An analysis of the cellular and humoral immune responses of *Galleria mellonella* larvae

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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

ACN	Acetonitrile
AMP	Anti-microbial Peptide
ApoLp-III	Apolipophorin-III
ApoE	Apolipoprotein E
APS	Ammonium persulphate
ATP	Adenosine triphosphate
β	beta
βGBP	beta-1, 3-glucan recognition protein
bp	Base Pair
BSA	Bovine serum albumin
°C	Degrees centigrade
cm	Centimeter
CR1	Complement receptor 1
d	Day
DAPI	4',6-diamidino-2-phenylindole
DAMP	Damage associated molecular pattern
DEPC	Dietyl pyrocarbonate
DH	Dehydrogenase
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide 5'-triphosphate
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra acetic acid
EF	Elongation factor
FACS	Fluorescence Activated Cell Sorter
fMLP	N-formyl-methionine- leucinephenylalanine
g	Grams
g	g-force
GST	Glutathione S transferase
GTP	Guanidine tri-phosphate
h	Hours

HCl	Hydrochloric acid
HP	High Power
HSP	Heat shock protein
IAA	Iodoacetamide
IEF	Iso-electric focusing
Ig	Immunoglobulin
IPG	Immobilised pH strips
IPS	Insect Physiological Saline
IMD	Immune Deficiency pathway
JHBP	Juvenile hormone binding protein precursor
k	Kilo
kDa	Kilodaltons
L	Litre
LPS	Lipopolysaccharide
LC/MS	Liquid chromatography mass spectrometry
M	Molar
MALDI ToF	Matrix-laser desorption/ionization-time of flight
mg	Milligrams
min	Minute
ml	Millilitre
mM	Millimolar
mRNA	Messenger ribonucleic acid
MRSA	Methicillin resistant *Staphylococcus aureus*
µg	Microgram
µl	Microlitre
µM	Micromolar
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
ng	Nanogram
nm	Nanometer
NO	Nitric oxide
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline

PCR	Polymerase Chain Reaction
pg	picogram
PG	Peptidoglycan
PIPES	Piperazine-N,N′-bis(2-ethanesulfonic acid)
PGRP	Peptidoglycan recognition protein
PMA	Phorbol 12-myristate 13-acetate
ProPO	Prophenoloxidase
PRR	Pattern recognition receptor
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive oxygen species
rpm	Revolutions per minute
RT	Reverse transcriptase
SBC3	1,3-dibenzyl-4,5-diphenyl-imidazol-2-ylidene silver(I) acetate
SDS	Sodium dodecyl sulfate
SOD	Superoxide dismutase
TAE	Tris acetate buffer
TCA	Tricarboxylic cycle
TCTP	Translationally-controlled tumor protein homolog
TEMED	N,N,N',N'-Tetramethlyethlenedimine
TER94	Transitional endoplasmic reticulum ATPase
TLR	Toll like receptor
U	Unit
UV	Ultraviolet
V	Volts
v	Volume
v/v	Volume per volume
V-ATPase	Vacuolar ATPase
w/v	Weight per volume
YEPD	Yeast Extract-Peptone-D-Glucose

Abstract

The invertebrate immune system is composed of the intertwined cellular and humoral components which have similar structural and functional attributes to the mammalian innate immune system. For this reason insects have served as useful screening tools in academic and industry research for the assessment of pathogenicity of microorganisms or the antimicrobial efficacy of drugs. Due to the low cost, fast turnover of results and lack of ethical constrictions the insect screening model has been used widely. The work presented here in this thesis sought to establish the effects of using *Galleria mellonella* larvae under different conditions to determine the impact these effects could have on larval survival following infection.

A number of abiotic factors known to influence the immune response of insects were assessed including temperature and food abundance or factors such as injury and age. *G.mellonella* larvae exposed to thermal and physical stress demonstrated increased survival to a lethal dose of *Aspergillus fumigatus* or *Staphylococcus aureus* when compared to control larvae. The thermal and physical stress induced an immune priming effect on larvae which coincided with an increased abundance of immune proteins from haemolymph and haemocytes, increased circulating haemocytes, altered expression of genes and a change in the composition of haemocyte populations. These changes peaked 24h after the stress event but declined 72h after the stress event, demonstrating the short term priming of the larval immune response to thermal and physical stress.

The effects of pre-incubation at 15°C on larval ability to survive an infection demonstrated a greater susceptibility to infection among larvae pre-incubated for > 3weeks. Larvae possessed reduced abundance of proteins associated with energy metabolism, immune function, decreased circulating haemocytes and AMP expression within larvae pre-incubated for periods of 3 weeks or more. The results highlight the use of larvae pre-incubated for different periods may impair inter-laboratory comparisons. Previous findings demonstrated that insufficient availability of nutrients for larvae also reduced survival to microbial infection when compared to larvae with adequate food. A reduced abundance of immune related proteins was detected in nutrient deprived larvae. The established use of *G.mellonella* in antimicrobial efficacy screening was demonstrated here through the assessment of a new silver based

antimicrobial which was capable of increasing the survival of larvae to a microbial challenge when compared to larvae that received no compound.

The assessment of similarities between the insect haemocytes NADPH oxidase activation profile to neutrophils demonstrated a number of proteins to be phosphorylated in response to fMLP and PMA stimulation which was previously demonstrated within mammalian neutrophils.

The results preformed here have highlighted the similarities shared between the insect and mammalian immune systems and how inter-laboratory variations in larval use may have consequences on larvae in terms of immune competence and priming. The utility of the larvae as a screening model to study the efficacy of antimicrobial compounds has also been demonstrated in this work.

Chapter 1

Introduction

1.0 Introduction

Insects are a very successful group of invertebrates, with approximately one million species that inhabit all ecological niches, apart from the sea (Hoffmann, 1995; Vilmos and Kurucz, 1998). The ability of insects to inhabit such a wide variety of environments indicates a highly efficient and versatile immune response (Bergin *et al.*, 2005; Lanot *et al.*, 2001; Pandey and Tiwari, 2012). Insects and vertebrates diverged approximately 500 million years ago, and while vertebrates have developed an adaptive immune response, the vertebrate innate immune system still retains strong structural and functional similarities to the insect immune system (Brennan *et al.*, 2002; Kavanagh and Reeves, 2004; Renwick *et al.*, 2007; Salzet, 2001). As a result of these conserved similarities a wide range of insects have been employed to study the virulence of bacterial and fungal pathogens and give results that are comparable with those obtained using vertebrates (e.g., mice) (Ben-Ami, 2011; Brennan *et al.*, 2002; Fleming *et al.*, 2006; Lemaitre and Hoffmann, 2007; Scully and Bidochka, 2006).

Within the last decade insect *in vivo* experimental models have proven to be a useful tool in assessing the *in vivo* host-pathogen interactions and as a system for drug efficacy screening. This surge in demand and interest in *in vivo* insect models has come about with the global increase in antibiotic and antifungal resistance as a result of more immunocompromised individuals who are susceptible to mostly commensal pathogenic organisms that include *Candida, Aspergillus*, and *Cryptococcus* species (Romani, 2004).

The use of the insect screening model is a more ethically appealing option as it offers a means to reduce the number of animal models employed and reduce the amount of suffering imposed on animals which share similar neurological and sentient capacities to humans. Although mammalian models are invaluable and traditionally employed for assessing antimicrobial compounds efficacy, they have certain drawbacks such as time, cost and ethical regulations (Kavanagh and Fallon, 2010). It is widely accepted that the use of mammalian models must be reduced to a minimum and to employ other systems more ethically acceptable when possible. This has been developed into the 3R policy (replace, reduce and refine) an approach adopted by many international funding agencies to curb less ethical approaches of scientific research in favour of alternatively valid and ethically acceptable screening systems.

Both mammalian and insect models are susceptible to similar pathogens and share common infection pathologies (Kemp and Massey, 2007). With this in mind the insect has a comparable innate immune system to mammals, sharing physiological and anatomical homologies, conserved across the phylogeny (Hoffmann *et al*., 1999).The insect screening model provides an innovative way to simultaneously examine the mechanisms of microbial pathogenicity and the activity of potential drug agents without the drawbacks of mammalian models.

This has led to a variety of insect host models being established for such purposes. These include the well characterised fruit fly *Drosophila melanogaster* (Lemaitre *et al*., 1996), wax moth *Galleria mellonella* (Kavanagh and Reeves, 2004; Mylonakis *et al*., 2005) and the silkworm *Bombyx mori* (Hamamoto *et al*., 2004) (Fig. 1.1).

1.1.1 *Drosophila melanogaster*

The most widely used and well established insect model for pathogenicity testing and antifungal screening is *D.melanogaster* (Fig. 1.1). *D.melanogaster* has the advantage of its genome being sequenced since 2000 (Adams *et al*., 2000), this has made the model ideal for the study of genes using a number of genetic tools and techniques. The well characterised immune system gives it a major advantage (Alarco *et al*., 2004) in generating knockout flies which have mutations in parts of the Toll signalling pathway making them susceptible to microbial pathogens and amenable to antimicrobial drug screening (Lionakis *et al*., 2005). Toll mutants show a strong correlation with the response in knockout mice models in pathogenic screening (Chamilos *et al*., 2006), making them amenable models for assessing drug efficacy.

D.melanogaster's genetic background, short generation time and commercial availability make it an ideal model for drug screening. But there are several drawbacks with this model which include its small size making it difficult to inoculate with pathogens or drugs and it is also limited in its ability to be biochemically analysed (Tzou *et al*., 2002). Although other methods of inoculation are possible such as rolling on a lawn of microbes or by feeding with sucrose (Mylonakis *et al*., 2005) these methods lack the accuracy of injection (Kavanagh and Fallon, 2010). The requirement for propagation between 22-25°C limits assessing interactions in both pathogenicity and antimicrobial drugs which would usually occur at 37°C in mammals
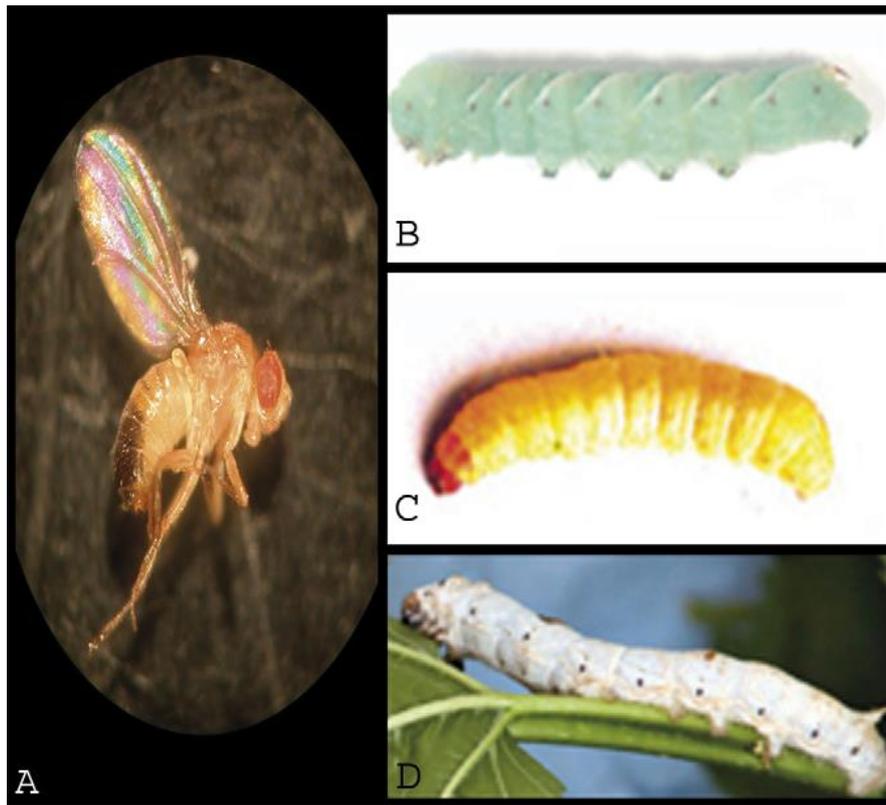
Fig. 1.1 Insects used as models for human pathogens.
(A) *Drosophila melanogaster*; (B) *Manduca sexta*; (C) *Galleria mellonella* and (D)
Bombyx mori (Kemp and Massey, 2007).

(Bhabhra *et al*., 2004). But despite these limitations *D.melanogaster* can provide a detailed analysis of host-pathogen interactions and deliver a high throughput screening system for antimicrobial drug testing (Kemp and Massey, 2007).

1.1.2 *Bombyx mori*

The silkworm *Bombyx mori* (Fig. 1.1) of the Lepidoptera order is a substantially large insect model at 5cm in length (Kaito *et al*., 2002). This makes for ease of handling and enables injection of specific amounts of compound or pathogen, making them amenable to drug pharmacodynamic studies through the ability to make haemolymph preparations, isolate organs (Hamamoto *et al*., 2004) and monitor proliferation through pathogen cell counts (Kaito *et al*., 2005). The ability to administer exact volumes is essential for quantitative evaluation of pathogenicity and efficacy of antimicrobial agents (Kaito and Sekimizu, 2007).

The silkworm has a circulatory system making it suitable for drug pharmacodynamics assessment, it also mirrors similar antibiotic effectiveness observed in both mice and humans against the same pathogens making them suitable mini host models (Kaito *et al*., 2002). They offer a high throughput system delivering results within 24-48h with vast amounts of the larvae commercially available cheaply due to the long history of silk production by the silk industry (Hamamoto *et al*., 2004). Since the completion of the silkworm genome project (Mita *et al*., 2004; Xia *et al*., 2004) the silkworm has become more genetically tractable in assessing host-pathogen virulence factors as seen with *D.melanogaster* and *Caenorhabditis elagans* (Kaito and Sekimizu, 2007). Although they do lack the well characterised immune response of *G.mellonella* and the genetic tools of *D.melanogaster* (Kemp and Massey, 2007).

1.1.3 *Manduca sexta*

Manduca sexta or the tobacco hornworm (Fig. 1.1) of the Lepidoptera order is 7cm in lenght, making them equivalent to some small mammals used in *in vivo* research and they possess a large volume of haemolymph (Kanost *et al*., 2004). This enables ease of handling and inoculation as well as the extraction of haemolymph and the ability to be incubated at 37°C (Horohov and Dunn, 1983; Kanost *et al*., 2004). The larvae are extensively used to study the innate immune pattern recognition

receptors (PRRs) (Fabrick *et al*., 2003; Yu *et al*., 2002) and proPO cascade (Tong and Kanost, 2005) involved in pathogen detection. Despite the numerous advantages these larvae offer they do lack a well characterised immune system and genome of other models such as *D.melanogaster* (Kemp and Massey, 2007) and are not as commercially available as other lepidoteran models such as *G.mellonella* and *B.mori*.

1.1.4 *Galleria mellonella*

The larvae of the greater wax moth *G.mellonella* are 1.5-2.5cm in length. Their size enables an easy means of inoculation with specific amounts of drug or pathogen via the pro-leg (Fig. 2.1) making *G.mellonella* more amenable to drug pharmacodynamics studies (Kavanagh and Fallon, 2010). It is also feasible to assess phagocytic cell function and immune responses to determine the virulence of pathogens (Cotter *et al*., 2000) and the actions of immunosuppressive molecules such as gliotoxin and fumigillin released from the fungus *A. fumigatus* on phagocytic functions (Fallon *et al*., 2011a; Renwick *et al*., 2007).

The assessment of haemocyte function gives comparable results to those seen in human neutrophils due to the functional homology of phagocytosing haemocytes in *G.mellonella* (Kavanagh and Reeves, 2004). A strong correlation is observed in microbial pathogenicity in *G.mellonella* and mammalian systems (Brennan *et al*., 2002; Jander *et al*., 2000). *G.mellonella* are particularly suited as *in vivo* models as they have a high throughput (Cotter *et al*., 2000) and can be incubated between 30-37°C enabling possible temperature dependent virulence factors to be studied (Mowlds and Kavanagh, 2008). Pathogen virulence and antimicrobial efficacy testing of agents can be analysed by using a number of parameters in *G.mellonella* including the degree of melanisation in response to a pathogen, larval death (Fig. 1.2), alterations in fungal burden, changes in haemocyte densities and changes in antimicrobial peptide expression (Bergin *et al*., 2003; Bergin *et al*., 2006).

Although *G.mellonella* do have several innate immune similarities to mammals they lack many defined organ systems preventing the full study of fungal dissemination and antifungal therapy which is possible in mammals (Kavanagh and Fallon, 2010). More recently the larvae have been employed to study the pathology of brain infections caused by *Listeria* which produces a comparable pathology to that
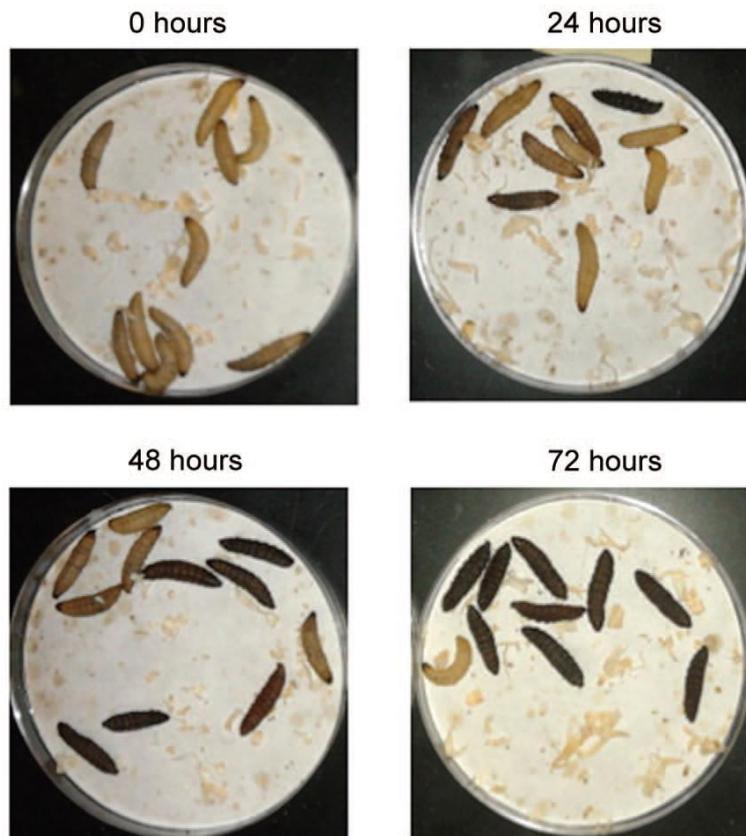
Fig. 1.2 Effect of *Staphylococcus aureus* on viability of *G. mellonella* larvae.

Larvae were inoculated with *S. aureus* and incubated at 37 °C. A mortality rate of 90% is observed at 72 h. The dark colour is due to the process of melanisation in response to the growth of the pathogen in the insect. Note that the larvae are supplied with a food source (wood shavings).

observed in humans (Mukherjee *et al*., 2013). Rowan *et al*. (2009) demonstrated how it is possible to distinguish between antimicrobial properties of a drug and the potential immune priming response a drug may induce when injected into *G.mellonella* larvae.

1.2 The innate immune system

The insect innate immune response consists of two tightly interconnected components, the cellular and the humoral responses (Hoffmann, 1995; Hultmark, 1993). The cellular response is mediated by haemocytes and involves responses such as phagocytosis, encapsulation, nodulation and clotting (Lavine and Strand, 2002). The humoral defences are composed of soluble effector molecules including anti-microbial peptides, complement-like proteins, melanin and products generated by proteolytic cascades such as the phenoloxidase (PO) pathway which immobilize or kill pathogens within the insect (Strand, 2008).

1.2.1 Humoral immune response

The haemolymph of insects is comparable in function to mammalian blood as it serves to transport nutrients, waste and signal molecules around the insect (Matha and Acek, 1984) without a function in gas exchange as seen in vertebrates. The haemolymph is the focal point of infection and immune response to infection. The humoral immune response is composed of a number of humoral components including proPO cascade, lysozyme, lectins (Dunphy *et al*., 1986) and PRRs (Lavine and Strand, 2002), as well as serine proteases (da Silva *et al*., 2000).

The insect humoral response has a number of similar mechanisms to counter pathogen invasion to mammals. Insects first line of defence is the cuticle which functions similarly to mammalian skin by preventing or slowing pathogen entry (Kavanagh and Reeves, 2004). Pathogens breaching the cuticle activate a number of humoral responses. These mechanisms and functions include wound healing to limit potential infection through the use of clottable lipophorin proteins from insects. These lipophorins have a homologous domain to the mammalian Von Willebrand's factor, which functions similarly to clot blood (Vilmos and Kurucz, 1998).

The melanisation response of insects results in the production of melanin which deposits on microbial surfaces and can be easily observed in infected *G.mellonella* larvae (Kavanagh and Fallon, 2010). Melanin formation is catalysed by phenoloxidase-monophenyl-L-dopa:oxygen oxidoreductase with proPO protein showing a similar sequence region to vertebrate complement proteins C3 and C4 (Söderhäll and Cerenius, 1998) which opsonise microbial surfaces. Melanisation is activated through PRRs binding to specific microbial surface proteins and structures causing a protease cascade activating PO that creates melanin (Söderhäll and Cerenius, 1998). Both insect and mammalian models display a strong resemblance in opsonisation activity through the use of similar PRRs (Royet *et al.*, 2005) which include lectins, integrins, apolipoproteins, scavenger receptors found in both mammals and insects. The Toll pathway of insects lack PRR ability observed in TLRs of mammals but both show similar pathway signalling. Spätzle is responsible for activating the toll dependent pathway of antimicrobial peptide encoding genes through NF-κB in the nucleus causing subsequent generation and release of AMPs (Hoffmann *et al.*, 1999) (Fig. 1.3). *D. melanogaster* has the added signalling pathway the Immuno deficiency (IMD) pathway analogous to the Tumour necrosis (TNF) signalling pathway in humans which functions in response to Gram negative and some Gram positive bacteria (Medzhitov *et al.*, 1997) (Fig. 1.3, 1.4).

