infection the neutrophil's actin cytoskeleton polymerizes to release chromatin fibres, which engage in immobilizing the opsonised microorganisms and allow microorganism engulfment to occur (Segal *et al.,* 1980). The microorganism

is taken into the vacuole, the constituents of the granules in the neutrophil are not only released to the exterior of the neutrophil which can lead to tissue damage, but they are also released into the vacuole containing the pathogen. There is no doubt that the concentration of the proteases in the vacuole reaches extremely high concentrations in order to break down the microorganism, some research has demonstrated the possibility that the concentration of proteases in the vacuole can reach 500mg/ml, which can be 40% of the total vacuole content (Reeves *et al.,* 2002). The neutrophil is a primary effector cell in the human's innate immune system and a contributor to the adaptive immune system. The importance of the neutrophil in innate immunity is further validated by the case of CGD patients or in an individual who has a lack of peripheral neutrophils (which occurs following chemotherapy), the clinical manifestations of infection can then be extremely detrimental (Vento and Cainelli, 2003). The body struggles to control infections and as a result, infection can become systemic.

1.2.2 Similarities between the Innate Immune response of Humans and the Insect Immune response

It is now well established that there exists many similarities between the innate immune responses of humans and the insect immune response. Although divergence occurred approximately 500 million years ago, there still exist many parallels between the two systems (Waterfield *et al*., 2004). These parallels have led to the emergence of insects as model organisms in biological and chemical research. For example both humans and insects have the ability to synthesize gene-encoded antimicrobial peptides (AMPs) that form an important part in the first line of defense against infection. The production of AMPs in humans and insects is conserved with

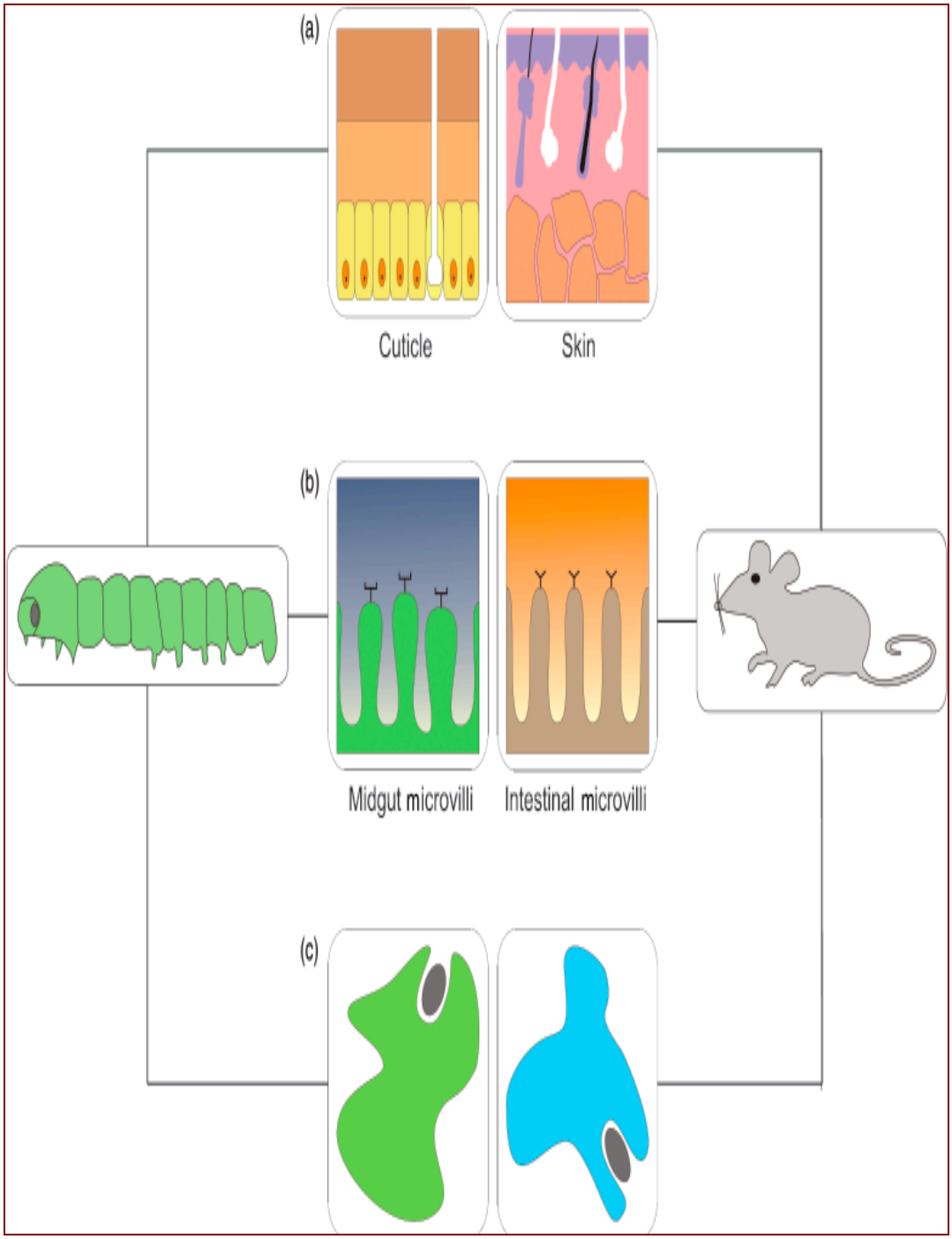


Figure 1.5 The similarities between the immune system of the insect model organism and the innate immune system of the mammalian model organism. The mammalian and insect innate immune cells can be seen in (C).

Adapted from Scully and Bidochka, 2006.

regard to molecular weight (usually less than 10kDa). AMPs in both humans and insects are hydrophobic and act on the membranes of microorganisms (Bulet *et al.,* 2004). Antimicrobial peptides are divided into three groups (1) alpha-helical conformational peptides, present in both insects (e.g. cerophin A in *Anopheles gambiae*) and humans (e.g. cathelicidin) (Ganz, 2002) (2) cyclic and open-ended cyclic peptides with pairs of cysteine residues, present in insects (drosomycin in *D. melanogaster*) and humans (beta-defensin) (Ehret-Sabatier *et al.,* 1996) (3) peptides with an over-representation of some amino acids are again present in insects (lebocin in *B. mori*) and humans (hepcidin) (Schuhmann *et al*., 2003; Hunter *et al*., 2002).

Another parallel between the two immune systems is that the human neutrophil and insect haemocyte bear many similar characteristics, e.g. both cells have the ability to produce pseudopodia (Segal *et al.*, 1980), phagocytose and engulf opsonised pathogens. Both the neutrophil and haemocyte produce reactive oxygen species such as superoxide and there exists homologies between the NADPH oxidase in both cells also. In fact the similarities are so comparable between the neutrophil and haemocyte that it has been found there exist 10 identical peptides in the insect haemocyte p67 to those in the human neutrophil $p67^{phox}$, it has also been found that there exist 7 identical peptides in insect haemocyte p47 to human neutrophil $p47^{phox}$ (Bergin *et al.,* 2005).

Probably the best-documented parallel between the insect immune system and the human immune system is the Toll pathway, which has been studied in *D. melanogaster* (Lemaitre *et al*., 1996).

There is also evidence that the molecular regulatory mechanisms in the human immune system are similar to those in the insect's immune system. In the moth *Hyalophora cecropia*, there exists a gene known as cecropia immunoresponsive factor (CIF) which is induced by invading microorganisms, it has been demonstrated that the protein CIF is similar in function to mammalian nuclear transcription factor (NF) –KB (Sun and Faye, 1992). It has also been found that the reactive oxygen species generated from haemocytes and neutrophils can activate CIF in a similar manner to the transcription factor NF-KB, which is heavily involved in the inflammatory innate immune response by stimulating the production of cytokines such as Interleukin-8 (Faye and Hultmark, 1993). These data suggest evolutionary similarites between insect and mammalian immune response factors.

These common themes at the molecular and the proteomic level between the human innate immune system and the immune system of insects further validate the use of insects in biological and chemical research. With the identification of even more similarities between the main effector cell of the human innate immune system and the immune cell of insects (Table 1.1), the idea of using insects as model organisms is further substantiated (Neeli *et al.,* 2009).

(Similarties can be seen in Figure 1.5, an electron micrograph of a neutrophil and a haemocyte can be visualised in Figures 1.6 and 1.7, respectively.)

Human Innate Immune System	Insect Immune System
Physical Barrier = skin	Physical Barrier = cuticle
Circulating blood; which transports nutrients, immune cells and AMPs.	Circulating haemolymph; which transports nutrients, the immune cells and AMPs.
Liver; aids in detoxifying the body and in the production of AMPs.	Fat body (Adipose tissue); aids in metabolism of proteins, the production of nutrients and AMPs.
Neutrophil; circulating immune cell where phagocytosis is lectin mediated, engulf and kill microorganisms, produce pseudopodia, contains the NADPH oxidase and can produce reactive oxygen species (ROS).	Haemocyte; circulating immune cell where phagocytosis is lectin mediated, engulf and kill microorganisms, produce pseudopodia, contains NADPH oxidase-like proteins and can produce reactive oxygen species (ROS).

Table 1.1 Table documenting the similarities between the human innate immune system and the insect immune system.

1.3 The importance of the Immune cell's actin cytoskeleton in fighting infection

The human neutrophil and the insect haemocyte are extremely significant cells in host immunity; their ability to migrate, produce pseudopodia, phagocytose and kill microorganisms is essential to survival. The cell's cytoskeleton plays an essential role in all of these processes. The defined structure of the cytoskeleton maintains the cell's shape and gives the cell the ability to change it's morphology. The cytoskeleton also allows mobility to occur and phagocytosis to become activated. These processes all contribute to microbial cell death. The cytoskeleton functions in the transport of vesicles, chromosome transport and macromolecule transport. The cytoskeleton of cells can also be linked through the cell surface to the extracellular matrix and help to form tissues.

The cytoskeleton of the neutrophil and haemocyte contains three types of filaments, these are the actin filaments, microtubules and the intermediate filaments (Castellano *et al.,* 2001). The cytoskeleton has been highly conserved throughout evolution, this conservation is most likely due to the critical role that the cytoskeleton plays in the architecture of the cell (Broderick *et al*. 2010) and even a small mutation in the sequence could completely disrupt the assembly of the cell.

Valerius *et al*., (1981) demonstrated the importance of the cytoskeleton in neutrophil phagosome development. Neutrophil cytoskeleton is not only important in the cell's locomotion, maintaining cellular shape and allowing degranulation to occur but it also plays a key role in the NADPH oxidase cascade which is crucial to the neutrophil's killing ability (Matute *et al.*, 2005), (as seen in Figures 1.8 and 1.9). It is also likely that the cytosolic phox proteins are critical to neutrophil cytoskeleton formation and regulation (Grogan *et al.,* 1997).

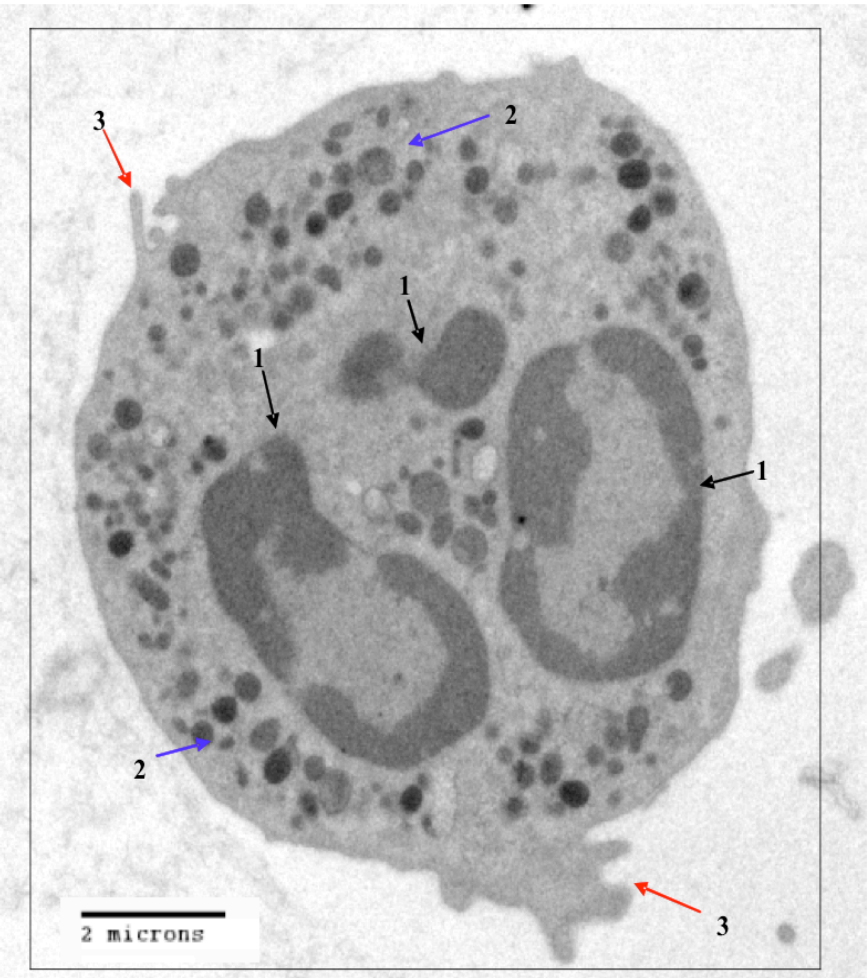


Figure 1.6 Electron micrograph of a human neutrophil, where the lobed nucleus is indicated (1), the granules are present in the cytoplasm (2) and the pseudopodia are indicated (3).

Sourced from Renwick *et al*., 2007.

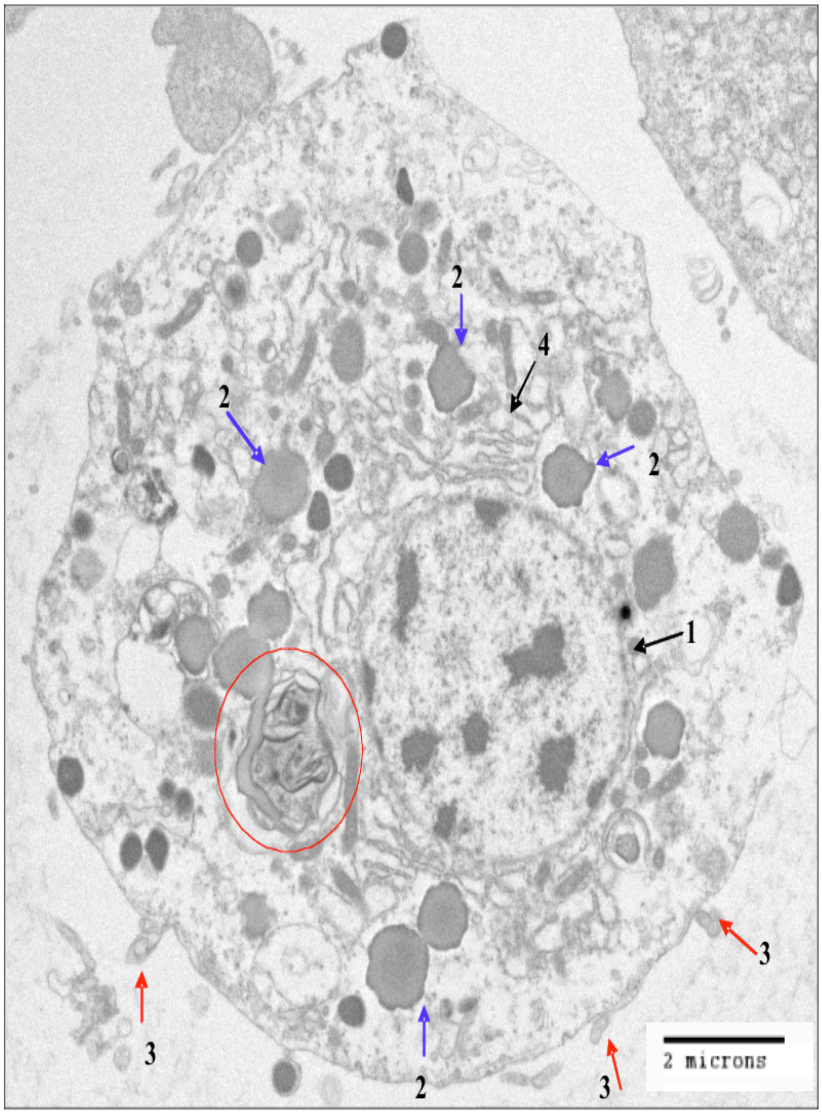


Figure 1.7 Electron micrograph of a haemocyte from *G. mellonella* larvae, where the large nucleus is indicated (1), there are granules present in the cytoplasm (2), there is evidence of pseudopodia (3), there is evidence of the endoplasmic rheticulum (4) and the red circle outlines a conidia that has been phagocytosed.

Sourced from Renwick *et al*., 2007.

It has also been well documented that neutrophil activation, which occurs upon binding of an opsonised microorganism to the receptors on the neutrophil's membrane, involves the actin filaments in the cytoskeleton becoming highly polymerized and the conversion of actin from a globular form to a filamentous form. There is a continuous process of depolymerisation and polymerization and this process essentially allows the neutrophil to be highly motile and alter it's shape immediately when it comes into contact with pathogens. The activation of protein kinase C, either by an opsonised particle binding to neutrophil receptors or by the activation of the neutrophil/haemocyte by artificial stimulants (such as phorbol 12-myristate 13-acetate (PMA) and N-formyl-methionine- leucine-phenylalanine (fMLP)), stimulates these polymerization events to occur.

Although there is less known about the make-up of the haemocyte cytoskeleton, it has been documented that chemical amines such as 5-hydroxytryptamine (5-HT) enhance *G. mellonella* larval haemocytes to undergo phagocytosis, enhance encapsulation events and the formation of nodules. It has been found that these amines are similar to mammalian amines; 5-HT activates levels of cyclic AMP, which mediate the process of phagocytosis in neutrophils. Diehl-Jones *et al.,* (1996) demonstrated that these amines also regulate haemocyte mobility and the polarization of the cytoskeleton. There is also evidence that there are of similar signaling pathways in insect haemocytes and in neutrophils, such as the pathways involved in focal adhesions and transmembrane integrin formation. Chen *et al*., (2005) demonstrated homologous proteins to the mammalian focal adhesion proteins Pyk2 and Fak in *D. melanogaster.* Focal adhesions are fundamental components of the phagocytosis process.

Given the importance of the cytoskeleton in the neutrophil's multilayered killing ability and the previously outlined similaratites between insect haemocytes and human neutrophils in processes such as phagocytosis, the NADPH oxidase constituents, pseudopodia formation and ROS production, it is likely that there are even more similarities between the neutrophil cytoskeleton and haemocyte cytoskeleton.

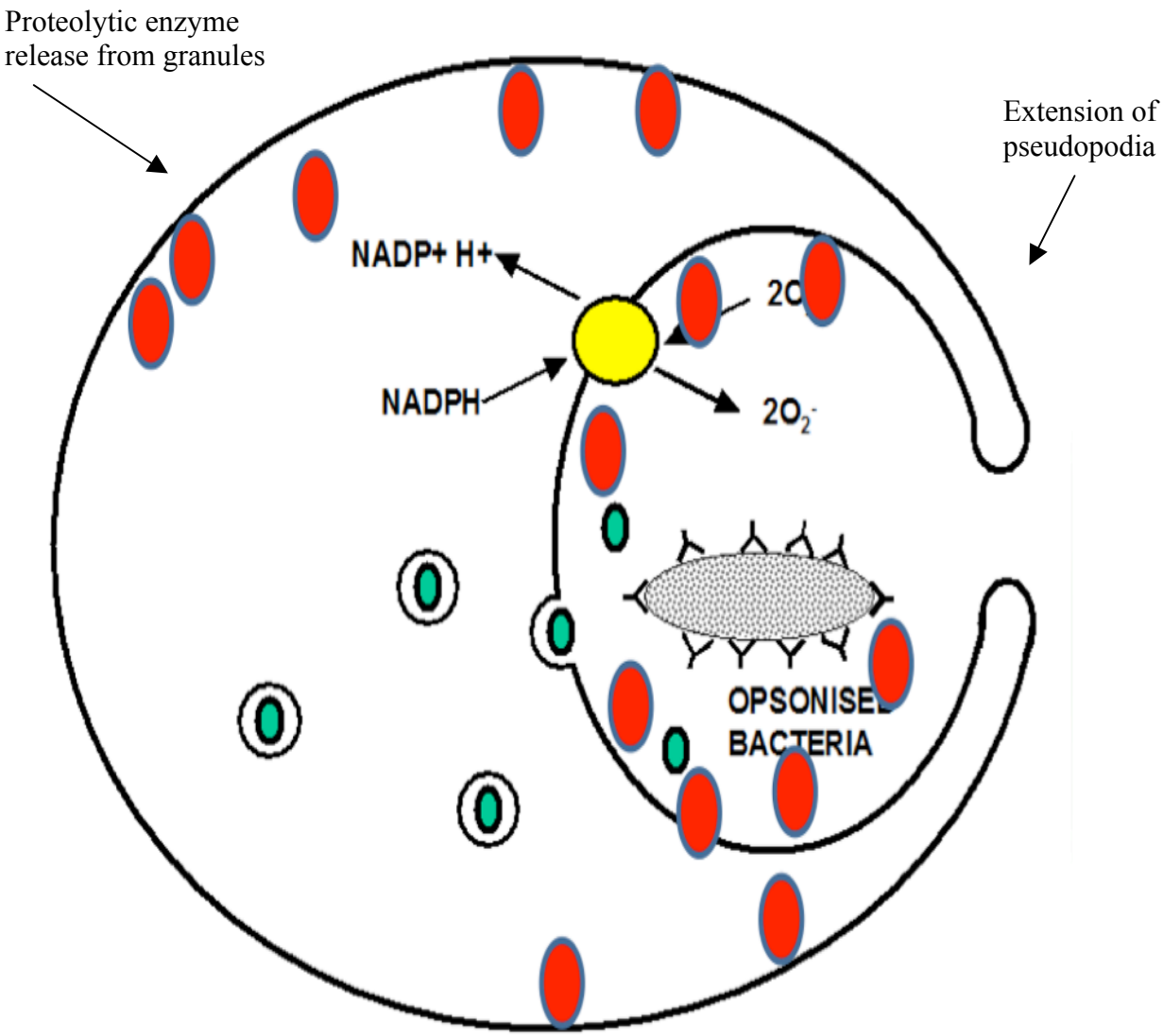


Figure 1.8 The NADPH oxidase cascade in a phagocytosing cell, triggering the release of proteolytic enzymes from the granules and the release of reactive oxygen species which help to degrade the microorganism.

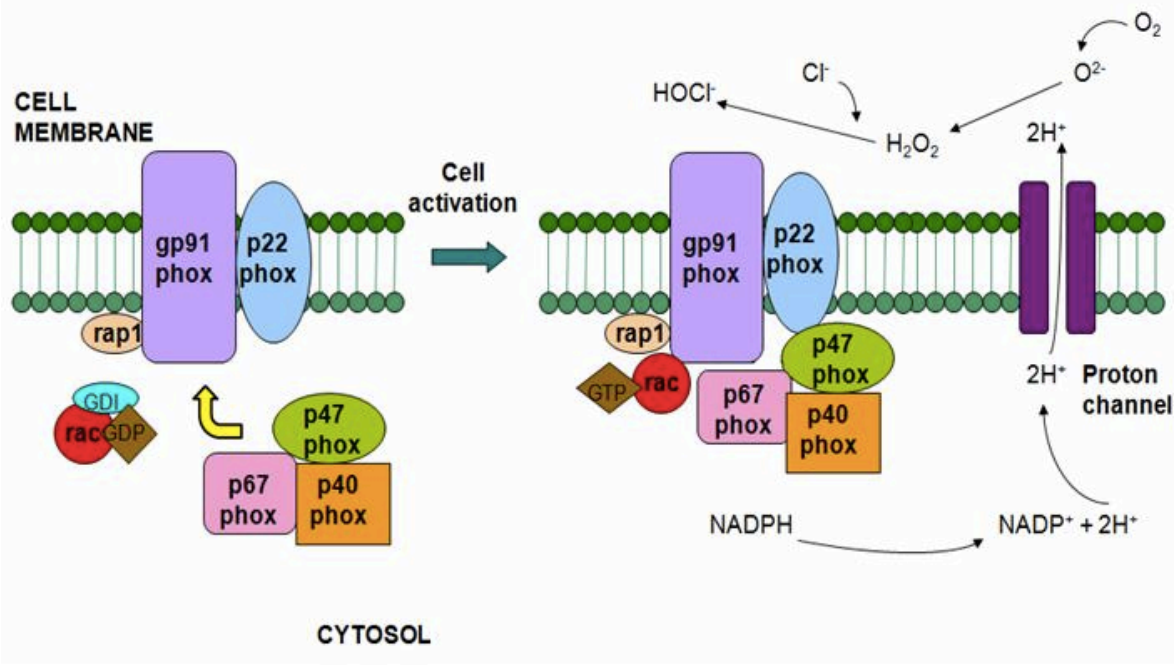


Figure 1.9 The activation of the NADPH oxidase cascade and ROS production in the membrane of a phagocytosing cell. The generation of ROS is a result of the translocation of NADPH oxidase components to the membrane.

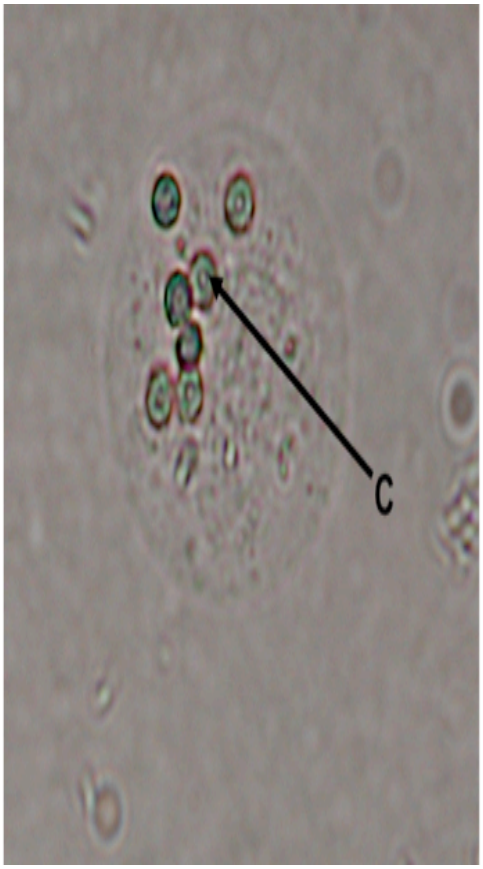
Sourced from Stefanska *et al.,* 2009.

1.3.1 The cytoskeleton inhibitors cytochalasin b and nocodazole

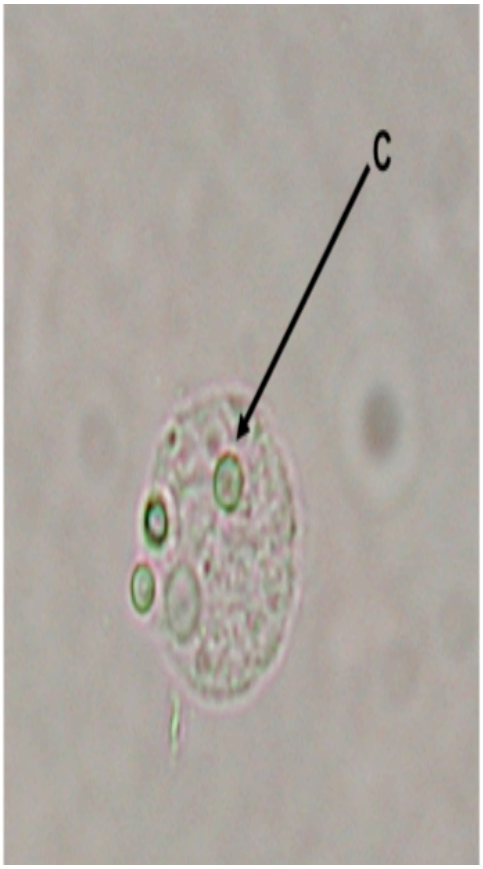
The ability of the actin filaments to polymerise is a tightly regulated process and is critical in the immune activity of the neutrophil and haemocyte. Cytochalasin b is a mycotoxin isolated from the fungus *Helminthosporium dermatioideum* that has been shown to inhibit the processes of phagocytosis, cell division and phagosome closure (Theodoropoulous *et al.,* 1994). It has been shown to drastically reduce the rate of actin polymerization (up to 90% reduction) by inhibiting the rate of monomer addition to the 'barbed' end of the actin filaments, which is where the monomers are added upon polymerization events. It is thought that cytochalasin b attached to this barbed end of the filaments, thus blocking the monomer binding sites (Maclean-Fletcher and Pollard, 1980). Cytochalasin b is often utilised in neutrophil studies, acting as an artificial inhibitor of the cell (Grogan *et al.,* 1997).

Microtubules make up networks that are important for the endoplasmic reticulum, golgi membrane and they are also important for the movement of vesicles and in the formation of organelles (Vasquez *et al.*, 1997). Nocodazole, which is a derivative of benzimidazole, was originally developed as an anticancer drug (Musa *et al.,* 2006). It is now widely used in studies of microtubules *in vitro* and *in vivo*. Nocodazole acts on the microtubule networks to prevent polymerization, as with cytochalasin b, nocodazole is used in the study of neutrophil cytoskeleton architecture (Vasquez *et al.,* 1997).

As there are similarities between the neutrophil and haemocyte cytoskeleton, cytochalasin b and nocodazole are valuable tools in the analysis of the haemocyte cytoskeleton's behavior. The chemical structures of cytochalasin b and nocodazole can be seen in Figure 1.11.

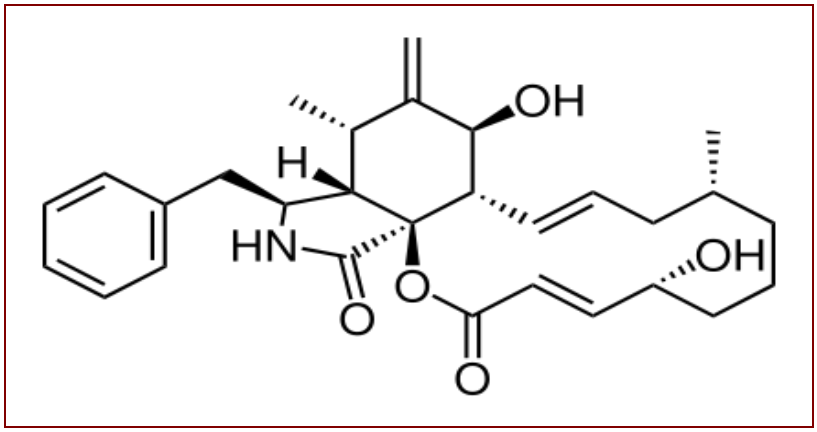


(A)

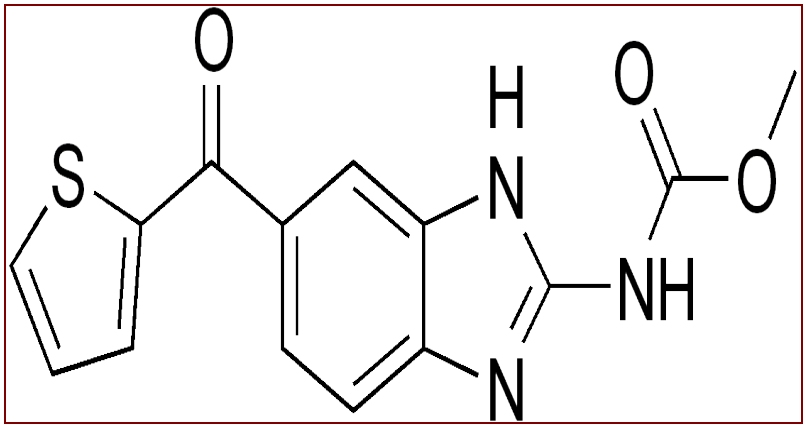


(B)

Figure 1.10 Conidia from *A. flavus* phagocytosed in a haemocyte from *G. mellonella* larvae (A), conidia from *A. flavus* phagocytosed in a human neutrophil (B). (C) = Conidia.



(A)



(B)

Figure 1.11 The chemical structures of the cytoskeleton filament inhibitors cytochalasin b (A) and nocodazole (B).

1.4 *Candida albicans*

The *Candida* genus is composed of approximately 200 yeast species, the species are found ubiquitously in the environment, they are generally not harmful, however if the immune system of an individual cannot fight a *Candida* infection, the infection can become systemic. *C. albicans* is the most common fungal species that is associated with human infections. *C. albicans* is a dimorphic yeast and can exist as budding yeast cells, chlamydospore or it can undergo phenotypic switching (Odds, 1994).

C. albicans utilizes both morphologies when infecting a host, that is it can undergo yeast-true hyphae transition (Ernst, 2000). *C. albicans* produces true hyphae (parralled sided walls and grows by continuous apical extension) and pseudohyphae (which appear in the extremities of the budding yeast and grow by unipolar budding). *C. albicans* has the ability to quickly adapt to environmental changes by changing the morphology of the cell, the switching of it's phenotype aids in the pathogenesis of the infection, especially in immunocompromised individuals (Ernst, 2000).

Candidiasis is a common opportunistic infection and *C. albicans* is the most prevalent fungal species associated with diseases such as cancer, HIV and is the fungal pathogen most commonly found in immunocompromised individuals. Mortality in individuals who are infected with *C. albicans* can reach 30% (Henderson and Hirvela, 1996).

C. albicans has the ability to express several different virulence factors that collectively contribute to it's pathogenicity. *C. albicans* can readily adhere to and infect host epithelial cells (i.e. the physical barrier of the innate immune system) and this means that the fungus can swiftly invade and colonise the host's immune system. *C. albicans* can readily colonise almost all of the body's surface, such as the pharynx, genitalia and the nails (Odds, 1988). The virulence of *C. albicans* is especially invasive if the host is immunocompromised due to factors such as chemotherapy or if a patient is taking immunosuppressive therapy. The reason for this is that the innate immune response of the host is especially important in counteracting an infection with *C. albicans*. The immune response to *C. albicans* infection is largely site specific, primarily involving the innate response of dendritic cells, mast cells and leukocytes (Caledrone and Fonzi, 2001). This importance of the innate immune response has been demonstrated in patients who have defective cellular immunity where the

clinical manifestations of *C. albicans* are particularly evident (Martinez *et al.,* 1998). *C. albicans* has the ability to switch it's phenotype in order to adapt to a variety of different environments, to adhere to host cells and tissues, to produce extracellular enzymes and to alter cell-surface hydrophobicity (Sudbery *et al.,* 2004). These factors greatly contribute to *C. albicans* pathogenicity.

There is a wide range of *C. albicans* infection types known to man. The infections can either manifest superficially or systemically (Sims *et al*., 2005). Superficial *C. albicans* infections include oral, skin, nail and genital infections such as oral thrush and vulvovaginitis. However basic hygiene and local treatment such as anti-fungal creams have been found to be effective against these infections (Eggimann *et al*., 2003). The systemic infections with *C. albicans* have been found to be the more serious of the two. It can occur in deep tissue and spread through the body via the bloodstream, mortality can be very high in individuals who have systemic candidosis and generally the methods of systemic infection diagnosis are made too late for effective intravenous treatments, this has led to mortality rates in some cases patients of approximately 80% (Eggimann, 2003). The antifungal therapies that exist for the treatment of *C. albicans* infections include polyenes (amphotericin B), azoles (itraconazole) and the echinocandins (caspufungin). The effect of therapeutic drugs such as caspofungin on *C. albicans* have been studied successfully by employing *G. mellonella* as model organisms (Kelly *et al.,* 2010).

The virulence of *C. albicans* has previously been studied in model organisms such as *D. melanogaster* (Glittenberg *et al.,* 2011) and *G. mellonella* (Bergin *et al.,* 2006; Brennan *et al.,* 2002). *G. mellonella* larvae provide a mechanism of detecting the effect of *C. albicans* on the host's physiology and the proteomic alterations that occur due to infection (Brennan *et al.,* 2002; Mowlds *et al.,* 2009), whereas *D. melanogaster* is also an extremely valuable model organisms especially in the study of the differences in the virulence of clinical isolates.

1.4.1 *Aspergillus fumigatus*

A. fumigatus is a filamentous fungus that is one of the most significant causes of deaths in individuals whose health is immunocompromised or in patients who are undergoing immuno-suppressive therapy. It is responsible for 4% of deaths in European hospitals (Latge, 1999).

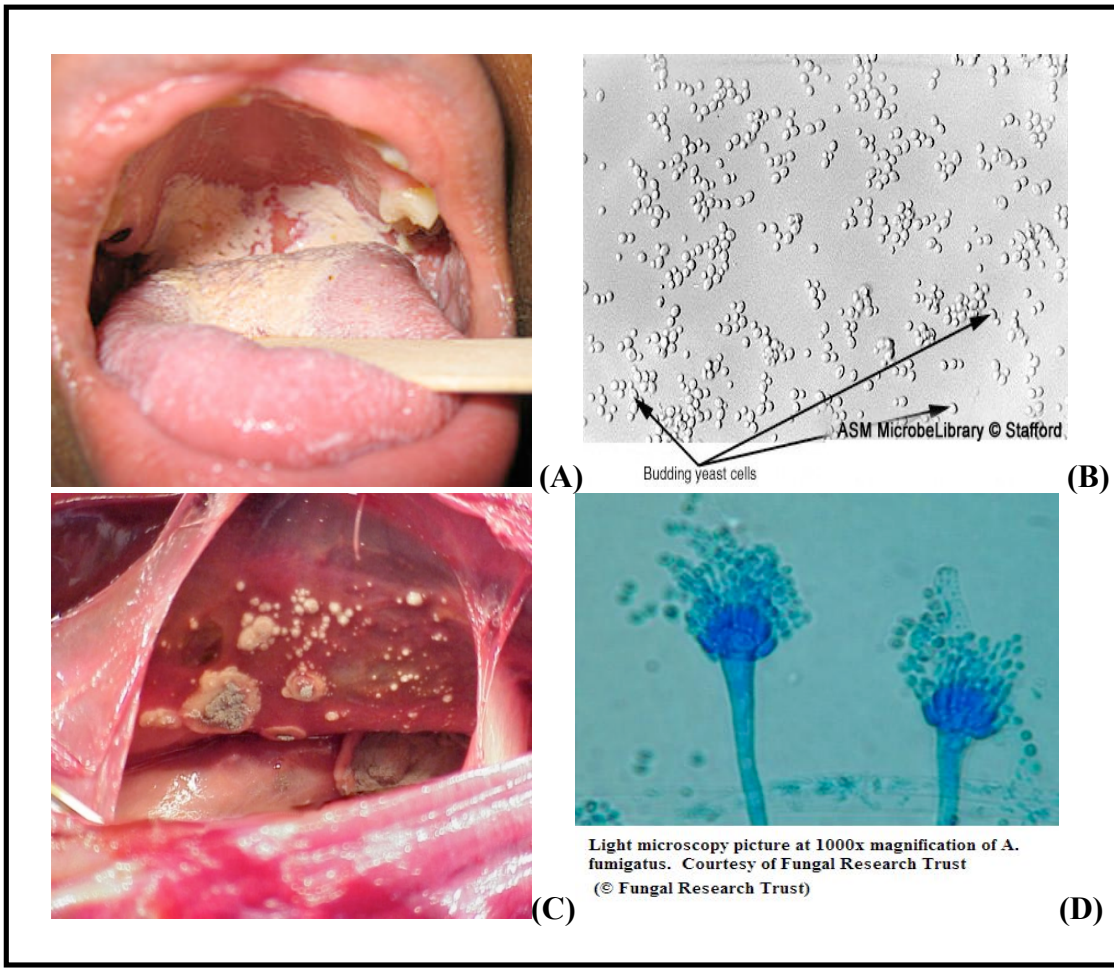


Figure 1.12 Examples of the fungal pathogens studied in *G. mellonella* larvae, *C. albicans* infection presenting as oral candidiasis (Thrush) (A), *C. albicans* cells (B), *A. fumigatus* infection presenting as pulmonary aspergillosis (C), *A. fumigatus* hyphae and conidia (D).

Sourced from http://www.mycology.adelaide.edu.au (A)

Sourced from http://www.pathmicro.med.sc.edu (B)

Sourced from http://www.michigan.gov (C)

Sourced from http://www.fungalresearchtrust.org D)

An infection with *A. fumigatus* is capable of inducing disease in individuals who have a pre-existing pulmonary malfunction, such as asthma, cystic fibrosis or emphysema (Denning, 1995). One of the reasons for this is that *A. fumigatus* cells can produce condia that are capable of being inhaled into the alveoli due to their small size (~ 3 micrometers). The alveoli of the lungs provide the perfect site for the conidia to initiate an aspergillosis infection (Raper *et al.,* 1965). Humans and animals constantly inhale condia however, it is only in individuals who have a weakened immune system where aspergillosis becomes invasive and potentially fatal. Other *Aspergillus* (such as *A. flavus* and *A. niger*) species also have the ability to cause a human infection, however of the human *Aspergillus* infections, 90% are caused by *A. fumigatus*.

There are three types of diseases associated with pulmonary *Aspergillus* infections. These are saprophytic, allergic or invasive. Saprophytic aspergillosis involves colonization of the airways, there is no invasion of the viable tissue, but there is a build up of inflammatory cells, fibrin and mycelium in the airways. Allergic aspergillosis is a potentially fatal infection in asthma and CF patients (also known as allergic bronchopulmonary aspergillosis), it involves a severe inflammatory immune response (Daly and Kavanagh, 2001). Invasive aspergillosis is the most common fungal infection in cancer patients; it can travel to organs and distant sites in the body (Bodey *et al.,* 1992).

As with *C. albicans* infection, the innate immune system plays a major role in fighting *A. fumigatus* infections. (*C. albicans* and *A. fumigatus* infections can be seen in Figure 1.12). The cilia present in the airways are an anatomical barrier to infection and aid in the sweeping of the airways to rid the body of particles such as dust, conidia and airborne pathogens (Latge, 1999). The hydrophilic conditions in the alveoli also aid in the innate immune system's defense against the pathogen (Latge, 1999). The neutrophil has also been widely documented as playing a primary role in the body's defense mechanism against *Aspergillus* spp. (Christin *et al*., 1998). The neutrophil has the ability to attach to the conidia hyphae and aids in the destruction of the hyphae by secreting reactive oxidant species, such as hydrogen peroxide and hydroxyl radicals. The neutrophil can also release the constituents of neutrophil granules, such as the proteolytic enzyme myeloperoxidase (Madan *et al*., 1997).

The use of *D. melanogaster* has been shown to be a fast and efficient model for the study of *A. fumigatus* virulence and also to study the efficacy of anti-fungal

drugs (Lionakis *et al.,* 2005). It has been well documented that there is a direct correlation between the conidia dosage concentration and the lethality of *A. fumigatus*, this has been seen in the classic murine models for studying *A. fumigatus* and has recently been demonstrated in *G. mellonella* larvae also (Mylonakis, 2008). *G. mellonella* larvae have recently been employed as model organisms to study the toxicity of *A. fumigatus* toxins, such as gliotoxin and fumagillin (Reeves *et al*., 2004; Fallon *et al*., 2011).

Reeves *et al*., (2004) demonstrated a correlation between the virulence of *A. fumigatus* and gliotoxin production in *G. mellonella* larvae. Gliotoxin is produced by *A. fumigatus* and aids in tissue destruction in the lungs, the utilization of *G. mellonella* larvae as the *in vivo* model indicated the toxic properties of gliotoxin. These studies further demonstrate the effectiveness of using *G. mellonella* as alternative models for the study of fungal infections. Cowen *et al*., (2009) studied the effect of inhibiting Hsp90's in *A. fumigatus* infections as an alternative therapy for the fungal infection by using *G. mellonella* larvae as the model organism.

(*G. mellonella* larvae infected with *A. fumigatus* can be visualised in Figure 1.13)

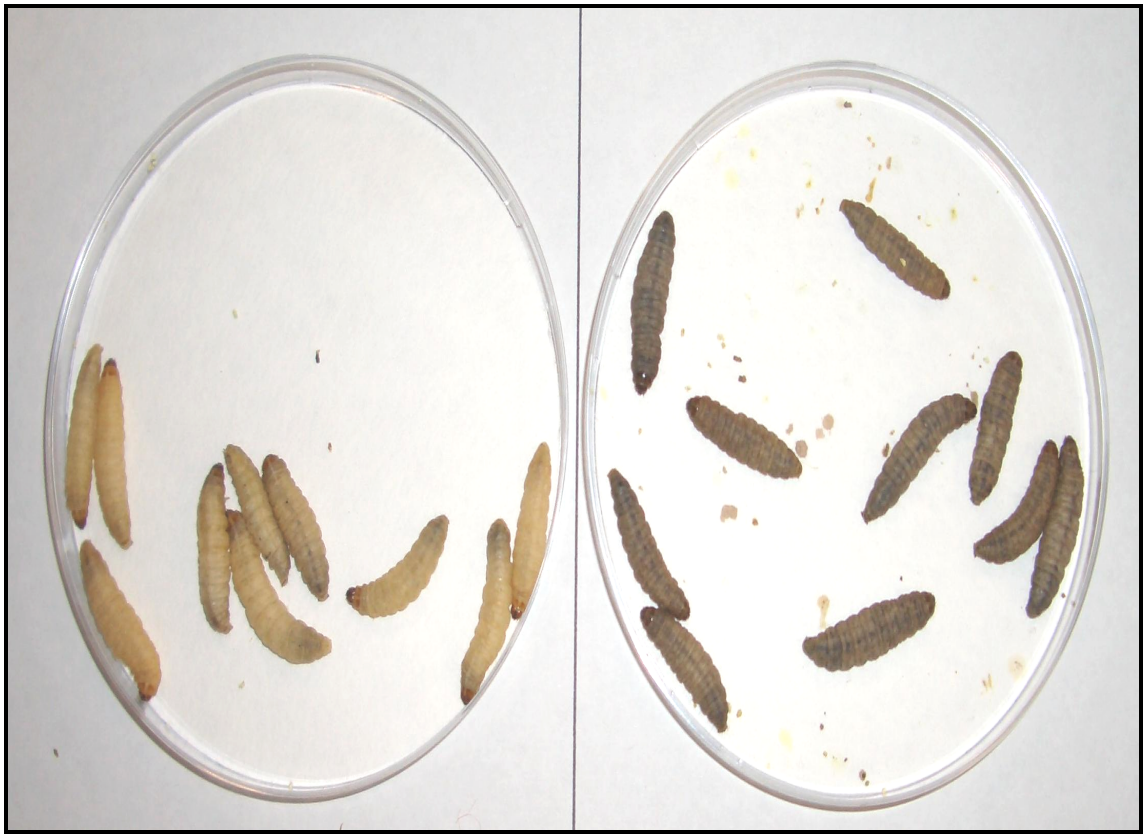


Figure 1.13 Healthy *G. mellonella* larvae and *G. mellonella* larvae infected with *A. fumigatus* spores, cuticle melanisation is visable in the infected larvae by the darker pigmentation.

1.4.2 Applications of proteomics in the study of biology

The improvements in proteomics have allowed significant advances in the analysis of cellular organizations and in the basic understanding of health and diseases. The combined use of 2-dimensional SDS-PAGE with LC-Mass Spectrometry has allowed researchers to separate, quantify and analyse the proteome content of tissues and cells (Brunner *et al*., 2007). Proteomics research is largely in the field of understanding the proteomic content of cells or tissues and how protein expression is altered under different physiological conditions. The importance of proteomics clinically is very significant; the reason for this is that the protein content in disease states is usually readily identified when compared to normal tissue. In the 1990s, genomics and biochemical studies were the main routes in the characterization of model organisms, such as *D. melanogaster*. However Walhout and Vidal, (2001) outlined the potential of utilising proteomics in comparative biology where protein-protein interactions and signaling pathways may be completely elucidated by the constantly evolving techniques.

Proteomics has been a useful tool in understanding neutrophil biology, for example proteomics has characterised much of the cytoskeleton (Xu *et al.,* 2009), the granule contents of neutrophils and the maturation of neutrophil phagosomes (Burlak *et al.,* 2006). Advances in the field of proteomics may allow complete mapping of the *G. mellonella* proteome, this may potentially demonstrate further comparisons between signaling cascades, protein-protein interactions and the physiological activities of the neutrophil and the insect haemocyte. This approach to comparative and developmental biology has been demonstrated with *D. melanogaster* (Brunner *et al.,* 2007). The advances in proteomics in areas such as label free quantitative protein identification methods and techniques such as shotgun proteomics are expedient tools in the development of alternative model organisms in research.

1.4 Objectives of this Study

The work presented in this Thesis sought to study four different areas in order to further validate *G. mellonella* larvae as useful tools in research. The main objectives were to:

(1) Identify and characterise an optimal nutritional status for *G. mellonella* larvae in the laboratory. To identify the effect of starvation, food source and larval weight on susceptibility to infection with the pathogens *C. albicans* and *A. fumigatus* and to characterise proteomic and molecular alterations in the larvae upon starvation.

(2) To investigate the effects of the artificial stimulators PMA and fMLP on *G. mellonella* larval haemocytes, to characterise and quantify the proteomic changes in the immune cell and compare the proteomic content to the human neutrophil. To investigate the phosphorylation events in the haemocyte and to establish similarities in the NADPH oxidase in the *G. mellonella* haemocyte and human neutrophil. To utilise the techniques of shotgun proteomics and multidimensional protein identification to further characterise the *G. mellonella* haemocyte proteome.

(3) To assess the effect that neutrophil cytoskeletal inhibitors have on the *G. mellonella* larvae's susceptibility to infection and the effect that the inhibitors have on the haemocyte's ability to kill. To characterise the components of the cytoskeleton in the haemocyte of *G. mellonella* larvae and compare the components to those in the human neutrophil. To utilise the combined techniques of flow cytometry and light microscopy to characterise the sub-populations of the haemocyte of *G. mellonella* larvae.

(4) To analyse the process of degranulation in the haemocyte and compare the process to degranulation in the human neutrophil. To assess the kinetics of proteolytic enzyme release from the haemocyte and to assess alterations in the presence of an MPO-like protein in different subpopulations of *G. mellonella* larval haemocytes.

Chapter 2

Materials and Methods

2.1 Chemicals

All chemicals and reagents were of the highest purity and were purchased from Sigma Aldrich Chemical Co. Ltd., Dorset, United Kingdom, unless otherwise stated.

2.2 Sterilisation Procedures

All growth media were sterilised prior to use by autoclaving in a Systec 3170 ELV autoclave at 121°C and 15 lb/sq.in. for 15 minutes. Any chemicals unsuitable for autoclaving were filter-sterilised using a filter with a pore size of 0.2 μm (Sarstedt, Nümbrecht, Germany). All cultures were autoclaved prior to disposal.

2.3 Microbial strains and culture conditions

2.3.1 *Candida albicans* growth conditions

C. albicans MEN (a kind gift from Prof D. Kerridge, Cambridge, United Kingdom) was cultured in YEPD broth (2% (w/v) glucose, 2% (w/v) Bactopeptone, 1 % (w/v) yeast extract (Oxoid Ltd., Basingstoke, England) at 30°C in an orbital shaker. Stocks were maintained on YEPD agar plates supplemented with erythromycin (1μg/mL).

2.3.2 *A. flavus* and *A. fumigatus* growth conditions

A. flavus and A. fumigatus were grown on Malt Extract agar plates (MEA; Oxoid Ltd.) at 37°C and sub-cultured every 4 weeks. Stocks were obtained from the American Type Culture Collection and were grown on malt extract agar (Difco Laboratories). To remove conidia, plates were washed with 0.1% (v/v) Tween 80,

harvested (2,056 x g for 10 minutes) on a Beckmann GS-6 bench centrifuge, washed in PBS and re-suspended in PBS, prior to diluting and counting on a haemocytometer

2.3.3 Ascertaining cell density

To determine the cell concentration, a haemocytometer (Neubauer, Germany) was employed. Liquid cultures were diluted 1/100 in sterile PBS, placed on a haemocytometer and counted on a light microscope using high power (x40) lens.

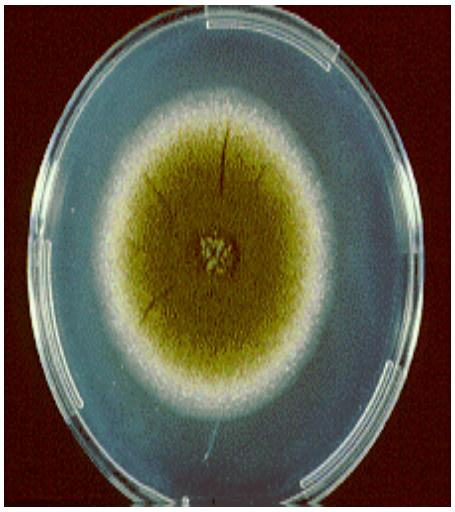
2.4 Larvae and Experimental Conditions

2.4.1 Insect Larvae

Sixth instar larvae of *Galleria mellonella* (Lepidoptera: pyralidae, greater wax moth) were obtained from the Mealworm Company (Sheffield, England) and stored in wood shavings in the dark at 15°C. Ten healthy larvae were placed in sterile 9 cm petri dishes with Whatman filter paper and 0.1g of wood shavings. Larvae were used within 3 weeks of receipt.

2.4.2 Determination of effect of differing weights on larval survival when inoculated with a pathogen

Sixth instar larvae of *G. mellonella* (Lepidoptera: pyralidae, greater wax moth) were stored in wood shavings in the dark at 15°C. Larvae chosen for experiments weighed 0.2g, 0.3g or 0.4g. Ten healthy larvae were placed in sterile 9 cm petri dishes with 1 Whatman filter paper and 0.1g of wood shavings.



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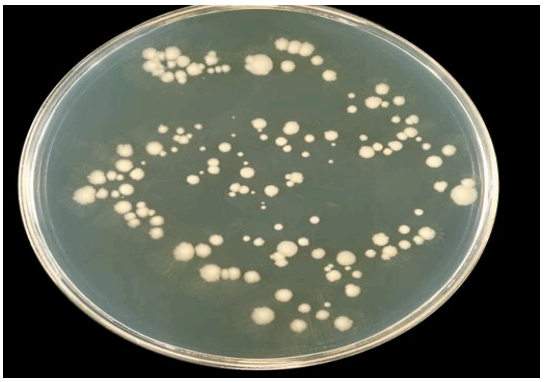


C

Figure 2.1 Appereance of fungal strains (A) *Aspergillus flavus* , (B) *Aspergillus fumigatus* , (C) *Candida albicans.*

2.4.3 Determination of effect of differing food sources on larval survival when inoculated with a pathogen

Larvae chosen for experiments weighed 0.3g. Ten healthy larvae were placed in sterile 9 cm petri dishes with 0.1g of wood shavings only, 0.1g of wood shavings and 1 Whatman filter paper or 1 Whatman filter paper only.

2.4.4 Determination of effect of starvations on larval survival when inoculated with a pathogen

Larvae chosen for experiments weighed 0.3g. Larvae were placed in a 9cm petri dish for 0, 2, 4, and 7 days without any food source and stored in the dark at 15°C.

2.4.5 Determination of haemocyte density of *G. mellonella*

Haemocyte density was calculated by piercing the head of three larvae with a sterile needle (gauge size of 23G, Terumo) and collecting the haemolymph into a pre-chilled Eppendorf tube. Haemolymph was diluted 1 in 10 in cold PBS containing 0.37 % (v/v) 2-Mercaptoethanol to reduce clotting and melanisation. Haemocytes were counted on a haemocytometer. Haemocyte density was calculated for larvae aftet (4 hr) injection with 10µM cytochalasin b, 10µM nocodazole, 1µg/mL PMA and 0.2µg/mL fMLP. Haemocyte density was also calculated for larvae that were given various feeds, larvae that were various weights and larvae that were starved for certain periods of time.

2.4.6 Protein extraction from *G. mellonella* haemolymph

Haemolymph (100 µL) was collected from larvae by piercing the head of insects that had been starved for 0 and 7 days and bleeding the haemolymph into a pre- chilled microcentrifuge tube. 2D electrophoresis was performed as described in section 2.10. Each 2D gel was scanned on a Hewlett Packard scanjet 5100c scanner and the images analysed with ImageMaster 2D Platinum v7 software (Amersham Biosciences UK Ltd.).

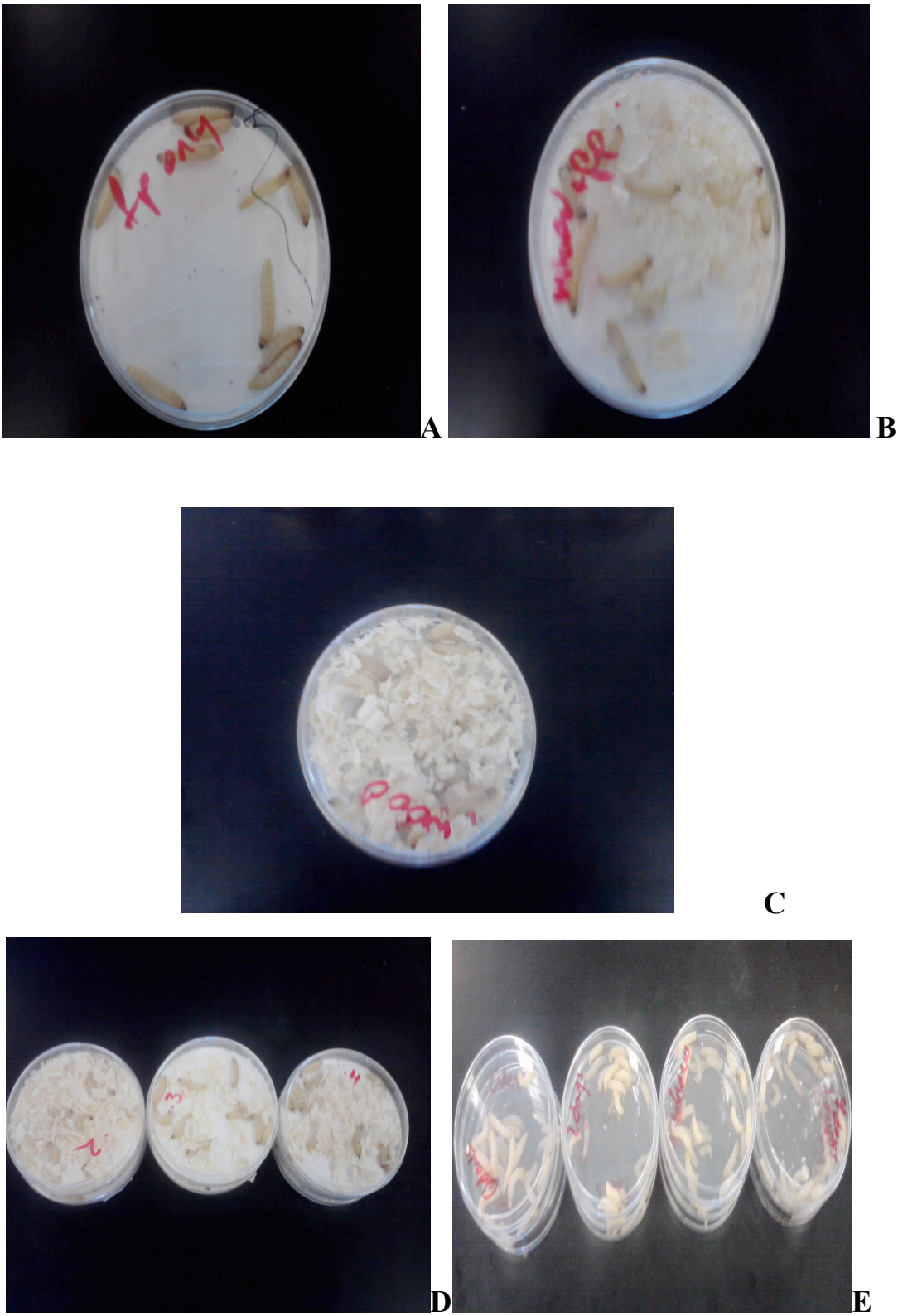


Figure 2.2 (A*) G. mellonella* on filter paper only (B) *G. mellonella* on wood shavings and filter paper (C) *G. mellonella* on wood shavings only (D) *G. mellonella* weighing 0.2g, 0.3g and 0.4g, respectively (E) *G. mellonella* that had been starved for 0, 2, 4 and 7 days respectively.

2.5 Effect of cytochalasin b and nocodazole

2.5.1 Assessment of the effect of cytochalasin b and nocodazole on viability of *G. mellonella*

Concentrations of cytochalasin b (dissolved in DMSO and diluted to 10µM, 5µM and 1µM) and nocodazole (dissolved in DMSO and diluted to 10µM, 5µM and 1µM) were made in sterile PBS, as described in Table 2.1. Larvae were inoculated through the last left pro-leg into the haemocoel with a volume of 20µl using a myjector syringe (Thermo Europe, Leuven, Belgium) and placed at 30°C in the dark for various amounts of time. All *in vivo* experiments included a control which consisted of injections with sterile PBS or 0.001% (v/v) DMSO. For assessment of larval death, larvae were probed with a needle and if no response was observed the larvae were deemed to be dead.

2.5.2 Inoculation of *G. mellonella* previously injected with cytochalasin b and nocodazole with the pathogen *C. albicans*

Larvae were injected with various concentrations of cytochalasin b and nocodazole. Four hours and twenty-four hours later larvae were inoculated with the pathogen *C. albicans* MEN. *C. albicans* MEN was grown to the stationary phase in YEPD liquid in an orbital shaker at 30°C. Cells were harvested and washed in PBS. Cells were resuspended in PBS at a cell density of 5 x 10^6 per 20µL. Larvae were inoculated through the last left pro-leg into the haemocoel with a volume of 20µL with a myjector syringe (Thermo Europe, Leuven, Belgium) and placed at 30°C in the dark for various amounts of time.

2.5.3 Assessment of effect of cytochalasin b and nocodazole on the ability of *G. mellonella* haemocytes to phagocytose

Haemocytes (5 x 10^6/mL) were isolated and incubated in the presence of cytochalasin b (10μM) and/or nocodazole (10μM) for 30 minutes at 37°C. Phagocytosis was measured by incubating pre-treated haemocytes (5 x 10^6/mL) with opsonised *A. flavus* conidia (1x10^7) in a final volume of 1 mL. This was stirred in a thermally controlled chamber at 37°C. An aliquot was removed at 30 minutes and at 90 minutes. Conidia of *A. flavus* were chosen for this experiment as their colour (dark green) made it easier to visualise when they had been internalised by the haemocytes. One hundred haemocytes were examined microscopically and the number containing phagocytosed conidia was ascertained on three independent occasions.

2.6 RNA extraction

2.6.1 Preparation of RNase free buffers and equipment

All glassware was baked at 220°C for 12 hours prior to use to reduce contamination by RNases. Diethyl pyrocarbonate (DEPC) is a strong inhibitor of RNases and was used at a concentration of 0.1% (v/v) to treat water, which was left stirring overnight followed by incubation at 37°C for a minimum of 4 hours prior to sterilization by autoclaving. This DEPC-treated water was used in the preparation of all buffers needed for RNA extraction. All bottle lids, O-rings and magnetic stirrers were soaked overnight in DEPC water and autoclaved before use. All chemicals were weighed without the use of a spatula. Gloves were worn at all times and changed regularly. Pipette tips and tubes were taken from freshly opened bags and autoclaved twice prior to use.

2.6.2 RNA Extraction from *G. mellonella*

Three larvae per treatment were selected and crushed into a fine powder using liquid nitrogen and a sterile pestle and mortar. To this 3 mL TRI®-Reagent was added, mixed and let rise to room temperature for 15 minutes. The mixture was placed in 1.5 mL tubes and centrifuged at 12,000 x g for 10 minutes at 4°C (Eppendorf centrifuge 5417R). The pellets were discarded and the supernatants collected in a new tube. Chloroform (200 µL-molecular grade) was added and mixed vigorously by vortexing. The solution was allowed to stand at room temperature for 10 minutes and centrifuged at 12,000 x g for 10 minutes at 4°C. The top clear layer was collected and placed in a new tube and 500 µL of 2-propanol (molecular grade) was added. The tube was inverted several times, left stand for 10 minutes and centrifuged at 12,000 x g for 10 minutes at 4°C. The supernatant was discarded and the resulting pellet was washed in 75% (v/v) ethanol (50 µL, molecular grade) and centrifuged at 12,000 x g for 10 minutes at 4°C. Supernatant was removed completely and the resulting pellet was air-dried and re-suspended in RNase-free water, aliquoted and stored at -70°C or used immediately. To check RNA purity, RNA extracted from *G. mellonella* was run on a 1% (w/v) agarose gel.

2.6.3 RNA Electrophoresis

Prior to use, the gel rig and tank were washed with 0.5% (w/v) SDS, rinsed with DEPC-treated water followed by ethanol and allowed to air dry. A 1% (w/v) agarose gel was prepared (1g agarose, 10 ml 10X FA buffer (200 mM 3-[N-morpholino]propanesulfonic acid (MOPS), 50 mM sodium acetate, 10 mM EDTA, pH 7) and DEPC water to make 100 mL). The mixture was heated until dissolved and allowed to cool to hand-hot. One point eight millilitres of 37% (v/v) formaldehyde plus 1 µL of a 10-mg/mL ethidium bromide solution was added prior to pouring the gel. Prior to running, the gel was equilibrated in 1X FA running buffer for at least 30 minutes. RNA (4 µL was added to 4 µL of 5X RNA gel loading dye; stock: 500 mM EDTA (80 µL, pH 8), 37% (v/v) formaldehyde (720

µL), glycerol (2 mL), formamide (3.084 mL), 10X FA buffer (4 mL) and 16 µL saturated aqueous bromophenol blue solution), heated to 65°C for 5 minutes and chilled on ice. Samples were loaded into the wells of the gel and run at 6 V/cm in 1X FA buffer. The gel was visualised using UV light.

2.7 cDNA synthesis and PCR analysis from *G. mellonella* RNA

2.7.1 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. RNA was added to an RNase and DNase-free tube and RNase-free water was added to bring the volume up to 8 µL. To this, 1 µL of 10 mM dNTP mix (provided) and 1 µL of 50 µM oligo (dT) (provided) were added. The solution was incubated at 65°C for 5 minutes, and then placed on ice for at least 1 minute. A master mix was prepared (Per reaction: 2 µL 10X RT buffer (supplied), 4 µL 25 mM $MgCl_2$, 2 µL 0.1 M DTT, 1µL RNaseOUT (supplied)) according to the number of reactions needed. Master mix (9 µL) was added to each of the RNA/primer mixes, mixed gently, incubated at 42°C for 2 minutes and held on ice. One microlitre of SuperScript III RT (200 U/µL, supplied) was added to each reaction tube and incubated as follows: 42°C for 50 minutes, 70°C for 5 minutes and held on ice. One microlitre of RNaseH (supplied) was added to each tube and further incubated at 37°C for 20 minutes. cDNA was aliquoted and stored at -20°C.

2.8 Real Time Polymerase Chain Reaction

2.8.1 Real Time PCR Conditions

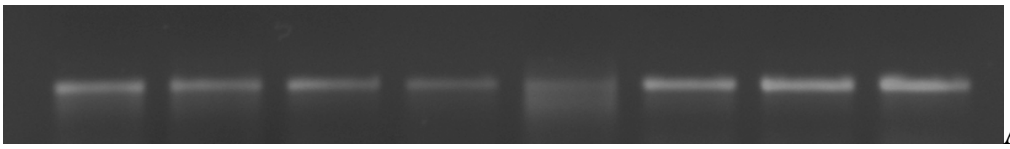
Real Time PCR was performed on a Light Cycler 480 system®(Roche) using the highest quality reagents obtained from Sigma and Roche. Cycle conditions were optimised for each primer set. The primers were obtained from literature (*G. mellonella* primers were obtained from Bergin *et al.* (2006) and Wojda and

Jakubowicz (2007)) and ordered from Sigma-Genosys. Primers were analyzed for secondary structures including dimer, hairpin-loop and palindrome formation using the Netprimer website available at (http://www.premierbiosoft.com/netprimer/netprlaunch/netprlaunch.html#) prior to ordering. Table 2.2 represents primer sequences and optimized cycle conditions for each primer set.

Primers were dissolved in molecular grade water to give a stock solution of 100 µM initially, which was diluted to a working solution of 10 µM. Each PCR reaction took place in a sterile 96 well plate (Roche) (9 µL volume) which contained 1 µg/µL cDNA, 5µL LightCycler® 480 SYBR Green, 1 µL water (molecular grade), 1 µL forward primer, 1 µL reverse primer. Solutions were mixed by gentle centrifugation and placed into the Light Cycler immediately. SYBR Green fluoresces as it associates with DNA. It forms bonds with the minor groove of dsDNA (Figure 2.3 (B)). SYBR green emits a 1000 greater fold signal when it is bound to DNA that when it is free in solution. The greater the quantity of DNA, the greater the emission spectra. The calculation was done by the deltadelta Ct method, whereby the Ct (Cycle time) is the time at which fluorescence reaches the threshold, the difference in Ct values between samples allows the software to quantify the DNA present (Valasek and Repa, 2005). As a standard curve, PCR amplification was performed with several dilutions of DNA template from uninjected larvae (S1=1000ng, S2= 250ng, S3=62.5ng, S4=15.625ng, S5=3.9ng). The cDNA prior to Real Time PCR amplification was of high quality as the RNA purity was assessed prior to synthesis. All RNA purity A260/A280 was above 1.88. All genes were standardised on the Roche Light Cycler 480® with effiecincy values of of 2.0 ± 0.4. All values and standardisations were performed on at least three occasions. The standard curves, melting peaks and amplification curves of these genes can be seen in Figures 2.4-2.8, inclusively.

2.8.2 PCR Product Visualisation and Quantification

The PCR product (4 µL) was visualised on a 1 % (w/v) Agarose gel. Gel images were analysed by densitometry to determine relative fold increase/decrease by using the array analysis tool in the ImageQuantL software.