Insects also have an anti-microbial peptide response synthesised by the fat body which is similar in function to the liver of mammals. AMPs are also synthesised by haemocytes and at environmentally exposed regions of the respiratory and genital tracts where AMPs target fungal cell walls and bacterial components (Ratcliffe, 1985). AMPs are similarly expressed in both mammalian and insect humoral responses and include cationic proteins (cathepsin and lysozyme), metalloproteinases, cecropin, gloverins, drosomycin and defensins (Kavanagh and Reeves, 2004)

1.2.2 Insect Haemocytes: structure, function, and diversity

There are at least eight types of haemocytes found in insects: prohaemocytes, plasmatocytes, granular cells, coagulocytes, crystal cells, spherulocytes, oenocytoids, and thrombocytoids (Pandey and Tiwari, 2012; Tanada and Kaya, 1993), however, the majority of insects do not possess all types of haemocytes (Fig. 1.5). Haemocytes are found circulating freely in the haemolymph or adhering to internal organs such
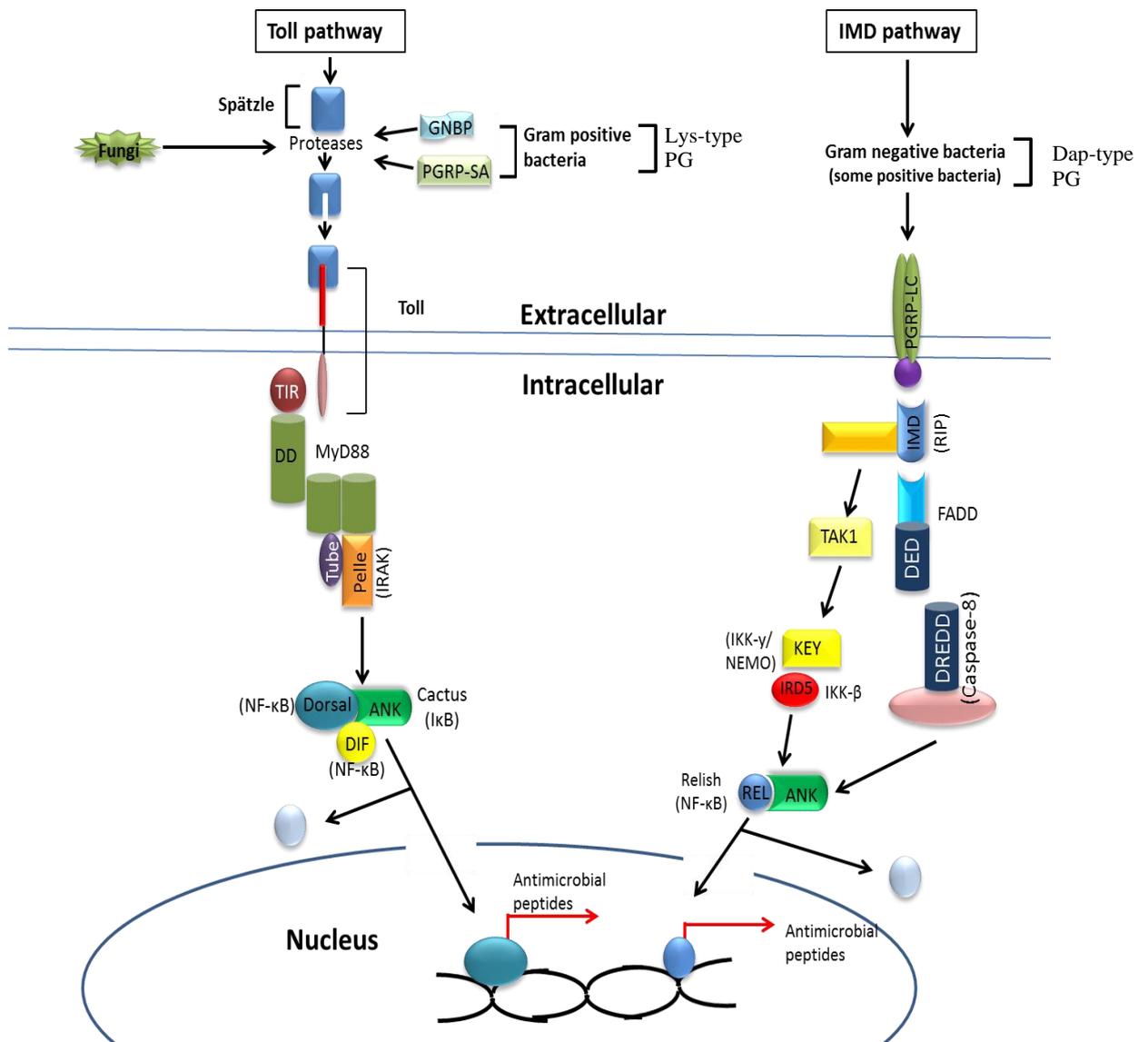
Fig. 1.3 Schematic summary of the Toll pathway (left) activated by mainly fungi and Gram positive bacteria, and (right) the IMD pathway mainly activated by Gram negative bacteria and some Gram positive bacteria.
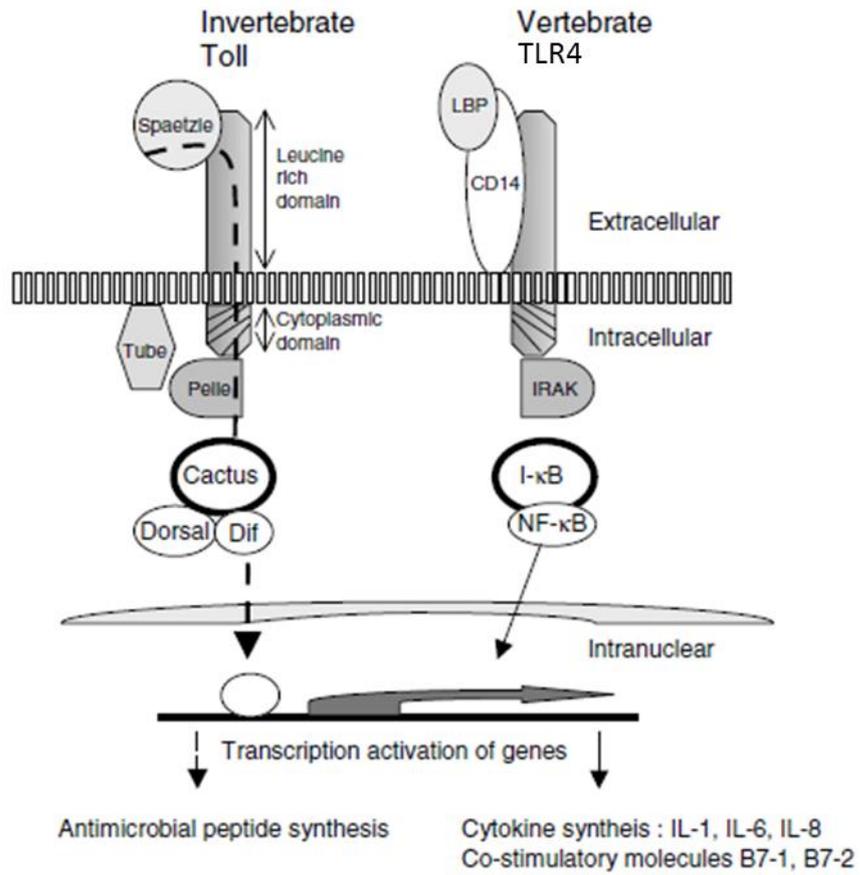
Fig. 1.4 Schematic summary of the invertebrate Toll pathway (left) and (right) the vertebrate TLR4 pathway (Kavanagh and Reeves, 2004).

as the fat body or the digestive tract of the insect (Kavanagh and Reeves, 2004). The density of haemocytes in the haemolymph varies during the life of the insect and also in response to the introduction of pathogens (Bergin *et al*., 2003).

Prohaemocytes are small circular cells with a large nucleus and a basophilic cytoplasm that can differentiate into a number of cell types. Plasmatocytes are the most common haemocyte, they are leaf like in shape and their cytoplasm contains lysosomal enzymes. They are usually larger than granular cells and are involved in capsule formation (Lavine and Strand, 2002). Granular cells have a small nucleus and many granules in the cytoplasm. Spherulocytes display a variety of differing shapes, with numerous small spherical inclusions (Fig. 1.5, 1.6). Oenocytoids are large, bi-nucleate, non-phagocytic cells that contain precursors of the proPO system, while coagulocytes participate in the clotting process. Haemocytes can recognize foreign material and also distinguish self from non-self during the cellular immune response in a similar manner to human immune cells (Izzetoglu, 2012; Lavine and Strand, 2002).

In Lepidopteran insects (e.g., *G. mellonella*) plasmatocytes and granular cells are involved in most cellular defence responses, whereas in *Drosophila* these responses include primarily plasmatocytes and lamellocytes (Lavine and Strand, 2002). Haemocyte-mediated phagocytosis is similar to that in mammalian neutrophils, the activity of the phagocytic cell in insects (the plasmatocyte) is enabled by opsonisation of microorganisms with complement-like proteins (Ben-Ami, 2011; Tojo *et al*., 2000). When a pathogen has been engulfed by a phagocytic cell, a membrane-bound enzyme system is activated which causes the translocation of electrons from cytosolic NADPH to extracellular oxygen, resulting in superoxide production (Fig. 1.7) (Baggiolini and Wymann, 1990). The respiratory burst is necessary for killing pathogens but also results in local tissue damage and inflammation in the area of infection due to the release of enzymes following degranulation (Arnhold, 2004; Baggiolini and Wymann, 1990). Phagocytosis depends upon the recognition of the target as foreign which activates downstream signalling and effector responses (Kavanagh and Reeves, 2004). Some pathogens are recognized by humoral pattern recognition molecules that bind to a target which increases its recognition by other receptors on the surface of haemocytes. Alternatively pathogens can be recognized directly by haemocyte surface receptors, e.g., calreticulin or apolipophorin. The phagocytic process itself is generally non-destructive to haemocytes, unlike
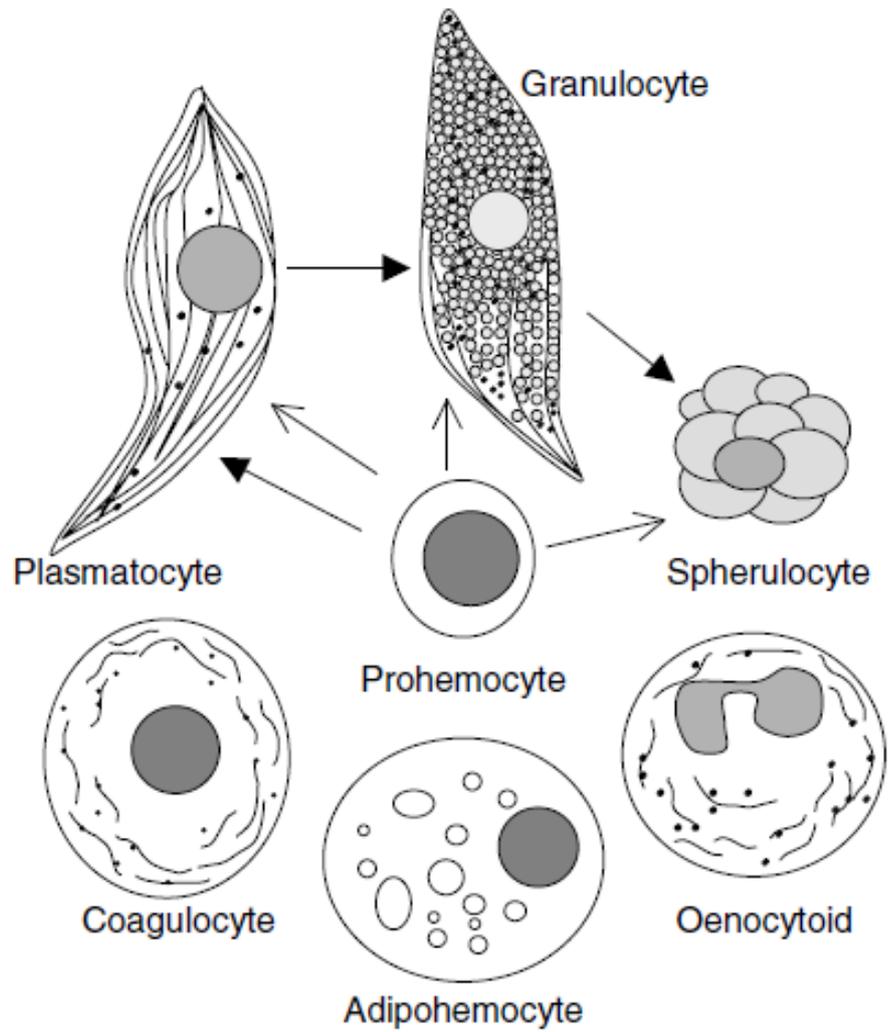
Fig. 1.5 Haemocytes found within *G.mellonella* larvae (Kavanagh and Reeves, 2004). *G.mellonella* has 6 types of haemocytes granulocyte (granular cell) and plasmatocyte (phagocytosing cells), oenocytoid and coalgulocytes (secondary roles involved in immune defence), spherulocyte and adipohaemocyte (energy storage).

melanisation, coagulation, and encapsulation, which are terminal events in the lifespan of a haemocyte (Oliver *et al*., 2011).

In order for phagocytosis to occur, opsonic ligands bind to molecules on the outside of the pathogen. Once the ligands are recognized, an intracellular cascade occurs resulting in the pathogen being internalized in the cell (Kavanagh and Reeves, 2004). The process of phagocytosis is lectin-mediated and lectins are found in the insect haemolymph, along with lysozyme (Kavanagh and Reeves, 2004). Lysozyme is an anti-microbial peptide that has been found within haemocytes. The presence of Gram-negative bacteria causes binding of N-acetylglucosamine (GlcNa)-specific lectins (e.g., BDL-2 lectins and PGBP 1/2) to peptidoglycans on the bacterial cell wall. After the lectins have bound to the surface, they then bind to plasmatocytes and induce phagocytosis of the bacterial cell (Kavanagh and Reeves, 2004). Lectins and lysozyme act simultaneously. While the lectins bind to the sugars on the bacterial cell surface, lysozyme breaks down the peptidoglycan layer, causing a release of sugars, revealing lipotechnoic acid and lipomannan, which in turn are recognized by BDL-1 lectins (Kavanagh and Reeves, 2004).

1.2.3 Nodulation

Nodulation occurs when multiple haemocytes bind to clusters of bacteria and fungi. This is the main insect cellular defence reaction following infection and allows large numbers of bacteria to be cleared from the haemolymph. Haemocytes begin to bind together, causing an overlapping sheath to form around the pathogen (Lavine and Strand, 2002). Nodulation is completed with the activation of prophenoloxidase and melanisation of mature nodules (Ratcliffe and Gagen, 1976). An equivalent nodulisation process does not occur in human phagocytes but complement displays a similar cascade event to melanisation (Cerenius *et al*., 2010).

1.2.4 Encapsulation

Encapsulation occurs in response to the entry of large structures such as protozoa, nematodes and eggs or larvae of parasitic insects into the haemolymph (Strand and Pech, 1995). There are two different types of encapsulation in insects:

Plasmatocyte

Spherulocyte
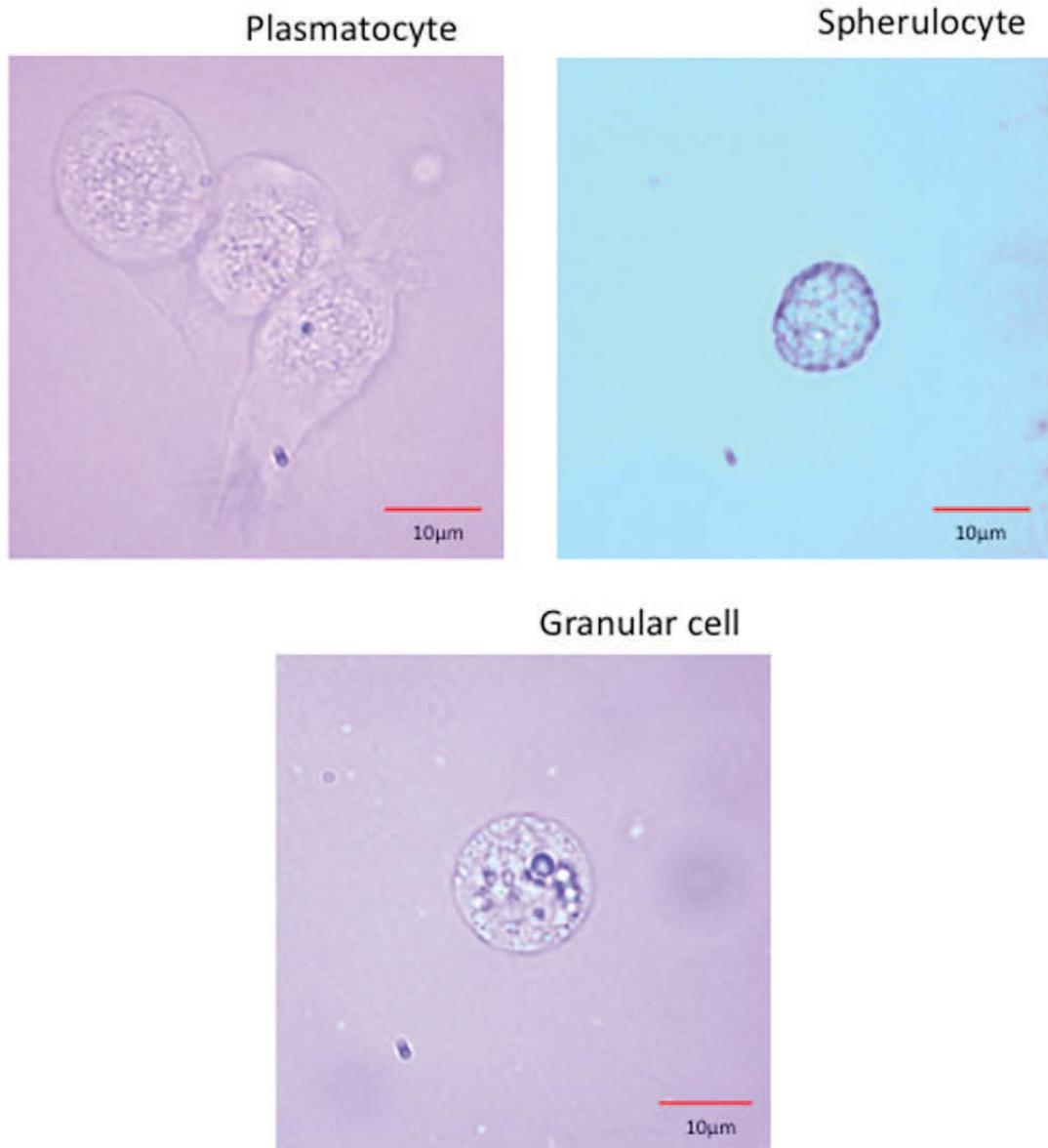
Granular cell

Fig. 1.6 Haemocytes of *G. mellonella* larvae.
Haemocytes were recovered from haemolymph of *G. mellonella* larvae and viewed using an Olympus Microscope. Images show plasmatocytes, spherulocyte, and granular cell (granulocyte).

cellular encapsulation mainly in Lepidoptera and humoral encapsulation mainly in Diptera. Humoral encapsulation can occur with or without haemocytes, and is always associated with phenoloxidase, cellular encapsulation can occur without melanisation. The encapsulation process in *G. mellonella* is led by the recognition of a foreign body by granular cells which upon contact lyse or degranulate releasing material that promotes plasmatocytes to attach (Pech and Strand, 1996). The attachment of multiple layers of plasmatocytes forms around the foreign body resulting in a smooth capsule of overlaid cells (Pech and Strand, 1996; Schmit and Ratcliffe, 1978). Granular cells and plasmatocytes are commonly observed in capsules in Lepidoptera, and in *Drosophila* lamellocytes were observed most frequently in capsules (Schmidt *et al.*, 2001).

1.3 Similarities between vertebrate and invertebrate innate immune responses

1.3.1 Similarities between cellular responses

Several aspects of insect and human immune responses exhibit functional similarities which suggests they both use similar effector and receptors, and have similar regulation of gene expression (Salzet, 2001). Various immune proteins in insects demonstrate a high degree of homology to proteins found in mammals, such as insect proteins malvolio and dSR-C1 which are similar to mouse natural resistance associated macrophage protein-1 (NRAMP-1) (Kavanagh and Reeves, 2004). The phagocytic cells in insects, the plasmatocytes and granular cells have receptors (e.g., calreticulin) on the surface which are similar to receptors on mammalian neutrophils (Kavanagh and Reeves, 2004; Renwick *et al.*, 2007). Structurally insect granular cells are slightly larger than neutrophils and do not have a multi-lobed nucleus. Haemocytes have a very granular cytoplasm compared with neutrophils (Renwick *et al.*, 2006). The phagocytic cells in both insects and humans engulf and kill pathogens and produce superoxide using similar p47 and p67 proteins (Bergin *et al.*, 2005; Renwick *et al.*, 2007). Neutrophils require the translocation of proteins p47^{phox} and p67^{phox} from the cytosol to the plasma membrane for the generation of a functional NADPH oxidase complex (Renwick *et al.*, 2007). This translocation event occurs when the cell is stimulated by exposure to a pathogen and activates flavocytochrome *b558* which is the redox centre of this enzyme system.

Haemocytes of *G. mellonella* have been shown to have proteins of 67 and 47kDa homologous to p67[phox] and p47[phox] proteins of the superoxide-forming NADPH oxidase complex of neutrophils (Fig. 1.7). The mode of superoxide production is so similar in insect haemocytes and human phagocytes that phorbol 12-myristate 13 acetate (PMA) induced superoxide generation in both. The translocation of haemocyte 47 and 67 kDa proteins from the cytosol to the plasma membrane of *G. mellonella* haemocytes can be suppressed by the fungal secondary metabolite gliotoxin (Renwick *et al*., 2007). Gliotoxin causes the same inhibition of translocation in human neutrophils, and thus inhibits the production of superoxide (Coméra *et al*., 2007; Tsunawaki *et al*., 2004). Fumagillin is also produced by *A. fumigatus* and was demonstrated to inhibit phagocytosis, superoxide production and degranulation in human neutrophils and *G. mellonella* haemocytes (Fallon *et al*., 2010, 2011a). Its primary mode of action was the inhibition of the conversion of G to F- actin in both cell types (Fallon *et al*., 2010, 2011a).

Cytochalasin b and nocodazole affect the activity and function of both neutrophils and haemocytes. In neutrophils these inhibitors disrupt the F-actin assembly which affects phagocytosis, the formation of NADPH oxidase and degranulation (Bengtsson *et al*., 1991; Möller *et al*., 2001; Neeli *et al*., 2009). Exposure of *G. mellonella* larvae to these inhibitors increased their susceptibility to infection, inhibited the phagocytosing ability of haemocytes and decreased the rate of F-actin formation, however the viability of the haemocytes was unaffected (Banville *et al*., 2011). The addition of the NADPH oxidase inhibitor diphenyleneiodonium chloride inhibited superoxide production and halted microbial killing in both haemocytes of *G. mellonella* and in human neutrophils (Renwick *et al*., 2007).

1.3.2 Humoral and signalling pathway similarities

Human immune cells express several Toll-like receptors (TLR), which directly recognize lipopolysaccharide and other components of the pathogen. These TLRs are considered cellular PRRs. The systems that mediate *Drosophila* Toll and mammalian IL-1 receptor-mediated signalling are very similar in structure and function (Medzhitov *et al*., 1997). Toll induces the production of antimicrobial peptides (e.g., drosomycin), an essential component of the insect immune response
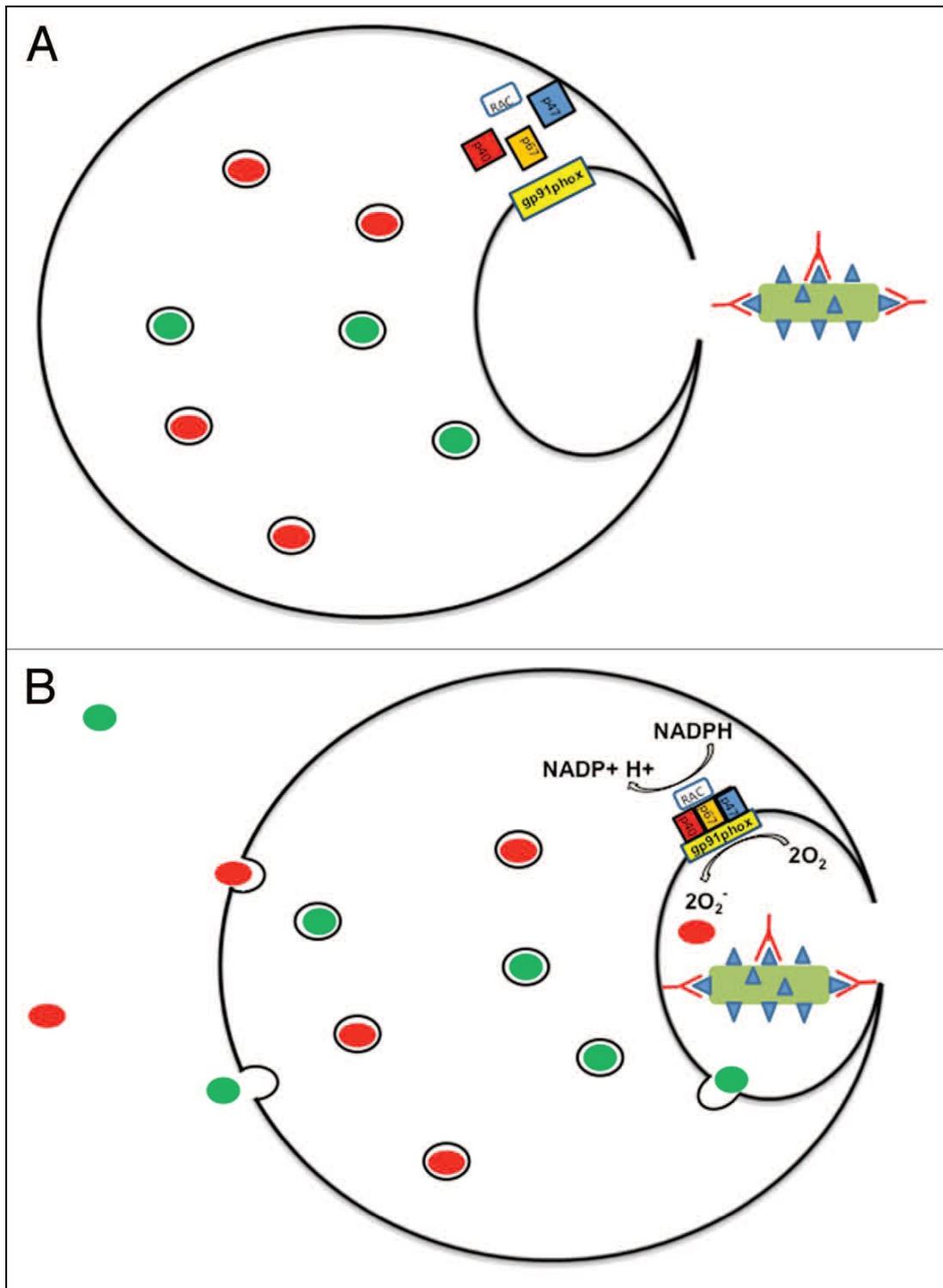
Fig. 1.7 Mode of action of neutrophil.

(A) Unactivated neutrophil: Opsonized bacterium is about to be phagocytosed, NADPH oxidase is not functional and all enzymes are retained on primary (red) and secondary (green) granules within cytoplasm. (B) Activated neutrophil: Opsonized bacterium has been phagocytosed, $p40^{phox}$, $p47^{phox}$, $p67^{phox}$, and rac have translocated from the cytoplasm to join $gp91^{phox}$ on the cell membrane and as a result superoxide is produced from oxygen with the consequent conversion of NADPH to NADP+ and H+. Degranulation has commenced releasing enzymes (red, green) from granules into the phagocytic vacuole and outside the cell.

(Fuchs and Mylonakis, 2006). The Toll and IMD pathways in insects activate two distinct NFκB-like transcription factors, which leads to the production of AMPs (Fig. 1.3). Human TLR, like *Drosophila* Toll protein (Toll), is a type I transmembrane protein that has an extracellular domain consisting of a leucine-rich repeat (LRR) domain and an intracellular domain that is homologous to the cytoplasmic domain of the human interleukin IL-1 receptor (Medzhitov *et al.*, 1997). *Drosophila* Toll and the IL-1 receptor signal through the NFκB pathway. Depending on the stage of the development in *Drosophila* the Toll protein serves different functions. In the embryonic stage it controls dorsal–ventral patterning and the activation of transcription factor Dorsal when it is bound to its ligand Spätzle. In the adult *Drosophila* the Toll/Dif signalling pathway is involved in anti-fungal immune response (Medzhitov *et al.*, 1997). The pathway induced by the IL-1 receptor (IL-IR) in mammalian cells mirrors the signalling pathway through Toll: IL-IR signals through the NFκB pathway and Dorsal and its inhibitor Cactus are homologous to NFκB and IκB proteins, respectively (Medzhitov *et al.*, 1997).

There is a major difference between mammalian Toll-like receptors (TLRs) and the *Drosophila* Toll protein, in that *Drosophila* Toll is not a pattern recognition receptor, instead it binds to the endogenous messenger Spätzle for the signalling cascade to occur (Ben-Ami, 2011). Recognition of the pathogen occurs upstream of Toll by secreted receptors which bind to their ligand and causes a cascade of serine proteases and cleavage of Spätzle, this forms dimers that bind to and activate Toll (Ben-Ami, 2011). In mammals, the expression of TLR is limited to immune-responsive cells, however, in insects expression is not only limited to immune cells (Ben-Ami, 2011) (Fig. 1.4). *Drosophila* has nine Toll-like genes; Toll, Toll 7 and Toll 9 have known immune functions. The expression of these genes is regulated in different tissues during embryogenesis, suggesting most Toll-like genes have developmental roles (Ben-Ami, 2011; Kambris *et al.*, 2002).

Similarly pathogen associated molecular patterns (PAMPs) are used by both insects and mammals to specifically detect pathogens through conserved microbial components including lipopolysaccharide and peptidoglycan (Dap-type PG) (Gram negative bacteria), peptidoglycan (Lys-type PG) and lipoteichoic acids (Gram positive bacteria), β-1, 3-glucans (fungi) and mannans (yeast) (Medzhitov and Janeway Jr, 1998). Similar immune molecules are observed in insects, e.g. apolipophorin III (apoLp-III) (PRR) and apolipoprotein E (apoE) (found in mammals) which is

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	Haemocytes	Neutrophils
Phagocytosis	Lectin-mediated	Lectin-mediated
ROS	$O2^-$, H_2O_2, NO^-	$O2^-$, H_2O_2, NO^-
Degranulation	yes	yes
AMPs	Peroxynectin, Transferrin, Lysozyme, Defensin	MPO, Transferrin, Lysozyme, Defensin
Receptors	Toll, B-1, 3-glucan, IL-IR calreticulin	TLRs, B-1, 3-glucan, IL-IR calreticulin
Transcription factors	NF-κß and I-κß (TF-inhibitor)	NF-κß and I-κß (TF-inhibitor)
Cascades	JNK, JAK/STAT	IMD, JNK, JAK/STAT
Kinases	p38 MAPK, ERK, PKC, PKA	p38 MAPK, ERK, PKC, PKA
Neutrophil extracellular nets (NET)	NET-like structures present	NETs present

Table 1.1 Summary of similarities between insect haemocytes and human neutrophils.

involved in LPD detoxification and phagocytosis (Zdybicka-Barabas and Cytryńska, 2011). The PRR calreticulin is also observed in both insects and mammals with conserved functions (Cho *et al*., 1999; Choi *et al*., 2002). The production of a wide range of antimicrobial peptides, which are crucial in combating infection, is similar in vertebrates and invertebrates (Table 1.1) (Vilmos and Kurucz, 1998).

1.3.3 Neutrophil extracellular trap

Neutrophils produce extracellular traps (NETs) containing nucleic acids and proteins to immobilise and kill pathogens (Brinkmann *et al*., 2004). A similar process has been observed to occur in haemocytes of *G.mellonella* in response to infection and has the effect of increasing the survival of the host (Altincicek *et al*., 2008). Interestingly the introduction of bacteria into *G.mellonella* larvae expressing nucleic acid hydrolysing enzymes allows the pathogen to escape entrapment and thus reduce the survival of the host. Human neutrophils and insect granular cells respond to inhibitors Cytochalasin b and nocodazole in a similar manner thus highlighting further similarities between the two cell types (Banville *et al*., 2011).

1.4 Immune priming

Insects within their natural environment may encounter a number of stresses including infection (Bergin *et al*., 2006), injury (Mowlds *et al*., 2008) or abiotic factors such as temperature fluctuations (Mowlds and Kavanagh, 2008) and changes in food availability (Banville *et al*., 2012). Although insects lack an immune response similar to the adaptive immune response of mammals they do have the ability to prime their immune response following a sub-lethal infection which prepares the insect to withstand a subsequent and potentially lethal infection. Insect immune priming has been shown to have a number of variations in insect species in terms of length of immune priming, specificity and overall cost to the insect's energy. It has been suggested that immune priming among insects is linked to a first encounter and an up-regulation of the relevant effector or receptor repertoires to this pathogen. This process has been observed in a number of species including *G. mellonella* (Bergin *et al*., 2006)*, D. melanogaster* (De Gregorio *et al*., 2001; Irving *et al*., 2001), *Anopheles*

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gambiae (mosquitoe) (Heard *et al*., 2005), *C. elgans* (Schulenburg *et al*., 2004) , molluscs and sea urchins (Zhang and Loker, 2004).

1.4.1 Stress and pathogen induced immune priming

Immune priming has obvious survival advantages for the host and it has previously been demonstrated that insects alter their immune response to pathogens they have been exposed to in the past resulting in better resistance to a subsequent infection by the pathogen as observed in *D. melanogaster* by an altered gene expression profile (De Gregorio *et al*., 2001; Irving *et al*., 2001). It has also been shown that insects may be primed against infection by certain pathogens based on prior exposure to antigens from the pathogen (Bergin *et al*., 2006) or to the pathogen itself (Little and Kraaijeveld, 2004).

Inoculation of *D. melanogaster* with a sub-lethal dose of *Streptococcus pneumoniae* protected against a subsequent lethal inoculum and was mediated by a change in the density of circulating phagocytic cells (Pham *et al*., 2007). In *Bombus terrestris* the protection and specificity of immune priming can last up to 22 days, which is long after transcription and the elevated production of antimicrobial peptides has ended (Sadd and Schmid-Hempel, 2006). Immune priming has been demonstrated in *G. mellonella* larvae following infection with sub-lethal doses of yeast (Bergin *et al*., 2006) or fungal cell wall components (Mowlds *et al*., 2010).

Antimicrobial drugs can stimulate an immune response due to their presence evoking a potential danger response within insects and has been demonstrated as an immune priming response within *G.mellonella* larvae to antifungal drugs, including silver derived drugs (Rowan *et al*., 2009) and caspofungin (Kelly and Kavanagh, 2011).

Abiotic factors such as thermal or physical stress and nutrient availability are factors that affect the insect in its environment. Larvae exposed to thermal (Mowlds and Kavanagh, 2008; Wojda and Jakubowicz, 2007) and physical (Mowlds *et al*., 2008) stress demonstrated significant immune priming effects which increased larval survival after subsequent infection. The availability of nutrients can also affect the insect immune response. Previous work demonstrated that dietary restriction can lead to an altered expression of a number of immune related genes and a delayed up-regulation of antimicrobial genes in *D. melanogaster* (Pletcher *et al*., 2002). Immune

priming has the advantage of protecting the insect from a subsequent potentially lethal infection but is costly and can result in death if compensatory feeding is unavailable (Moret, 2006).

1.4.2 Specificity of immune response

Both *D. melanogaster* and *A. gambiae* have been shown to generate specificity through Dscam immunoglobulin via alternative exon splicing which can potentially produce up to 30,000 isoforms in both insects (Dong *et al*., 2006; Watson *et al*., 2005) although these isoform repertoires have to be proven to recognize and inhibit different strains of the same pathogen species (Schulenburg *et al*., 2007). It has been further suggested by Rodrigues *et al*. (2010) that mosquito's differentiation and proliferation of a subset of haemocytes (phagocytic cells) are responsible for the improved survival and decreased *Plasmodium* oocyst numbers encysted in the gut epithelium upon secondary challenge. It was also possible to transfer resistance to *Plasmodium* by transplanting haemocytes from primed to naïve mosquitoes (Rodrigues *et al*., 2010).

1.4.3 Trans-generational immune priming

The ability to transfer immunity from parent to offspring has also been demonstrated following microbial challenge in a number of publications both maternally (Freitak *et al*., 2009; Little *et al*., 2003; Moret, 2006; Roth *et al*., 2010; Sadd *et al*., 2005; Tidbury *et al*., 2011) and paternally for the first time in the *Tribolium castaneum* (red flour beetle) (Roth *et al*., 2010). This parental transfer of immunity demonstrated that males previously exposed to either *Escherichia coli* or *Bacillus thuringiensis* produce offspring better able to survive that particular infection (Roth *et al*., 2010). This effect of immune priming transfer may be due to parental experiences and highlights the potential for epigenetic transfer (Chambers and Schneider, 2012). Interestingly it has been shown that gender specific immune responses occur as with the *T. castaneum*, where females demonstrated a higher immune competency than males (Freitak *et al*., 2012). The hypothesis of Bateman's principle in immunity, predicts gender-specific immune responses where females gain

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fitness through increased longevity (i.e. ability to live long enough to produce more offspring by possessing a stronger immunity), whereas males gain fitness by increasing mating rates.

1.4.4 Colony based immunity and immunity transfer

Social immunity in insects has been suggested in a number of colony based communities similar to that of the *Apis* colony which *G. mellonella* larvae inhabit. Social immunity has been observed within infected ants from a colony which could regurgitate droplets which were capable of transferring immune factors once consumed by other ants (Hamilton *et al.*, 2011). Previous work by Traniello *et al.* (2002) demonstrated how naïve termites (*Zootermopsis angusticollis*) reared alongside termites previously exposed to pathogens (entomopathogenic fungus *Metarhizium anisopliae*) can improve the subsequent resistance of the naïve termites to the fungal pathogen.

G. mellonella inhabiting bee hives may transfer resistance to a certain pathogen through proximity to other naïve larvae and as a result activate a short elevation of the naïve larvae's immune response enabling larvae to overcome a subsequent lethal infection. This mechanism may operate through the absorption of small amounts of pathogen fragments or immuno-active molecules produced by the infected host and subsequently stimulating immune defences of naïve termites (Traniello *et al.*, 2002). Schal *et al.* (1998) suggested that the cuticle has an active association with the haemolymph, where compounds from the haemolymph (which can include immuno-active molecules released by fat bodies) are readily transported to the outer epicuticular surface found in *Blattella germanica* (Fan *et al.*, 2003). Although such a mechanism of immune-active molecule transport has been suggested (Siva-Jothy *et al.*, 2005) it requires further investigation to establish the potential transfer of immune priming to other insects within a colony. It has been observed that the accumulation of *G. mellonella* larvae within beehives produces temperatures that can reach up to 40°C (Schmolz and Schulz, 1995). Other authors suggest a potential role of so-called ''behavioural fever'', where insects alter their temperature through thermoregulatory behaviour, leading to an increased ability to fight off pathogens (Blanford *et al.*, 1998; Elliot *et al.*, 2002; Thomas and Blanford, 2003; Watson *et al.*, 1993).

1.5 Proteomic analysis applied to *G.mellonella*

The use of proteomic methods to analyse the proteome of insects has established a greater understanding of the insect immune response and the proteins shared with the mammalian immune response (Bergin *et al*., 2006; Zdybicka-Barabas and Cytryńska, 2011). This has developed from the use of simple 1-Dimensional gels to more complex 2-Dimensional gel analysis techniques enabling an analysis of the proteomic changes that may occur at a given time within an insect. The development of mass spectrometers including the MALDI ToF and LC/MS gave greater identification of proteins in combination with greater availability of sequenced insect genomes (Bergin *et al*., 2006; Yanay *et al*., 2008). This ability to identify proteins in combination with software capable of assessing fold changes of proteins between treatments using Progenesis SameSpot Software enabled insight into proteomic changes within the insect (Kelly and Kavanagh, 2011). The most recent addition to this proteomic analysis is the label free approach using the Thermo Scientific™ Q Exactive™ which does not utilise gels but only the protein from the insect to distinguish changes in expression across a broader range and quantity of proteins. It is also possible to detect protein post translational modifications with a high accuracy (Megger *et al*., 2013).

1.5.1 Haemocyte population identification using FACS analysis

It has been through the use of percoll gradients that haemocyte populations have been separated on the basis of size using centrifugation followed by morphological identification of haemocyte cell layers by microscopy and staining (McKenzie and Preston, 1992; Mead *et al*., 1986). The identification of sub-populations of haemocytes within insects by different research groups has been difficult due to the lack of morphological features in haemocytes between different insects and due to changes in haemocyte populations that can occur at different developmental stages of the insect (Nardi *et al*., 2003). Efforts to establish what haematopoetic stem cells produced certain lineages of haemocytes has led to the use of structural identification in combination with labelling patterns using specific antibodies (Gardiner and Strand, 1999; Willott *et al*., 1994). Although these

techniques have been applied, considerable confusion still remains on the classification of haemocytes from literature (Strand, 2008).

The more recent use of flow cytometry or fluorescence activated cell sorter (FACS) analysis has led to a greater degree of clarity and consistency between haemocyte densities in the same insect species or different insect species (García-García *et al*., 2009). Flow cytometry uses the measurement of light scatter differences and fluorescence emission from cells to distinguish haemocyte cell types (Parks and Herzenberg, 1989). The ability to study a variety of parameters of haemocytes such as size and granularity and the use of fluorescent labels allows cells to be analysed with greater specificity and speed compared to alternative methods using percoll gradient separation. The sorter component of FACS enables the separation of haemocyte populations based on their morphology or staining into individual containers that allows the individual populations to be viewed by microscopy in the presence or absence of fluorescent staining. For example, Fig. 1.8 shows the image of a granular haemocyte isolated from *G.mellonella* by FACS and subsequently stained with DAPI and rhodamine prior to visualisation.

Due to the large haemolymph volume possessed by most lepidopteran species e.g. *G.mellonella*, they have been extensively used to study haemocytes when compared to smaller insects such as *Drosophila*. The method of identifying phagocytosing haemocytes has been successful using FACS for comparing a number of species (García-García *et al*., 2009), as well as identifying different haemocyte populations within *M. sexta* (Nardi *et al*., 2003). Although phagocytosing haemocytes have been shown in *G.mellonella* (Fuchs *et al*., 2010; Mowlds *et al*., 2010), they lack an in-depth identification of haemocyte sub-populations within the insect.

1.6 Pathogens assessed within *G.mellonella* larvae

G.mellonella larvae have been used extensively to study the virulence of both bacterial (Mukherjee *et al*., 2010) and fungal pathogens (Cotter *et al*., 2000). Invertebrate models such as *G.mellonella* are popular for the screening of drugs and virulence of pathogens as they offer fast preliminary insight into the pathogenesis and resistance of pathogens to novel drugs. For these reasons and the growing resistance of pathogens to current drugs *G.mellonella* is increasingly being used to assess pathogen virulence and the efficacy of antimicrobial drugs. A variety of pathogens
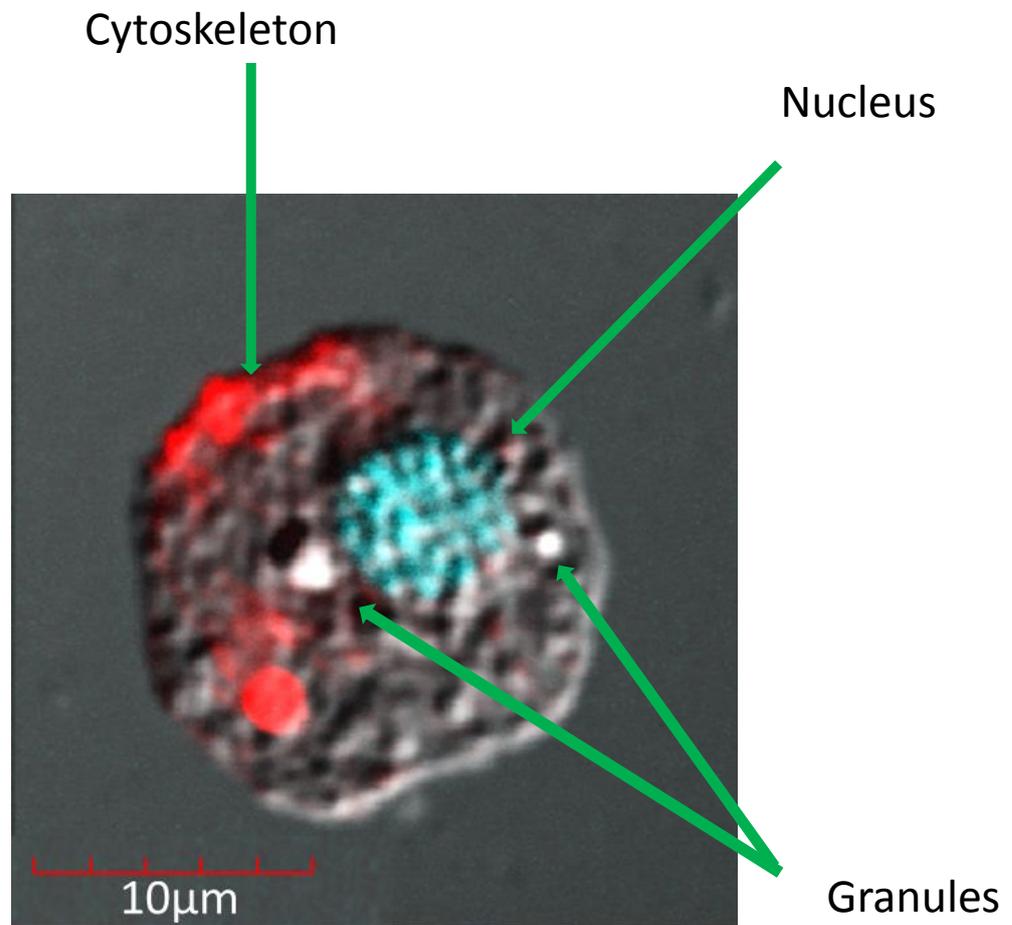
Fig 1.8 Granular haemocyte from *G. mellonella* larvae.