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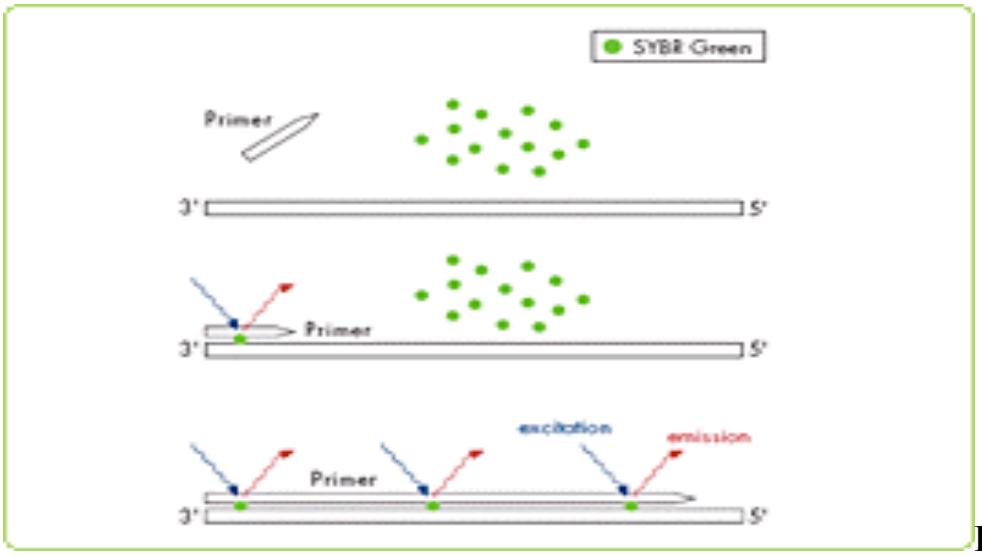
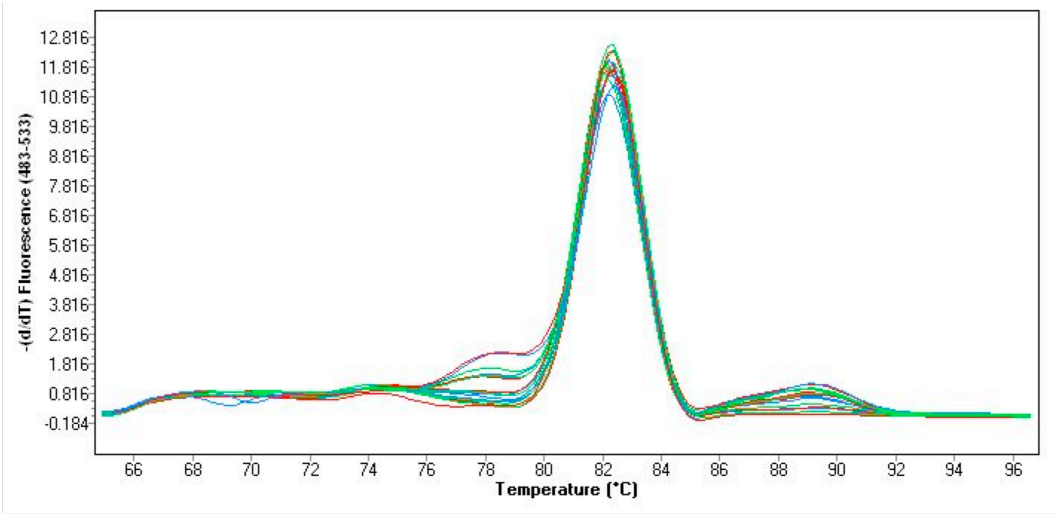


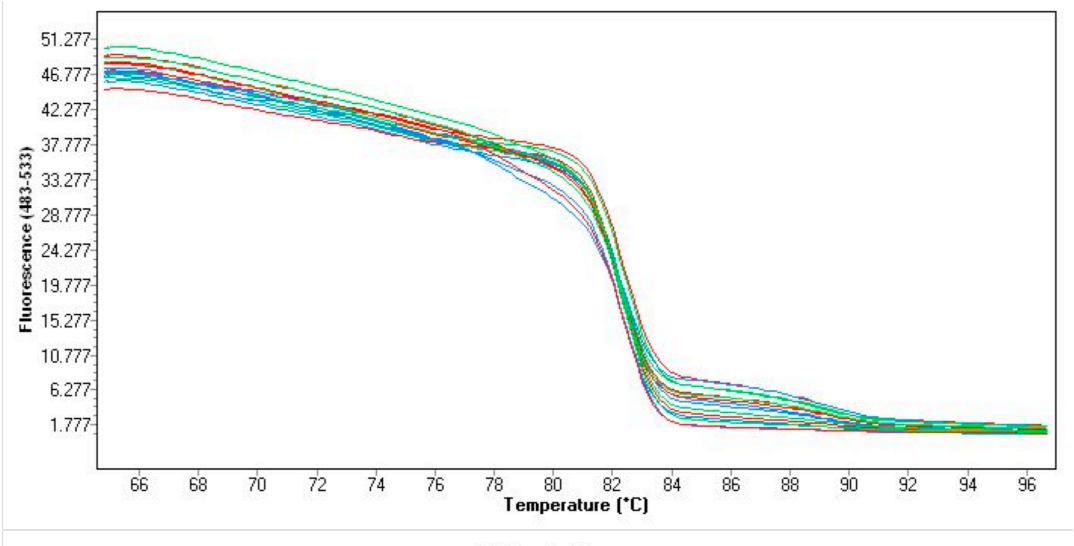
B

C

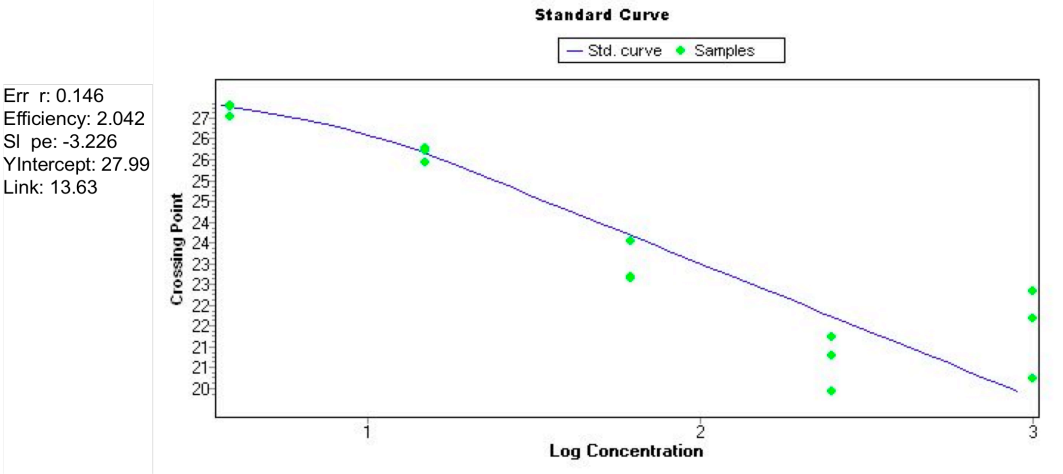
Figure 2.3 (A) *G. mellonella* RNA on a 1% agarose gel visualized under UV light (B) The intercalation of SYBR Green with DNA and the fluorescent emmision (Section 2.8.1) (C) Roche ® Light Cycler 480 used in this work for Real Time PCR (Section 2.8.1)



(A)

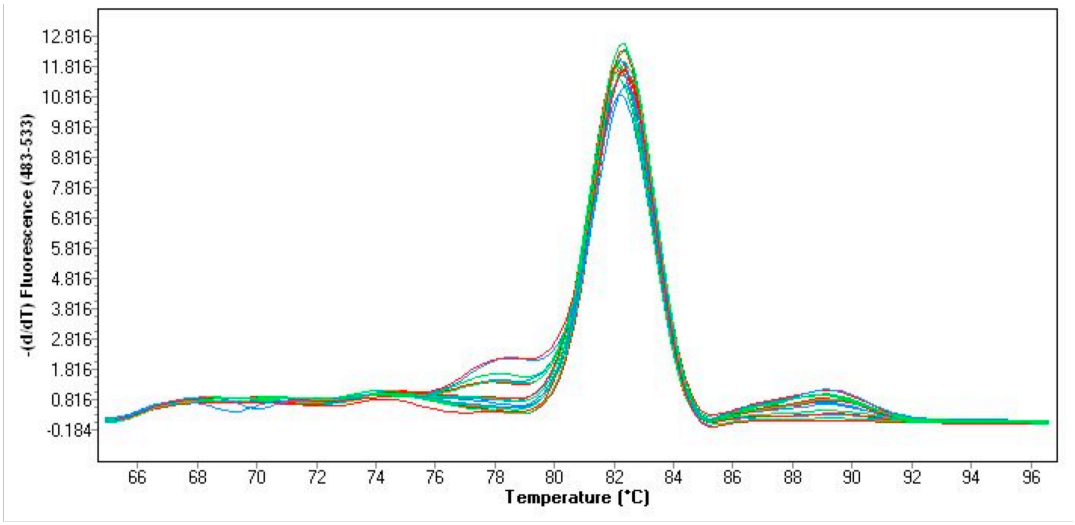


(B)

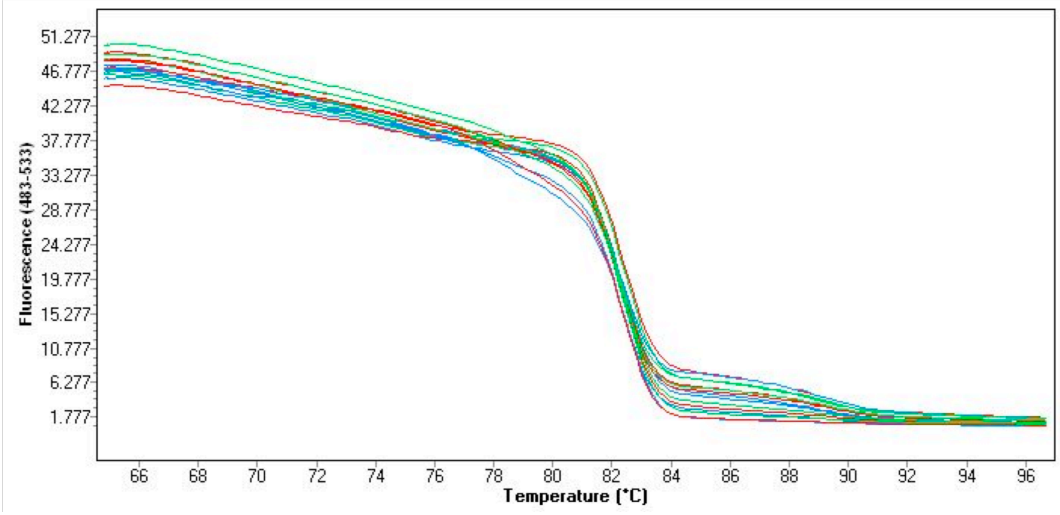


(C)

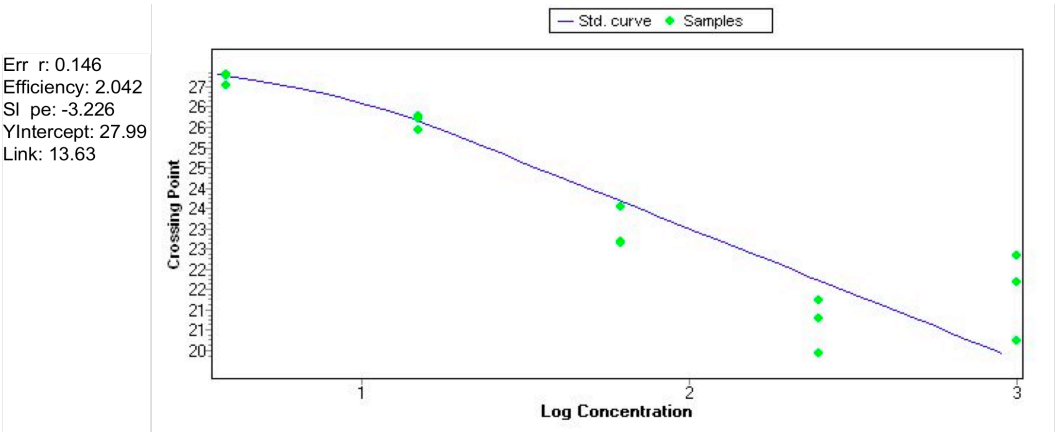
Figure 2.4 Melting Peak (A), Melting Curve (B) and Standard Curve (C) of *Galleriomicin* Gene. Data retrieved from the standardisation of *Galleriomycin*.



(A)

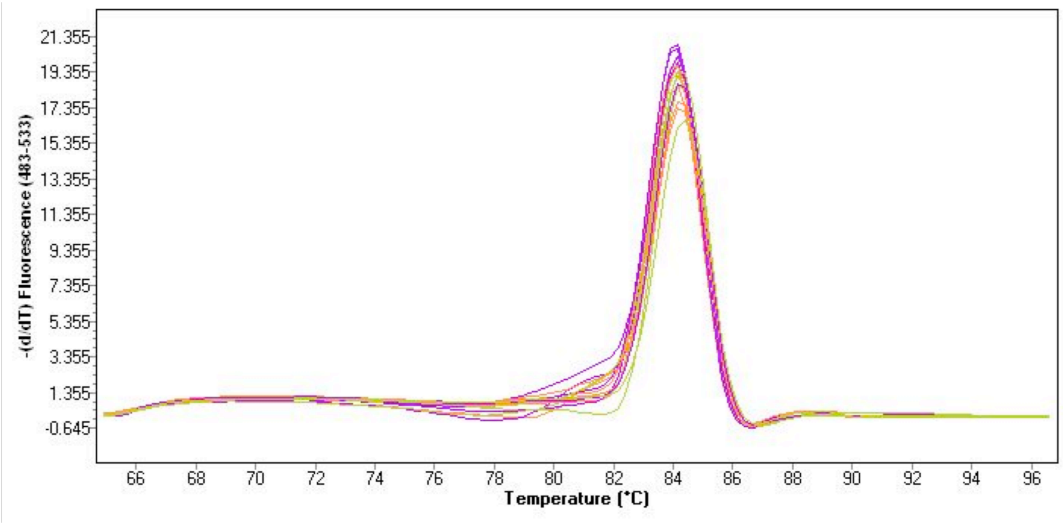


(B)

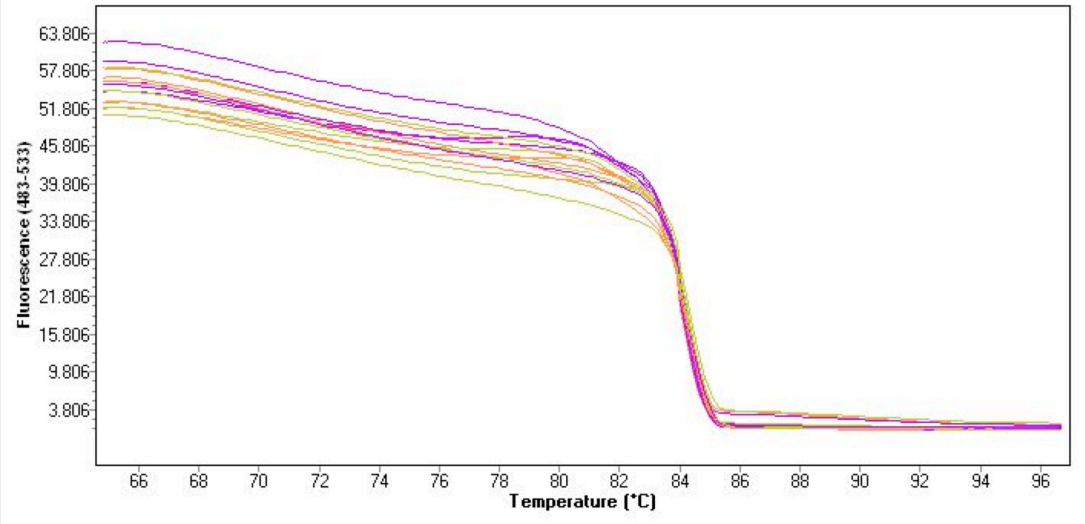


(C)

Figure 2.5 Melting Peak (A), Melting Curve (B) and Standard Curve (C) of *Galiomicin* Gene. Data retrieved from the standardisation of *Galiomicin*.



(A)



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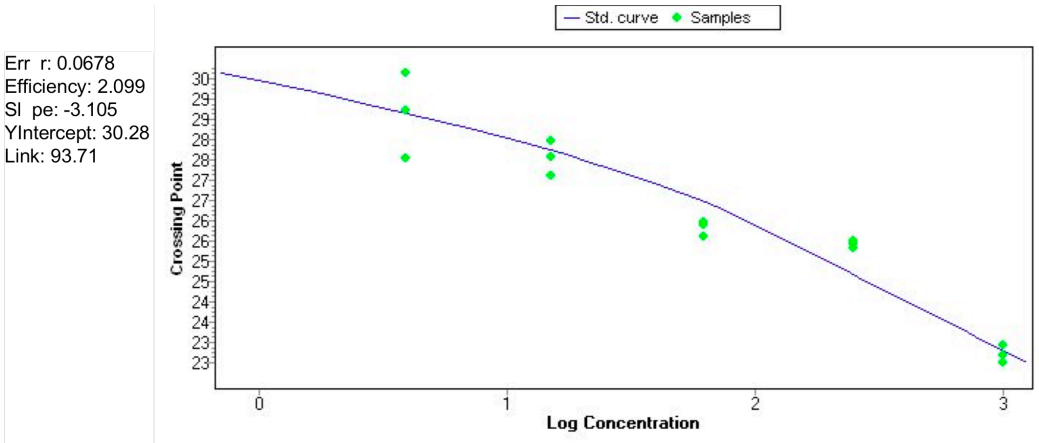
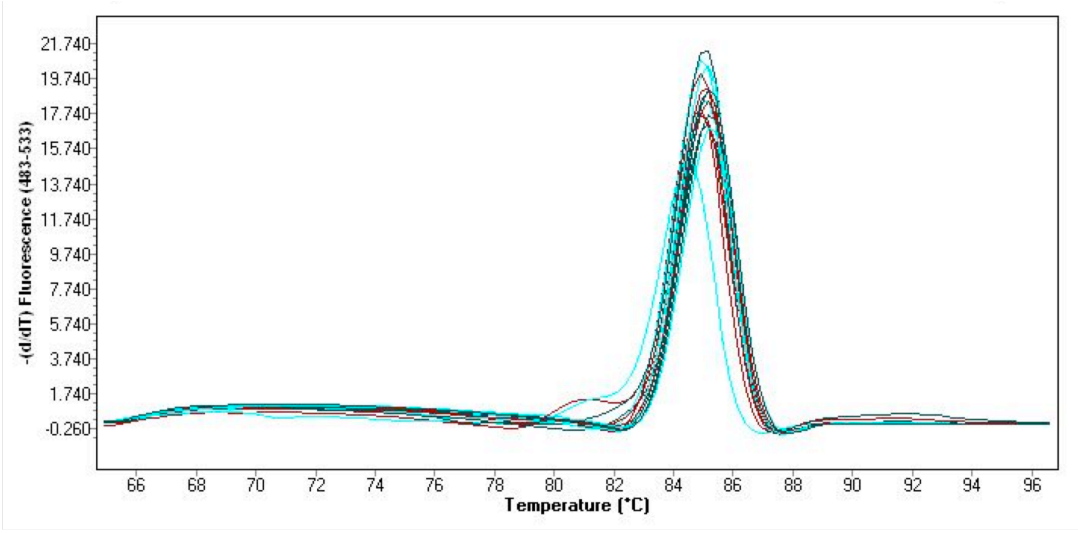
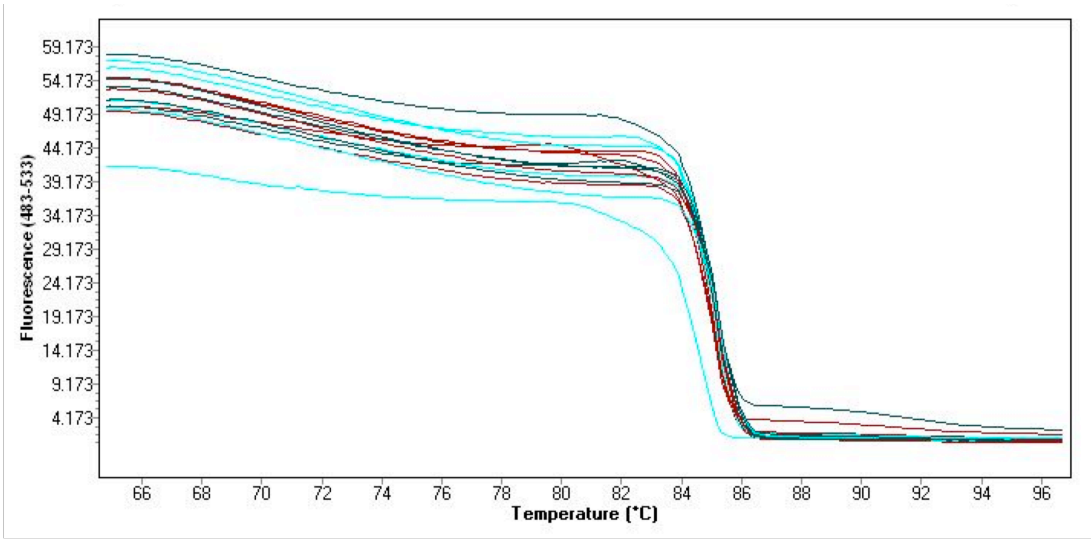


(C)

Figure 2.6 Melting Peak (A), Melting Curve (B) and Standard Curve (C) of *IMPI* Gene. Data retrieved from the standardisation of *IMPI*.



(A)



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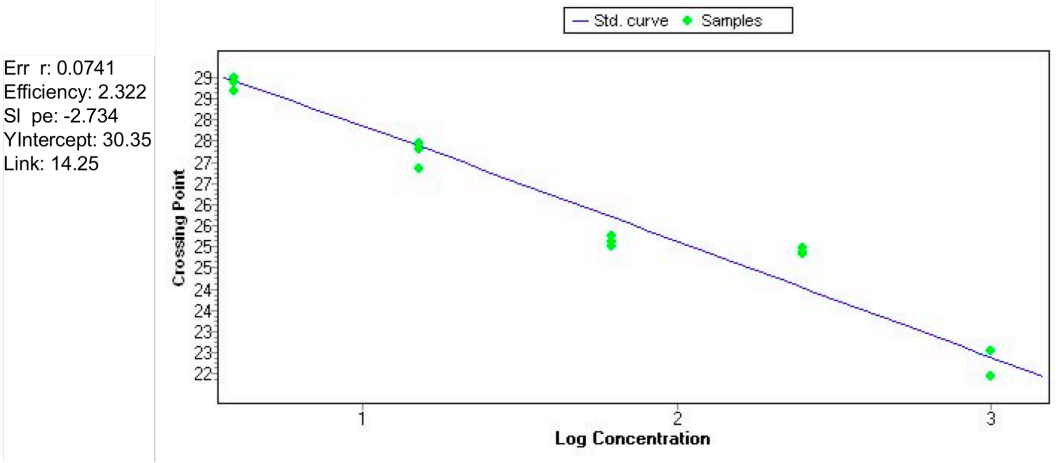
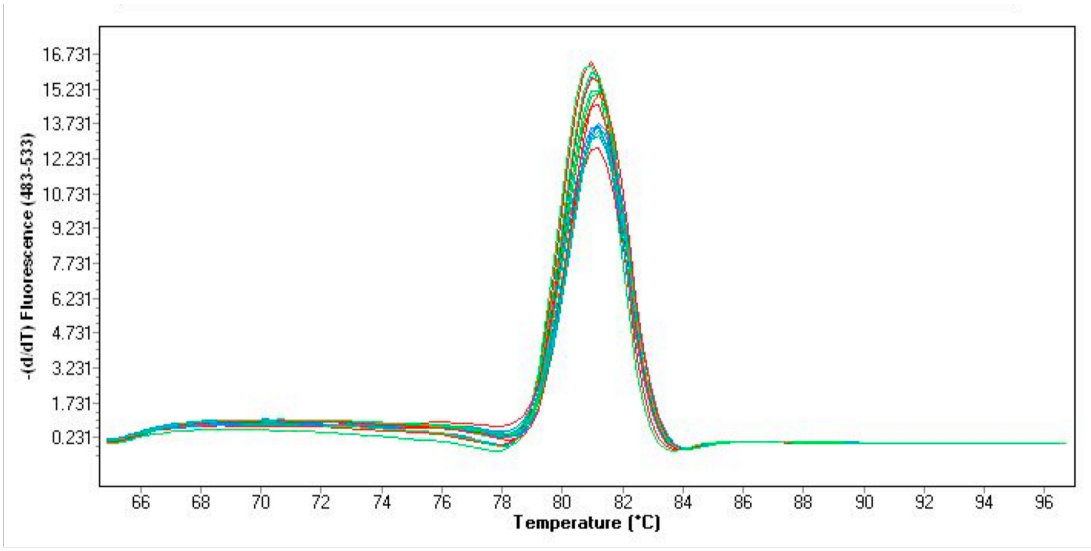
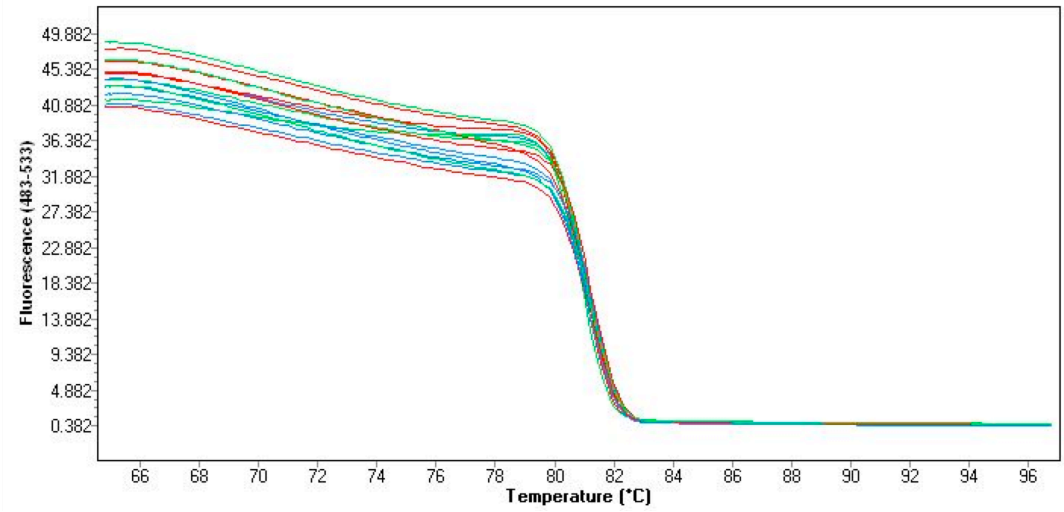


(C)

Figure 2.7 Melting Peak (A), Melting Curve (B) and Standard Curve (C) of *Transferrin* Gene. Data retrieved from the standardization of *Transferrin*.



(A)



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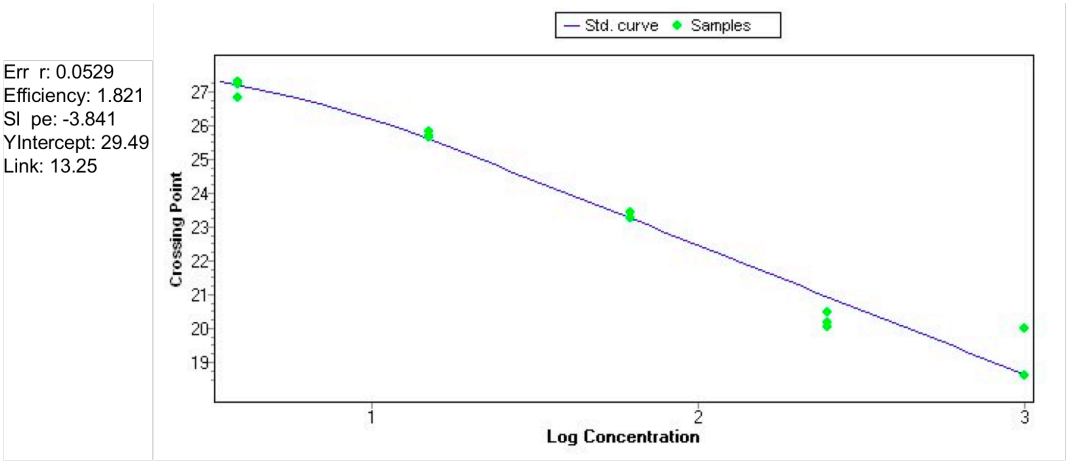

(C)

Figure 2.8 Melting Peak (A), Melting Curve (B) and Standard Curve (C) of *Se7* (Housekeeping Gene). Data retrieved from the standardization of *Se7*.

2.9 Isolation of Human Neutrophils and Insect Haemcytes (and the Proteomic Applications)

2.9.1 Isolation of Human Neutrophils

Neutrophils were isolated from blood collected from healthy donors. Blood was quickly added to equal volume of sterile 1X Saline (0.9%w/v) and 5mls of sterile 10% w/v sterile Dextran Sulphate was added. Buffers were heparinised (5u/ml). The tube was inverted and left standing for 30 minutes, at room temperature, to separate the red blood cells. The top layer was transferred to a fresh tube which was underlaid with 5mls of Lymphoprep (Axis-Shield) and centrifuged at 2,000xg for 10 minutes. Supernatant was removed and 25mls of dH_2O was added, the tube was then inverted 3-4 times and 25mls 2X Saline (1.8%) was added. The cells were centrifuged at 1,500xg for 5 minutes and the pellets were resuspended in 5mM PBS Glucose.

2.9.2 Isolation of Insect Haemocytes

Insect haemocytes were harvested from sixth-instar larvae of *G. mellonella*. Larvae were stored in wood shavings in the dark at 15°C and insects weighing 0.4g were selected. Haemocytes were extracted by bleeding 10 larvae into 10mL of sterile anti-coagulant buffer; IPS (150mM NaCl, 5mM KCL, 0.1M Tris-HCL, 10mM EDTA and 30mM Trisodium citrate in dH_2O, pH6.9). Cells were centrifuged at 1,500xg, washed once and finally resuspended in 5mM PBS-Glucose containing 1mg/ml Pepstatin A, 1 mg/ml Aprotitin, 1mM PMSF and 1mg/ml Leupeptin.

2.9.3 Viability of Haemocytes and Neutrophils

Viability of Haemocytes and Neutrophils was assayed using the Trypan Blue assay (Degel *et al.,* 2010).

2.9.4 Preparation of Whole cell Lysates from Haemocytes and Neutrophils

Haemocytes and neutrophils (1 x 10^8) were extracted from *G. mellonella* by piercing insects with a 23G BD Microlance needle, or in the case of neutrophils; from healthy donors. The cells were resuspended in Breaks Buffer (10mM KCL, 3mM NaCl, 4mM $MgCl_2$ and 10mM PIPEs, pH7.2) containing 1mg/mL Pepstatin A, 1mg/mL Aprotinin, 1mM PMSF and 1mg/mL Leupeptin. Neutrophils were extracted as described in section 2.9.1. PMA was added to cells at a concentration of 1µg/mL, and incubated at 37°C for 6 minutes. fMLP was added to haemocytes at a concentration of 0.2µg/mL and incubated at 37°C for 30 minutes. Cells were then sonicated for 3x5 seconds at 20% power (Bedelin Senepuls, Bendelin Electronics, Germany). The resultant lysates were centrifuged at 700xg for 5 minutes at 4°C and prepared for 1D and 2D electrophoresis.

2.9.5 *In vitro* phagocytosis of *C. albicans* by insect haemocytes

C. albicans was opsonised using cell free haemolymph diluted in IPS (1:10 dilution factor). Phagocytosis was measured by incubating 2 x 10^5 yeast cells with 1 x 10^5 haemocytes in a stirred chamber at 37°C, with a final volume of 2 mls. An aliquot was removed immediately after addition of the yeast cells (time zero) and after 20, 40, 60 and 80 minutes incubation, diluted in Minimum Essential Medium and plated onto YEPD plates supplemented with erythromycin at a concentration of 1µg/ml. The viability of cells was assessed by determining colony number and the results were determined in triplicate for each sample. The viability data are expressed as percentage viability at time zero. This assay was performed for haemocytes for *G. mellonella* that had been starved for 0 and 7 days and for haemocytes exposed to the agents in Table 2.1.

Compound	Concentrations Used	Preincubation Time
DMSO	0.001%v/v	30 minutes at 37°C
Cytochalasin B	10μM, 5 μM and 1 μM	30 minutes at 37°C
Nocodazole	10μM, 5 μM and 1 μM	30 minutes at 37°C
DPI	5 μM	30 minutes at 37°C
SOD	50μg/mL	30 minutes at 37°C

Table 2.1 List of Chemicals and concentrations involved in survival assays in Section 2.9.5.

2.9.6 Assessment of Neutrophil and Haemocyte Degranulation

Neutrophils and Haemocytes were extracted as described in sections 2.9.1 and 2.9.2 and on separate occasions cells were added to a sterile stirred chamber at 37°C. PMA was added at a concentration of 1µg/mL and samples were removed from the chamber at 0, 3, 6 and 9 minutes and placed on ice. Cells were centrifuged at 700 xg for 10 minutes and the protein content of the supernatant was quantified using the Bradford Assay (as described in section 2.9.9) for both 1 dimensional and 2 dimensional gel electrophoresis.

2.9.7 Fractionation of Cytosolic and Membrane Components from Haemocytes and Neutrophils

Haemocytes and neutrophils were isolated as indicated (Section 2.9.2 and Section 2.9.1) and 1 x 10^8 cells were resuspended in Breaks Buffer (10mM KCl, 3mM NaCl, 4mM $MgCl_2$ and 10mM Pipes, pH7.2) containing 1mg/mL Pepstatin A, 1 mg/mL Aprotitin, 1mM PMSF and 1mg/mL Leupeptin. PMA was added to a suspension of Haemocytes at a concentration of 1µg/mL and incubated at 37° for 6 minutes. Cells were fractionated into membrane and cytosolic components as mentioned in Renwick *et al.*, 2007.

2.9.8 Precipitation of Proteins

To precipitate protein, three times the volume of 100% cold acetone was added to each protein sample and allowed to precipitate at -20°C overnight. Samples were then centrifuged at 12,000xg for 30 minutes at 4°C. Samples were washed twice with 80% acetone and centrifuged again before the pellet was processed.

2.9.9 Bradford Assay

Sterile PBS was used to make a dilution of bovine serum albumin and this was used to make a range of standards (0.05-1.5mg/mL). All samples used were diluted in sterile PBS. Twenty µL of sample was placed in a 1mL cuvette. To this 980µL of Biorad Bradford protein assay reagent (Bio-Rad, Hercules, California, USA) was added. Samples were allowed to incubate for 5 minutes at room temperature before being read in an Eppendorf Bio-photometer. The quantity of protein was based on the OD590nm readings of the standards as in Figure 2.9.

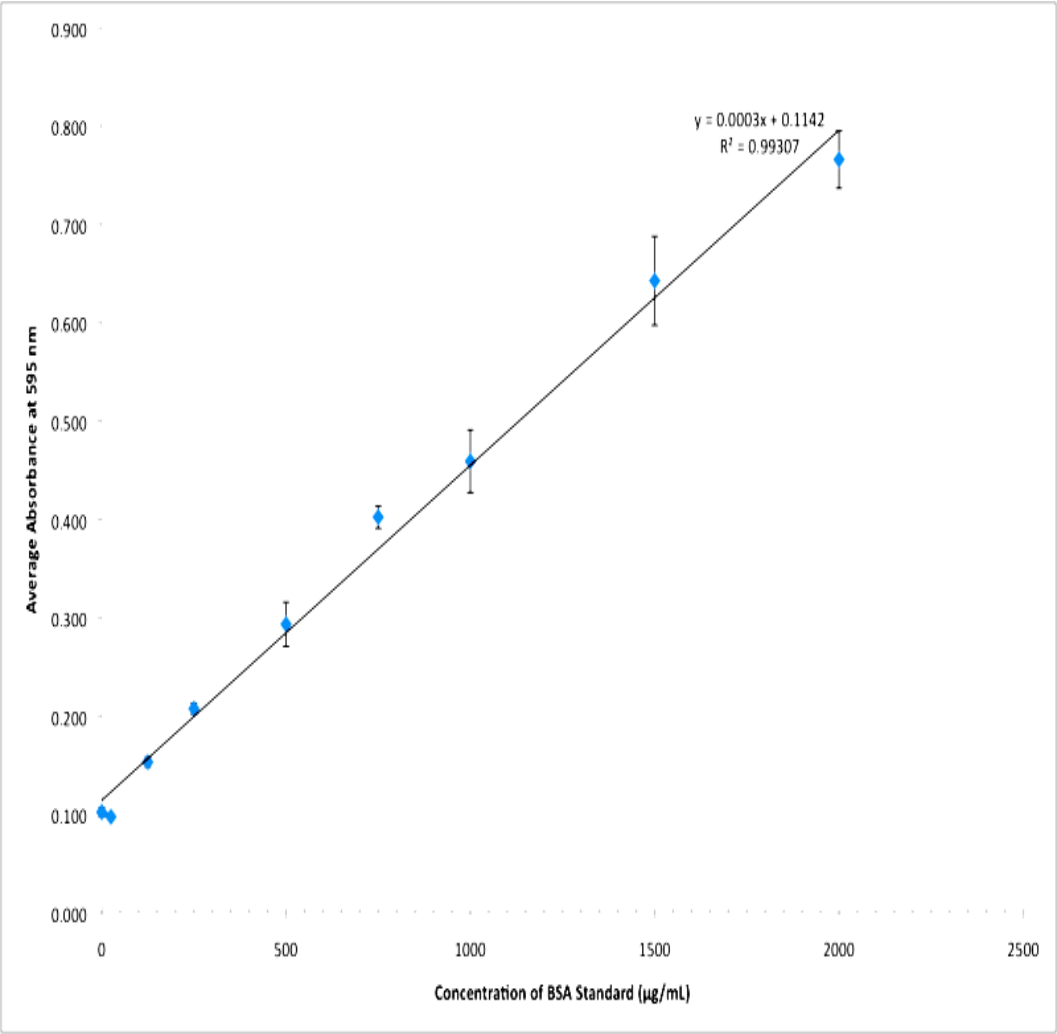

Figure 2.9 Standard curve of Bovine serum albumin used to determine unknown protein concentrations

2.10 Electrophoresis

2.10.1 1 D Electrophoresis

Solutions and buffers used for 1- and 2- dimensional protein analysis are listed below.

Stacking Gel Buffer:

- Tris-HCl 0.5 M, pH 6.8

Separating Gel Buffer

- Tris-HCl

1.5 M, pH 8.8

5% Polyacrylamide Stacking gel (for 4 gels):

- 0.5 M Tris-HCl 1.26 ml, pH 6.8
- Deionised water 6.8 mL
- 30% Bis-acrylamide (Protogel) 1.66 mL
- 10% SDS 0.1 mL
- 10% APS 0.1 mL
- TEMED 0.01 mL

12.5% Polyacrylamide Separating gel (for 4 gels):

- 1.5 M Tris-HCl 6 ml, pH 8.8
- Deionised water 7.6 mL
- 30% Bis-acrylamide (Protogel) 10 mL
- 10% SDS 0.24 mL
- 10% APS 0.15 mL
- TEMED 0.006 mL

The amount was multiplied according to number of gels required.

Running Buffer (10X):

· Tris-HCl	25 mM
· Glycine	250 mM
· SDS	0.1% (w/v)

pH 8.3

Running buffer was diluted to 1X in distilled water prior to running gels.

Ammonium Persulphate (APS):

· APS	10% (w/v) in distilled water.

Sodium Dodecyl sulphate (SDS):

· SDS	10% (w/v) in distilled water.

Sample buffer (5X);

· SDS	1.6 ml of a 10% (w/v) solution
· 2-Mercaptoethanol	0.4 mL
· Glycerol	10 mL
· 0.5 M Tris-HCl	1 ml, pH 6.8
· 0.5% (w/v) Bromophenol blue	0.2 mL
· Distilled water	4 mL

Coomassie Blue Stain Solution:

· Brilliant Blue R	0.2% (w/v)
· Acetic Acid	10% (v/v)
· Methanol	45% (v/v)
· Distilled water	45% (v/v)

Destain Solution:

· Acetic Acid	10% (v/v)
· Methanol	20% (v/v)
· Distilled water	70% (v/v)

Colloidal Coomassie Fixing Solution:

- Ethanol 50% (v/v)
- Phosphoric acid 3% (v/v)
- Distilled water 47% (v/v)

Colloidal Coomassie Stain Solution:

- Methanol 34% (v/v)
- Phosphoric acid 3% (v/v)
- Ammonium Sulphate 17% (w/v)
- SERVA Blue G (SERVA) 0.1 g
- Distilled water 4% (v/v)

5% Polyacrylamide Stacking gel (for 4 gels):

- 0.5 M Tris-HCl 1.26 mL, pH 6.8
- Deionised water 6.8 mL
- 30% Bis-acrylamide (Protogel) 1.66 mL
- 10% SDS 0.1 mL
- 10% APS 0.1 mL
- TEMED 0.01 mL

12.5% Polyacrylamide Separating gel (for 4 gels):

- 1.5 M Tris-HCl 6 ml, pH 8.8
- Deionised water 7.6 mL
- 30% Bis-acrylamide (Protogel) 10 mL
- 10% SDS 0.24 mL
- 10% APS 0.15 mL
- TEMED 0.006 mL

Ingredients were multiplied according to number of gels required.

Agarose sealing solution:

- Agarose 1% (w/v)
- Bromophenol blue 0.5% (w/v)
- 1X Running buffer 100 mL

Samples were run on SDS-polyacrylamide gel electrophoresis (PAGE) mini-gels (12.5%) and protein profiles were visualised by Colloidal Coomassie blue staining.

2.10.2 Colloidal Coomassie Blue Staining

Gels were immediately incubated in fixing solution overnight on a rotary shaker. Gels were then washed three times in deionised water for 10 minutes each time, on a rotary shaker. Gels were pre-incubated for 20 minutes in pre-incubation buffer (as described in Section 2.10.1) and Serva blue stain was added. To wash gels, pre-incubation buffer was poured off and gels were continuously washed with deionised water and scanned on a HP scanjet 5400C.

2.10.3 Western Blotting

The protein was transferred from the mini-gels to a nitrocellulose membrane using a wet blotter for 70 minutes at 100Volts. Rabbit polyclonal antisera raised against human epitopes (purchased from Genway Scientific, United States.) were used as is described in Table 2.2.

Horseradish peroxidase conjugated donkey anti-rabbit antibody was used at a concentration of 1/2,500 for 1 hour at room temperature to detect reactive bands by enhanced chemiluminesence in all western blots (PerkinElmer Biotechnology Inc.).

Antibody	**Concentrations Used**	**Company**	**Incubation Time**
Myeloperoxidase	1/500	Genway Biotech	Overnight at 4°C
Lactoferrin	1/500	Genway Biotech	Overnight at 4°C
Vinculin	1/200	Santa Cruz Technologies	Overnight at 4°C
Coronin	1/200	Santa Cruz Technologies	Overnight at 4°C
Actin	1/200	Santa Cruz Technologies	Overnight at 4°C
p47	1/1000	Bergin *et al*., 2005.	4 hours at R/T

Table 2.2 List of Antibody Concentrations and incubation periods involved in this work.

Separating Gel	Chemicals
60 mL	1.5M Tris HCl
0	0.5M Tris HCl
76mL	ddH_2O
100mL	Acrylamide
2.4mL	SDS
1.5mL	APS
60µL	TEMED

Table 2.3 Ingredients for 3x 13cm Gels

Separating Gel	Chemicals
150mL	1.5M Tris HCl
0	0.5M Tris HCl
190mL	ddH_2O
250mL	Acrylamide
6mL	SDS
3.75mL	APS
130µL	TEMED

Table 2.4 Ingredients for 3x 24cm Gels

2.10.4 2 D Electrophoresis

Protein extracted samples were acetone precipitated and centrifuged at 14,000xg, IEF buffer was added to the pellets (8M Urea, 2M Thiourea, 4% (w/v) CHAPS, 1% (vol/vol) Trion X-100, 65mM DTT, 10mM Trizma Base and 0.8% (vol/vol) Ampholyes (Amersham Bioscience, United Kingdom). The protein was quantified using the Bradford Assay and the protein solution was brought to 150µL for 7cm (250µL for 13cm, 340µL and 450µL for 24cm) IPG strips with the IEF buffer. Strips were allowed to rehydrate on the bench overnight. Protein (100µg)

was loaded onto the 7cm strips, 300µg was loaded onto 13cm strips and 450µg was loaded onto 24cm strips. Iso-electric focusing was performed on an Ettan IPGphor II (Amersham Biosciences, NJ, USA) system using the following program:

1. 50 Volts Step and Hold 10 Hours
2. 250 Volts Step and Hold 0.15 Hours
3. 8000 Volts Gradient 5 Hours
4. 8000 Volts Step and Hold 8 Hours

Following iso-electric focusing (IEF), strips were equilibrated in reducing buffer (50 mM Tris-HCl, 6 M Urea, 2% (w/v) SDS, 30% (v/v) Glycerol, 2% (w/v) DTT, pH 6.8) for 15 minutes at room temperature, followed by equilibration in alkylation buffer (50 mM Tris-HCl, 6 M Urea, 2% (w/v) SDS, 30% (v/v) Glycerol, 2.5% iodoacetamide (IAA) and trace bromophenol blue, pH 6.8) for 15 minutes. Strips were placed on top of homogeneous 12.5% SDS-PAGE gels (cast on the PROTEAN II casting unit (Bio-Rad)). SDS-PAGE Standards, low range (Bio-Rad) were boiled for 5 minutes in 2X sample buffer and loaded near the anodic side of the strip. Strips were overlaid with hand-warm sealing solution. Gels were electrophoresed on the Amersham Biosciences Dalt cell system for 20 hours at 100V or until the bromophenol indication layer was at the bottom of the gel. Gels were stained with colloidal Coomassie stain for several days and destained with destaining solution, until all the background blue was removed, as described.

Mass spectrometry of trypsin digested proteins was performed using an Agilent ESI Trap LC/MS. Resulting spectra were analysed using MASCOT, Matrix Sciences with a score over 67 considered significant.

2.10.5 In gel trypsin digestion of spots/bands

All tubes and pipette tips were washed with 100%(v/v) LC/MS grade Acetonitrile (ACN). Coomassie Destain: Ammonium Bicarbonate (100mM)/ACN, 1:1 dilution, was added to the spots for 30 minutes shaking at room temperature to destain samples. Following this 30-minute incubation period 500µls of ACN was added for 10 minutes shaking at room temperature.

Reduction and Alkylation: Samples were centrifuged at 20,000xg for 10 minutes and the supernatant was removed. DTT (50µL of 10mM) in 100mM Ammonium

Bicarbonate was added to each spot for 30 minutes at 56°C. Tubes were chilled to room temperature and centrifuged at 20,000xg for 10 minutes. The supernatant was removed. IAA (50µL of 55mM) in 100mM Ammonium Bicarbonate was added for 20 minutes shaking at room temperature.

Tryptic Digestion: Trypsin (20µgs-Promega) was reconstituted in 100µL of reconstitution buffer (10mM Ammonium Bicarbonate containing 10% ACN). This was aliquoted into 10x10µL aliquots and to each of these 500µL of 50mM Ammonium Bicarbonate was added. The trypsin solution (50µL) was added to each gel plug. This was incubated at 4°C for 1 hour and at 37°C overnight.

Peptide recovery: Samples are centrifuged at 20,000xg for 10 minutes and supernatant was transferred to a fresh 1.5mL tube. Extraction buffer (1:2 5% Formic Acid (v/v): ACN) was added to the plugs (50µL) and incubated at 37°C for 15 minutes. Samples were centrifuged at 20,000xg for 10 minutes and added to the supernatant. Extracts were dried in a vacuum centrifuge overnight. Dried peptides were resuspended in 20µL of 0.1% Formic Acid and sonicated for 2 minutes. Samples were filter sterilized and supernatants were added to mass spectroscopy vials (Agilent Technologies, USA). Samples were analysed on a 6340 Ion Trap LC/MS spectrometer (Agilent Technologies) using BSA as external standards. The mass lists generated were blasted using the MASCOT MS/MS ion search program available at http://www.matrixscience.com.

2.11 Confocal Immunofluoresence Microscopy

Haemocytes and Neutrohils ($1x10^5$) were isolated and allowed to adhere to glass slides for 5 minutes. Slides were incubated at 37°C for 10 minutes with PBS and 6 minutes PMA (1µg/mL). Slides were fixed for 10mins with 4% (v/v) paraformaldehyde and washed in PBS for 10 minutes. Cells were permeabilised with 0.2% (v/v)Triton X 100 in PBS for 10 minutes and blocked with 1% BSA in PBS (v/v) for 1 hour. This was followed by a 10 minutes wash in PBS. Cells were incubated with rabbit primary antibody overnight against Myeloperoxidase at 4°C at a concentration of 1/500 to assess degranulation. Slides were washed with PBS and incubated with (TRITC) goat anti rabbit (The Jackson Laboratory, Bar Harbor, Maine, United States.) at a concentration of 1/250. The controls for this experiment included cells alone and cells exposed to secondary antibody only.

Cells were also incubated with the cytoskeletal inhibitors cytochalasin b and nocodazole at a concentration of 10µM for 30 minutes at 37°C prior to PMA (1µg/mL) stimulation. The slides were washed and incubated with rabbit polyclonal antisera raised against the actin proteins vinculin, coronin, globular actin and filamentous actin at concentrations of 1/200. Slides were washed with PBS and incubated with (TRITC) goat anti rabbit (The Jackson Laboratory, Maine, United States.). The controls for this experiment were unstimulated cells and cells exposed to secondary antibody only. Slides were also prepared for Rhodamine Phalloidin (Biotium, Inc.) staining, which is used for staining F-actin *in vitro*. Cells were treated as described and fixed with 4% paraformaldehyde. Following fixation, slides were washed with PBS and permeabilised with 0.2% Triton X 100 in PBS for 10 minutes at room temperature for 10 minutes. Cells were PMA (1µg/ml) stimulated before being treated with the relevant inhibitors. Slides were washed and blocked with 1% BSA in PBS (v/v) for 1 hour. Slides were washed in PBS, 10µL of methanolic stock solution of the phalloidin was diluted in 200µL PBS with 1% BSA per slide. This was added for 20 minutes at room temperature. The slides were washed 2-3 times with PBS and the coverslip was added and sealed.

2.12 Shotgun Proteomics utilising Multidimensional Protein Identification

$1x10^8$ cells were isolated and whole cell lysates were acquired by sonicating isolated haemocytes at cycle 6 for 10 seconds at 10%, 3 times using a Bandelin Senoplus HD2200 sonicator. Cells were centrifuged at 700xg for 10 minutes at 4°C. Supernatant was quantified using the Bradford assay as described in Section 2.9.9 and 500µg of protein was added to a 1.5mL tube with a final volume being 100µL. DTT (10µL of 1M stock) was added to the tube. Tube was incubated at 56°C for 30 minutes. Tubes were allowed to cool to room temperature and 55µL of 1M Iodoacetamide (IAA) was added to the lysate. Tubes were incubated at room temperature for 20 minutes. Samples were dialysed twice with ice-cold 100mM ammonium bicarbonate, (2x50ml volumes in a 50ml tube), for at least three hours with occasional stirring, at 4°C. From this point on, all tubes were washed with LC-MS grade acetonitrile. Denatured protein solution was added to the tube and 5µls of

Trysin (Promega) in 10mM ammonium bicarbonate containing 10% acetonitrile was added. Samples were allowed to digest overnight at 37°C. Peptides were recovered as described above. Samples were diluted to a 1/10 dilution in 0.1% formic acid prior to loading onto the LC-MS. Samples were analysed on a 6340 Ion Trap LC/MS spectrometer (Agilent Technologies) using BSA as external standards as in Figure 2.9. The mass lists generated were blasted using the MASCOT MS/MS ion search program available at http://www.matrixscience.com.

2.13 ProQ Diamond Phosphorylation Stain for 1 Dimensional and 2 Dimensional gels

Gels were immersed in ~50mL (100mL for 2D gels) of fixing solution (50% methanol and 10% acetic acid) and incubated at room temperature for 30 minutes, with gentle agitation. Fixation step was repeated once more to ensure that all the SDS was washed out of the gel. Gels were washed with ultrapure water for 10 minutes, with gentle agitation. This step was repeated twice for a total of three washes. Gels were incubated in ~50mL (100mL for 2D gels) of Pro-Q® Diamond stain for a 90 minute period. The gels were destained in 20% acetonitrile (v/v), 50mM sodium acetate (pH 4) a total of 3 times at 30 minutes per incubation, to reduce background signal. Gels were washed twice with ultrapure water, for 10 minutes per wash step. Gels were then imaged using a Typhoon Trio+ (GE Healthcare Life Science ®), with excitation source at 532nm and emision filter at 560nm.

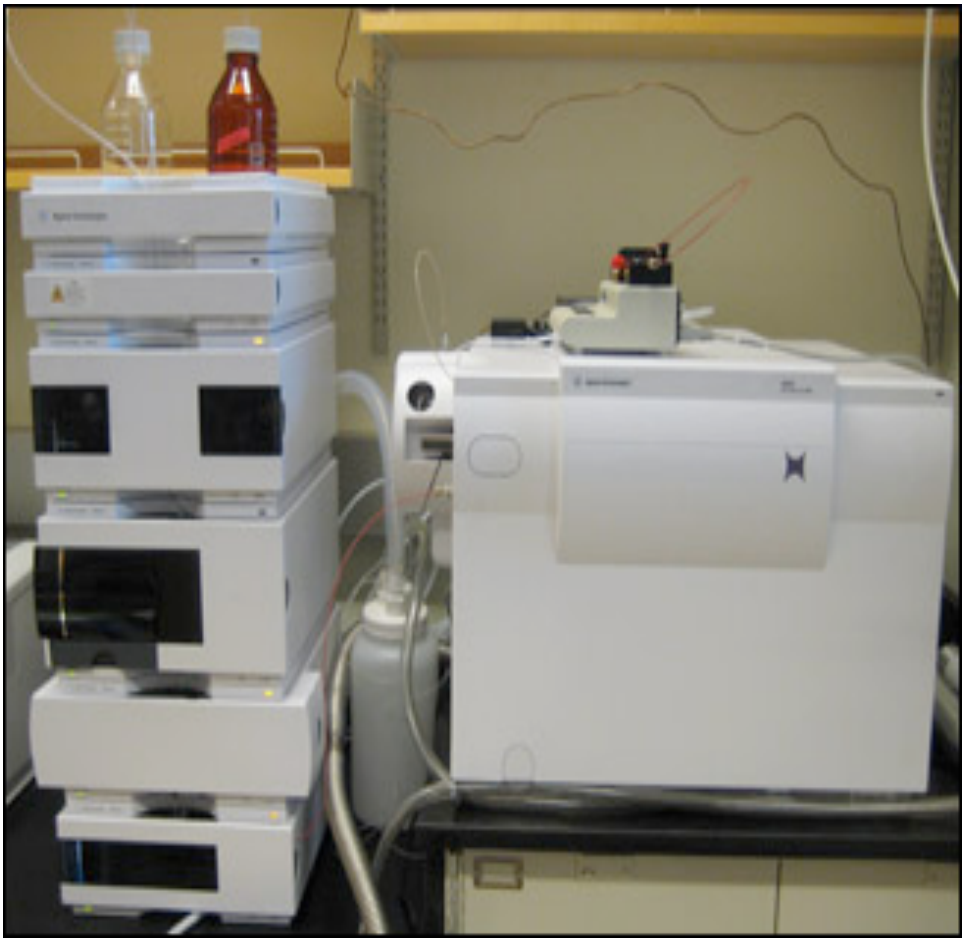

Figure 2.10 Agilent 6340 LC-MS Ion Trap used in this work to help identify peptides

2.14 MPO Activity Assay

Haemocyes and neutrophils were isolated and induced to degranulated as described in Section 2.9.6. Degranulation sample (100µL) at the relevant time points was added to a 96 well microtitre plate. Tetramethylbenzidine (100µL of 2mM 3, 3', 5, 5' –TMB) was added and allowed to incubate at room temperature for 10 minutes. The optical density (OD) of each well was then read using 525/25nM excitation filter and at 590/25nm emmision filter using a microtitre plate spectrophotometer. To ensure that activity was due to MPO release due to degranulation and not due to cell lysis, lysed cells were also assayed.

2.15 Flow Cytometry

Haemocytes ($1x10^5$) were isolated in IPS Buffer as described in Section 2.9.2, cells were resuspended in 5mM PBS-Glucose containing 1mg/mL Pepstatin A, 1 mg/mL Aprotitin, 1mM PMSF and 1mg/mL Leupeptin. Cells were PMA (1µg/mL) stimulated and treated with the relevant inhibitors as before. Following relevant treatments, cells were centrifuged at 2,000xg and resuspended in 1% (w/v) BSA/PBS. Cells were immediately fixed with 3.7% formaldehyde at 4°C for 10 minutes. Cells were washed twice with 1% (w/v) BSA/PBS and resuspended at final concentrations of $1x10^4$ cells per FACS tube.

2.15.1 Labeling of cells with antibodies for Size versus Granularity analysis

Cells were incubated at 4°C overnight with anti- vinculin, anti-coronin, anti-actin and anti-MPO at concentrations of 1/200. Cells were washed twice with 1% (w/v) BSA/PBS and incubated with secondary Alexa Flour 488 Anti-human antibody at a concentration of 1/1000 for 1 hour at room temperature. Cells were washed twice with 1% (w/v) BSA/PBS and analysed on a Becton Dickinson® FACS Calibur (San Jose, California, USA) using FL1 parameters to detect flouresence.

2.15.2 Preparation of cells for sub-population sorting

Haemocytes ($1x10^8$) were isolated and treated with PMA (1µg/ml) for 6 minutes; a control group was left unstimulated. Cells were immediately fixed with 3.7% (v/v) formaldehyde at 4°C for 10 minutes. Cells were washed with 1% (w/v) BSA/PBS twice and resuspended in 1% (w/v) BSA/PBS at a final concentration of $1x10^7$. Cells were analysed on a Becton Dickinson® FACS ARIA, National Institute of Cellular Biotechnology, Dublin City University.

A

B

Figure 2.11 (A) BD FACS Calibur ® used in this work to analyse granularity and size in cells,

(B) BD FACS Aria ® used in this work to sort and fractionate cells into sub-populations.

2.16 Statistical Analysis

All assays were performed on three independent occasions. Results are expressed as the mean ±SE. Statistical comparisons were made with a one tailed, two sample, equal variance (homoscedastic), students t test.

Differences were considered significant at $p<0.05$.

Gene	Primer	Reference	Sequence 5' - 3'	Product Size (bp)
Se7	**Se7 F**	Wojda and Jakubowicz (2007)	**ATGTGCCAATGCCCAGTTG**	131
	Se7 R		**GTGGCTAGGCTTGGGAAGAAT**	
Transferrin	**TRANS F**	Bergin *et al*., (2006)	**CCCGAAGATGAACGATCAC**	535
	TRANS R		**CGAAAGGCCTAGAACGTTTG**	
IMPI	**IMPI F**	Bergin *et al*., (2006)	**ATTTGTAACGGTGGACACGA**	409
	IMPI R		**CGCAAATTGGTATGCATGG**	
Galiomicin	**GALIO F**	Bergin *et al*., (2006)	**CCTCTGATTGCAATGCTGAGTG**	359
	GALIO R		**GCTGCCAAGTTAGTCAACAGG**	
Gallerimycin	**GALLER F**	Bergin *et al*., (2006)	**GAAGATCGCTTTCATAGTCGC**	175
	GALLER R		**TACTCCTGCAGTTAGCAATGC**	

Real Time PCR Conditions: 95°C, 3 min, (95°C, 15 seconds)

60°C, 15 seconds

72°C, (1 min) x 29 cycles

72°C, 10 min.

Table 2.5 Real Time PCR conditions and Primer Designs

Chapter 3

Effect of nutritional status of *G. mellonella* on larval susceptibility to pathogen infection

3.0 Effect of nutritional status of *G. mellonella* on larval susceptibility to pathogen infection

The main objectives of this Chapter were to characterise the nutritional state of *G. mellonella* larvae so that the insect can be accurately studied and utilised as a model organism and also to study the relationship between larval nutrition and insect survival when the immune system is attacked by pathogens. *G. mellonella* larvae are an excellent model organism due to the many similarities between the insect immune response and the human innate immune response (Mukherjee *et al*., 2010; Kavanagh and Reeves, 2004). *G. mellonella* possess an immune system that can rapidly alter itself to deal with the invasion of pathogens and they have an interconnecting web of humoral and cellular immune responses waiting to be activated. There are similarities throughout these signaling cascades with the human innate immune system.

The ability of mammals to survive is largely dependent upon factors such as food and therefore energy levels, the degree of basal immune system ability and the individual's weight and strength (Gluckman *et al.*, 2007). This is an area that has received an immense amount of study, whereas insect nutritional status has been predominantly left untouched, even though there has been an increase in the use of insects as *in vivo* and *ex vivo* models in research and development (Scully *et al.,* 2006).

It has previously been documented that *G. mellonella* larvae display the ability to prime their immune system when exposed to thermal and physical stress (Mowlds *et al.*, 2008), therefore a reduction/alteration or even elimination of food may also lead to priming or conversely increased cell death and insect melanisation upon infection. Similarly, the insect's weight may be a factor in the ability to withstand an infection

This Chapter sought to consider the nutritional variables that may affect *G. mellonella*'s ability to survive upon infection with *C. albicans* and *A. fumigatus*. Food sources provide energy for the larvae to survive and also lead to the formation of a basal immune system, therefore it was decided to study the effect of nutritional variation and total nutritional depletion on the larvae's ability to survive infection with a pathogen.

Due to the variation of larval weight in experimental science (Brennan *et al*., 2002; Reeves *et al*., 2004; Kopacek *et al*., 1995; Bratti *et al*., 1995), a study focused on the relationship between larval weight and ability to survive was also carried out. Changes in haemocyte density, which is a fundamental indicator of infection as an increase in density can be indicative of an elevated immune response, was assessed, as was the expression of certain gene encoding antimicrobial peptides involved in insect immunity.

3.1 Assessment of the effect of different food sources on the ability of *G. mellonella* larvae to withstand infection

The relationship between food source and the larvae's ability to survive infection was examined as described in section 2.4.3. The larvae were inoculated with 1 x 10^6 /20 µL *C. albicans* cells or 1 x 10^5/20 µL *A. fumigatus* spores. The percentage survival of *G. mellonella,* when inoculated with each pathogen following seven days of being exposed to each food source, is expressed with the respective standard errors. The statistics are based upon the values obtained from feeding the larvae filter paper only.

It was found that larvae fed a combination of wood and filter paper and later inoculated with *C. albicans* cells displayed a reduction in survival over a 48 hour period from 90% ± 3.33% to 80% ± 0% ($p < 0.01$). Larvae that were fed wood shavings only, displayed a reduction from 70% ± 5.77% to 60% ± 5.77% ($p < 0.05$). *G. mellonella* larvae that were fed on just filter paper demonstrated survival that remained at 40% from 24 hours to 48 hours. These data are presented in Figure 3.1.

A parallel study was carried out with the fungal pathogen *A. fumigatus*. In this study larvae that were fed a combination of wood shavings and filter paper demonstrated a decrease in survival from 43% ± 3.33% at 24 hours to 11.55% ± 6.67% ($p < 0.05$) at 48 hours. Larvae that were fed a diet of wood shavings only, showed a reduction from 30% ± 0% to 10% ± 5.77% ($p < 0.1$) at 48 hours. The larvae that were fed filter paper only maintained survival at 33% ± 6.67%, 24 hours after inoculation. This fell to 3% ± 1.33% 48 hours following initial inoculation (Figure 3.2).

This study demonstrated that a combination of wood shavings and filter paper will maintain a higher larval survival rate 48-hours post inoculation, indicating it to be the most nutritious dietary source.

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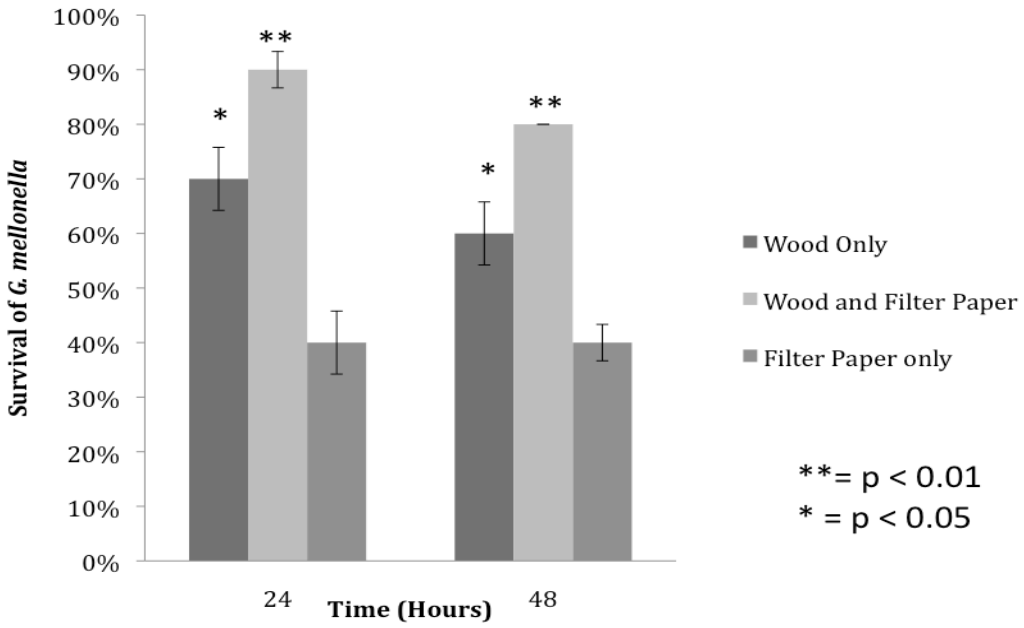


Figure 3.1 Survival of *G. mellonella* larvae fed on different food sources and inoculated with 1 x 10^{6}/20 µL *C. albicans* cells (n=3).

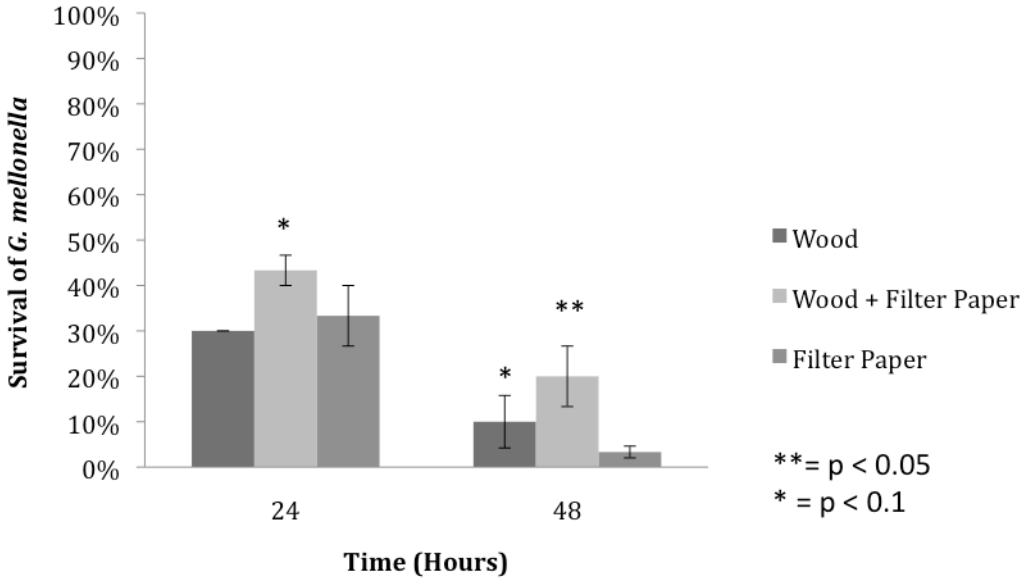


Figure 3.2 Survival of *G. mellonella* larvae fed on different food sources and innoculated with 1 x 10^{5}/20 µL *A. fumigatus* spores (n=3).
(Statistics are based on the larvae that were fed filter paper only.)

3.2 Assessment of the effect of different starvation periods on the ability of *G. mellonella* larvae to withstand infection

G. mellonella larvae were starved for 2, 4, or 7 days as described in section 2.4.4. A control group of larvae was left unstarved (0 Days). To investigate the relationship between the starvation period and immune status the larvae were inoculated with 1 x 10^6 /20 µL *C. albicans* cells or with 1 x 10^5/20 µL *A. fumigatus* spores. Again, the percentage survival of *G. mellonella,* when inoculated with each pathogen, is expressed with the respective standard errors. The statistics are based on the values obtained from the control; i.e. the unstarved larvae.

Upon infection with *C. albicans,* the unstarved larvae displayed a survival rate of 66.67% ± 5.09% after 24 hours. This value fell to 60% ± 5.77% 48 hours post inoculation. Larvae starved for 2 days prior to infection survived at 53.33% ± 5.09% after 24 hours ($p < 0.5$), but 48 hours post inoculation this value fell to 30% ± 3.33% ($p < 0.1$). An initial starvation period of 4 days left larval survival at 53.33% ± 1.92% 24 hours post inoculation ($p < 0.5$) and survival fell to 50% ± 0% after 48 hours ($p < 0.5$). The larvae starved for 7 days showed a survival rate at 33.33% ± 1.92% after 24 hours ($p < 0.1$), survival remained at 33.33% ± 1.92% 48 hours post inoculation with *C. albicans* ($p < 0.1$). (Figure 3.3)

With regard to infection with *A. fumigatus* spores, the unstarved larvae displayed a survival rate of 30% ± 5.77% after 24 hours; this value was 10% after 48 hours (Figure 3.4). Larvae starved for 2 days initially survived at 27% ± 1.92% after 24 hours (no significant difference), survival was 6.67% ± 1.92% 48 hours following infection ($p < 0.5$). The larvae starved for 4 days and inoculated with *A. fumigatus* spores showed a survival rate of 27% ± 1.92% 24 hours following infection (no significant difference), at 48 hours survival was 10% ± 3.33% (no significant difference). A period without food for seven days demonstrated larval survival of 20% ± 3.33% at 24 hours (no significant difference); this value fell to 0% survival after 48 hours ($p < 0.05$).

These results indicated that starving the larvae prior to infection for any period of time makes the larvae more susceptible to infection.

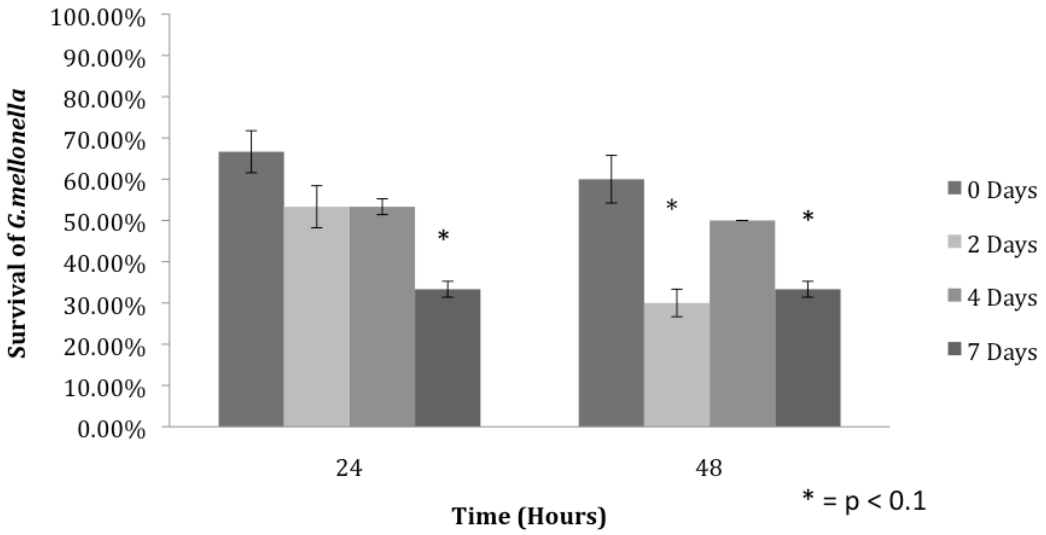


Figure 3.3 Survival of *G. mellonella* larvae starved for 0, 2, 4 or 7 days prior to inoculation with 1 x 10^{6} /20 µL *C. albicans* cells (n=3).

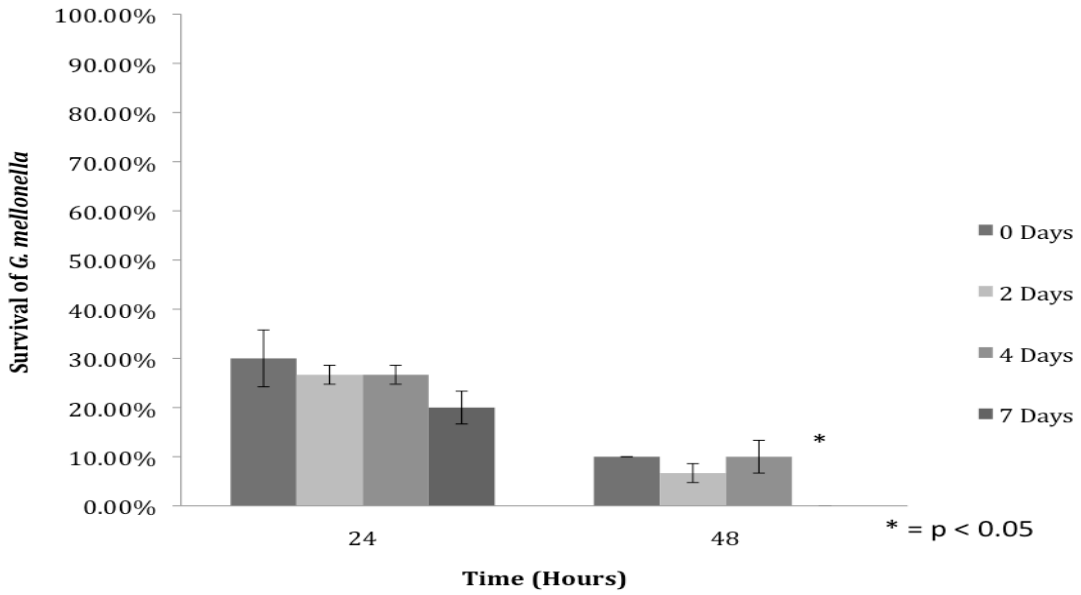


Figure 3.4 Survival of *G. mellonella* larvae starved for 0, 2, 4 or 7 days prior to inoculation with 1 x 10^{5}/20 µL *A. fumigatus* spores (n=3).
(The statistics were based on the values obtained from the unstarved larvae.)

3.3 Assessment of survival rates of various weights of *G. mellonella* larvae when infected with *C. albicans* or *A. fumigatus*

G. mellonella larvae weighing 0.2g, 0.3g and 0.4g were inoculated with 1 x 10^6 /20 µL *C. albicans* cell or with 1 x 10^5/20 µL *A. fumigatus* spores. The survival of the larvae was assessed over a 48-hour period, post inoculation. The statistics are based on the lightest larvae studied, which were 0.2g in weight.

The larvae weighing 0.2g and that were inoculated with *C. albicans* demonstrated a survival rate of 80% ± 5.77% at 24 hours and at 30% ± 5.77% at 48 hours. Larvae weighing 0.3g survived at a rate of 93% ± 3.33% after 24 hours ($p < 0.2$) and this fell to 46% ± 11.55% 48 hours post infection ($p < 0.2$). Larvae weighing 0.4g demonstrated survival rates of 97% ± 3.33% at 24 hours ($p < 0.2$) and 47% ± 5.77% 48 hours after infection ($p < 0.05$).

G. mellonella larvae weighing 0.2g infected with *A. fumigatus* survived at a rate of 17% ± 8.82% 24 hours post inoculation but this fell to 3% ± 0.33% at 48 hours ($p < 0.5$). Larvae weighing 0.3g survived at 43% ± 6.66% after 24 hours ($p < 0.1$), this value fell to 13% ± 8.82 after 48 hours ($p < 0.5$). The heaviest larvae, weighing 0.4g, survived at a rate of 33% ± 3.33% after 24 hours ($p < 0.5$) and 17% ± 5.77% after 48 hours ($p < 0.5$). These data can be visualised in Figures 3.5 and 3.6, respectively.

These results demonstrate that lighter larvae are more susceptible to infection.

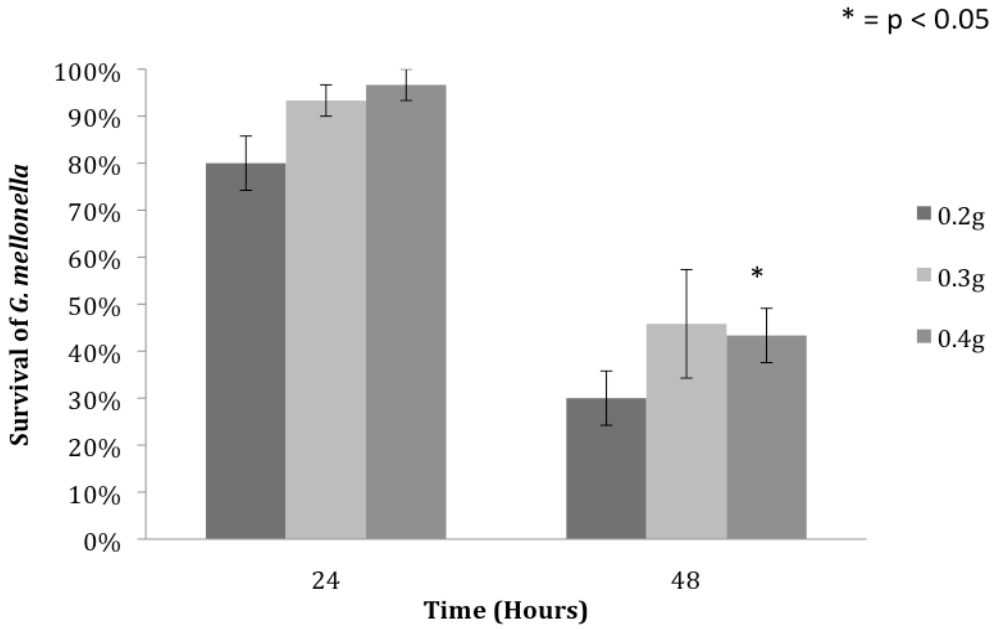


Figure 3.5 Survival of *G. mellonella* larvae weighing 0.2g, 0.3g and 0.4g, upon inoculation with 1 x 10^{6} /20 µL *C. albicans* cells (n=3).