Haemocytes were separated by FACS ARIA, stained with DAPI nuclear stain (Blue) and Rhodamine Cytoskeletal stain (Red) and viewed using an Olympus Microscope.

were used to assess the ability of larvae to overcome a pathogen infection when larvae were pre-stressed, pre-incubated for long periods or administered an antimicrobial compound, these pathogens included *Candida albicans*, *Aspergillus fumigatus* and *Staphylococcus aureus*.

1.6.1 *Candida albicans*

C.albicans is an ubiquitous opportunistic pathogen that is the leading cause of hospital acquired fungal infections within humans accounting for a near 40% mortality rate among patients with systemic candidiasis (Gudlaugsson *et al*., 2003; Pfaller and Diekema, 2007). *Candida* infections are particularly prevalent among immunocompromised patients who lack the ability to launch an effective adaptive immune response against the pathogen due to the effects of immune suppressive drugs on transplant patients or chemotherapy on cancer patients (Blumberg *et al*., 2001; Hajjeh *et al*., 2004; Pfaller and Diekema, 2004). *C. albicans* remains the predominant cause of Invasive Candidasis (IC), accounting for over half of all cases (Pfaller and Diekema, 2007) (Fig. 1.9 A). The increase in immunocompromised patients has required a greater use of antifungal drugs and has resulted in the development of antifungal drug resistance becoming a serious problem with fewer drugs available to tackle *Candida* infections (Cannon *et al*., 2007).

Resistance is also compounded by the issue of reduced antifungal drug development as well as the problem of fungal drugs often having severe toxicity effects on humans as well as fungi (Worth *et al*., 2008). *C.albicans* is also capable of adhering to epithelial cells and phenotypic switching from a unicellular form to an invasive multicellular filamentous form capable of penetrating through host cells and entering the bloodstream enabling systemic infection (Kumamoto and Vinces, 2005) (Fig. 1.9 B). These combined factors have made treatment and development of new antifungal therapies problematic and has resulted in a greater demand to develop new drug therapies in both academia and industry which has driven the development of invertebrate models to assess the efficacy of novel drug potential.

G.mellonella larvae have been used in *in vivo* assessments of novel antifungals against *Candida* species particularly as a result of the strong correlation demonstrated between the *G.mellonella* and mice models (Bergin *et al*., 2006). The larvae have been used to assess the virulence of *Candida* strains
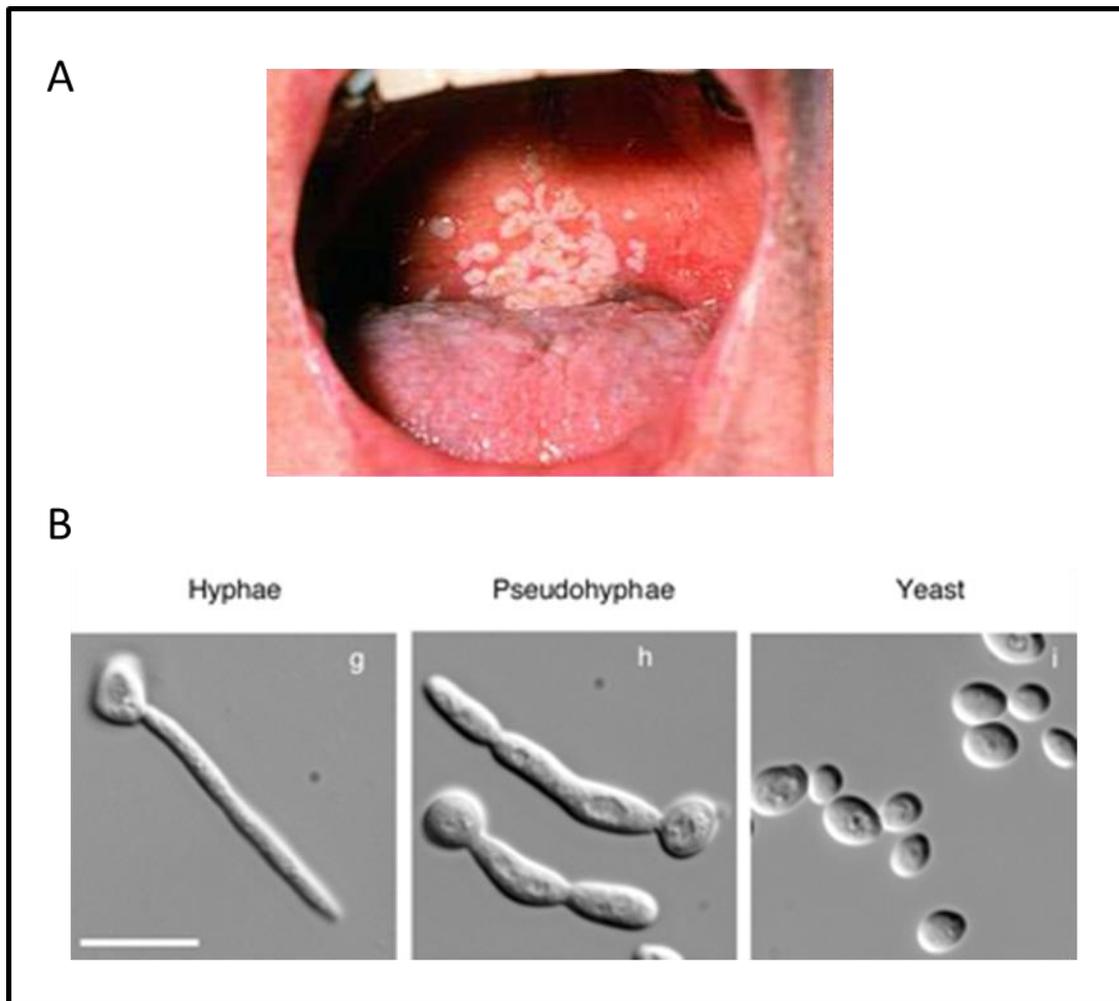
Fig. 1.9 *C. albicans* infection presenting as oral candidiasis (Thrush) (A), *C. albicans* cells stages (B).

Sourced from; (A) http://web.mst.edu/~microbio/BIO221_2009/C_albicans.html, (B) (Wightman *et al*., 2004).

(Bergin *et al*., 2006; Brennan *et al*., 2002) and to assess the physiologically changes that occur in larvae in response to infection through proteomic analysis (Brennan *et al*., 2002). The fact that larvae lack an adaptive immune response makes them more suitable models to compare with immunocompromised patients. This principle was demonstrated when only *C.albicans* but not *C.glabrata* were observed to be virulent in immunocompromised patients (Fridkin and Jarvis, 1996) and was similarly demonstrated within *G.mellonella* larvae (Cotter *et al*., 2000). *G.mellonella* have been used to assess the efficacy of the antifungal drug, caspofungin (Kelly and Kavanagh, 2011) and silver based compounds (Rowan *et al*., 2009) against *Candida*. This has made *G.mellonella* larvae a useful tool in the discovery of new antifungal drugs and into mechanisms of *Candida* infection.

1.6.2 *Aspergillus fumigatus*

A.fumigatus is a fungal pathogen that is commonly found within the air as conidia (Fig. 1.10 B) and is a leading cause of mortality within immunocompromised individuals (Denning *et al*., 1998; Marr *et al*., 2002). Since 1997 mortality associated with invasive aspergillosis has continued to decline (Pfaller and Diekema, 2007). Humans and animals routinely inhale conidia although only individuals who have a weakened immune system can develop aspergillosis where invasive *A.fumigatus* invades through the pulmonary tissue and is potentially fatal (Fig. 1.10 A). Allergic bronchopulmonary aspergillosis can potentially be fatal to asthmatic and cystic fibrosis (CF) patients as it triggers a severe inflammatory immune response within the lung (Daly and Kavanagh, 2000). Contributing to *A.fumigatus* virulence is its ability to secrete a number of secondary metabolites that impede competing fungal strains and help to evade the immune response of the host (Keller *et al*., 2005; Rohlfs *et al*., 2007).

These secondary metabolites have the ability to inhibit the phagocytosing abilities of both mammalian neutrophils (Coméra *et al*., 2007; Fallon *et al*., 2010; Tsunawaki *et al*., 2004) and invertebrate granular haemocytes (Fallon *et al*., 2011a; Renwick *et al*., 2007) due to their conserved structures and functions. This has demonstrated how useful *G.mellonella* larvae are in the study of *A.fumigatus* due to the pathogenesis in *G.mellonella* causing inhibition of the granular phagocytosis as observed in immunocompromised patients (Fallon *et al*., 2011a). The insect larvae
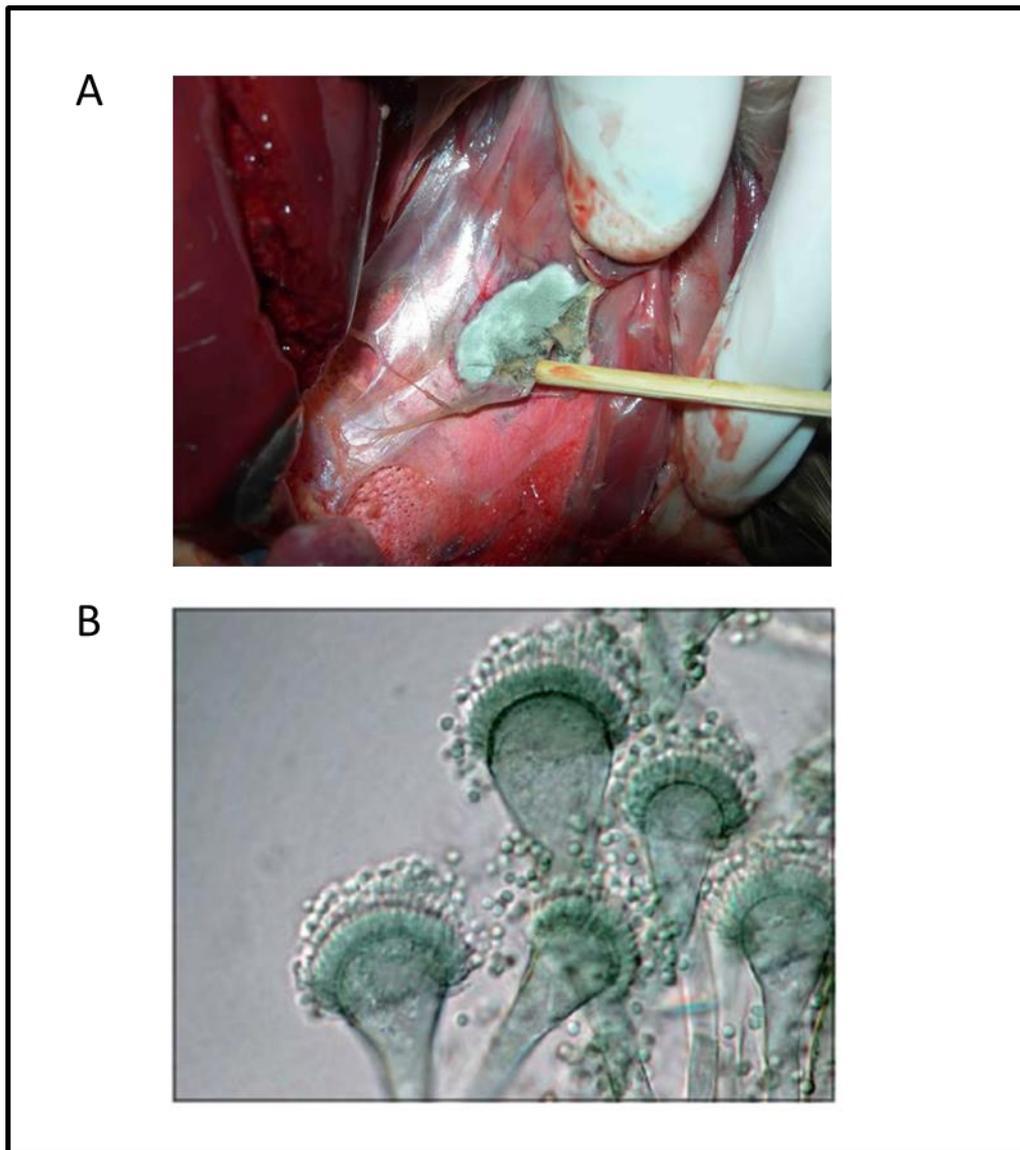
Fig 1.10 *A. fumigatus* infection presenting as pulmonary aspergillosis (A), *A. fumigatus* hyphae and conidia (B).

Sourced from; (A) http://forum.backyardpoultry.com/viewtopic.php?f=5&t=8019606,

(B) (Cruz *et al.*, 2012),

have similar PRRs to mammals which are used to detect *A.fumigatus* conidia. These parallels have made the *G.mellonella* model particularly suited to studying the pathogen's mechanism of virulence as well as assessing the efficacy of new or current antifungal therapies (Cowen *et al*., 2009).

1.6.3 *Staphylococcus aureus*

S.aureus is a common bacteria capable of causing infections in humans and is a leading bacterium type to develop resistance to antibiotics (e.g. MRSA) (Heijer *et al*., 2013). *S.aureus* is the most prevalent bacterial infection within hospitals due to a number of factors contributing to its pathogenesis such as the ability to form a capsule, secretion of a number of toxins and immunomodulators as well as the expression of adhesins on its surface (Smith *et al*., 2013). Infection caused by *S.aureus* can produce a number of cytotoxins which have detrimental effects on the host immune response (Dinges *et al*., 2000). These factors have led to its prevalence in infections of the soft tissue and skin (Kirby *et al*., 2002) which can lead to more severe conditions including sepsis, toxic shock syndrome and scalded skin syndrome (Plano, 2004) (Fig. 1.11 A). The versatility of *S.aureus* has been associated with both hospital and community based infections (García-Lara *et al*., 2005). The virulence and prevalence of *S.aureus* infection has made it difficult to treat and control with up to 4% of patient infections resulting in mortality (García-Lara *et al*., 2005).

The increased resistance of *S.aureus* to antibiotics has highlighted the urgency to develop new drugs that are capable of overcoming infections that have become resistant to the most potent antibiotics (e.g. vancomycin). This has increased the use of invertebrate *in vivo* models in preliminary screening to assess the pathogenicity of strains (Gao *et al*., 2010) as well as the efficacy of new antibacterial compounds against *S.aureus* (Peleg *et al*., 2009). *B.mori* has been used extensively to assess the efficacy of current antibiotics, methicillin (Akimitsu *et al*., 1999), vancomycin and kanamycin (Hamamoto *et al*., 2004) against *S.aureus* infection. More recent emphasis has been focused on the antimicrobial effects of new metal ion derived compounds most notably the silver ion which has been used in a number of compounds demonstrating good activity against *S.aureus* (Patil *et al*., 2011a; Rowan *et al*., 2006). Silver derived compounds have also been assessed in *G.mellonella* demonstrating low
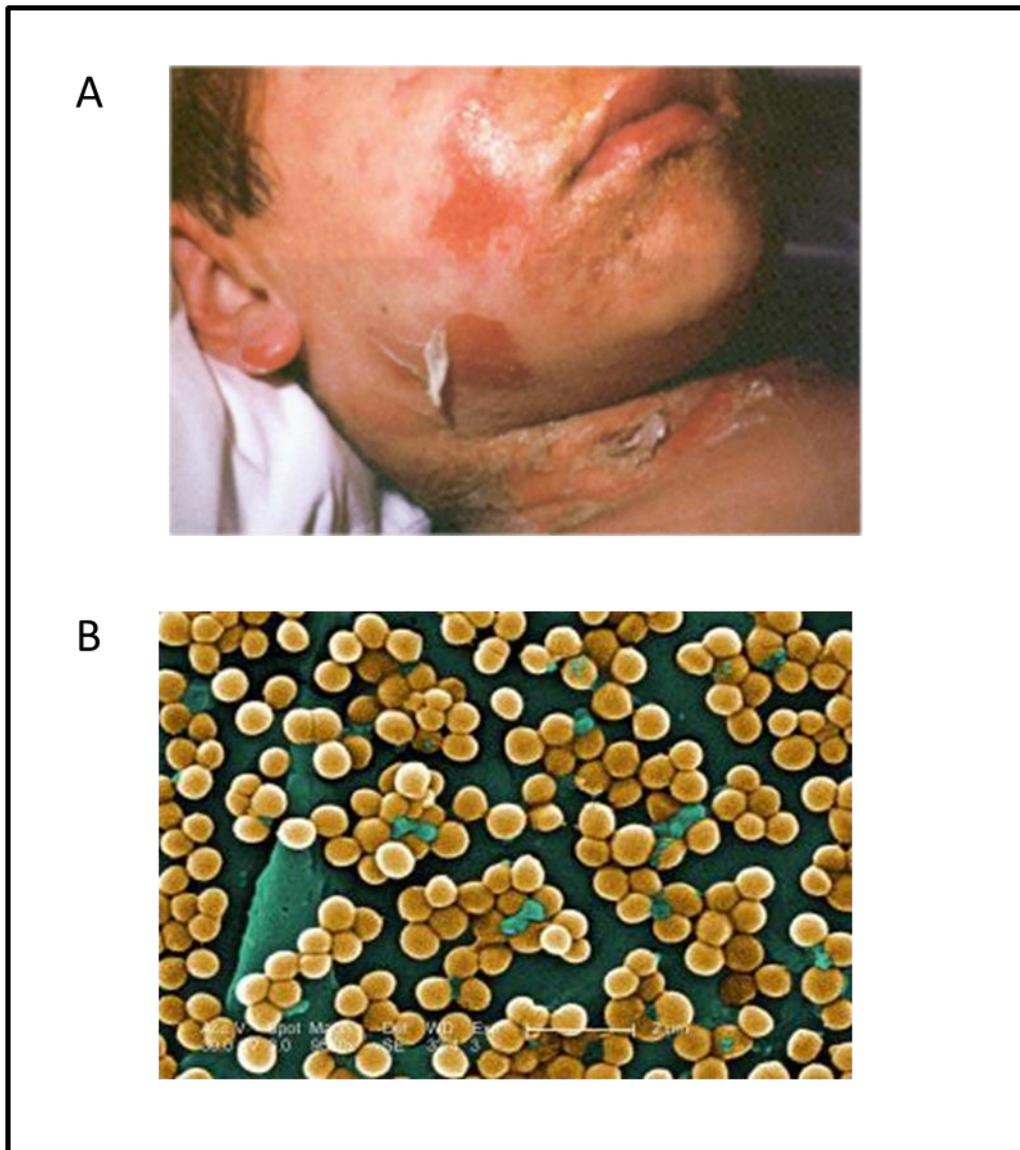
Fig. 1.11 *S.aureus* infection presenting as scalded skin syndrome (A), *S.aureus* cells (B).

Sourced from; (A) http://scmsociety.typepad.com/mrsa/mrsa-patient-information.html, (B) http://sciencelife.uchospitals.edu/2010/08/16/turning-mrsas-weapons-on-itself/.

to no toxicity while demonstrating potency against the *S.aureus* infection *in vivo* (Rowan *et al*., 2009).

1.7 Objectives of this study

The Objectives of this study can be summarised as follows:

    (1) The characterisation of innate immune proteins from *G.mellonella* and human sera. To establish the effects of thermal and physical stress on *G.mellonella* larvae and on the activity of immune proteins.

    (2) To assess the humoral and cellular responses of larvae to thermal and physical stress. To establish how nutrient deprivation alters the larval haemolymph proteome and the larva's ability to mount an immune response.

    (3) To identify haemocyte proteins phosphorylated in response to fMLP and PMA, and to determine if similar activation pathways are activated as observed in mammalian neutrophils.

    (4) To determine the effect of pre-incubation of larvae for up to 10 weeks on the larva's ability to tolerate infection.

    (5) To establish the utility of *G. mellonella* larvae for *in vivo* screening of a novel silver based antimicrobial agent (SBC3).

Chapter 2

Materials and Methods

2.0 General laboratory practice and sterilisation procedures

2.1.1 Chemicals and reagents

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

2.1.2 Statistical analysis

All experiments were performed on three independent occasions and results are expressed as the mean ± SE. Changes in *G.mellonella* survival were analysed with log rank (Mantel-Cox) method using GraphPad Prism version 5.00. Analysis of significant changes in protein expression or cell density was performed by Two-way ANOVA using GraphPad Prism version 5.00 for Windows 8, GraphPad Software, San Diego California USA, (www.graphpad.com). For all experimentation a p-value of < 0.05 was deemed statistically significant.

2.2 Bacterial and fungal strains

2.2.1 *Aspergillus* strain culture

A. fumigatus ATCC 26933 (obtained from the American Type Culture Collection) was grown and maintained on Malt Extract Agar (Oxoid) at 37ºC in thermally controlled incubator for 4-8 days. Plates were inspected on a daily basis for the level of growth. Once conidial development was achieved the plates were wrapped in parafilm and stored at 4ºC.

2.2.2 *C. albicans* liquid culture and cell harvest

A single colony of *C. albicans* (MEN) (serotype B, wild-type originally isolated from an eye infection, Dr. D. Kerridge, Cambridge, UK) was transferred to sterile YEPD broth using a sterile inoculating loop. The flask was re-plugged with cotton and incubated at 30ºC at 200 rpm overnight. The cell suspension was transferred to a sterile 50 ml tube, cells were diluted in sterile PBS, enumerated by haemocytometry and adjusted in PBS to the correct concentration prior to inoculation.

2.2.3 *Staphylococcus aureus* liquid culture

S. aureus (Clinical isolate) was transferred aseptically using a sterile loop from a single colony grown on nutrient agar to nutrient broth (Oxoid) and grown overnight at 37°C and 200 rpm to the early stationary phase.

2.3 Microbial culture and quantification

2.3.1 Sterilisation procedures

All micro-centrifuge tubes and pipette tips were stored in a sealed container and autoclaved at 121ºC for 15 min prior to use. All liquid growth media and agar media were autoclaved at 121ºC for 15 min prior to use. Molten agar medium was allowed to cool until hand hot (~50ºC) and poured under sterile conditions using sterile 90mm petri-dishes. *S. aureus* and *C. albicans* MEN were sub-cultured by aseptic techniques while all work involving *A. fumigatus* ATCC 26933 was performed using a class II safety cabinet.