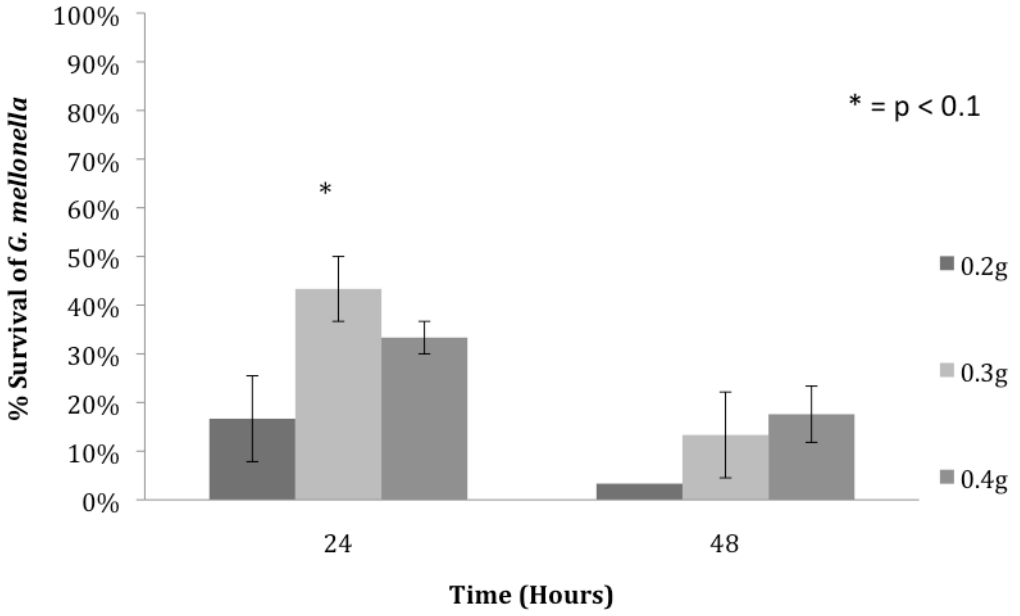


Figure 3.6 Survival of *G. mellonella* larvae weighing 0.2g, 0.3g and 0.4g, upon inoculation with 1 x 10^{5} /20 µL *A. fumigatus* spores (n=3).

(The statistics were based on the values obtained from the larvae weighing 0.2g.)

3.3.1 Summary

It can be deduced that there is a direct correlation between larval nutritional status and the ability to survive infection with fungal pathogens. The results were similar with both *C. albicans* and *A. fumigatus* in terms of larval survival over the 48-hour period. These results provide evidence that the food source, length of starvation period and larval weight are factors that need to be considered when beginning an experimental trial with *G. mellonella* larvae. It is clear that the combined diet of wood shavings and filter paper is the most nutritious. The heavier larvae are best able to withstand infection and a starvation period of more than two days will lead to a decreased ability to fight infection.

3.4 Investigation of the effect of food source, starvation period and larval weight on haemocyte density and the survival of an opsonised target

There exists a strong correlation between haemocyte density and the immune system's ability to withstand infection as seen in Mowlds *et al*., (2008). In this experiment haemocyte density was calculated as in section 2.4.5.

Haemocyte density was calculated for larvae fed on wood shavings only, larvae fed on wood shavings and filter paper and for larvae fed on filter paper only for a period of 7 days. The haemocyte densities were 2.16 ± 0.60 (x 10^7) per 1 mL, 2.35 ± 0.38 (x 10^7) per 1 mL and 1.83 ± 0.49 (x 10^7) per 1mL, respectively. These values were not significantly different and can be seen in Figure 3.7.

The haemocyte densities in unstarved larvae (0 Days) and larvae starved for 2, 4 and 7 days were 2.2 ± 0.49 (x 10^7) per 1 mL, 2.03 ± 0.41 (x 10^7) per 1 mL, 1.75 ± 0.08 (x 10^7) per 1 mL and 1.44 ± 0.14 (x 10^7) haemocytes per 1 mL, respectively (Figure 3.8). There was a marginal decrease in density depending on how long the larvae were starved for, however the differences were not significant.

Figure 3.9 demonstrates the haemocyte densities of the three weights of *G. mellonella* larvae. Larvae weighing 0.2g had an average haemocyte density of 1.46±0.47 (x 10^7) per 1 mL, larvae weighing 0.3g had 2.28±0.14 (x 10^7) haemocytes per 1 mL, and larvae weighing 0.4g had an average of 1.5±0.34 (x $10^{7)}$ haemocytes per 1 mL. Weight loss of the larvae was also calculated, which can be seen in Figure 3.10**.** Larval weight began at 0.3g and this was reduced to 0.25 ±0.018g after 7 days of starvation ($p<0.1$).

To determine whether the rate of haemocyte-mediated killing of a pathogen was affected by larval starvation, a survival assay using opsonised *C. albicans* cells was performed. There were no significant differences between the rate of killing by haemocytes taken from unstarved larvae and those from starved larvae. Twenty minutes subsequent to incubation the survival of *C. albicans* incubated with haemocytes from unstarved larvae was 58.23 ± 19.1%, survival of *C. albicans* incubated with haemocytes from the 7 day starved larvae was 57.42 ± 6.2% (no significant difference). At 60 minutes following incubation, survival of *C. albicans* incubated with haemocytes from unstarved larvae was 31.13 ± 9.8%, this value was 31.08 ± 4.6% with haemocytes from 7 day starved larvae (no significant difference). Following an 80-minute incubation period with the opsonised *C. albicans* cells, the

rate of survival of the yeast cells with haemocytes from unstarved *G. mellonella* larvae was 10.28 ± 2.5%, this value was 8.33 ± 1.33% in haemocytes from larvae starved for 7 days (no significant difference).

These results suggest that although the haemocyte density is reduced with the limiting food sources, longer periods of starvation and is dependent on larval weight, the ability of the haemocyte to kill is not affected.

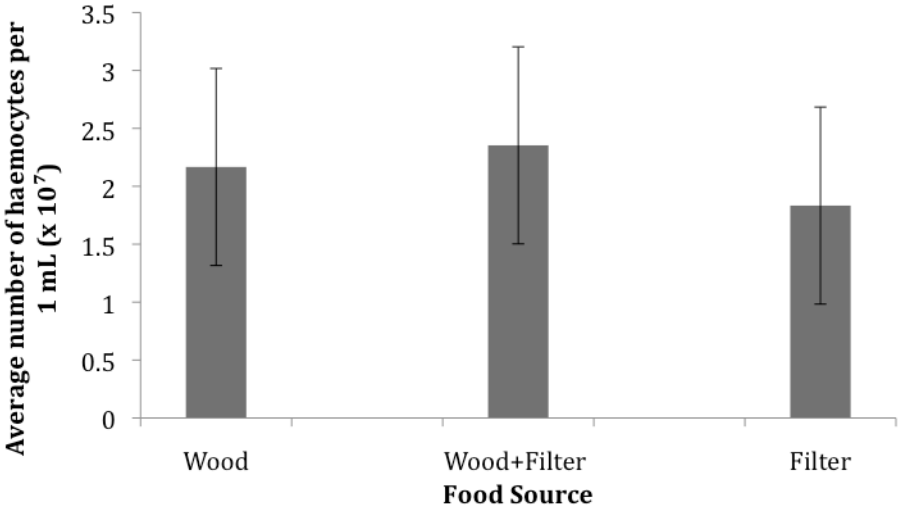


Figure 3.7 Haemocyte density in *G. mellonella* larvae that were fed diets of wood shavings only, wood shavings and filter paper or filter paper only (n=3). (There were no significant differences in haemocyte density between the food sources)

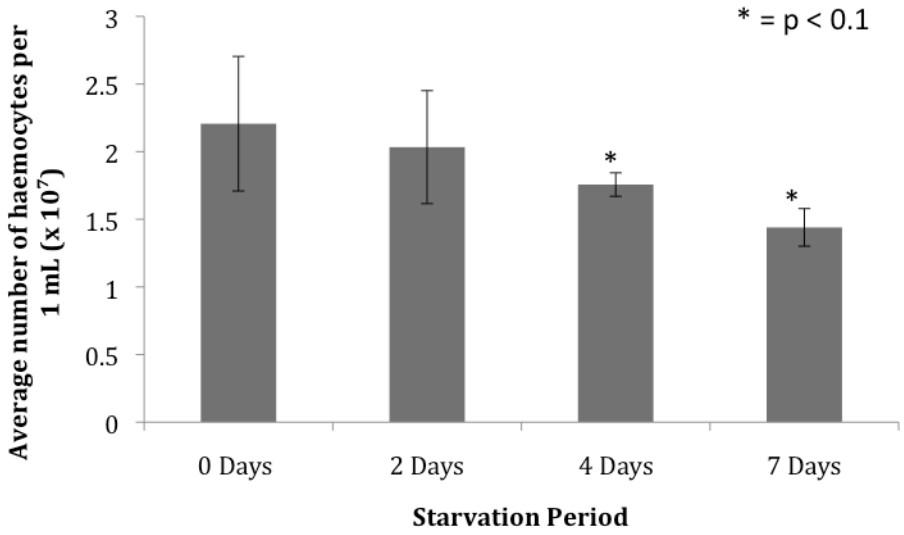


Figure 3.8 Haemocyte density in *G. mellonella* larvae that were starved for 0, 2, 4 and 7 days (n=3). (The statistics were based on the values obtained from the unstarved larvae.)

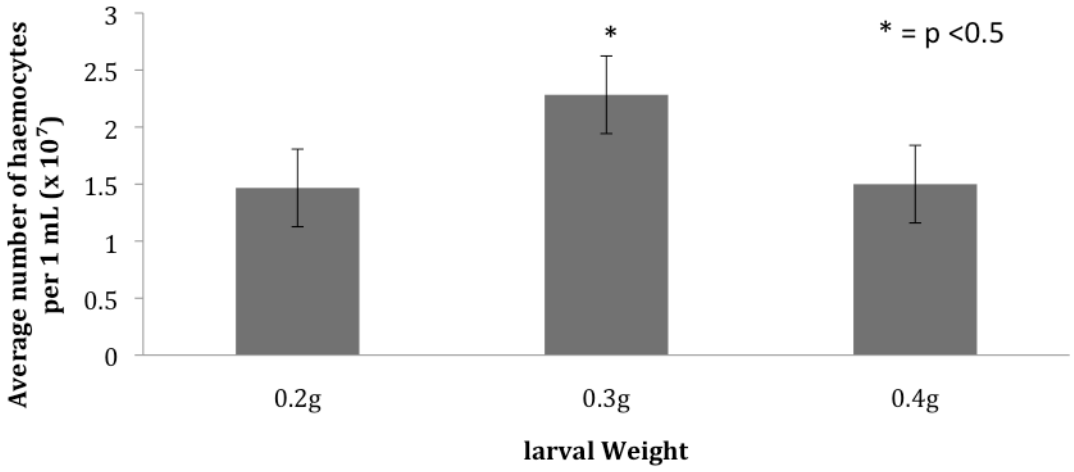


Figure 3.9 Haemocyte density in *G. mellonella* larvae that weigh 0.2g, 0.3 g and 0.4 g (n=3). (The statistics were based on the values obtained from the larvae weighing 0.2g and there were no significant differences between varying weights of *G. mellonella* larvae)

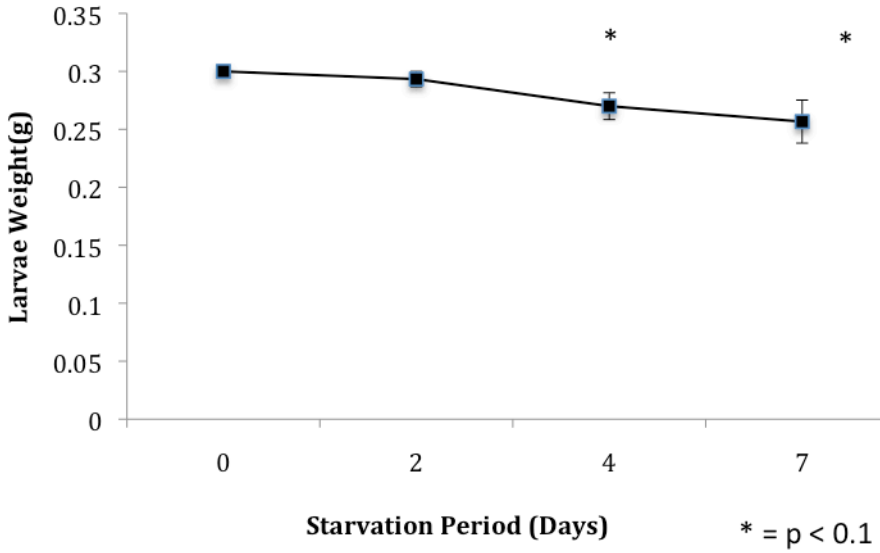


Figure 3.10 The weight loss of the larvae over the 7 day period (n=3). (The statistics were based on the values obtained from the unstarved larvae.)

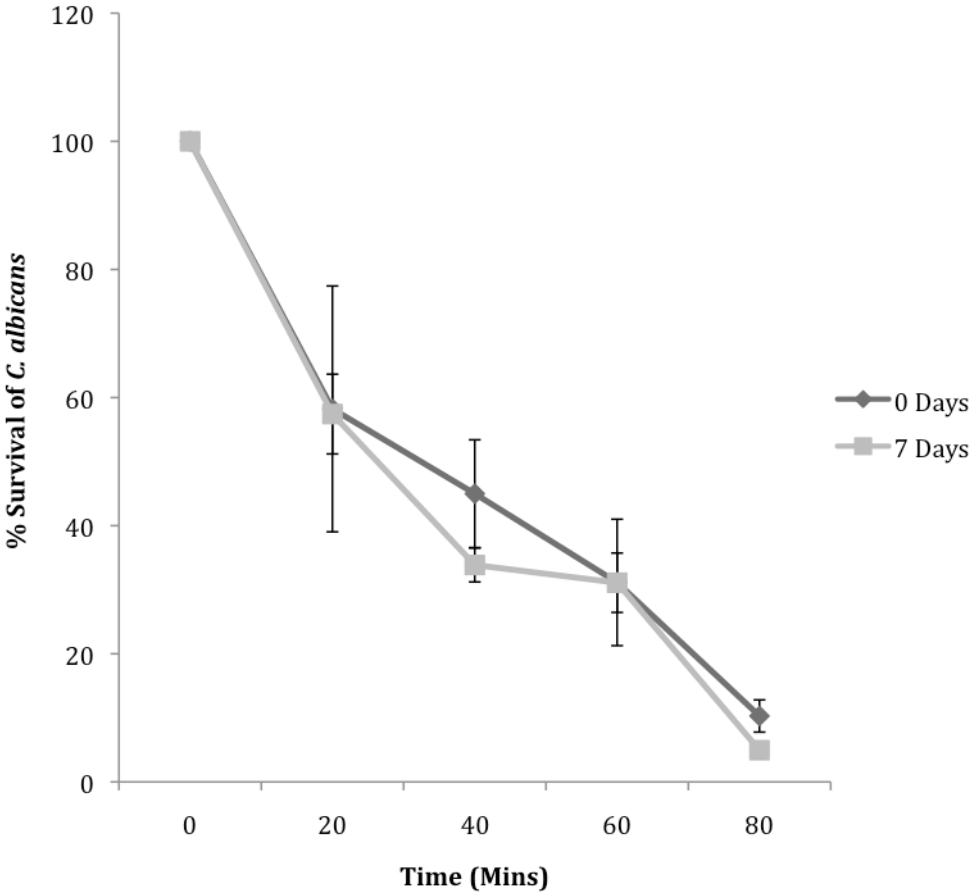


Figure 3.11 Survival of *C. albicans* incubated with haemocytes over 80 minutes when insects are starved for 0 and 7 days, no significance between unstarved and starved larval haemocytes (n=3).

3.5 Proteomic analysis of haemolymph of unstarved larvae and larvae starved for 7 days

In order to investigate the effect of starvation on the humoral immune response, haemolypmh was extracted from the larvae and the protein profiles were examined by means of 1 D electrophoresis. Protein profiles of haemolypmh samples provide insight into the effect of variables on protein expression (Zdybicka-Barabas & Cytryńska, 2010). The samples from unstarved and starved larvae were visualised using colloidal coomassie staining (Figure 3.12). There was little difference obvious between unstarved and starved larval haemolymph by performing 1-dimensional SDS-PAGE so it was decided to utilize 2-dimensional SDS-PAGE.

To further study the proteomic response of the larvae to nutritional depletion the haemolymph was analysed using 2-dimensional SDS-PAGE which separates peptides in the first dimension based on their isoelectric points, i.e. the pH at which the peptide has a neutral charge, and in the second dimension based on the molecular weight of the peptide, as described in section 2.10. The putative identity of these peptides was characterised by utilising mass spectrometry and the MASCOT database. These gels can be visualised in Figure 3.13 with the relevant spots numbered.

Table 3.1 lists the identities and fold decreases of the spots relative to the haemolymph from unstarved larvae. Each listed putative peptide decreased at least 1 fold in the starved larvae haemolypmh when compared to the intensity of the protein from unstarved larvae haemolymph. Fold changes were identified using Progenesis SameSpots Software®.

Serine protease (Spot number 11) was found to be decreased 1.6 fold in expression in haemolypmh when the larvae were starved. Serine proteases are important in activation of the toll pathway and prophenoloxidase (PPO) pathway (Brown *et al.,* 2009) and therefore a decrease in expression will ensure a more likely chance of an infection becoming fatal although further validation on specific serine protease activity would confirm this as many there exists many serine proteases in mammalian immune responses. Apolipophorin (Spot number 10) functions in the haemolymph to transport lipids and aids in commencement of the innate immune response (Gupta *et al.,* 2010), it is downregulated 1.3 fold when starvation occurs. Arylphorin (Spot number 9) was found to be downregulated by 1.1 fold, it primarily

functions in storage of the aromatic amino acids, but it may play a role in insect immunity as evident in Beresford *et al*., (1997). Hexamerin (Spot number 8) was detected in the unstarved haemolypmh and was not detected in haemolymph from larvae starved for 7 days, it is also a storage protein that stores amino acids (Liu *et al*., 2009).

Cobaltcheletase acts as a chelator of cobalt and functions in synthesis of vitamin B12 and is involved in haemolymph organisation (Spot number 4). In the starved larvae cobaltchelatase was down regulated 1 fold, relative to that in unstarved larvae. ERRC-4 (Spot number 5), an excision repair protein, is conserved across most species, (McDaniel and Schultz, 2008) and down regulated 1.1 fold in starved larval haemolypmh. Other putative proteins that were only identified in the unstarved haemolypmh were juvenile hormone binding protein (Spot number 7), 14-3-3- like protein (Spot number 3), a hypothetical protein (Spot number 1), and 27kDa haemolymph protein (Spot number 6) were identified again, albeit with a lower sequence coverage.

The proteomic results indicated a generalised decrease in the expression of a range of proteins in larvae deprived of food for 7 days. Given the importance of the humoral immune response in defending insects from infection, this decrease in the expression of antimicrobial peptides, in particular, would make the larvae more susceptible to infection.

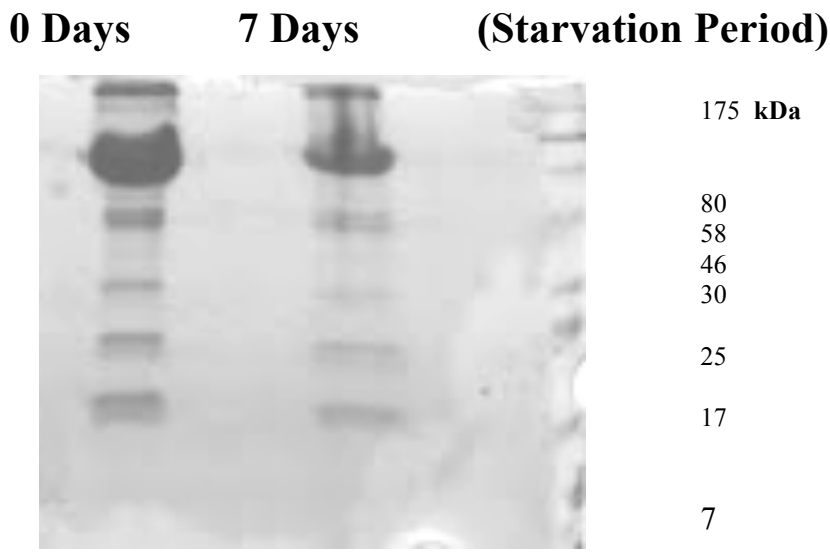


Figure 3.12 The 1-dimensional protein profile of the haemolymph from *G. mellonella* larvae that were starved for 0 and 7 days respectively (n=2).

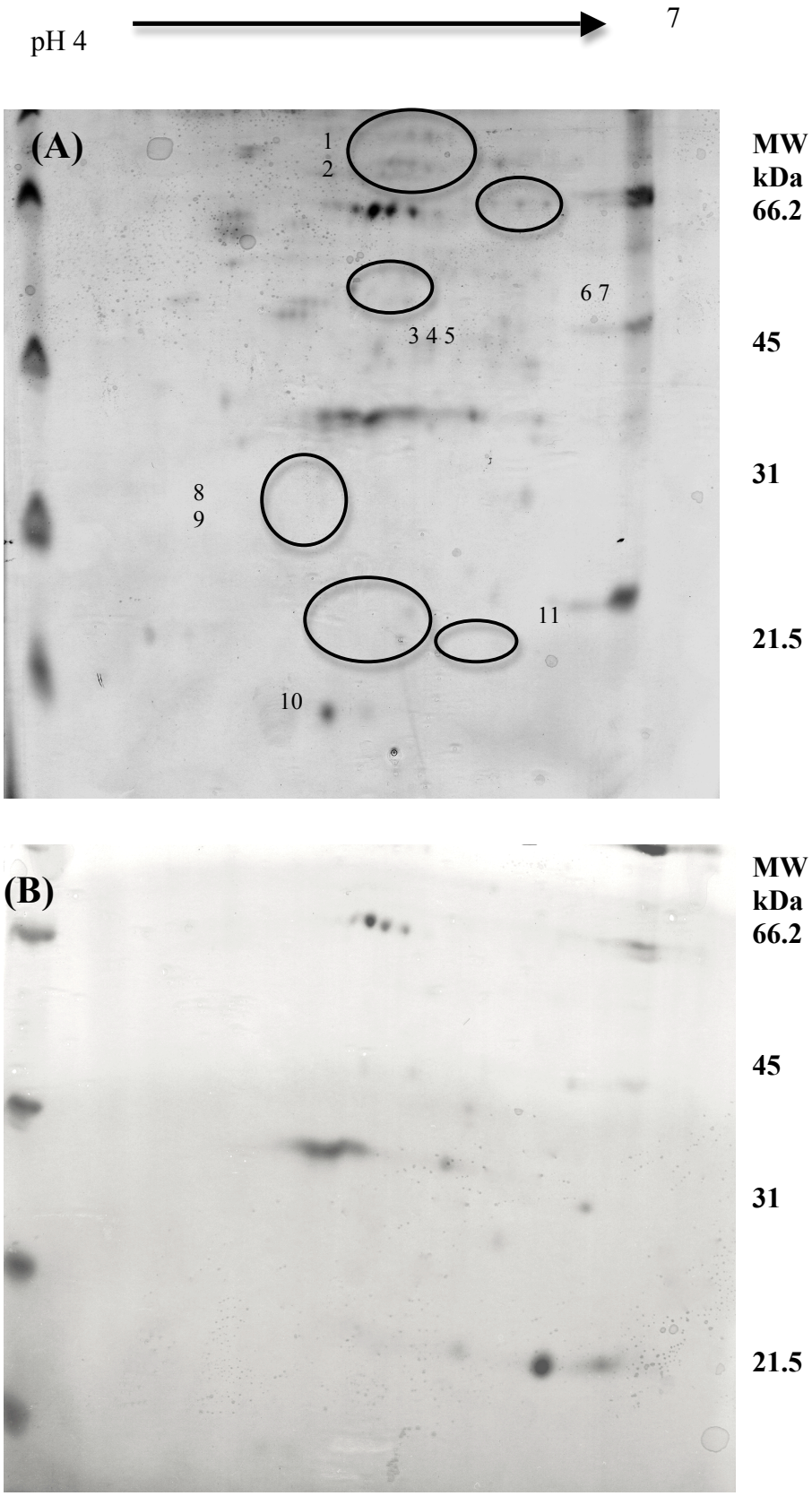


Figure 3.13 Proteomic profiles from haemolymph of unstarved larvae (A) and larvae starved for 7 days (B) (n=4).
(300 µgs/250µLs was loaded onto each Immobiline Drystrip).

Table 3.1 Mass spectrometry results detailing the peptide matches from the spots on Gel (A). The fold decrease indicated is relative to Gel (A).

Protein Name	**Mascot score**	**% Coverage**	**Accession No.**	**Spot no.**	**Function**	**Fold decrease**
Hypothetical protein	72	2%	XP_001747315	1	Unknown	Absent
14-3-3-like protein	113	7%	CAC20378	2	Conserved proteins, involved in binding signaling molecules such as kinases	Absent
27kDa Protein	92	8%	P83632	3	Unknown	1.6
Cobaltchelatease	67	1%	YP_002363537	4	Functions in Vitamin B12 biosynthesis which plays a key roll in haemolypmh formation	1
ERCC4	67	2%	YP_003405258	5	Essential protein in the nucleotide excision repair (NER) pathway	1
27kDa Protein	220	16%	P83632	6	Unknown	Absent
Juvenile hormone binding protein	73	7%	AAS94224	7	Regulates insect growth and development	Absent
Hexamerin	146	5%	AAA19801	8	Ecdysteroid carrier in the haemolymph	Absent
Arlyphorin	240	8%	AAA74229	9	Storage protein that replenishes the amino acid contents	1.1
Apolipophorin-3	74	7%	Q6VU70	10	Involved in lipid transport and immune function	1.3
Protease, serine, 1	73	8%	NP_444473	11	Functions in blood clotting, the immune system, and inflammation.	1.6

3.6 Summary

The results presented in this section demonstrated that nutritional variation, a complete depletion of a food source and larval weight affect the density of haemocytes. Most strikingly, the longer the time period that the larvae were starved for, the less dense that the haemolypmh was with respect to the circulating haemocytes. This may be due to the total weight loss of the larvae over the 7 day period. It was found that there was an average loss of 0.05 grams per larvae when the food source was removed and this had a direct correlation with the immune cell concentration, therefore larval death was more likely to occur upon infection. However, the killing ability of haemocytes was not affected, as demonstrated with the low survival of *C. albicans* when the yeast cells were incubated with haemocytes from starved larvae.

Mass spectrometry demonstrated the putative identification of immuno-relevant peptides that showed a decrease in expression upon starvation in the haemolypmh. The haemolymph is the primary source of immune activators upon infection in *G. mellonella* larvae.

3.7 Real Time PCR analysis of immune gene expression in unstarved and 7 day starved *G. mellonella* larvae upon infection with *C. albicans*

Following the proteomic findings in the haemolypmh of larvae starved for 7 days, it was decided to investigate the expression of immune relevant genes in unstarved larvae and larvae that were starved for 7 days prior to an infection with the yeast *C. albicans.* RNA extraction and cDNA synthesis were carried out as specified in section 2.6. Primers for *Galiomicin, Gallerimycin, Transferrin, IMPI* and *S7e* were designed based on previous literature (Wojda and Jakubowicz, (2007) and Bergin *et al*., (2006). *Gallerimycin* codes for a novel antifungal peptide (Langen *et al*., 2006). *Galiomicin* is a defensin indentified as a key player in the immune response (Lee *et al*., 2004). *IMPI* codes for an inducible metalloproteinase inhibitor (Mukherjee *et al.*, 2010). *Transferrin* is widely known for its ability to remove essential iron ions from invading pathogens (Seitz *et al*., 2003). The housekeeping gene *S7e* was used to normalise expression of all genes. A control set of larvae were left uninfected.

The decision to proceed with real time PCR was due to the heightened sensitivity of the techniques with regard to amplification and melting curve analysis; therefore comparisons could be accurately made between the genes and also between the treatments. The expression of each gene can be accurately quantified as well as the amplification curves, which reveal data regarding the kinetics and speed of amplification, which is unique for each gene. Melting curves reveal the characteristics of the final amplified product (Valasek and Repa, 2005).

3.7.1 Real time PCR analysis of *Gallerimycin* in unstarved (0 Days) and 7 day starved larvae infected with *C. albicans*

It was found that *Gallerimycin* gene expression in the unstarved (0 Days) and *C. albicans* infected larvae was found to increase 157.23 ± 0.0026 times when compared to the unstarved, uninfected larvae ($p < 0.05$). In contrast to this, the expression of *Gallerimycin,* in the larvae starved for 7 days and subsequently infected with *C. albicans* increased 2.55 ± 0.46 times when it was compared to the uninfected larvae starved for 7 days ($p < 0.05$) (Figures 3.14). A relatively high standard error was encountered in thes study which may be due to the differnential change in gut microbiota when using *Galleria mellonella* biological replicates in these experiments.

To clarify the findings from the real time PCR data, all products from real time PCR were run on a 1% agarose gel following 30 cycles, as described in section 2.8.2 (Figure 3.15). *Gallerimycin* intensity increased 2.38 fold when the unstarved and *C. albicans* infected larvae were compared to the unstarved, uninfected larvae ($p < 0.05$). The intensity in the larvae starved for 7 days and infected with *C. albicans* increased 1.77 fold ($p < 0.05$), when compared to larvae starved for 7 days and left uninfected (Figures 3.16).

The upregulation of *Gallerimycin* gene expression is one of *G. mellonella's* larvae's defense mechanisms (Langen *et al*., 2006). An increase in *Gallerimycin* expression has been seen in *G. mellonella* larvae when the larvae were infected with *C. albicans (Bergin et al.,* 2006) and also with pathogenic fungi (Altincicek *et al.,* 2007). These results demonstrate the inability of the starved larvae to increase the expression of *Gallerimycin* upon infection, which indicated a weaker immune response in the starved larvae compared to the unstarved larvae. This was highlighted by the significant difference in gene expression between the starved and unstarved larvae, again this highlights the requirement for food when working with *G. mellonella* larvae.

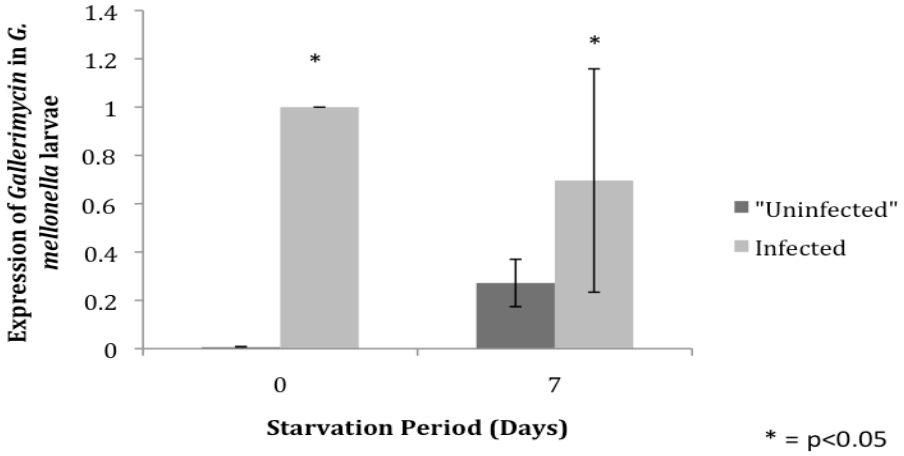


Figure 3.14 Normalised values of *Gallerimycin* when unstarved, *C.albicans* infected larvae were taken as the basal level of gene, all values were normalised using *S7e* housekeeping gene (n=3).

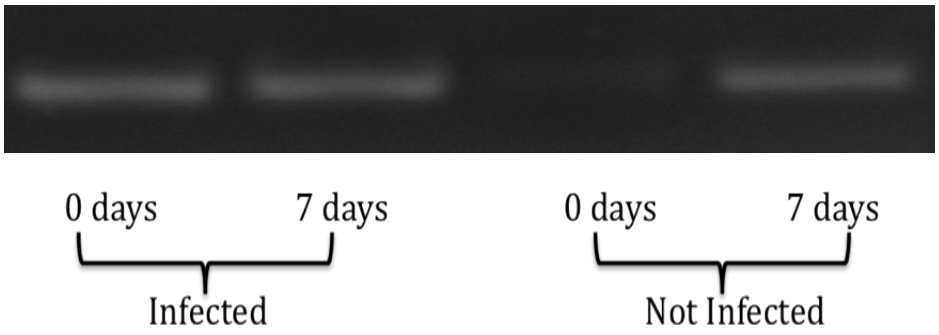


Figure 3.15 *Gallerimycin* PCR products were run on 1% agarose gels and viewed under UV light (n=3).

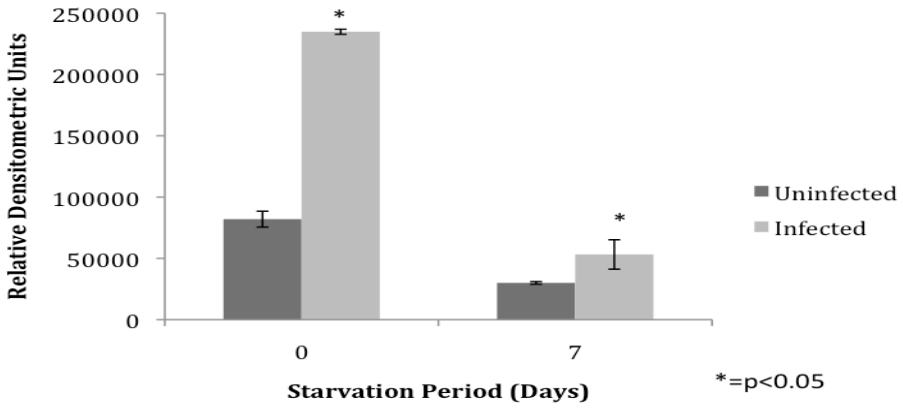


Figure 3.16 Relative expression of *Gallerimycin* agarose gel images analysed using Image Quant Software® (n=3).

3.7.2 Real Time PCR analysis of *Galiomicin* in unstarved (0 Days) and 7 day starved larvae infected with *C. albicans*

The expression of *Galiomicin* increased 270.2 ± 0.19 times when unstarved and *C. albicans* infected larvae were compared to unstarved larvae that were left uninfected ($p < 0.05$). *Galiomicin* expression in the larvae starved for 7 days and infected with *C. albicans* increased 2.7 ± 0.16 fold when compared to the uninfected larvae starved for 7 days ($p < 0.05$) (Figures 3.17).

Galiomicin band intensity was elevated 7.9 times when the unstarved and uninfected larvae were compared with the unstarved, *C. albicans* infected larvae ($p < 0.05$). Whereas, in the 7 day starved and infected larvae, *Galiomicin* demonstrated an increase of 1.5 fold in comparison to the uninfected cDNA from 7 day starved larvae ($p < 0.05$) (Figure 3.18 and 3.19).

Previously, factors such as physical and thermal stress and infection with yeast and fungal pathogens have induced the upregulation of *Galiomicin* in *G. mellonella* larvae (Mowlds *et al*., 2008; Bergin *et al*., 2006). These results show that there was a sizeable difference in the ability of larvae to boost the expression of *Galiomicin* between the larvae that were starved and the larvae that were left unstarved. These results display the importance of nutritional status in allowing the larvae to launch the defense response against microorganisms and boost expression of important immuno-relevant genes.

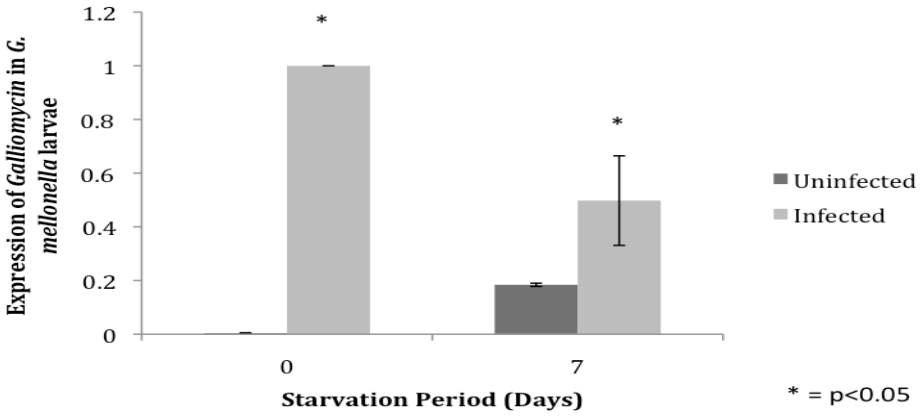


Figure 3.17 Normalised values of *Galiomicin* when unstarved and *C. albicans* infected larvae were taken as the basal level of gene, values were normalised using *S7e* housekeeping gene (n=3).

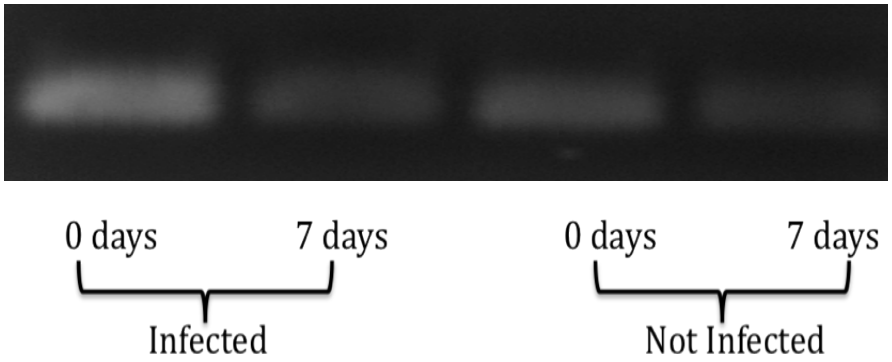


Figure 3.18 *Galiomicin* PCR products were run on 1% agarose gels and viewed under UV light (n=3).

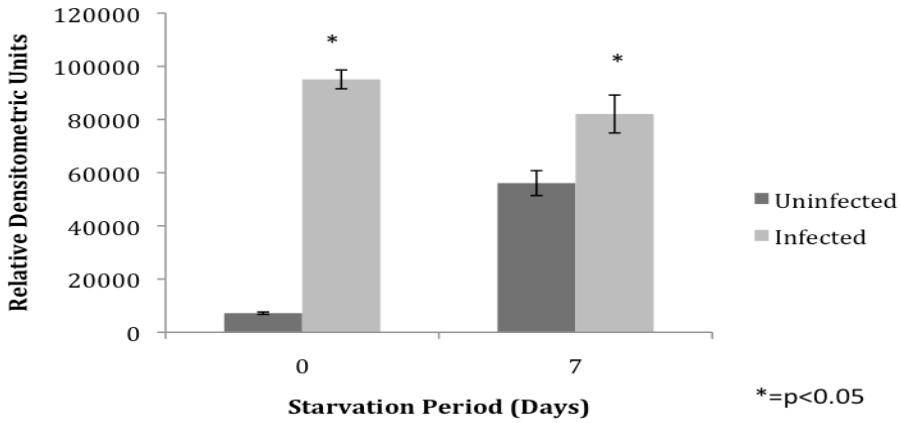


Figure 3.19 Relative expression of *Galiomicin* agarose gel images analysed using Image Quant Software® (n=3).

3.7.3 Real Time PCR analysis of *IMPI* in unstarved (0 Days) and 7 day starved larvae infected with *C. albicans*

The *IMPI* gene encodes two distinct inhibitors; one, which inhibits microbial metalloproteinases and is an important aspect of the immune response of *G. mellonella* larvae, the other regulates endogenous MMPs (matrix metalloproteinases) during larval metamorphosis (Wedde *et al.,* 2006).

In the case of *IMPI* gene expression, the unstarved larvae that were infected with *C. albicans* demonstrated an expression increase of 3.28 fold ± 0.06 relative to unstarved, uninfected larvae ($p < 0.05$). The gene expression in the larvae starved for 7 days and infected with *C. albicans* increased 13.5 ± 0.012 fold compared with uninfected larvae starved for 7 days ($p < 0.05$) (Figures 3.20).

The *IMPI* intensity in the unstarved, infected larvae demonstrated an increase of 7.79 fold, when compared to unstarved, uninfected larvae ($p < 0.05$). Larvae starved for seven days and infected with *C. albicans* increased the expression of *IMPI* by 2.88 times when compared with uninfected, 7 day starved larvae ($p < 0.05$) (Figures 3.21 and 3.22).

The increase in *IMPI* expression was not as significant as demonstrated with the other genes, this may be due to the fact that *IMPI* inducement can be associated more with combating bacterial pathogens rather that the yeast *C. albicans* (Mukherjee *et al*., 2010).

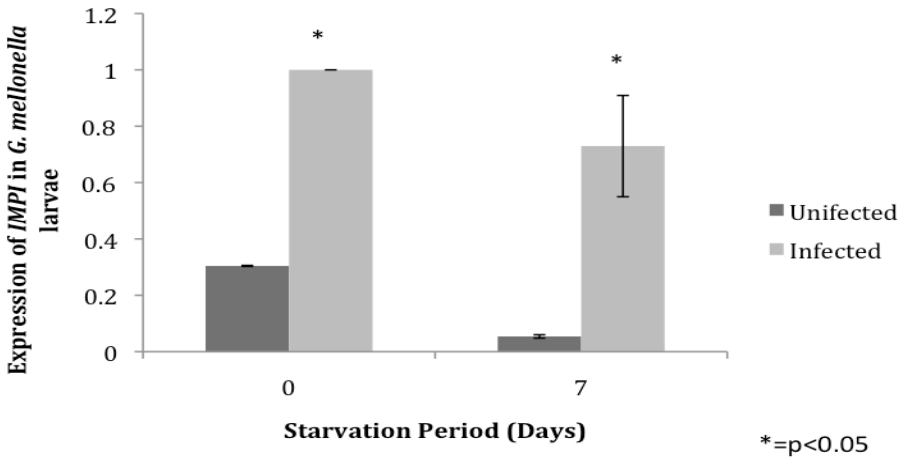


Figure 3.20 Normalised values of *IMPI* when the unstarved and *C. albicans* infected larvae were taken as the basal level of gene, values were normalised using *S7e* housekeeping gene (n=3).

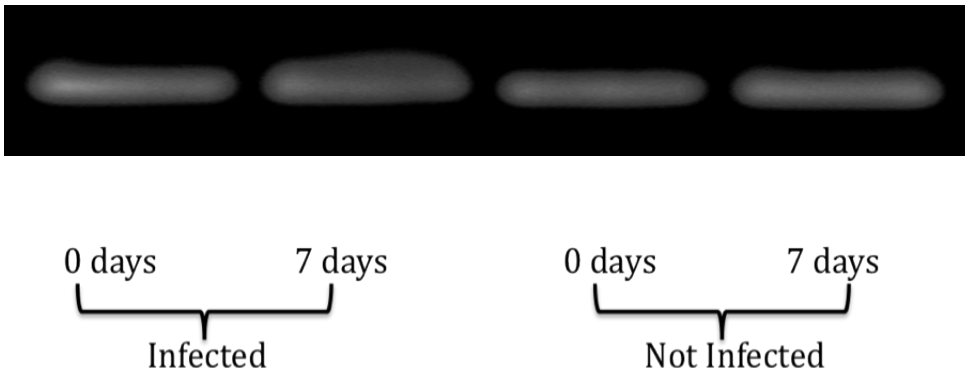


Figure 3.21 *IMPI* PCR products were run on 1% agarose gels and viewed under UV light (n=3).

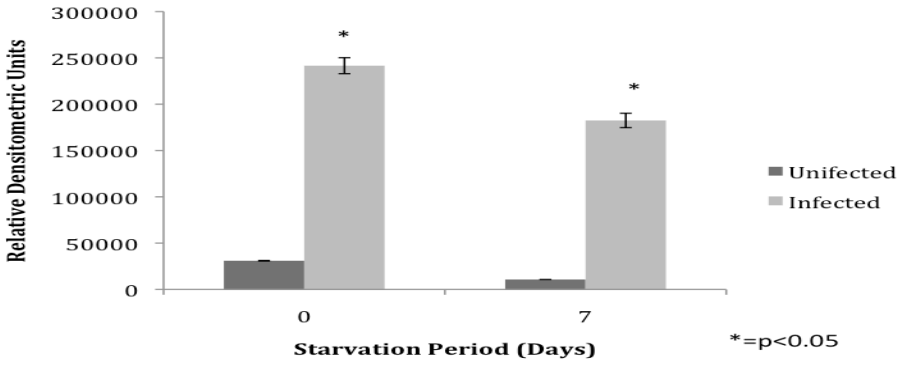


Figure 3.22 Relative expression of *IMPI* agarose gel images analysed using Image Quant Software® (n=3).

3.7.4 Real Time PCR analysis of *Transferrin* in unstarved (0 Days) and 7 day starved larvae infected with *C. albicans*

Transferrin is responsible for iron sequesterisation and is a fundamental factor in *G. mellonalla* larvae (and other insect models) immunity (Bergin *et al.*, 2006). The ability of larvae to upregulate the expression of *Transferrin* aids in the destruction of microorganisms by capturing iron ions.

Tranferrin expression followed a similar trend to *Gallerymicin* and *Galiomicin* in that the expression levels rose by 277.7 ± 0.11 times in the larvae that were unstarved and infected with *C. albicans* compared to the uninfected, unstarved larvae. *Transferrin* expression in larvae starved for 7 days and infected with *C. albicans* rose by just 3.24 ± 0.057 fold ($p < 0.05$), again relative to larvae starved for 7 days and left uninfected (Figures 3.23).

The intensity of the *Transferrin* band increased 12.29 fold ($p < 0.05$), from the unstarved, uninfected larvae relative to unstarved, infected larvae. Band intensity in larvae starved for 7 days prior to infection was 1.13 times higher than larvae starved for 7 days and not infected (No significant difference) (Figures 3.24 and 3.25).

These results further indicate the importance of consistent nutritional status in *G. mellonella* larvae for the maintenance of an efficient immune response.

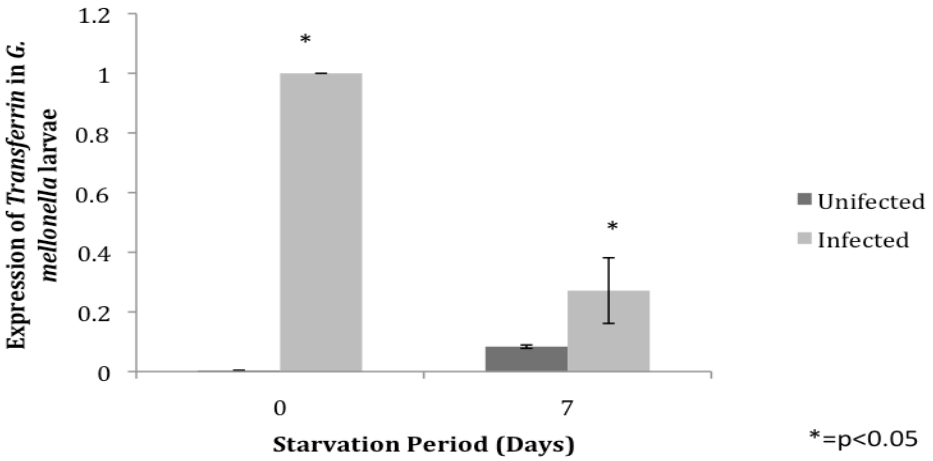


Figure 3.23 Normalised values of *Transferrin* when unstarved and *C. albicans* larvae were taken as the basal level of gene, values normalised using *S7e* housekeeping gene (n=3).

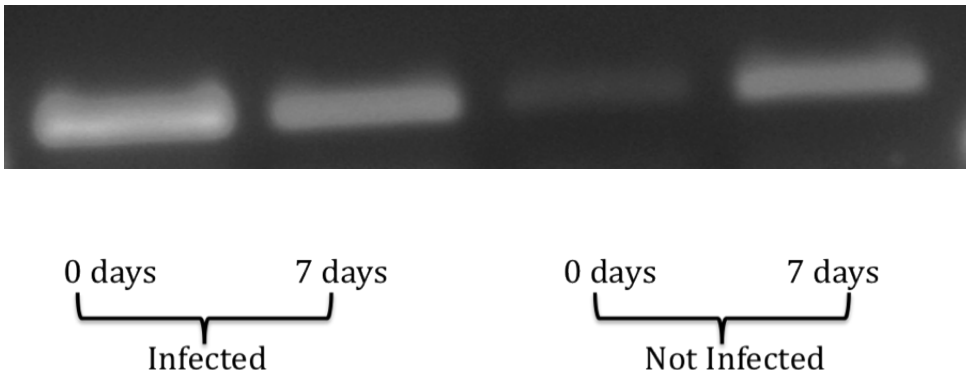


Figure 3.24 *Transferrin* PCR products were run on 1% agarose gels and viewed under UV light (n=3).

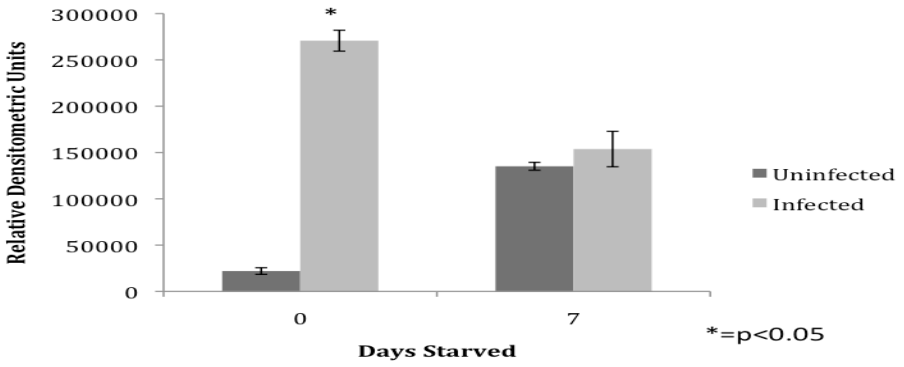


Figure 3.25 Relative expression of *Transferrin* agarose gel images analysed using Image Quant Software® (n=3).

3.7.5 Real Time PCR analysis of the housekeeping gene *S7e* in unstarved (0 Days) and 7 day starved larvae infected with *C. albicans*

The ribosomal protein *S7e* was utilised as the housekeeping gene in the real time PCR, as described by Wojda *et al.,* (2007), and therefore should remain constant in all treatments. Relative intensity values retrieved from Image Quant Software were 54800 ± 3389 for unstarved and infected larvae, 58274 ± 2102 for larvae starved for 7 days prior to infection, 47931 ± 11981 for unstarved, uninfected larvae and 66012 ± 3305 for larvae starved for 7 days and uninfected, there was no significant difference between the treatments ($p > 0.2$) (Figures 3.26 and 3.27).

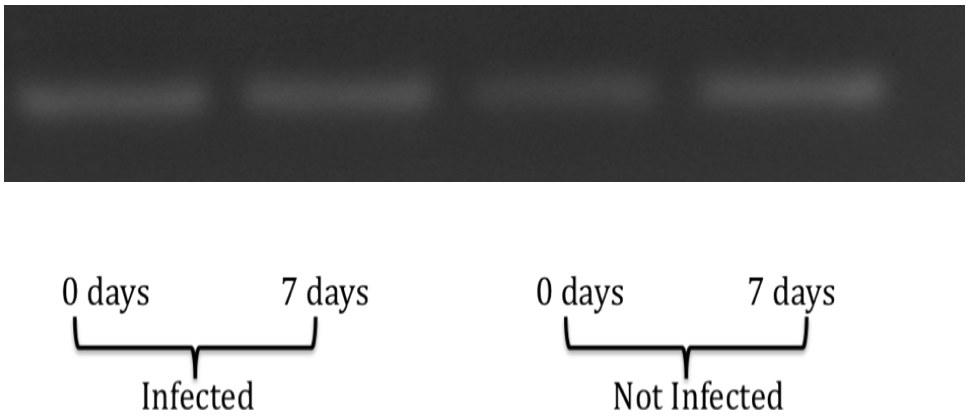


Figure 3.26 *S7e* PCR products were run on 1% Agarose gels and viewed under UV light (n=3).

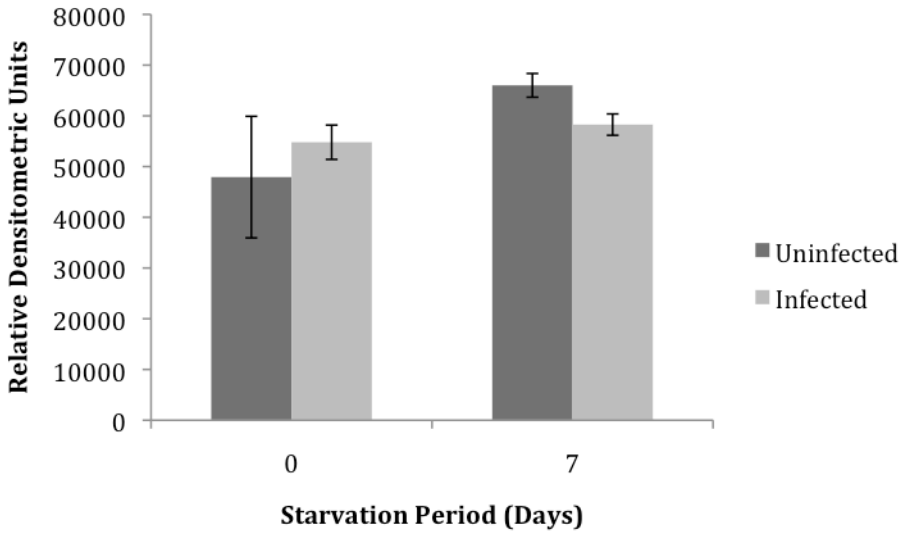


Figure 3.27 Relative expression of *S7e* agarose gel images analysed using Image Quant Software® (n=3).

3.8 Summary

This section examined the expression of genes encoding proteins that have been implicated in the immune response of *G. mellonella* larvae. Expression levels of genes were investigated in 0 (unstarved) and 7 day starved larvae infected with *C. albicans* and compared with those in 0 (unstarved) and 7 day starved larvae that were not infected with a pathogen.

The general trend was that the maximum expression of each gene was found in the cDNA sample of the unstarved (0 Days), infected larvae. The greatest fold increase in gene expression from unstarved, uninfected to unstarved and *C. albicans* infected larvae was seen with *Transferrin*. *IMPI* demonstrated the smallest difference in expression between unstarved and *C. albicans* infected larvae and unstarved, uninfected larvae. *IMPI* was found to show the highest increase from uninfected larvae starved for 7 days to infected larvae that had been starved for 7 days. This may imply that the expression of *IMPI* is not required as much as *Transferrin, Galiomicin* and *Gallerimycin* upon infection with *C. albicans* cells.

Larvae that were starved for 7 days were unable to increase the expression of these immune-relevant genes to the same degree as the unstarved larvae.

3.9 Discussion

The primary goal of the work presented in this Chapter was to examine how the nutritional status of *G. mellonella* larvae affected susceptibility to infection, so that the larvae could be exploited as a model organism in research and industry. The effect of nutritional source, larval weight and effect of starvation over a number of days on *G. mellonella* larvae's susceptibility to infection were measured. These are the considerations that must be taken into account when providing evidence for an optimal model so comparative experiments may be performed accurately.

The provision of a food source provides energy and this energy will influence the immune response. Previously, it has been demonstrated that larvae can prime their immune system in response to thermal and physical stress (Mowlds *et al*., 2008) and also with the pre-injection with drugs such as echinocandins (Kelly and Kavanagh, 2010). While it has been shown that the thermal environment is an important factor in *G. mellonella* larvae's ability to be used as a host and studied (Śmietanko *et al*., 1988), the effects of different food sources and weight have not been accurately studied. Immune priming in the absence of compensatory feeding in bumblebees has been shown to be fatal (Moret *et al.,* 2000) indicating an important link between nutritional status and survival. This has implications in the bee's natural environment, where resources can be limited depending on the environment and therefore, the bee colony numbers are affected.

The results presented in this Chapter demonstrate a clear relationship between the larvae's access to food and the ability to retard microbial infection.

The use of *G. mellonella* larvae to study infections with bacteria, fungi and yeast (Fuchs *et al*., 2010) has rendered it important to identify parameters that need to be constant for the larvae to be utilised in the laboratory. It was therefore decided to investigate these variables and their effect on larval ability to survive an infection with the pathogens *A. fumigatus* and *C. albicans.*

It was evident from the decrease in survival of the larvae when the food sources were limited that the combination of filter paper and wood shavings are the best source for storing larvae in during an experiment. Filter paper alone is more difficult for the insect to eat, due to adherence to the petri dish and this caused an inability of the larvae to consume a sufficient quantity. The indications are that the

food source needs to be accounted for when devising a model experiment using *G. mellonella* larvae.

The effect of varying starvation periods was also studied in *G. mellonella* larvae to assess whether the lack of food would have a damaging effect on larval immune response. Larvae were starved for 0, 2, 4 and 7 days, prior to inoculation with *C. albicans* and *A. fumigatus.* Starvation is a stress that may be counteracted by the lipid content in the fat body or storage proteins by the slow release of amino acids from these storage proteins. This has been documented in *D. melanogaster* (Gibbs *et al*., 2009) where starvation negatively affected the metabolic rate. If metabolism slows down, the production of proteins such as antimicrobial peptides is negatively affected (Gilchrist *et al*., 1999). This was demonstrated over the seven day long starvation period with the larvae, the longer the starvation length the lighter the larvae at the end. This led to an increased rate of death in the larvae that had been starved for a longer time period, especially upon infection with *A. fumigatus.* However, the activity of haemocytes in the haemolymph may not be affected. To clarify this, a survival assay was carried out using *C. albicans* as an opsonised target. The rate of death of *C. albicans* over 80 minutes was very similar in both sets of haemocytes (haemocytes from larvae that had been starved for 0 days (i.e. unstarved) and haemocytes from larvae that had been starved for 7 days). The activity of the haemocyte is therefore unchanged, but larval weight loss had a negative effect on survival, subsequent to inoculation with an infectious pathogen.

Experiments utilising *G. mellonella* larvae usually use larvae that weigh between 0.2g and 0.4g (Aperis *et al.*, 2007; Brennan *et al*., 2002). When mammalian models are used, standardisations are adhered to at the beginning of the study and variations are very few (Willmann *et al*., 2009). When dealing with *G. mellonella* larvae a difference of 0.1gram can account for 50% of larval weight, therefore experiments need to be conducted stringently with regard to the initial weight of the larvae. This study identified the differential survival of three varying weights of *G. mellonella* larvae upon infection with *C. albicans* or *A. fumigatus*. The ability of the heavier larvae to survive at an elevated level, when compared to the lightest insect, denotes a positive correlation between resistance to infection and weight, presumably due to a higher volume of haemolymph and a larger fat body, allowing the innate immune response in heavier larvae to be faster acting and better equipped

compared to that in the lighter larvae. Therefore, studies using *G. mellonella* larvae as model organisms should adhere to standards with regard to larval weight.

The change in haemocyte density demonstrated in this Chapter reflects the slower metabolism studied in *D. melanogaster* (Djawdan *et al.,* 1997) where the removal of food (and application of other stresses such as food variation and desiccation) from the insect led to marked decrease in the mass-specific metabolic rate. The density change may be attributed to this, as larval weight suffered a decrease over the starvation period but the activity of the haemocyte was not affected when haemocytes were challenged with the opsonised target, as demonstrated with the survival of *C. albicans* when incubated with haemocytes from both starved and unstarved larvae (Figure 3.11).

Analysis of the haemolypmh proteome provided an insight into the quantitative changes in the haemolymph upon stress (Vierstraete *et al.,* 2003). One dimensional electrophoresis, Western blotting, two-dimensional electrophoresis and mass spectrometry were engaged to identify these changes in the larvae proteome. Comparisons were made between unstarved larvae and larvae that had been depleted of food for a week. One-dimensional SDS-PAGE revealed little or no change between the proteomes of the unstarved and starved larvae. However, to get a clearer view of the events in cell free haemolypmh the samples were analysed using 2-dimensional SDS-PAGE and mass spectrometry, utilising peptide separation based on the PI (Isoelectric Point) and the molecular weight. Due to reduced metabolism of the starved larvae there was a marked fold difference between the expressions of proteins such as cobaltchelatase (spot number 4). The decrease in cobaltchelatase in starved larvae may be due to the fact that synthesis of vitamin B12 depends largely on ATP and energy, derived from a food source. Vitamin B12 has been shown to potentiate larval growth in *Aedes aegypti.* It was demonstrated here that an exhaustion of a food source could reduce growth by directly preventing vitamin synthesis, which is a reaction that heavily relies on metal chelatases (Singh and Brown, 2003).

The reduction of mass-specific metabolism in *D. melanogaster* shown by Djawdan *et al.* (1997) is evidence that stress has implications on lipid transport. Apolipophorin 3 (spot number 10) is heavily involved in lipid transport and its level in the haemolymph reaches the highest at the end of the feeding phase of the larvae

prior to pupation (Halwani *et al*., 2001). This, in effect may explain the decrease in Apolipophorin 3 in the starved larvae.

Arlyphorin (spot 9) has recently been demonstrated to become down regulated in the Lepidopteran model *B. mori* when a calorie restriction was applied to the insect's diet, as were other storage proteins such as lipoprotein (Yljia *et al*., 2009). The down regulation of arylphorin in *G. mellonella* larvae upon the 7 day starvation period was 1.3 fold and therefore it is evident that this phenonomen is conserved among insects and that the storage protein content of the larvae is decreased upon starvation.

Serine protease (spot number 11) was also down regulated upon depletion of food. Serine protease cascades function in the haemolypmh as a pre-requisite step to the prophenoloxidase series of events; the primary signalling cascade involved in fighting infection (Rolff *et al.,* 2009). The decline of serine protease by 1.6 fold further implies that starvation may affect innate immunity in the haemolypmh.

The nucleotide excision repair pathway is critical when the organism is stressed in any way. The removal of food is a form of stress that can affect this pathway. ERCC-4 (spot number 5) is conserved through most species and is produced extensively when cells become stressed (Camenisch and Nägel, 2008) to help repair damaged DNA. It was found that starvation for 7 days decreased expression by 1.1 fold, thus exposing DNA to damage that may not be repairable.

Other putative peptides were only identified in haemolymph from unstarved larvae, such as hexamerin (a storage protein, containing amino acids), juvenile hormone binding protein and 14-3-3 like protein. There may not have been sufficient metabolic resources to produce these proteins in larvae that were starved for 7 days.

The use of real time PCR allowed a highly sensitive study of the effect of starvation on the basal gene level, with normalisation using a ribosomal housekeeping gene. Real time PCR proves extremely advantageous in that it allows the detection of target sequences as low as five copies, with the key to real time PCR being that it allows quantification of DNA in real time, as amplification takes place. Real time PCR is rapid and accurate and for these reasons it was decided to exploit real time PCR to analyse immune gene expression in larvae, upon starvation and infection with a pathogen (Valasek and Repa, 2005). Larvae were starved for 7 days and infected with the yeast *C. albicans* in order to determine whether starvation

affected the expression of immuno-relevant genes such as*, Galleriomycin, Galiomicin*, *IMPI* and *Transferrin*.

Gallerimycin demonstrates defensin like activities (Mukherjee *et al*., 2010) and has been demonstrated to increase in expression when the larvae are exposed to mild heat stresses (Wojda & Jakubowicz, 2007). In terms of aiding in the defense against pathogens and maintenance of a strong immune system, the ability of larvae to boost *Gallerimycin* expression is imperative. Starvation negatively affected *Gallerimycin's* ability to boost it's expression in larvae that were infected with a pathogen, which may ultimately lead to reduced survival.

The up-regulation in expression of these genes by such a large extent in the unstarved larvae is indicative of an active and healthy immune system. The fact that the unstarved larvae can boost their immune response to such an extent that it is much more active than unfed/starved larvae by, in some cases 100 fold, again demonstrates the need for a calorific diet when experimenting with *G. mellonella.* An increase in *Galiomicin* expression has been implicated in both antibacterial and antifungal infections in *G. mellonella* (Lee *et al*., 2004). *Galiomicin* expression has not only been shown to increase in expression when larvae are stressed by a pathogen, but also when *G. mellonella* larvae are physically stressed (Mowlds *et al*., 2008). The stress involved in the depletion of the primary energy source seems to allow this inducement, however not to such an increase as when larvae are nourished. This is almost certainly accountable for by the fact that metabolism in larvae is lower when larvae are starved and a food source is removed.

The insect metalloproteinase inhibitor (*IMPI*) has previously been shown to be inducible in *G. mellonella* larvae upon infection with pathogens such as *C. albicans* and *Listeria monocytogenes* (Bergin *et al*., 2006; Mukherjee *et al.*, 2010). Interestingly the alteration in expression of *IMPI* was not as significant as the other genes studied, which may be due to the fact that there is some DNA damage. There is also some evidence in the literature that metalloproteinases are, perhaps, more active when larvae are challenged with bacterial pathogens that produce virulence factors such as aureolysin and pseudolysin (Altincicek *et al*., 2006) rather than when challenged with a yeast infection such as *C. albicans*. This may account for the less significant increase in *IMPI* expression in the larvae compared to the other genes that were studied.

Transferrin is an important iron binding protein and has been shown to be increased in expression in *G. mellonella* larvae upon infection with *C. albicans* (Bergin *et al*., 2005). The inability of the starved larvae to increase the expression of this important immune-genes most likely reflects a reduced metabolism compared to the unstarved *G. mellonella* larvae.

Real time PCR allowed accurate and sensitive readings with regard to all of the genes, during the amplification step. From the real time PCR analysis results in this Chapter it appeared that the larvae that were not starved and infected with *C. albicans* had the resources to launch an effective immune response that yielded a much greater expression of the immune-genes required to target an infection, when compared with the 7 day starved larvae, which were also infected. The overall implications of this would mean that the 7 day starved larvae can not fight an infection as well as the unstarved larvae, resulting in greater susceptibility to fungal infection.

The key objectives of this study were to examine the larvae's abilty to survive when nutritionally stressed and whether the stresses that were applied could affect an experimental outcome. The variables that were studied allowed larval diet and weight to be assessed with regard to survival when infected with a pathogen. The increase in use of *G. mellonella* larvae in research underlines the requirement for standardised experimental conditions.

3.10 Conclusion

Experimental conditions will almost always affect the environment a model organism is in, however to continuously perform comparative experiments there are variables which need to be consistent. Temperature, raw materials and an abundance of these raw materials (i.e. food) are of vital importance. *G. mellonella* larvae are being used widely in research. (Aperis *et al*., 2007; Mylonakis *et al*., 2005; Brennan *et al*., 2002) and an increase in their use has allowed the determination of a set of variables, which researchers should consider prior to experimental use.

The decrease in survival of nutritionally challenged larvae which were injected with pathogens revealed that the food source needs to be consistent. Larval weight and starvation were also found to be significant variables that need to be

taken into account when studying *G. mellonella* larvae. The down-regulation of several proteins over a starvation period of 7 days and the inability of starved larvae to increase expression of immuno-relevant genes to such an extent as unstarved larvae increases their susceptibility of infection.

The results presented in this Chapter describe the optimisation of factors that researchers can exploit when devising a model or a comparative biology experiment with *G. mellonella* larvae. It may be postulated that larval weight should always be equivalent, there should not be exhaustion in nutrition and that the combination of wood shavings and filter paper as a food source will allow consistency in research.

Chapter 4

Analysis of the proteome of *G. mellonella* haemocytes and a comparison to the human neutrophil

4.0 Analysis of the proteome of *G. mellonella* haemocytes and a comparison to the human neutrophil

G. mellonella larvae are one of the most widely used Lepidotera models in the world for studying pathogen virulence and chemical toxicity (Fuchs *et al*., 2010; Seed & Dennis, 2008; Rowan *et al*., 2008). This Chapter endeavored to characterise the proteomic similarities between the haemocyte of *G. mellonella* and the human neutrophil. The establishment of such relationships would underline the similarities that have been previously demonstrated between the insect immune response and the human immune response (Bergin *et al*., 2005; Scully and Bidocka, 2006).

The human neutrophil is involved in the body's first line of defence. The characterisation of a parallel proteomic relationship between the neutrophil and the haemocyte will allow more detailed research to be conducted with *G. mellonella* larvae and further rationalise the similarities between both cells. Previous studies have provided evidence that there are simialar proteins in the haemocyte to some of the proteins involved in the NADPH oxidase cascade of neutrophils, which leads to the production of free radicals, ultimately leading to death of an opsonised microbial target (Renwick *et al*., 2006; Bergin *et al*., 2005). The activation of this pathway involves a series of phosphorylation events. This Chapter investigated the phosphorylation events involved in the haemocyte by using a fluorescent stain. The comparative effect of two artificial stimulants was also described. Phorbol 12-myristate 13-acetate (PMA) activates the NADPH oxidase cascade via Protein Kinase C and is extensively used in neutophil studies (Bylund *et al*., 2004). The effect of N-formyl-methionine- leucine-phenylalanine (FMLP), a chemoattractant, on the haemocyte was also studied and a comparative analysis was made to the response that was elicited by PMA.

Two dimensional SDS-PAGE, Western blotting and confocal immunofluoresence were utilised to characterise the proteins that are activated in the haemocyte upon stimulation and to identify similarities with human neutrophil proteins. A 'shotgun' approach to proteomics is extremely useful in studies of cells and quantitative analysis of proteins in cellular pathways (Chen *et al.*, 2009). Shotgun proteomics allows rapid identification of complex samples by utilising multidimensional protein identifcation technology (MUDPIT). The combination of

shotgun proteomics and MUDPIT, which takes advantage of the databases available, has advanced proteomics.

The aim of the work described in this Chapter was to further characterise the proteomic similarities between the human neutrophil and the insect haemocyte. This would further justify *G. mellonella* larvae's use as model organisms in research.

4.1 Assessment of the effect of PMA and fMLP on haemocyte density and larval survival

As a change in haemocyte denisty can be indicative of the immune activity of *G. mellonella* larvae (Mowlds *et al.,* 2010), PMA and fMLP were injected into the larvae and the haemocyte density was calculated. This determined whether the artificial stimulants induced an increase in haemocyte density relative to the controls which were PBS and 0.001% (v/v) DMSO.

The results demonstrated an average number of 7 ± 0.35 (x 10^6) haemocytes per 100 µL in controls. PMA treatment resulted in an average haemocyte density of 17 ± 0.57 (x 10^6) per 100 µL ($p < 0.05$). fMLP also induced an increase in haemocyte density resulting in almost double the number of haemocytes compared to the control, 14 ± 0.58 (x 10^6) per 100 µL ($p < 0.05$) (Figure 4.1).

In order to determine the potency of the treatments used in the haemocyte density assay, treated and control *G.mellonella* larvae (1µg/mL PMA, 0.2 µg/mL fMLP, 0.001% (v/v) DMSO and PBS) were assessed for their survival by visualisation of movement and melanisation 24 hours following injection. Results demonstrated that all larvae displayed high survival rates indicating no toxicity ($p = 1$) (Figure 4.2).

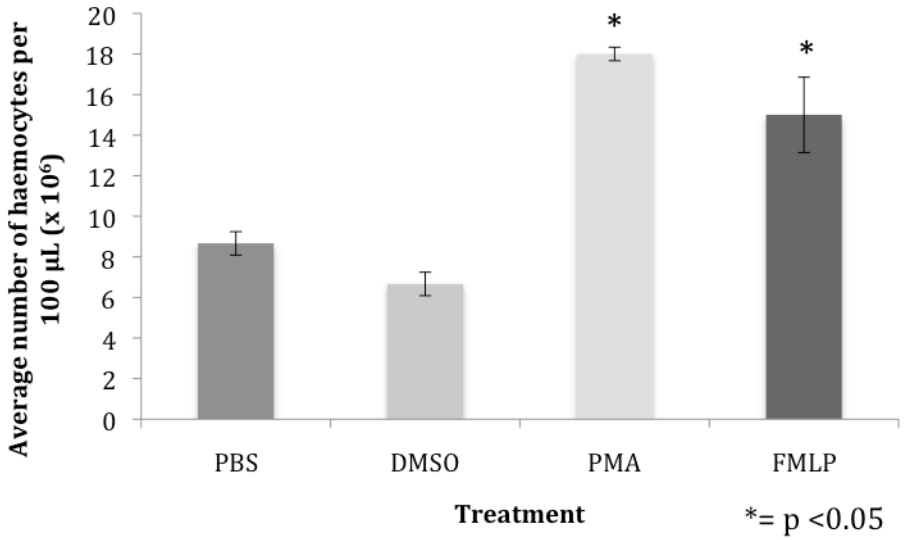


Figure 4.1 Haemocyte density in larvae injected with PBS, 0.001% (v/v) DMSO, 1µg/mL PMA or 0.2 µg/mL FMLP 24 hours following inoculation (All statistics are relative to PBS control) (n=3).

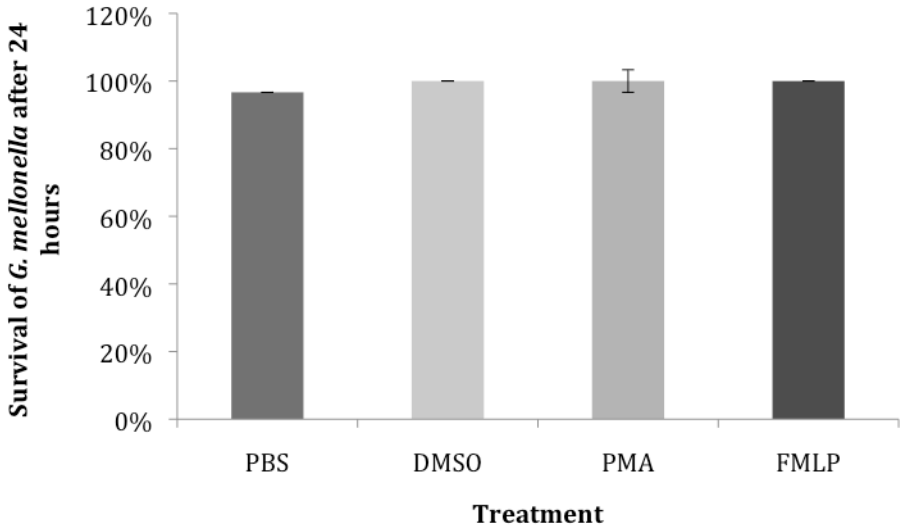


Figure 4.2 Survival of larvae injected with PBS, 0.001% (v/v) DMSO, 1µg/mL PMA, 0.2 µg/mL or FMLP at 24 hours. (No significant difference, p>0.1) 24 hours following inoculation (All statistics are relative to PBS control) (n=3).

4.2 One dimensional analysis of haemocytes, stimulated with PMA and fMLP

One dimensional gel electrophoresis was performed using haemocyte samples extracted from larvae of *G. mellonella* and stimulated with fMLP (0.2 µg/mL) or PMA (1µg/mL) for a total of 6 minutes. Haemocyte extracts treated with 0.001% (v/v) DMSO were used as experimental controls. Colloidal coomassie staining was used to visualise protein bands and allow for the comparisons between protein expression in stimulated haemocytes and the controls (Figure 4.3).

The results presented here demonstrated a clear upregulation in the expression of a variety of proteins when compared to the DMSO control, which can be seen in Figure 4.3. PMA stimulation induced a greater upregulation of protein expression in contrast to fMLP and unstimulated haemocytes. These upregulated proteins were later excised and identified using LC-mass spectrometry (Figure 4.4 and Table 4.1). These results suggest that the haemocyte of *G. mellonella* larvae may increase the expression of certain proteins upon stimulation, which have a specific function in the insect immune response against infection.

Upon excision and identification, band number 1 (Figure 4.4) displayed homology to phenoloxidase; a vital component of the insect melanisation process.. Phenoloxidase is involved in the prophenoloxidase cascade and melanisation process (Pfaller, 2002; Li *et al.,* 1992). It was decreased 0.17 fold in fMLP stimulated haemocytes and 0.37 fold in control haemocytes compared to the PMA stimulated haemocytes (Figure 4.4 (B)). It has been shown to be induced upon immune challenge with PMA in haemocytes of the Sydney Rock Oyster (Aladaileh *et al.,* 2007). fMLP has also been found to affect phenoloxidase expression in insect haemocytes (García-García *et al.*, 2009).

Band number 2 (Figure 4.4) was identified as being homologous to GF21232, an amino acid storage protein, from *D. melanogaster* (Clark, 2007). This band was demonstrated to be reduced 0.10 fold in fMLP stimulated haemocytes and 0.40 in the control haemocytes. Transferrin was identified as band number 3, transferrin is also found to be contained in the human neutrophil (Brieland and Fantone, 1991) and it functions in the binding of iron. Increases in transferrin expression can be indicative of an upregulation in the activity of the haemocytes immune response (Slater *et al*., 2010; Bergin *et al*., 2006). Neutrophil transferrin has been implicated in the maturation of neutrophil granulocytes (Evans, 1986), in

addition to binding iron. Transferrin was reduced 0.17 fold in fMLP stimulated haemocytes and 0.31 fold in control haemocytes, compared to the PMA stimulated haemocytes.

The fourth identified peptide (band number 4) was homologous to the hexameric glycoprotein arlyphorin, arylphorin has been identified in haemocytes of *M. sexta* (Beetz *et al*., 2007). Beresford *et al.* (1997) found that arylphorins are implicated in the immune response of *G. mellonella* larvae and that there are similarities with it and with peptides from murine blood. Arlyphorin was demonstrated to be reduced 0.31 fold in fMLP stimulated haemocytes, compared to the PMA stimulated haemocytes and it did not appear in control haemocytes.

Prophenoloxidase, transferrin precursor and arlyphorin were detected, each of these have been implicated in the insect cellular immune response and are located in the haemocyte (Gillespie *et al*., 1997; Bergin *et al*., 2006; Beresford *et al*., 1997). These results demonstrate the complexity of the haemocyte. This haemocyte can attack pathogens by the up-regulation in expression of different proteins, these results also demonstrate that PMA is the most active stimulator of the haemocyte compared to fMLP.

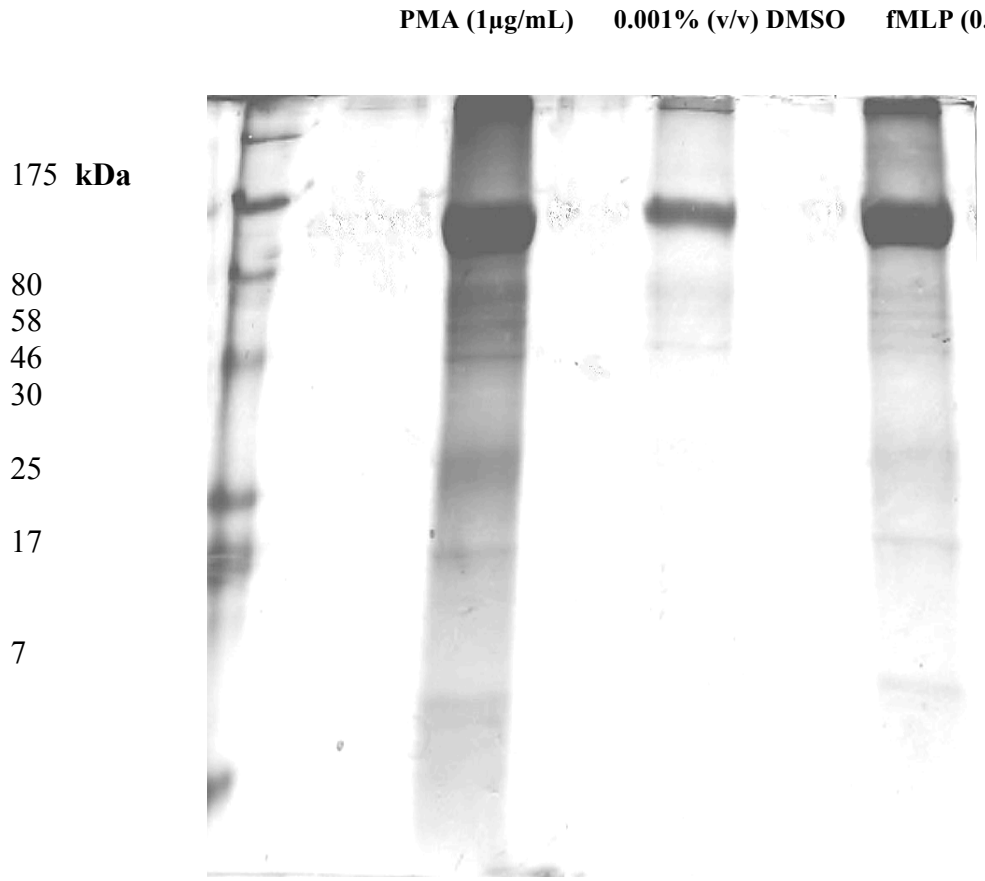
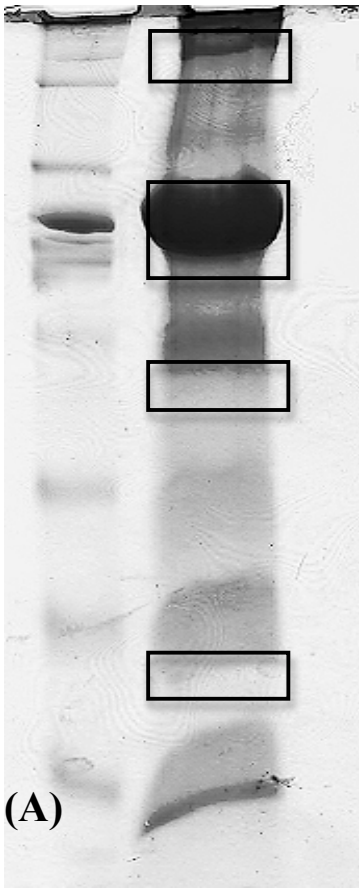


Figure 4.3 One dimensional analysis of haemocyte lysates using colloidal Coomassie staining (20µg/20µL) (n=2).



Band no	PMA	fMLP	Control
1	2.32	1.26	1
2	1.66	1.5	1
3	1.45	1.20	1
4	1	0.69	0

(B)

Figure 4.4 One dimensional SDS-PAGE gel of PMA stimulated haemocyte lystaes (A) (n=1), fold decreases in labeled bands relative to PMA stimulated haemocytes,

Band no:	Protein name:	Suggested protein function:	% Coverage:	Mascot Score:	Accession No.:
1	Prophenoloxidase	Involved in the insect melanisation process	9	201	AAK64363
2	GF21232	Storage Protein	1	74	XP_001963839
3	Transferrin Precursor	An iron binding glycoprotein	15	420	AAQ63970
4	Arylphorin	Hexameric glycoprotein found in haemolymph	17	385	ZP07873717

referring to Figure 4.3 (B)

Table 4.1 Mass spectrometry identification of peptides from haemocyte of *G. mellonella* larvae

4.2.1 Summary

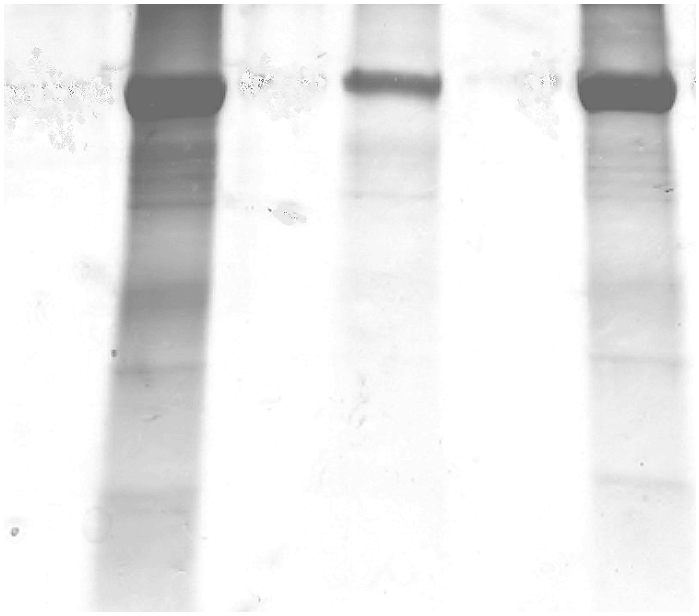
This section of results assessed the effect of the artificial cellular stimulants PMA and fMLP on *G. mellonella* larval survival and protein expression in whole cell 1-Dimensional SDS PAGE. The stimulants have no toxic effect on the larvae, however the injection of the compounds increased haemocyte density significantly. PMA had a slightly greater effect on the number of haemocytes in larvae. Proteomic analysis showed that PMA also increased protein expression in haemocytes. Some of these proteins were identified as immuno-relevant putative peptides such as prophenoloxidase, transferrin precursor and arlyphorin, all of which have previously been implicated in haemocyte immune function.

4.3 Assessment of the phosphorylation events in haemocytes using Western Blotting and ProQ Diamond Phospho-Staining

The invasion of pathogens leads to many phosphorylation events in the insect immune response (Gillespie *et al.*, 1997), such as the prophenoloxidase cascade and the activation of Protein Kinase C leading to NADPH oxidase activation (Renwick *et al*., 2007). Encapsulation is also very likely to be mediated by phosphorylation events in *G. mellonella* larvae (Strand, 2008). These immune events signify the importance of phosphorylation in *G. mellonella* larvae. Phosphorylation in the neutrophil is of fundamental importance leading to cellular activation and the translocation of the phox proteins (Bennas *et al.*, 1994).

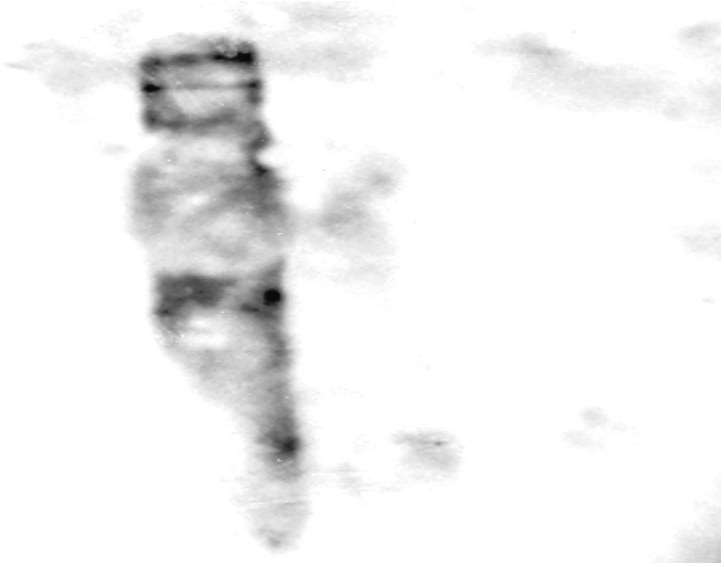
Phosphorylation events in haemocyte extracts were first assessed to specifically identify the proteins phosphorylated by the AGC-family kinases. Phospho-(Ser/Thr) PKA substrate antibody detects the PKC and PKA proteins, which are 90% homologous and this antibody was utilised in this section. fMLP, PMA and unstimulated haemocyte samples were run on 1-D SDS-PAGE gels and transferred to a nitrocellulose membrane. Reactivity to the Phospho-(Ser/Thr) PKA substrate antibody was only visualised in PMA stimulated haemocytes (Figure 4.5).

As neutrophil and haemocyte activation is associated with several phosphorylation events involving protein kinases (Cytrynska *et al*., 2007), gels were stained with the Pro-Q Diamond Phosphoprotein stain to detect phosphate groups attached to serine, threonine or tyrosine residues. The image displays a low level of phosphorylation in the fMLP and unstimulated haemocytes of *G. mellonella* larvae when compared to the PMA treated cells (Figure 4.6). These results indicate that PKA and PKC play a role in the PMA activation of haemocytes. Due to the fact that no reactivity was observed in fMLP treated or control haemocytes, comparisons could not be made in order to determine whether the proteins identified were specifically phosphorylated upon immune challenge or whether they were constantly phosphorylated in the cell.



(A)

PMA (1µg/mL) 0.001% (v/v) DMSO fMLP (0.2 µg/mL).



(B)

Figure 4.5 PMA stimulated haemocytes displayed reactivity in proteins of molecular masses ~ 80 kDa, 70 kDa, 55 kDa, 35 kDa and 25 kDa.
Colloidal coomassie stained gel of haemocytes (A).
Western blot analysis of haemocytes of *G. mellonella* larvae using phospho-(Ser/Thr) PKA substrate antibody (20µg/20µL) (B).
Lane 1: PMA (1µg/mL), Lane 2: 0.001% (v/v) DMSO and Lane 3: fMLP (0.2 µg/mL) (n=4).

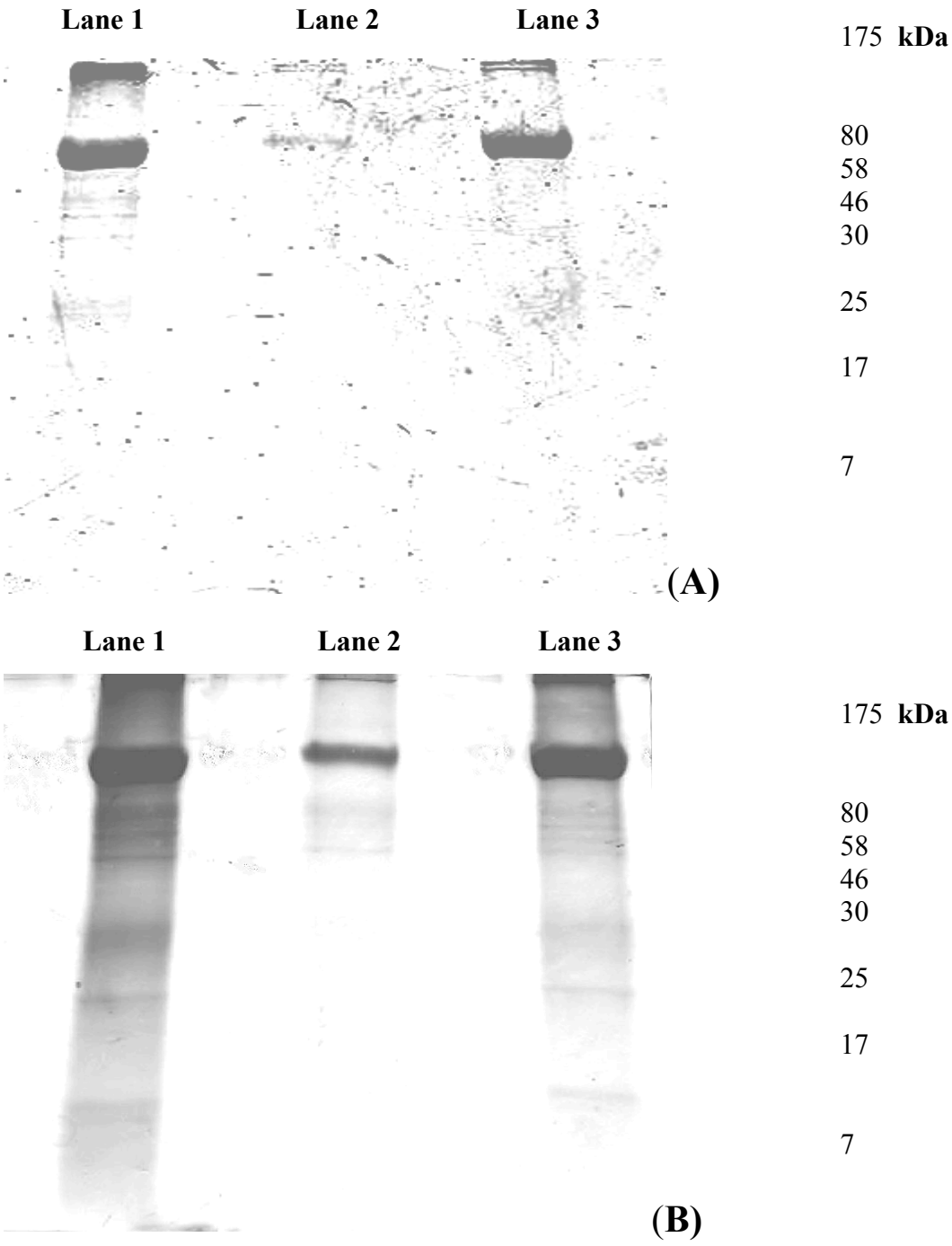


Figure 4.6 One dimensional gel of haemocyte samples (20µg/20µL) stained with Pro-Q Diamond Gel stain for the visualisation of phosphoproteins (A). Parallel gel stained with colloidal coomassie Stain (B).

Lane 1: PMA (1µg/mL), Lane 2: 0.001% (v/v) DMSO and Lane 3: fMLP (0.2 µg/mL) (n=4).

4.4 Two-Dimensional SDS-PAGE of fMLP and PMA stimulated haemocytes

Two-dimensional SDS-PAGE was used to identify alterations in protein expression in immune challenged and control haemocytes. Unstimulated haemocytes, fMLP stimulated haemocytes and PMA stimulated haemocytes were colloidal coomassie stained as shown in Figure 4.7. Changes in protein expression between the control and PMA treated gel were then examined using Progenesis SameSpots software® and proteins of interest were excised from the PMA gel and trypsin digested prior to identification by LC-Mass spectrometry as demonstrated in Figure 4.8. Twenty-one proteins were identified and are listed in Table 4.2. A Mascot score over 67 is considered significant.

Transferrin precursor (spot number 2), GF21232 protein (spot number 6), transthyretin precursor (spot number 21) and a 27kDa haemolymph protein (spot number 16) were identified and all were found to have significantly different expression levels in PMA and FMLP treated cells in comparison to the control. Spot 1 displayed homologies to phenoloxidase, which catalyses the synthesis of melanin (Strand, 2008) and has a vital role in the melanisation cascade defending against infection (Broderick *et al.,* 2010). Spot 2 was identified as a transferrin precursor (Vogel *et al*., 2011) and expression was significantly increased (2.8 fold) upon immune challenge by PMA. There was no significant change in arylphorin (spot number 3) expression, an amino acid storage protein (Telfer and Kunkel, 1991).

Spot 18 displayed homologies with a heat shock protein, a small stress induced protein. Spot 19 and 20 displayed homology with serine protease, which increased in expression upon stimulation with PMA (+1.6) but interestingly demonstrated a decrease in expression upon stimulation with FMLP (-1.4).

Proteins of unknown function were also identified. A 27kDa haemolymph protein with an unknown function was identified as spots 15 and 16, which displayed a 2.2 fold increase upon stimulation with PMA and a 1.2 fold decrease upon stimulation with FMLP.

These results suggest that PMA up regulates the expression of many immune relevant proteins in haemocytes, in a comparable manner to an opsonised microorganism.

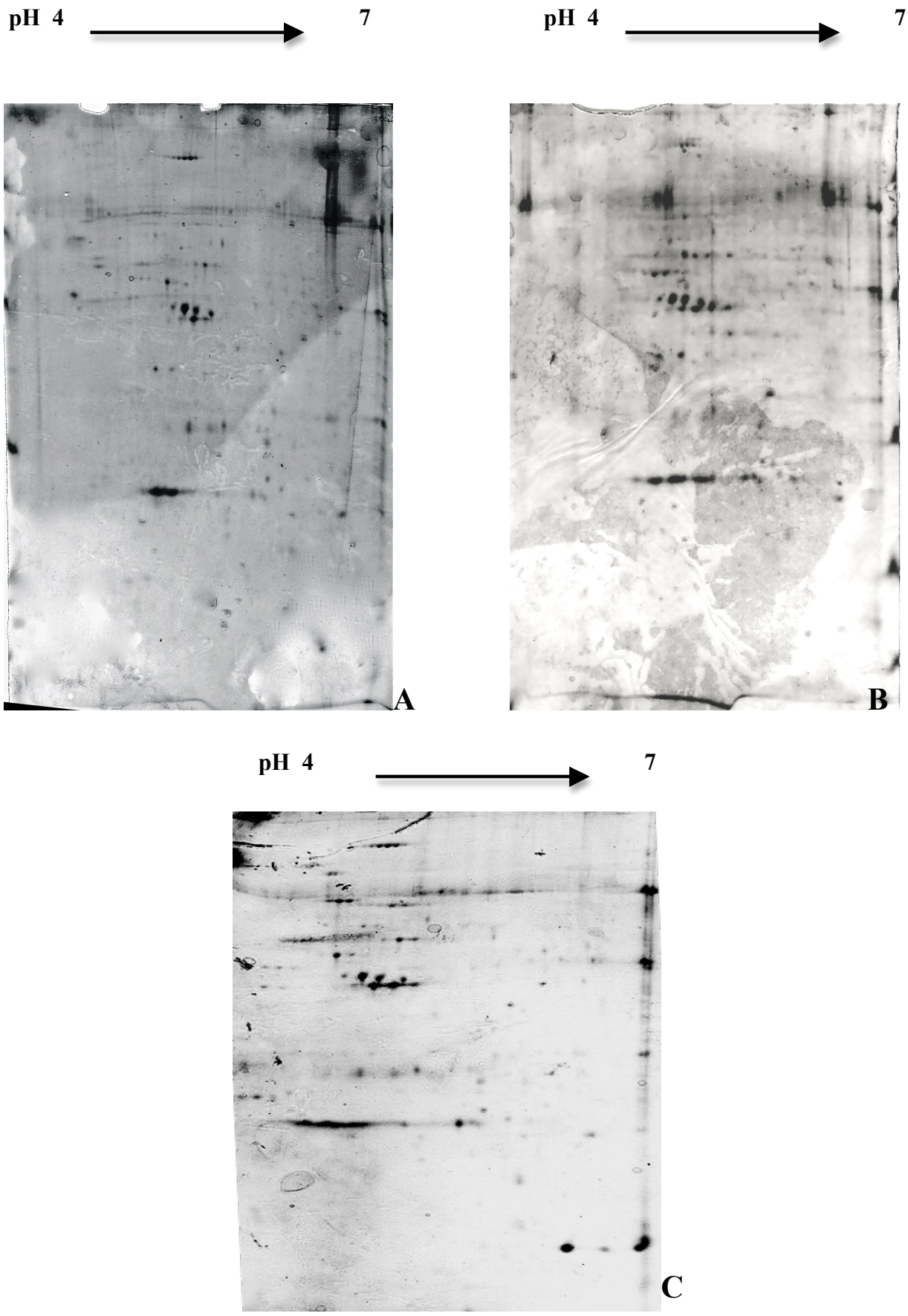


Figure 4.7 **A**: Two dimensional SDS-PAGE gels from PMA stimulated Haemocyte lysates; **B**: fMLP stimulated Haemocyte lysates; **C**: Unstimulated Haemocyte Lysates (0.001% DMSO) (n=3)

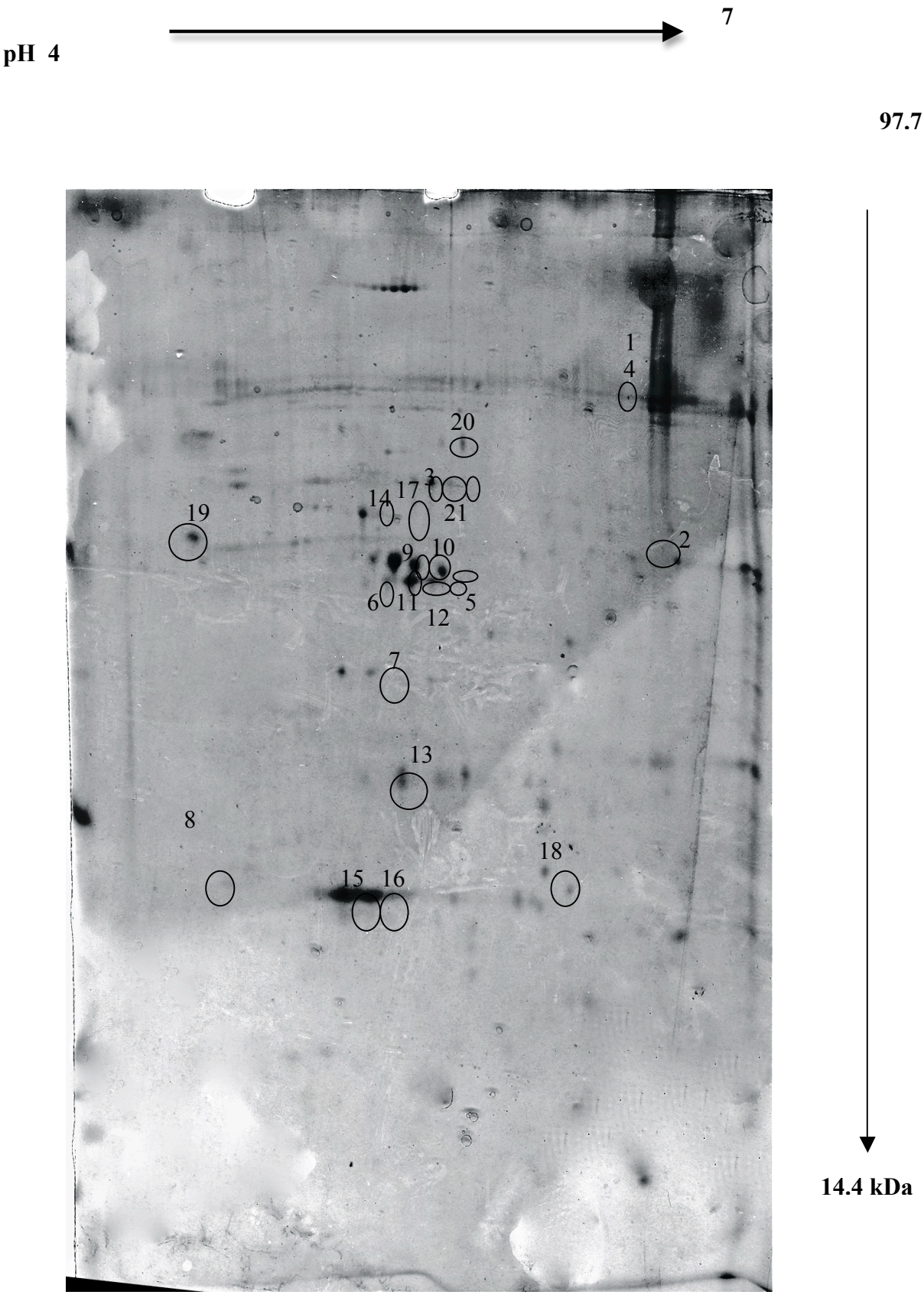


Figure 4.8 Reference 2-dimensional SDS-PAGE gel of PMA stimulated haemocyte samples (300 µg/250 µL) with identified numbered peptides (n=3).

Spot No:	Protein Identity	Organism	Mr	PI	Score	Sequence Coverage	Accession No
Spot No:	Protein Identity	*Organism*	Mr	PI	Score	Sequence Coverage	Accession No
1	Prophenoloxidase	*Galleria mellonella*	79076	6.84	201	9%	AAK64363
12	actin	*Haemaphysalis longicornis*	42204	5.3	273	21%	AAP81255
2	Transferrin precursor	*Galleria mellonella*	77238	6.76	420	15%	AAQ63970
13	DNA ligase	*Arsenophonus nasoniae*	75121	6.7	68	5%	CBA72055
3	Arylphorin	*Galleria mellonella*	83651	5.23	68	8%	AAA74229
14	unnamed protein product	*Homo sapiens*	71281	5.92	90	6%	CAA23753
4	Prophenoloxidase 27 kDa subunit 2	*Galleria mellonella*	80198	5.95	292	12%	AAQ75026
15	glycoprotein precursor	*Bombyx mori*	25582	5.12	87	8	NP_001037687 8
5	NADP-dependent malic enzyme, putative	*Pediculus humanus Corporis*	132548	8.01	70	9%	XP_00242668 0
16	hemolymph protein	*Galleria mellonella*	26387	5.17	311	35%	P83632
6	GF21232	*Drosophila ananassae*	113624	8.42	70	9%	XP_00196383 9
17	ATP synthase	*Helicoverpa zea*	55254	5.21	227	13%	ADJ95799
7	Arylphorin	*Galleria mellonella*	83651	5.23	385	17%	AAA74229
18	heat shock protein hsp21.4	*Bombyx mori*	21391	5.79	185	25%	NP_001036985
8	Pyridoxamine kinase	*Deinococcus proteolyticus*	33580	5.01	69	6%	YP_00425481 6
19	protease, serine	*Mus musculus*	26814	4.75	79	8%	NP_444473
9	TetR-family transcriptional regulator	*Sphingobium japonicum*	21974	9.69	80	9%	YP_00355946 9
20	protease, serine	*Mus musculus*	26814	4.75	88	11%	NP_444473
10	transthyretin precursor	*Bos taurus*	15833	5.91	139	40%	NP_776392
21	small ribosomal protein 4	*Wuchereria mollis*	21831	9.97	84	9%	AAR06541
Spot No:	Protein Identity	*Organism*	Mr	PI	Score	Sequence Coverage	Accession No
11	alpha-actin	*Homo sapiens*	42487	5.23	77	7%	AAA51577

12	actin	*Haemaphysalis longicornis*	42204	5.3	273	21%	AAP81255
13	DNA ligase	*Arsenophonus nasoniae*	75121	6.7	68	5%	CBA72055
14	unnamed protein product	*Homo sapiens*	71281	5.92	90	6%	CAA23753
15	27 kDa glycoprotein precursor	*Bombyx mori*	25582	5.12	87	8	NP_001036878
16	27 kDa hemolymph protein	*Galleria mellonella*	26387	5.17	311	35%	P83632
17	ATP synthase	*Helicoverpa zea*	55254	5.21	227	13%	ADJ95799
18	heat shock protein hsp21.4	*Bombyx mori*	21391	5.79	185	25%	NP_001036985
19	protease, serine	*Mus musculus*	26814	4.75	79	8%	NP_444473
20	protease, serine	*Mus musculus*	26814	4.75	88	11%	NP_444473
21	transthyretin precursor	*Bos taurus*	15833	5.91	139	40%	NP_776392

Table 4.2 Identification of proteins by LC-Mass spectrometry.

Spot No:	Protein	Fold change (PMA)	Fold change (FMLP)	P value	Function
1	Prophenoloxidase	-	-	-	Enzyme which plays an important role in the production of melanin. This protein is also involved in cuticle sclerotization and wound healing.
2	Transferrin precursor	+ 2.8	+1.5	0.018	Role in the innate immune system. Transferrin binds to iron which is essential for bacterial growth. The lack of free iron impedes microbial growth.
3	Arylphorin	-	-	-	A storage protein found in insects.
4	Prophenoloxidase subunit 2	-	-	-	Protein family found in arthropods; includes hemocyanins and insect larval storage proteins.
5	NADP-dependent malic enzyme, putative	+3	+3	0.859	Catalyses the oxidative decarboxylation of L-malate to pyruvate in the presence of cations and is dependent on the reduction of NAD+ or NADP+.
6	GF21232	+2.1	+2.1	0.010	N-terminal DNA binding fragment found in eukaryotic DNA topoisomerase (topo) IB proteins similar to human topo I.
8	Pyridoxamine kinase	-	-	-	Enzyme which is involved in the synthesis of pyridoxal-5'-phosphate (PLP). It induces the phosphorylation of the precursor vitamin B6 in the presence of Zn2+ and ATP.
9	TetR-family transcriptional regulator	+2.3	-2	0.461	Single-stranded DNA binding proteins which are involved in DNA replication, repair and recombination
10	Small ribosomal protein S4	0	0	0.995	Found in stressed proteins, ribosomal proteins and tRNA synthetases. Function remians unknown.
11	Alpha-actin	+1.1	-1.9	0.31	Component of the cytoskeleton. It is important for a variety of cellular processes such as cell migration and cytokinesis.
15	27 kDa glycoprotein precursor	+2.2	-1.2	0.077	Family of insect proteins. Function remains unknown
16	27 kDa hemolymph protein	+2.2	+2.1	0.010	Family of insect proteins. Function remains unknown
17	ATP synthase	-1.5	-1.5	0.051	Homologous to the F1 ATP synthase beta subunit, nucleotide-binding domain. It has a role in ATP synthesis.
18	Heat shock protein, hsp21.4	-	-	-	Small stress induced proteins (12-43 kDa) which are active as large oligomers consisting of multiple subunits. It has been suggested that these proteins function as ATP-independent chaperones which prevent aggregation.
19	Protease, serine	+1.6	-1.4	0.257	Enzymes that are synthesized as inactive precursor zymogens and are cleaved by proteolysis into their active forms.
21	Transthyretin precursor	+4.4	+4.8	0.049	Transthyretin (TTR) is a 55 kDa protein responsible for the transport of thyroid hormones and retinol in vertebrates. A fraction of plasma TTR is carried in high density lipoproteins by binding to apolipoprotein A.

Table 4.3 Identities of putative peptides from Reference PMA gel (Fold change is relative to control). (- indicates no fold change in the identified putative peptide)

4.5 Assessment of phosphorlyation events in stimulated haemocytes

The Pro-Q diamond phosphoprotein stain is a non-specific phosphorylation stain, which allows for in gel detection of phosphate groups attached to serine, threonine or tyrosine residues (Steinberg *et al.,* 2003). No upregulation in phosphorylation was detected in unstimulated and FMLP stimulated haemocytes, as no protein spots were visible on these gels (results not presented). Several phosphorylated proteins were observed in PMA stimulated samples. The area of this gel marked within a box outlines an area of high intensity spots which have been chosen to be analysed and identified using LC-Mass Spectrometry (Figure 4.9).

When cross-referenced with the reference gel (Figure 4.8), which was used to identify the proteins via mass spectrometry, 8 proteins identified were determined to undergo phosphorylation upon PMA stimulation. Spots 3, 5, 9, 10, 11, 13, 18 and 21 correspond to the PMA colloidal coomassie image, these phosphorylated peptides are aryliphorin, TetR transcriptional regulator, NADP-dependent enzyme, small ribosomal protein, alpha actin, 27kDa protein, heat shock protein (HSP 21-4) and transthyretin precursor, respectively (Figure 4.8).

Transthyretin (spot number 21) has been identified in the neutrophil and enhances ROS production. It's expression has previously been shown to be activated by PKC activators, indicating that PMA would increase its expression and evidently, phosphorylation of the protein (Mezosi *et al.,* 2005). Arylphorin (spot number 3) phosphorylation has been suggested to be responsible for the release of ecdysteroid hormones, which are responsible for insect development (Arif *et al.,* 2003).

The phosphorylation of alpha-actin (Spot number 11) correlates with the stimulation of the cell by PMA causing major actin reassembly (Grogan *et al*., 1997; Fallon *et al*., 2011) in order to allow processes such as phagocytosis and degranulation to occur. Heat shock proteins (spot number 18) are required for the process of actin polymerisation in the neutrophil and phosphorylation is required for the activation of these proteins (Howare and Oresajo, 1985).

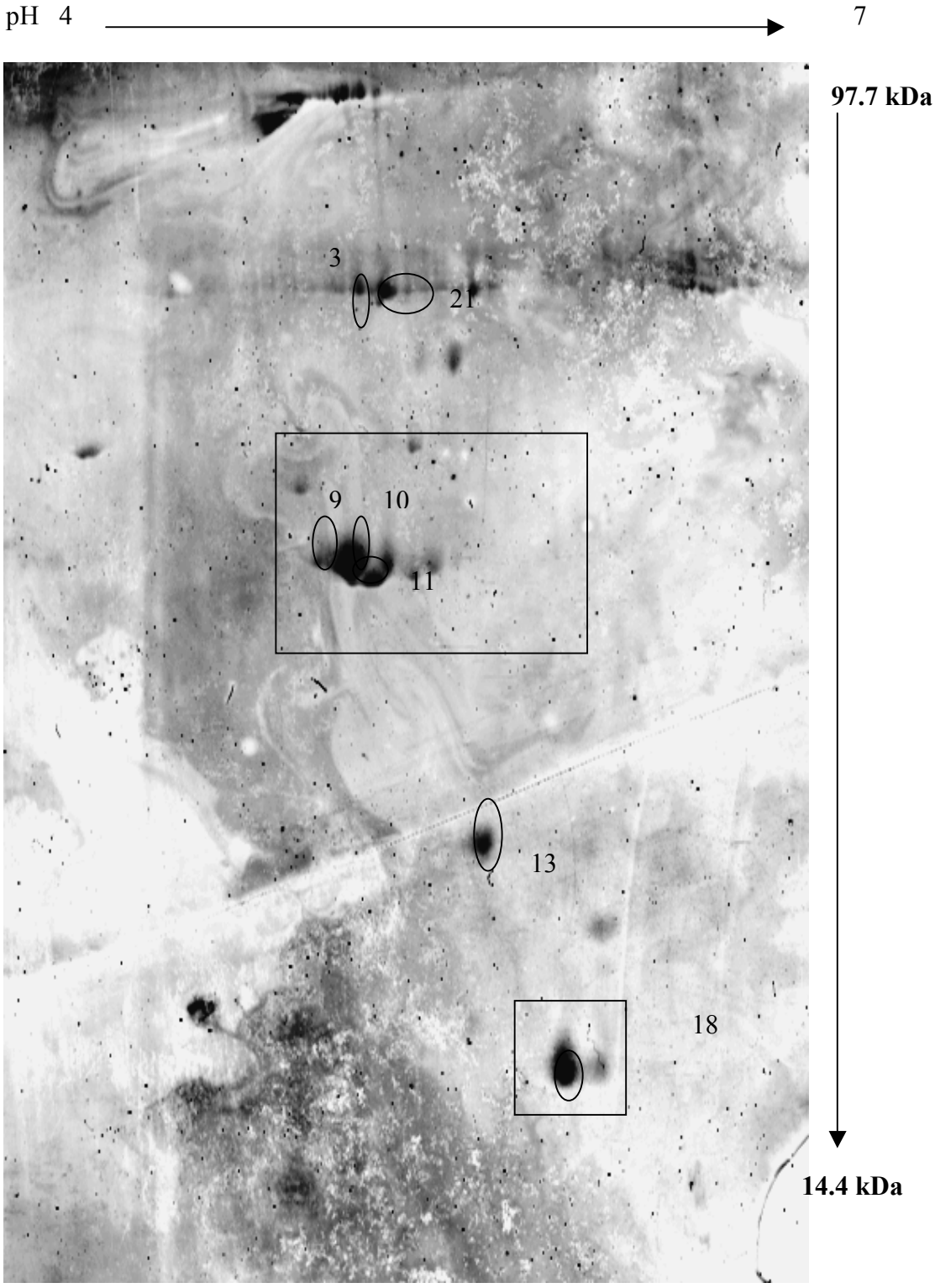


Figure 4.9 Two dimensional SDS-PAGE image of PMA (1µg/mL) stimulated haemocyte samples (300µg/250µL) with identified numbered peptides stained with Pro-Q Diamond Gel stain for the visualisation of phosphoproteins. No phosphorylated regions were identified on fMLP stimulated haemocyte gels or unstimulated gels. The labelled spots refer to those that are identified and quantified in Table 4.3 (n=4).

4.5.1 Summary

The experiments presented in this section sought to characterise the effect that PMA and fMLP had on the haemocyte proteome. PMA stimulation of the cells caused an up regulation in kinase activity, demonstrated by the Western blot and one-dimensional Pro-Q diamond staining.

Cells that were unstimulated or those stimulated with fMLP did not display any phosphorylation at this stage. Colloidal coomassie stained 2-dimensional gels allowed the visualisation of the haemocyte proteome. PMA and fMLP stimulation altered the protein expression compared to the unstimulated, control cells. Twenty-one proteins were identified using LC-mass spectrometry. Some of these were implicated in the immune response of *G. mellonella* larvae. Pro-Q diamond staining demonstrated that phosphorylation only seemed to be occurring in the PMA stimulated haemocytes with no visual up regulation in haemocyte phosphorylation occurring in the unstimulated and fMLP gels.

4.6 Characterisation of translocation events in the haemocyte of *G. mellonella* larvae and comparison with the neutrophil

Upon activation of the respiratory burst in the neutrophil, either by an opsonised particle or by a chemotactic comound such as PMA, a number of proteins (such as the phox proteins, $p47^{phox}$ and $p67^{phox}$) translocate from the cytoplasm to the membrane, (Bennas *et al.*, 1994; Renwick *et al*., 2007).

One-dimensional electrophoresis and Western blotting was used to assess this event in the haemocyte of *G. mellonella* larvae. The colloidal coomassie stained one-dimensional profiles of the translocation events in the neutrophil and the haemocyte can be seen in Figures 4.10 and 4.11, respectively. This was carried out as described in section 2.9.7. Western blotting and confocal immunofluoresence were employed to assess the translocation of the protein $p47^{phox}$ in both cell types. PMA was used as the chemotactic activator due to its predominance in the assessment of phosphorylation in the haemocyte.

An increase in $p47^{phox}$ expression was demonstrated in both PMA stimulated neutrophil and haemocyte membrane fractions, indicating a translocation event. This was assessed by Western blotting where the neutrophil $p47^{phox}$ membrane intensity increased significantly ($p < 0.05$), from 196 ± 0.33 to 241 ± 0.66 upon PMA stimulation. Haemocyte $p47^{phox}$ membrane intensity increased from 68 ± 8.08 to 114 ± 15.04 ($p < 0.05$), upon stimulation with PMA.

This was further characterised by confocal immunofluoresence as described in section 2.11, where fluoresence intensity in PMA stimulated cells translocated from cytosolic regions to be concentrated in the cell periphery (Figure 4.13), the level of intensity in the neutrophils and haemocytes rose slightly upon PMA stimulation.

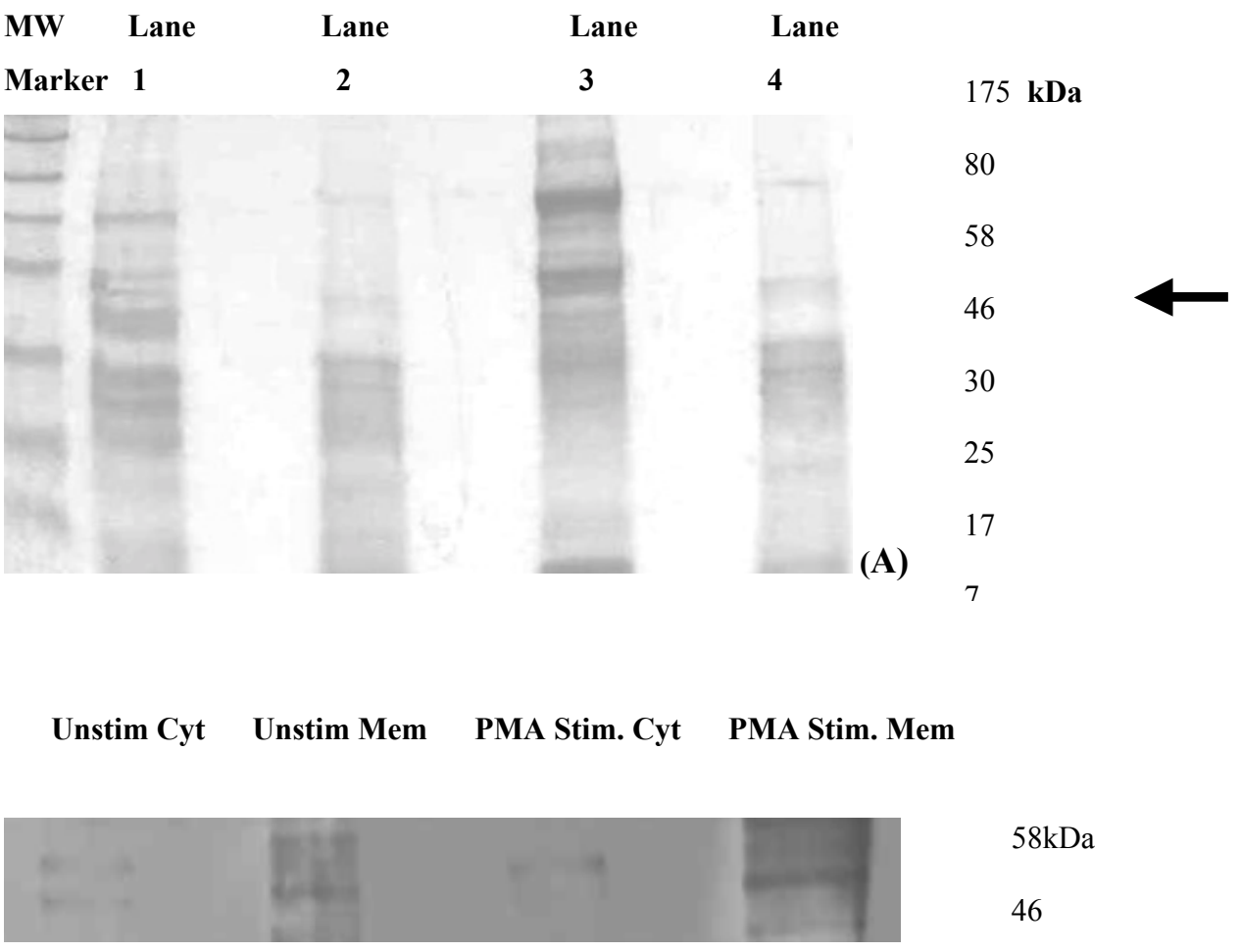


Figure 4.10 (A) Colloidal coomassie stained 1 dimensional gel of unstimulated and PMA (1µg/mL) stimulated neutrophil cytosolic and membrane components (20µg/20µL). Arrow points to 47kDa point in 1D SDS PAGE gel.

(B) Western blot of $p47^{phox}$ protein, displaying translocation from cytosol to membrane upon PMA stimulation (20µg/20µL) (n=3).

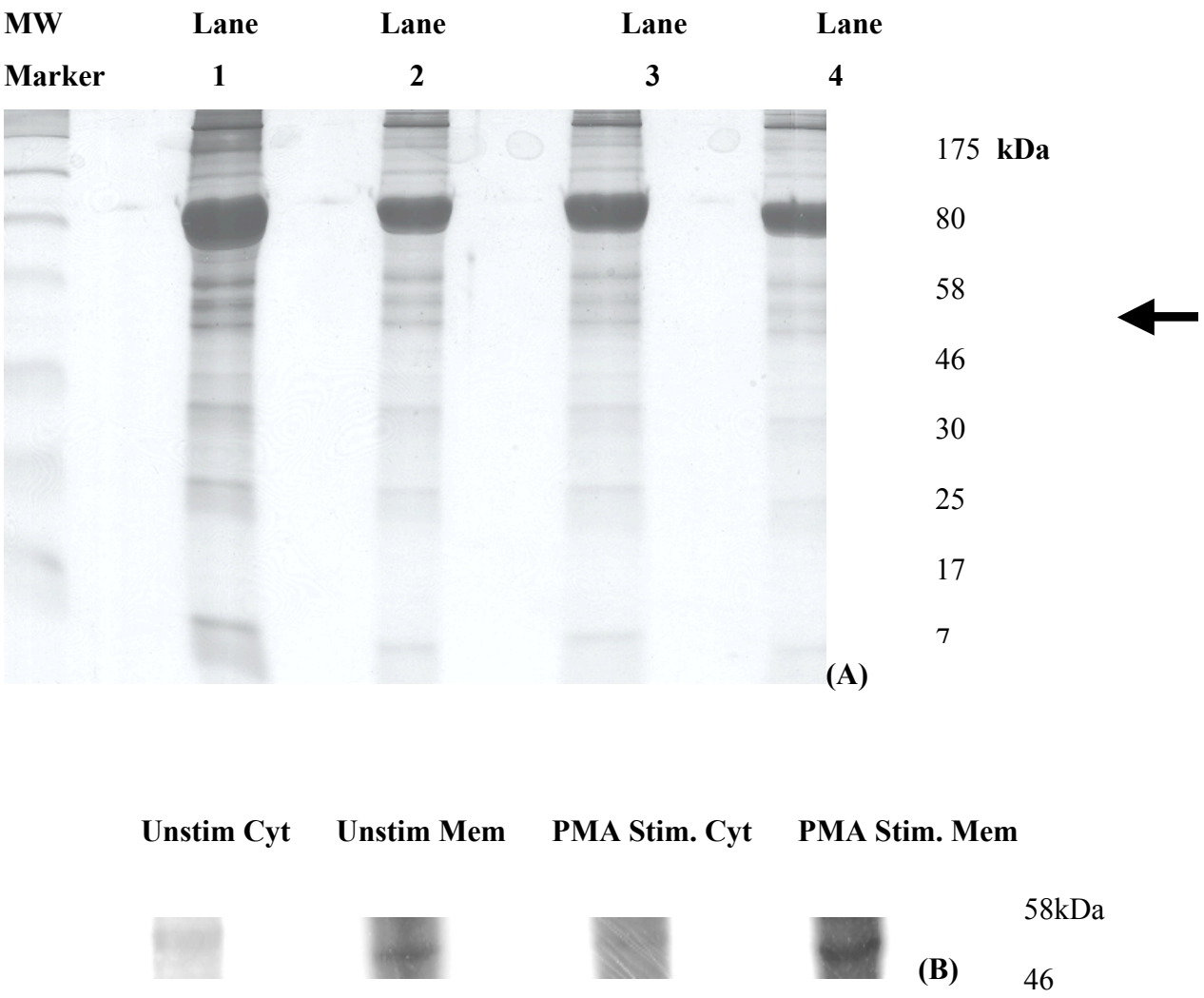


Figure 4.11 (A) Colloidal coomassie stained 1 dimensional SDS-PAGE gel of unstimulated and PMA (1µg/mL) stimulated haemocyte cytosolic and membrane components (20µg/20µL). Arrow points to 47kDa point in 1D SDS PAGE gel.
(B) Western blot of $p47^{phox}$ protein, displaying translocation from cytosol to membrane upon PMA stimulation (20µg/20µL) (n=3).

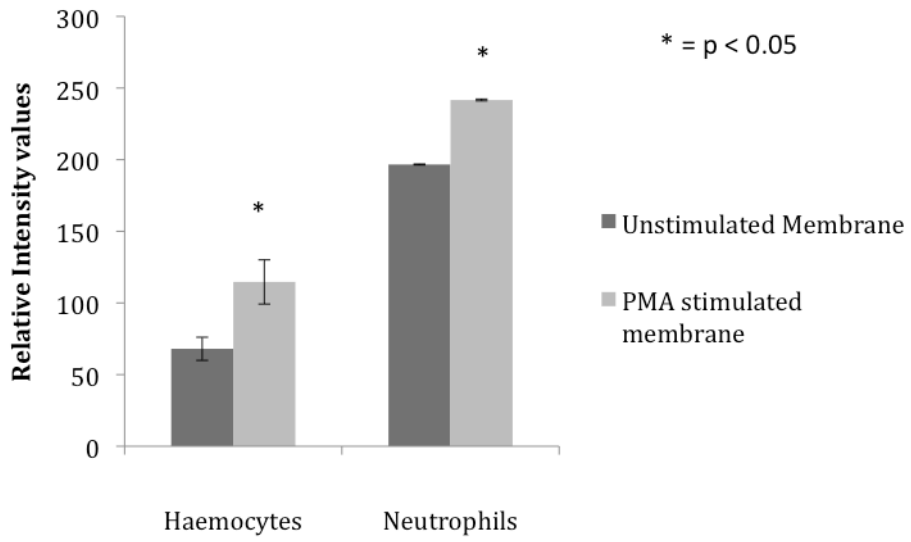


Figure 4.12 Graph demonstrating the relative intensity of the $p47^{phox}$ bands in membrane fraction when analysed using Image J ® software (n=3).

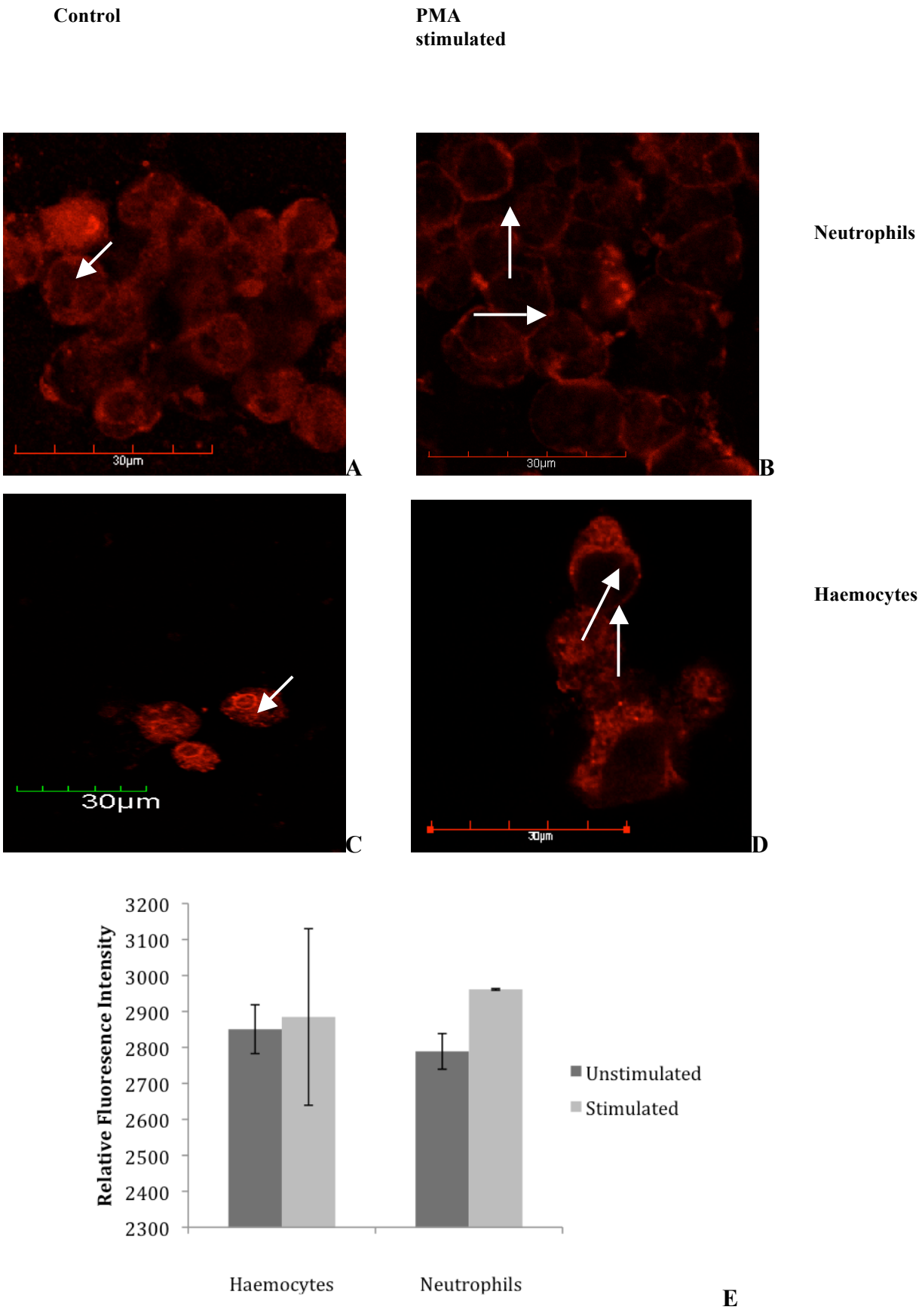


Figure 4.13 Confocal Immunofluoresence images of $p47^{phox}$ in unstimulated neutrophils (A), PMA (1µg/mL) stimulated neutrophils (B), unstimulated haemocytes (C), and PMA (1µg/mL) stimulated haemocytes (D).
Graph showing relative intensity of fluorescence in the cells (E). Arrows point to areas of fluorescence (n=3).

4.6.1 Summary

The experiments in this section focused on the translocation events occurring in the NADPH oxidase cascade which is an essential part of the neutrophil's and haemocyte's killing ability as demonstrated by Renwick *et al.,* (2007).

It can be deduced that homologous proteins translocate from the cytosol to the membrane upon PMA stimulation in both cell types. The protein $p47^{phox}$ was demonstrated to undergo translocation in the haemocyte in a similar manner to the neutrophil. This was demonstrated by Western blotting, and at the cellular level by confocal immunofluoresence.

These findings demonstrate another similarity between the neutrophil and haemocyte in the cascade of events that is required for the formation of a functional NADPH oxidase cascade.

4.7 Utilisation of shotgun proteomics to identify peptides in haemocytes of *G. mellonalla* larvae

Shotgun proteomics is a term derived from shotgun sequencing of DNA. Shotgun proteomics has allowed complex mixtures of peptides to be characterised using a combination of LC-mass spectrometry and the manipulation of sequence databases (Washburn *et al.,* 2001). The aim of this section was to determine whether 'shotgun' proteomics could be utilised to investigate the proteome of the haemocyte of *G. mellonella* larvae. Shotgun proteomics has been used in the identification of peptides from *B. mori* (Chen *et al*., 2009) and *D. melanogaster* (Brunner *et al*., 2007).

The haemocytes were digested and dialysed as described in section 2.12. Optimisation was required to identify the ideal concentration of protein to load onto the 6340 Ion Trap LC/MS spectrometer (Agilent Technologies) without overloading and blocking the nano-chip. During this trial a concentration of 10 µg was also tried, however very few matches were found. A final concentration of protein lysate of 50µg was used in the experiment. Table 4.4 details the peptides identified from this study, with indications of 32 identities established (Mascot score > 67, indicating a p value <0.05 with regard to identity).

There is a clear abundance of storage proteins in the haemocyte with the identification of putative storage peptides such as arylphorin, hexamerin and methionine rich storage proteins, which are all members of the insect storage family. Arylphorin is an abundant storage protein in *G. mellonella* larvae, it is synthesised in the fat body but has been found to be incorporated into the haemocyte (Marmaras and Tsakas, 1988). This has been implicated in the immune response of *Trichoplusia ni* larvae to bacteria (Freitak et al., 2007). Arylphorin has tyrosine and phenylalanine components totalling 15% of the amino acid content (Telfer and Kunkel, 1991). Methionine rich storage proteins are those in which the methionine amino acid content is over 4% (Telfer and Kunkel, 1991). An increase in hexamerin has been identified in *Sarcophaga bullata* larvae following pathogen infection (Masova, 2010).

Putative actin was identified 10 times, indicating a significant abundance of actin in the proteome of the haemocyte. Actin can constitute 50% of the total cellular protein (Numaguchi *et al*., 1999) and this is investigated further in Chapter

5. Tpr is a nuclear pore complex–associated protein that is organised into filaments extending 100–350 nm into the nucleus and may be involved in mRNA export (Pederson, 2000).

The immuno-relevant protein apolipophorin 3 was also identified. Apolipophorin 3 has been implicated in recognition responses of *G. mellonella* larvae to components of bacteria and fungal cell wall components (Leon *et al*., 2006). Apolipophorin 3 is homologous to the mammalian protein Apolipophorin E, which has been found to be involved in LPS detoxification, pattern recognition and phagocytosis (Whitten *et al*., 2004). Another identified protein was the PhoH family protein, which is a cytoplasmic protein, speculated to be involved in lipid transport and RNA synthesis (Kazakov *et al*., 2003). An occuldin like protein was identified also, it is known to be located in the transmembrane and to carry a marvel domain, however it's exact cellular function is not yet known (Blasig *et al*., 2011). An abnormal spindle-like microcephaly associated protein homolog (ASPM) was also identified; it is involved in the coordination of mitotic processes in cells (Zhong *et al*., 2005). There was also the prediction of many hypothetical proteins, these are proteins whose existence has been predicted in the peptide database, however there is no experimental or biological evidence for these proteins in literature (Zarembinski *et al.,* 1998).

Twenty- one peptides matches were also made with scores > 52. However, these had no protein matches, most likely due to the proteomic limits with *G. mellonella* larvae where there is no full protein sequence available. It is likely that most of these were also hypothetical proteins that are not yet known to have a function in the haemocyte due to the fact that their sequence is not yet fully annotated and a cellular function has not yet been assigned.

Hit Number	Protein Name	Score	Sequence Coverage	Protein Function	Organism
1	Arylphorin	1734	51%	Insect Storage Protein	*G.mellonella*
12	Actin isoform	311	24%	Nucleation, polymerization	*H. glaberrima*
2	Arylphorin	995	36%	Insect Storage Protein	*G.mellonella (sea cucumber)*
13	Actin	293	20%	Nucleation, polymerization	*T. languginosus*
3	Apolipophorin 3	1709	28%	Involved in pattern recognition and multicellular encapsulation reactions in the innate immune response of insects	*G. mellonlella*
14	Actin	280	20%	Nucleation, polymerization	*D. dendriticum*
15	Unnamed protein product	271	18%	Unkown	*M. musculus*
16	Actin	223	19%	Nucleation, polymerization	*G. max (soybean)*
4	Hexamerin	1049	24%	Insect Storage Protein	*G. mellonella*
17	Type I actin	203	12%	Nucleation, polymerization	*P. carterae (algae)*
5	Hexamerin	606	19%	Insect Storage Protein	*G. mellonella*
18	Unnamed Protein	190	19%	Unknown	*D. ananassae (fly)*
6	27kDa haemolymph protein	507	51%	Unknown	*G. mellonella*
19	Hexamerin receptor	172	4%	Receptor for hexamerin, becomes phosphorylated	*C. cepalonica*
7	27kDa haemolymph protein	278	34%	Unknown	*G. mellonella*
20	Unnamed protein	171	16%	Unknown	*N. nectensis*
8	Actin	355	26%	Nucleation, polymerisation	*H. longicornis)*
9	Actin	464	38%	Nucleation, polymerisation	*D. melonagaster*
21	Unnamed protein	162	10%	Unknown	*D. discoideium*
10	Apolipophorin 3	332	40%	Involved in pattern recognition and multicellular encapsulation reactions in the innate immune response of insects	*G. mellonella*
22	Actin	142	28%	Nucleation, polymerization	*D. melonagaster*
23	Methionine rich storage protein	132	3%	Amino Acid Storage	*S. cerivisiae*
24	Actin	145	10%	Polymerisation	*D. melonagaster*
25	Muscle actin	111	13%	Polymerisation	*L. japonium*
26	Apolipophori n 3	93	24%	Involved in pattern recognition and multicellular encapsulation	*G. mellonella*
11	Actin	319	23%	Nucleation, polymerisation	*D. melonagaster*

26	Apolipophorin 3	93	24%	Involved in pattern recognition and multicellular encapsulation reactions in the innate immune response of insects	*G. mellonella*
27	TPR Nucleoprotein	90	1%	Component of the cytoplasmic fibrils of nuclear pore complex	*P. abelii*
28	PhoH family protein	84	4%	Cytoplasmic protein and predicted ATPase	*O. granulosus*
29	Hypothetical larval protein	77	10%	Unknown	*G. mellonella*
30	Hypothetical protein	73	1%	Unknown	*C. glabrata*
31	Occuldin-like protein	116	64%	Transmembrane protein, function unknown	*D. melonogaster*
32	Abnormal	94	49%	Probable role in	*A. geoffroyi*

Table 4.4 Identities of putative peptides in the haemocyte of *G. mellonella* larvae from Shotgun Proteomics approach to protein identification (n=1).

4.7.1 Summary

Over fifty peptides were found in haemocytes of *G. mellonella*, however a limiting resource with *G. mellonella* larvae is the lack of a publically available sequenced proteome, without this, proteomics is constrained and limited. This section exploited shotgun with MUDPIT (Multidimenional Protein Identification Technology) by employing the MASCOT science database and NCBI database. Many of these putative peptides are membrane bound or are functional in the transmembrane, the majority of the other peptides identified are storage proteins and have been identified as being immuno-relevant. This section optimised the protein concentration that the 6340 Ion Trap LC/MS spectrometer (Agilent Technologies) nano-chip can grasp and 32 putative proteins were identified. There was a large quantity of storage proteins such as arlyphorin and hexamerin, the immuno-relevant protein apolipophorin 3, and actin in the haemocyte. Shotgun proteomics is advantageous in that it is not time consuming and is much more time efficient than performing in-gel digestion of peptides, however proteomics with *G. mellonella* larvae is still restricted.

4.8 Discussion

The innate immune system is the primary defense system of both insects and mammals, comprising physical barriers (the skin and the cuticle), the humoral response and the cellular response. In mammals the cellular component of the innate immune response is largely dependent on the neutrophil, which comprises 65% of circulating leukocytes (Cassatella, 2003). The neutrophil is the primary phagocytic cell, neutrophils aid in killing by secreting cytokines and inflammatory mediators. Insect haemocytes are structurally and functionally similar to neutrophils (Kavanagh and Reeves, 2004), with the production of reactive oxygen species (ROS) in both cell types, phagocytosis being lectin mediated and the production of antimicrobial peptides, also. Degranulation is also a major aspect of the neutrophil's killing ability (Jog *et al*., 2011), which is further investigated in Chapter 6 where the haemocyte's ability to degranulate is analysed. Comparative analysis of the neutrophil and haemocyte would aid in the validation of *G. mellonella* larvae as model organisms. Utilisation of insects as models and an analysis of their response to pathogens can therefore be indicative of mammalian responses.

PMA (Phorbol 12-Myristate 13-Acetate) and fMLP (N-formyl-methionine-leucine-phenylalanine) are commonly used in neutrophil activation studies as chemotactic activators and stimulators of the NADPH oxidase cascade, (Grogan *et al.,* 1997; Sengel *et al*., 1993). PMA is the most commonly used phorbol ester; it binds and activates Protein Kinase C (PKC) (Lee *et al*., 2002). Nauseef *et al.,* (1991) demonstrated the importance of the activation of PKC in activation of the oxidase components and therefore, PMA can be used as an artificial stimulant when studying the immune cell. fMLP is also commonly used, in the activation of neutrophils *in vitro* (Bennas *et al*., 1994; García-García *et al*., 2009). PMA has been demonstrated to activate neutrophils in a receptor independent manner, PMA induces PKC translocation to the neutrophil membrane and the activation of the reactive oxygen burst. In contrast to this fMLP has been shown to activate the neutrophil through a receptor dependent manner by binding heptahelical membrane receptors, known as G-protein coupled receptors (Zu *et al.,* 1996). However, the literature referenced in this Chapter supports the idea that both fMLP and PMA are likely to regulate universal downstream pathways (Cockraft and Stutschfield, 1989; Bokock and Gilman, 1984).

Previously in our laboratory PMA has been used as the activator in neutrophil and haemocyte studies (Fallon *et al*., 2011; Bergin *et al*., 2005; Renwick *et al*., 2007). Here, it was decided to investigate the effect of PMA and fMLP on the haemocyte and to examine which stimulator would be the more efficient in future studies. The survival of *G. mellonella* larvae when inoculated with PMA and fMLP confirmed that the compounds were not toxic to the larvae, as there was no sign of melanisation or deterioration in the larvae. Increased numbers of haemocytes in the larvae have been found to correlate with increased survival and this was the result in this study with PMA and fMLP. These findings suggest that the larvae are capable of increasing the number of circulating haemocytes upon immune challenge in order to fight off infection. *In vitro* investigation using artificial stimulants should be analogous to the cell being challenged with an opsonised pathogen, therefore these findings further validate PMA and fMLP use. This increase in haemocytes has been suggested to be due to either haemocyte proliferation or the release of haemocytes bound to internal organs (Mowlds *et al*., 2010; Ling *et al.,* 2005).

Subsequent to these findings, it was hoped to determine if any change in protein expression involved in the immune response would occur after PMA and fMLP stimulation. Alterations in protein expression can occur in *G. mellonella* larvae upon activation of a protective immune response (Wiesner *et al*., 1996). Haemocytes stimulated with PMA and fMLP were assessed for differing alterations in protein expression. Bands that displayed alterations in expression, when compared to control haemocytes, were excised and identified using LC-mass spectrometry. Cells stimulated with PMA demonstrated an increase in expression of peptides at the 60kDa, 55kDa, 46kDa, 30kDa, 25kDa and 20kDa molecular masses. An upregulation of these was also observed in fMLP treated cells, with the exception of the 20kDa band, however this upregulation was not to the same extent as seen in the PMA treated cells as visualised in the one-dimensional profiles. This difference indicates that PMA and fMLP may be acting on different pathways in the haemocyte, similar to what has been seen in the neutrophil, where the initial activation of the neutrophil is different in that PMA activates the cell in a receptor independent manner and fMLP activates in a receptor dependent manner where it binds to heptha-helical receptors resulting in the activation of MAPKinases (Zu *et al*., 1998).

Mass spectrometry revealed that prophenoloxidase was identified as band number 1 in Figure 4.4. This is essential for the phenoloxidase cascade, which culminates in melanisation and destruction of foreign particles (Strand, 2008). The activation of this cascade has previously been demonstrated to be similar to the complement system in humans (Rus *et al.,* 2005). Transferrin precursor (band number 3) was also detected and aids in sequesterisation of iron (Bergin *et al.*, 2006). Arylphorin (band number 4) has been proposed to play a role in the immune response in the haemolymph when larvae are immune challenged, it is also taken up into the haemocyte to act as an amino acid rich storage protein (Freitak *et al.*, 2007).

In light of these proteomic findings it was decided to study the phosphorylation activity in the haemocyte upon PMA and fMLP stimulation. In both the neutrophil and haemocyte, cellular activation relies heavily on protein phosphorylation (Xu *et al.* , 2009; Gillespie *et al.,* 1997). ProQ diamond phosphoprotein stain was employed to identify changes in the haemocyte upon stimulation with PMA and fMLP compared to control haemocytes. A protein that becomes more heavily phosphorylated upon immune activation may play an important role in regulation of the immune respone of *G. mellonella* larvae.

The phosphorylation of a band at the 70kDa molecular weight was observed in the PMA and fMLP activated haemocyte samples. As the intensity of the band increased upon stimulation with both PMA and fMLP it may be assumed that the stimulants result in a greater degree of phosphorylation. (However it is possible the increase in intensity of the band may also be due to an increased level of protein expression rather than actual phosphorylation events.) Perhaps advanced quantitative phospho-analysis would solve this issue (Gruhler *et al.,* 2005). Previous studies with neutrophils have identified phosphorylation of a protein at the 70kDa mark upon stimulation with fMLP; this was identified as L-plastin, which functions in the regulation of cell movement (Paclet *et al*., 2004).

As PMA is a well-known activator of PKC (Volz, 1993), phosphorylation events in the PMA treated lysates were expected to take place, several phosphorylated bands were observed in Figure 4.5. The 70kDa band appeared in this sample also, as did a band at approximately the 46kDa mark. Neutrophil phosphorylation studies have found heavy phosphorylation of a similar weight protein upon PMA stimulation (Andrews and Babior, 1983). There was also phosphorylation occurring in the lower range (60-25kDa), however these were not

as evident. As activation of the NADPH oxidase cascade results from a series of phosphorylation events (Matute *et al*. , 2005) one can assume that these findings in the haemocyte one dimensional SDS-PAGE profiles indicate that similar processes are occurring in the haemocyte upon challenge with these chemotactic stimulators.

It was then decided to study the presence of peptides that have been identified in haemocytes to be involved in regulation of the cellular and humoral response. The antibody used in this study detects the substrates of the AGC kinase superfamily whose members are PKC and PKA (Cytrynska *et al.,* 2007). In haemocyte lysates, five potential substrates were detected at the 80, 70, 50, 30 and 25 kDa mark by the antibody. The up-regulation in phosphorylation of these peptides was similar to what Cytrynska *et al.,* (2007) found upon immune challenging haemocytes from larvae of *G. mellonella* with LPS and pathogens. Comparable phosphorylation events have been found in PMA stimulated neutrophils (Ohira *et al*., 2003). The $p47^{phox}$ protein is heavily phosphorylated in the neutrophil upon PMA stimulation; this may be the identity of the band appearing in the 45-50kDa ranges in the PMA stimulated haemocyte lysate. No reactivity was visualised in the fMLP and control haemocyte lysate samples, which makes it difficult to determine whether the phosphorylation is specific to PMA stimulation or if it is constant in the cell. As phosphorylation events are due to the combination of kinases (PKC/PKA) and phosphatases, which dephosphorylate proteins (Bradford and Soltoff, 1998), the examination of the role of phosphatases in the haemocyte may uncover further similarities between the haemocyte and the neutrophil at the protein modifications level.