2.3.2 Cell enumeration and haemocytometer

Cell concentrations were determined using a haemocytometer (Neubauer, Germany). Cells were diluted by a factor of 1/100 in sterile PBS, pipetted up and down until suspension was homogenous and cell suspension (10 µl) was loaded under the cover slide of the haemocytometer before being counted under a light microscope at a magnification of 40x. All determinations were calculated from the average of three counts.

2.4 *A. fumigatus* culture conditions

2.4.1 Malt Extract agar

Malt extract agar (Oxoid Ltd Basingstoke, UK) was prepared by adding 50 grams of extract per litre of deionised water and autoclaving at 115ºC for 15 min. Once the agar was hand hot it was poured into Petri-dish plates.

2.4.2 *Aspergillus* Trace Elements

$Na_2B_4O_7.7H_2O$ (0.04 g), $CuSO_4.(5H_2O)$ (0.7 g), $FeSO_4.7H_2O$ (1.2 g), $MnSO_4$ (0.7 g), $NaMoO.2H_2O$ (0.8 g) and $ZnSO_4.7H_2O$ (10 g) were added to 800 ml of

distilled water and dissolved. The solution was made up to 1 L with distilled water and subsequently autoclaved. The solution was aliquoted in 50 ml volumes and stored at -20ºC.

2.4.3 *Aspergillus* Salt Solution

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

2.4.4 Ammonium Tartrate

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

2.4.5 *Aspergillus* Minimal Medium (AMM)

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

2.4.6 Phosphate Buffered Saline 0.01% (v/v) Tween

One PBS tablet was dissolved in 100 ml deionised water and autoclaved at 121ºC for 15 min. PBS was stored at room temperature. For PBS-T, PBS was supplemented with 0.01% Tween-80 (Merck), and was vortexed until mixed homogenously. PBS-T was filtered through a 0.22 μm filter (Millipore) prior to use.

2.4.7 Harvesting of *Aspergillus* conidia

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

2.4.8 Liquid Culture of *A. fumigatus*

For *Aspergillus* culture was grown in AMM sterile medium by inoculating conidia at concentration of 5 x 10^7 cells per 100 ml culture and incubated at 37^oC for 7h at 200rpm to facilitate germination of conidia.

2.5 *C. albicans* culture media

YEPD agar was prepared by dissolving Glucose (2% w/v), Yeast extract (1% w/v) (Oxoid), and bacteriological peptone (2% w/v) (Difco), in deionised water and autoclaving at 121ºC for 15 min. For agar plates 2% (w/v) agar was added and autoclaved as described. Erythromycin was added to the hand warm agar prior to pouring to control bacterial contamination. This was prepared by dissolving 1 mg of erythromycin in 1000ml agar solution when hand warm. Once in the agar solution the plates were poured as per normal and stored at 4ºC. All erythromycin supplemented plates were used within 3-5 days.

2.6 *S. aureus* growth and harvest

2.6.1 Nutrient broth / agar

Nutrient broth (Oxoid) was prepared by adding (13g/L) of powder to deionised water and autoclaving at 121ºC for 15 min. Nutrient agar was prepared using nutrient agar powder (13g/L) in deionised water and autoclaved as before. The agar solution was poured aseptically into sterile petri dishes once hand warm and allowed to set before sealing and storing the plates at 4ºC.

2.6.2 Calculation of *S. aureus* culture cell concentration

S. aureus was grown in nutrient broth (Oxoid) at 37°C and 200 rpm to the early stationary phase. A bacterial culture was measured on a spectrophotometer using an OD_{600} adjusted to a value of 1 that was diluted 1/10 and re-adjusted in PBS until the OD_{600} read 0.1, equivalent to $4.01x10^7$ cells per ml prior to inoculation into larvae.

2.6.3 Maintaining long term microbial stocks

Storage of bacterial and fungal strains over the longer term was achieved by freezing stocks of liquid culture in 50% (v/v) glycerol and placing at -80^oC.

2.7 *G. mellonella* larval storage and experimental conditions

2.7.1 *G. mellonella* storage and food

Sixth - instar larvae of the greater wax moth *G. mellonella* (Lepidoptera: Pyralidae, the Greater Wax Moth) (Mealworm Company, Sheffield, England) were purchased and stored in wood shavings in the dark at $15^{o}C$ to prevent pupation (Cotter *et al*., 2000; Hornsey and Wareham, 2011). Larvae weighed between (0.25-0.3g) and were excluded if there was evidence of localised melanisation or infection.

2.7.2 Inoculation of *G.mellonella* larvae

Larvae were injected through the last left pro-leg (Fig. 2.1), with a Myjector U-100 insulin syringe, (Terumo Europe N.V., 3001 Leuven, Belgium), while applying mild pressure to the insect to allow the opening of pro-legs. Larvae were kept in 9cm petri-dishes with 0.45 mm Whatmann filter paper inserted on the lids and some wood shavings for all experiments (Fig. 1.2). Great care was taken with each injection to maintain a high accuracy of drug or pathogen delivery. All *in vivo* assays were performed on three separate occasions using a group size of 10 larvae per dish and average results were calculated. For viability studies larvae were injected with sterile PBS as a control for the injection of larvae.

2.7.3 *G. mellonella* larval thermal and physical stress treatments

Larvae were selected and exposed to thermal stress of $15^{o}C$, $30^{o}C$ or $37^{o}C$ for 24h or physically stressed by gentle shaking of larvae in a cupped hands back and forth over a distance of 25cm for 80 repetitions per minute for a total of 2 min and subsequently placed at $30^{o}C$ for 24h prior to use (Mowlds *et al*., 2008). All larvae were kept in the dark at $15^{o}C$. Larvae were thermally and physically stressed for 24, 48 or 72h and haemocytes or protein was extracted and assessed at these points, gene expression of larvae thermally and physically stressed was measured after 1, 4, 12, 24, 48 and 72h. Alternatively larvae were inoculated with a pathogen 24h after physical and thermal stress (short term stressing) treatment placed at $30^{o}C$ and larval survivals were recorded after 24, 48 and 72h. For long term stressing larvae were thermally and physically stressed for 24h and subsequently placed at $30^{o}C$ for 48h prior to inoculation and subsequent survivals monitored at 24, 48 and 72h points.

2.7.4 Assessment of pre-incubation period on *G.mellonella* larvae

Larvae were stored in wood shavings in the dark at 15°C for 1, 3, 6 and 10 weeks from point of arrival. The responses of the larvae were compared for variations in haemocytes density, protein expression or survival following bacterial or fungal infection.

2.7.5 Determining the effects of starvation on larvae

Larvae were stored in petri dishes without a food source for 0 or 7 days (~20°C) prior to extraction of protein (300µg) and separation on 2-Dimensional gel.

Fig. 2.1 Inoculation of *G. mellonella* larvae through the last left pro-leg.

Inoculation through the pro-leg enables introduction of the pathogen or drug into the insect haemocoel (Kavanagh and Fallon, 2010).

2.8 1-D and 2-D gel electrophoresis

2.8.1 1.5 M Tris–HCl

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

2.8.2 0.5 M Tris – HCl

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

2.8.3 10% w/v Sodium Dodecyl Sulphate (SDS)

Sodium Dodecyl Sulphate (SDS), (10% w/v) was prepared by placing 5 g SDS into a 50ml falcon and filling upto 50 ml mark with deionised water and vortexed until all SDS had been solubilised and the solution was stored at room temperature.

2.8.4 10% Ammonium Persulphate (APS)

APS (10% w/v) was prepared by adding 0.1 g APS placed into microcentrifuge tube and filled upto 1ml with deionised water and vortexed briefly to achieve solubility. APS (10% w/v) stocks were frozen.

2.8.5 10X electrode Running Buffer

Running buffer (10X), (electrode buffer), was prepared by adding; Tris Base 30 g/l, Glycine 144 g/l and SDS 10 g/l dissolved using distilled water filled upto 1000ml mark and the mixture was stirred until the solution was solubilised. Electrode running buffer (10X) stock was diluted to 1X concentration by making 1/10 dilution with distilled water when required.

2.8.6 5X Solubilisation Buffer for 1-D SDS–PAGE

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

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

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

2.8.7 Preparation of SDS–PAGE minigels

All glass plates were washed in warm soapy water and cleaned thoroughly with 70% (v/v) ethanol prior to use. SDS–PAGE minigels (cast using the Mini-Protean II gel casting apparatus), were made of acrylamide with 12.5% Bis-acrylamide in all experiments.

Seperating gel composition

12.5% Bis-Acrylamide solution

- 1.5 M Tris-HCl (pH 8.9)   3 ml
- Deionised water   3.8 ml
- 30% Bis-Acrylamide   5 ml
- 10% v/v SDS   120 µl
- 10% v/v APS   75 µl
- TEMED   3 µl

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

Stacking gel composition

The following components were mixed together and applied on top of the separating gel;

- Deionised water   3.4 ml
- 30% Bis-Acrylamide   830 μl
- 0.5 M Tris–HCl (pH 6.8)   630 μl
- 10% v/v SDS   50 μl
- 10% v/v APS   50 μl
- TEMED   5 μl

Combs were placed in the gel matrix before it set to create wells for sample loading. These volumes were sufficient to make 2 minigels and volumes were adjusted accordingly where a larger volume was required.

2.8.8 1-D SDS-PAGE sample loading and voltages

SDS-PAGE gels 12.5% were immersed in 1X running buffer. Samples varied in volume and protein concentration depending on the experiment but were aliquoted using a sterile gel loading tip. The gels were electrophoresed at 40V initially and the voltage was increased to 60 V once the protein had travelled sufficiently through the stacking gel. Once the blue tracking dye had moved to the bottom of the gel, the gels were transferred to a clean staining dish.

2.9 2-D IEF/SDS-PAGE preparation and analysis

2.9.1 Isoelectric focussing (IEF) Buffer

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

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

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

2.9.2 Equilibration Buffer

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

The solution was adjusted to pH 6.8 and aliquoted in 40 ml volumes prior to storage at -20ºC. For equilibration the buffer was modified as either reducing or alkylating. For reduction, DTT (0.01 g/ml) was added and dissolved thoroughly. For alkylation, IAA (0.025 g/ml) was dissolved thoroughly in the buffer.

2.9.3 Agarose sealing solution (1% w/v)

Agarose   1% (w/v)

1X running Buffer   100 ml

Bromophenol blue   0.5% (w/v)

2.9.4 Gel preparation for 2-D electrophoresis

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

- Tris – HCl (1.5 M)   60 ml
- Deionised water   76 ml
- Bis-Acrylamide (30% w/v)   100 ml
- SDS (10% w/v)   2.4 ml
- APS (10% w/v)   1.5 ml
- TEMED   60 μl

2.9.5 2-Dimensional Gel Electrophoresis

Protein was extracted from haemolymph of *G. mellonella* larvae and 400 μg (mini 2-D gels 200 μg) of protein was obtained via quantification by the Bradford

method (2.10.1). The required aliquot of haemolymph was added to 100 μl (mini 2-D gels 50 μl) of IEF buffer and allowed to solubilise fully with regular vortexing for 1 hour. Ampholytes (Immobline pH 4-7) 2 μl (mini 2-D gels 1 μl) were added directly to the sample and a further 150 μl (mini 2-D gels 100 μl) of IEF buffer with a few grains of bromophenol blue was added to the sample. The sample was briefly vortexed and applied to a 13cm (mini 2-D gels 7cm) Immobline DryStrip pH 4-7 (G.E. Healthcare). Prior to isoelectric focusing the strips were covered in Plus One drystrip cover fluid, (GE Healthcare). Focussing was performed on an Ettan IPGphor II (Amersham Biosciences, NJ, USA) (Fig. 2.2) system using the following programme.

(4 Step process)

1. 50 Volts	Step and Hold	10 hours
2. 250 Volts	Step and Hold	15 min
3. 8000 Volts	Gradient	5 hours
4. 8000 Volts	Step and Hold	8 hours
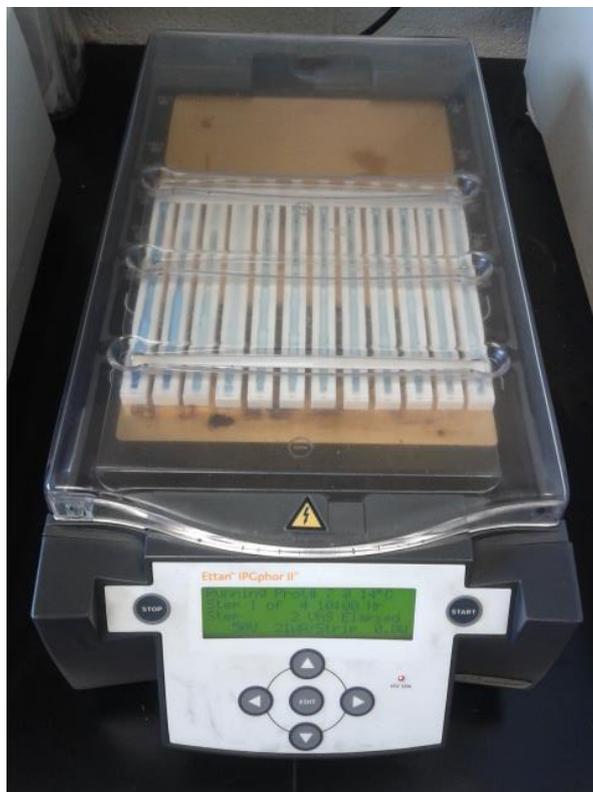
Fig. 2.2. Ettan IPGphor II (Amersham Biosciences, NJ, USA).

Following IEF IPG strips were frozen at -70ºC for long term storage, -20ºC for short term storage or were transferred directly to equilibration. Strips were initially equilibrated in 10 ml reducing equilibration buffer (DTT) (0.01 g/ml) for 15 min. Strips were transferred to the alkylation buffer (IAA) (0.025 g/ml) for 15 min. Following equilibration IPG strips were rinsed briefly in 1X electrode running buffer. Strips were placed on top of homogenous 12.5% SDS-PAGE gels and sealed with 1% w/v agarose sealing solution once hand hot. For a molecular marker a piece of straw with filter paper was inserted to the left of the IPG strip and an aliquot of molecular weight marker was placed into the filter prior to sealing.

The second dimension of protein separation was achieved by placing the gels in a Protean Plus^{TM} Dodeca^{TM} Cell gel rig with horizontal electrophoresis cells. The chamber was filled to mid-way on the top grey glass plate spacer of all the gel glass plates with 1X electrode running buffer to ensure the buffer covered above the acrylamide gel level for proper running. Gels were initially electrophoresed for 1 hour at 1 Watt per gel, and then increased to 1.5 Watts per gel if left overnight provided the gel rig coolant system was kept between 9-10ºC to prevent faster protein migration. The following day gels were increased from 1.5-2.5Watts per gel depending on how far and fast the blue tracking dye line migrated. Gels were monitored at regular intervals to assess the level of electrophoresis and once complete were transferred to a staining dish which was previously washed with soap and 70% ethanol to prevent any contamination to the gels during staining.

2.9.6 Comparative analysis of protein expression

2-D electrophoresis gel images (In triplicate) were analysed using Progenesis SameSpots software (Nonlinear Dynamics) in order to assess the fold change in protein expression in larvae subjected to different treatments. The level of differential expression was analysed by ANOVA with p-values of ≤ 0.05 considered statistically significant for changes in expression.

2.10 General Protein Methodology

2.10.1 Bradford Protein assay

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

2.10.2 Acetone precipitation of protein samples

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

2.10.3 Colloidal Coomassie staining of 1-Dimensional and 2-Dimensional SDS-PAGE gels

Upon completion of electrophoresis, gels were removed and immediately placed in staining dish with the following treatments;

Step	Solution	1-D gel (2-D gel)	Time
Fixation	50% EtOH, 3% phosphoric acid	100 mL (250 mL)	4h
Washing	Deionised water	100 mL (250 mL)	3x20min
Pre-stain	34% MeOH, 3% phosphoric acid and 17% (w/v) ammonium sulphate	100 mL (250 mL)	3-5days
Serva Blue	Add 1 spatula tip of serva blue	1 spatula tip (2)	after pre-stain 1h
Destaining	Deionised water	100 mL (250 mL)	3x20min

2.10.4 Coomassie Brilliant Blue (CBB) Stain

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

- Methanol 45% (v/v)
- Deionised Water 44.8% (v/v)
- Acetic acid 10% (v/v)
- CBB (Brilliant Blue) 0.2% (w/v)

2.10.5 Coomassie Destain

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

- Methanol 25%
- Deionised Water 65%
- Acetic Acid 10%

2.11 Peptide extraction from *G. mellonella* haemolymph

2.11.1 Extraction of *G.mellonella* haemolymph

The extraction of haemolymph from larvae was achieved by piercing and bleeding through the anterior end with a sterile 23G needle and haemolymph was squeezed into a pre-chilled eppendorf tube. A 1/10 dilutionin PBS was carried out of pure haemolymph using a pipette tip with the top cut off to enable up take of the viscous haemolymph. Haemocytes were pelleted out of solution by centrifugation at 1500 x g for 5 min and the protein supernatant was transferred to a fresh eppendorf and diluted a further 1/10 (1/100 of original). Protein was quantified using the Bradford assay 2.10.1 and protein adjusted according to 20 µg (1-D gel well), 200 µg (mini 2-D gel) or 400 µg (large 2-D gel).

2.11.2 ProQDiamond Sample preparation

Protein was extracted from haemocytes stimulated as described in section 2.14.3 or unstimulated and fractionated with the use of phosphatase inhibitor cocktail 2.14.1.

2.11.3 Pro-Q staining of 1-Dimensional and 2-Dimensional SDS-PAGE gels

Once gel electrophoresis was complete the gels were removed and immediately treated as follows using the modified technique from (Agrawal and Thelen, 2005);

Step	Solution	1-D gel (2-D gel) Volume	Time
Fixation	50% methanol, 10% acetic acid	100 mL (250 mL)	2x30 min
Washing	Deionized water	100 mL (250 mL)	2x15 min
Staining	"3-fold diluted" Pro-Q DPS in deionized H_2O	65 mL (150 mL)	120 min
Destaining	50 mM sodium acetate, (pH 4.0) 20% ACN)	100 mL (250 mL)	4x30 min
Washing	Deionized water	100 mL (250 mL)	2x5 min

N.B. Steps, staining to washing, should be carried out in the dark.

Images were immediately scanned on the Typhoon Scanner (9400) (Amersham Biosciences, NJ, USA) (Fig. 2.9) set at an emission filter of 580BP 30 on a 532nm green laser. Once gel images were scanned they could be stained in colloidal coomassie stain to enable visualisation of spots for cutting to identify phosphorylated proteins on the LC/MS.

2.12 In-gel trypsin digestion and Liquid chromatography–mass spectrometry (LC/MS) analysis

2.12.1 Preparation of micro-centrifuge tubes

All micro-centrifuge tubes were fresh from the bag or placed in a sealed container and autoclaved before use. Tips were autoclaved and cut to varied lengths for spot cutting and subsequently tips and all other utensils in contact with the gel,

scalpels, and blunt needles were soaked in acetonitrile prior to use to eliminate keratin contamination.

2.12.2 Trypsin digestion of 1 and 2-D SDS-PAGE protein bands/spots for LC/MS analysis

Processing of bands and spots for LC/MS analysis was achieved by following the method of Shevchenko *et al.* (2007). Gel pieces were cut and transferred to sterile micro-centrifuge tubes and gel pieces were cut if too large (approximately 1-2 mm) using a scalpel when required to ensure full saturation of gel piece for de-staining and digestion process. Gel pieces were de-stained by addition of 100 μl of 100 mM Ammonium bicarbonate: Acetonitrile 1:1 ratio and subsequent vortexing every 10-15min (3X).

Acetonitrile (500 μl) was added to dehydrate and shrink gel pieces to the point they became white. Acetonitrile was removed and the samples were stored at -20ºC or processed further immediately. Tryptic digestion was achieved with the addition of approximately 20-30 μl of trypsin buffer (~10 ng/μl, trypsin enzyme prepared in trypsin reconstitution buffer, 10 mM ammonium bicarbonate, 10% (v/v) acetonitrile). Samples were placed at 4^oC to prevent trypsin auto-digestion and allow for the penetration of trypsin buffer into the gel piece. Gel pieces were checked after 30 min to ensure they were sufficiently covered or if more trypsin buffer was required. Samples were rechecked after a further 30 min for adequate coverage of gel pieces. If required gel pieces were topped up with 10 mM ammonium bicarbonate/10% (v/v) acetonitrile to prevent drying out. The samples were transferred to the 37^oC orbital incubator overnight and set to 160-180 revolutions per minute.