In order to study the proteomics of the cell in more depth, and to identify the cellular components, quantitive 2 dimensional electrophoresis paired with Progenesis SameSpot software® was performed. Some of the proteins identified have been implicated extensively in the literature to be involved in the cellular immune response of *G. mellonella* larvae. Phenoloxidase was indentified as spot number 1 in Figure 4.8; it is the last component of the prophenoloxidase activation system and is involved in the cross-linking and melanisation of proteins (Sideri *et al.*, 2007). Spot number 2 displayed homology to transferrin precursor; expression of this spot increased 2.8 fold upon PMA stimulation. Yang *et al*., (2009) revealed the release of transferrin precursor upon degranulation from the neutrophil, arlyphorin (spot number 3) is also able to bind to molecules such as LPS and thus, is

involved in the immune response (Freitak *et al.,* 2007). Several transcriptional regulators were also identified such as GF21232 (PMA and fMLP stimulation increased this 2.1 fold), TetR-family transcriptional regulator (increased 2.3 fold with PMA and decreased 2 fold with fMLP) and small ribosomal protein-S4 (displayed no increase/decrease). Spot number 4 was identified as the subunit 2 of prophenoloxidase, which is involved in the melanisation process (Strand, 2008). Putative NADP-dependent malic enzyme was identified as spot number 5, this increased 3 fold in both treatments, indicating that both fMLP and PMA stimulation have the same up regulating effect on this enzyme. This enzyme has been demonstrated to be involved in the inflammatory response in humans and is dependent on the reduction of NAD+ to NADP+ (Garcia *et al.,* 2007). Putative pyridoxamine kinase was identified as spot number 8, it displayed no change upon stimulation with either PMA/fMLP and it is an enzyme that catalyses the phosphorylation of vitamin B6. This enzyme has been identified and characterised in the insect *B. mori* (Huang *et al*., 2011). Interestingly alpha-actin increased in expression upon PMA stimulation (+1.1) but decreased in expression when the cells were stimulated with fMLP. This may be due to the rapid change in shape of the cells when stimulated with differing chemotactic stimulators, and the difference in kinetic activity of PMA and fMLP (Klink and Sulowska, 2007).

The expression of ATP synthase was decreased 1.5 fold in both PMA and fMLP stimulated cells but the precise reason for this is unclear. However, there is evidence in the literature that arachidonic acid (AA), a second messenger molecule produced from activated cell membranes in the neutrophil, may negatively affect some of the ATP synthases (Klink and Sulowska, 2007). This may be what is occurring in the haemocyte also. A putative heat shock protein (hsp 21.4) was identified as spot number 18 and there were no changes in expression upon stimulation, however Wojda and Jakubowicz (2007) postulated that hsps may be involved in the immune response of *G. mellonella* larvae, hence the positive identification of this protein in the immune cell. They showed that hsp90 is involved in *G. mellonella* larvae's humoral immune response and that there may be a relationship between MAP ERK kinases and heat shock proteins. Hickman and Hilderbarnd (2004) also reported involvement of heat shock proteins in the immune response of *G. mellonella* larvae. The identification of hsp 21.4 in the haemocyte indicates that it has a connection in *G. mellonella's* immune response also.

Another immuno-relevant putative peptide identified was serine protease (spots 19 and 20 displayed homology with this in Figure 4.8). Serine protease increased in expression with PMA 1.6 fold but expression fell with fMLP stimulation by 1.4 fold, when compared to the control haemocytes. Serine protease homologs (SPHs) have previously been identified as components of the prophenoloxidase cascade system in insects also (Strand, 2008). Serine protease has also been implicated in the neutrophil's immune response (Meyer-Hoffert and Wiedow, 2011). Transthyretin precursor was increased 4.4 fold in the PMA stimulated haemocytes and 4.8 fold in the fMLP stimulated haemocytes, it has been shown to be associated with apolipoprotein A (which bears homologies to Apolipophorin 3 in insects), there is also evidence that human transthyretin functions in capturing toxic compounds (Ingenbleek and Young, 1994).

To analyse phosphorylation events in the haemocytes, gels were stained with ProQ diamond phospho stain and proteins that appeared to be phosphorylated were cross-referenced with the PMA reference gel to identify them. Arylphorin (Figure 4.9) became highly phosphorylated upon ProQ diamond staining. The nucleoproteins TetR-family transcriptional regulator and small ribosomal protein s4 also appeared to become phosphorylated upon PMA stimulation (Spots 9 and 10, respectively).

The phosphorylation of alpha-actin (Spot number 11) correlates with the stimulation of the cell by PMA which causes major actin reassembly (Grogan *et al*., 1997; Fallon *et al*., 2011) in order to allow processes such as phagocytosis and degranulation to occur. DNA ligase was identified as Spot number 13 and it appeared to undergo phosphorylation when the cells were stimulated with PMA. In mammalian cells, DNA encounters continuous attack as a result of the generation of ROS, this can induce stress on the DNA and DNA ligase is therefore essential. Wang *et al*., (2004) demonstrated that the phosphorylation of DNA ligase is essential for it's stability. As ROS production in insects has been documented to be an essential feature of the immune response (Gillespie *et al*., 1997), DNA ligase may also aid in the repair of the DNA damage in the haemocyte. Heat shock proteins have been demonstrated in the neutrophil to undergo phosphorylation on serine residues (Jog *et al*., 2007). Spot number 18 displayed homology with HSP21, and phosphorylation of the peptide appears to bear similarities to the phsophorylation of HSP27 in the neutrophil, which is required for actin

reorganisation and polymerisation (Howard and Oresajo, 1985). The visualisation of spot number 21 upon ProQ diamond staining indicates it undergoes phosphorylation upon PMA stimulation of the haemocyte. This protein was identified as transthyretin precursor and was shown to be up-regulated 4.4 fold when the haemocytes were stimulated. The phosphorylation of transthyretin is most likely due to the fact that it enhances ROS production in the neutrophil (Mezosi *et al.,* 2005).

The proteomic findings indicate that the identified putative proteins all collectively play a role in the haemocytes ability to activate the immune response and kill microorganisms; the phosphorylation caused by stimulation with PMA indicates that PMA activates post-translational modifications, which act in the immune response. No phosphorylation was identified when the cells were unstimulated or fMLP stimulated. In light of this study, the fMLP-induced cascade of events in the haemocyte may not activate the haemocyte to the extent that PMA stimulation does and as a result of this it was decided to carry out subsequent comparative experiments using PMA stimulation.

As a consequence of these findings, it was decided to investigate the effect of PMA on the translocation of the $p47^{phox}$ from the cytosol to the membrane in the haemocyte and also in the neutrophil. The reason for this is that the NADPH cascade is an integral component of the immune response in the neutrophil, allowing for degranulation of proteolytic enzymes (and phagocytosis) to occur, ultimately resulting in destruction of an opsonised target (Bokoch, 2009). The respiratory burst of ROS is dependent upon the activation of NADPH oxidase, which is in turn dependent on the translocation of a number of proteins from the cytoplasm to the membrane, where NADPH oxidase resides (Grogan *et al.*, 1997; Bokoch, 2009). The protein $p47^{phox}$ is one of these proteins and is a component of the NADPH oxidase, it's importance in neutrophil function is demonstrated by the fact that it's absence or dysfunction can lead to a potentially fatal condition known as chronic granulomatous disease (CGD) (Thrasher *et al.,* 1994). The use of Western blotting and confocal immunoflouresence microscopy in this section revealed similarities, when both the neutrophil and the haemocyte were probed with Anti-Human $p47^{phox}$ antibodies. The translocation of the protein from the cytosol to the membrane was evident in both cell types, indicating that the translocation of homologous proteins occurs in the neutrophil and haemocyte in a similar manner.

Recently proteomic technology has improved exponentially with the rise in

use of shotgun proteomics and MUDPIT technology. It has been used to characterise the proteomes of *D. melanogaser* and *B. mori* (Shimomura *et al*., 2004; Brunner *et al.*, 2007). Essentially, shotgun proteomics allows the identification of complex protein mixtures in a simple experiment (Wu and MacCross, 2002) and in most cases eliminating the use of gel electrophoresis, which unfortunately can limit proteomics with the loss of small molecular weight peptides and the lack of consistency with staining protocols (Rabilloud *et al.,* 2009). The results of this study demonstrated the wide range of opportunities that shotgun proteomics allows scientists. Here it was decidied to employ this tactic and to further characterise the haemocyte's proteome, again aiming to further emphasise similarities with the neutrophil. With a 5µL sample loading onto a 6340 Ion Trap LC/MS spectrophotometer, it was possible to identify 32 putative peptides with mascot scores > 67, indicating homology. These identities included proteins that are implicated in the literature to be involved in *G. mellonella's* immune response, such as apolipophorin 3 (Altincicek *et al.*, 2007), arylphorin (Freitak *et al.*, 2007), hexamerin (Telfer and Kunkel, 1991) and there was also an abundance of actin. There are limitations with the *G. mellonella* larval proteome, in that there is no full sequence available on protein databases. Until a sequence is publically available, comparative biology is restricted. This study did identify a novel approach to studying the immune cell of the larvae of *G. mellonella*, nonetheless.

4.9 Conclusion

This results presented in this Chapter have increased our understanding of the similarities that exist between the haemocyte of *G. mellonella* larvae and the human neutrophil. Post-translational modifications and characterisations of the haemocyte have been studied, utilising a number of proteomic techniques, with the identification of immuno-relevant proteins in the haemocyte that become increased in expression upon immune stimulation with the chemotactic activators, PMA and fMLP, which are routinely used in *in vitro* experiments with the neutrophil.

The results presented here indicate PMA to be the more active of these compounds. Analysis of the components of the NADPH oxidase enzyme showed similar translocation events in both the neutrophil and the haemocyte, with kinetics

that appear to be similar.

Utilisation of shotgun proteomics provided a novel means of identifying haemocyte proteins. It was demonstrated that complex protein matrices could be studied and quantified. Optimisation of the experimental protocol and the release of a full sequence would allow further insights into the haemocyte proteome.

Chapter 5

Examination of the architecture of the cytoskeleton of the haemocyte and the sub-fractionation of the haemocyte population

5.0 Examination of the architecture of the cytoskeleton of the haemocyte and the sub-fractionation of the haemocyte population

The haemocyte is the immune cell of *G. mellonella* larvae and it is the cellular component of the immune response, which is comprised of a complex relationship between humoral and cellular components (Strand, 2008). The haemocyte's cytoskeleton plays a key role in processes such as degranulation, phagocytosis and production of reactive oxygen species (Gillespie and Kanost, 1997). The cytoskeleton maintains cellular shape, the integrity of the cell and it allows the neutrophil and haemocyte to undergo the morphological changes required for the immune cells to kill microorganisms (Xu *et al*., 2009). Despite the increase in use of *G. mellonella* larvae as model organisms, the architecture of the cytoskeleton is poorly understood (Scully and Bidochka, 2006). This Chapter investigates the composition of the cytoskeleton and the presence of the proteins vinculin, coronin, F-actin and G-actin, employing techniques such as confocal immunofluorescence microscopy, flow cytometry and proteomics. The composition of the haemocyte's cytoskeleton is also compared to that of the neutrophil in order to compare the similarities between the haemocyte and the neutrophil.

This Chapter investigates this phenonomen in the haemocyte in order to analyse whether there is a change in F-actin expression with PMA stimulation.

This Chapter examines the effects that the inhibitors cytochalasin b and nocodazole have on the haemocyte and compares these effects to what has been demonstrated to occur in the neutrophil.

Haemocyte functions are well characterised in the literature (Wojda & Jakubowicz, 2007; Fallon *et al*., 2010; Strand, 2008), however detailed analysis of each population subset has yet to be characterised. Recently, flow cytometric analysis has been employed to classify the subsets of haemocyte population from insects such as *B. mori* and *M. sexta* (Nakahara *et al.,* 2009; Nardi *et al*., 2003). The final objective of this Chapter was to utilise flow cytometry techniques to sort the haemocyte populations of *G. mellonella* larvae into distinct sub-populations based on cellular size and granularity and to examine which of these subsets is most similar to the human neutrophil.

5.1 Assessment of the effect of cytochalasin b and nocodazole on larval survival and haemocyte density

Cytochalasin b is a cell permeable mycotoxin that has been used extensively in neutrophil cytoskeletal studies (Sengel *et al.*, 1993; Jog *et al*., 2011) and it has been shown to inferfere with the actin filaments in the neutrophil (Bylund *et al*., 2004). Nocodazole is an antineoplastic agent that negatively affects the cell's microtubules (Vasquez *et al.,* 1999) and it has been demonstrated to diminish the neutrophil's killing ability by inhibiting actin filamentation (Neeli *et al*., 2009).

Cytochalasin b and nocodazole were injected into healthy larvae as described in section 2.5.1 at concentrations of 10μM, 5μM and 1μM to assess whether the agents had a toxic effect on the larvae over a 72 hour period. There was no significant change in larval survival ($p = 1$, Figure 5.1) and therefore it was decided to use the highest concentration of cytochalasin b and nocodazole in subsequent experiments (10μM).

It was also hoped to establish whether there was a change in the haemocyte density in the larvae when 10μM cytochalasin b or 10μM nocodazole or a 10μM cytochalasin b/10μM nocodazole combination were injected and haemocyte density was calculated 4 hours after initial inoculation (Figure 5.2). There was no significant difference in haemocyte densities between treatments. The results show an average haemocyte density of $1.43 \pm 0.1 \times 10^7$ per 100 μL in larvae injected with PBS, $1.1 \pm 0.2 \times 10^7$ per 100 μL haemocytes present in larvae injected with 0.001% (v/v) DMSO, $1.86 \pm 0.13 \times 10^7$ per 100 μL in larvae injected with 10μM cytochalasin b ($p = 1$), $1.23 \pm 0.16 \times 10^7$ per 100 μL in larvae injected with 10μM nocodazole ($p = 1$) and $1.33 \pm 0.33 \times 10^7$ per 100 μL in larvae injected with a 10μM cytochalasin b/10μM nocodazole combination ($p = 1$). Alterations in haemocyte densities can be indicative of an immune response (Mowlds *et al*., 2010), however as there was not a significant change in haemocyte density with the injection of the compounds, it is evident that there is not an immune response to the inhibitors.

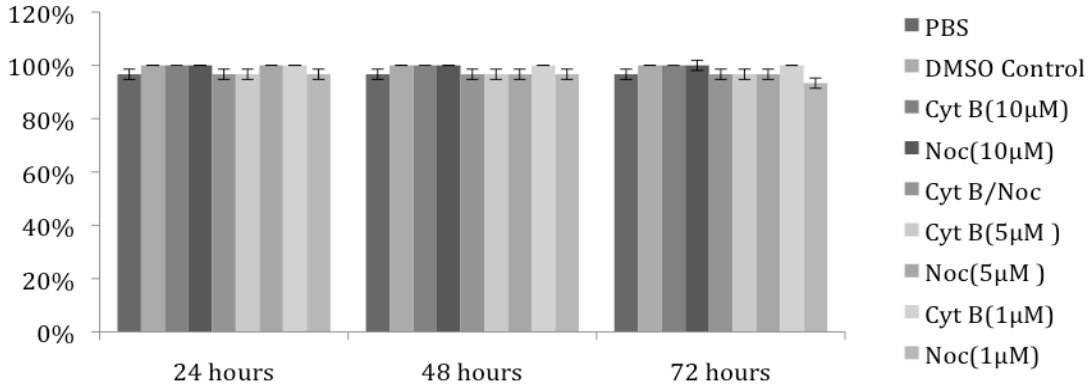


Figure 5.1 Survival of larvae injected with 10µM, 5µM and 1µM cytochalasin b and nocodazole, a combination of 10µM cytochalasin b and 10µM nocodazole, 0.001% (v/v) DMSO and PBS (No significant difference) at 24 hours, 48 hours and 72 hours (n=3).

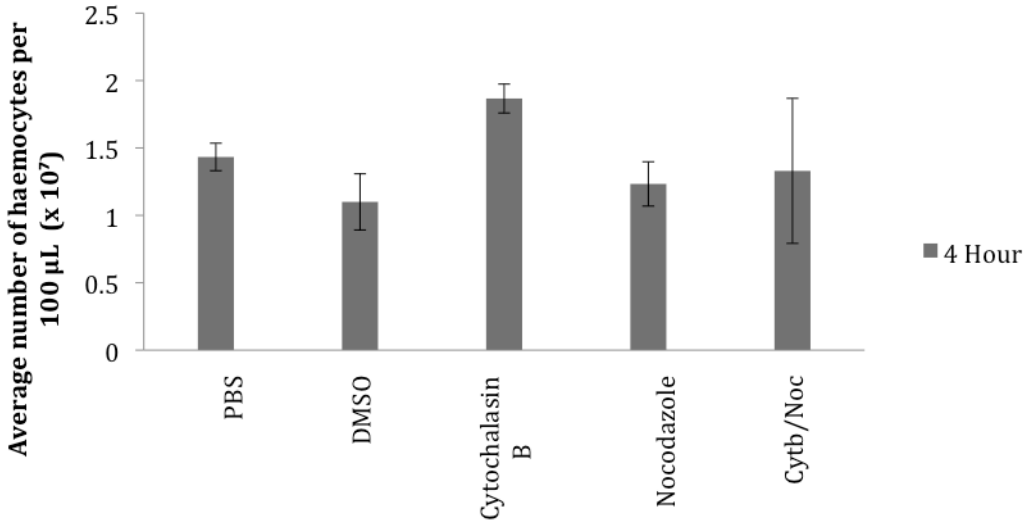


Figure 5.2 Haemocyte density in larvae injected with 10µM cytochalasin b, 10µM nocodazole, 0.001% (v/v) DMSO, PBS and a combination of 10µM cytochalasin b and 10µM nocodazole (No significant difference) at 4 hours (n=3).

5.2 Analysis of the effect of 10µM cytochalasin b and 10µM nocodazole on larval survival when injected with 1 x 10^6 *C. albicans* cells/20 µL

Four hours after injection with cytochalasin b or nocodazole (section 2.5.2), the larvae were injected with 1 x 10^6 /20 µL *C. albicans* cells. Survival of larvae was assessed at 30 minutes, 1 hour, 3 hours and 24 hours and significances are displayed against the 0.001% (v/v) DMSO control (Figure 5.3).

At three hours post inoculation PBS control larvae survived at 60 ± 7%, DMSO control larvae survived at 36 ± 11%, the survival of cytochalasin b treated larvae was 20 ± 0.01% ($P< 0.05$), nocodazole treated larvae survived at 10 ± 2% and cytochalasin b/nocodazole treated larvae survived at 6.7 ± 0.01%.

Larval survival was also assessed 24 hours post inoculation where the PBS control larvae survived at 40 ± 7%, DMSO control larvae survived at 26 ± 11%, the survival of cytochalasin b treated larvae was 6.7 ± %, nocodazole treated larvae survived at 10 ± 2% and cytochalasin b/nocodazole treated larvae survived at 3.33 ± 2%.

A parallel assay was performed 24 hours post injection with cytochalasin b and nocodazole where the larvae were injected with *C. albicans*.

Assessement of larvae three hours after inoculation demonstrated cytochalasin b treated larvae survival rates at 87 ± 2.3%, the larvae treated with nocodazole survival was 87 ± 1.9%, the cytochalasin b/nocodazole larvae survival was 83 ± 0.01 ($p<0.05$). Twenty-four hours following inoculation survival of larvae was also assessed, cytochalasin b larvae survival was 37 ± 9%, nocodazole treated larvae survival was 17 ± 9% ($p<0.05$, relative to control larvae). The survival of cytochalasin b/nocodazole treated larvae was 40 ± 9% (Figure 5.4).

5.3 Analysis of the effect of cytochalasin b and nocodazole on the ability of the haemocyte to phagocytose an opsonised target

To assess what effect cytochalasin b and nocodazole had on the haemocyte's ability to phagocytose a target, an assay using *A. flavus* conidia was performed as described in section 2.5.3. Cytochalasin b and nocodazole have both been demonstrated to inhibit phagocytosis in the neutrophil (Peng *et al*., 2010; Silver and Harrison, 2011). Conidia and haemocytes were incubated together and phagocytosis was determined following 30 and 90 minutes (Figure 5.5). Thirty minutes following incubation, the % of PBS-treated haemocytes containing conidia was 33.33 ± 2%, of the DMSO treated haemocytes 27 ± 1.2% contained conidia, 12 ± 2% cytochalasin b treated haemocytes contained conidia, 17.3 ± 2% nocodazole treated haemocytes contained conidia with 22.3 ± 1.8% cytochalasin b/nocodazole treated haemocytes containing conidia.

Phagocytosis was also assessed after 90 minutes and 55 ± 4% PBS and 61.3 ± 1.1% DMSO controls contained conidia, 21.3 ± 1.3% of cytochalasin b and 21 ± 2 of nocodazole treated haemocytes contained condia ($p<0.05$), 31.7 ± 3.3% of cytochalasin b/nocodazole treated haemocytes contained conidia ($p<0.05$).
These results indicate that pretreatment of the haemocytes with cytochalasin b or nocodazole negatively affected the cells ability to phagocytose an opsonised target.

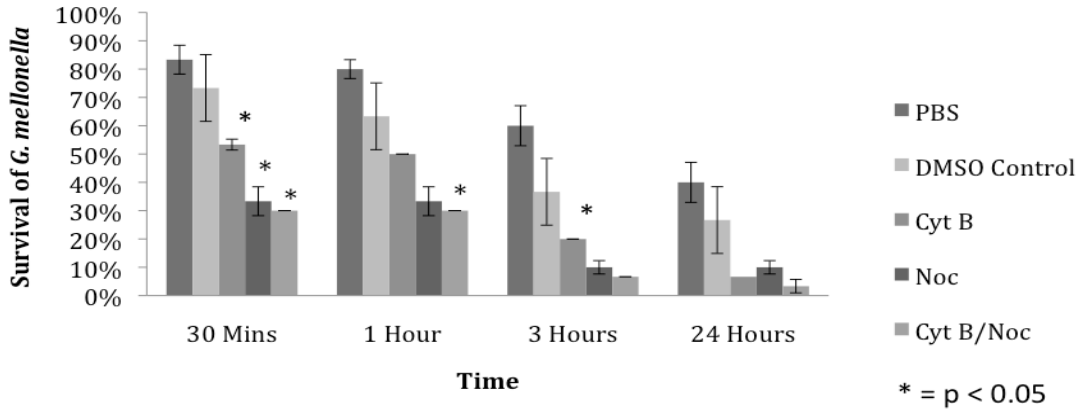


Figure 5.3 Survival of *G. mellonella* larvae injected with cytochalasin b and nocodozole and inoculated with 1 x 10^6 *C. albicans* cells/20 µL 4 hours later (n=3).

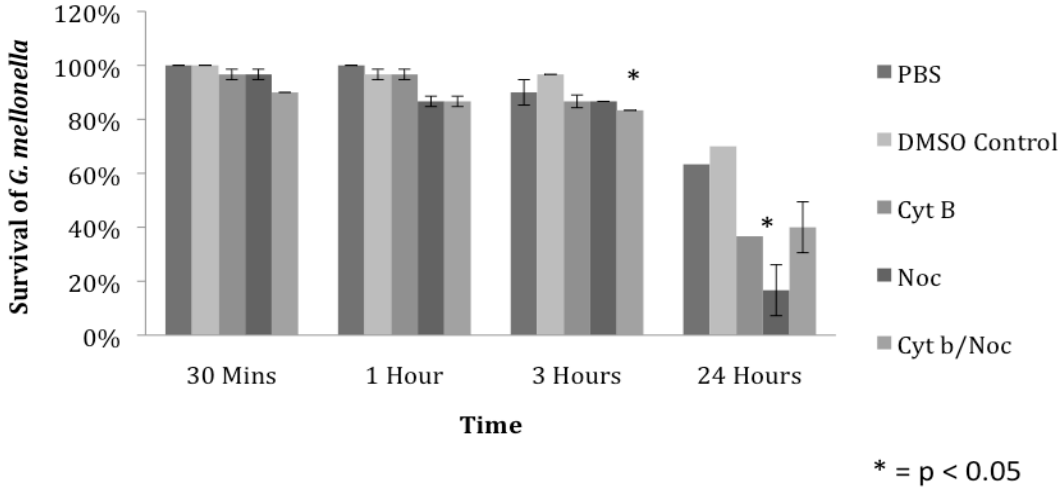


Figure 5.4 Survival of *G. mellonella* larvae injected with cytochalasin b and nocodozole and inoculated with 1 x 10^6 *C. albicans* cells/20 µL 24 hours later (n=3).

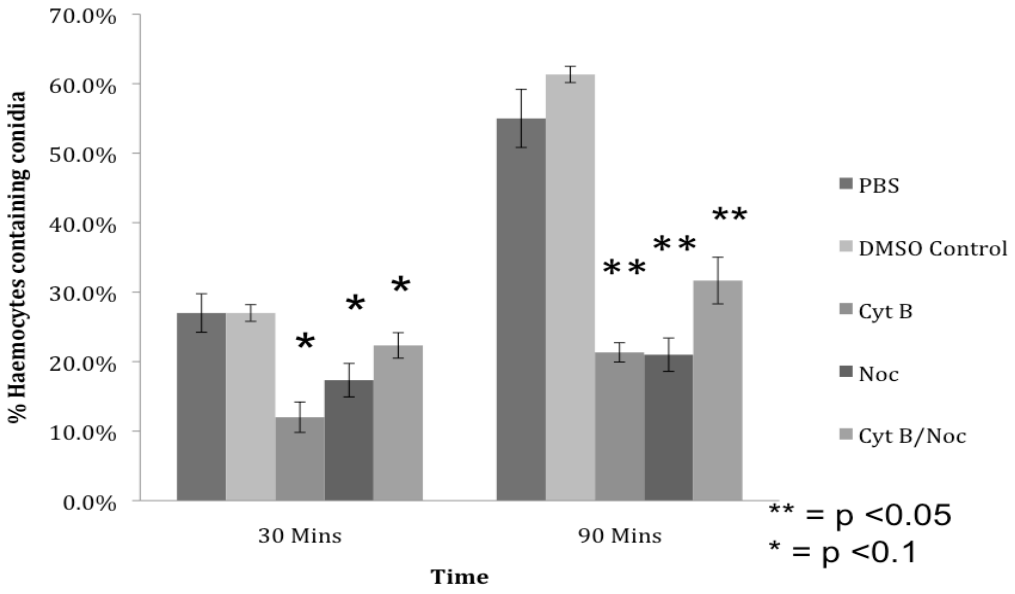


Figure 5.5 Haemocytes (%) that have internalised *A. flavus* conidia which were treated with cytochalasin b and nocodozole containing conidia (n=3).

5.3.1 Summary

The results in this section demonstrate that concentrations of 10µM, 5µM and 1µM of cytochalasin b and nocodazole are not potent when injected into healthy *G. mellonella* larvae at 24, 48 or 72 hours. A 10µM cytochalasin b/10µM nocodazole combination was also not toxic to the larvae. There was no significant difference in haemocyte densities between treatments and controls. However, when larvae were injected with 10µM cytochalasin b, 10µM nocodazole or a 10µM cytochalasin b/10µM nocodazole combination they became more susceptible to infection with *C. albicans.* Cytochalasin b and nocodazole were also demonstrated to negatively affect the haemocytes ability to phagocytose an opsonised target.

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5.4 Analysis of the survival of *C. albicans* when incubated with haemocytes pre-treated with the inhibitors cytochalasin b, nocodazole, Superoxide Dismutase (SOD), and (Diphenyleneiodonium Chloride) DPI

This section examined the effect of pre-treating haemocytes of *G. mellonella* larvae with cytochalasin b, nocodazole, SOD, DPI, or combinations of these, on the ability of the haemocyte to kill an opsonised target, *C. albicans*, as described in section 2.9.5. DPI and SOD were used in this section as DPI has been shown to make neutrophils defective in their ability to kill a target. DPI is a redox inhibitor (Decleva *et al*., 2006) and SOD is a scavenger of superoxide, which is a product of the NADPH oxidase cascade in the neutrophil and haemocyte (Bergin *et al*., 2006).

Pre-treatment of the haemocytes is decribed in section 2.5.3. The survival of *C. albicans* cells 20 minutes following incubation with control haemocytes was 51 ± 4%, after 60 minutes survival of *C. albicans* was 15 ± 7.5% and after 80 minutes survival of *C. albicans* was 15 ± 11%.

The survival of *C. albicans* cells incubated with cytochalasin b pre-treated haemocytes was 100% at 20 minutes ($p<0.05$), 92.33 ± 7% at 40 minutes ($p<0.05$), 70.6 ± 25 following 60 minutes ($p<0.05$) and 50.6 ± 9% after 80 minutes ($p<0.05$).

Haemocytes pre-treated with nocodazole demonstrated survival of *C. albicans* 80.4 ± 29% at 40 minutes and 52.4 ± 13.8% ($p<0.05$) at 80 minutes. Haemocytes were also pre-treated with a combination of cytochalasin b and nocodazole prior to incubation with *C. albicans.* Survival of the yeast cells at 20 minutes was 100 ± 14% ($p<0.05$), 40 minutes following incubation survival was 92.66 ±19% ($p<0.05$) and 80 minutes following incubation survival was 100 ± 11% ($p<0.05$). These results can be seen in Figure 5.6.

SOD and DPI also reduced the ability of the haemocytes to kill the yeast cells but not to the extent as cytochalasin b and nocodazole pre-treatment. The survival of *C. albicans* when incubated with SOD pre-treated haemocytes was 41.3 ± 12% at 60 minutes ($p<0.05$) and 38 ± 9% at 80 minutes (Figure 5.7).

When incubated with haemocytes that had been pre-treated with DPI, the survival of *C. albicans* was 42 ± 10.5% ($p<0.05$) at 60 minutes and 40 ± 13% at 80 minutes. (Figure 5.7)

Haemocytes treated with the SOD and DPI combination demonstrated *C. albicans* survival rates of 79 ± 3% ($p<0.05$) at 20 minutes, 81.9 ± 6% at 60 minutes

($p<0.05$) and a rate of 64.5 ± 7% at 80 minutes ($p<0.05$). When SOD and nocodazole were combined and haemocytes were pre-treated, survival of *C. albicans* was 71.5 ± 2.5% at 60 minutes ($p<0.05$) and 65.35 ± 1.7% at 80 minutes ($p<0.05$). A SOD and cytochalasin b combination demonstrated *C. albicans* survival rates of 60.7 ± 19.7% at 60 minutes ($p<0.05$) and 71.6 ± 18% at 80 minutes ($p<0.05$). The combination of cytochalasin b and DPI produced *C. albicans* survival rates of 81.8 ± 11% ($p<0.05$) at 20 minutes, 66.15 ± 16.7% at 60 minutes ($p<0.05$) and 77.08 ± 21% at 80 minutes ($p<0.05$). Nocodazole and DPI were also combined; this combination did not significantly alter survival rates when compared to the control with survival at 13.22 ± 3.2% at 60 minutes and 15.66 ± 7.53% at 80 minutes. These data are presented in Figure 5.8 (A) and (B).

To ensure that the viability of the haemocyte was not compromised over the 80 minute time period a viability assay was performed in the stirred chamber as described in section 2.9.3. There was not a significant change in viability of the haemocytes over the 80 minutes ($p=1$). After 40 minutes haemocyte viability was 83.8 ± 7%, 60 minutes demonstrated haemocyte viability of 73 ± 3.4% and after 80 minutes haemocyte viability was 81.4 ± 8.1% (Figure 5.9).

As cytochalasin b and nocodazole are used extensively in these studies, haemocyte viability was also assessed before and after treatment with cytochalasin b and nocodazole (Figure 5.10). Following 30 minutes incubation with 10μM cytochalasin b haemocyte viability was 75.75 ± 4.7%, while 30 minutes incubation with 10μM nocodazole demonstrated a haemocyte viability of 82.92 ± 7.3% ($p=1$, with regard to viability at time 0).

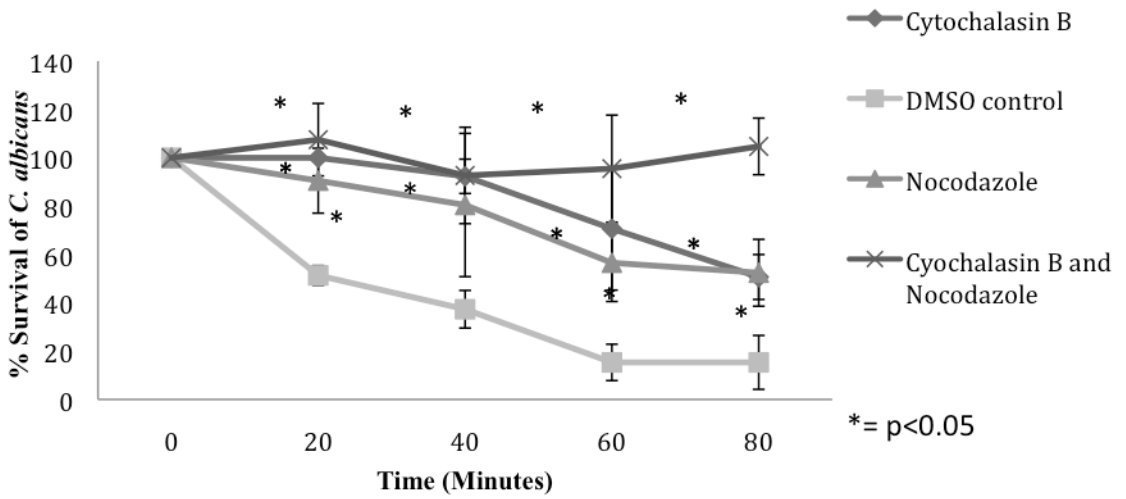


Figure 5.6 Survival (%) of *C. albicans* when incubated with haemocytes pre-treated with cytochalasin b, nocodazole and cytochalasin b/nocodazole combination (n=3).

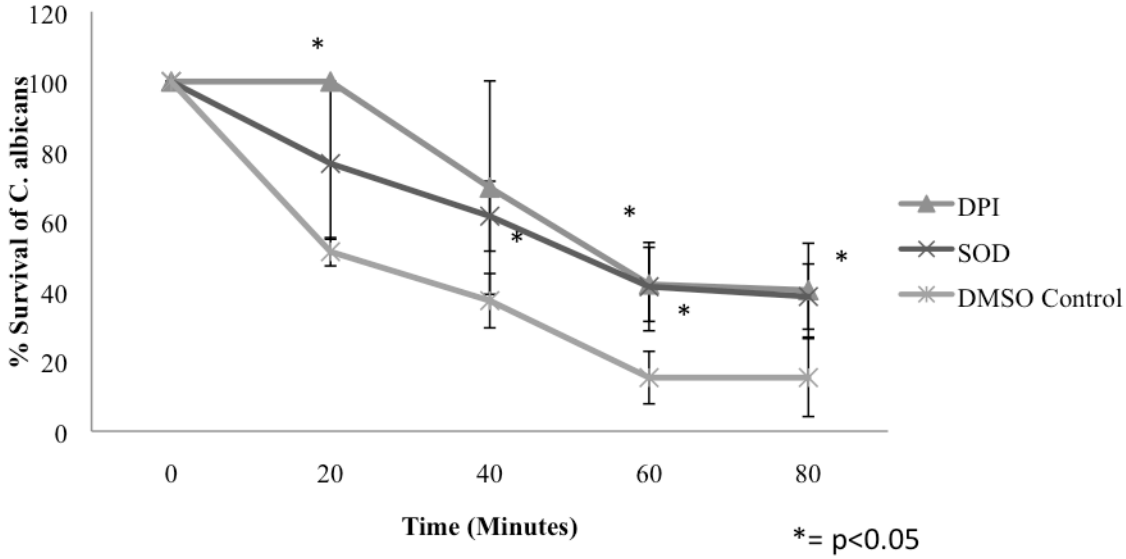
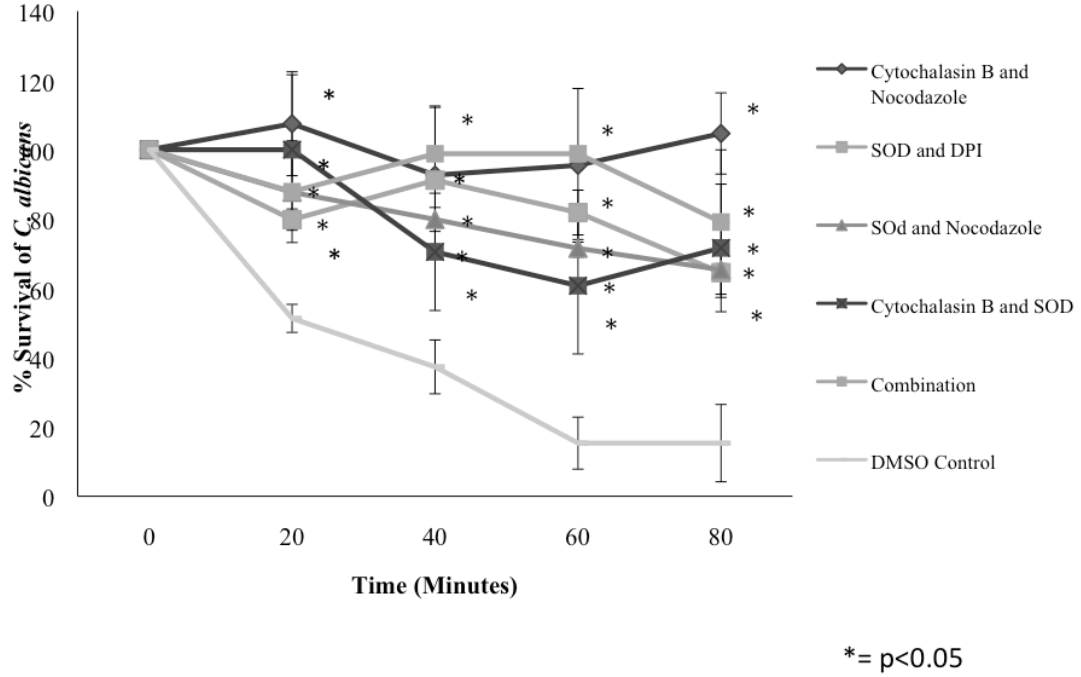
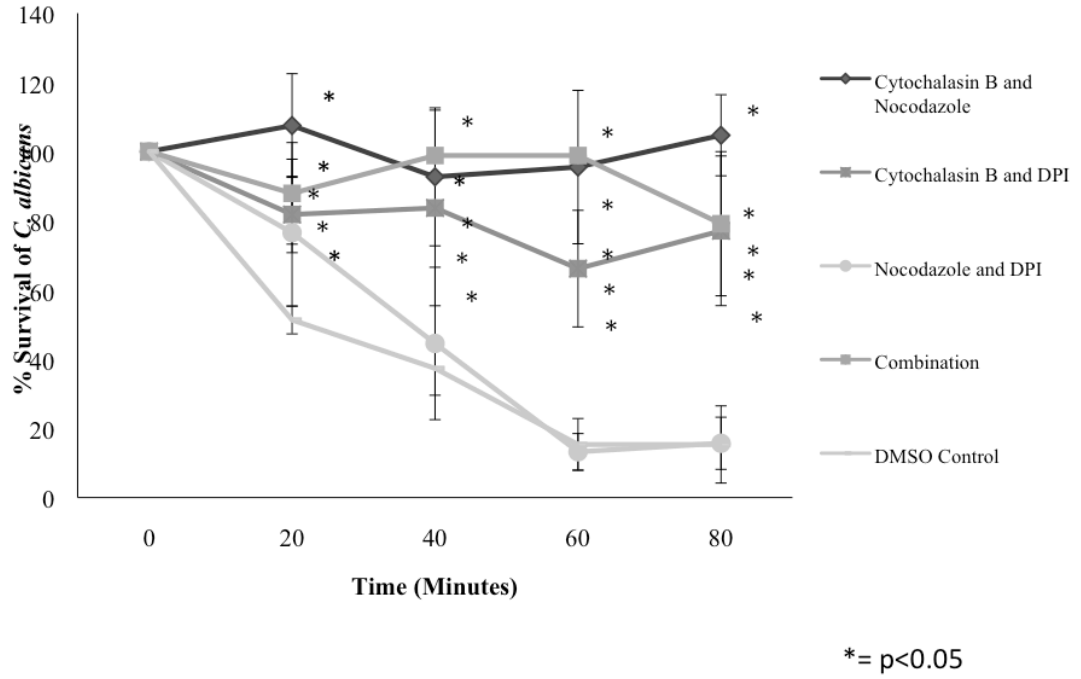


Figure 5.7 Survival (%) of *C. albicans* when incubated with haemocytes pre-treated with SOD and DPI (n=3).



(A)



(B)

Figure 5.8 Survival (%) of *C. albicans* when incubated with haemocytes pre-treated with combinations of all agents involved in survival assays (n=3).

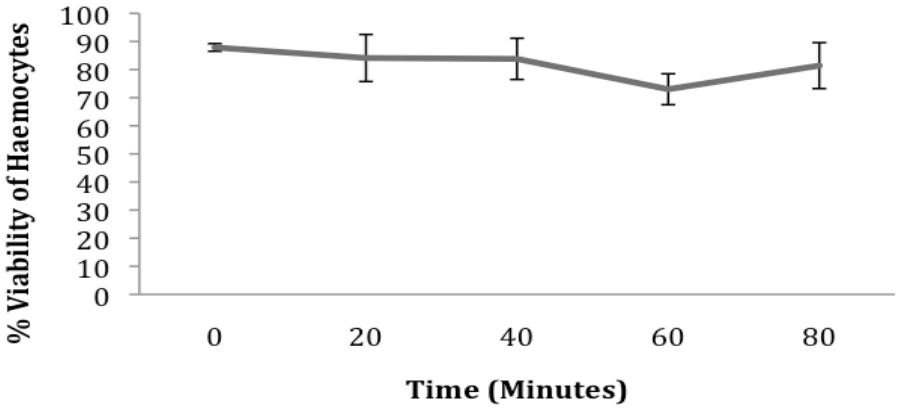


Figure 5.9 Viability of Haemocytes over 80 minutes in the stirred chamber (p=1, no statistical change in viability) (n=3).

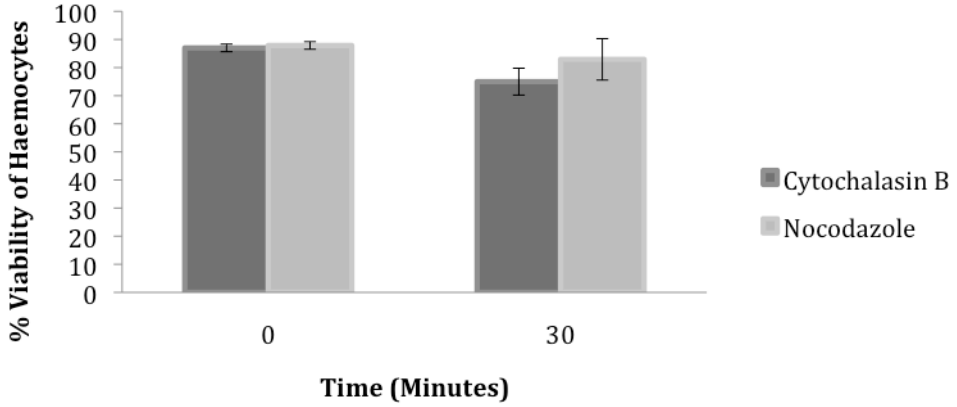


Figure 5.10 Viability of Haemocytes when incubated with cytochalasin b and nocodazole, (p=1, no statistical change in viability) (n=3).

5.4.1 Summary

The data presented in this section indicate that the pre-treatment of haemocytes with cytochalasin b, nocodazole, SOD, DPI and combinations of these agents disrupts the function of the haemocyte but not to the extent that the viability of the haemocyte is compromised. Cytochalasin b (10μM) pre-treatment of haemocytes had a more significant effect on the survival of the *C. albicans* cells, indicating that cytochalasin b renders the haemocyte less able to alter its cytoskeleton to allow phagocytosis and ROS production to occur. The combination of cytochalasin b and nocodazole pre-treatment of the haemocyte had the most significant effect on reduction of haemocyte function. These results indicate that prior exposure of haemocytes to cytochalasin b, nocodazole, SOD, DPI or a combination of these agents significantly reduced the phagocytic and microbiocidal abilities of haemocytes without significantly affecting haemocyte viability.

5.5.1 Examination of the distribution of vinculin in the haemocyte of *G. mellonella* larvae and the effect of cytochalasin b and nocodazole on this protein

In the neutrophil, vinculin acts as a linker molecule for the actin filaments in the cell membrane, it is also associated with the cellular junctions (Peng *et al*., 2010) such as pseudopodia production, which are important for the neutrophil to circulate and attack a target (Entschladen and Zänker, 2000).

Analysis of the presence of cytoskeletal linker protein vinculin in the haemocyte was investigated by utilising confocal immunofluoresence microscopy. The study used Anti-Human antibodies at all times. The intensity values and images of the distribution of vinculin upon PMA stimulation and cytochalasin b and nocodazole treatment were compared to those that were found in the neutrophil, as described in section 2.11.

There is an increase in intensity of vinculin fluorescence in the haemocyte and the neutrophil upon PMA stimulation, when compared to the unstimulated cells (Figure 5.11). The average intensity in the haemocyte increases from 3198.3 ± 138 to 3324 ± 129 upon PMA stimulation, the intensity in the cytochalasin b (+ PMA) treated cells was 2827 ± 172 and intensity in the nocodazole treated cells (+ PMA) was 2627 ± 192 (Figure 5.12). Although vinculin expression decreased when haemocytes were pre-treated with cytochalasin b and nocodazole, there was not a significant difference, p=1 (Figure 5.12).

Vinculin was also analysed in isolated neutrophils, where it was shown that PMA stimulation of neutrophils increased intensity from 3025 ± 43 to 3244 ± 71, cytochalasin b (+ PMA) pretreated cells demonstrated intensities of 3068 ± 73, with nocodazole (+ PMA) pretreated neutrophils demonstrating intensities of 3023 ± 74. Again, there was not a significant difference in intensities, however the average values did decrease when the neutrophils were pretreated with the inhibitors (Figure 5.11). Figure 5.13 details the comparative intensities of the neutrophils and the haemocytes upon treatment. It is likely that the activation of the haemocyte and the neutrophil upon PMA stimulation is causing an increase in the vinculin availability in the cell and throughout the actin filaments. Yuruker and Niggli (1992) demonstrated changes in vinculin re-organisation in the neutrophil upon actin filamentation, which is a result of PMA stimulation.

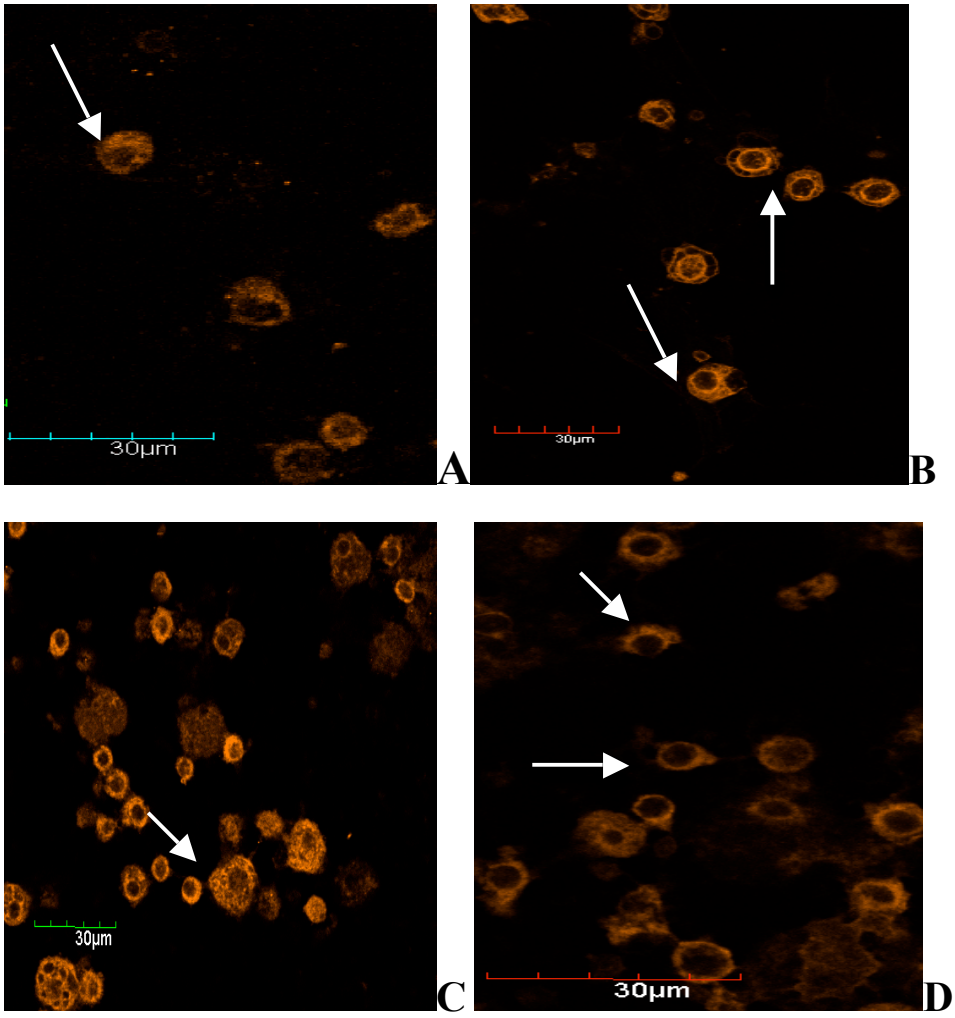


Figure 5.11 Confocal immunofluoresence images of vinculin in unstimulated haemocytes (using Anti human Vinculin antibody) (A), PMA stimulated haemocytes (B), cytochalasin b + PMA treated haemocytes (C) and nocodazole + PMA treated Haemocytes (D) (n=3).

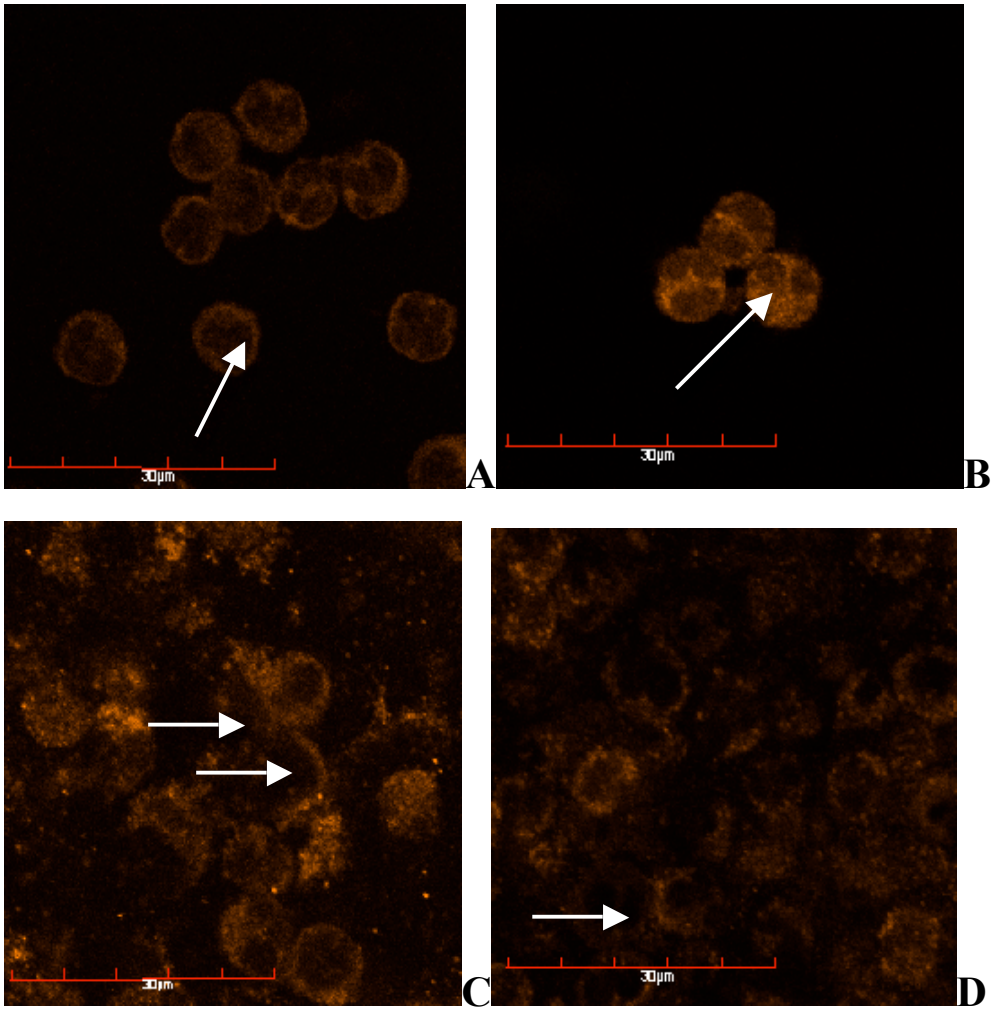


Figure 5.12 Confocal immunofluoresence images of vinculin in unstimulated neutrophils (using Anti human Vinculin antibody) (A), PMA stimulated neutrophils (B), cytochalasin b + PMA treated neutrophils (C) and nocodazole + PMA treated neutrophils (D) (n=3).

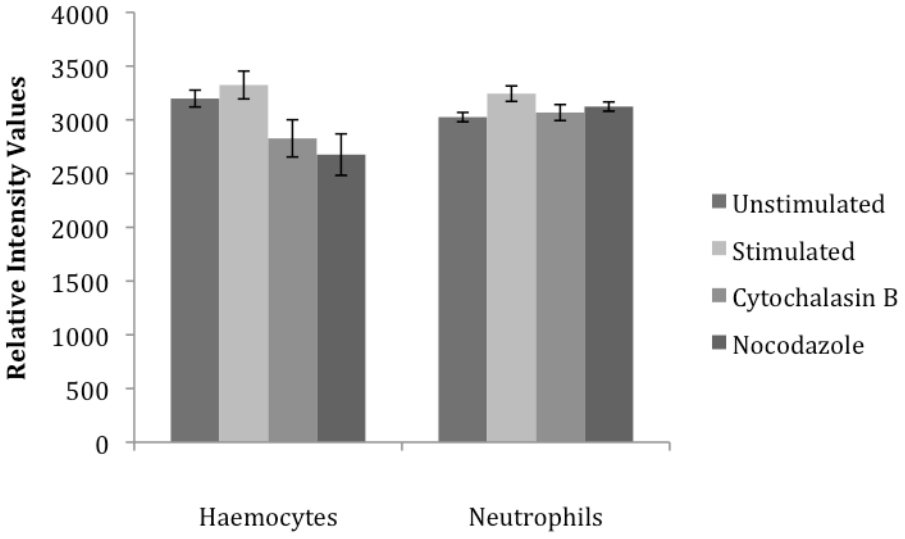


Figure 5.13 Confocal immunofluoresence relative intensity values of vinculin in neutrophils and haemocytes (n=3).

5.5.2 Analysis of the distribution of vinculin in 3 sub-populations of haemocytes in *G. mellonella* larvae

It has been speculated that there are different sub-populations of *G. mellonella* larvae haemocytes (García-García *et al,* 2009; Gillespie *et al* , 1997). These sub-populations are likely to differ in their sizes and their granularity, thus performing differing functions in the haemolypmh. This section studied the expression of the linker protein vinculin, which was shown previously in this Chapter to be distributed in the cell differently depending on the treatment or stimulation.

It was determined, as described in section 2.14, that there exists three distinct sub-populations of haemocytes, differing in size (FSH) and granularity (SCH) (Figure 5.14). These sub-populations were further analysed for the presence of vinculin and how vinculin expression changed when the cells were PMA stimulated, cytochalasin b + PMA treated or nocodazole + PMA treated (Figures 5.14 and 5.15).

In the population of cells located in gate 1, which was determined to be the smallest and most granular cells (Figure 5.13), the vinculin expression increased from 87.95 ± 7.45% to 94 ± 1.99% upon PMA stimulation, expression was 92 ± 5% when the cells were treated with cytochalasin b + PMA and 79 ± 15% when the haemocytes were nocodazole + PMA treated.

Gate 2, which is displayed as R2 in Figure 5.13, demonstrated the expression of vinculin in the unstimulated cells to be 79 ± 16%, PMA stimulated haemocytes showed an expression of 78.26 ± 16.7%, cytochalasin b+ PMA haemocytes have 81.2 ± 14.2% expression and nocodazole + PMA haemocytes demonstrate an expression of 71.6 ± 19%. In gate 3 which contains the largest cell sub-population of cells (Figure 5.14), unstimulated cells express vinculin at 96.9 ± 3% in this gate, PMA stimulated cells demonstrated expression at 90.4 ± 8.17%, cytochalasin b + PMA demonstrate expression at 97.05 ± 2.62% and nocodazole + PMA demonstrated expression at 91.3 ± 8%. The cells in gate 1 displayed the highest expression of vinculin in the PMA stimulated haemocytes. This was of interest as this sub-population is the smallest size and displayed the highest granularity and is therefore the most similar to the small, highly granular neutrophil. A representative FACs Calibur analysis plot of vinculin in haemocytes can be visualised in Figure 5.16.

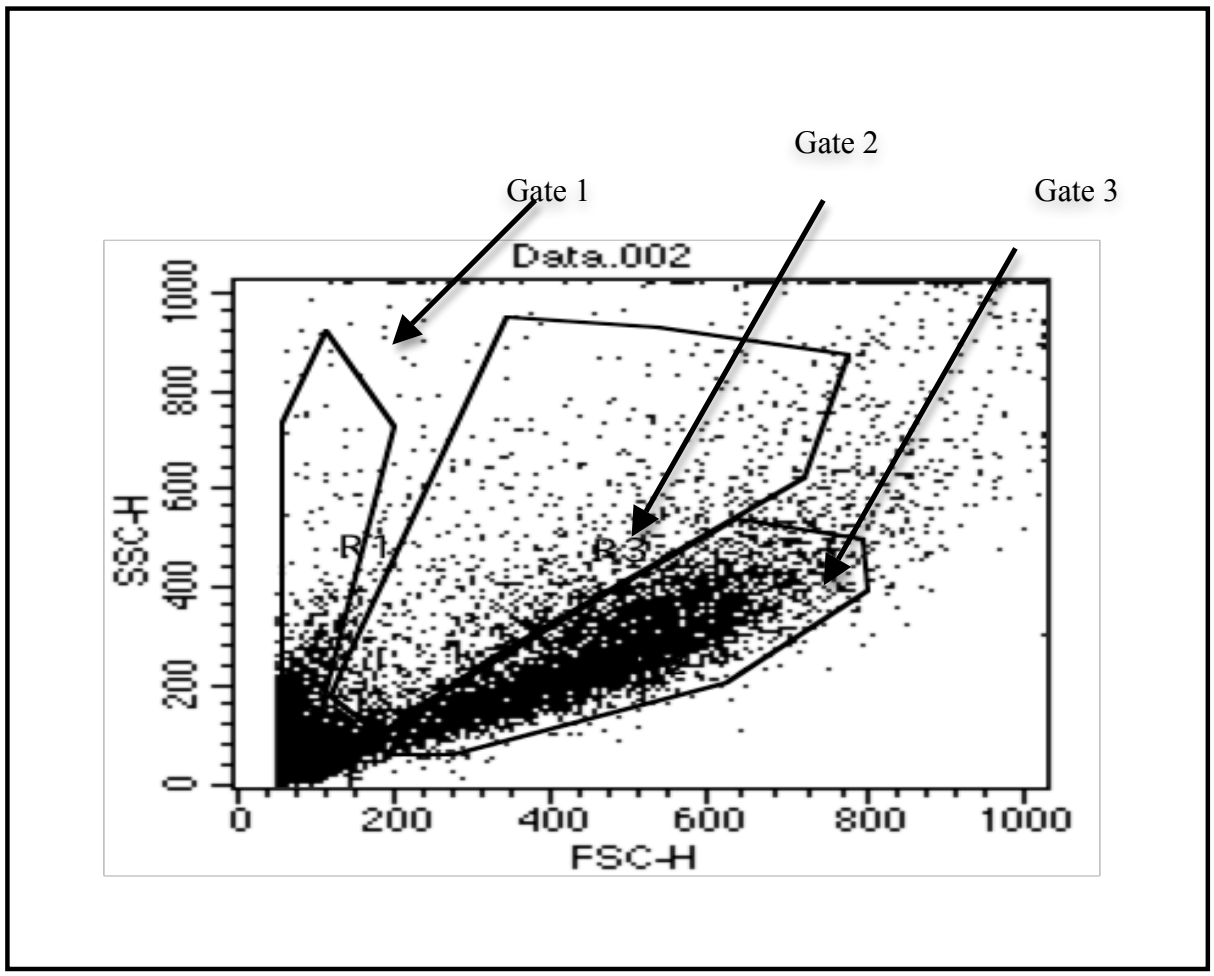


Figure 5.14 FACs Calibur analysis of the sub-populations in the haemocytes of *G. mellonella* larvae and the identification of 3 sub-populations differing in size (FSC-H) and granularity (SSC-H).

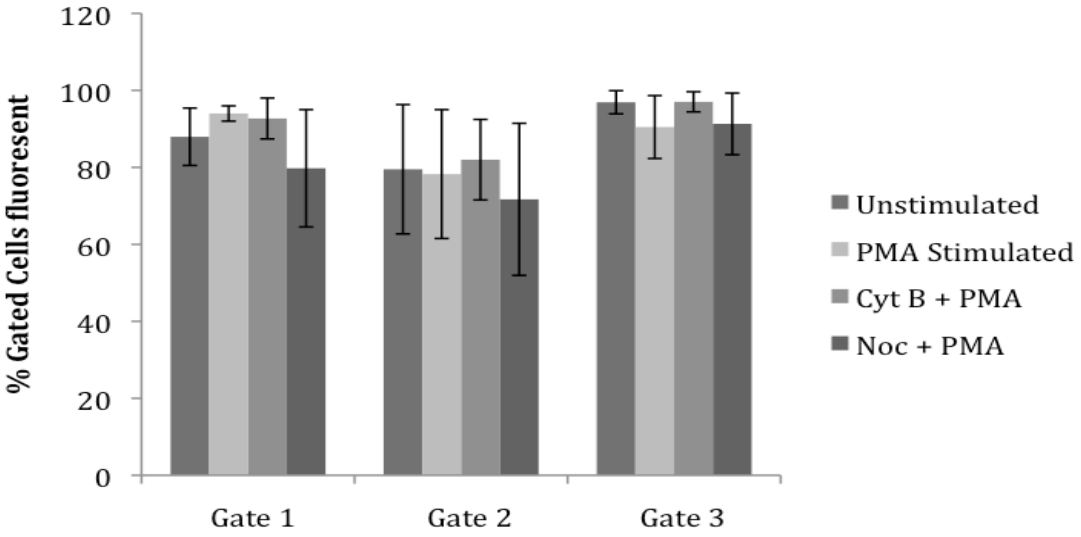
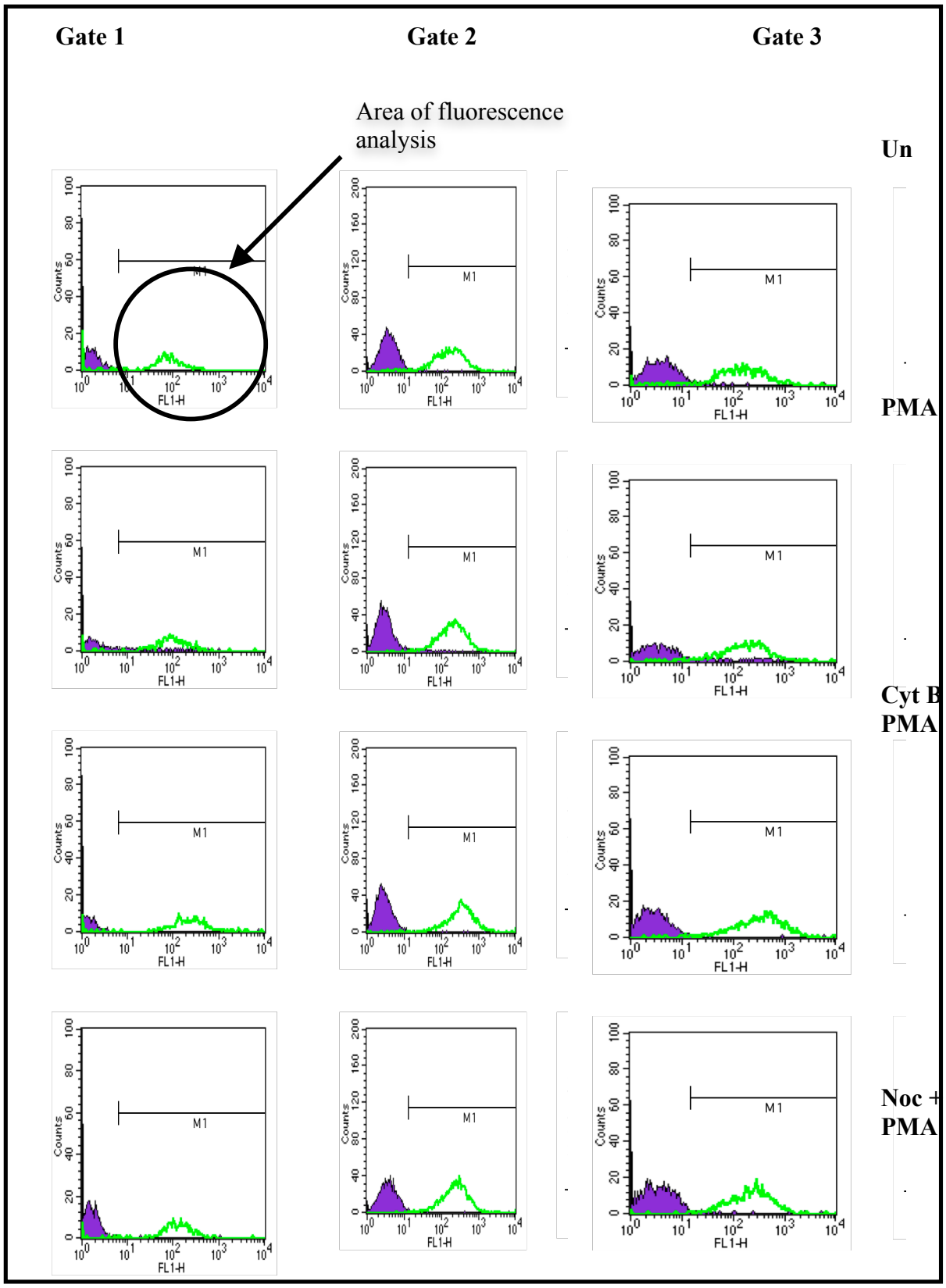


Figure 5.15 Analysis of the 3-gated populations in haemocytes of *G. mellonella* larvae expressing vinculin (n=3).



5.16 Representative analysis of fluorescence in sub-populations of haemocytes of *G. mellonella* larvae using fluorescently tagged Anti-vinculin (n=3).

5.6.1 Evaluation of the distribution of coronin in the haemocyte of *G. mellonella* larvae and the effect of cytochalasin b and nocodazole on this protein

Coronin is a highly conserved protein from yeast to mammals; it is essential for the assembly of actin in the cytoskeleton for roles such as chemotaxis and phagocytosis (Yan *et al.,* 2007). Both of these processes have been demonstrated to occur in the haemocyte (Wiesner, 1996; Strand, 2008). Yan *et al.* (2007) showed that neutrophil adhesion and migration did not occur in the absence of coronin.

Coronin has been identified in the cytoskeleton of the neutrophil as an important actin binding protein and also, it has been shown to interact with the phox proteins (Grogan *et al*., 1997), which have been described in the haemocyte in Chapter 4. This section examines a 'coronin like protein' in the haemocyte, and analyses the expression of this protein upon treatment with the inhibitors cytochalasin b and nocodazole prior to PMA stimulation. Grogan *et al.,* (1997) detailed the increase in coronin expression in the neutrophil upon PMA stimulation.

This section also compares the expression of coronin in the haemocye to what occurs in the neutophil. The haemocytes can be visualised in Figure 5.17 and the neutrophils can be visualised in Figure 5.18. There was a significant difference between fluorescent intensity in unstimulated haemocytes and PMA stimulated haemocytes, where intensities rose from 2578 ± 25 to 3219 ±92 ($p<0.05$). Cytochalasin b + PMA treated haemocytes demonstrated intensities of 2878 ± 258 and nocodazole + PMA treated haemocytes showed intensity at 2991 ± 13 (Figure 5.18). The variances were similar to what occurred in the neutrophil, where upon PMA stimulation the intensity rose from 3161 ± 38 to 3305 ± 24 ($p<0.05$). Cytochalasin B + PMA treated haemocytes demonstrated fluorescent intensities at 2888.3 ± 223 and nocodazole + PMA treated haemocytes demonstrated fluorescent intensities at 2836 ± 107 ($p<0.05$). These data can be visualised in Figure 5.19. Again, it is likely that coronin availability increased with PMA stimulation to the haemocyte, allowing alterations in cytoskeletol conformation to occur.

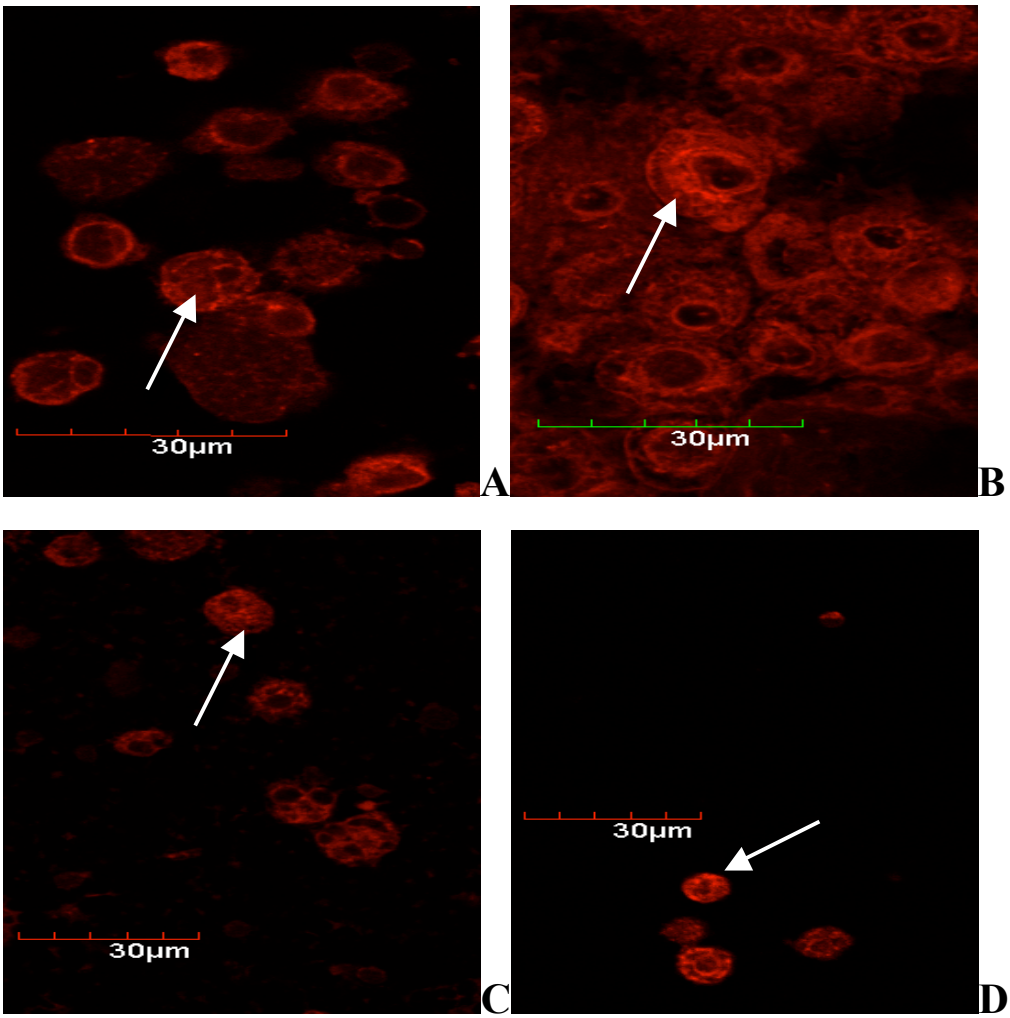


Figure 5.17 Confocal immunofluoresence images of coronin in unstimulated haemocytes(using Anti human Coronin antibody) (A), PMA stimulated haemocytes (B), cytochalasin b + PMA treated haemocytes (C) and nocodazole + PMA treated haemocytes (D) (n=3).

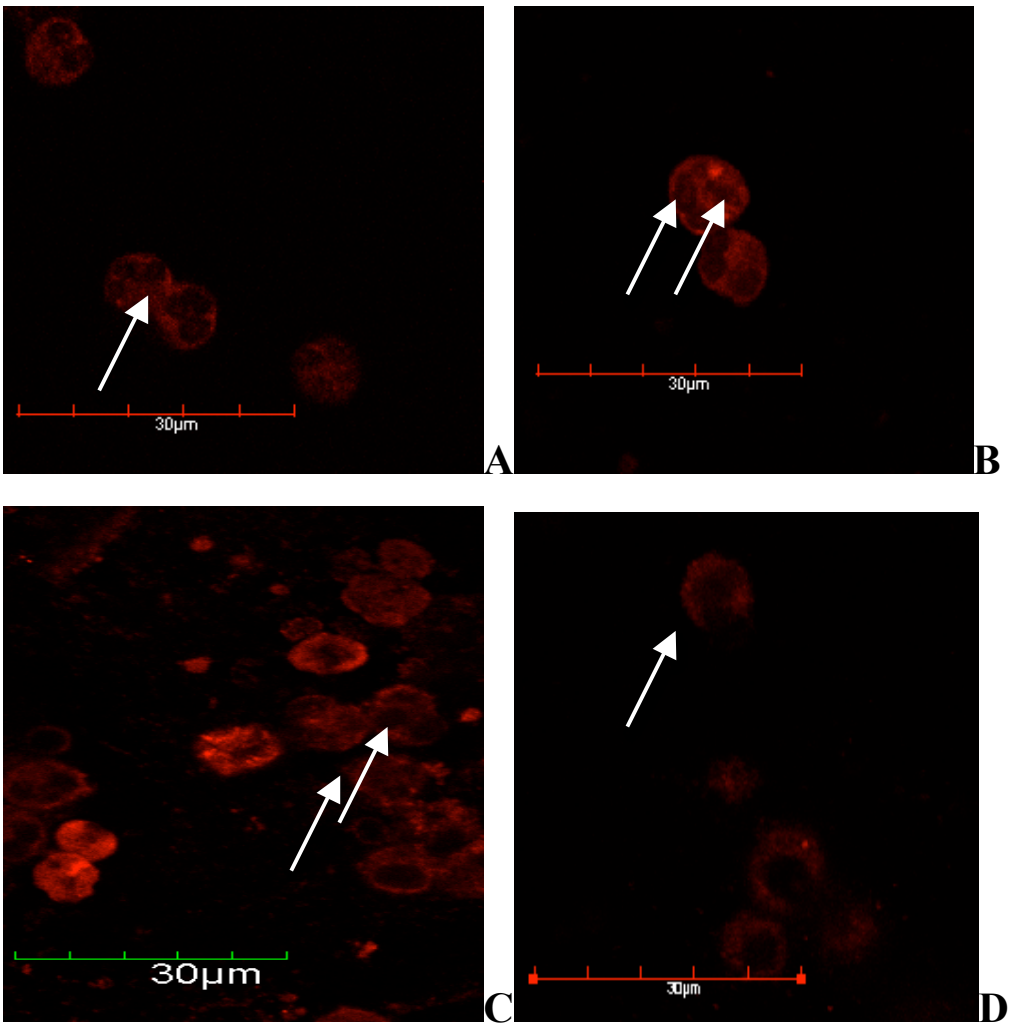


Figure 5.18 Confocal immunofluoresence images of coronin in unstimulated neutrophils (using Anti human Coronin antibody) (A), PMA stimulated neutrophils (B), cytochalasin b + PMA treated neutrophils (C) and nocodazole + PMA treated neutrophils (D) (n=3).

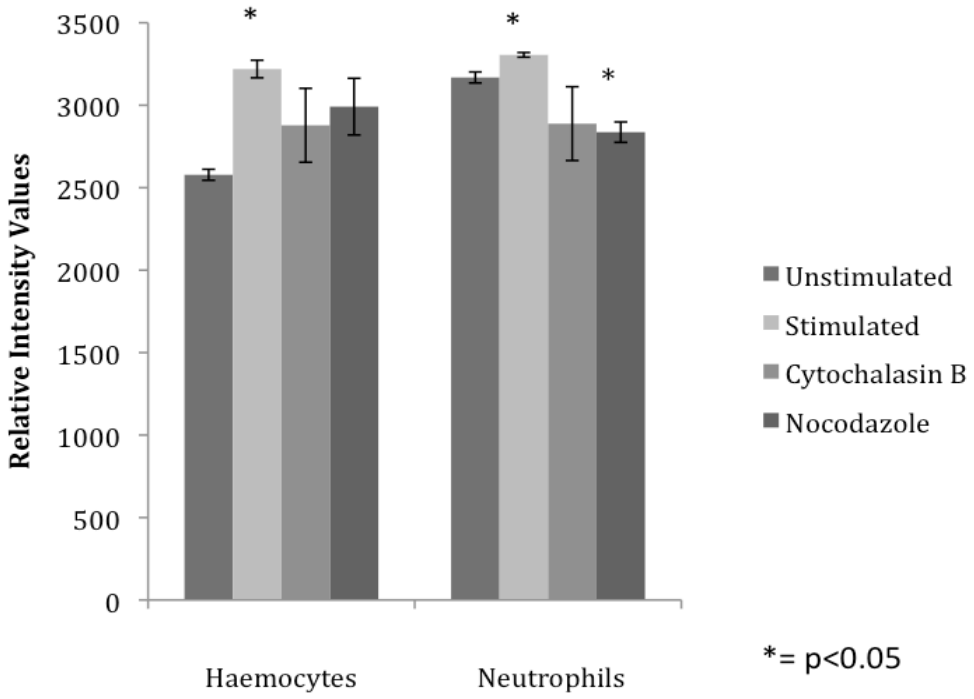


Figure 5.19 Confocal immunofluoresence relative intensity values of coronin in neutrophils and haemocytes (n=3).

5.6.2 Analysis of the distribution of coronin in 3 sub-populations of haemocytes of *G. mellonella* larvae

To determine if coronin expression was altered in the sub-populations of *G. mellonella* larvae, flow cytometry analysis was employed and the sub-populations were compared with each other.

Coronin was found to be expressed in all three sub-populations. Gate number 1 which contains the smallest and most granular cell population, expression of coronin was found to be 97.5 ± 0.5% in the unstimulated cells, 96 ± 3% in PMA stimulated cells, 72.5 ± 5.5% in cytochalasin b + PMA stimulated cells and 68.5 ± 16.5% in nocodazole + PMA stimulated cells. Although there were variances in the populations, there was not a significant difference between unstimulated, control haemocytes and the treatments (p=1).

Figure 5.20 also identifies a second sub-population of haemocytes which is larger than those in gate 1 and not as granular (gate 2). Unstimulated cells demonstrated expression of coronin in this sub-population at 99%, PMA stimulated cells demonstrated expression of coronin at 97.5 ± 0.5%, cytochalasin b + PMA demonstrated expression of coronin at 93 ± 6% and expression of coronin in nocodazole + PMA treated haemocytes was 98.5 ± 0.5% (p=1).

The largest sub-population of haemocytes (gate number 3) showed expression of coronin at 99% in unstimulated cells, 97% in PMA stimulated cells, 70.5 ± 28.5% in cytochalasin b + PMA treated cells and 98.5 ± 0.5% in nocodazole + PMA treated cells (Figure 5.21). There were no significant differences in these variances (p=1), although there does appear to be a change in the protein expression in the sub-populations when the haemocytes were PMA treated or treated with cytochalasin b and nocodazole prior to PMA stimulation.

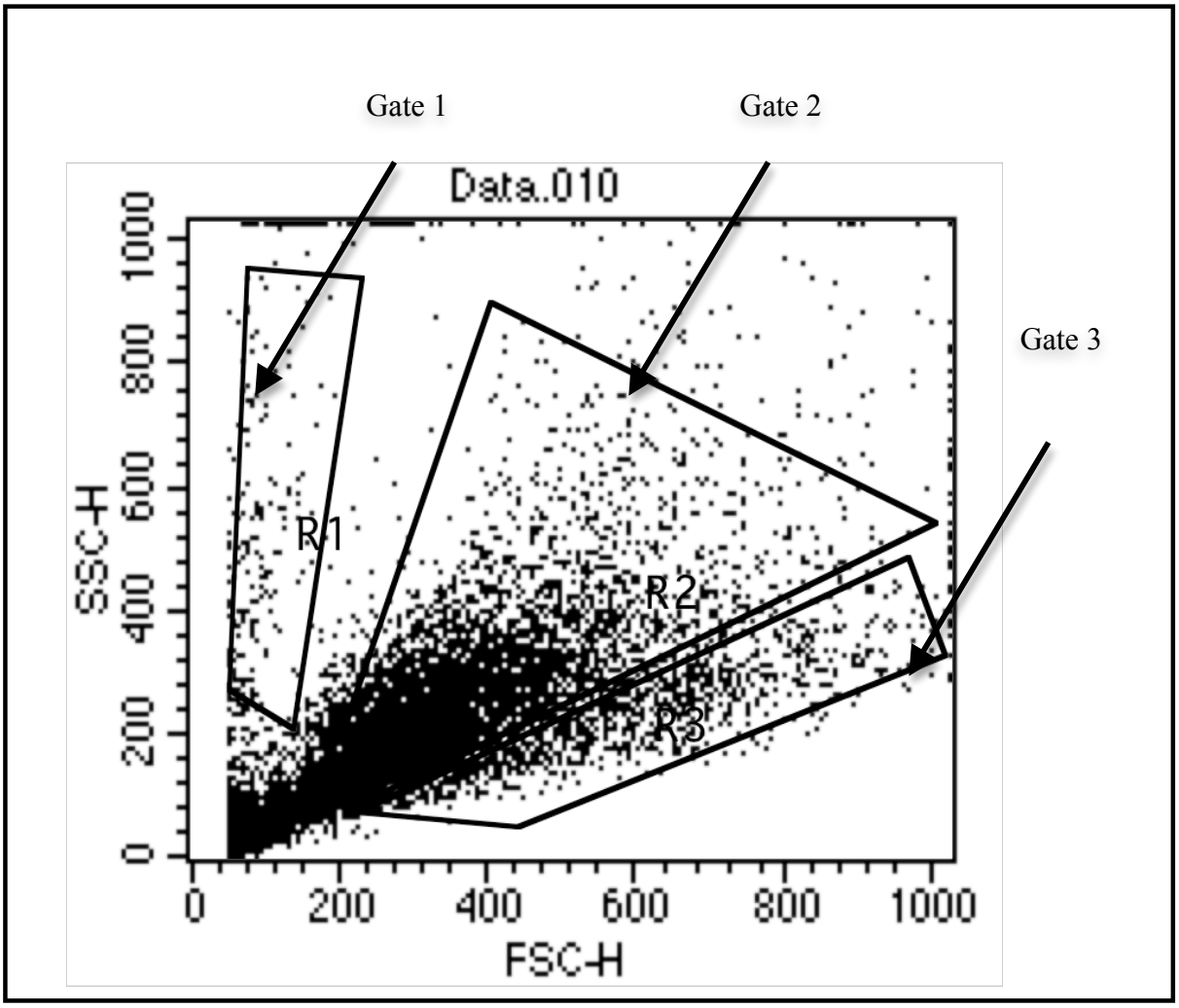


Figure 5.20 FACs Calibur analysis of the sub-populations in the haemocytes *G. mellonella* larvae and identification of 3 sub-populations differing in size (FSC-H) and granularity (SSC-H), further analysed for expression of coronin.

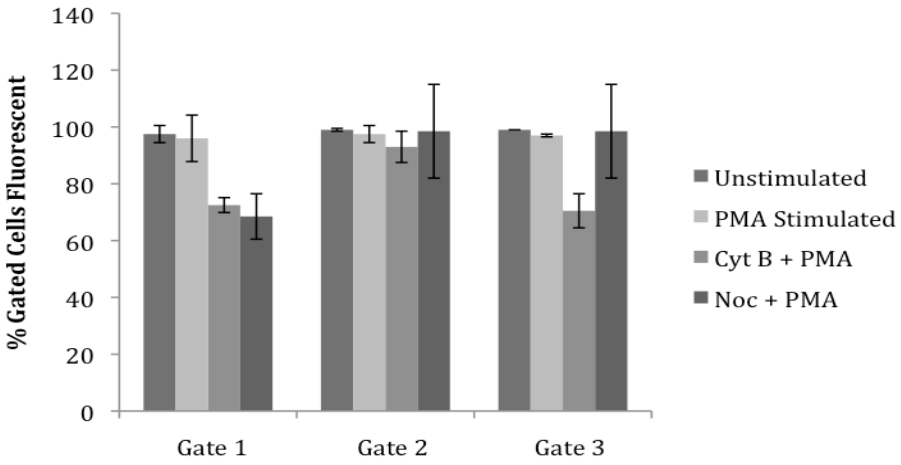


Figure 5.21 Analysis of the 3-gated populations in haemocytes of *G. mellonella* larvae expressing coronin (n=3).

5.6.3 Summary

The objectives of this study were to determine if the cytoskeletal linker protein vinculin is present in the haemocyte of *G. mellonella* larvae and compare its presence to that in the neutrophil. This section also studied the expression of coronin in the haemocyte of *G.mellonella* larvae and analysed the expression of coronin in human neutrophils. Upon analysing vinculin and coronin expression in the haemocyte and the neutrophil, by employing confocal immunofluoresence techniques, it was found that the there are similar proteins to neutrophil vinculin and coronin present in the haemocyte. The distribution of the proteins was parallel to what was observed in the neutrophil. When haemocytes were treated with the cytoskeletal inhibiors cytochalasin b and nocodazole, the distribution of vinculin and coronin resembled closely what was observed in the neutrophil.

This section also deduced that there are three sub-populations of haemocytes in *G. mellonella* larvae, these were shown to differ in size and granularity. The expression of vinculin and coronin in these sub-populations was also examined, with the pre-treatment of the inhibitors cytochalasin b and nocodazole prior to PMA stimulation.

5.7.1 Evaluation of the distribution of globular actin in the haemocyte of *G. mellonella* larvae and the effect of cytochalasin b and nocodazole on this protein

Actin is an essential component of the neutrophil (and the haemocyte) where the filaments can comprise 50% of total cellular protein (Numiguchi *et al*., 1999). In the neutrophil motility is determined by the decrease in G-actin and increase in F-actin expression. It has been demonstrated, in the neutrophil, that artificial stimulation of the cell with chemotactic activators such as PMA increases F-actin expression (Howard and Oresajo, 1985). Globular actin (G-actin) content in the neutrophil is decreased when the cell becomes activated by interacting with a foreign particle (Howard and Meyer, 1984). This coincides with an increase in the filamentous actin (F-actin) content in cells, allowing cell shape changes to occur (Stie and Jesaitis, 2007).

This section examined G-actin changes in the haemocyte and compared to the neutrophil by employing confocal immunofluorescent technology. The intensity of G-actin in unstimulated haemocytes was 2906 ± 77, PMA stimulated cells demonstrated intensity at 2850 ± 27, cytochalasin b + PMA stimulated cells intensity was 2827 ± 222 and nocodazole + PMA stimulated cells demonstrated intensity at 2898 ± 91 (Figure 5.22). There were no significant differences between haemocyte treatments ($p=1$), although it is evident that G-actin content decreased in the PMA stimulated haemocytes.

G-actin intensity was also analysed in isolated neutrophils. The intensity in unstimulated neutrophils was 3242 ± 65, PMA stimulated neutrophils revealed intensity at 3105 ± 102, cytochalasin b + PMA treated cells demonstrated intensity at 3010 ± 32 ($p < 0.05$) and nocodazole + PMA treated cells demonstrated intensity at 3159 ± 23 (Figure 5.23 and 5.24).

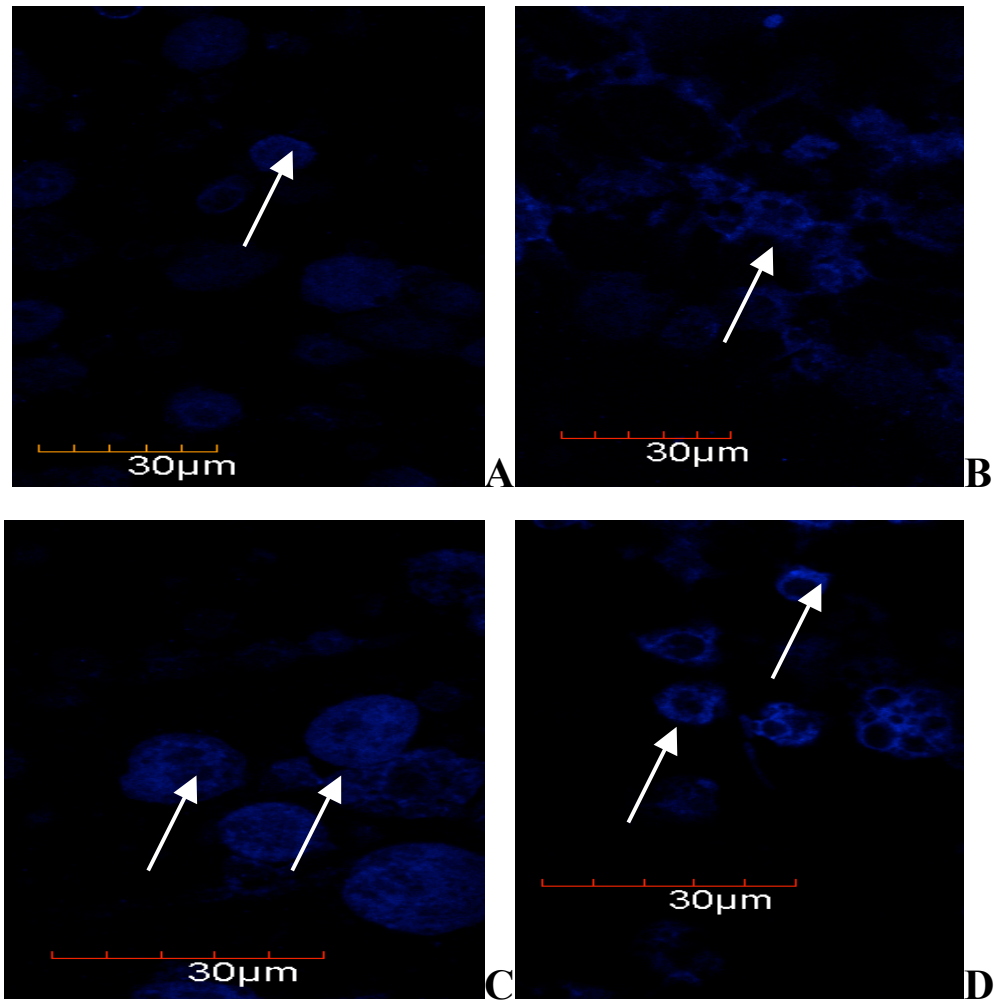


Figure 5.22 Confocal immunofluoresence images of G-actin (using Anti human G-actin antibody) in unstimulated haemocytes (A), PMA stimulated haemocytes (B), cytochalasin b + PMA treated haemocytes (C) and nocodazole + PMA treated haemocytes (D) (n=3).

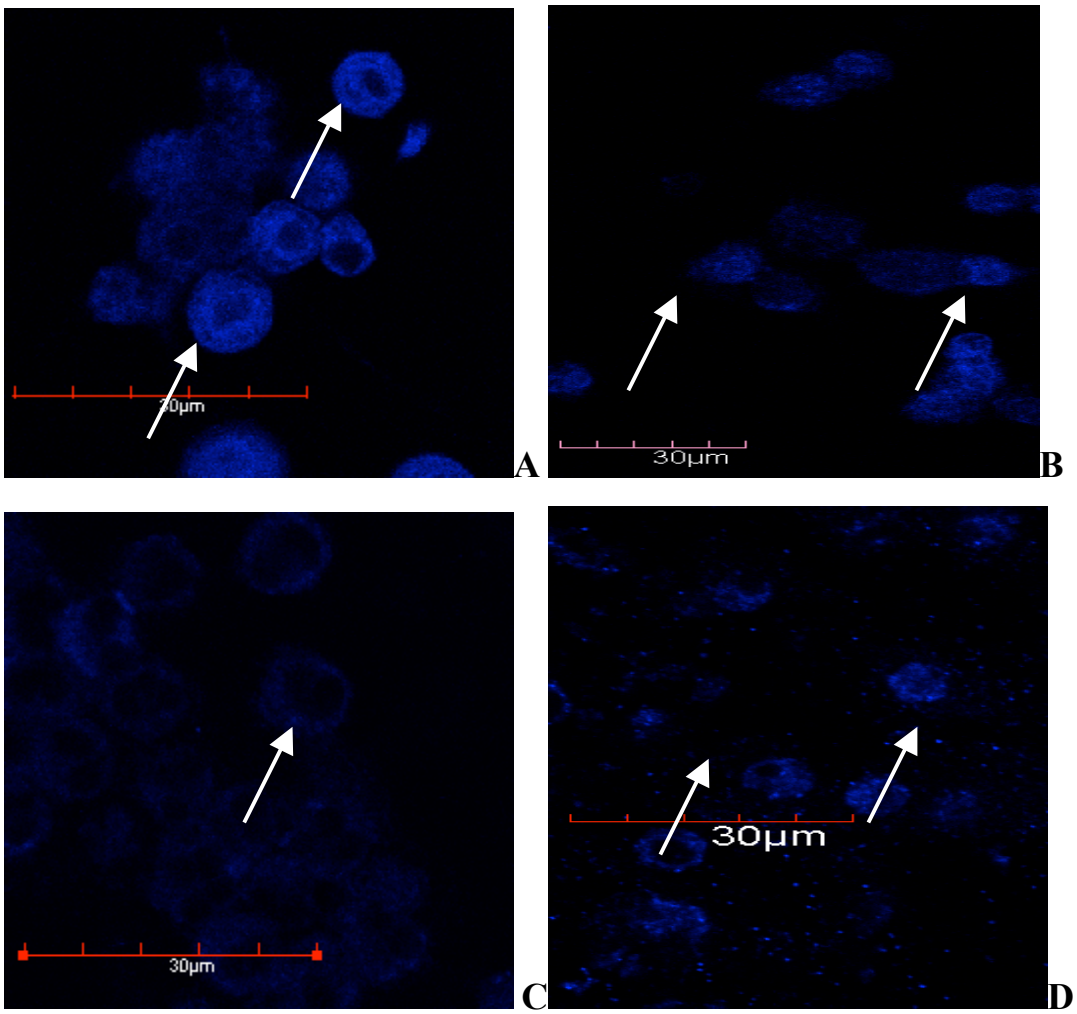


Figure 5.23 Confocal immunofluoresence images of G-actin (using Anti human G-actin antibody) in unstimulated neutrophils (A), PMA stimulated neutrophils (B), cytochalasin b + PMA treated neutrophils (C) and nocodazole + PMA treated neutrophils (D) (n=3).

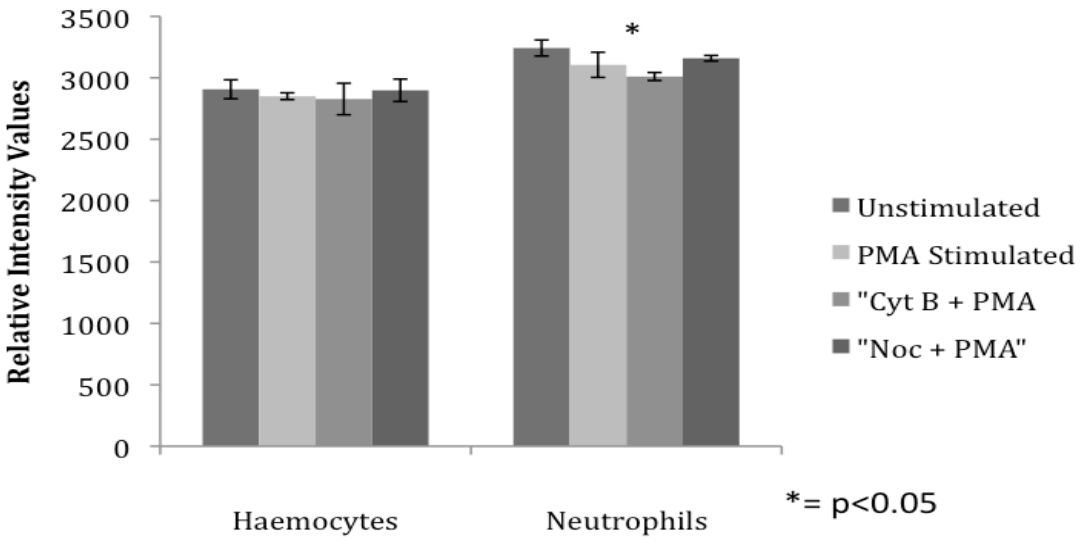


Figure 5.24 Confocal immunofluoresence relative intensity values of G-actin in neutrophils and haemocytes (n=3).

5.7.2 Analysis of the distribution of globular actin in 3 sub-populations of haemocytes of *G. mellonella* larvae

As detailed in the previous sections, FACs calibur analysis of isolated haemocytes identified three sub-populations in haemocytes. These were further analysed for the presence of G-actin in the sub-populations. Globular actin was identified in gate number 1 (smallest and most granular cell population) at a level of 98.6 ± 0.53% in unstimulated haemocytes, 98.17 ± 0.95% in PMA stimulated haemocytes, 99.28 ± 0.15% in cytochalasin b + PMA treated haemocytes and 98.38 ± 0.21% in nocodazole + PMA treated haemocytes, flow cytometry demonstrated no significant variances in G-actin expression in this gated population (p=1).

The cells in gate number 2 demonstrated expression of G-actin at a rate of 99.39 ± 0.21% in unstimulated haemocytes, 99.09 ± 0.48% in PMA stimulated haemocytes, 99.73 ± 0.22% in cytochalasin b + PMA stimulated haemocytes and 99.83 ± 0.06% in nocodazole + PMA stimulated haemocytes. There was no significant difference between the treatments in this population (p=1).

Haemocytes in gate number 3, as demonstrated in Figure 5.25 and 5.26, are the largest population of haemocytes. It was here that unstimulated haemocytes demonstrated an expression of 99.39 ± 0.21% of G-actin, PMA stimulated haemocytes demonstrated expression of 99.09 ± 0.49%, cytochalasin b + PMA treated haemocytes demonstrated G-actin expression of 99.73 ± 0.22% and nocodazole + PMA treated haemocytes demonstrated expression of 99.83% ± 0.06%. Again, there were no significant differences in G-actin expression in cells between treatments.

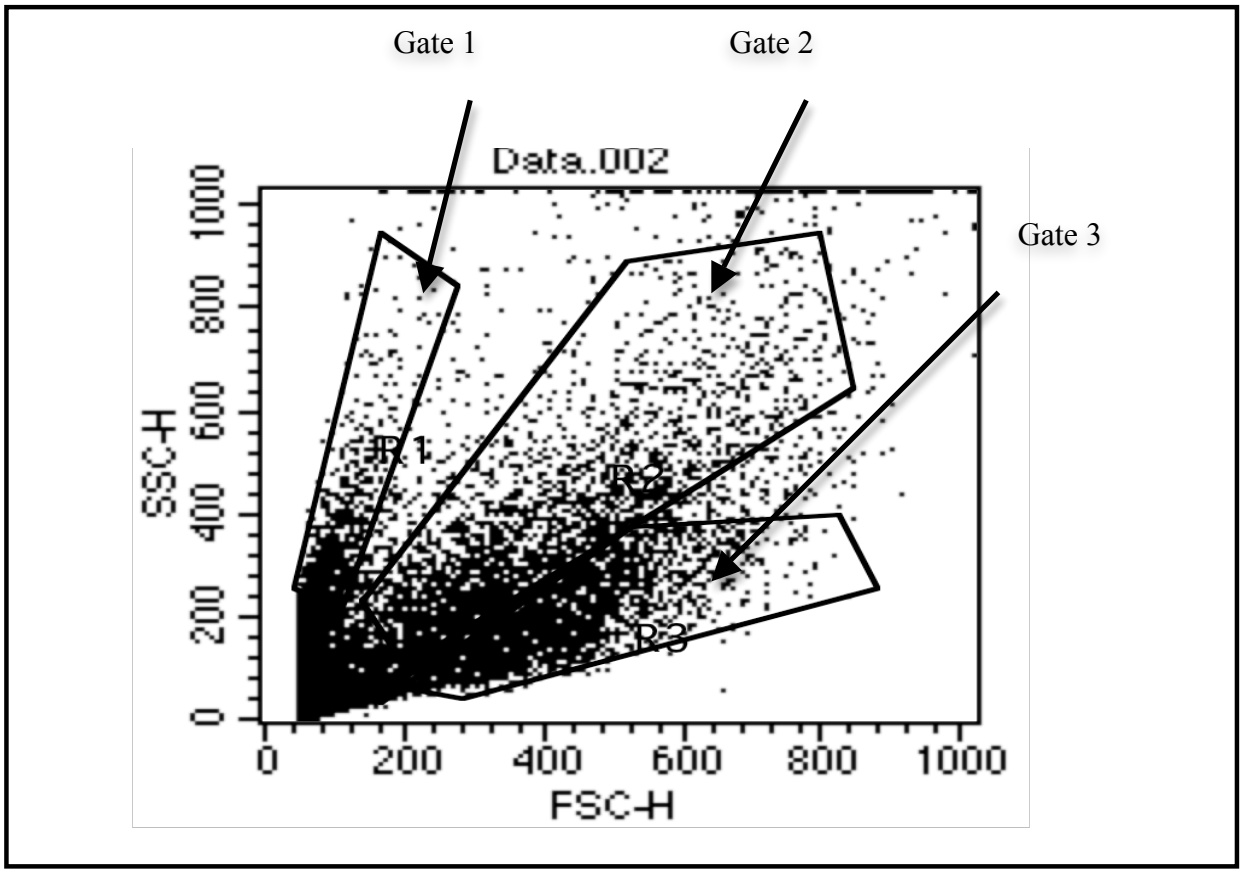


Figure 5.25 FACs Calibur analysis of the sub-populations in the haemocytes *G. mellonella* larvae and identification of 3 sub-populations differing in size (FSC-H) and granularity (SSC-H), further analysed for expression of G-actin.

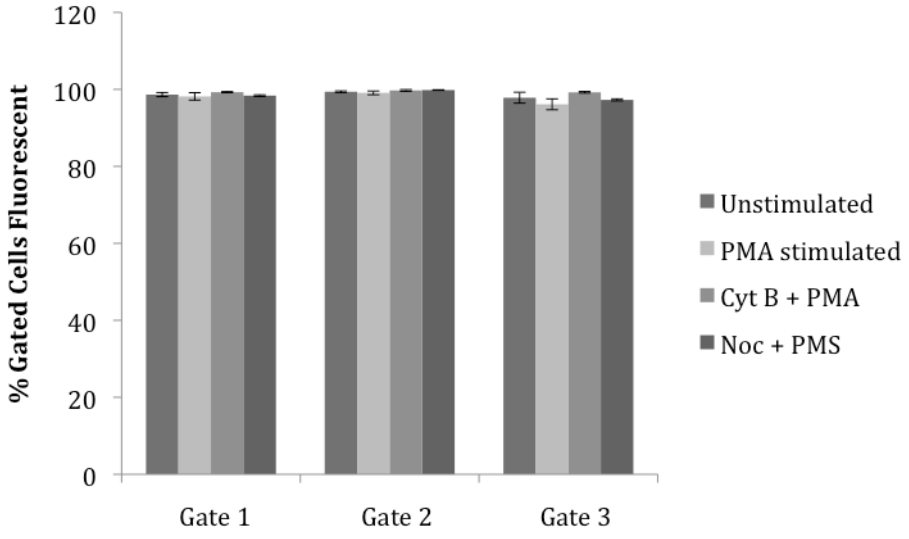


Figure 5.26 Analysis of the 3 gated populations in haemocytes of *G. mellonella* larvae expressing G-actin (n=3).

5.8 Examination of the presence of filamentous actin in the haemocyte of *G. mellonella* larvae and the effect of cytochalasin b and nocodazole on this protein using Rhodamine Phalloidin Staining

Rhodamine phalloidin is a non-species specific stain for filamentous actin (F-actin) in cells. It has been used widely with neutrophils (Sun *et al*., 2004; Grogan *et al.*, 1997). This section details F-actin distribution in the neutrophil and compared the results to those shown in the previous section where G-actin was studied. F-actin expression has been demonstrated to increase when the neutrophil is activated to begin phagocytosis and destruction of an opsonised target (Grogan *et al*., 1997).

Figure 5.27 displays images of haemocytes that were unstimulated, PMA stimulated, cytochalasin b + PMA treated and nocodazole + PMA treated. Upon PMA stimulation of isolated haemocytes, relative intensity on the haemocyte increased from 2797 ± 45.89 to 3249 ± 7.35 ($p<0.05$). Cytochalasin b + PMA demonstrated an intensity of 2783.6 ± 39 and nocodazole + PMA demonstrated intensity at 2823 ± 12.9.

Intensity of F-actin in unstimulated neutrophils was 3075 ± 24, PMA stimulated neutrophils demonstrated intensity at 3455 ± 245, cytochalasin b + PMA treated neutrophils had intensity levels of 2725 ± 306 and nocodazole + PMA treated neutrophils had intensity levels at 2938 ± 353. There was not a significant difference between intensity levels in the neutrophil, however there was an increase in intensity. These data can be seen in Figures 5.28 and 5.29.

These results demonstrate that the inhibitors have a negative effect on F-actin expression, therefore prevent cytoskeletal changes such as phagocytosis to occur. These data also demonstrated the similar effect that PMA had on the neutrophil and the haemocyte in activating the G-actin polymerization to the F-actin state in the cell. Figure 5.30 demonstrates the filamentous actin content in a PMA stimulated haemocyte, the fluorescence can be seen throughout the cell periphery as the haemocyte is sectioned from bottom of cell to top of cell.

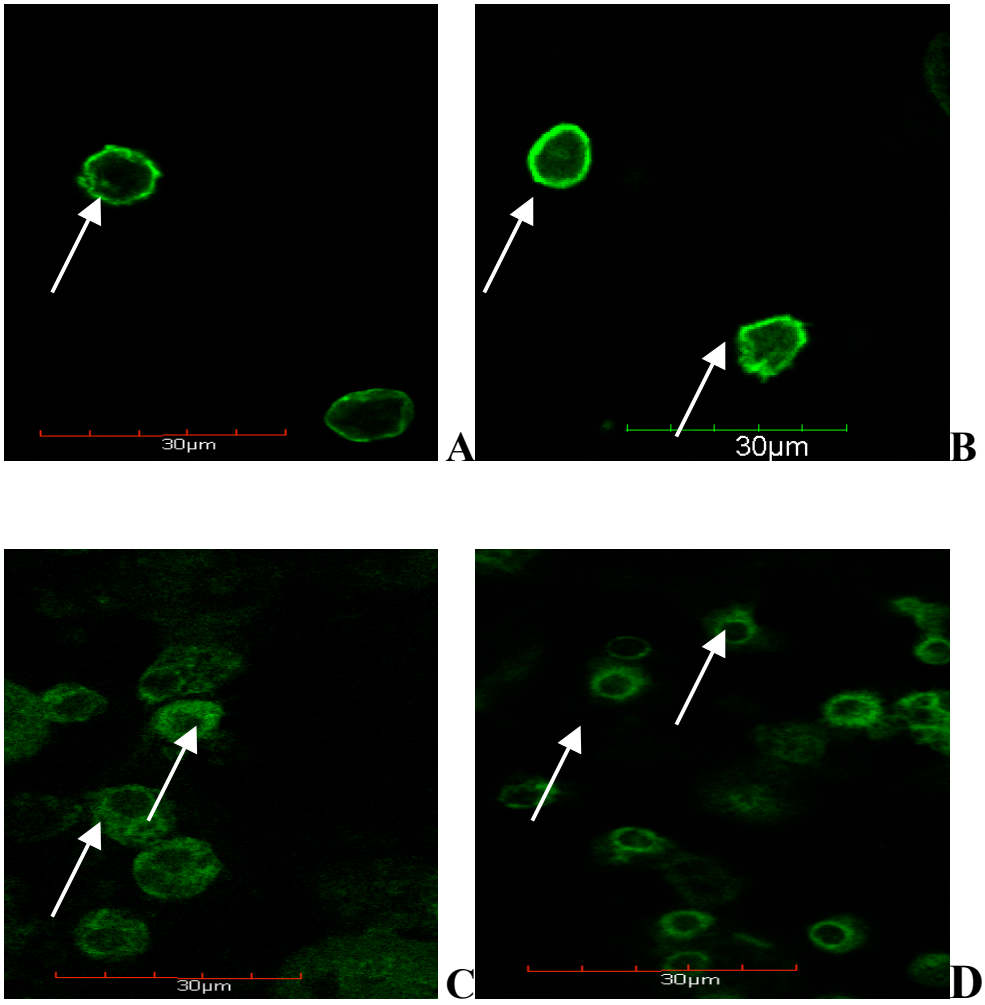


Figure 5.27 Confocal immunofluoresence images of filamentous actin (using Rhodamine Phalloidin stain) in unstimulated haemocytes (A), PMA stimulated haemocytes (B), cytochalasin b + PMA treated Haemocytes (C) and nocodazole + PMA treated haemocytes (D) (n=3).

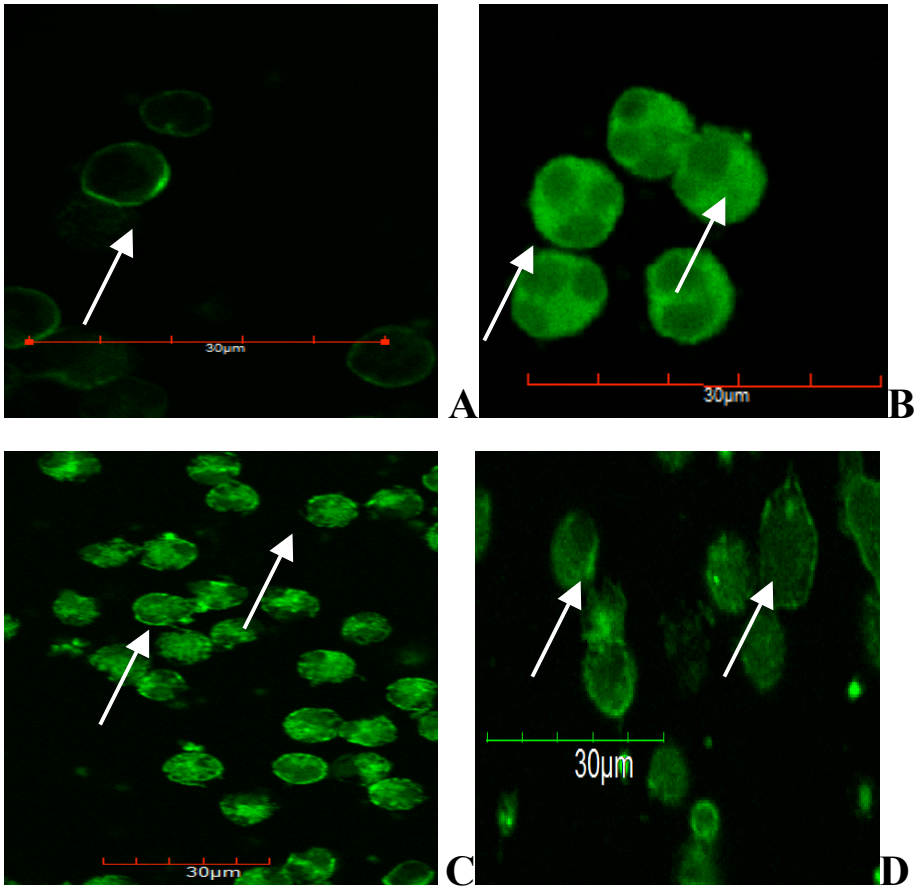


Figure 5.28 Confocal immunofluoresence images of filamentous actin (using Rhodamine Phalloidin stain) in unstimulated neutrophils (A), PMA stimulated neutrophils (B), cytochalasin b + PMA treated neutrophils (C) and nocodazole + PMA treated neutrophils (D) (n=3).

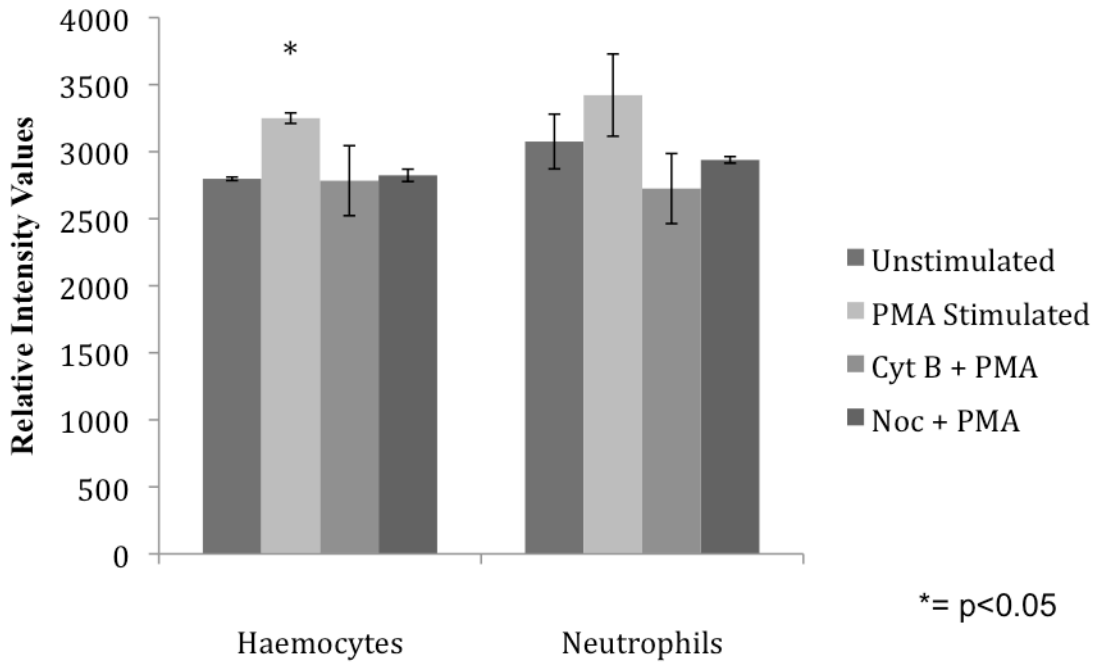


Figure 5.29 Confocal immunofluoresence relative intensity values of filamentous actin in neutrophils and haemocytes (n=3).

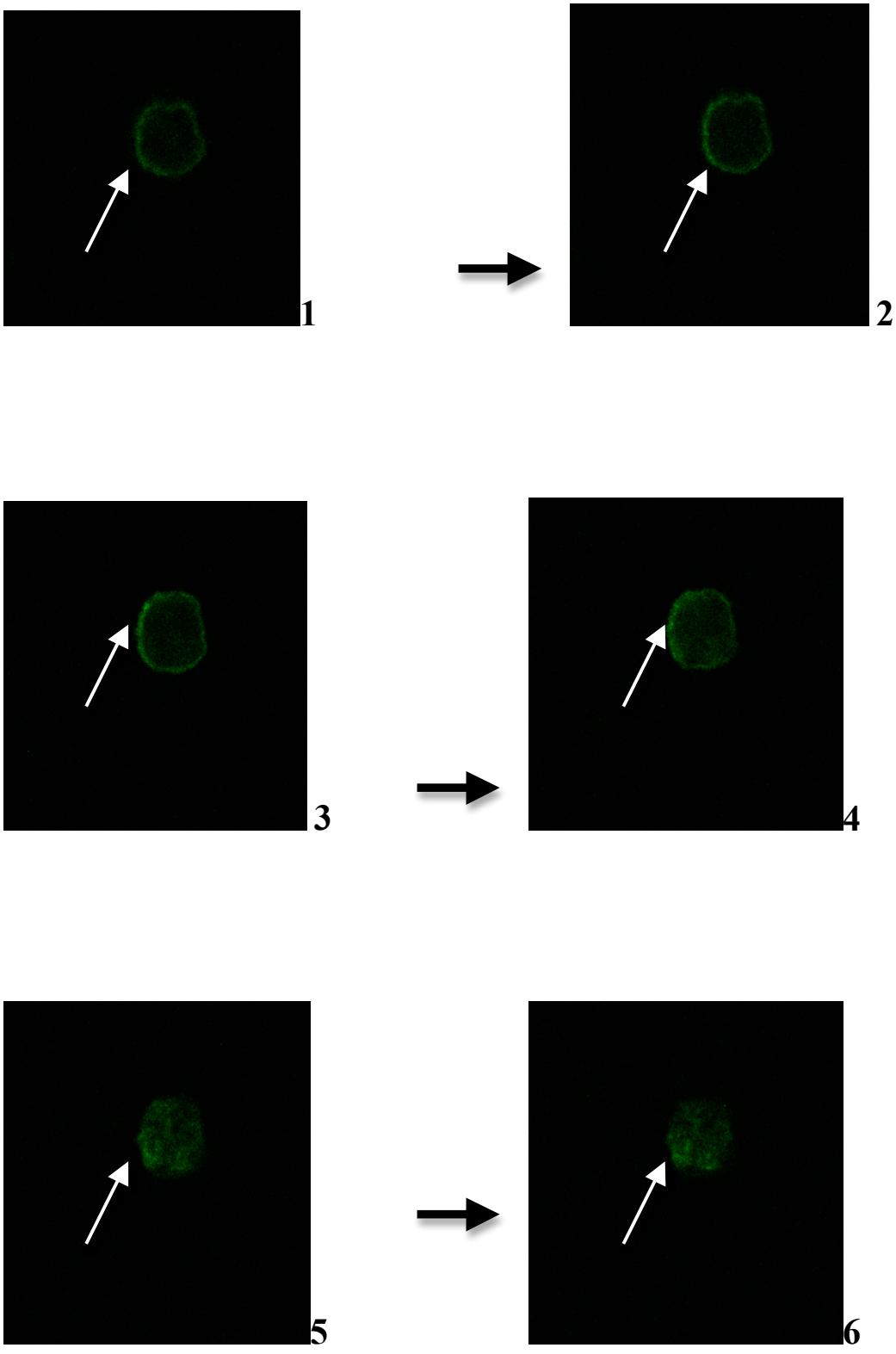


Figure 5.30 A series of images from a PMA stimulated haemoctye, where filamentous actin is evident in cell's periphery, stained with rhodamine phalliodin, 1= bottom of cell through to 6= top of cell. Arrows show the areas of F-actin distribution throughout the haemocyte.

5.8.1 Summary

This section examined the presence of globular actin and filamentous actin in the haemocyte and compared the expression to that in the neutrophil, further demonstrating similarities between the two cell types. It was found that G-actin expression in the haemocyte decreased upon PMA stimulation (however it was not significant), this occurred in the neutrophil also whilst the expression of F-actin increased in both cell types. This is most likely due to the fact that G-actin content decreases as F-actin content increases in the immune cell as cellular activation occurs (Linssen *et al.,* 2008). Globular and filamentous actin expression were also varied in the neutrophil and haemocyte when the cells were pre-treated with the inhibitors cytochalasin b and nocodazole prior to PMA stimulation. There was a significant change in G-actin expression in the neutrophil, indicating that the inhibitors affected the polymerisation of G-actin. G-actin was further analysed using flow cytometry, three gates that varied in size and granularity were compared to each other with respect to G-actin distribution. It was found that expression was very similar in all gated populations; almost all cells expressed positive fluorescence for G-actin in their cytoskeleton. Confocal immunofluoresence analysis revealed similar expression levels of F-actin in the haemocyte and the neutrophil. Figure 5.30 displayed F-actin location in the haemocyte when the haemocyte is sectioned through, it was demonstrated that F-actin lies in the cell's membrane when the cell is PMA stimulated and activated to begin ROS production and phagocytosis, allowing an alteration in the cell's cytoskeleton.

5.9 Utilising flow cytometry methods to characterise and sort the haemocyte population into distinct sub-populations

Based on the findings from the previous sections, it was decided to sort the sub-populations by utilising an advanced flow cytometer and cell sorter, the BD FACs Aria®. Another objective was to view these sub-populations microscopically in order to visualise the size and granularity differences.

Haemocyte sub-populations have recently been characterised in the insect *B. mori* by utilising flow cytometry (Nakahara *et al.,* 2009). It is likely that the whole haemocyte population of *G. mellonella* larvae is comprised of smaller sub-populations that differ in function. This section also examines whether the PMA stimulation of the haemocytes results in a shift in population density in each sub-set.

It was found that there exists four distinct sub-populations of haemocytes in *G. mellonella* larvae. This differs slightly to what was detailed earlier in this Chapter where three populations were identified by a BD FACs Calibur flow cytometer, and analysed for the presence of different proteins. Figure 5.31 demonstrates the populations of unstimulated haemocytes that were sorted into the sub-sets differing in size and granularity by employing the BD FACs Aria® cell sorter which are named Gate 1- Gate 4. Figure 5.31 demonstrates the populations of PMA stimulated haemocytes that were also into these subsets.

PMA stimulation of haemocytes was found to induce a shift in cell numbers in the populations. The population analysed in gate 1 increased in cell density from 5.87 ± 0.42% to 7.3 ± 0.12% of the total cell count ($p<0.05$). The cells that were analysed in gate 2 were 12.87 ± 2.94% of the total cell count in the unstimulated haemocytes and this increased to 17.37 ± 2.93% in the PMA stimulated haemocytes. The cells in gate 3, which are larger than the previous two populations, demonstrated 13.72 ± 2.42% of the total cells in the unstimulated haemocye population. This was changed to 9.53 ± 0.97% in the PMA stimulated haemocytes. The fourth gate, which is also a physically large subset, was 10.4% in unstimulated haemocytes and 6.9 ± 0.78% in PMA stimulated haemocytes ($p<0.05$).

The trend in the populations was that PMA stimulation increased the percentage of cells that are in the smaller and more granular subsets, these subsets may be the most similar to the neutrophil, with regard to cell size and granularity content and cellular function (Figure 5.33).

The cells from each sub-population were imaged using light microscopy and can be viewed in Figure 5.34. There are clear differences in the cell populations, as is obvious from the cell size and presence of granule-like organelles. These sub-populations possibly have different functions in the insect's defense system. It is probable that gate 1 is the sub-population most similar to the neutrophil, due to the granules and to the small size. The addition of PMA to the isolated haemocytes also increased the cell density in this population significantly, indicating the PMA is activating the cell granules, as it does when neutrophils are stimulated with PMA (Bylund *et al.,* 2004). Cells in gate 2, 3 and 4 are larger than cells in gate 1 and may participate in processes such as wound healing and nutritional processes as described by Ratcliff *et al.,* (1985).

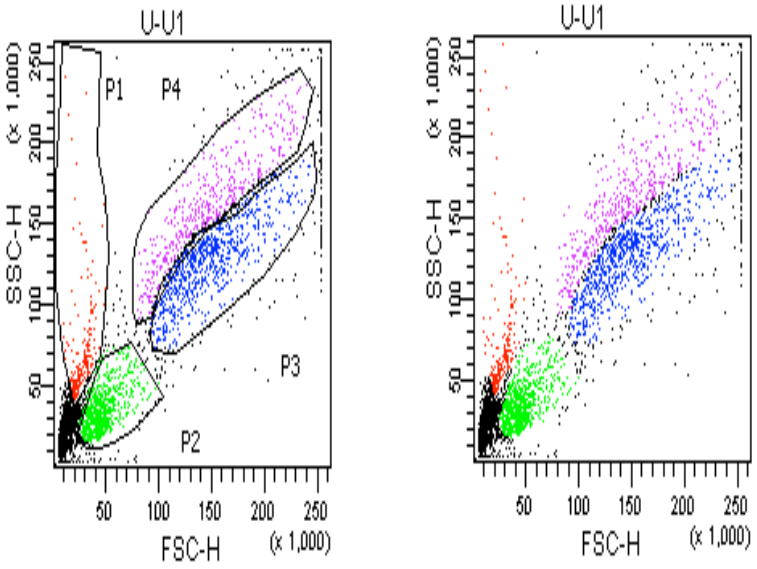


Figure 5.31 Unstimulated haemocytes sorted into 4 sub-populations using FACs Aria Flow Cytometer, BD ® (n=4).

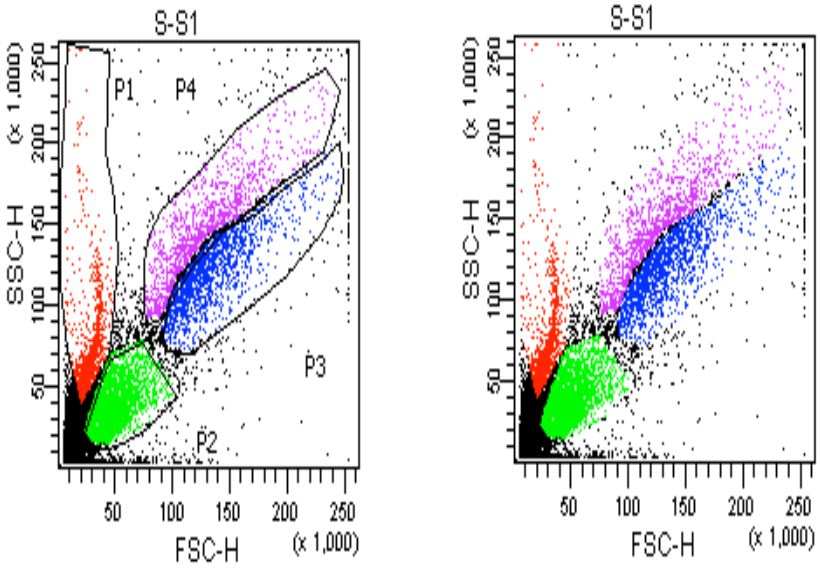


Figure 5.32 PMA stimulated haemocytes sorted into 4 sub-populations using FACs Aria Flow Cytometer, BD ® (n=4).

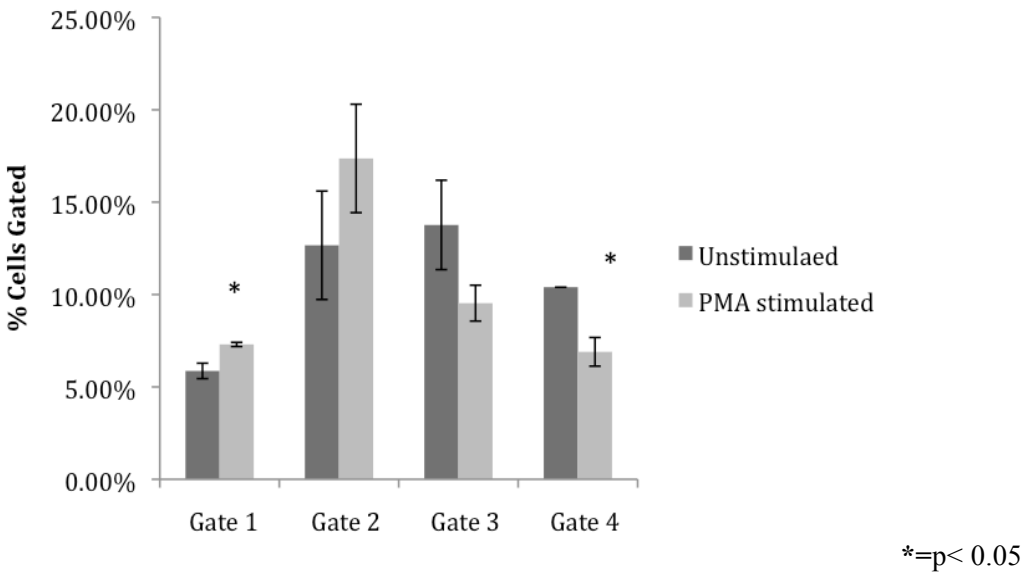


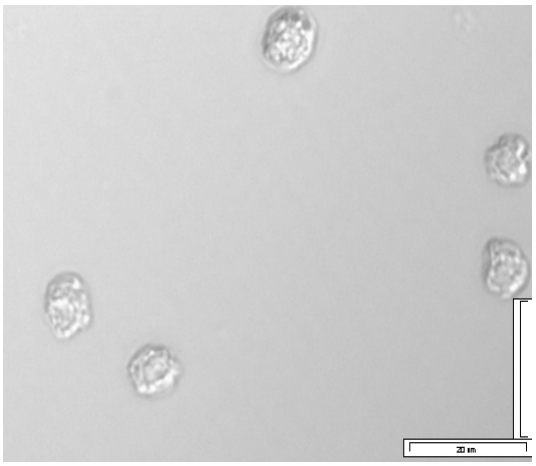
Figure 5.33 Analysis of the four sorted populations detailing % of total cells in each sub-population (n=4).

Gate 1 = P 1.

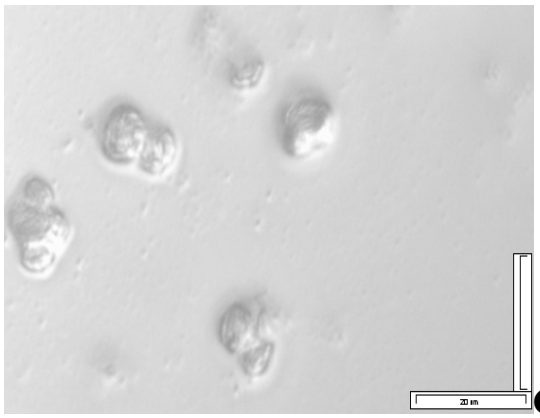
Gate 2 = P 2.

Gate 3 = P 3.

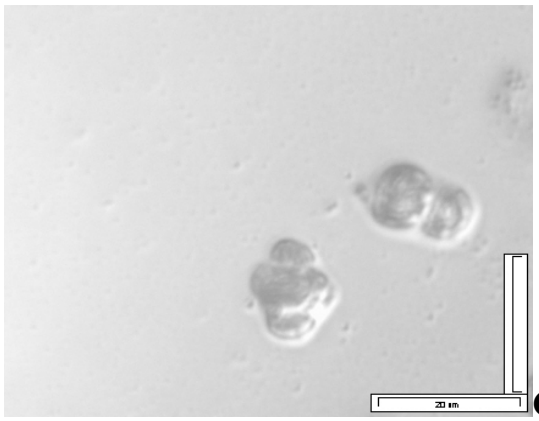
Gate 4 = P 4.



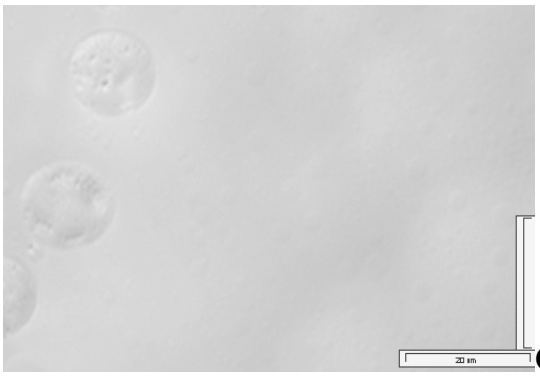
Gate 1/ P 1



Gate 2/ P 2



Gate 3/ P 3



Gate 4/ P 4

Figure 5.34 Gated sub-populations 1-4 viewed under the light microscope (x 400) (Scale = 20 microns)

5.9.1 Summary

This section employed cell sorting to characterise the haemocyte population of *G. mellonella* larvae. Four populations were identified using advanced flow cytometry and sorted into distinct categories, varying in their cell size and granular content. It was evident that the addition of PMA to isolated haemocytes caused a shift in the cell populations, this may be due to the effect that PMA has on the granular content, releasing granules from lipid rafts and activating proteolytic enzymes contained in the granules. The populations were subsequently viewed using light microscopy and different characteristics were obvious from these images.

5.10 Discussion

G. mellonella larvae possess an immune cell, the haemocyte, which plays a vital role in the larval immune system and constantly migrates through the haemolymph (Strand, 2008). The haemocyte's cytoskeleton has an essential role to play in the response of the cell to invading microorganisms, in order to phagocytose and produce ROS production the composition of the cytoskeleton and expression of various proteins must change (Vilcinkas *et al*., 1997).

The cytoskeleton of the cell is essentially the scaffolding of the cell and ensures that the cellular components are protected. This allows the cell to change it's morphology when the cell becomes activated. When the neutrophil is activated to begin phagocytosis, a cascade of events takes place, such as particle binding, actin assembly, membrane remodeling, pseudopodia extension and phagosome enclosure (Anes *et al*., 2003). The haemocyte of *G. mellonella* larvae has the ability to phagocytose particles (Zdybicka-Barabas and Cytryńska, 2010) and therefore the reassembly of the membrane that occurs in the neutrophil is likely to occur in the haemocyte also. Gillespie *et al*., (1997) described phagocytosis in the haemocyte and it is remarkably similar to the steps that occur in the neutrophil, i.e. there is attachment, recognition, signal transduction, pseudopodia formation, ingestion and phagosome ingestion.

The objectives of this Chapter were to characterise similarities between the neutrophil and the haemocyte, to further understand the haemocyte's cytoskeleton and to characterise the sub-populations of the haemocyte.

Cytochalasin b is a secondary metabolite isolated from *Drechslera domatiodea* (Cheung, 1989) and is widely used in neutrophil studies (Grogan *et al*., 1997; Bylund *et al.*, 2004) where it has been found to disrupt the cytoskeleton. Cytochalasin b has been shown to weaken cytoskeletal components and affect the neutrophil's ability to phagocytose and produce pseudopodia (Neeli *et al.,* 2009). Nocodazole is a tubulin polymerization inhibitor that inhibits particle uptake (Bershadsky *et al.,* 1995). F-actin formation plays a central role in exocytosis of granules and the inhibition of this process by compounds such as cytochalasin b and nocodazole reduces neutrophil mediated exocytosis (Bylund *et al*., 2004).

This Chapter first determined whether cytochalasin b and nocodazole, and a combination of these agents had a toxic or negative effect on larval survival. There

was no significant toxic effect on the larvae, with larval survival remaining over 90% at all times (Figure 5.1). This also allowed the determination of the concentration of the agents that could be used in subsequent haemocyte experiments.

G. mellonella larvae are widely used in biological and chemical assays, where the virulence of many pathogens may be assessed and screened (Seed and Dennis, 2008). In some cases, immune priming can also be assessed in order to determine whether an agent alters the immune response prior to infection with a pathogen (Mowlds *et al.*, 2010). Changes in the haemocyte density of larvae injected with cytochalasin b and nocodazole was also investigated, particularly because of the significance of haemocytes in the immune system of the larvae (Wiesner, 1996). There were no significant changes in the density of haemocytes in larvae that had been injected with cytochalasin b or nocodazole.

Pre-injection of larvae with cytochalasin b and nocodazole affected the immune system negatively, as demonstrated by inoculation with the yeast *C. albicans* resulting in faster death when larvae had been pre-injected with cytochalasin b and nocodozole. This may occur due to the inhibitors affecting the haemocytes ability to kill, by disrupting events such as phagocytosis, ROS production, encapsulation and nodule formation (Sideri *et al*., 2007; Renwick *et al*., 2006). Cytochalasin b affects the neutrophil in a manner that slows the phagocytosis process, the cytoskeleton is unable to change to induce phagocytosis and allow particle killing to occur (Yost *et al*., 2009). Nocodazole inhibits particle uptake during phagocytosis by disrupting microtubule formation (Koebnigskinect and Landreth, 2004). However, when *G. mellonella* larvae were injected with cytochalasin b and nocodazole and incubated for 24 hours prior to *C. albicans* inoculation, the death rate was found to not be as significant as it was when larvae were injected with the inhibitors 4 hours prior to *C. albicans* inoculation. This may be due to the larvae generating more healthy haemocytes in this longer time period and becoming more able to combat infection.

In order to further study the influence of cytochalasin b and nocodazole on the haemocytes ability to phagocytose, an assay was designed where phagocytosis could be monitored over time. The study of phagocytosis is often employed to monitor the uptake of particles (García-García *et al.*, 2009) and in these studies the target used was *A. flavus* conidia, which can easily be viewed under the light

microscope. Approximately 60% of control haemocytes had engulfed conidia after 90 minutes compared to 20% of those haemocytes that had been exposed to nocodazole or cytochalasin b ($p<0.05$). This suggests that, while the concentration used here did not adversely affect the viability of haemocytes (Figure 5.10), the agents reduce the ability of haemocytes to undergo the morphological changes required to engulf a target.

Treatment of haemocytes with cytochalasin b or nocodazole also severely reduced the ability of haemocytes to kill a pathogenic target. Indeed exposure of haemocytes to both inhibitors simultaneously completely abolished their ability to kill without significantly affecting their viability. *C. albicans* survival was significantly higher with control haemocytes when compared to haemocytes that were pre-treated with cytochalasin b, nocodazole and the agents SOD and DPI. SOD (super oxide dismutase) is a scavenger of the reactive oxygen species O_2^- and therefore affects the haemocyte's killing ability (Stie and Jesaitis, 2007). DPI (diphenyleneiodonium) also negatively affect the haemocyte by inhibiting NADPH oxidase (Bergin *et al.*, 2005). Even though these agents did not negatively affect haemocyte viability, the ability of the cell to engulf a target was significantly affected. The results of these assays indicated that cytochalasin b and nocodazole pre-treatment of the haemocyte of *G. mellonella* larvae negatively affect the killing ability of the haemocyte. Co-administration of these agents further affected the haemocyte's killing ability and resulted in a higher survival of *C. albicans* cells, indicating that the co-treatment with the inhibitors was more destructive to the haemocyte than the inhibitors on their own. Cytochalasin b has been shown not to compromise the viability of the neutrophil when employed in experiments (Jog *et al.,* 2011), and although neutrophil activity is affected by the addition of nocodazole, viability is not (Neeli *et al*., 2009).

Confocal microscopy is widely used to analyse and study cellular activity and cell contents, it is broadly used when studying haemocytes (Gallardo-Escárate *et al.*, 2007; Zdybicka-Barabas and Cytryńska, 2010). This Chapter utilised confocal immunofluoresence to study the presence of proteins such as vinculin, coronin, G-actin and F-actin in the cell membrane. As discussed earlier in this Chapter, the integrity of the cell is very much dependent upon the actin filaments. Vinculin is a component of the filaments, involved in cell-cell junctions and actin membrane associations. The change in vinculin in neutrophils is similar to those changes that

occur during actin polymerisation (Bershadsky *et al.,* 1995). Vinculin expression in the neutrophil has previously been found to be decreased by cytochalasin b and nocodazole treatment (Volz, 1993). The results demonstrated here detailed the presence of a vinculin homolog in the haemocyte. Vinculin expression also increased in the haemocyte and the neutrophil with the addition of the chemotactic activator PMA. Volz (1993) demonstrated that this occurs when protein kinase C is activated in the neutrophil. Lacchini *et al.* (2006) demonstrated that activated protein kinase C ineteracts with vinculin and actin in the haemocyte of the gastropod snail *Lymnaea stagnalis.* As discussed in Chapter 4, PMA is an activator of protein kinase C. The pre-addition of cytochalasin b and nocodazole to the haemocyte caused similar effects in the haemocyte and neutrophil, at the intensity level and at the visual level, where the fluorescence is distorted in both cell types. These results indicated another similarity between both cell types.

Due to the increasing use of flow cytometry as an analytic technique (Linssen *et al.*, 2008; Sideri *et al*., 2007), it was decided to utilise it in a novel manner and further gain knowledge on the expression of vinculin in sub-populations of haemocytes. Three gates were chosen to analyse the expression of the protein. The cells in these gates differed in size and granularity. Cells in gate one (Figure 5.14) demonstrated a small increase in fluorescence in the PMA stimulated haemocytes compared to unstimulated haemocytes and those that had been pre-treated with cytochalasin b and nocodazole. This may be due to this particular sub-population of cells undergoing degranulation, a well characterised phenonomen that the neutrophil undergoes when stimulated (Stie and Jesaitis, 2007). Due to the physically smaller size of this population when compared to the other two sub-populations, and the higher granularity, it indicates that these cells are similar in morphology to the neutrophil. Degranulation leads to a vast reorganisation of the cell membrane (Linssen *et al.*, 2008) and this results in vinculin expression increasing.

Coronin was also studied in the haemocyte. Coronin is a 57kDa protein (Grogan *et al.*, 1997) that actively binds actin, it is important for processes such as phagocytosis and chemotaxis to occur in the neutrophil (Matute *et al.*, 2005). Cytochalasin b has been shown to detach coronin from neutrophil membranes, thus negatively affecting it (Grogan *et al.,* 1997). Nocodazole has also been shown to negatively affect coronin in the neutrophil (Neeli *et al*., 2009). When haemocytes

were PMA stimulated coronin expression increased significantly in both cell types, coronin diminished significantly in the neutrophil when the cell was pre-treated with nocodazole. In Grogan *et al.,* (1997) it was shown that coronin activation is closely associated with the phox proteins (p47 and p67), which translocate from the neutrophil cytosol to the membrane with PMA stimulation and coronin activation also occurs. Chapter 4 demonstrated the presence of $p47^{phox}$ in the haemocyte and this Chapter demonstrates a human coronin homolog in the haemocyte also.

Coronin was also analysed using a FACs Calibur Flow Cytometer. There was no difference between unstimulated and PMA stimulated cells in any of the populations, however the greatest difference was the decrease in expression upon pre-treatment of the neutrophil with cytochalasin b and nocodazole. This reduced availabilty of coronin in the population in gate 1 was found to be more than the other two populations (gate 1 and gate 2). It is evident that the agents have a negative effect on the haemocyte populations but the smallest sub-population was the sub-population that had the most obvious differences in coronin expression. The results in this section indicated another similarity at the cell membrane level between neutrophils and haemocytes.

Actin is the most abundant intracellular protein in most eukaryotic cells. Actin exists as a globular monomer called G-actin and a filamentous polymer called F-actin (Schmid *et al*., 1997). The ability of the neutrophil to interconvert the actin filaments between G-actin and F-actin is crucial to the cell's ability to phagocytose and change it's shape when it comes into contact with a pathogen. It is also essential for neutrophil motility (Tse *et al.,* 2004). It was detailed in this section that there was a decrease in G-actin expression between unstimulated haemocytes and PMA stimulated haemocytes. G-actin expression in both the neutrophil and the haemocyte remained low with cytochalasin b and nocodazole pre-treatment. G-actin was further analysed in the haemocyte sub-populations using flow cytometry. Here, it was found that G-actin decreased expression in all 3 gates upon PMA stimulation indicating that G-actin becomes polymerised to F-actin in the haemocyte when the cell is stimulated with PMA. The localisation of F-actin in the neutrophil was identified with the use of rhodamine coupled phalloidin staining. Phalloidin is a stabiliser of polymerised actin (F-actin) and Rhodamine is a fluorescent stain (Bershadsky *et al.*, 1995). Cytochalasin b treatment of neutrophils has been shown to cause disruption to F-actin polymerization and cause a change in F-actin so that it fails to condense

around the cell's periphery (Grogan *et al*., 1997). Nocodazole has been shown to completely disassemble microtubule filaments in the neutrophil to a polarised state (Volz, 1993). The haemocyte F-actin disassembly upon treatment with cytochalasin b and nocodazole demonstrated in Figure 5.28 is comparable to what occurs in the neutrophils (Figure 5.29). This Chapter indicates that inhibitors of neutrophil function also disrupt the function of insect haemocytes, which highlights the similarities between the two cells types. These results establish further similarities between mammalian neutrophils and insect haemocytes and reinforces the validity of using insects as models for studying virulence of human microbial pathogens (Scully and Bidochka, 2006; Aperis *et al.*, 2007).

The haemocyte is responsible for phagocytosis, encapsulation (Gillespie *et al*., 1997), for pathogen recognition and the stimulation of differential signaling pathways (Strand, 2008). However this component of the immune response, as of yet, is not fully understood. There exists sub-populations of haemocytes that have historically been identified using morphological phenotypes and histochemical methods (Gupta, 1985: Brehelin and Zachary, 1986). Flow cytometric techniques are fast becoming the leading route for the separation of haemocyte sub-populations (Gallardo-Escárate *et al*., 2007; Nakahara *et al*., 2009). *D. mellanogaster* larvae are the most well defined when it comes to haemocyte cell lines, and have been found to have at least 3 different types of haemocytes (Wertheim *et al.,* 2005). Strand (2008) documented that there are four sub-populations of haemocytes in larval stage lepidoterans such as *M. sexta* and *B. mori.* These consist of the phagocytic granulocytes, capsule forming plasmatocytes, spherule cells and phenoloxidase containing oenocytoids. The sub-populations that were sorted in section 5.9 may fall into these categories. The largest populations of cells were seen between gates 2 and 3 (Figure 5.31 and 5.32), these were large and granular and may be the phagocytic granulocytes which are the most abundant species in *Anopheles gambiae* and *Ae. Aegypti* (Castillo *et al*., 2006). Granulocytes have been shown to adhere strongly to foreign surfaces and spread symmetrically (Strand *et al.,* 2006). The population of cells in gate 1 (Figure 5.31) appeared to be the most granular sub-population from *G. mellonella* larvae and interestingly this population significantly increased when the haemocytes were PMA stimulated. It is for this reason that it may be deduced that this population is most like the neutrophil, with its small size and high granule content (Linssen *et al*., 2008). It is possible that the cells in the fourth population

analysed (gate 4) are the oenocytoids due to the clearly uniform cell shape, this has also been demonstrated in the mosquito (Castillo *et al*., 2006). Plasmatocytes spread asymmetrically on foreign surfaces and are involved in capsule-forming (Strand *et al.,* 2006), it is for this reason that, morphologically, the cells in gates 2 and 3 (Figure 5.34) appear more like the plasmatocyte mentioned in the literature (Strand, 2008).

The humoral insect response is better understood than the cellular response (Kim *et al.*, 2004; Telfer and Kunkel, 1991; Gillespie *et al.*, 1997). However, with technologies such as flow cytometry and confocal microscopy, the sub-populations of larvae of *G. mellonella* are now closer to full characterisation.

This section described evidence for the presence of four different haemocyte sub-populations in larvae. Light microscopy revealed the morphology and shape of the cells, however further microscopy analysis, for example the utilisation of confocal immunofluoresence in conjunction with molecular markers would reveal further information on specific functions of the sub-sets. The main challenge that characterising insect sub-populations face is that the collection of haemolymph is difficult and sorting can cause a large loss of cells.

5.11 Conclusion

The results presented in this Chapter further illustrate similarities between the haemocyte of *G. mellonella* larvae and the human neutrophil. Both cell types are susceptible to the same disruption by the inhibitors cytochalasin b and nocodazole. Confocal microscopy and flow cytometry allowed detection of well-characterised neutrophil cytoskeletal proteins in the haemocyte and the visualisation of the effect of cytochalasin B and nocodazole on coronin, vinculin, G-actin and F-actin, in the haemocyte. Comparable effects were shown in the haemocyte and the neutrophil. Flow cytometry allowed the sub-cellular sorting of the haemocyte into four sub-populations. The sub-populations sorted in this Chapter reveal striking similarities to what has been documented in other Lepidopteran models (Lavine and Strand, 2002; Ribeiro and Brehelin, 2006).

Chapter 6

Analysis of the process of degranulation in the haemocyte of *G. mellonella* larvae

6.0 Analysis of the process of degranulation in the haemocyte of *G. mellonella* larvae

The human innate immune system is comprised of cellular and humoral components and the cellular component consists of circulating leukocytes. The neutrophil is the most abundant leukocyte in the human body and it represents about 65% of all circulating leukocytes (Cassatella, 2003). The primary function of the neutrophil is to engulf an opsonised particle or pathogen into a vacuole known as the phagosome in a process known as phagocytosis. Once the pathogen is contained within this phagosome, the neutrophil releases an avalanche of toxic agents, including proteases, hydrolytic enzymes and oxidants in order to destroy the pathogen (Gabrilovich, 1999). The release of these toxic agents is cellular 'degranulation' and neutrophil degranulation occurs in conjunction with the processes of phagocytosis and ROS production (Stie and Jesaitis, 2007).