Digested samples were centrifuged on a desk top Tomy centrifuge and the supernatant was transferred to clean micro-centrifuge tube. For a double extraction the original gel piece was extracted further with 5% (v/v) Formic Acid in HPLC grade H_2O. Samples were incubated at 37ºC in the orbital shaker and all supernatant was transferred to the tryptic digest supernatant. The samples were lyophilised in a vacuum centrifuge and stored at -20^oC or immediately re-suspended in 20 μl 0.1% formic acid and sonicated. All samples were filtered through 0.22 μm cellulose acetate spin filter tubes (Spin-X®, Costar) and transferred to mass spectrometry vials. Analysis of digested peptides was achieved using an Agilent 6340 Ion Trap LC/MS using acetonitrile elution (Fig. 2.3)

2.12.3 Bioinformatic analysis of LC/MS results

The fragmented protein samples were eluted by LC/MS (Aglient 6340 Ion Trap) which determines the relative charge to mass ratio from detected ionized particles. These data were analysed using the mascot search engine to identify the protein (www.matrixscience.com) or via Spectrum Mill MS Proteomics. MASCOT scores above 67 were deemed to have a significant match ($p < 0.05$). The mass error tolerance was 1 Da allowing for a maximum of no more than two missed cleavages. Verification of protein sequences was confirmed by blasting the protein sequence on the Uniprot (www.uniprot.org) and NCBI (www.ncbi.nlm.nih.gov) websites.
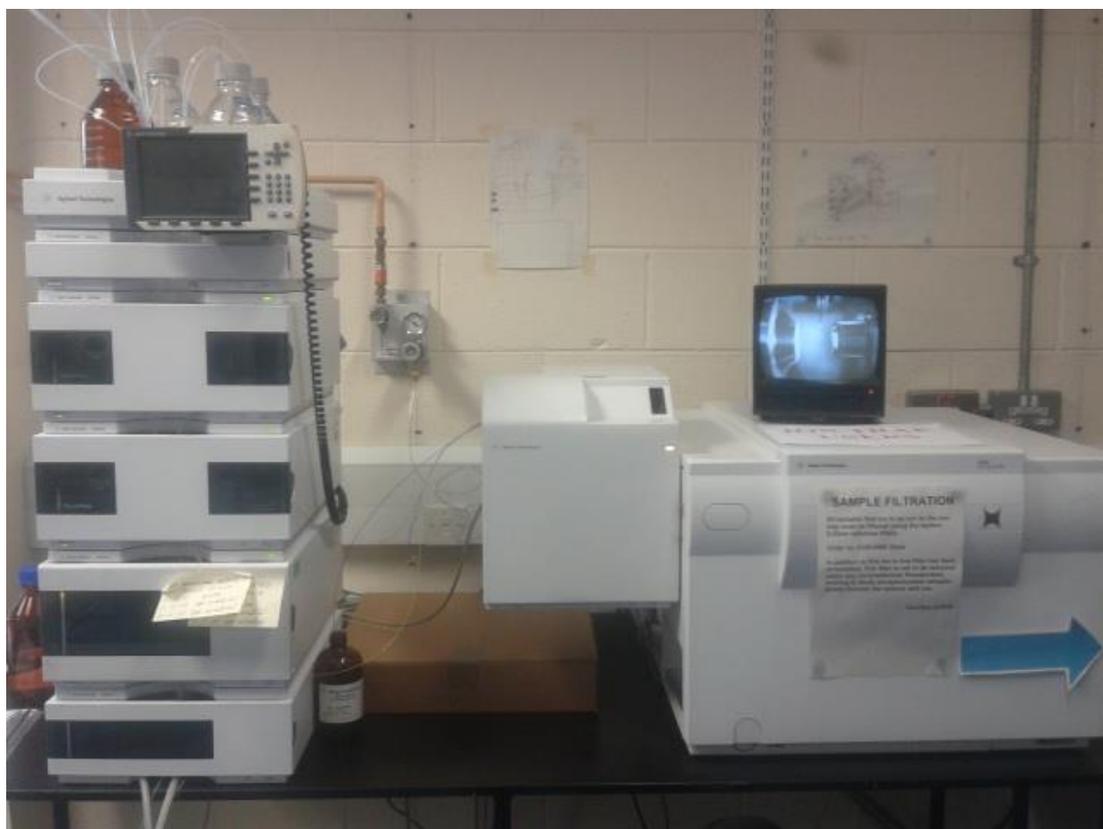
Fig. 2.3. Aglient 6340 Ion Trap LC/MS used for protein identification in this thesis.

2.12.4 Label Free Quantification

Label free shotgun quantitative proteomics was conducted on the haemolymph of 1 week and 10 week pre-incubated larvae. Protein (75 μg) was reduced with dithiotreitol (DTT; 200 mM), alkylated with iodoacetamide (IAA; 1 M) and digested with sequence grade trypsin (Promega, Ireland) at a trypsin:protein ratio of 1:40, overnight at 37 °C. Tryptic peptides were purified for mass spectrometry using C18 spin filters (Medical Supply Company, Ireland) and 1 μg of peptide mix was eluted onto a QExactive (ThermoFisher Scientific) high resolution accurate mass spectrometer connected to a Dionex Ultimate 3000 (RSLCnano) chromatography system. Peptides were separated by an increasing acetonitrile gradient on a Biobasic C18 PicofritTM column (100 mm length, 75 mm ID), using a 120 min reverse phase gradient at a flow rate of 250 nL min-1. All data was acquired with the mass spectrometer operating in automatic data dependent switching mode. A high resolution MS scan (300-2000 Dalton) was performed using the Orbitrap to select the 15 most intense ions prior to MS/MS.

Protein identification from the MS/MS data was performed using the Andromeda search engine (Cox *et al*., 2011) in MaxQuant (version 1.2.2.5; http://maxquant.org/) to correlate the data against a 6-frame translation of the EST contigs for *G. mellonella* (Vogel *et al*., 2011). The following search parameters were used: first search peptide tolerance of 20 ppm, second search peptide tolerance 4.5 ppm with cysteine carbamidomethylation as a fixed modification and N-acetylation of protein and oxidation of methionine as variable modifications and a maximum of 2 missed cleavage sites allowed. False Discovery Rates (FDR) were set to 1% for both peptides and proteins and the FDR was estimated following searches against a target-decoy database. Peptides with minimum length of seven amino acid length were considered for identification and proteins were only considered indentified when more than one unique peptide for each protein was observed.

Results processing, statistical analyses and graphics generation were conducted using Persues v. 1.5.0.31. LFQ intensities were log2-transformed and ANOVA of significance and t-tests between the haemolymph proteomes of week 1 and week 10 larvae was performed using a p-value of 0.05 and significance was determined using FDR correction (Benjamini-Hochberg). Proteins that had non-existent values (indicative of absence or very low abundance in a sample) were included in the study only when they were completely absent from one group and

present in at least three of the four replicates in the second group (hereafter referred to as qualitatively differentially abundant proteins). The Blast2GO suite of software tools was utilised to assign gene ontology terms (GO terms) relating to biological processes, molecular function and cellular component. Enzyme commission (EC) numbers and Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway mapping was performed as part of the Blast2GO annotation pipeline (Conesa and Götz, 2008).

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

2.13.1 5 mM PBS – Glucose (PBS-G)

PBS – Glucose (5 mM) was prepared by adding 0.9 g of glucose to 1 L container and dissolving in PBS upto the 1L mark. This was filter sterilised through a 0.22 μm filter and kept at room temperature.

2.13.2 Trypan Blue exclusion assay

The trypan blue assay was used to establish the viability of haemocytes, (Eichner *et al*., 1986). Unhealthy cells will not form an intact membrane and pores will allow the leakage of the dye into the cell which can be observed under light microscopy. Cells ($\sim1x10^6$) were suspended in trypan blue solution at the following volumes: 20 μl cells, 60 μl PBS and 20 μl 0.4% (v/v) Trypan Blue solution (made by dissolving trypan blue powder in PBS and sterilising through a 0.22 μm filter). Solution allowed stand for 3 min at room temperature and the cells were counted and recorded with regard to the percentage viability of the cell suspension.

2.13.3 Insect Physiological Saline (IPS)

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

2.13.4 Extraction of haemocytes from *G. mellonella* larvae

Larvae were bled through the anterior region as previously stated (Section 2.11.1) and haemolymph was squeezed through the head region into a pre-chilled eppendorf containing ice cold PBS and pipetted several times. Haemocytes were pelleted by centrifugation at 1500 x g for 5 min and washed in 5 ml PBS to remove any excess haemolymph (x2). Following a further centrifugation step at 1500 x g for 5 min, the remaining supernatant was removed and the mixed cells were re-suspended gently in PBS or 5 mM PBS-Glucose for haemocyte functional assessments.

2.14 Cellular fractionation of haemocytes

2.14.1 Lysis Buffer

PIPES 20mM, NaCl 5mM, dissolve in distilled water adjusted to pH 7.2, autoclaved and TritonX 100 (0.2% v/v) was added aseptically. Prior to use lysis buffer was supplemented with phosphatase inhibitor cocktail 2 (10 μl/ml) (Sigma-Aldrich) which contains acid and alkaline phosphatase as well as tyrosine protein phosphatase inhibitors (Table 2.1).

Phosphatase	Inhibitor
Alkaline phosphatase	Imidazole
Acid Phosphatase	Sodium molybdate, sodium tartrate
Phosphotyrosyl phosphatases (PTPs) (general inhibitor)	Sodium Orthovanadate (Vanadate, Na_3VO_4)

Table 2.1 Phosphatase inhibitor cocktail 2 (Sigma-Aldrich) contents of phosphatase inhibitors

2.14.2 Haemocyte membrane solubilisation

Haemocyte membrane solubilisation buffer (0.2% v/v Triton X–100 in PBS) was made to solubilise membranes to enable internal cell staining.

2.14.3 Haemocyte cell degranulation

Haemocytes, (5×10^7), were pre-treated for 10 min in the presence of 0.5 µg/ml Formyl-Methionyl-Leucyl-Phenylalanine (fMLP) or pre-treated for 6 min with (1 µg/ml) Phorbol myristate acetate (PMA) at 37ºC in PBS-G. To slow down cell stimulation 500µl of ice cold PBS was added to haemocytes before being centrifuged at 1500 x g for 5 min at 4ºC to pellet the cells and this pellet was washed twice to remove excess (fMLP) or (PMA).

The cells were re-suspended in ice cold Lysis buffer (Section 2.14.1) and left for 10 min, the cells were briefly sonicated for 5 min. The cells were centrifuged at 1500 x g for 5 min to pellet the cells, the supernatant was discarded and the pellet re-suspended in PBS. The samples were analysed for protein concentration using the Bradford assay, and were concentrated by acetone precipitation prior to electrophoresis.

2.14.4 Haemocyte cell preparation for Flow Cytometry analysis

Larvae were bled into a sterile pre-chilled centrifuge tube containing 800 µl of chilled PBS. Haemolymph was mixed with the PBS by pipetting slowly several times. Larval haemolymph was centrifuged at 1500 x g for 5mins at 4ºC, supernatant was removed and pelleted cells were re-suspended in 1ml of ice cold PBS. Cells were washed a further 2-3 times using the above method. Re-suspended cells were enumerated and viability was checked using the Trypan blue exclusion assay. Cells were adjusted to a cell density of 1×10^6 per 500 µl. Cells were fixed in 3.7% formaldehyde in PBS for 10min at 4ºC. The cells were centrifuged at 1500xg for 5min and re-suspended in 1% BSA/PBS and washed 1-2 times before final re-suspension in 500 µl 1% (w/v) BSA/PBS. Cells can be stored at 4ºC for ~3 days prior to flow cytometry analysis. Cells were transferred into a FACS tube with a 35 µm cell strainer cap to reduce potential blockage of the instrument by cell clumps and debris.

2.14.5 Flow Cytometry analysis of Haemocyte cell populations

Cells were analysed on a Becton Dickson® FACS ARIA, with cell populations grouped based on their forward scatter (size) and side scatter (granularity). Selected populations were sorted into vials of their own individual populations based on their size and morphology using the cell sorting application at the National Institute of Cellular Biotechnology, Dublin City University under the

supervision of Dr. Clair Gallagher, these cells were stained and identified by microscopy as stated (Section 2.15).

2.15 Confocal Immunofluorescence microscopy

2.15.1 Confocal microscopy of haemocytes

Haemocytes (1×10^6 ml) were extracted from larvae pre-treated with different thermal or physical conditions and washed twice in sterile PBS. Cells were fixed in 3.7% formaldehyde for 10min at 4^oC, washed twice, permeabilised in 0.2% Triton X-100 for 10 min and washed twice. Cells were stained overnight at 4^oC with diamidino-2-phenylindole (DAPI) or Rhodamine phalloidin. The cells were washed twice and 10 µl aliquot was taken from a stock (1×10^6 ml) and placed on a slide with a cover slide placed on top, excess liquid was drawn out by placing Whattman paper at the edge of the cover slip this stopped cells from floating freely under the slide. The cover slides were then fixed *in-situ* by applying a clear sealing solution around the perimeter of the slide which also prevented the sample drying out. The controls for this experiment included cells alone and stain alone with no cells. Cells were viewed with an Olympus Flouview 1000 confocal microscope under the supervision of Dr. Ica Dix, Biology Dept, NUIM.

2.16 *G.mellonella* haemolymph protein binding assay

2.16.1 Comparative analysis of binding of haemolymph proteins to germinated and non-germinated *A. fumigatus* conidia, *C. albicans*, *S. aureus*

A.fumigatus conidia were harvested, washed and pelleted (5×10^8 conidia) as previously described (Section 2.4.7). *S.aureus* grown to the stationary phase and an $OD_{600} = 2.0$ was pelleted after washing twice in PBS, 1500 x g for 5min, supernatant discarded. A stock of *C.albicans* (1×10^9 cells/ml) was washed in PBS (3x) to remove media, resuspended in PBS and 1ml (1×10^9 cells/ml) was pelleted. Microbial cell pellets, *Aspergillus* conidia, *S.aureus* and *C.albicans* cells were re-suspended in 1 ml of cell free larval haemolymph (Section 2.11.1) at a concentration of 10mg/ml in ice cold IPS.

The microbial cell/ haemolymph suspension was mixed gently for 40 min at 4ºC on a Stuart Rotator SB2 and immediately centrifuged at 1500 x g for 5 min to

pellet microbial cells. Cells or conidia were washed 3 times with 1 ml of sterile IPS to remove any non-specifically bound proteins. Protein was then removed from the cell surfaces by the addition of 5X solubilisation buffer and brief sonication in a water bath followed by boiling at 95°C for 5 min. The samples were centrifuged at 1500 x g for 5 min at 4ºC and the protein supernatant was transferred to a pre-chilled micro-centrifuge tube. Samples were frozen immediately at -20ºC or subjected to 1-D SDS-PAGE. For analysis of human serum binding proteins, serum was pooled from multiple donors and the quantity of protein was ascertained and adjusted (10mg/ml) in sterile PBS and the binding protocol was performed the same as for the haemolymph assessment.

2.16.2 Comparative analysis of protein binding of haemolymph proteins to β-1, 3-Glucan and Lipopolysaccharide

β-1, 3-Glucan (G5011 Sigma-Aldrich, *Saccharomyces cerevisiae*) (10mg/ml) stock was made using a powder solution added to PBS followed by vigorous vortexing until the solution resembled a homogenous liquid suspension. A sample (100μl, (1mg)) was pelleted and re-suspended in larval haemolymph from thermally or physically stressed larvae. A LPS (Sigma-Aldrich) stock was made at the same concentration of 10mg/ml with 100 μl (1mg) sample pelleted and resuspended as above in larval haemolymph.

G. mellonella larvae were bled through the anterior region into a pre-chilled micro-centrifuge tube and centrifuged immediately at 1500 x *g* for 5 min at 4ºC. Cell free haemolymph was removed and protein concentration determined by Bradford assay. Approximately 100 μl of cell free haemolymph (10 mg of protein) was diluted in 900 μl ice-cold IPS and the β-1, 3-Glucan pellet or the LPS pellet was re-suspended in the 10mg of haemolymph protein. The β-1, 3-Glucan or LPS/haemolymph suspension was gently mixed for 40 min at 4ºC on a Stuart Rotator SB2 and immediately centrifuged at 1500 x *g* or 13000 x *g* for 5 min to pellet β-1, 3-Glucan or LPS, respectively. The cell wall constituents were washed 3 times with 1 ml sterile IPS to remove any non-specifically bound proteins. Haemolymph protein bound to cell wall constituents were separated by the addition of 5X solubilisation buffer, brief sonication and boiling at 95ºC for 5 min.

The samples were centrifuged at 1500 x *g* for 5 min for at 4°C for β-1, 3-Glucan or 13000 x *g* for LPS, the protein supernatant was transferred to a pre-chilled

micro-centrifuge tube. Samples were frozen immediately at -20ºC or subjected to 1-D SDS-PAGE. For analysis of human serum binding proteins, serum was pooled from multiple donors and the quantity of protein was ascertained and adjusted (10mg/ml) in sterile PBS and the binding protocol was performed as for the haemolymph assessment.

2.17 RT-PCR analysis of larval antimicrobial peptide gene expression

2.17.1 RNase treatment of water and laboratory apparatus

All water was nuclease free and was purchased from (Sigma-Aldrich). Buffers and solutions were made with nuclease free water treated with Diethylpyrocarbonate (DEPC), pestles and mortars were left to soak in a large beaker of nuclease free water prior to being wrapped in aluminium foil and oven baked at 220°C for 12h prior to use. RNase free filter tips (Fisher) were used and all eppendorf tubes were taken from a previously unopened bag and autoclaved twice in sealed container with eppendorf lids closed prior to use. For RNA visualisation all gel casting, and gel electrophoresis equipment was washed in nuclease free water supplemented with 2% (v/v) hydrogen peroxide. Gloves were worn at all times and changed at regular intervals.

2.17.2 RNA extraction from *G. mellonella*

Three larvae per treatment were selected at random from a plate of ten and crushed to a fine powder using liquid nitrogen and a pestle and mortar. TRI-Reagent (3 ml) (Sigma-Aldrich) was added, mixed and allowed to rise to room temperature for 15 min. The mixture was transferred to a pre-chilled micro-centrifuge tube and centrifuged at 12,000 x g for 10 min at 4°C (Eppendorf centrifuge 5417R). The supernatants were transferred to a fresh tube and the pellets were discarded. Chloroform (200 µl) (molecular grade; Sigma-Aldrich) was added and mixed vigorously.

The solution was allowed to stand at room temperature for 10 min and centrifuged for 10 min for 12,000 x g at 4°C. The top layer was transferred to a fresh tube and 500µl 2-propanol (molecular grade; Sigma-Aldrich) was added. The tube was inverted 10 times and left to stand for ten min at room temperature. Samples were centrifuged at 12,000 x g for 10 min at 4°C. The supernatant was discarded and the pellet was washed in 50 µl of 75% (v/v) ethanol (molecular grade; Sigma-Aldrich)

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and centrifuged at 12,000 x g for 10 min at 4°C. The supernatant was removed and the resulting pellet was allowed to air dry prior to re-suspending in molecular grade RNase/nuclease free water. RNA was aliquoted and stored at -70^{0}C or used immediately.

2.17.3 DNase digestion of RNA

Contaminating DNA was removed by DNase treating the RNA extract with a Deoxyribonuclease I (AMP-D1) kit. RNA (4 µg) quantified by spectrometry (A260/A280), was brought up to a volume of 8 µl using DEPC treated water. This was followed by the addition of 1 µl 10X reaction buffer and 1 µl DNase 1 (1 unit/µl) to the micro centrifuge tube containing the RNA mixture, prior to incubation at room temperature for 15 min. The enzyme activity was stopped by adding 1µl of Stop Solution (50 mM EDTA) to the RNA mixture and mixed by pulsing on the micro centrifuge. Samples were incubated at 70ºC for ten min and cooled on ice. RNA was further processed for cDNA synthesis or frozen at -70ºC.

2.17.4 cDNA synthesis

cDNA was synthesised using the Superscript III First Strand Synthesis System for RT-PCR kit from Invitrogen (CA, USA). RNA concentration was determined and all samples contained equal amounts of RNA prior to cDNA synthesis following adjustment to 8 µl using nuclease free water. RNA used for cDNA synthesis was of high quality, with all RNA A260/A280 readings of above 1.97 (Section 2.7.4) and 1.95 (Section 2.7.3). The 1 µl of dNTP (10 mM) and 1 µl mM oligo (dT) were added to the RNA. The solution was incubated at 65ºC for 5 min and then cooled to 4ºC. A master-mix was prepared (Per reaction: 2 µl 10X RT Buffer, 4 µl 25 mM $MgCl_2$, 2 µl 0.1 mM DTT, 1 µl RNaseOUT) according to the number of reactions necessary.

Master-mix (9 µl) was added to each of the RNA samples and mixed gently followed by incubation at 42ºC for 2 min and then 4ºC for 5 min. Superscript III RT (1 µl) was added to each reaction tube and incubated as follows: 42ºC for 50 min, 70ºC for 5 min, and held on ice. RNase H (1 µl) was added to each tube and incubated for 20 min at 37ºC. cDNA was aliquoted and stored at -20ºC.

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	

Table 2.2. Primers used in this work.

2.17.5 DNA gel electrophoresis of PCR products

DNA was visualised by running samples on a 1% agarose gel. Agarose (1 g) (Melford Labs Ltd., Ipswich England) was dissolved in 100 ml 1 X TAE Buffer, (1/50 dilution of 50X stock: 24.2% (w/v) Tris-Base, 5.71% (v/v) Acetic acid, 0.05 M EDTA (pH8), with the addition of 4 µl of 5 mg/ml ethidium bromide. Samples (8 µl) were mixed with 2 µl of Blue Orange 6X loading dye (Promega). The sample was placed

into the wells of the agarose gel. Samples were run at 80 V Embi-Tech and viewed using a UV-transilluminator (Alpha Innotech).

2.17.6 RNA electrophoresis

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

2.17.7 Real Time Polymerase Chain Reaction

Real Time PCR was carried out on the Light Cycler® 480 (Roche) (Fig 2.4) with high quality reagents sourced from Anachem and Sigma. The cycle conditions were optimised for each primer set. Primers for target genes and cycle conditions were as used by Bergin *et al*. (2006), primers for reference gene (housekeeping) and cycle conditions as used by Wojda and Jakubowicz (2007). Primers were assessed for secondary structures as dimer, hairpin-loop and palindrome formation using the (http://www.premierbiosoft.com/netprimer/netprlaunch/netprlaunch.html#) Netprimer website.

Table 2.2 represents primer sequences for each primer set. Each PCR reaction (biological triplicates) was carried out on a 96 well plate (Anachem) containing 1 µg/µL of cDNA, 5 µL SYBR (KAPA SYBR® FAST) 1 µL H_2O (nuclease free), 1 µL forward primer and 1 µL reverse primer (9 µL volume). A negative control consisted of the same reagents but cDNA was replaced with nuclease free H_2O. Plates containing the reaction mix were centrifuged gently prior to placing in the Light Cycler.

DNA quantification was assessed from the measurement of fluorescence when it passed a threshold or Ct (cycle time) which was expressed as a graphed line (Valasek and Repa, 2005). Cycle conditions optimized for Real-time qPCR: 95 ^{o}C 3 min, 45× (95°C 10s, 60°C 30s, 72°C 30s). The calculation was performed using the

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delta-delta Ct method. As a standard curve, PCR amplification was performed with 5 serial dilutions of DNA template (Biological triplicate) from 1 week old larvae (control) stored at 15°C (efficiencies of 2.0 ± 0.18) (Fig. 2.4-2.8). All values were expressed against the gene expression in larvae incubated at15^{o}C.