Neutrophils contain at least four different types of granules: (1) primary granules, also known as azurophilic granules; (2) secondary granules, also known as specific granules; (3) tertiary granules; and (4) secretory vesicles (Lacy, 2011). Primary granules contain elastase, myeloperoxidase and defensins (Witko-Sarsat *et al.,* 2000). The secondary granules contain lactoferrin (an iron binding protein), lysozyme and collagenase along with many other antimicrobial agents (Borregaard *et al.,* 1995). Secondary and tertiary granules contain overlapping enzymes and these differ from each other based on their buoyancy density (Lacy, 2006). The secretory granules contain serum albumin and provide a valuable reservoir of membrane components that can replenish the plasma membrane, which becomes consumed during phagocytosis (Segal, 2005). *G. mellonella* larval haemocytes possess similar characteristics to the human neutrophil, such as phagocytosis, pseudopodia production (Whitten *et al.*, 2011) and reactive oxygen species production (Bergin *et al.*, 2005).

This Chapter investigated whether haemocytes released proteolytic enzymes when simulated with PMA, and whether the release was comparable to neutrophil degranulation. This Chapter also describes work, which sought to establish whether the haemocyte of *G. mellonella* larvae released proteolytic enzymes in a kinetic manner that is similar to what is known to occur in the human neutrophil.

It is also examined whether haemocyte pre-treatment with the cytoskeletal inhibitors cytochalasin b and nocodazole altered the ability of the haemocyte to release important immune proteins upon activation by PMA.

6.1 Assessment of neutrophil and haemocyte viability following isolation

To optimise the isolation of neutrophils and haemocytes and ultimately to determine how long both cell types can be utilised in experiments over a time period, the viability of the cells was ascertained as described in section 2.9.

Upon isolation of the cells the viability was ascertained regularly over 48 hours, as detailed in Figure 6.1. One hour following isolation, the viability of the neutrophils was 95 ± 0.02%, after 4 hours viability was 86 ± 0.01% ($p < 0.05$) and 5 hours following isolation viability was 88 ± 0.01% ($p<0.05$). Twenty-four hours following isolation viability was 89 ± 0.04%, and 48 hours following neutrophil isolation cellular viability was 38 ± 0.04% ($p<0.05$).

The viability of *G. mellonella* larval haemocytes was also analysed and viability was shown to decrease at a faster rate than neutrophil viability. One hour following isolation haemocyte viability was 95 ± 0.02% and after 4 hours viability was 60 ± 0.07% ($p<0.05$). However, following 24 hours, cell viability was 1 ± 0.008% and this fell to 0% after 48 hours. These viability studies allowed all future studies to be carried out in the time frame at which both cell types were viable.

Cell viability was also assessed over a 9 minute time period in a 37° C stirring chamber to ensure cell viability was not compromised during degranulation experiments. At 3 minutes haemocyte viability in the chamber was 86.5 ± 1.7%, following 6 minutes in the temperature controlled chamber viability was 74 ± 0.7% and by 9 minutes viability was 78 ± 1.4% (No significant differences). Neutrophil viability at 3 minutes was 88.5 ± 4.5%, at 6 minutes in the chamber the cellular viability was 68.5 ± 9.5% and 9 minutes in the chamber viability was 79.5 ± 2.4% (Figure 6.2). All significances are in relation to cellular viability at time 0.

These results indicate that viability was not compromised significantly in the temperature-controlled chamber that was used for degranulation studies.

6.2 Western Blot analysis of MPO-like and lactoferrin-like material from haemocytes of *G. mellonella* larvae

Haemocytes were isolated as described in section 2.9. In order to determine if there was material released from PMA stimulated haemocytes that was similar to neutrophil myeloperoxidase (MPO) and lactoferrin proteins, Western blot analysis was carried out on the precipitated degranulated fractions from the haemocytes.

MPO-like material was characterised in the sectretome of unstimulated and PMA stimulated haemocytes. Anti-human MPO antibodies were used in these haemocyte studies. It was found that at 0, 3 and 6 minutes in the unstimulated secretome the intensity was low, at 9 minutes the total intensity at the 59kDa and 13kDa ranges was higher, but not significantly. MPO-like material in the PMA stimulated haemocyte secretome increased significantly at 3 and 9 minutes post-PMA stimulation, when compared to the same unstimulated secretome time points (Figures 6.3 and 6.4). The MPO-like material that was detected in the haemocyte secretomes was subsequently analysed by LC- mass spectrometry and it was revealed to be prophenoloxidase (Fallon *et al.,* 2011).

The release of lactoferrin-like material from the isolated haemocytes was also studied. In the unstimulated haemocytes there was very little detected at 0, 3 and 6 minutes and at 9 minutes it was found that there was higher reactivity than the previous time points. When the haemocyte secretome was PMA stimulated there was a significant difference at 0, 3 and 9 minutes compared to the unstimulated secretome at the same time points (Figures 6.3 and 6.5).

These results demonstrate that there is similar proteolytic enzyme release from the haemocyte and neutrophil upon PMA stimulation.

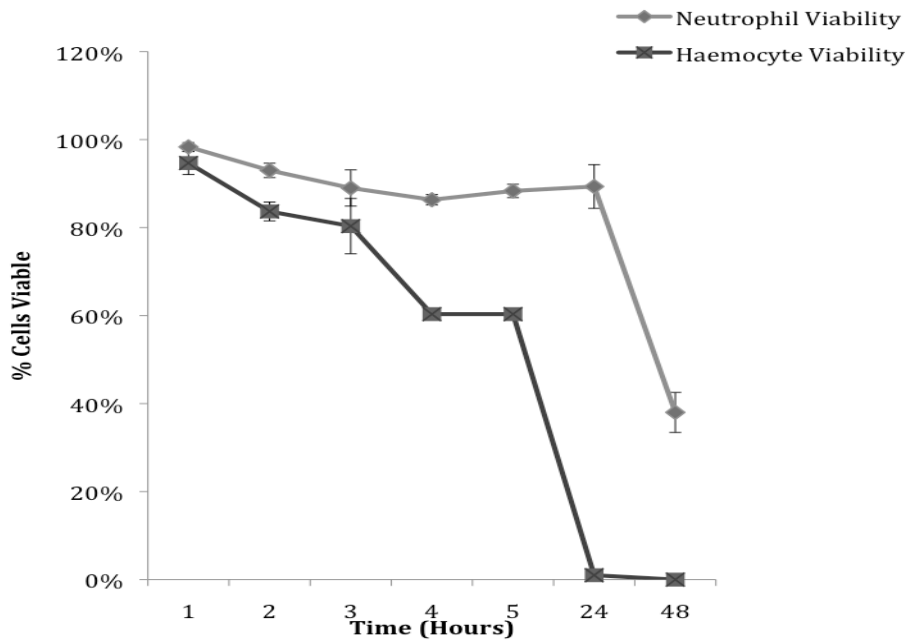


Figure 6.1 Viability of isolated neutrophils and haemocytes over 48 hours. Isolated cells kept at room temperature, no significant difference (p=1) (n=3).

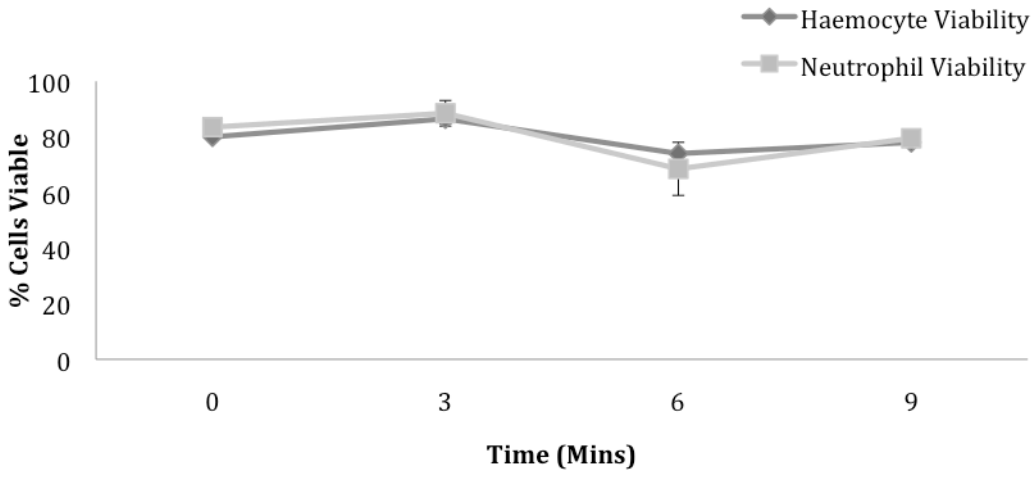


Figure 6.2 Viability of isolated neutrophils and haemocytes over 9 minutes in stirred chamber at 37° C, no significant difference (p=1) (n=3).

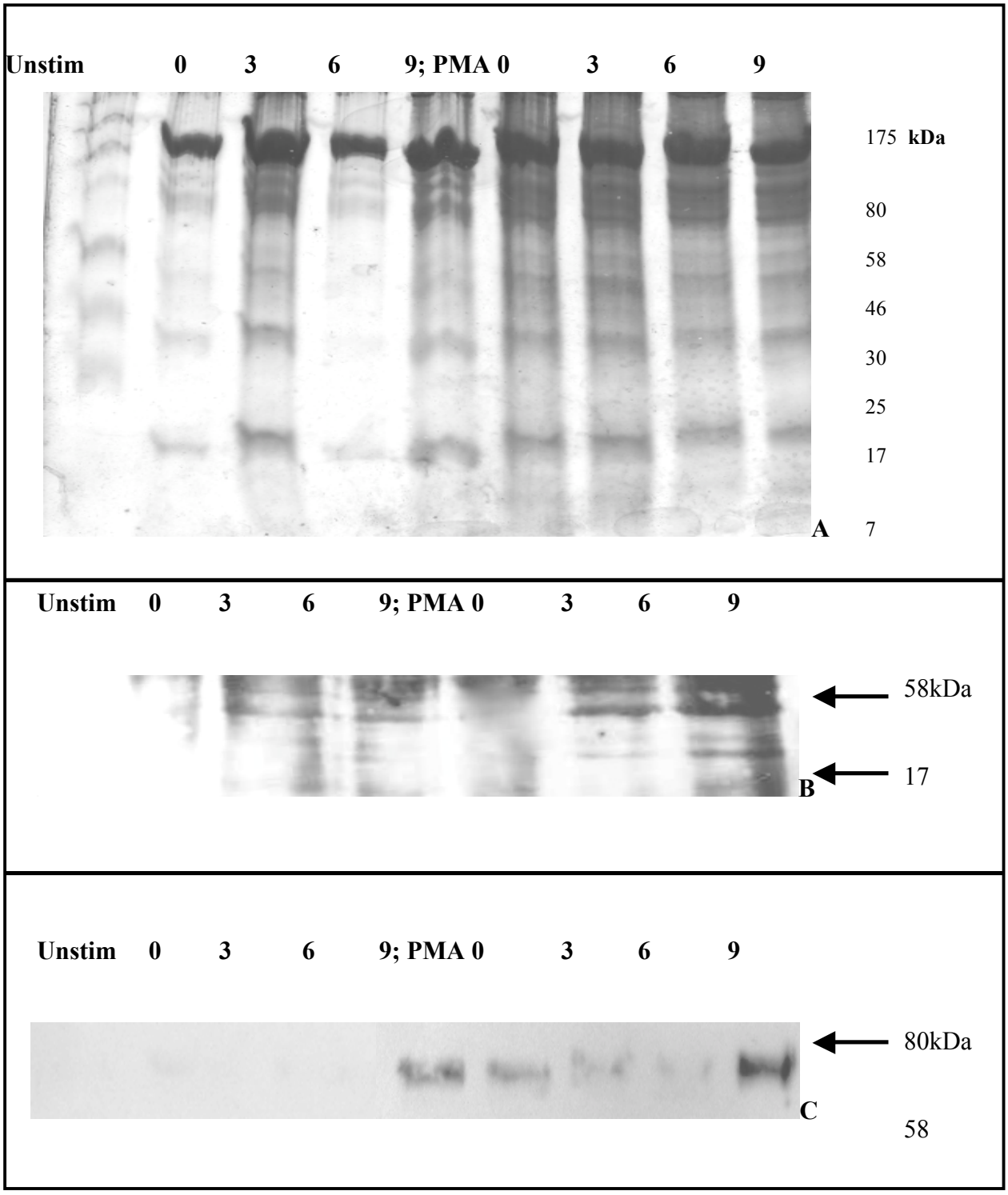


Figure 6.3 One-dimensional gel of unstimulated and PMA stimulated haemocyte secretomes at 0, 3, 6, and 9 minutes (A),
Western blot of MPO-like material from haemocyte secretomes (unstimulated and PMA stimulated) at 0, 3, 6 and 9 Minutes (B), Western blot of lactoferrin- like material from haemocyte secretomes (unstimulated and PMA Stimulated) at 0, 3, 6 and 9 Minutes (C).

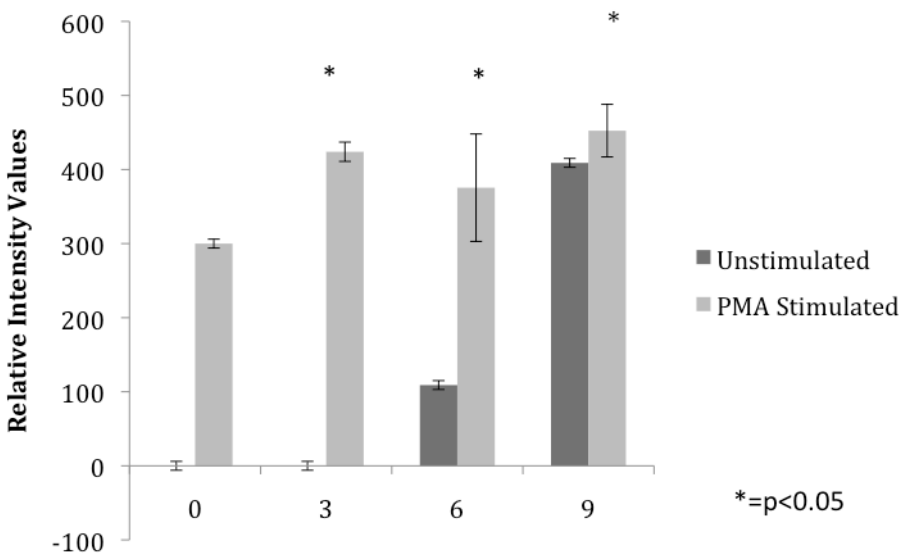


Figure 6.4 The relative intensity of the haemocyte MPO-like bands, analysis was carried out using Image J ® software. Significances are in relation to the unstimulated time points (T=0) (n=3).

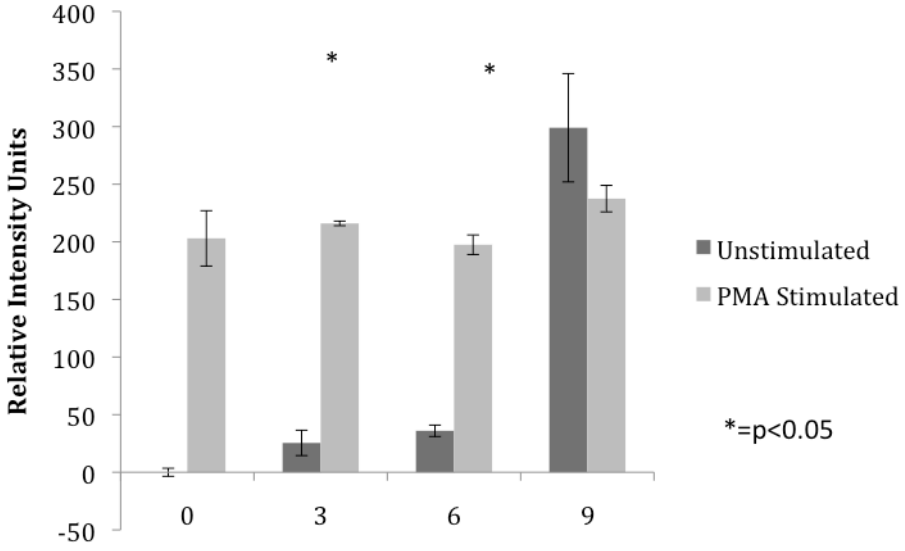


Figure 6.5 Graph demonstrating the relative intensity of the haemocyte lactoferrin-like band, analysis was carried out using Image J ® software. Significances are in relation to the unstimulated time point (T=0) (n=3).

6.3 Confocal immunofluoresence analysis of MPO-like material in haemocytes of *G. mellonella* larvae and a comparison to the human neutrophil

In order to visualise the presence of MPO-like material in the haemocyte and compare this to what was seen in the neutrophil, confocal immunofluorescence was carried out. It was found that upon PMA stimulation in both neutrophils and haemocytes, there was an increase in fluorescence of granule like bundles, which was very evident in neutrophils in Figure 6.6 and this is also evident in the cross section of the PMA stimulated haemocyte that is presented in Figure 6.7.

The intensity of the fluorescence in cells was examined by intersecting individual cells with a 20-micron bar and determining the fluorescence value in representative cells that were from different slides. The cells were analysed for the increase in fluorescence and it was found that fluorescence increased significantly in the neutrophil upon PMA stimulation, from 3293 ± 33 to 3797 ± 49 ($p<0.05$). In the isolated haemocytes, the intensity increased from 2748 ± 108 to 3026 ± 107, but this was not significant (Figure 6.6). The cross section of the PMA stimulated haemocyte that is presented in Figure 6.7 indicates that the MPO-like material detected in the haemocyte may be held on granules similar to the granule contents in the neutrophil (Heyworth *et al.*, 1989). These granules become activated and release their proteolytic enzymes if the immune cell is targeted by an opsonised microorganism or by a chemotactic activator such as PMA (Ai & Gilchrist, 2011).

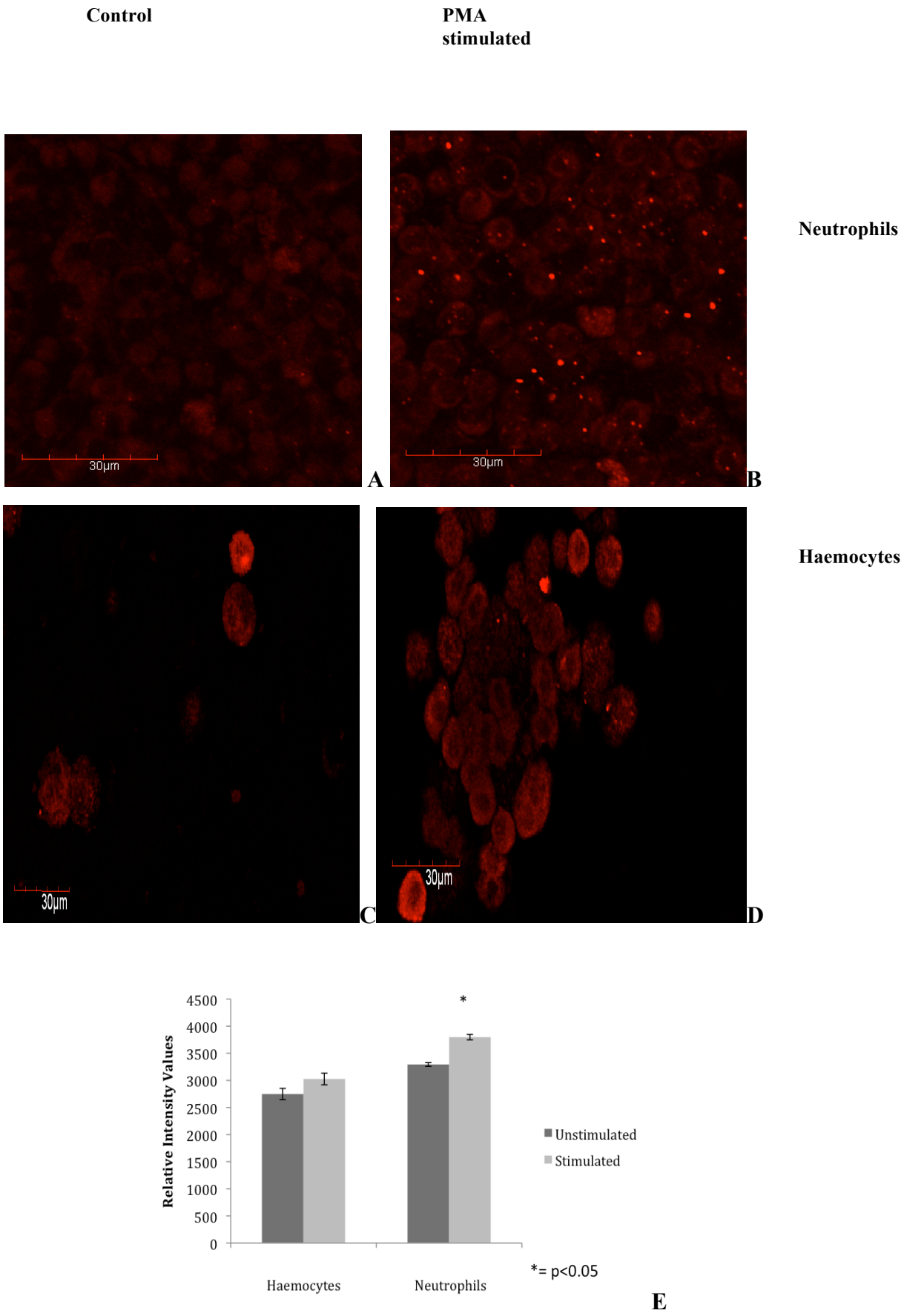


Figure 6.6 Confocal Immunofluoresence images of Anti-MPO antibody reactivity in unstimulated neutrophils (A), PMA (1µg/ml) stimulated neutrophils (B), unstimulated haemocytes (C), and PMA (1µg/ml) stimulated haemocytes (D). Graph showing relative intensity of fluorescence in the cells (E) ($p < 0.05$) (n=3).

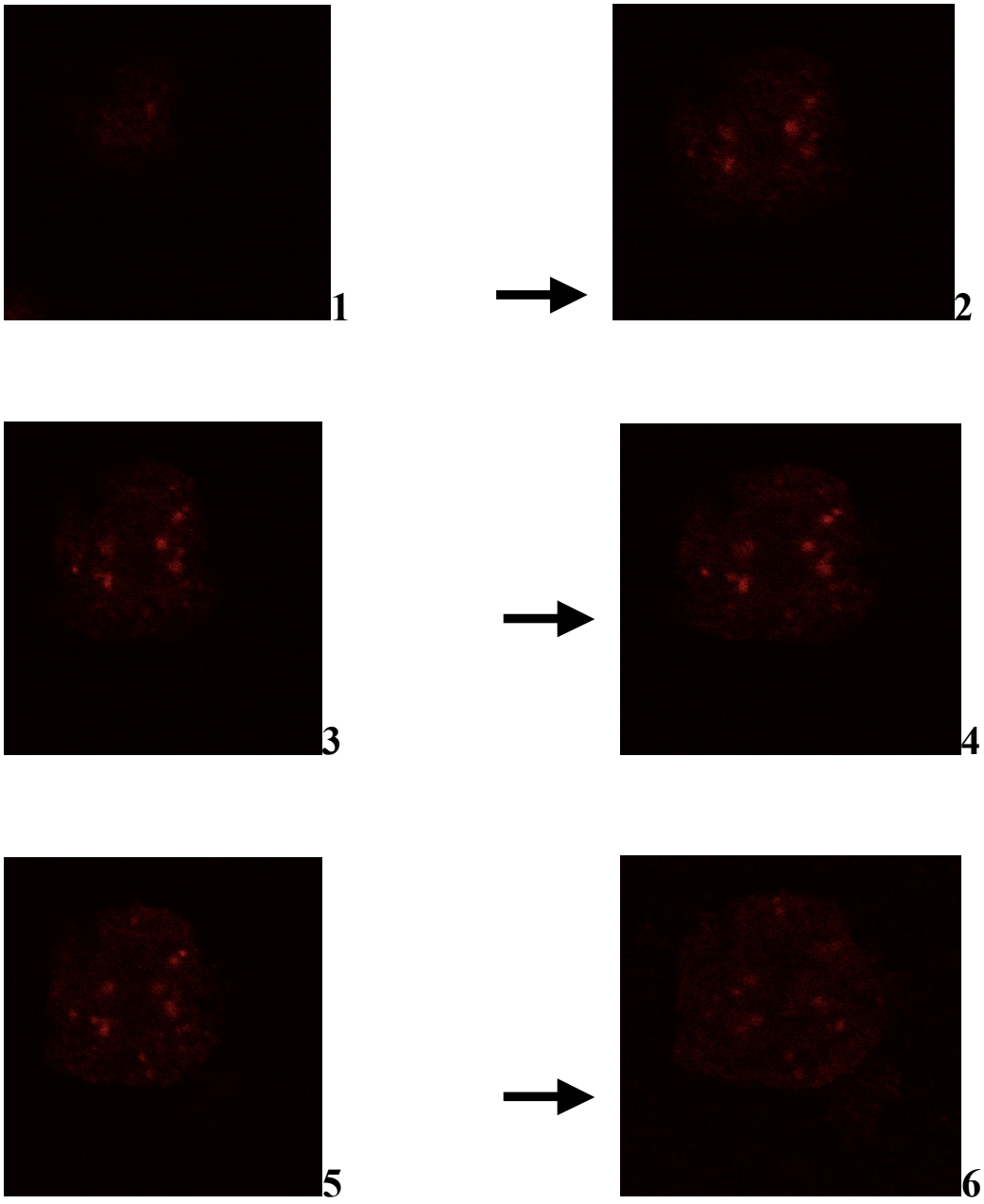


Figure 6.7 A series of images from a PMA stimulated haemocyte, where MPO-like material is evident in granules throughout the cell, 1= bottom of cell through to 6= top of cell.

6.3.1 Summary

The results presented in this section detailed the optimisation of the isolation of neutrophils and haemocytes and it was revealed that once cellular isolation takes place cells should be experimented with as soon as possible as viability decreases significantly after 4-5 hours. As degranulation studies were carried out in a temperature controlled chamber, it was decided to determine whether this had any effect on cellular viability and it was determined that there was no significant loss in cellular viability in haemocytes or neutrophils.

Degranulation was assessed by Westerm Blotting and confocal microscopy. MPO-like material was detected in haemocyte secretomes, with an increase in the release of this protein when the cells were PMA stimulated. Lactoferrin-like material was also detected and PMA stimulation also resulted in an increase in the secretion of this material.

Confocal immunofluoresence was employed to view MPO-like materail in the neutrophil and haemocyte and this demonstrated similar granules that were present in both cell types, again demonstrating similarities between the neutrophil and haemocyte.

6.4 Analysis of the peroxidase activity of haemocytes of *G. mellonella* larvae and of human neutrophils

MPO activity may be determined by employing the reagent TMB (Tetramethylbenzidine) as a substrate, (Andrews and Krinsky, 1982, section 2.13). Neutrophils and haemocytes were allowed to degranulate, as previously described in section 2.9.6.

The increases in activity relative to the activity at 0 minutes for neutrophils were found to be higher than those found for haemocytes. In unstimulated neutrophils the increase in % activity of peroxidase was 6.82 ± 1.57% at 3 minutes, 5.5 ± 1.65% at 6 minutes and 2.52 ± 0.5% at 9 minutes, relative to the peroxidase activity at 0 minutes. Neutrophils that were PMA stimulated demonstrated a peroxidase activity increase of 14.23 ± 0.07% at 3 minutes, 5.95 ± 1.64% at 6 minutes and 7.02 ± 2.5% at 9 minutes ($p=0.06$), relative to the activity of the cells at 0 minutes. There was an increase in peroxidase activity when PMA stimulated neutrophils were compared to unstimulated neutrophils, however it was not significant (Figure 6.8).

Unstimulated haemocytes were also analysed for peroxidase activity and it was found that at 3 minutes the % peroxidase activity was 1.62 ± 0.6%, however at 6 and 9 minutes there was no peroxidase activity increase relative to 0 minutes. The peroxidase activity for PMA stimulated haemocytes was also analysed, at 3 minutes and this was 3.3 ± 0.5% increased, at 6 minutes the activity was 7.07 ± 0.07% increased and at 9 minutes this increased to 10.46 ± 1.02% ($p=0.06$). There was an obvious increase between unstimulated and PMA stimulated haemocytes, as demonstrated in Figure 6.9.

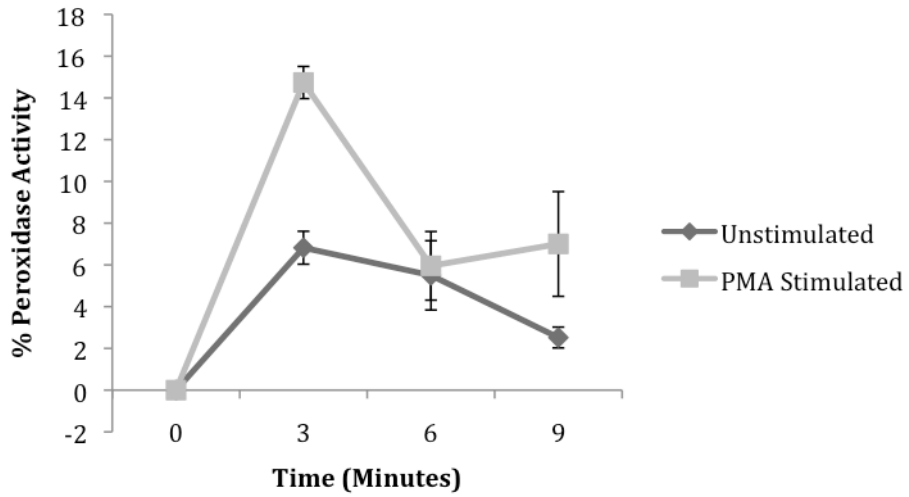


Figure 6.8 Peroxidase activity in unstimulated and PMA (1µg/mL) stimulated neutrophils, increase is relative to the control (n=3).

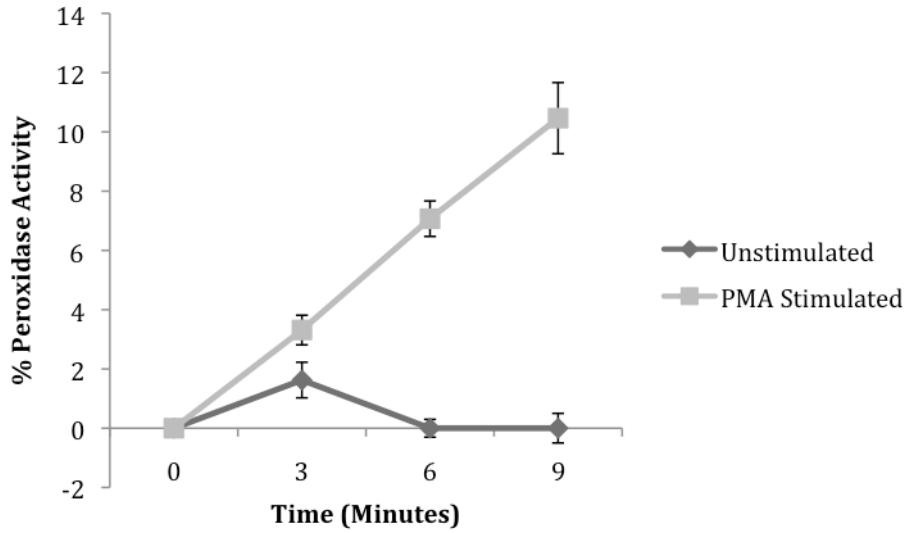


Figure 6.9 Peroxidase activity (%) in unstimulated and PMA (1µg/mL) stimulated haemocytes, increase is relative to the control (n=3).

6.5 Analysis of the distribution of MPO-like material in 3 sub-populations of *G. mellonella* larvae haemocytes

Chapter 5 detailed the analysis of proteins in the cytoskeletal architecture of the haemocyte by employing the technique of flow cytometry. This section details the study of the presence of MPO-like material in three sub-populations of haemocytes by utilising flow cytometry. The inhibitors cytochalasin b and nocodazole which were used in Chapter 5 were also utilised in this section, in order to determine whether they affected the MPO-like material in the negative manner that they affected coronin, vinculin, G and F-actin in Chapter 5.

As in the previous chapter, three sub-populations were analysed, which had variations in cellular size and granularity. The cells in Gate 1, which is the smallest population with regard to cell size and it is also the most granular, demonstrated 95.9 ± 0.085% of cells displaying MPO-fluorescence in the unstimulated control cells, 98.995 ± 0.005% in PMA stimulated cells ($p<0.05$), 97.79 ± 0.21% displaying fluorescence in cytochalasin b + PMA treated cells ($p<0.05$) and 97.8 ± 0.3% in nocodazole + PMA treated cells ($p<0.05$).

Gate 2 contained cells that were larger but not as granular as the cells contained in Gate 1. In this population of cells 96.9 ± 1.01% of cells displayed positive fluorescence in the unstimulated cells, 99.01 ± 0.7% cells displayed fluorescence in PMA stimulated cells, 99.1% cells were fluorescent in cytochalasin b + PMA treated cells, and 98.64% cells were fluorescent in nocodazole + PMA treated cells. There was no significance between the treatments and controls in this population.

The population of cells in Gate 3 were the largest sub-population analysed in terms of cellular size. Unstimulated cells demonstrated 98.52 ± 0.5% fluorescence, PMA stimulated cells demonstrated 99.11 ± 0.11% fluorescence, cytochalasin b + PMA treated cells displayed 99.28 ± 0.28% fluorescence and nocodazole + PMA demonstrated 99.115 ± 0.35% fluorescence.

As in the population of cells in Gate 2 there was not a significant difference between control cells and treatments. These data can be visualised in Figure 6.10. Figure 6.11 is a representative acquisition of the information from MPO activity analysis using the FACs Calibur® machine. There are significant differences in the population of cells in Gate 1 where PMA stimulation, cytochalasin b and nocodazole

treatments cause cell numbers displaying fluorescence to be significantly altered in this small and granular sub-set.

This data revealed that it is the smallest population of cells in terms of size and the most granular, which is the population that may be the most similar to the neutrophil with regard to secreting an MPO-like protein. These data reflect the cell sorting analysis that was demonstrated in Chapter 5.

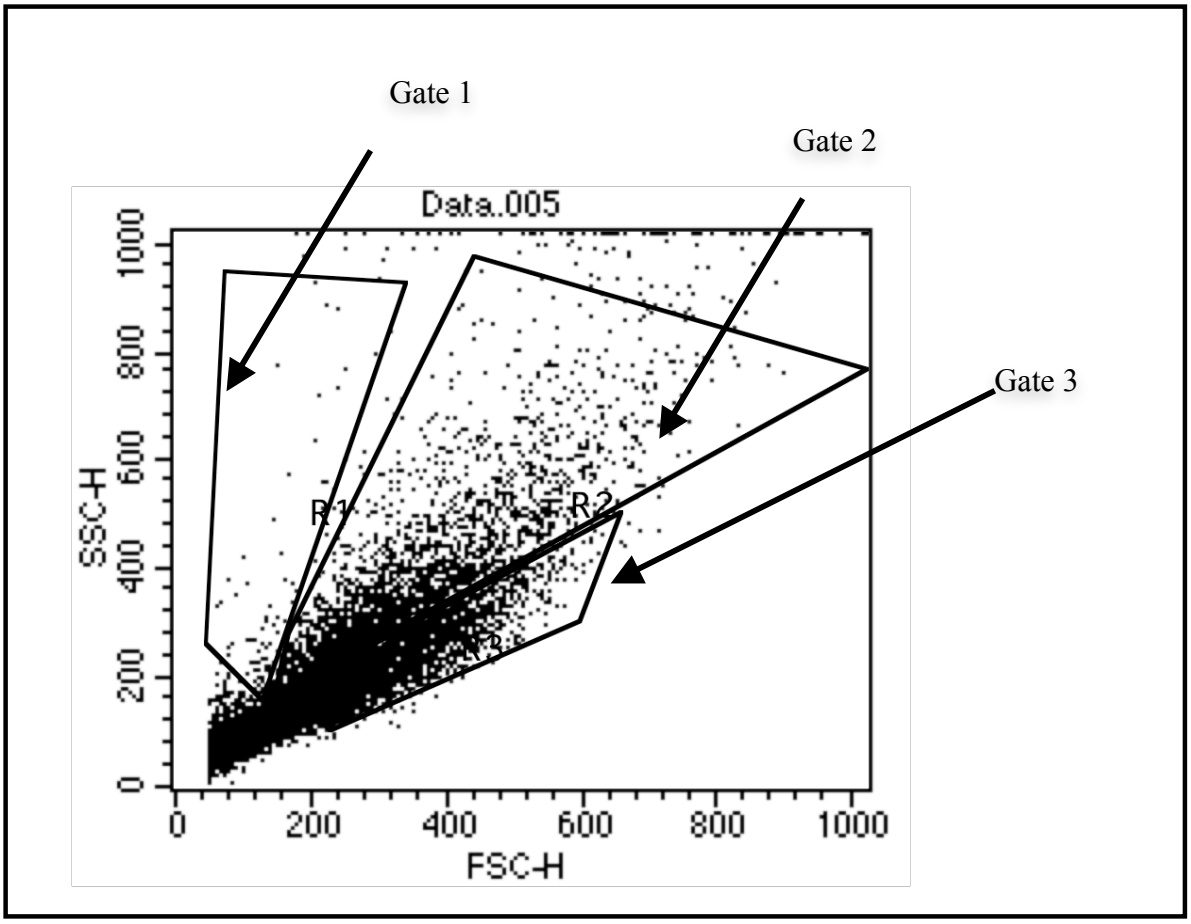


Figure 6.10 Representative FACs Calibur analysis of MPO-like material in the sub-populations of *G. mellonella* larvae haemocytes.

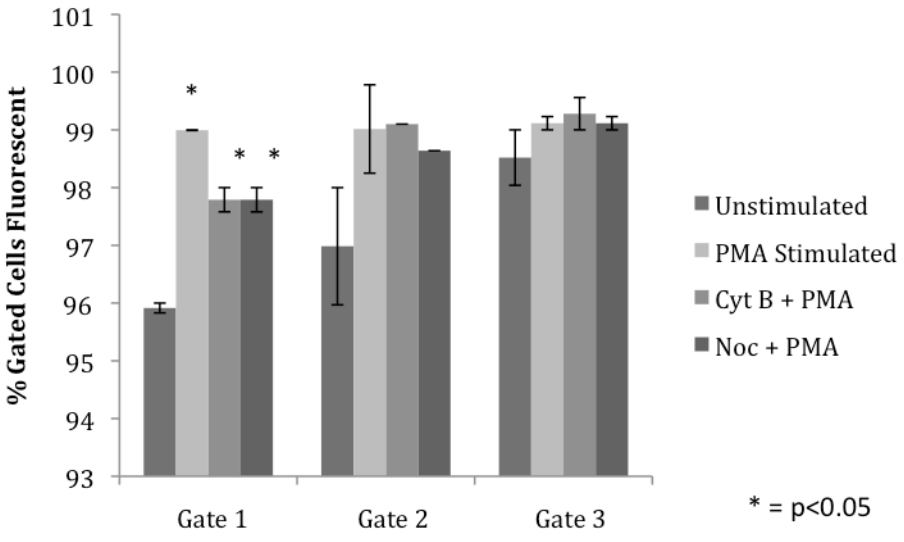


Figure 6.11 Analysis of the 3 gated populations of haemocytes of *G. mellonella* larvae displaying fluorescently tagged MPO-like material (n=3).

6.6 The optimisation of proteomic techniques for degranulation studies with haemocytes of *G. mellonella* larvae

To further analyse the secretome that is released from haemocytes upon PMA stimulation it was decided to perform 2D SDS PAGE coupled with LC Mass Spectrometry. It was first decided to compare two staining techniques: silver staining and colloidal coomassie staining. It was found that although silver staining required less quantity of protein to be loaded onto the Immobiline DryStrip, the procedure was extremely tedious and time consuming and further analysis of spots by LCMS proved difficult due to loss of peptides whilst reducing and alkylating the spots (Figure 6.12). Colloidal coomassie staining induced similar results, however it was not as laborious as the silver staining. It was therefore decided to continue the subsequent studies with coomassie staining protocols (Figure 6.13).

This optimisation step also allowed the clarification of an appropriate pH range to continue experimental studies with. It was found that the 24cm pH strips which were utilised with silver staining protocols made the SDS PAGE gels extremely brittle and the tendancy for the gels to break was a lot higher than with the 13cm strips that were utilised with colloidal coomassie studies in Figure 6.13. Due to the pinched peptide content in the centre of the pH 3-10 images, it was decided to continue studies with the smaller range of pH 4-7.

6.7 Analysis of the effect of cytochalasin b and nocodazole on protein release from PMA stimulated haemocytes

Cytochalasin b and nocodazole have previously been shown to inhibit the process of degranulation in the neutrophil (Bengtsson *et al.,* 1994; Volz *et al.,* 1993) and were therefore employed in this section to determine whether they negatively affected the haemocyte's ability to release peptides that are important in the immune response of *G. mellonella* larvae. Haemocytes were exposed to PMA to induce degranulation. The PMA image in Figure 6.14 was used as the reference image for spot picking and allowed a comparative analysis for the cytochalasin B + PMA and nocodazole + PMA treated haemocytes.

A number of peptides were identified and those that are outlined in this section show similarities to neutrophil proteins (Table 6.1). It was found that upon pre-treatment of the haemocytes with cytochalasin b, spot 1, which was identified as apolipophorin 3, which plays an essential role in the encapsulation reactions (Whitten *et al.*, 2011), was found to reduce release by 53%. Nocodazole pre-treatment reduced apolipophorin 3 secretion by 60%, relative to PMA stimulated haemocytes. Ferritin was identified as spot number 2. Nocodazole caused a 45% reduction in ferritin release and cytochalasin B caused a 33% inhibition of release of this protein. Apoferritin exists in the haemocytes of insects and is involved in iron sequesterisation (Locke *et al*., 1991). Spot 3 showed homology to serine protease and cytochalasin B caused a 22% decrease in the release of serine protease, while nocodazole caused a 10% decrease in release of this protein from haemocytes. Serine protease inhibition has been shown to cause an inhibition of neutrophil killing activity (Standish and Weisner, 2010). Spot number 4 was identified as a putative transferrin precursor and it's release was reduced by approximately 60% with both cytochalasin B and nocodazole treatment. Transferrin has been shown to be upregulated in the haemocyte of *C. quinquefasciatus* upon infection (Paily *et al*., 2007). Triosephosphate isomerase (spot number 5) is an enzyme that is associated with neutrophil phagosome formation (Burlak *et al.,* 2006), it's release was inhibited by 10% and 18% by cytochalasin b and nocodazole treatment, respectively. It is enriched in the neutrophil phagosome and is essential for microbial uptake. The decrease in expression that was demonstrated with cytochalasin b and nocodazole treatment would reduce the efficacy of the cell (Ballarin *et al.*, 2008).

Juvenile hormone binding protein was identified as spot number 6 and is involved in larval maturation in insects (Chen *et al*., 2009). It was found to be decreased in secretion by 21% and 8% by cytochalasin b and nocodasole treatment, respectively. Spot 7 showed homology to aldo keto reductase, which is released upon degranulation by neutrophils and is responsible for detoxifying the cell (Rakita *et al.*, 2010). Haemocytes treated with nocodazole and cytochalasin B demonstrated a 21% and 9% inhibition of release, respectively, as demonstrated in Figure 6.14.

Spot number 8 showed homology to enolase, which is located on the neutrophil cytoskeleton and released upon degranulation (Xu *et al.*, 2009). The release of this enzyme from haemocytes was inhibited by 31% and 18% by pre-treatment with cytochalasin B or nocodazole, respectively. These results indicate

that cytochalasin b and nocodazole retard the degranulation of important immune-proteins from the haemocyte and thus negatively affect degranulation (Figure 6.15).

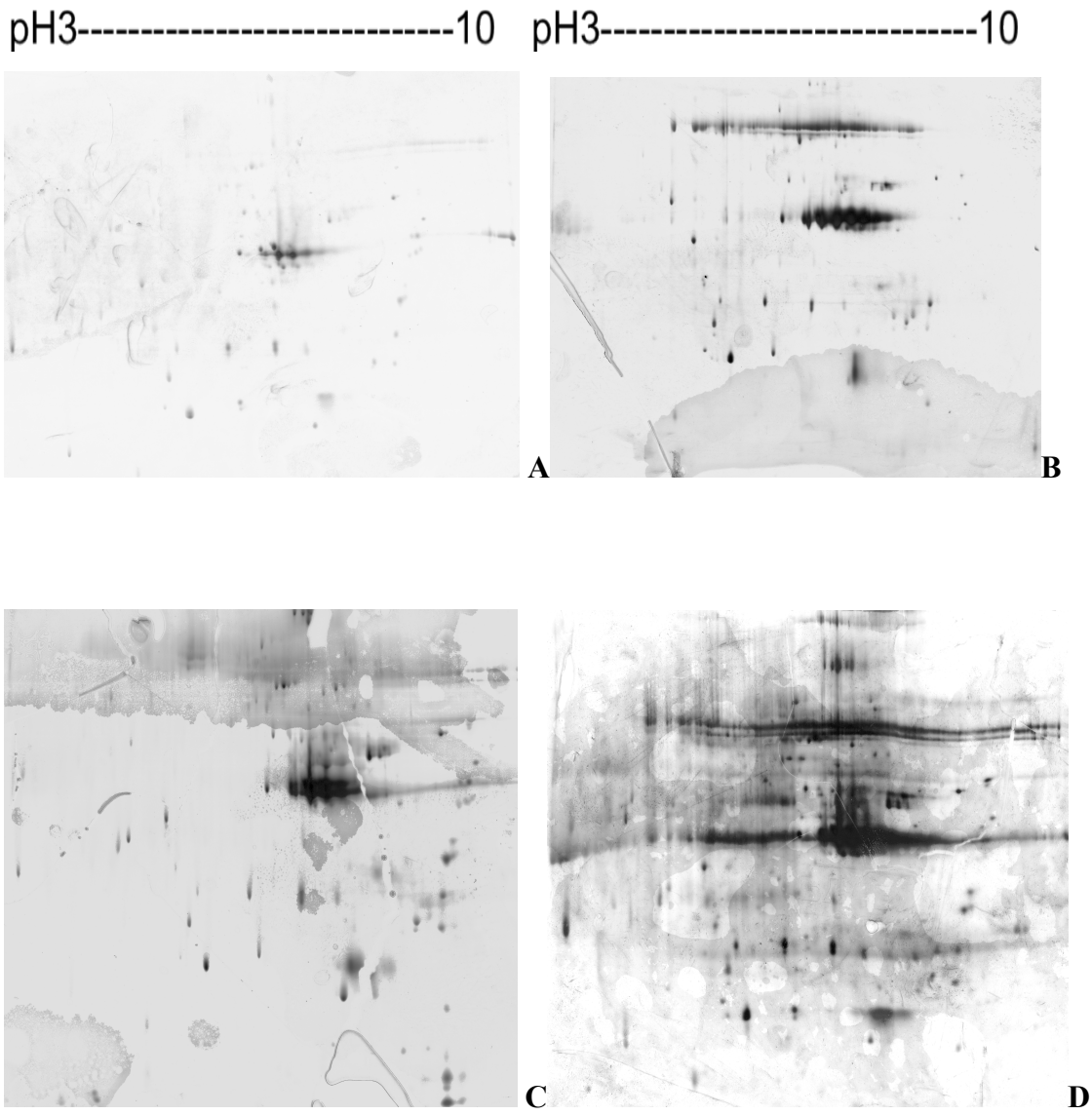


Figure 6.12 Silver stained SDS gel images of PMA stimulated isolated haemocyte secretomes at (A) 0 Minutes, (B) 3 Minutes, (C) 6 Minutes and (D) 9 Minutes using 24cm pH 3-10 Immobiline DryStrips (n=2).

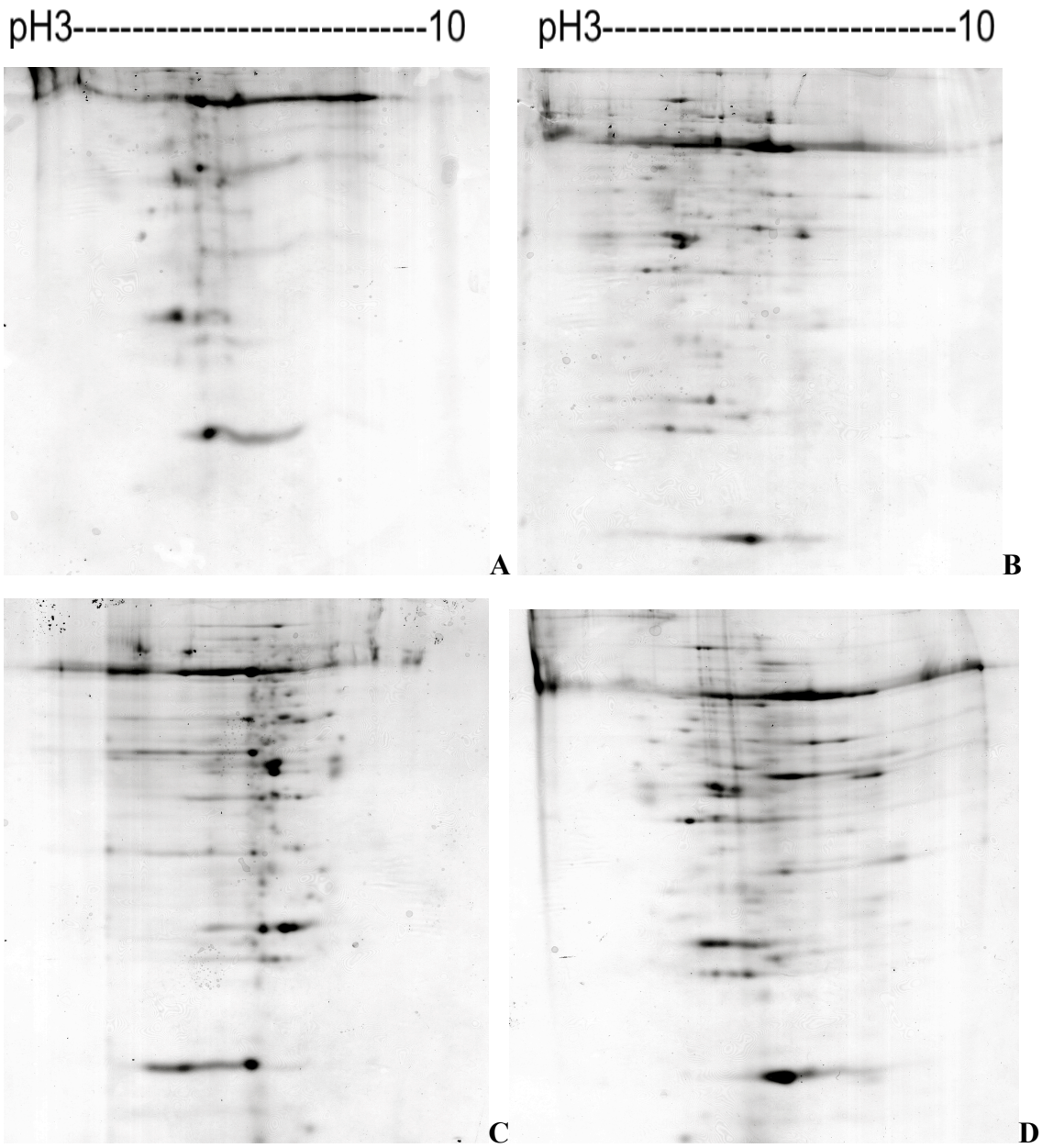


Figure 6.13 Colloidal coomassie stained SDS gel images of PMA stimulated isolated haemocyte secretomes at (A) 0 Minutes, (B) 3 Minutes, (C) 6 Minutes, (D) 9 Minutes using 13cm pH 3-10 Immobiline Dry Strips (n=2).

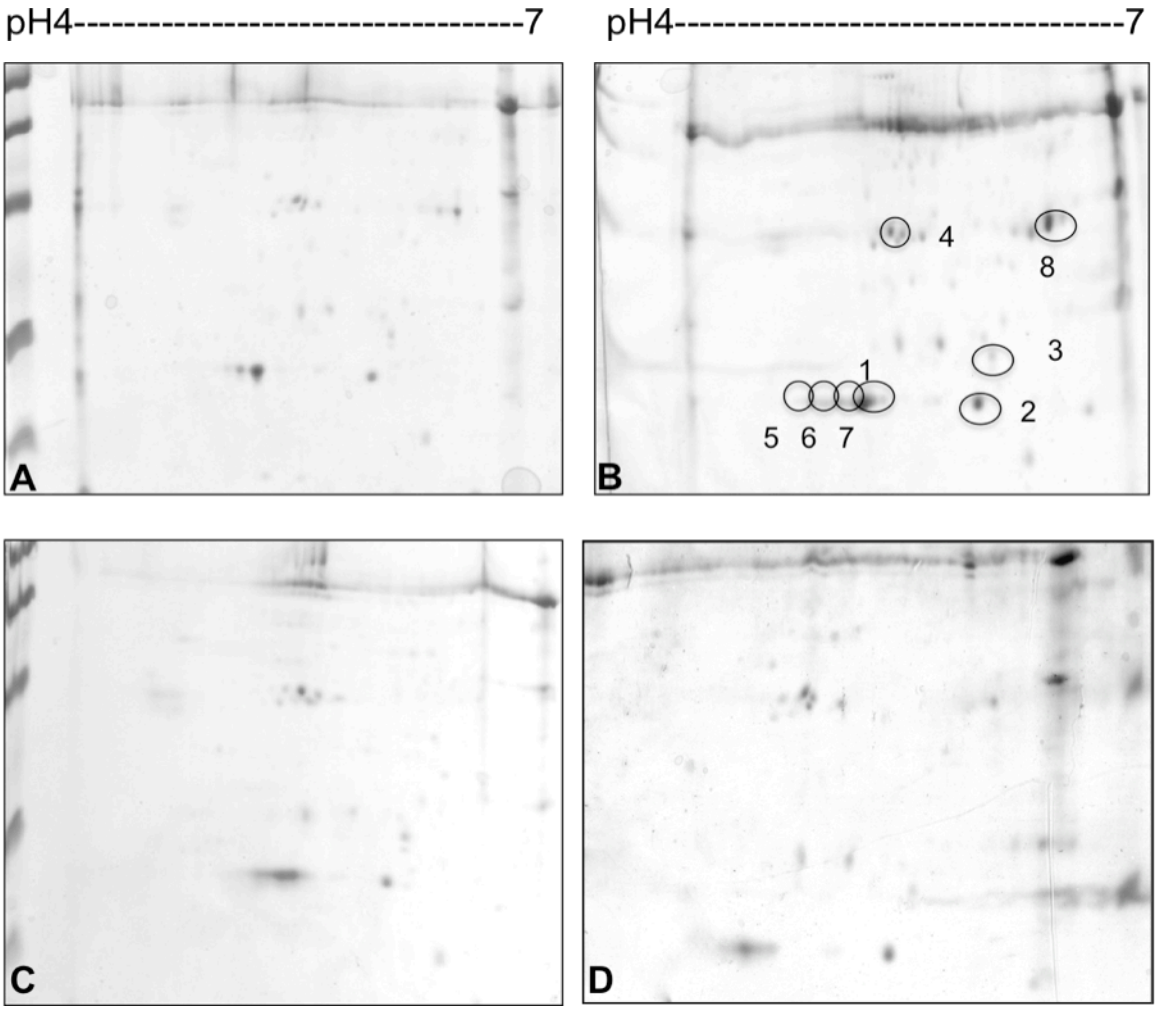


Figure 6.14 Two-dimensional gels of peptides released from *G. mellonella* haemocytes. Haemocytes were induced to degranulate and the secretomes were compared.

(A) Unstimulated control, (B) reference PMA stimulated, (C) cytochalasin b + PMA and (D) nocodazole + PMA (n=3).

Spot No:	Protein Identity	Organism	Mr	PI	Score	Sequence Coverage	Accession No
1	Apolipophorin 3	*Galleria mellonella*	20498	8.59	299	54%	P80703
2	Ferritin	*Galleria mellonella*	77238	5.69	554	39%	AAG41120
3	Serine Protease	*M musculus*	26802	4.75	105	8%	NP_44473
4	Transferrin Precursor	*G. mellonella*	77212	6.76	159	5%	AA06379
5	Triosephosphate isomerase	*C. pomonella*	30288	5.57	105	15%	ABV81297
6	Juvenile Hormone Binding Protein	*G. mellonella*	23627	5.57	320	32%	AF413772
7	Aldo Keto Reductase	*M. musculus*	27488	5.52	86	2%	BAA8687
8	Enolase	*B. morii*	47000	6.5	121	9%	AB27388

Table 6.1 List of peptides isolated, digestion and identified from Reference PMA gel and identified using LC- Mass Spectrometry.

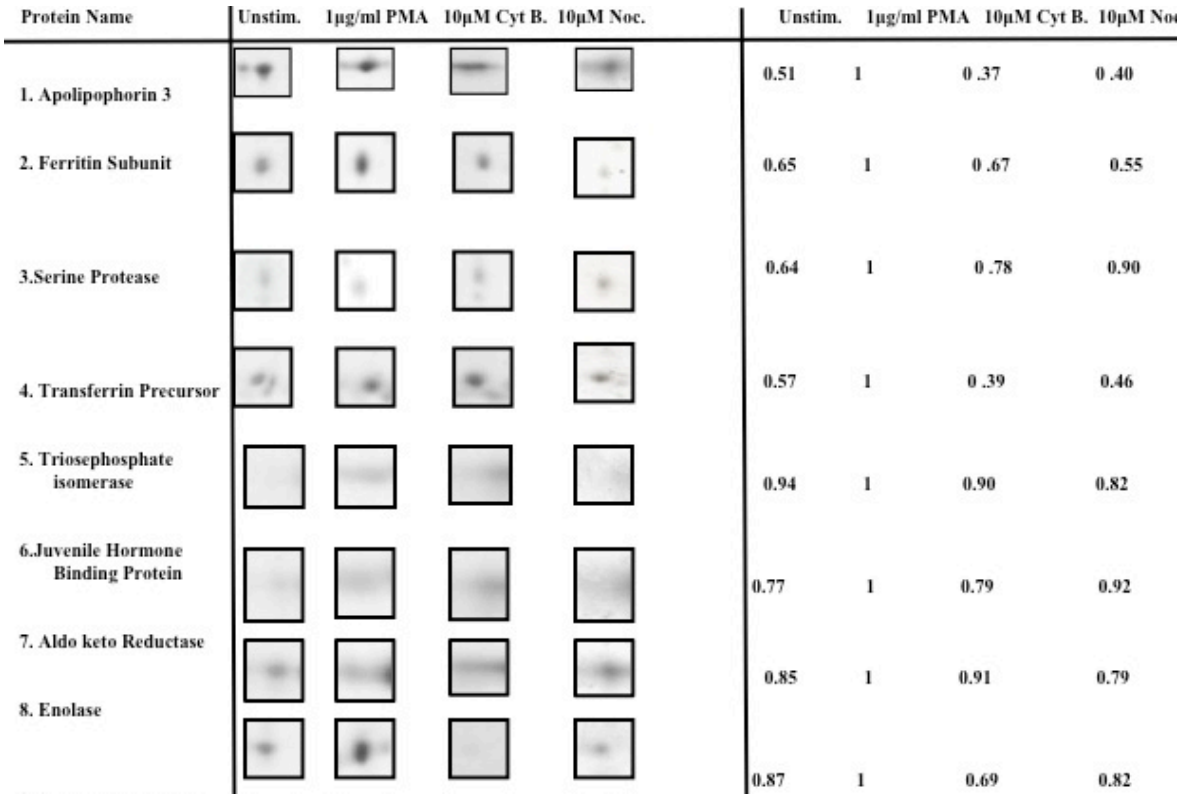


Figure 6.15 Relative expression of selected peptides released from *G. mellonella* haemocytes. Spots 1-8 refer to spots marked in Figure 6.14 (B).

6.7.1 Summary

This section demonstrated that there was an increase in peroxidase release from the PMA stimulated neutrophils and haemocytes compared to the unstimulated cells, over the 9 minutes. Thus, indicating that the haemocyte does release a similar protein to the well-characterised neutrophil MPO. Flow cytometry was also utilised to study the presence of MPO in haemocyte sub-types. This method demonstrated significantly more MPO-like material in the smallest and most granular subset of haemocytes in *G. mellonella* larvae. This coincides with the flow cytometric analysis contained in Chapter 5 that detailed this sub-set of haemocytes to potentially be the most similar haemocyte population to the neutrophil. It was shown that the pre-treatment of haemocytes with the inhibitors cytochalasin B and nocodazole prior to PMA stimulation negatively influenced immune protein release from the haemocyte in a manner that is similar to neutrophil degranulation. A number of these putative peptides bear similarities to neutrophil proteins which are released upon degranulation. These results indicate further similarities between neutrophils and haemocytes.

6.8 Discussion

The importance of the neutrophil in the human immune defence system's response is underlined by the fact that they are the first cells to arrive to the site of bacterial or fungal infections (Stie and Jesaitis, 2007). Once the pathogen is engulfed by the neutrophil, within the phagosome, the neutrophil releases an avalanche of toxic agents, including proteases, hydrolytic enzymes and oxidants in order to destroy the pathogen (Gabrilovich, 1999). Neutrophils are rapidly mobilised from the bone marrow and become activated when appropriate signals are received. These signals can include cytokines (TNF-α, IL-1), chemokines (IL-8), bacterial proteins, complement proteins or other inflammatory stimuli (Bajt *et al.,* 2001).

Upon arrival to the scene of infection, a number of neutrophil processes become activated including phagocytosis of the opsonised particle, degranulation of proteolytic enzymes and the production of superoxide, all of these processes will result in microbial death (Sengelov *et al*., 1993). The neutrophil contains distinct granule subsets, which have differing functions in terms of microbial killing. Two thirds of neutrophil killing is mediated by the release of granules, while one third is mediated by the NADPH oxidase (Bokoch, 2009). The granule content is comprised of primary granules (azurophilic), secondary granules (specific) and tertiary granules (secretory). There are also storage granules located in the cell cytoplasm (Stie and Jesaitis, 2007). Upon activation of the granules, the fusion of granule membrane with neutrophil plasma membrane results in the exocytosis of the proteolytic enzyme contents (Sengelov *et al.,* 1993).

These enzymes include myeloperoxidase (MPO), which is contained in the primary granules and can comprise almost 5% of total neutrophil protein content. MPO catalyses the reaction of H_2O_2 with chloride ions, thus producing the highly antiseptic hypochlorous acid (HOCl) (Test *et al.,* 1984). The secondary granules contain the iron binding protein lactoferrin, lysozyme and collagenase along with many other antimicrobial agents (Borregaard *et al.,* 1995). Wang *et al*., (1995) identified lactoferrin as being capable of inactivating the LPS content of invading microorganisms.

Prior to the analysis of MPO and lactoferrin activity in the haemocyte, this Chapter investigated the viability of isolated neutrophils and haemocytes *in vitro* with a view to characterising an optimum time during which experiments could be

performed. It was found that with neutrophils there was a significant loss in viability 4 hours after isolation, while haemocytes also demonstrated a significant loss in viability 4 hours following isolation. This section also demonstrated that the neutrophil and the haemocyte could be induced to degranulate in a 37°C stirring chamber without loss of viability which would have compromised the experimental results.

Due to the significance of MPO activity in the neutrophil's ability to kill, it was decided to investigate whether a comparable enzyme existed in the haemocyte. MPO is extremely versatile and has been shown to possess peroxidase, catalase, superoxide dismutase, oxidase and DNA binding activities in the neutrophil (Humphries *et al.,* 1989). Using a combination of Western Blotting and confocal immunofluoresence microscopy, this Chapter sought to identify if such activities (Humpries *et al.,* 1989), occurred in the haemocyte secretome. The haemocytes were induced to degranulate using PMA, (Figure 6.3). The haemocyte secretomes demonstrated significant increases in expression of these proteins upon stimulation with PMA compared to unstimulated haemocytes (Figure 6.4). PMA has been shown to stimulate increases in MPO degranulation from neutrophils by activating protein kinase C (Fallon *et al*., 2010; Klink & Sulowska, 2007). The region displaying reactivity in the haemocyte sectretomes was subsequently identified using LC-Mass Spectrometry as prophenoloxidase (PPO), with a mascot score of 501 and sequence coverage of 24% by Fallon *et al.,* (2011). This indicates that the significance of MPO in the neutrophil is comparable to the significance of PPO in the haemocyte. In fresh water crayfish and shrimps it has been demonstrated that there is release of a protein called peroxinectin from haemocytes, which is involved in the encapsulation process, which is a mediator of insect immunity (Gillespie *et al.*, 1997). Peroxinectin appears to work in parallel with PPO and has been identified as an MPO homolog (Lin *et al.,* 2007). Lin *et al.,* (2007) also demonstrated similarities between activation of peroxinectin and the activation of phenoloxidase. The results presented in Chapter 4 correlate with this finding, where the protein content of the haemocyte was investigated and identified prophenoloxidase as a significant component of the haemocyte.

To visually analyse and compare the neutrophil and haemocyte with regard to MPO-like material, confocal immunofluoresence was employed. It was shown in Figure 6.6 that PMA stimulation of the haemocytes produced comparable results to

those that occur in the neutrophil. The visualisation of granules in the haemocyte upon PMA stimulation also revealed the possibility of similarity to the neutrophil granules where the proteolytic enzymes are contained until cellular stimulation. Interestingly the level of fluorescence in the neutrophils and haemocytes were similar upon PMA stimulation (Figure 6.6). When the neutrophil becomes activated, the respiratory burst that occurs and leads to the release of reactive oxygen species, indirectly leads to a change in the membrane potential, which activates the granules to release their contents (Klink and Sulowska, 2007), confocal immunofluoresence images in this section indicate that similar events are occurring in the haemocyte and the neutrophil.

Detailed flow cytometric analysis was also performed in Chapter 5 and this was developed further in this Chapter where the presence of MPO-like material was studied in the three sub-populations that had been analysed in the previous Chapter. In the final section of Chapter 5 it was hypothesised that the smallest sub-population of haemocytes in *G. mellonella* larvae is the most similar to the human neutrophil. Interestingly it was this sub-population that was found to demonstrate significant differences in positive MPO-fluorescence between PMA stimulated haemocytes, cytochalasin b + PMA treated haemocytes and nocodazole + PMA treated haemocytes, compared with the unstimulated, control haemocytes. It is this population, which was the smallest sub-population and was the most granular (Figure 6.10). Two other cell populations were also analysed (gate 2 and gate 3) and these did not demonstrate significant differences in MPO expression. PMA stimulates neutrophil granules to release their contents and is extensively used as an artificial stimulant (Grogan *et al*., 1997; Bylund *et al.*, 2004). The results in presented in this section demonstrate that PMA affects the release of MPO-like material from the haemocyte in a similar manner to release of MPO from the neutrophil.

The activity of peroxidases was studied by employing the substrate TMB (3,3', 5,5'-tetramethylbenzidine). TMB is routinely used in neutrophil studies to quantify or assay MPO activity experimentally *in vitro* (Bozeman *et al.,* 1990; Van der Vliet *et al.,* 2000). TMB is also implicated in the study of the insect immune response and has been utilised to assay PPO activity (Guangyou *et al.,* 1999). This Chapter identified the increase in peroxidase activity upon PMA stimulation in the neutrophil and the haemocyte (Figures 6.8 and 6.9). This was demonstrated to a

lesser extent in the haemocyte. The reason for this may be due to the fact that the haemocyte sub-sets analysis revealed in Chapter 5 demonstrated the smallest population (with regard to size) to potentially be more closely related to the neutrophil. It is possible that this subset is the only sub-set producing peroxidase like activity and would result in lesser activity values in an assay. An efficient cell sorting system would clarify as to whether this is the case.

As mentioned previously, lactoferrin is an iron binding protein, held in secondary bundles in the neutrophil (Stie and Jesaitis, 2007). Lactoferrin has anti-microbial as well as anti-inflammatory properties and it belongs to the transferrin family (Wong *et al.,* 2009). PMA has been demonstrated to increase levels of lactoferrin release from neutrophils (Standish and Weisner, 2009). PMA was also demonstrated to increase the prominence of a transferrin precursor in Chapter 4 (section 4.4) in the haemocyte and in the final section of this Chapter (section 6.7). The results indicate that an increase in lactoferrin-like material release from the haemocyte upon stimulation with PMA, in a manner similar to the neutrophil and was significant when compared with the unstimulated, control haemocytes (Figures 6.3 and 6.5).

The apparent differences in the rate of secretion of lactoferrin and MPO from the neutrophil is most likely due to differential kinetics in terms of the granule release. Segal *et al*., (1980) investigated the kinetics of release and found that lactoferrin was secreted quicker when compared to MPO release, however the relationship between the primary and secondary vesicles is extremely close with regard to the degranulation time. It is likely that there are similar kinetics occurring in the haemocyte when the cell is activated.

Two dimensional electrophoresis was also employed to further analyse the content of the secretome from the PMA activated haemocyte. However, optimistaion was first investigated with regard to the pH range that would be studied and also what would be the most efficient staining technique. To determine the pH range that would allow the better identification of peptides, a broad pH 3-10 range was examined. This saw the localisation of most of the peptides in the centre of the gels, and therefore it was decided to continue with the narrower range of pH 4-7. Comparative analysis of silver staining and colloidal coomassie blue staining was also performed. Despite the heightened sensitivity with silver staining, it was found to be less efficient than coomassie

staining in these studies. Silver staining also proved difficult when proceeding to identify the spots. The practical advantages when using colloidal coomassie were evident and are outlined in Anderson *et al*., (2005) where colloidal coomassie staining is described as being (1) cost effective (2) fast in terms of being non time consuming (3) highly reducible and (4) the reagents are reusable. Colloidal coomassie staining is still cited as being the most widely used protein detection method (Westermeler, 2006). The reliability with regard to mass spectrometry compatibility when the gels were stained with colloidal coomassie blue also made the technique more attractive than silver staining (Figure 6.12 and 6.13). The toxicity of silver nitrate and the problems faced when disposing the silver reagents are also disadvantageous (Ratte, 2009). Upon determining the optimum methods for secretome characterisation, it was decided to compare the secretome of haemocytes that had been pre-treated with nocodazole or cytochalasin B prior to PMA stimulation with those that were PMA stimulated and the control haemocytes (which were unstimulated). It was found that upon pre-treatment of haemocytes with cytochalasin B and nocodazole, the secretion of a number of putative peptides was reduced. Nishida *et al*., (2005) found that the pre-treatment of mast cells with nocodazole not only inhibited microtubule formation but also found that degranulation was down regulated. Nocodazole treatment of the neutrophil has also been demonstrated to inhibit degranulation, underlining the importance for microtubule formation in degranulation and destruction of foreign particles (Mollinedo *et al.,* 1989). Cytochalasin b has also been shown to negatively affect degranulation (Mayo *et al.,* 1997; Tschesche *et al.,* 1994).

When the SDS PAGE gels were compared to each other, with the PMA stimulated haemocyte gel being the reference gel, a number of peptides were identified as being down-regulated in terms of secretion. These included apolipophorin 3 (spot number 1), which has been cited as forming an important component of the insect immune system (Gupta *et al.,* 2010). The putative protein ferritin was also inhibited (spot number 2). Serine protease was identified as spot number 3, it is an important factor in the Prophenoloxidase cascade (Strand, 2008) but serine proteases are also crucial in the neutrophils ability to kill targets such as *Staphylococcos aureus* (Standish and Weisner, 2009). Transferrin precursor was identified as spot number 4. If the haemocyte is

unable to secrete sufficient amounts of transferrin, the ability of the immune cell to kill is decreased (Yoshiga *et al.*, 1999).

Triosephosphate isomerase (TPI) is involved in the maturation of the neutrophil's phagosome, an essential element of the killing process (Burlak *et al.,* 2006), it was shown to be secreted by PMA stimulated haemocytes but on pre-treatment of the haemocyte's with nocodazole or cytochalasin B, TPI was shown to be reduced in secretion (spot number 5). Juvenile hormone binding protein was identified as spot number 6 and has been demonstrated to be localized in the haemocyte of the moth *Trichophisia ni* upon viral infection (Booth *et al.,* 1992). Spot number 7 was Aldo Keto Reductase (AKR), an enzyme that is involved in the production of myeloperoxidase in the human neutrophil (Rakita *et al.,* 1990).

Enolase (spot number 8) is an important glycolytic enzyme and has been associated with the neutrophil cytoskeleton (Xu *et al.*, 2009). Decreased levels of secretion when the haemocyte is pre-treated with cytoskeletal inhibitors reflects another neutrophil-haemocyte parallel (Figure 6.15).

The results presented in this section strongly indicate that cytochalasin b and nocodazole affect the haemocyte in a manner that inhibits the secretion of important immuno-relevant proteins, this process is similar to the well-established process of degranulation in the human neutrophil.

6.9 Conclusion

The results presented in this Chapter investigated the process of degranulation in the haemocyte of *G. mellonella* larvae and compared the events to what is known to occur in the human neutrophil. It was found that MPO-like material and lactoferrin-like material are released from the haemocyte with similar kinetics to the human neutrophil. The peroxidase activity in the haemocyte and neutrophil were increased when both cell types were stimulated with PMA.

The results detailed the differences in haemocyte sub-populations in haemocytes of *G. mellonella* larvae and the potential for one of these to be more

similar to the neutrophil. The results also demonstrated that the smallest sub-population had the most significant differences in human MPO-like expression.

It was also demonstrated that the cytoskeletal inhibitors cytochalasin B and nocodazole inhibit the sectretion of important proteins from the haemocyte, similar to what has been demonstrated in the neutrophil upon treatment with these inhibitors.