(A)
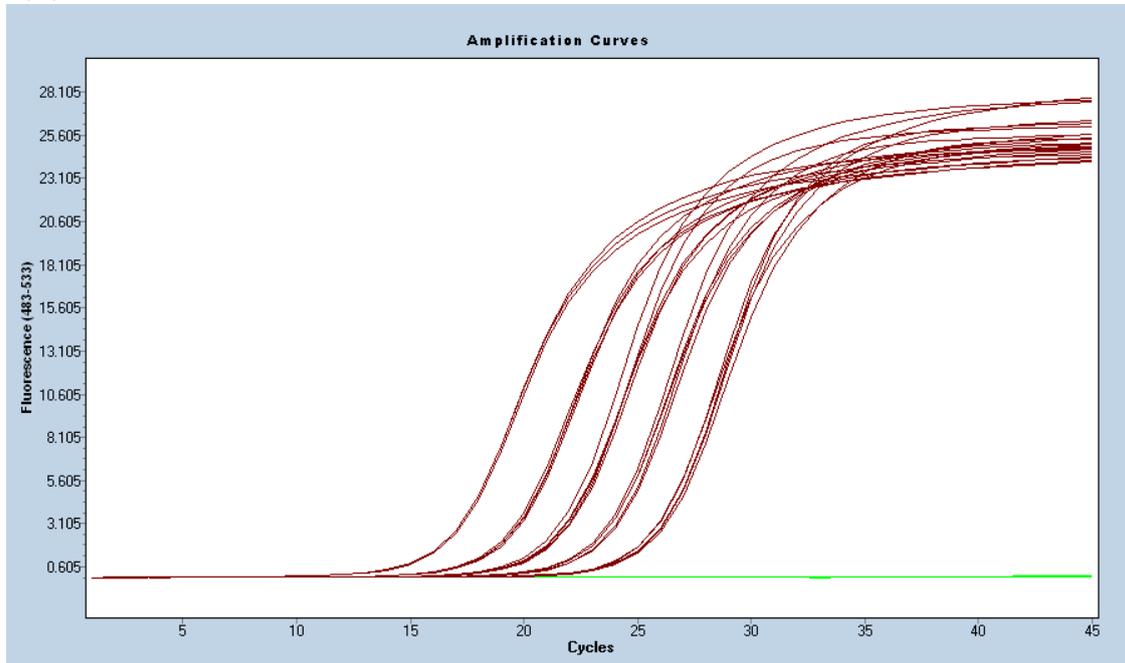
(B)
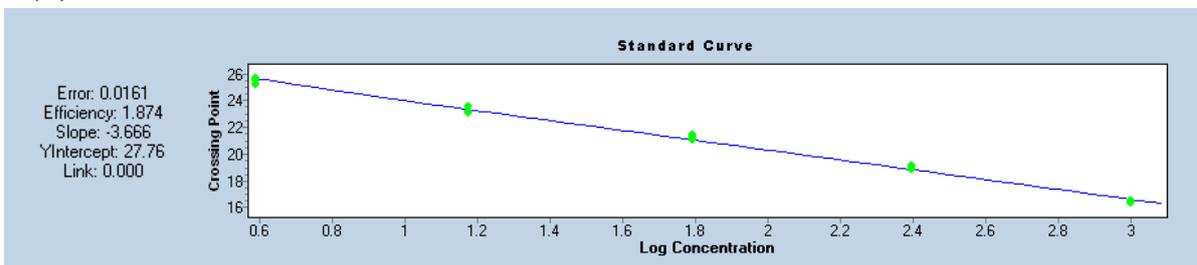
Fig. 2.4 Standardisation of *S7e* reference gene run on a Light Cycler® 480 (Roche). (A) Amplification curves and (B) Standard curve.

(A)
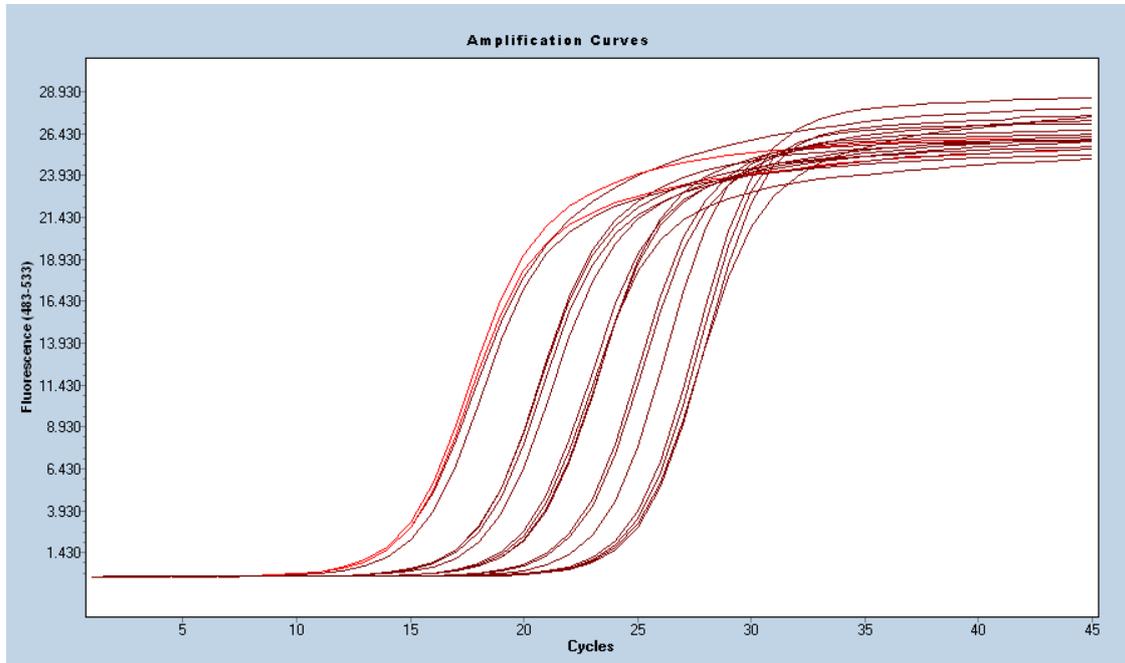
(B)
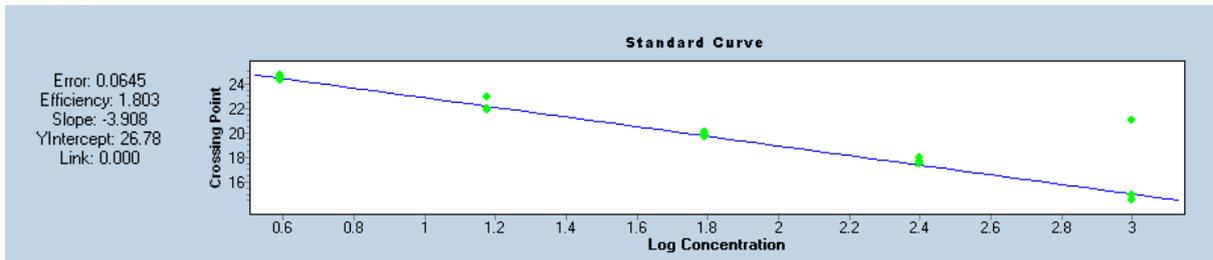
Fig. 2.5 Standardisation of *Galiomicin* reference gene run on a Light Cycler® 480 (Roche). (A) Amplification curves and (B) Standard curve.

(A)
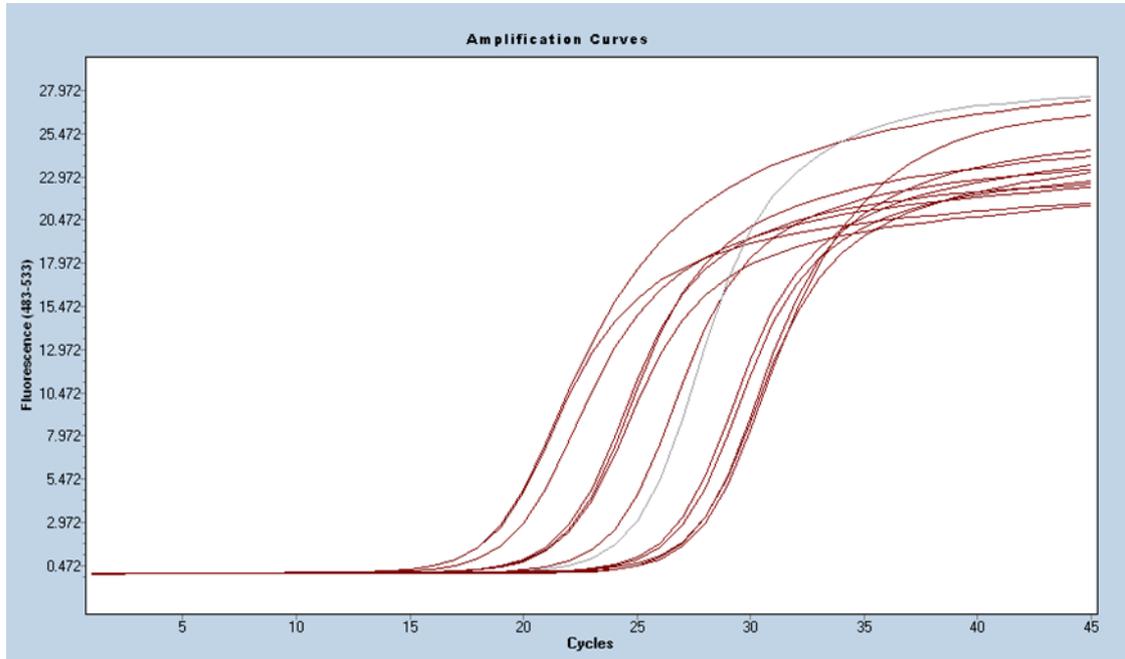
(B)
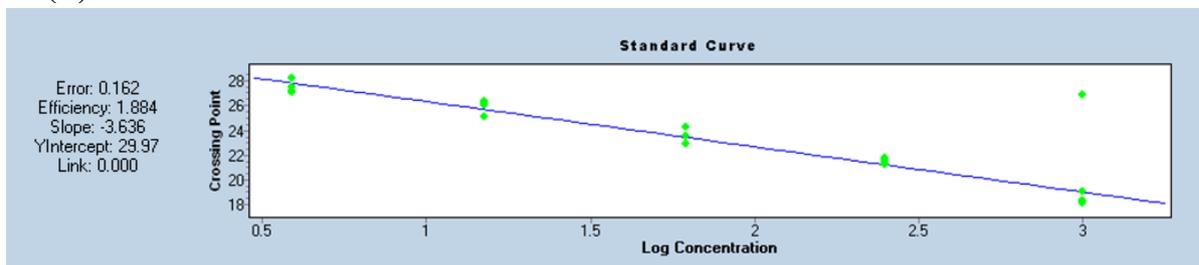
Fig. 2.6 Standardisation of *Gallerimycin* reference gene run on a Light Cycler® 480 (Roche). (A) Amplification curves and (B) Standard curve.

(A)
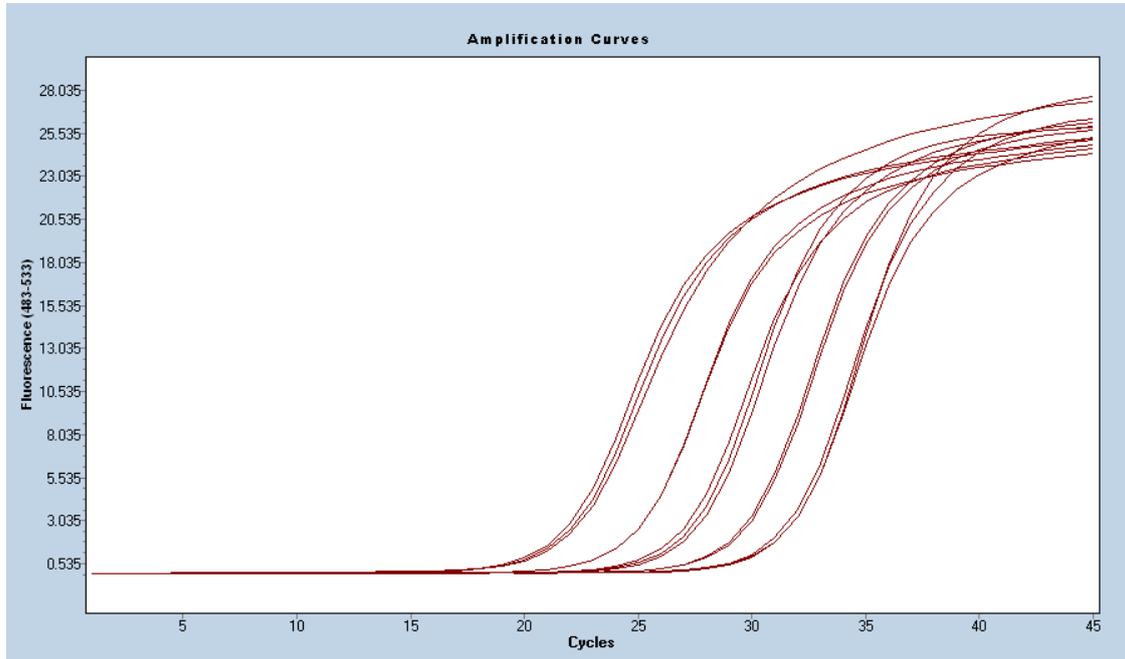
(B)
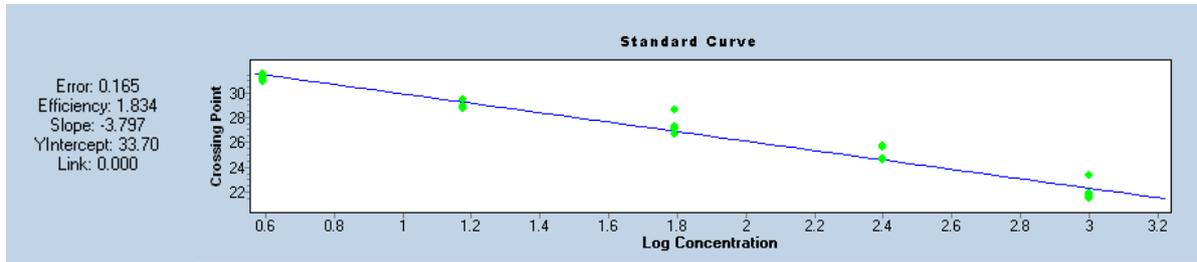
Fig. 2.7 Standardisation of *IMPI* reference gene run on a Light Cycler® 480 (Roche). (A) Amplification curves and (B) Standard curve.

(A)
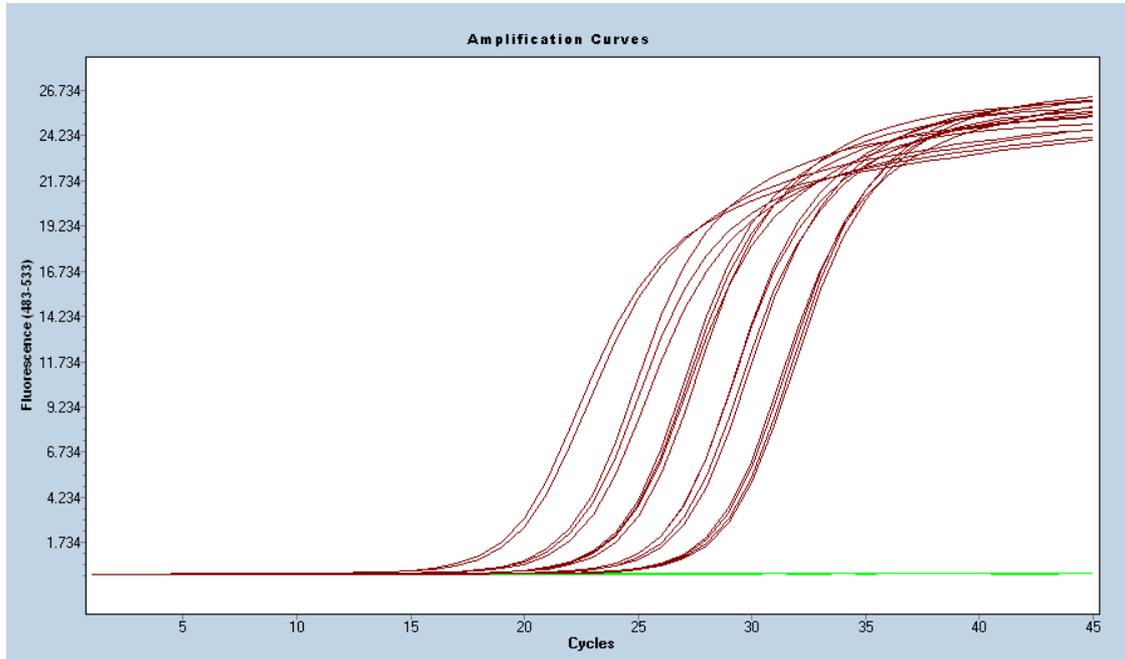
(B)
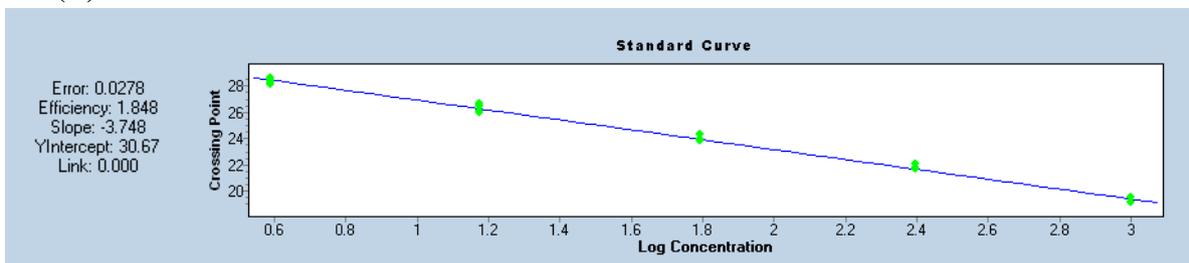
Fig. 2.8 Standardisation of *Transferrin* reference gene run on a Light Cycler® 480 (Roche). (A) Amplification curves and (B) Standard curve.

Fig. 2.9 Typhoon Scanner (9400) (Amersham Biosciences, NJ, USA).

2.18 Assessment of the efficacy of SBC3 in *G.mellonella*

2.18.1 SBC3

SBC3 (1,3-dibenzyl-4,5-diphenyl-imidazol-2-ylidene silver(I) acetate) was provided (Patil *et al*., 2011) (Fig. 6.1) and dissolved in a minimal amount of DMSO (dimethylsulfoxide) before dilution with water-based media as described in later experimental sections.

2.18.2 *In vitro* toxicity assessment of SBC3 against *S. aureus* and *C. albicans*

To each well of a 96–well plate (Sarsdedt), 100 µl of fresh nutrient broth medium or minimal medium (2% glucose, 0.17% yeast nitrogen base and 0.5% ammonium sulphate) was added. One hundred µl of SBC3 stock solution was added to each well of row 3 on the plate and a serial dilution was carried out across the plate giving a concentration range of 0.39 – 100µg/ml. Cells (100 µl of *S. aureus* ($OD_{600} = 0.1$/ $4.01x10^7$ cells/ml) or *C. albicans* (1×10^6 cells/ml)) were added to each

well and the plates were incubated at 37˚C or 30˚C respectively for 24 hours. The OD$_{570nm}$ of the cultures was determined using a microplate reader (Bio-Tek, Synergy HT). All susceptibility assays were performed on three separate occasions.

2.18.3 *In vivo* toxicity assessment of SBC3 on *G.mellonella* larvae

Larvae were injected with 20 µl of SBC3 solution (10, 100, 250, 500 or 1000 µg/ml) or 5% (v/v) DMSO through the last left pro-leg as described. Larvae were incubated at 30˚C for 24 h prior to quantifying survival.

Chapter 3

Identification of innate immune proteins of *G.mellonella* and humans that bind to microbial surfaces

3.0 Identification of innate immune proteins of *G.mellonella* and humans that bind to microbial surfaces

The aim of the experiments described in this Chapter was to identify humoral immune proteins of the innate immune response of *G.mellonella* that can bind to microbial surfaces. The insect innate immune response is composed of the cellular immune response which enables the phagocytosing and neutralising of pathogens (Lavine and Strand, 2002) and the humoral immune responses. The humoral immune response is the second arm of the innate immune response and produces a range of proteins that can recognise pathogen associated molecular patterns (PAMPs) through PRR binding or arrest pathogens through antimicrobial peptide production or immune cascades (Janeway, 1989).

The first section of this Chapter focused on how abiotic factors such as thermal and physical stress affected the insect immune response in terms of immune binding proteins. The aim was to determine how abiotic factors may influence *G.mellonella* and how this may impact experimental outcomes when larvae were incubated at different temperatures or handled prior to use.

It has previously been documented that incubation of larvae at different temperatures had the effect of priming their immune system enabling them to overcome an infection with *C.albicans* (Mowlds and Kavanagh, 2008). It was similarly observed by Wojda and Jakubowicz, (2007) that incubation at 38°C enhanced the humoral immune response to infection when compared to incubation at 28°C. Mowlds *et al*. (2008) demonstrated that physical stress improved resistance of larvae to infection when compared to control larvae. It has not been previously demonstrated how the abundance of binding proteins is influenced by thermal and physical stress.

The second section of this Chapter analysed the binding proteins from cell free haemolymph of *G.mellonella* and humans to microbial surfaces in order to identify similar innate immune binding proteins that are conserved between the insect and mammal. It is well established that highly conserved components of the insect innate immune system are shared with those of the innate immune system of mammals despite almost 500 million years of evolutionary divergence (Kavanagh and Reeves, 2004; Sackton *et al*., 2007). The similarities shared include conserved regions within the melanisation cascade proteins of insects and the mammalian complement proteins

of the complement cascade (Kavanagh and Reeves, 2004). A high level of conserved signal pathways exist within the innate immune response and include the Toll like pathway (Lemaitre *et al*., 1996) and the insect IMD pathway with the mammalian TNFα pathway (Khush *et al*., 2001). This work aimed to characterise the similarities of insect and mammalian innate immune systems both structurally and functionally therefore highlighting the insect's utility as a preliminary screening model for pathogens and/or for screening antimicrobial drugs.

3.1 Binding assessment of thermally and physically stressed *G.mellonella* haemolymph to microbial surfaces

Larvae were thermally or physically stressed as described (2.7.3). Larvae were bled and the haemolymph was exposed to a known quantity of microbial cells i.e. (5×10^8 cells/ml) (Section 2.16.1) or cell wall constituent (e.g. LPS or β-1,3-Glucan) (Section 2.16.2). Bound *G.mellonella* haemolymph proteins were separated from microbial cells/cell wall constituents and equal amounts of protein (solution) was separated on a 1-Dimensional gel to enable visualisation and identification of proteins via LC/MS. Quantification was carried out using Image Quant TL and statistical analysis was carried out by Two-Way ANOVA using Graph Pad Prism 5.

3.2 Binding assessment of haemolymph proteins of thermally and physically stressed *G.mellonella* to Lipopolysaccharide

Haemolymph from larvae thermally and physically stressed were exposed to LPS and a number of proteins bound (Fig. 3.1). A high level of prophenoloxidase subunit 2 (Band 8, Table 3.1) binding to LPS was observed from haemolymph of larvae incubated at 37^oC or physically stressed (93k±5.6k, *p*<0.001) and (80k±5.4k, p<0.001), respectively (Fig. 3.2). ProPO in the proPO cascade can be activated by extremely low quantities (pg/l) of microbial cell wall components such as LPS, β-1, 3-glucans or peptidoglycans (PG) resulting in the production of melanin in the insect cuticle (Sugumaran and Kanost, 1993; Söderhäll, 1982). ProPO was also suggested to crosslink LPS to a LPS receptor present on the surface of haemocytes although this receptor was not characterised (Charalambidis *et al*., 1996). It was also observed that masquerade-like serine proteinase (Band 12, Table 3.1) had increased presence in

haemolymph from larvae pre-incubated at 37$^{\text{o}}$C (108k±3.6k, $p<0.01$) or physically stressed (110k±11.0k, $p<0.01$) compared to those incubated at 15$^{\text{o}}$C (74k±17.0k). It is known that serine proteases activate proPO and therefore bind to and cleave the proPO to active PO (Söderhäll, 1982).