Chapter 7

General Discussion

7.0 General Discussion

This project sought to establish a series of parallels between the haemocyte of *G. mellonella* larvae and the human neutrophil. In addition to this, this work sought to further characterise the haemocyte of *G. mellonella* larvae in a bid to advance the understanding of the cellular infrastructure and to distinguish and identify sub-types of haemocytes in the larva. The reasoning behind this project's rational was due to the recent emergence of *G. mellonella* larvae as alternative models in research and industry. They are now commonly used in the study of pathogen virulence (Aperis *et al*., 2007; Mukherjee *et al*., 2010), to study drug toxicity and in the development of novel therapeutics (Ballarin *et al.,* 2008). The hypothesis was that if there are a number of defined similarities between the human immune cell and the immune cell of *G. mellonella* larvae, the Lepidopteran model is an efficient alternative model organism, which may be used in research rather than ethically challenging mammalian models.

When researching with a mammalian host or model there are the obvious ethical concerns that need to be dealt with (Sully and Bidochka, 2006), but aside from these ethical negativities there are further inconveniences with regard to the experimental cost, the time consumed during studies and the high maintenance concerns (Willmann *et al*., 2009). The use of insects is becoming a more appealing alternative due to the fact that they are much less expensive, there are fewer ethical issues, insects can be used in high numbers and the time required for pathogenicity or toxicity testing of a microbe or substance is a lot quicker, thus yielding faster results. It is for these reasons that *G. mellonella* larvae have been utilised for measuring the virulence of bacterial (Mukherjee *et al.,* 2010), fungi (Fuchs *et al*., 2010) and yeast (Bergin *et al.,* 2005) and to study novel therapeutic/compound toxicity or efficacy (Rowan *et al*., 2009). Their use is increasing, as is evident by the increasing numbers of citations of *G. mellonella* larvae as experimental models (Mylonakis and Aballay, 2005).

However it was evident from the literature that there were many deviations from a set of standard conditions with regard to experimental protocol (Barabas and Crtynska, 2011; Bergin *et al*., 2005). It was therefore decided to study a set of variables that may lead to different experimental outcomes (Chapter 3), these were food, starvation period and larval weight. In order to understand how these may

affect a result, larvae were infected with the fungal pathogens *A. fumigatus* and *C. albicans.* Here it was noted that the nutritional status of the larvae was of vital importance prior to a study that would be carried out using *G. mellonella* larvae. The faster rate of larval death that was observed in the nutritionally challenged larvae indicated that this does have an impact on the experimental results. To further analyse the effects of food source on *G. mellonella* larvae's susceptibility to infection, the haemolymph was proteomically studied by utilising 2-dimensional electrophoresis. The reason for this is that the haemolypmh is the primary source of immune activators in *G. mellonella* larvae, harboring the circulating immune cell, the haemocyte, and many antimicrobial peptides (Gillespie *et al.,* 1997). It was determined in Chapter 3, that despite the activity of the haemocyte or haemocyte numbers not being affected by the removal of food, the expression of immue-relevant proteins, such as serine protease (Brown *et al.,* 2009), apolipophorin 3 (Gupta *et al.,* 2010) and aryliphorin (Beresford *et al*., (1997), was severely down regulated. The use of quantitative real time PCR, revealed that the larvae starved for seven days and subsequently infected with *C. albicans* were unable to boost their immune systems to the level that the unstarved larvae were. The expression of the genes: *Transferrin, Galleriomicin, Galliomycin* and *IMPI* were significantly lower in larvae that were starved compared with the larvae that were not starved prior to a *C. albicans* infection. It was therefore possible to state that the nutritional status of the larvae has a strong bearing on experimental outcome. This Chapter also revealed that the larval weight and the type of food is of vital importance prior to beginning an experimental study.

The haemocyte is the circulating immune cell of *G. mellonella* larvae and is the basis for the insect's cellular immune response. The haemocyte has been widely documented in the literature to perform many processes that aid in inducing microbial cell death such as the production of reactive oxygen species (Mukherjee *et al*., 2010). Haemocytes have been demonstrated to perform phagocytosis of opsonised particles (Ehlers *et al.,* 1992) and to form nodules, which are aggregations of haemocytes that can trap large numbers of microbes with extracellular material (Sideri *et al.,* 2007). The haemocyte can also perform cellular encapsulation. Little is known of the precise cellular signaling cascades involved in cellular encapsulation, however it has been documented that Lepidopteran encapsulation occurs such that many haemocytes form layers on a foreign particle and the inner

layers melanise contributing to microbial death (Gillespie *et al*., 1997). The haemocyte is also a source of the phenoloxidase cascade components, ultimately leading to the accumulation of melanin around the pathogen (Wiesner *et al.,* 1996). The work presented in Chapter 4 of this thesis sought to characterise the proteomic content of haemocytes in *G. mellonella* larvae and to establish similarities with the human neutrophil in terms of cellular structure and protein content. It was observed that the phorbol-ester protein kinase C activator PMA was the most active chemotactic activator when it was compared with fMLP. This was in terms of stimulating a haemocyte response that was analogous to the response that would occur when the haemocyte would come into contact with an opsonised target. PMA also induced an upregulation in the number of proteins that were phosphorylated in the haemocyte, as demonstrated by both one dimensional and two dimensional electrophoresis. It was decided to study the phosphorylation events in the haemocyte as an increase in phoshphorylation events on proteins in mammalian phagocytes has been observed when they come into contact with an opsonised target (Wojda and Jakubowicz, 2007). Sideri *et al.,* (2007) demonstrated that *Ceratitis capitata* larvae recognition of non-self particles involved an up-regulation of phosphorylation events on intracellular proteins, also. It was noted in this Chapter that there was an increase in the phosphorylation of immune-relevant proteins in the haemocyte that showed similarity to some neutrophil proteins, such as transthyretin and alpha actin (Mezosi *et al.,* 2005; Grogan *et al*., 1997).

In addition to these findings, quantitative two dimensional electrophoresis also identified the upregulation of many internal immune-relevant proteins in the haemocyte following PMA stimulation, when compared to the proteomic profiles observed with fMLP stimulation of the haemocytes and the control. The vast majority of identified proteins could be related to neutrophil proteins. The translocation of a $p47^{phox}$ like protein from the cytosol to the membrane in the haemocyte upon PMA stimulation also revealed further similarities between the neutrophil and haemocyte. This translocation process is critical in the neutrophil when targeting an opsonised particle and its importance in the neutrophil is underlined by a disease known as chronic granulomatous disease (Grogan *et al.,* 1997), which is characterised by defects in components of the NADPH oxidase multiprotein, one of which is the $p47^{phox}$ protein (Bennas *et al.,* 1994).

The data in the literature indicate an increase in the use of shotgun proteomics in recent years (Chen *et al.,* 2009). This technique allows in depth analysis of complex protein matrices based on an extremely efficient approach. It also eliminates two dimensional gel electrophoresis which can lead to the loss of many small molecular weight peptides (Brunner *et al.,* 2007). Shotgun proteomics has lead to characterisation of the proteome of *B. mori* and *D. melanogaster* (Chen *et al.,* 2009; Brunner *et al*., 2007). Chapter 4 sought to establish further knowledge of the haemocyte proteome of *G. mellonella* larvae by employing this 'shotgun' approach. However, there is currently a lack of a fully sequenced *G. mellonella* larval protein sequence publically available and it can cause limitations when attempting to study proteomics in *G. mellonella* larvae. Nonetheless, almost 30 proteins were identified in the order of abundance in the cell when this technique was utilised with the haemocytes, moreover this experiment revealed the power of shotgun proteomics and the usefulness of the technique. Future research in the area would improve substantially with the release of the sequenced proteome to a public database and the employment of further quantitative mass spectrometric techniques such as peak counting.

The cytoskeleton of both the neutrophil and the haemocyte has a fundamental role in processes such as phagocytosis, ROS production and degranulation (Xu *et al.,* 2009). Cellular shape and division also relies on the architecture of the cytoskeleton (Jog *et al.,* 2007). Despite it's primary role in the haemocyte's ability to kill microbes, the structure of the cytoskeleton in haemocyte's of *G. mellonella* is not well characterised. Chapter 5 endeavored to characterise the significance of the cytoskeleton by employing two inhibitors of cytoskeletal processes that are widely used in neutrophil studies: cytochalasin b and nocodazole. It was evident from the studies in this Chapter that the inhibitors negatively affected the haemocyte's ability to kill an opsonised target or to alter the cell shape to allow phagocytosis to occur. These results were in agreement with neutrophil studies in the literature that have also utilised cytochalasin b and nocodazole (Bylund *et al.,* 2004; Volz, 1993). In addition to these findings it was also demonstrated that there exists similar proteins in the haemocyte cytoskeleton to those that are present in the neutrophil cytoskeleton. These proteins, vinculin, coronin, F-actin and G-actin, were found to be affected in similar manners in both

cell types by the pre-treatment of cells with cytochalasin b and nocodazole, as evaluated by confocal immunofluoresence microscopy.

Another conclusion from this Chapter was that F-actin distribution was increased in both cells with PMA stimulation, further confirming that PMA stimulates the haemocytes and neutrophils in a manner analogous to an opsonised target. The decrease in G-actin and increase in F-actin upon PMA stimulation also agrees with cytoskeleton studies regarding the neutrophil when the cell is activated with an opsonised particle (Howard and Oresajo, 1985).

It was also determined from flow cytometric techniques that there exists at least three different sub-populations of haemocytes in *G. mellonella* larvae. This finding agrees with results documented in the literature (Garcia-Garcia *et al.,* 2009) where the phagocytosis of particles was investigated. The presence of coronin, vinculin and actin was investigated in the three sub-populations. This Chapter also examined how the inhibitors affected the different populations. It was found that the most significant sub-population that was undergoing changes upon treatment with the inhibitors was the small, granular sub-population. This was interesting as it was this sub-population that was most likely the most similar to the small and granular neutrophil (Linssen *et al.,* 2008).

It was therefore decided to further employ an advanced flow cytometric technique to sort the sub-populations of haemocytes of *G. mellonella* larvae and to view the cells under the light microscope to fully characterise and understand the size and granularity differences. It was found that upon analysis with the BD FACs ARIA®, that four sub-populations existed, again differing in size and granularity. It was also noted that the smallest sub-population referred to in the previous flow cytometry results section of this Chapter had a significant increase in cell numbers when the haemocytes were stimulated with PMA. This may be due to the fact that PMA is a known activator of neutrophil granules (Klink and Sulowska, 2007), and the results demonstrated here correlated with the hypothesis that the smallest and most granular haemocytes are the most similar haemocyte sub-population to neutrophils. These results also agree with Strand (2008), where it was postulated that Lepidopteran larvae most likely possess four sub-populations of haemocytes. The most difficult aspect of using cell sorting techniques to study *G. mellonella* haemocytes fully is the limitation with regard to haemolymph quantity and inefficiency of haemolymph collection prior to cell sorting.

As discussed elsewhere in this thesis the process of degranulation in the neutrophil is essential to the killing ability of the cell. Degranulation ultimately results in the ordered exocytosis of proteolytic enzymes that aid in the killing of invading microorganisms (Stie and Jesaitis, 2007). Degranulation is well documented as being a fundamental process in the neutrophil's defence mechanisms. The results presented in Chapter 6 sought to investigate whether the haemocyte degranulates enzymes that are similar to the enzymes that are contained in the neutrophil granules and how similar the release kinetics were in both cell types.

Following cellular viability assays and the optimistaion of the time that the isolated neutrophils and haemocytes could be experimented with, it was found that there was a release of similar proteins to neutrophil myeloperoxidase and lactoferrin. Myeloperoxidase (MPO) is involved in the production of the potent agent hypochlorous acid (Johnson *et al.,* 1987) while lactoferrin, which is a member of the transferrin family, has the ability to bind iron from the invading microorganism (Adlerova *et al*., 2008). Western blotting and confocal immunofluorescence allowed the identification of an MPO-like protein to be present in the haemocyte. Confocal immunofluoresence images also demonstrated PMA stimulation of the haemocyte, which revealed this protein to appear in granule-like bundles, similar to what occurs in the neutrophil upon stimulation. This protein was later identified to be prophenoloxidase (Bergin *et al.*, 2005), by mass spectrometry analysis, which is an enzyme that catalyses reactions similar to neutrophil MPO (Lin *et al.,* 2007).

Lactoferrin-like material was also identified to be released from the haemocyte upon PMA stimulation. Lactoferrin is closely related to transferrin (Bergin *et al.,* 2005), which was documented in Chapter 4 as being one of the immuno-releavant proteins that is held in the haemocyte. In addition to these findings, peroxidase activity techniques revealed an increase in peroxidase activity in the neutrophil and haemocyte upon PMA stimulation. Interestingly the increase in peroxidase activity in both cell types was similar. Flow cytometry analysis also demonstrated the sub-population that was discussed in Chapter 5 as being the most similar to the neutrophil and the results in Chapter 6 revealed the neutrophil-MPO-like activity to be significantly altered upon PMA stimulation. This further confirmed that degranulation-like activity occurs in the haemocyte of *G. mellonella* larvae.

Finally it was decided to employ two dimensional electrophoresis to characterise and analyse the secretion of immune-relevant proteins from the haemocyte. It has previously been demonstrated that neutrophil degranulation is negatively affected by treatment with cytochalasin b and nocodazole (Bengtsson *et al.,* 1994; Volz *et al.,* 1993). The effect of these inhibitors on the haemocyte's ability to secrete important proteins upon PMA stimulation was therefore evaluated. However, prior to this it was decided to optimise the proteomic technique for studying this process with haemocytes. Isoelectric focusing and staining techniques were compared. Due to efficiency, reproducibility and the high compatibility with mass spectrometry it was decided to continue the studies with colloidal coomassie staining. Colloidal coomassie is still the most common laboratory used stain in proteomic research and is cited as being highly sensitive and quantitative (Westermeler, 2006).

It was determined that the pre-treatment of the haemocyte with the inhibitors also decreased secretion of important proteins, such as serine protease, apolipophorin 3, aldo keto reductase and transferrin (which, as discussed previously is a member of the same protein family as lactoferrin). The majority of these released proteins are homologous to neutrophil proteins that are important in the mammalian immune response. It is therefore possible to state, based on these findings, that the haemocyte degranulates in a similar manner to neutrophils, when activated, either artificially or by an opsonised microbe.

As work throughout this Thesis demonstrates, the immune response of *G. mellonella* larvae is complex and it is evident that there are numerous similarities to the innate immune response of humans. The similarities demonstrated in the Chapters were shown by proteomic, molecular and biological assays carried out on the larvae and the isolated haemocytes. These studies have demonstrated many novel characteristics of the haemocyte and have evaluated many processes in the cell, while comparing the processes to the human neutrophil. This work has provided evidence for the effectiveness of *G. mellonella* larvae as an alternative model for researchers to use in comparative biology and for use in biological and chemical testing.

Future research with *G. mellonella* larvae may focus on areas such as cell sorting and utilising molecular markers to fully characterise the haemocyte sub-populations. It is also evident that there is a need for a standardised system in terms

of naming haemocyte sub-populations in the literature as there are some conflicting approaches to naming the sub-populations at present (Ribeiro and Brehelin, 2006).

If methods and techniques become more sensitive in terms of preventing cell loss when sorting haemocytes by flow cytometry the current challenge that is faced with haemocyte collections may be overcome.

Further analysis of the cell free serum may also be evaluated, with a possible comparison to human serum. This would result in the identification of biological markers and immune markers that are potentially analogous to those in human serum. This approach may involve further real time PCR analysis and advanced proteomic techniques. If a publically accessible protein sequence database becomes available for *G. mellonella* larvae, the shotgun approach to proteomics in correlation with quantitative techniques would also facilitate a greater insight into the non-cellular aspect of the insect immune response.

Chapter 8

References

Ai, Y., & Gilchrist, A. (2011). Activation of MAP kinase-activated protein kinase 2 in human neutrophils after phorbol ester or fMLP peptide stimulation. *Cell*, 5287-5296.

Adlerova, L., Bartoskova, A., & Faldyna, M. (2008). Lactoferrin : a review. *Pharmaceutical Sciences*, 2008(7), 457-468.

Akira, S., Takeda K. and Kaisho, T. (2001) Toll-like receptors: critical proteins linking innate and acquired immunity. *Nature Immunology,* 2: 675–680.

Aladaileh, S., Nair, V.F., Birch, D. and Raftos, D.A. (2007) Sydney rock oyster (*Saccostrea glomerata*) hemocytes: Morphology and function. *Journal of Invertebrate Pathology,* 96, 1, 48-63.

Alarco, A. M. A., Marcil J., Chen B., Suter D., Thomas P., Whiteway M. (2004). Immune-deficient *Drosophila melanogaster*: a model for the innate immune response to human fungal pathogens. *Journal of Immunology*, 172, 5622–5628.

Altincicek, B., Linder, M., Linder, D., Preissner, K. T., & Vilcinskas, A. (2007). Microbial Metalloproteinases Mediate Sensing of Invading Pathogens and Activate Innate Immune Responses in the Lepidopteran Model Host *Galleria mellonella* . *Infection and Immunity,* 75 (1), 175-183.

Andrews, P.C., and Krinsky N.I. (1982) A kinetic analysis of the interaction of human myeloperoxidase with hydrogen peroxide, chloride ions, and protons. *Journal of Biological Chemistry*, 257, 13240–13245.

Anderson, N. L., Matheson, A. D., & Steiner, S. (2000). Proteomics : applications in basic and applied biology. *Current Opinions in Biotechnology*, 408-412.

Andrews, P. and Babior, B. (1983). Endogenous protein phosphorylation by resting and activated human neutrophils. *Blood*, 61, 333-34.

Anes, E., M.P. Kuhnel, E. Bos, J. Moniz-Pereira, A. Habermann, and G. Griffiths. (2003). Selected lipids activate phagosome actin assembly and maturation resulting in killing of pathogenic mycobacteria. *Nature Cell Biology* 5, 793–802.

Anggraeni, T. and Ratcliffe, N.A. (1991). Studies on cell-cell cooperation during phagocytosis by purified haemocyte populations of the wax moth, *Galleria mellonella*. *Journal of Insect Physiology*. 37, 453–60.

Aperis, G., Fuchs, B. B., Anderson, C. a, Warner, J. E., Calderwood, S. B., & Mylonakis, E. (2007). *Galleria mellonella* as a model host to study infection by the Francisella tularensis live vaccine strain. *Microbes and infection*, 9(6), 729-34.

Arif, A., Scheller, K. and Dutta-Gupta, A. (2003). Tyrosine kinase mediated phosphorylation of the hexamerin receptor in the rice moth *Corcyra cephalonica* by ecdysteroids. *Insect Biochemistry and Molecular Biology*, 33, 921-928.

Bainton, D.F., Ullyot, J.L. and Farquhar, M.G. (1971) The development of neutrophilic polymorphonuclear leukocytes in the bone marrow, *The Journal of experimental medicine*, 134, 907-934.

Ballarin, L., Cammarata, M., Cima, F., Grimaldi, A., Lorenzon, S., Malagoli, D., & Ottaviani, E. (2008). Immune-neuroendocrine biology of invertebrates : a collection of methods. *Galleria Rassegna Bimestrale Di Cultura*, 192-215.

Bajt, M.L., Farhood, A. and Jaeschke, H. (2001) Effects of CXC chemokines on neutrophil activation and sequestration in hepatic vasculature. *American Journol Physiological Gastrointestinal Liver physiology,* 281, 1188–1195.

Beetz, S., Brinkmann, M. and Trenczek, T. (2004) Differences between larval and pupal hemocytes of the tobacco hornworm, *Manduca sexta*, determined by monoclonal antibodies and density centrifugation. *Journal of Insect Physiology*, 50, 805- 819.

Bengtsson T., Dahlgren C., Stendahl O. and Andersson T. (1991). Actin assembly and regulation of neutrophil function: effects of cytochalasin B and tetracaine on chemotactic peptide-induced O2- production and degranulation. *Journal of Leukocyte Biology,* 49, 236-244

Bennas, J. E., Faust, L. P., & Babioro, B. M. (1994). The Phosphorylation of the Respiratory Burst Oxidase Component. *Biochemistry*, 269 (38), 23431-23436.

Beresford, P.J., Basinski-Gray, J.M., Chiu, J.K., Chadwick, J.S., Aston, W.P. (1997). Characterization of hemolytic and cytotoxic Gallysins: a relationship with arylphorins. *Developmental and Comparative Immunology*, 21, 253–266.

Bergin, D., Murphy, L., Keenan, J., Clynes, M., and Kavanagh, K. (2006). Pre-exposure to yeast protects larvae of Galleria mellonella from a subsequent lethal infection by Candida albicans and is mediated by the increased expression of antimicrobial peptides. *Microbes and infection* , 8(8), 2105-12.

Bergin, D., Reeves, E. P., Renwick, J., Wientjes, F. B., and Kavanagh, K. (2005). Superoxide Production in Galleria mellonella Hemocytes : Identification of Proteins Homologous to the NADPH Oxidase Complex of Human Neutrophils. *Infection and Immunity*, 73(7), 4161-4170.

Bershadsky, A.D., Glück, U., Denisenko, O. N., Sklyarova, T. V., Spector, I., & Ben-Ze'ev, A. (1995). The state of actin assembly regulates actin and vinculin expression by a feedback loop. *Journal of cell scienc*e, 108, 1183-93.

Blasig, I.E., Bellmann, C., Cording, J., Del Vecchio, G., Zwanziger, D., Huber, O., and Haseloff, R.F. (2011) Occludin protein family: oxidative stress and reducing conditions. *Antioxidant Redox Signal.* 15(5), 1195-219.

Bodey, G., B. Bueltmann, W. Duguid, D. Gibbs, H. Hanak, M. Hotchi, G. Mall, P.Martino, F. Meunier, S. Milliken, S. Naoe, M. Okudaira, D. Scevola, and J. Van't Wout. (1992). Fungal infections in cancer patients: an international autopsy survey. *European Journal of Clinical Microbiology and Infectious Disease,* 11, 99–109.

Bokoch, G.M. and Gilman, A.G. (1984). Inhibition of receptor-mediated release of arachidonic acid by pertussis toxin. *Cell,* 39, 301.

Bokoch, G. M. (2009). NADPH oxidases in innate immunity. *Journal of Innate Immunity*, 1(6), 507-8.

Booth, T.F. and Bonning, B.C. (1992) Hammock Localization of juvenile hormone esterase during development in normal and in recombinant baculovirus-infected larvae of the moth *Trichoplusia ni. Tissue and Cell,* 24 (2), 267-282

Bozeman, P.M., Learn, D.B. and Thomas, E.L. (1990) Assay of the human leukocyte enzymes myeloperoxidase and eosinophil peroxidase, *Journal of Immunological Methods*, 126, (1), 125-133

Broderick, N. A., Raffa, K. F., and Handelsman, J. (2010). Chemical modulators of the innate immune response alter gypsy moth larval. *Plant Pathology.* 10 (129), 1-35.

Brown, K.L., Christenson, K., Karlsson, A., Dahl- gren, C. and Bylund, J. (2009). Divergent effects on phago- cytosis by macrophage-derived oxygen radi- cals. *Journal of Innate Immunity* 1, 592–598.

Brackman, G., Celen, S., Baruah, K., Bossier, P., Van Calenbergh, S., Nelis, H. J., and Coenye, T. (2009). AI-2 quorum-sensing inhibitors affect the starvation response and reduce virulence in several *Vibrio* species, most likely by interfering with LuxPQ. *Microbiology* (Reading, England), 155, 4114-22.

Bradford, M. D. and Soltoff, S. P. (1998) Involvement of protein kinases and phosphatases in tyrosine phosphorylation of PKCd in rat parotid acinar cells exposed to secretory stimuli. *European Journal of Morphology,* 36, 176-180.

Bratti, A. and Coulibaly, A.K. (1995) In vitro rearing of Exorista larvarum on tissue culture-based diets. *Entomologica Experimentas et Applicata*, 74, (1) 47-53.

Brehelin, M. and Zachary, D. (1986) Insect haemocytes, a new classification to rule out controversy. *Immunity in Inverte brates* (ed. M. Brehelin), 36-38.

Brennan, M., Thomas, DY., Whiteway, M. and Kavanagh, K. (2002) Correlation between virulence of *Candida albicans* mutants in mice and *Galleria mellonella* larvae. *Medical Mycology*, 34, 153-157.

Brieland, J.K. and Fantone, J.C. (1991) Ferrous iron release from transferrin by human neutrophil-derived superoxide anion: Effect of pH and iron saturation. *Archives of Biochemistry and Biophysics*, 284, 1, 78-83.

Brunner, E., Ahrens, C. H., Mohanty, S., Baetschmann, H., Loevenich, S., Potthast, F., Deutsch, E. W., et al. (2007). A high-quality catalog of the *Drosophila melanogaster* proteome. *Nature Biotechnology*, 25(5), 576-83.

Bulet, P., Hetro, C., Dimarqo J.C., and Hoffmann, D. (1999). Antimicrobial peptides in insects; structure and function. *Developmental and Comparative Immunolology* , 23, 329-344.

Burlak, C., Whitney, A. R., Mead, D. J., Hackstadt, T. and Deleo, F. R. (2006) Maturation of human neutrophil phagosomes includes incorporation of molecular chaperones and endoplasmic reticulum quality control machinery. *Molecular and Cellular Proteomics*, 5, 620–634

Bylund, J., Pellmé, S., Fu, H., Mellqvist, U.-H., Hellstrand, K., Karlsson, A., & Dahlgren, C. (2004). Cytochalasin B triggers a novel pertussis toxin sensitive pathway in TNF-alpha primed neutrophils. *BMC cell biology*, 5, 21.

Calderone, R. A. & Fonzi, W. A. (2001). Virulence factors of *Candida albicans*. *Trends Microbiol* 9, 327–335.

Cassatella, M.A. (2003) The neutrophil: An emerging regulator of inflammatory and immune response, Casatlella eds.

Castellano, F., Chavrier, P. and Caron, E. (2001) Actin dynamics during phagocytosis. *Semininars in Immunology*, 13, 347–355.

Castillo, J.C., Robertson, A.E. and Strand, M.R. (2006) Characterization of hemocytes from the mosquitoes *Anopheles gambiae* and *Aedes aegypti*. *Insect Biochemistry and Molecular Biology*, 36, 891-903.

Chen, M., Wei, H., and Zhong, B.-x. (2009). Shotgun proteomics approach to characterizing the embryonic proteome of the silkworm , *Bombyx mori* , at labrum appearance stage. *Insect Molecular Biology*, 18, 649-660.

Chen, G.C., Turano, B., Ruest, P.J., Hagel, M., Settleman, J. and Thomas, S.M. (2005) Regulation of Rho and Rac signaling to the actin cytoskeleton by paxillin during *Drosophila development*. *Molecular and Cellular Biology*, 25, 979-987.

Cheung., P.T. and Hammermann., M.R. Cytochalasin B binding to rabbit proximal tubular basolateral membranes. *Kidney International,* 35, 1290—1294.

Christin, L., D. R. Wysong, T. Meshulam, R. Hastey, E. R. Simons, and R. D. Diamond. (1998). Human platelets damage *Aspergillus fumigatus* hy- phae and may supplement killing by neutrophils. *Infection and Immunity*, 66, 1181– 1189.

Clark, S.R., Ma, A.C., Tavener, S.A., McDonald, B., Goodarzi, Z., Kelly, M.M., Patel, K.D., Chakra- barti, S., McAvoy, E., Sinclair, G.D., Keys, E.M., Allen-Vercoe, E., Devinney, R., Doig, C.J., Green, F.H. and Kubes P. (2007). Platelet TLR4 activates neutrophil extracellular traps to ensnare bacteria in septic blood. *Nature Medicine*, 13, 463–469.

Cockroft, S. and Stutschfield, J. (1989) The receptors for ATP and fMet- Leu-Phe are independently coupled to phospholipases C and A2 via G-protein(s). Relationship between phospholipase C and A2 activa- tion and exocytosis in HL60 cells and human neutrophils. *Journal of Biochemistry*, 263, 715.

Cotter, G., S. Doyle, and K. Kavanagh. (2000). Development of an insect model for the *in vivo* pathogenicity testing of yeasts. *FEMS Immunology and Medical Microbiology,* 27, 163–169.

Cowen, L. E., S. D. Singh, J. R. Kohler, C. Collins, A. K. Zaas, W. A. Schell, H. Aziz, E. Mylonakis, J. R. Perfect, L. Whitesell, and S. Lindquist. (2009). Harnessing Hsp90 function as a powerful, broadly effective therapeutic strategy for fungal infectious disease. *PNAS,* 106, 2818– 2823

Daly, P. & Kavanagh, K. (2002). Immobilization of *Aspergillus fumigatus* colonies in a soft agar matrix allows visualisation of A549 cell detachment and death. *Medical Mycology*, 40, 27–33

Davey, H. M., & Kell, D. B. (1996). Flow Cytometry and Cell Sorting of Heterogeneous Microbial Populations : the Importance of Single-Cell Analyses. *Microbiology*, 60(4), 641-696

Decleva, E., Menegazzi, R., Busetto, S., Patriarca, P. and Pietro, D. (2006) Common methodology is inadequate for studies on the microbicidal activity of neutrophils. *Journal of Leukocyte Biology.* 79, 87-94.

Denning, D. W. (1995). Issues in the management of invasive aspergillosis. *Annals of Internal Medicine,* 146,106–110.

Desbois, A.P. and Coote, P. (2011) Wax moth larva (*Galleria mellonella*): an in vivo model for assessing the efficacy of antistaphylococcal agents . *Antimicrobial Chemotherapy*, 66, 1785-1790.

Diehl-Jones, W., Mandato, C.A., Whent, G., Downer, R.G.H. (1996). Monoaminergic regulation of hemocyte activity. *Journal of Insect Physiology*, 42, 13–19.

Dionne, M. and Scheider, D.S. (2008). Models of infectious diseases in the fruit fly Drosophila melanogaster. *Disease Models & Mechanisms,* 1, 1, 43-49.

Djawdan, M., M. R. Rose, and T. J. Bradley. (1997). Does selection for stress resistance lower metabolic rate? *Ecology,* 78, 828–837.

Dong, Y.M., Taylor, H.E. and Dimopoulos, G. (2006) AgDscam, a hypervariable immunoglobulin domain-containing receptor of the *Anopheles gambiae* immune system. *PLos Biology*, 4, 1137-1146.

Eggimann, P., Garbinob, J. Pittet, A. Epidemiology of Candida species infections in critically ill non-immunosuppressed patients. *The Lancet Infectious Diseases*, 11, 685-702.

Ehlers, D., Zosel, B., Mohrig, W., Kauschke, E., & Ehlers, M. (1992). Comparison of *in vivo* and *in vitro* phagocytosis in *Galleria mellonella*. *Parasitology Research,* 78, 354-359.

Ehret-Sabatier, L., Loew, D., Goy.on, M., Fehlbaum, P., Hoffmann, J.A., Van Dorsselaer, A., Bulet, **P**. (1996). Characterization of novel cysteine-rich antimicrobial peptides from scorpion blood. *Journal of Biological Chemistry* 271, 29537-29544.

English, D., and B.R. Anderson. (1974). Single-step separation of red blood cells, granulocytes and mononuclear leukocytes on discontinuous density gradients of ficoll-hypaque. *Journal of Immunological Methods.* 5, 249–252.

Entschladen F., Zanker K.S. (2000) Locomotion of tumor cells. a molecular comparison to migrating pre- and postmitotic leukocytes. *Journal of Cancer Research and Clinical Oncology*, 126, 671–681.

Evans, W.H., Wilson, S.M., Mage, M.G. (1986) Transferrin induces maturation of neutrophil granulocyte precursors in vitro. *Leukocyte Research*, 10, 4, 429-436.

Fallon, J. P., Reeves, E. P., & Kavanagh, K. (2010). Inhibition of neutrophil function following exposure to the *Aspergillus fumigatus* toxin fumagillin. *Journal of Medical Microbiology*, 59(Pt 6), 625-33.

Faye, I. and Kanost, M. (1996). Function and regulation of hemolin, *Molecular Mechanisms of Immune Responses in Insects,* (P.T. Brey and D. Hultmark, eds) Chapman and Hall, New York, 173-188.

Freitak, D., Wheat, C. W., Heckel, D. G., & Vogel, H. (2007). Immune system responses and fitness costs associated with consumption of bacteria in larvae of *Trichoplusia ni*. *BMC Biology*, 13, 1-13.

Fuchs, B. B., O'Brien, E., El Khoury, J. B., & Mylonakis, E. (2010). Methods for using *Galleria mellonella* as a model host to study fungal pathogenesis. *Virulence*, 1(6), 475-482.

Gabrilovich, D.I. (1999) The Neutrophils: New outlook for old cells. London: *Imperial college press.*

Gallardo-Escárate, C., Alvarez-Borrego, J., Von Brand, E., Dupré, E., & Del Río-Portilla, M. A. (2007). Relationship between DAPI-fluorescence fading and nuclear DNA content: An alternative method to DNA quantification? *Biological research,* 40 (1), 29-40.

Ganz, T. (2002) Epithelia: Not just physical barriers. *PNAS*, 99 (6) 3357-3358.

García-García, E., García-García, P. L., & Rosales, C. (2009). An fMLP receptor is involved in activation of phagocytosis by hemocytes from specific insect species. *Developmental and Comparative Immunology*, 33(6), 728-39.

Gibbs, A.G. and Gefen, E. (2009) Physiological adaption in laboratory environments. Chapter 17, Experimental Evolution: Concepts, Methods, and Applications of Selection Experiments.

Gilchrist, G. W., and R. B. Huey. (1999)**.** The direct response of *Drosophila melanogaster* to selection on knockdown temperature. *Heredity* 83:15–29

Gillespie, J. P., Kanost, M. R., & Trenczek, T. (1997). Biological mediators of insect immunity. *Annual review of entomology*, 42, 611-43.

Glittenberg, M.T., Kounatidis, I., Christensen, D., Kostov, M., Kimber, S., Roberts, I., Ligoxygakis, P. Pathogen and host factors are needed to provoke a systemic host response to gastrointestinal infection of *Drosophila larvae* by *Candida albicans. Disease and Model Mechanisms*. 2011, 5, 515-25.

Gluckman, P. D., Lillycrop, K. A., Vickers, M. H., Pleasants, A. B., Phillips, E. S., Beedle, A. S., Burdge, G. C., *et al*. (2007). Metabolic plasticity during mammalian development is directionally dependent on early nutritional status. *PNAS*, 104 (31), 11-15.

Goldman AS, Prabhakar BS (1996). The Complement System. *in: Baron's Medical Microbiology* (Baron S et al., eds.) (4th ed.). Univ of Texas Medical Branch.

Greenberger, J.S., Sakakeeny, M.S., Humphries, R.S., Eaves, C.S. and Eckner. R.J. (1983). Demonstration of permanent factor-dependent multipotential (erythroid/neutrophil/basophil) hematopoietic progenitor cell lines. *PNAS*, 80, 1.

Grogan, A, Reeves, E., Keep, N., Wientjes, F., Totty, N. F., Burlingame, a L., Hsuan, J. J., et al. (1997). Cytosolic phox proteins interact with and regulate the assembly of coronin in neutrophils. *Journal of Cell Science*, 110 (Pt 2), 3071-81.

Gruhler, A., Olsen, J. V., Mohammed, S., Mortensen, P., Faergeman, N. J., Mann, M., and Jensen, O. N. (2005) Quantitative phosphoproteomics applied to the yeast pheromone signaling pathway. *Molecular Cellular Proteomics*, 4, 310–327.

Gucker, F. T., C. O'Konski, H. B. Pickard, and J. N. Pitts. 1947. A photoelectric counter for colloidal particles. *American Journal of Chemistry*, 69, 2422–2431.

Hushan, S. and Guangyou, L. (1999) Phenoloxidase and myeloperoxidase activity in the haemocytes and serum of Chlamys farreri. *Journal of fisheries sciences ,China*.

Gupta, L., Noh, J. Y., Jo, Y. H., Oh, S. H., Kumar, S., Noh, M. Y., Lee, Y. S., et al. (2010). Apolipophorin-III mediates antiplasmodial epithelial responses in *Anopheles gambiae* (G3) mosquitoes. *PloS one,* 5(11), e15410.

Halwani, A.E., Niven, D.F., Dunphy, G.B., 2001. Apolipophorin-III in the greater wax moth, Galleria mellonella (Lepidoptera: Pyr- alidae). *Archives of Insect Biochemistry.* 48, 135–143.

Heyworth, P. G., Shrimpton, C. F., & Segal, A. W. (1989). Localization of the 47 kDa phosphoprotein involved in the respiratory-burst NADPH oxidase of phagocytic cells, 260, 243-248.

Hedges, S. B. 2002. The origin and evolution of model organisms. *Nature Reviews in Genetics.* 3, 838–849.

Henderson VJ, Hirvela ER. (1996) Emerging and reemerging microbial threats. Nosocomial fungal infections. *Archives of Surgery*, 131: 330-7.

Heemskerk J., Tobin A.J., Ravina B. (2002). From chemical to drug: neurodegeneration drug screening and the ethics of clinical trials *Nature Neuroscience,* 5, 1027–1029.

Hickman-Miller HD, Hildebrand WH. (2004) The immune response under stress: the role of HSP-derived peptides. *Trends in Immunology.* 2004, (8), 427-33.

Hoffmann JA, Janeway CA Jr, Natori S, eds. (1994). Phylogenetic Perspectives in Immunity: The Insect Host Defense. Boca Raton: CRC Press.

Hoffmann, J. A., Kafatos, F. C., Jr, C. A. J., & Ezekowitz, R. A. B. (1999). Phylogenetic Perspectives in Innate Immunity. *Science,* 284(May), 1313-1318

Hoffmann, J. A. & Reichkart, J.-M. 2002. *Drosophila i*nnate immunity: an evolutionary perspective. *Nature Immunology,* 3, 121-126

Howard, T. H., & Oresajo, C. O. (1985). The Kinetics of Chemotactic Peptide-induced Change in F-Actin Content , F-Actin Distribution, and the Shape of Neutrophils. *Cell*, 101.

Huang, N., Clem, R.J. and George F. Rohrmann. (2011) Characterization of cDNAs encoding p53 of *Bombyx mori* and *Spodoptera frugiperda, Insect Biochemistry and Molecular Biology*, 41, (8), 613-619.

Hultmark, D. 1994. Insect immunology: ancient relationships. *Nature,* 367:116.

Humphreys, J.M., Davies, B., Hart, A. and Edwards, S.W. (1989) Role of myeloperoxidase in the killing of staphylococcus aureus by human neutrophils: studies with the myeloperoxidase inhibitor salicylhydroxamic acid. *Journal of general microbiology*, 135, 1187-1193.

Hunter, H.N., Fulton, D.B., Ganz, T. and Vogel, H.J. (2002). The solution structure of human hepcidin, a peptide hormone with antimicrobial activity that is involved in iron uptake and hereditary hemochromatosis. *Journal of Biological Chemistry,* 277: 597-603

Imler, J-L. and Zheng, L. (2005) Biology of Toll receptors, lessons from insects and mammals. *Journal of Leukocyte Biology*, 74, 18-26.

Ingenbleek, M. and Young, V. (1994)**.** Transthyretin (prealbumin) in health and disease: nutritional implications. *Annual Reviews in Nutrition*, 14, 495–533.

Jander, G., Rahme, G. and Ausbel, G. (2000) Positive correlation between virulence of *Pseudomonas aeruginosa* mutants in mice and insects. *Journal of Bacteriology*, 182, 3843-3845

Jog, N. R., Jala, V. R., Ward, R. A., Rane, M. J., Haribabu, B., and R, K. (2011). Heat Shock Protein 27 Regulates Neutrophil Chemotaxis and Exocytosis through Two Independent Mechanisms. *The Journal of Immunology*. 178, 2421-2428.

Johnson, R.J., Klebanoff, S., Ochi1, R.F., Adler, S., Baker, P., Sparks, L. and Couse W.G. (1987). Participation of the myeloperoxidase-H2O2-halide system in immune complex nephritis. *Kidney International*, 32, 342–349

Kazakov, A.E., Vassieva, O., Gelfand, M., Osterman, A. and Overbeek, R. (2003). Bioinformatics Classification and Functional Analysis of PhoH Homologs. *In silico biology*. 3, 3-15

Kavanagh, K. and Reeves, E.P. (2004) Exploiting the potential of Insects for i*n vivo* pathogenicity testing of microbial pathogens. *FEMS Microbiol Reviews*. 28, 101-112.

Kelly, J. and Kavanagh, K. (2011). Caspofungin primes the immune response of the larvae of *Galleria mellonella* and induces a non-specific antimicrobial response. *Journal of Medical Microbiology*, 60, 189-196.

Kemp, M.W. (2007). The use of insect models to study human pathogens. *Drug Discovery Today: Disease Models,* 4, 105-110

Kim, H. J., Je, H. J., Park, S. Y., Lee, I. H., Jin, B. R., Yun, H. K., Yun, C. Y., et al. (2004). Immune activation of apolipophorin-III and its distribution in hemocyte from *Hyphantria cunea. Insect biochemistry and molecular biology*, 34(10), 1011-23.

Klink, M., & Sulowska, Z. (2007). Effects of Nitric Oxide Donors on the Biological Activity of Human Neutrophils *in Vitro*. *Letters in Drug Discovery and Design*, 4, 55-66.

Koenigsknecht, J. and Landreth, G. (2004) Microglial phagocytosis of fibrillar beta-amyloid through a beta1 integrin-dependent mechanism. *Journal of Neuroscience,* 24, 9838 –9846.

Kopácek, P., Weise, C. & Götz, P. (1995). The prophenoloxidase from the wax moth *Galleria mellonella*: purification and characterization of the proenzyme. *Insect Biochemistry and Molecular Biology*, 25, 1081–1091.

Lacchini, A.H, Davies A.J., Mackintosh, D. and Walker, A.J. (2006) Beta-1 3-glucan modulates PKC signalling in Lymnaea stagnalis defence cells: a role for PKC in H2O2 production and downstream ERK activation. *Journal of Experimental Biology*, 209, 4829–40.

Lacy, P. and Stow, JL. (2011) Cytokine release from innate immune cells: association with diverse membrane trafficking pathways. *Blood*,118 (1) 9-18.

Lackie, A.M. (1988) Haemocyte behaviour. Advances in Insect Physiology, 21, 85-178

Langen, G., Imani, J., Altincicek, B., Kieseritzky, G., Kogel, K.-H., & Vilcinskas, A. (2006). Transgenic expression of *gallerimycin,* a novel antifungal insect defensin from the greater wax moth *Galleria mellonella*, confers resistance to pathogenic fungi in tobacco. *Biological chemistry*. 87 (5): 1431-6730.

Latgé, J.P. 1999. *Aspergillus fumigatus* and aspergillosis. *Clinical Microbiolobiology Reviews.* 12:310-350

Lavine, M.D. and Strand, M.R. (2002) Insect hemocytes and their role in cellular immune responses. *Insect Biochemistry and Molecular Biology*, 32, 1237-1242.

Lavine, M.D. and Strand, M.R. (2003) Hemocytes from *Pseudoplusia includens* express multiple alpha and beta integrin subunits. *Insect Molecular Biology*, 12, 441-452

Lavine, M.D., Chen, G. and Strand, M.R. (2005) Immune challenge differentially affects transcript abundance of three anti- microbial peptides in hemocytes from the moth *Pseudoplusia includens*. *Insect Biochemistry and Molecular Biology,* 35, 1335-1346.

Lee, Y.S., Yun, E.K., Jang, W.S., Kim, I., Lee, J.H., Park, S.Y., Ryu, K.S., Seo, S.J., Kim, C.H., Lee, I.H., 2004. Purification, cDNA cloning and expression of an insect defensin from the great wax moth, *Galleria mellonella*. *Insect Molecular Biology,* 13, 65–72

Lemaitre B., Nicolas E., Michaut L., Reichhart J.M., and Hoffmann J.A. (1996) The dorsoventral regulatory gene cassette spätzle/Toll/cactus controls the potent antifungal response in Drosophila adults. *Cell*, 86, 973-83.

Leon, L.J., Idangodage, H., Wan, Ch.-P.L., Weers, P.M.M., (2006). Apolipophorin III: lipopolysaccharide binding requires helix bundle opening. *Biochemical and Biophysical Communications*. 348, 1328–1333

Li J, Tracy JW, Christensen BM. (1992). Phenol oxidase activity in hemolymph compartments of *Aedes aegypti* during melanotic encapsulation reactions against microfilariae. *Developemental and Comparative. Immunology*. 16:41–48.

Li, J.Y., Chen, X., Fan,W., Hosseini Moghaddam, S.H., Chen, M., Zhou, Z.H. et al. (2009). Proteomic and bioinformatic analysis onendocrineorgans of domesticated silkworm, *Bombyx mori L*. for a comprehensive understanding of their roles and relations. *Journal of Proteome Research*, 8: 2620–2632.

Ling, E., Shirai, K., Kanekatsu, R. and Kiguchi, K. (2005) Hemocyte differentiation in the hematopoietic organs of the silkworm, *Bombyx mori*: prohemocytes have the function of phagocytosis. *Cell and Tissue Research*, 320, 535-543.

Linssen, J., Aderhold, S., Nierhaus, a, Frings, D., Kaltschmidt, C., & Zänker, K. (2008). Automation and validation of a rapid method to assess neutrophil and monocyte activation by routine fluorescence flow cytometry in vitro. *Cytometry Part B: Clinical Cytometry*, 74B(5), 295-309.

Lionakis, M. S. and Kontoyiannis, D. P. (2005). Fruit flies as a minihost model for studying drug activity and virulence in *Aspergillus*. *Medical Mycology*, (1), S111–S114.

Liu, C.T., Hou, R.F. and Chen, C.C. (1998) Formation of basement membrane-like structure terminates the cellular encapsulation of microfilariae in the haemocoel of *Anopheles quadrimaculatus*. *Parasitology*, 116, 511-518.

Locke, M., Ketola-Pirie, C., Leung, H. and Nichol, H. (1991). Vacuolar apoferritin synthesis by the fat body of an insect (*Calpodes ethlius*). *Journal of Insect Physiology*. 34, 297-309.

Madan, T., P. Eggleton, U. Kishore, P. Strong, S. Aggrawal, P. U. Sarma, and K. B. M. Reid. (1997). Binding of pulmonary surfactant proteins A and Dto

Aspergillus fumigatus conidia enhances phagocytosis and killing by human neutrophils and alveolar macrophages. *Infection and Immunity*. 65:3171– 3179.

MacLean-Fletcher. S and Pollard, T.D. (1980). Identification of a factor in conventional muscle actin preparations which inhibits actin filament self-association. *Biochemical and Biophysical Research Communications.* 96, 18-27.

Maeda, H. (1996) Role of microbial proteases in pathogenesis. *Microbiological Immunology,* 40: 685–699

Matha, V., Mracek, Z. (1984) Changes in haemocyte counts in *Galleria mellonella* (L) (*Lepidoptera: Galleriidae*) larvae infected with *Steinernema* sp. (Nematoda: steinernematidae). *Nematology*, 30, 86–89

Martínez, J.P., Luisa Gil, M., López-Ribot, J.P. and LaJean, W. (1998). Chaffin Serologic Response to Cell Wall Mannoproteins and Proteins of *Candida albicans. Clinical Microbiological Reviews.* 11, 121–141.

Marmaras, V.J. and Tsakas, S. Temporally regulated protein synthesis in cultured haemocytes of the Mediterranean fruit fly *Ceratitis capitata* during larval and prepupal development: Internalization of larval serum proteins into the haemocytes, *Developmental Biology*, 129, 294-303.

Masova, A., Sanda, M., Jiracek, J. and Selicharova, I. (2010) Changes in the proteomes of the hemocytes and fat bodies of the flesh fly *Sarcophaga bullata* larvae after infection by *Escherichia coli*. *Proteome Science*, 8:1.

Matute, J. D., Arias, A. a, Dinauer, M. C., & Patiño, P. J. (2005). p40phox: the last NADPH oxidase subunit. *Blood cells, molecules & diseases*, 35(2), 291-302.

Mayo, J.C., Sáinz, R.M., Antolín, I., Uría, H., Menéndez-Peláez, A. and Rodríguez, C.. (1997) Androgen-dependent mast cell degranulation in the Harderian gland of female *Syrian hamsters*: *in vivo* and organ culture evidence. *Anatatomical Embryology (Berl).* 196(2), 133-40.

McDaniel, L.D., Schultz, R.A. (2008) XPF/ERCC4 and ERCC1: their products and biological roles. *Advances in Experimental Medical Biology.* 637:65-82

McKenzie ANJ, Preston TM. (1992). Functional studies on *Calliphora vomitoria* haemocyte subpopulations defined by lectin staining and density centrifugation. *Developmental and Comparative Immunology*. 16:19–30

Mead, G.P., Ratcliffe, N.A. and Renwrantz, L.R. (1986) The separation of insect hemocyte types on Percoll gradients; methodology and problems. *Journal of Insect Physiology*, 25, 795-803

Medzhitiov R. (2008) Origin and physiological roles of inflammation. *Nature,* 454, 428-435.

Meyer-Hoffert ,U. and Wiedow, O. (2010) Neutrophil serine proteases: mediators of innate immune responses. *Journal of Internal Medicine*, 257, 319-328.

Mezosi, E., Szabo, J., Nagy, E. V., Borbaly, A., Varge, E., Paragh, G. & Varga, Z. (2005). Nongenomic effect of thyroid hormone on free-radical production in human polymorphonuclear leukocytes. *Journal of Endocrinology,* 185, 121-129

Mollinedo, F., Borregaard, N. and Boxer, L.A. (1999) "Novel trends in neutrophil structure, function and development", *Trends in immunology today*, 20(12), 535-537

Mowlds, P., Barron, A. and Kavanagh, K. (2008) Physical stress primes the immune response of *Galleria mellonella* larvae to infection by *Candida albicans*. *Microbes and Infection.* 10, 628-634.

Mowlds, P., Coates, C., Renwick, J., & Kavanagh, K. (2010). Dose-dependent cellular and humoral responses in *Galleria mellonella* larvae following b-glucan inoculation. *Microbes and Infection*, 12(2), 146-153.

Moret, Y. (2000). Survival for Immunity: The Price of Immune System Activation for Bumblebee Workers. *Science*, 290(5494), 1166-1168.

Mukherjee, K., Altincicek, B., Hain, T., Domann, E., Vilcinskas, A., & Chakraborty, T. (2010). *Galleria mellonella* as a model system for studying *Listeria pathogenesis*. *Applied and Environmental Microbiology,* 76(1), 310-7.

Musa, H., Orton, C., Morrison, E. E., Peckham, M., Unit, M., & James, S. (2006). Microtubule assembly in cultured myoblasts and myotubes following nocodazole induced microtubule depolymerisation. *Cell*, 24, 301-308.

Mylonakis, E. and Aballay, A. (2005) Worms and flies as genetically tractable animal models to study host–pathogen interactions. *Infection and Immunity* 73: 3833–3841.

Mylonakis, E. (2008). *Galleria mellonella* and the study of fungal pathogenesis: making the case for another genetically tractable model host. *Mycopathologia*, 165, 1–3.

Nakahara, Y., Matsumoto, H., Kanamori, Y., Kataoka, H., Mizoguchi, A., Kiuchi, M. and Kamimura, M. (2006) Insulin signaling is involved in hematopoietic regulation in an insect hematopoietic organ. *Journal of Insect Physiology*, 52, 105- 111.

Nappi, C. 2005. The hemocyte does everything. *Insect Biochemistry and Molecular Biology,* 35, 443-459.

Nardi, J.B., Ujhelyi, E., Pilas, B., Garsha, K. and Kanost, M.R. (2003) Hematopoietic organs of *Manduca sexta* and hemocyte lineages. *Development Genes and Evolution*, 213, 477-491.

Nardi, J.B. (2004) Embryonic origins of the two main classes of hemocytes-granular cells and plasmatocytes in *Manduca sexta*. *Development Genes and Evolution*, 214, 19-28.

Nauseef, W. M., Volpp, B. D., McCormick, S., Leidal, K. G. and Clark, R. A. (1991). Assembly of the neutrophil respiratory burst oxidase. Protein kinase C promotes cytoskeletal and membrane association of cytosolic oxidase components. *Journal of Biolical Chemistry*, 266, 5911-5917.

Neeli, I., Dwivedi, N., Khan, S., & Radic, M. (2009). Regulation of Extracellular Chromatin Release from Neutrophils. *Journal of Innate Immunity*, 1, 194-201.

Nishida, K., Yamasaki, S., Ito, Y., Kabu, K., Hattori, K., Tezuka, T., Nishizumi, H., Kitamura, D.,Goitsuka, R.,Geha, R.S., Yamamoto, T., Yagi, T. and Hirano, T. (1999) FcεRI-mediated mast cell degranulation requires calcium-independent microtubule-dependent translocation of granules to the plasma membrane. *Journal of Cell Biology.* 170 (1), 115.

Numaguchi, K., Eguchi, S., Yamakawa, T., Motley, E.D. and Inagami, T. (1999) Mechanotransduction of Rat Aortic Vascular Smooth Muscle Cells Requires RhoA and Intact Actin Filaments. *Circulation Research*, 85, 5-11.

Odds, F.C. (1988) *Candida* and Candidosis, 2nd edn.London: Bailliere Tindal.

Odds, F.C. (1994) *Candida* species and virulence. *American Society of Microbiology News*, 60: 313-318.

Ohira, T., Zhan, Q., Ge, Q., Vandyke, T. & Badwey, J. A. (2003). Protein phosphorylation in neutrophils monitored with phosphospecific antibodies. *Journal of Immunological Methods,* 281, 79-94.

Paclet, M. H., Davis, C., Kotosis, P., Godovac-Zimmermann, J., Segal, A. W. & Dekker, L. V. (2004). N-Formyl peptide receptor subtypes in human neutrophils activate L-plastin phosphorylation through different signal transduction intermediates. *The Biochemical journal*, 377, 469-77.

Paily, P., Agiesh Kumar, P. and Balaraman, K. Transferrin in the mosquito, *Culex quinquefasciatus Say* (Diptera: Culicidae), up-regulated upon infection and development of the filarial parasite, *Wuchereria bancrofti* (Cobbold) (Spirurida: Onchocercidae). (2007) *Parasitology Research*. 101, 325–330.

Parks, D.R., Lanier, L.L., Herzenberg, L.A. (1986) Flow cytometry and fluorescence activated cell sorting (FACS). In: Weir DM (ed): *Handbook of Experimental Immunology, 4th ed. Volume 1: Immunochemistry.* p.29.1, Oxford, Blackwell.

Park, S.J., Hidemitsu Kitamura, N, Atsumi, T., Kamon, H., Sawa, S., Kamimura, Naoko Ueda, D., Iwakura, Y., Ishihara, K., Murakami, M. and Hirano, T. (2004) IL-6 Regulates *In Vivo* Dendritic Cell Differentiation through STAT3 Activation. *The Journal of Immunology*, 173, 3844-3854.

Pederson, T. (2000). Half a century of "the nuclear matrix". *Molecular biology of the cell*, 11(3), 799-805.

Peng, X., L.E. Cuff, C.D. Lawton and K.A. DeMali. (2010). Vinculin regulates cell-surface E-cadherin expression by binding to beta-catenin. *Journal of Cell Science*. 123, 567–577

Pfaller, M.A. and Diekema, D.J. (2004) Rare and emerging opportunistic fungal pathogens: concern for resistance beyond *Candida albicans* and *Aspergillus fumigatus*. *Journal of Clinical Microbiology,* 42, 4419–4431.

Rabilloud, T., Vaezzadeh, A.R., Potier, N., Lelong, C., Leize-Wagner, E. and Chevallet, M. (2009) Power and limitations of electrophoretic separations in proteomics strategies. *Mass Spectrometry Reviews* 28, 5, 816-843.

Rakita, R. M., B. R. Michel, and H. Rosen. (1990). Differential inactivation of Escherichia coli membrane dehydrogenases by a myeloperoxidase-mediated antimicrobial system. *Biochemistry,* 29, 1075-1080.

Raper, K. B., and D. I. Fennell. (1965). *Aspergillus fumigatus* group, p. 238– 268. In K. B. Raper and D. I. Fennell (ed.), *The genus Aspergillus*. The William & Wilkins Co., Baltimore, Md.

Ratcliffe N. A., Rowley A. F., Fitzgerald S. W. and Rhodes C. P. (1985). Invertebrate immunity: basic concepts and recent advances. International Reviews in Cytology, 97, 183-350.

Ratte, H.T. (1999). Bioaccumulation and toxicity of silver compounds: A review. *Environmental Toxicology and Chemistry,* 18, 1, 89–108.

Reeves, E.P, Lu, H., Jacobs, H.L., Messina, C.G.M., Bolsover, S., Gabellak, G., Potma, E.O., Warley, A., RoesI, J. and Segal, A.W. (2002) Killing activity of neutrophils is mediated through activation of proteases by K+ flux. *Nature*, 416, 291-297.

Renwick, J., Reeves, E. P., Wientjes, F. B. and Kavanagh, K. (2007). Translocation of proteins homologous to human neutrophil p47phox and p67phox to the cell membrane in activated hemocytes of *Galleria mellonella*. *Developmental and Comparative Immunology*, 31, 347-35.

Rizki, T. 1984. Insect Ultrastructure, New York, Plenum Publishing Corporation.

Riberio, C. & Brehelin, M. (2006). Insect haemocytes: What type of cell is that? *Journal of Insect Physiology*, 52, 417-429

Rohloff, L.H. and Wiesner, A.G. (1994) Fluorescence assay demonstrating stimulation of phagocytosis by haemolymph molecules of *Galleria mellonella.* *Journal of Insect Physiology*, 40, 1045–1049.

Rowan, R., Moran, C., McCann, M. & Kavanagh, K. (2009). Use of *Galleria mellonella* larvae to evaluate the *in vivo* anti-fungal activity of [Ag2(mal)(phen)3]. *Biometals,* 22, 461–467.

Rus, H., Cudrici, C., and Niculescu, F. (2005). The role of the complement system in innate immunity. *Journal of Immunological Research*, 33(2): 103-112

Ryan, R.P., McCarthy, Y., W 1 att, S.A., Niehaus, K., and Dow, J.M. (2009). Intraspecies signalling involving the diffusible signal factor BDSF (cis-2-dodecenoic 3 acid) influences virulence in *Burkholderia cenocepacia*. *Journal of Bacteriology.* 191, 5013-5019

Sandler, B., Nikonova, L., Leal, W. and Clardy, J. (2000) Sexual attraction in the silkworm moth: structure of the pheromone-bindingprotein–bombykol complex. *Chemical Biology*, 7, 143–151

Schmidt, S., Sohrmann, M., Hofmann, K., Woollard, A. and Simanis, V. (1997). The Spg1p GTPase is an essential dosage-dependent inducer of septum formation in *Schizosaccharomyces pombe*. *Genes Developmental,* 11, 1519-1534

Schneider, D. (2000). Using *Drosophila* as a model insect. *Genetics*, 1, 218-226.

Schuhmann, B., Seitz, V., Vilcinskas, A., and Podsiadlowski, L. (2003). Cloning and expression of gallerimycin, an antifungal peptide expressed in immune response of greater wax moth larvae, *Galleria melonella*. *Archives of Insect Biochemistry and Physiology,* 53, 125–133.

Scully, L. R., and Bidochka, M. J. (2006). Developing insect models for the study of current and emerging human pathogens. *FEMS microbiology letters*, 263(1), 1-9.

Seed, K.M. and Dennis, J.J. (2009) Experimental bacteriophage therapy increases survival of *Galleria mellonella* larvae. infected with clinically relevant strains of the *Burkholderia cepacia* complex. *Antimicrobial Agents and Chemotherapy.* 53, 5, 2205-2208.

Seed, K. D., and Dennis, J. J. (2008). Development of *Galleria mellonella* as an alternative infection model for the *Burkholderia cepacia* complex. *Infection and immunity*, 76(3), 1267-75.

Segal, a W., Dorling, J., and Coade, S. (1980). Kinetics of fusion of the cytoplasmic granules with phagocytic vacuoles in human polymorphonuclear leukocytes. Biochemical and morphological studies. *The Journal of cell biology*, 85(1), 42-59.

Segal, A.W. (2005) How neutrophils kill microbes, *Annual review of immunology*, 23, 197-223

Seitz, V., Clermont, A., Wedde, M., Hummel, M., Vilcinskas, A., Schlatterer, K. and Podsiadlowski, L. (2003). Identification of immuno- relevant genes from greater wax moth (*Galleria mellonella*)by substractive hybridization approach. *Developmental and Comparative Immunology* 27, 207–215.

Sengel, H., Kjeldsen, L., & Borregaard, N. (1993). Early Neutrophil Activation, *Journal of Immunology*, 150(4), 1535-1543.

Shimomura, M., Minami, H., Suetsugu, Y., Ohyanagi, H., Satoh, C., Antonio, B., Nagamura, Y., et al. (2004). KAIKObase : An integrated silkworm genome database and data mining tool. *BMC Genomics*, 8, 1-8.

Sideri, M., Tsakas, S., Lampropoulou, M., and Marmaras, V. J. (2007). Innate immunity in insects : surface-associated dopa decarboxylase-dependent pathways regulate phagocytosis , nodulation and melanization in medfly haemocytes. *Journal of Immunlogy,* 528-537.

Silva, C. P., Waterfield, N. R., Daborn, P. J., Dean, P., Chilver, T., Au, C. P. Y., Sharma, S., et al. (2002). Bacterial infection of a model insect : *Photorhabdus luminescens* and *Manduca sexta*. *Cellular Microbiology,* 4, 329-339.

Silver, K.E, and Harrison, R.E. Kinesin 5B Is Necessary for Delivery of

Membrane and Receptors during FcγR-Mediated Phagocytosis. *The Journal of Immunology,* 186, 816-825.

Sims, C. R., Ostrosky-Zeichner, L. and Rex, J. H. (2005). Invasive candidiasis in immunocompromised hospitalized patients. *Archives Medical Research*, 36, 660–671.

Singh, D.P., Kumar, R., Malik, V. and Tyagi, P. (2007) Template synthesis, spectroscopic studies and biological activities of macrocyclic complexes derived from thiocarbohydrazide and glyoxal. *Journal of Enzyme Inhibition and Medicinal Chemistry.* 22, 2, 177-182.

Slater, J. L., L. Gregson, D. W. Denning, and P. A. Warn. 2011. Pathogenicity of *Aspergillus fumigatus* mutants assessed in *Galleria mellonella* matches that in mice. *Medical Mycology,* 49, 107–S113

Śmietankoa, A., Wiśniewski, J.R., Cymborowski, B. (1989). Effect of low rearing temperature on development of *Galleria mellonella* larvae and their sensitivity to juvenilizing treatment. *Comparative Biochemistry and Physiology Part A: Physiology,* 92, 163-169

Standish, A. and Weisner, J. (2010)**.** Human Neutrophils Kill Streptococcus pneumoniae via Serine Proteases. *Journal of Immunology*, 185, 2602-2609

Stefanska, J. and Pawliczak, R. (2008) Apocynin: molecular aptitudes. *Mediators of Inflammatory*, 106-507.

Steinberg, T.H., Agnew, B.J., Gee, K.R., Wai-Yee Leung, W.L., Goodman, T., Schulenberg, B., Hendrickson, J., Beechem, J.M., Haugland, R.P. and Patton, W.F. (2003) Global quantitative phosphoprotein analysis using Multiplexed Proteomics technology, *Proteomics,* Special Issue: Multiplexing Technologies, 3, 7, 1128–1144 .

Stie, J., and Jesaitis, A. J. (2007). Reorganization of the human neutrophil plasma membrane is associated with functional priming : implications for neutrophil preparations. *Journal of Leukocyte Biology*, 81, 672-685.

Strand, M.R., Beck, M.H. and Lavine, M.D. (2006) Microplitis demolitor bracovirus inhibits phagocytosis by hemocytes from *Pseudoplusia includens. Archives of Insect Biochemistry and Physiology*, 61, 134-145.

Strand, M. R. (2008). Invited review The insect cellular immune response. *Insect Science*, 1 - 14.

Styrt, B. and Klempner, M. S. (1982) Internal pH of human neutrophil lysosomes. *FEBS Letters,* 149:113-116.

Sudbery, P., Gow, N. and Berman, J. (2004) The distinct morphogenic states of *Candida albicans*, *Trends in Microbiology*, 12, 7, 317-324

Sun, S.C. and Faye, I. (1992). Affinity purification and characterization of CIF, an insect immunoresponsive factor with NF-κB-like properties. *Comparative Biochemistry and Physiology.* 103, 225–33.

Sun, C. X., Downey, G. P., Zhu, F., Koh, A. L. Y., Thang, H., and Glogauer, M. (2004). Rac1 is the small GTPase responsible for regulating the neutrophil chemotaxis compass. *Blood,* 104(12), 3758-65.

Theopold, U., Schmidt, O., Soderhall, K. and Dushay, M.S. (2004) Coagulation in arthropods, defence, wound closure and healing. *Trends in Immunology*, 25, 289-294.

Theodoropoulos, P. A., Gravanis, A., Tsapara, A., Margioris, A. N., Papadogiorgaki, E., Galanopoulos, V. and Stournaras, C. (1994). Cytochalasin B may shorten actin filaments by a mechanism independent of barbed end capping. *Biochemical Pharmacology*, 47, 1875-1881

Thrasher, A. J., Keep, N. H., Wientjes, F. and Segal, A. W. (1994). Chronic granulomatous disease. *Biochemical and Biophysical Reviews*, 1227, 1-24.

Telfer, W. H., and Kunkel, J. G. (1991). The function and evolution of insect storage hexamers. *Annual review of Entomology*, 36, 205-28.

Test, S.T., Lampert, M.B., Ossanna, P.J., Thoene, J.G. and Weiss, S.J. (1984) Generation of nitrogen chlorine oxidants by human phagocytes. *Journal of Clinical Investigations*, 74(4), 1341-1349.

Tschesche, H., Kopps, C., Horln, W.H., and Hempelmann, U. (1994) Inhibition of Degranulation of Polymorphonuclear Leukocytes by Angiogenin and Its Tryptic Fragment. *The Journal of Biological Chemistry,* 269, 48, 30274-30280.

Tse, W.Y., Nash, G.B., Hewins, P., Savage, C.S. and Adu, D. (2005) ANCA-induced neutrophil F-actin polymerization: Implications for microvascular inflammation. *Kidney International*, 67, 130–139.

Valasek, M.A., Joyce, J. and Repa, J.A. (2005) The power of real-time PCR. *Advances in Physiology Education,* 29: 151–159, 2005.

Valerius, N. H., Stendahl, O., Hartwig, J. H. and Stossel, T. P. (1981). Distribution of actin-binding protein and myosin in polymorphonuclear leukocytes during locomotion and phagocytosis. *Cell,* 24, 195-202

van der Vliet A, Eiserich, J.P. and Cross, C.E. (2000) Nitric oxide: a proinflammatory mediator in lung disease? *Respiratory Research*, 1, 67-72.

Vasquez, R. J., Howell, B., Wadsworth, P., & Cassimeris, L. (1997). Nanornolar Concentrations of Nocodazole Alter Microtubule Dynamic Instability In Vivo and *In Vitro*. *Molecular Biology of the Cell*, 8, 973-985.

Vento S, Cainelli F. Infections in patients with cancer undergoing chemotherapy: aetiology, prevention, and treatment. *Lancet Oncology*. 2003;4:595-604

Vierstraete, E., Verleyen, P., Baggerman, G., D'Hertog, W., Van den Bergh, G., Arckens, L., DeLoof, A. and Schoofs, L.A. (2004) Proteomic approach for the analysis of instantly released wound and immune proteins in *Drosophila melanogaster* hemolymph. *PNAS, U S A*, 101, 470-475.

Vilcinskas, A. and Wedde, M. Insect inhibitors of metalloproteinases. *IUBMB Life,* 54, 339-43.

Vogel, H., Altincicek, B., Glöckner, G., & Vilcinskas, A. (2011). A comprehensive transcriptome and immune-gene repertoire of the lepidopteran model host *Galleria mellonella*. *BMC genomics*, 12, 1, 308.

Volz, A. (1993). Regulation of CD18 expression in human neutrophils as related to shape changes. *Journal of Cell Science*, 502.

Walhout, A. J. M., and Vidal, M. (2001). Protein interaction Maps for model organisms. *Cell*, 2, 55-62.

Wang, Y.G., Nnawke, C., Lane, W. S., Modesti, M. and Frank, K. M. (2004). Phosphorylation and Regulation of DNA Ligase IV Stability by DNA-dependent Protein Kinase. *Journal of Biological Chemistry*, 279, 37282-37290

Washburn, M.P., Wolters, D. and Yates, J.R., III. (2001) Large-scale analysis of the yeast proteome by multidimensional protein identification technology. *Nature Biotechnology.* 19, 242–247.

Waterfield NR,Wren BWand Ffrench-Constant RH (2004) Invertebrates as a source of emerging human pathogens. *Nature Reviews Microbiology*, 2, 833–841.

Wedde, M., C. Weise, C. Nuck, B. Altincicek, and A. Vilcinskas. (2006). The insect metalloproteinase inhibitor gene of the lepidopteran *Galleria mellonella* encodes for two distinct inhibitors. *Journal of Biological Chemistry,* 388, 119-127.

Wertheim, B., Kraaijeveld, A.R., Schuster, E., Blanc, E., Hopkins, M., Pletcher, S.D., Strand, M.R., Godfray, H.C.J. and Partridge, L. (2005) Genome wide expression in response to parasitoid attack in *Drosophila melanogaster*. *Genome Biology*, 6, 94, 1-20.

Westermeler, R. (2006) Sensitive, Quantitative, and Fast Modifications for Coomassie Blue Staining of Polyacrylamide Gels . *Proteomics,* 6, 61-64.

Whitten, M. M.A., Tew, I.F., Lee, B.L., and Ratcliffe, N.A. (2004). A novel role for an insect apolipoprotein (apolipophorin III) in b-1,3-glucan pattern recognition and cellular encapsulation reactions. *Journal of Immunology.* 172, 2177–2185

Witko-Sarsat, V., Riev, P., Descamps-Latscha, B., Lesaure, P. and Halbwachs-Mecarelli, L. (2000). Neutrophils: molecules, functions and pathophysiological aspects. *Laboratory investigation*, 80(5), 617-653.

Wiesner, A. (1996). A small phagocytosis stimulating factor is released by and acts on phagocytosing *Galleria mellonella* haemocytes *in vitro*. *Journal of Insect Physiology,* 42(9), 829-835.

Wiesner, A., Losen, S., Kopaćek, P., Weise, C. and Götz, P. (1997). Isolated apolipophorin III from *Galleria mellonella* stimulates the immune reactions of this insect. *Journal of Insect Physiology*, 43, 383–391.

Willmann, R., Possekel, S., Dubach-Powell, J., Meier, T., and Ruegg, M. (2009). Mammalian animal models for Duchenne muscular dystrophy. *Neuromuscular disorders : NMD*, 19(4), 241-9.

Wojda, I., & Jakubowicz, T. (2007). Humoral immune response upon mild heat-shock conditions in *Galleria mellonella* larvae. *Journal of insect physiology,* 53(11), 1134-44.

Wong, S. H., Francis, N., Chahal, H., Raza, K., Salmon, M., Scheel-Toellner, D., and Lord, J. M. (2009). Lactoferrin is a survival factor for neutrophils in rheumatoid synovial fluid. *Rheumatology (Oxford, England)*, 48(1), 39-44.

Wu, C.C and MacCoss, M.J. (2002) Shotgun proteomics: tools for the analysis of complex biological systems. *Current Opinions in Molecular Therapeutics,* 4, 242-250 .

Xu, P., Crawford, M., Way, M., & Godovac-zimmermann, J. (2009). Subproteome analysis of the neutrophil cytoskeleton. *Proteomics*, 2037-2049.

Yan, M., Di Ciano-Oliveira, C., Grinstein, S. and Trimble, W.S. (2007) Coronin function is required for chemotaxis and phagocytosis in human neutrophils. J*ournal of Immunolology*, 178, 5769– 5778.

Yang, D., De La Rosa, G., Tewary, P. and Oppeinheim, J. J. (2009). Alarmins link neutrophils and dendritic cells. *Trends in Immunology*, 30, 531-537.

Yoshiga, T. , Georgieva, T., Dunkov, C., Harizanova, N., Ralchev, K. and Law, L.H. (1999). *Drosophila melanogaster* transferrin. Cloning, deduced protein sequence, expression during the life cycle, gene localization and up- regulationon bacterial infection. *European Journal of Biochemistry*, 260, 2, 414-420

Yost, C.H., Cody, M., Harris, E.S., Thornton, N., McInturff, A.M. *et al.* (2009). Impaired neutrophil extracellular trap (NET) formation: a novel innate immune deficiency of human neonates. *Blood*, 113, 6419-6427.

Yürüker, B. and Niggli, B. (1992) a -Actinin and vinculin in human neutrophils: Reorganization during adhesion and relation to the actin network. *Journal of Cell Science*, 101, 403-414.

Zarembinski, T. I., Hung, L. W., Mueller-Dieckmann, H. J., Kim,K. K., Yokota, H., Kim, R. and Kim, S. H. (1998) Structure based assignment of the biochemical function of a hypothetical protein: a test case of structural genomics. *PNAS*, 95, 15189-15193.

Zdybicka-Barabas, A., and Cytryńska, M. (2010). Involvement of apolipophorin III in antibacterial defense of *Galleria mellonella* larvae. *Comparative biochemistry and physiology. Part B, Biochemistry & molecular biology*, 158(1), 90-98.

Zhong, B.X., Li, J.K., Lin, J.R., Liang, J.S., Su, S.K., Xu, H.S. et al. (2005) Possible effect of 30 K proteins in embryonic development of silkworm Bombyx mori. *Biochemistry and Biophysiology*, 37, 355–361.

Zhao, L. and Kanost, M.R. 1996. In search of a function for hemolin, a hemolymph protein from the immunoglobulin super- family. *Journal of Insect Physiology*, 42:73–80.

Zu, Y. L., J. Qi, A. Gilchrist, G. A. Fernandez, D. Vazquez-Abad, D. L. Kreutzer, C. K. Huang, and R. I. Sha'afi. (1998). p38 mitogen-activated protein kinase ac- tivation is required for human neutrophil function triggered by TNF stimulation. *Journal of Immunology*, 160: 1982–1989.