Apolipophorin III (apoLp-III) (Band 14, Table 3.1) demonstrated a significant increase in binding to LPS from larvae incubated at 37$^{\text{o}}$C (103k±7.1k, $p<0.001$) compared to binding in haemolymph from larvae incubated at 30$^{\text{o}}$C (70k±5.9k) or 15$^{\text{o}}$C (56k±12.1k) (Fig. 3.2). ApoLp-III has previously been shown to recognize a range of cell wall components including LPS, and has roles in pathogen recognition and microbial cell wall detoxification (Halwani $et\ al$., 2000; Leon $et\ al$., 2006a; Leon $et\ al$., 2006b; Ma $et\ al$., 2006).

The peptidoglycan recognition protein B (PGRP-B) (Band 13, Table 3.1) showed increased binding to LPS in haemolymph from larvae incubated at 37$^{\text{o}}$C (142k± 9.8, $p<0.001$) or physically stressed (115k±23.3). Peptidoglycan-binding protein has been shown to be a mediator of peptidoglycan activation of the prophenoloxidase system of $B.mori$ (Yoshida $et\ al$., 1996).

Calreticulin (Band 7, Table 3.1) was also found in haemolymph from all larval treatments and was bound to the surface of LPS although levels did not vary significantly (Fig. 3.2). It has been reported that calreticulin partially localises on the surface of neutrophils, and anti-microbial bound calreticulin transmits a signal into cells via a G-protein to activate neutrophils that subsequently could generate superoxide anions (Yoshida $et\ al$., 1996).

The protein arylphorin (Band 5, Table 3.1) showed a significant presence in haemolymph from the 37$^{\text{o}}$C incubated (118k± 21.1k, $p<0.001$) and physically stressed larvae (98k±15.1k, $p<0.001$) compared to the control (46k±7.7k). Arylphorin contains a region of 20 amino acids within the protein that have been suggested to function in the insect antimicrobial response and has sequence homology with the N-terminus of gallysin-1.

The binding of HSP 70 (Band 4, Table 3.1) was found to steadily increase in haemolymph from larvae incubated at 30$^{\text{o}}$C (101k±9.0k), 37$^{\text{o}}$C (103k±20.1k) and physically stressed (118k±9.0k, $p<0.01$) when compared to the control larvae (83k±5.9k). This was not observed with HSP 90 (Band 2, Table 3.1) which showed a decrease in haemolymph from the 30$^{\text{o}}$C incubated larvae (58k±8.1k) but remained relatively unchanged in the 37$^{\text{o}}$C and physically stressed larvae (Fig. 3.2). Heat shock

proteins as HSP 70 have been suggested to be a damage associated molecular pattern (DAMP) protein activating Toll like receptors (TLR) 2 and 4 (Asea *et al.*, 2002). The main function of HSP70 is in binding to newly synthesised proteins and subsequently linking to HSP90 which stabilises the peptide in heat stress treatments (Wojda and Jakubowicz, 2007). It has been suggested that heat-shock stress may induce cross communication between the signalling pathways activated by increased temperature and immunogens (Wojda and Jakubowicz, 2007).

3.3 Binding assessment of thermally and physically stressed *G.mellonella* extracted haemolymph to β-1, 3-Glucan

β–glucan is a major component of fungal cell walls and it was observed that β-glucan had a number of *G.mellonella* haemolymph proteins that bound to it (Fig. 3.3). An increased binding of hexamerin (Band 1, Table 3.2), HSP 70 (Band 4, Table 3.2) and prophenoloxidase subunit 2 (Band 5, Table 3.2) to β-glucan was recorded in haemolymph from larvae incubated at 37°C or physically stressed (Fig. 3.4). There was a high level of binding of proPO subunit 2 to β-glucan in haemolymph from larvae incubated at 37°C ($84k\pm10.1k$, $p<0.001$), physically stressed ($94k\pm6.9k$, $p<0.01$) or incubated at 30°C ($68k\pm7.6k$, $p<0.05$). Binding of proPO subunit 2 to β-glucan was not detected in haemolymph from larvae incubated at 15°C.

HSP 70 (Band 4, Table 3.2) showed elevated binding to β-glucan in haemolymph from larvae incubated at 30°C ($38k\pm3.3k$, $p<0.01$), physically stressed ($61k\pm10.0k$, $p<0.001$) and 37°C ($49k\pm11.2k$, $p<0.001$). β-glucan bound arylphorin (Band 4) demonstrated significantly higher binding levels in haemolymph from larvae incubated at 30°C ($183k\pm46.4k$ $p<0.001$) when compared to the control ($73k\pm8.0k$) but no bound arylphorin was detectable in the larvae incubated at 37°C or physically stressed. Insect GAPDH (Band 9, Table 3.2), demonstrated very little change in abundance between haemolymph samples incubated at different temperatures or physically stressed. GAPDH has been suggested to sequentially phosphorylate signalling cascades which includes serine proteases (Sirover, 2011).
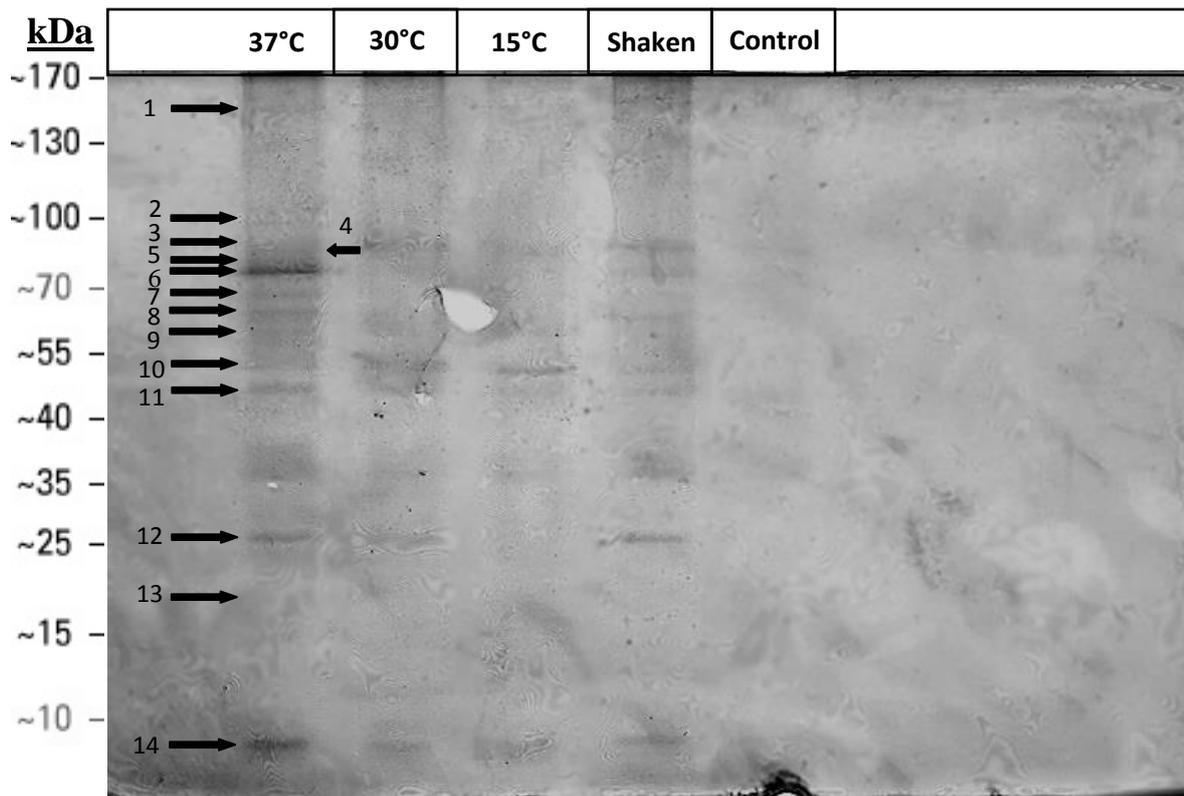
Fig. 3.1 1-Dimensional gel of separated *G.mellonella* larval haemolymph proteins bound to LPS.

Lanes loaded with 20µl of haemolymph proteins separated from the microbial cell constituent LPS by denaturing bound proteins. Samples were loaded from left to right 37°C, 30°C, 15°C, physically stressed larvae and negative control (IPS and LPS), replicates (n=3). Proteins bands were cut and identified by LC/MS and identities numbered on the gel and listed in Table. 3.1.

Band no.	Protein Identity	Organism	Mr	Score	Seq. coverage (%)	Accession no.	Protein function
1	Hexamerin	*G. mellonella*	81807	238	9	AAA19801	Amino acid storage and immune functions.
2*	Heat shock protein 90	*Loxostege sticticalis*	83004	226	8	ABW87791	Chaperone protein involved in protein folding and heat stress stability.
3	CG9319 (CoA-transferases)	*D. melanogaster*	41992	68	2	NP_610054	Catalyse reversible transfer reactions of coenzyme A groups from CoA-thioesters to free acids.
4*	Heat shock 70 kDa protein cognate precursor	*B. mori*	73168	127	4	NP_001036 837	Chaperone protein involved in protein folding and heat stress stability.
5*	Arylphorin	*G. mellonella*	83651	136	6	AAA74229	Amino acid storage and immune functions.
6	Prophenoloxidase subunit 2	*G. mellonella*	80198	110	2	AAQ75026	Component of melanisation cascade.
7*	Calreticulin	*G. mellonella*	46343	62	4	BAB79277	Binding protein for anti-microbial peptides.
8*	Prophenoloxidase subunit 2	*G. mellonella*	80198	407	16	AAQ75026	Component of melanisation cascade.
9	Beta-tubulin	*B. mori*	50590	453	21	BAA32102	Component of microtubules of the cytoskeleton.
10	Hypothetical protein KGM_17939 (Gelsolin)	*Danaus plexippus*	35099	76	4	EHJ66212	Either Ca2+ or actin binding regulate actin assembly.
11	Actin	*Haemaphysalis longicornis*	42204	576	36	AAP81255	Nucleotide-Binding Domain of the sugar kinase/ HSP70/actin superfamily.
12*	Masquerade-like serine proteinase	*Pieris rapae*	46063	234	9	ACZ68116	Enzymes that cleave key components of the melanisation cascade.

13*	Peptidoglycan recognition-like protein B	*G. mellonella*	16458	144	29	AAN15786	Pattern recognition protein for bacterial cell wall component peptidoglycan.
14*	Apolipophorin-III	*G. mellonella*	20499	170	20	P80703	PRR of microbial components.

Table 3.1 Proteins identified by LC/MS from thermally and physically stressed *G.mellonella* larvae bound to LPS.

Proteins 1-14 excised and identified by LC/MS. * representing protein with fold change represented in Fig. 3.2.
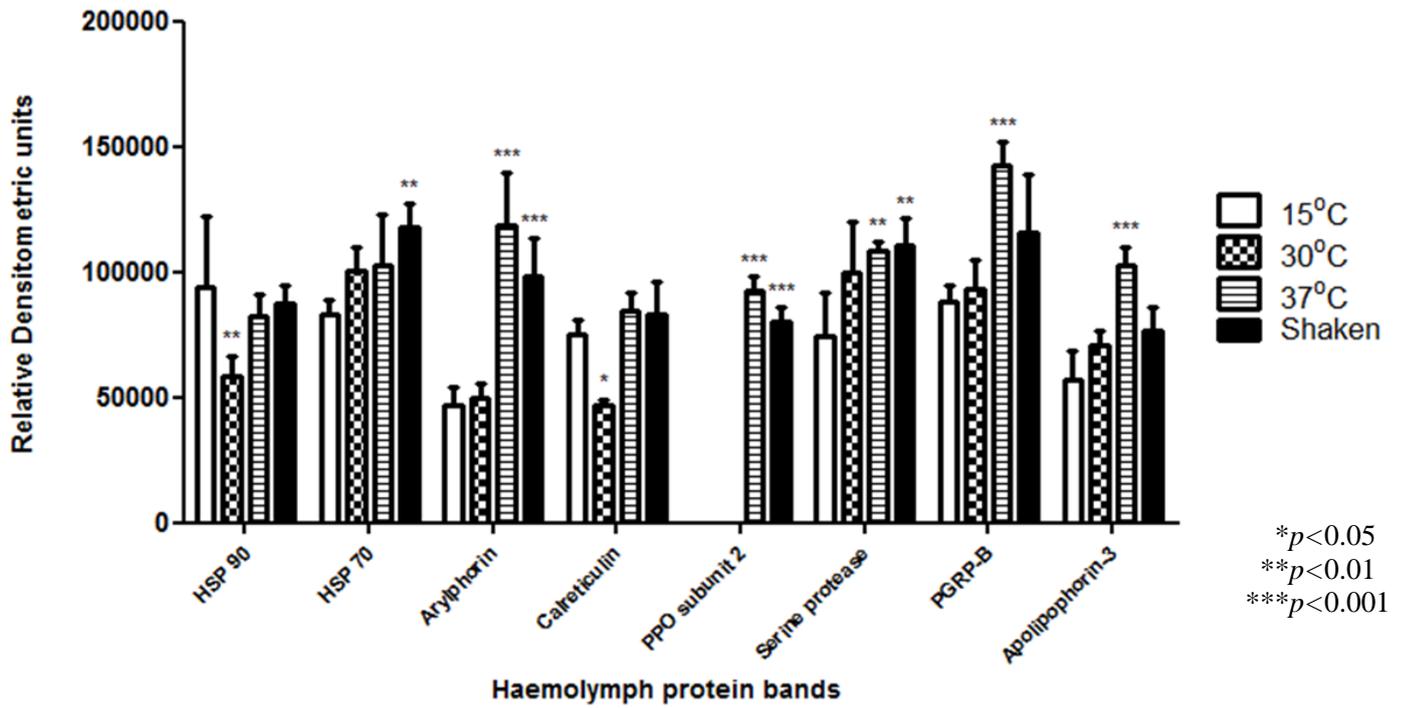
Fig. 3.2 Bound haemolymph proteins to LPS, identified by LC/MS showing the relative abundance of each protein from larvae exposed to different thermal or physical treatment.
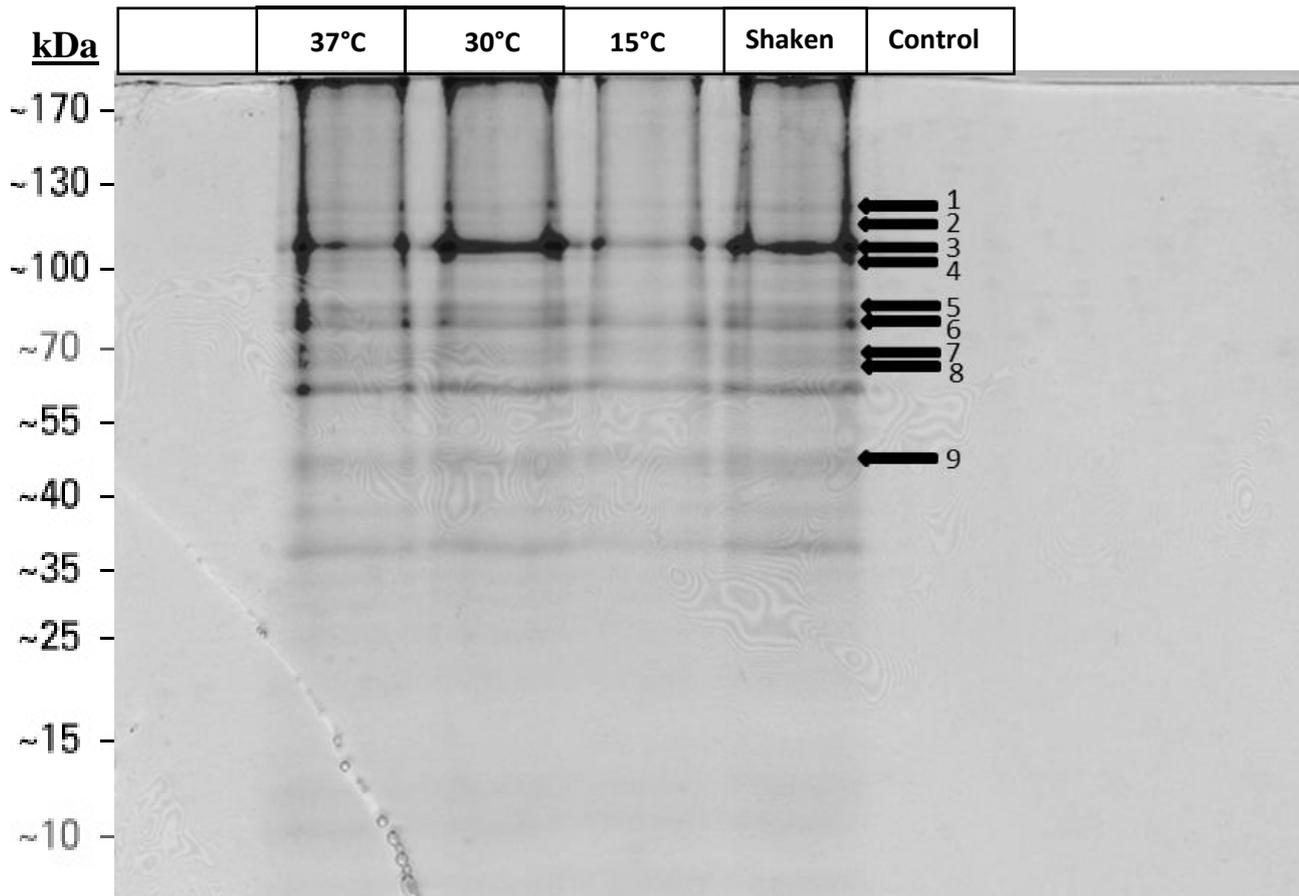
Fig. 3.3 1-Dimensional gel of separated *G.mellonella* larval haemolymph proteins bound to β-1, 3-Glucan.

Lanes loaded with 20µl of haemolymph proteins separated from the microbial cell constituent β-1, 3-Glucan by denaturing bound proteins. Samples were loaded from left to right 37°C, 30°C, 15°C, physically stressed larvae and negative control (IPS and β-1, 3-Glucan), replicates (n=3). Proteins bands were cut and identified by LC/MS and identities numbered on the gel and listed in Table. 3.2.

Band no.	Protein Identity	Organism	Mr	Score	Seq. coverage (%)	Accession no.	Protein function
1*	Hexamerin	*G. mellonella*	81807	232	6	AAA19801	Amino acid storage and immune functions.
2	Heat shock protein 90	*Pteromalus puparum*	82515	237	6	ACO57617	Chaperone protein involved in protein folding and heat stress stability.
3*	Arylphorin	*G. mellonella*	83651	324	9	AAA74229	Amino acid storage and immune functions.
4*	Heat shock protein 70	*Trichinella britovi*	71867	217	5	CAA73574	Role in maintaining unfolded precursor proteins in the cytosol and in transport.
5*	Prophenoloxidase subunit 2	*G. mellonella*	80198	202	7	AAQ75026	Component of melanisation cascade.
6	Tubulin beta-1 chain	*M. sexta*	50662	455	16	O17449	Component of microtubules of the cytoskeleton.
7	GL25088 (ATPase)	*Drosophila persimilis*	71632	193	5	XP_002020970	Synthesises ATP from ADP.
8	Actin-related protein 66B	*D. melanogaster*	47467	69	2	NP_523968	Nucleotide-Binding Domain of the sugar kinase/HSP70/actin superfamily.
9*	Glyceraldehyde-3-phosphate dehydrogenase	*B. mori*	35522	128	6	BAE96011	Enzyme of the glycolysis pathway.

Table 3.2 Proteins identified by LC/MS from thermally and physically stressed *G.mellonella* larvae bound to β-1, 3-Glucan.
Proteins 1-9 excised and identified by LC/MS. * representing protein with fold change represented in Fig. 3.4.
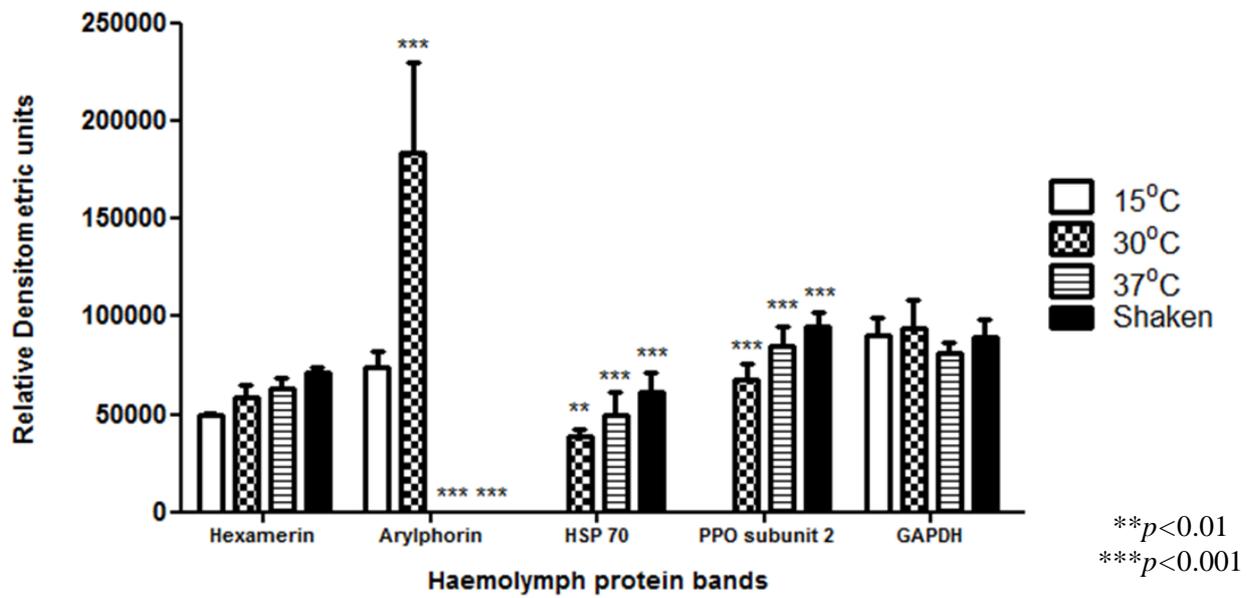
Fig. 3.4 Bound haemolymph proteins to β-1, 3-Glucan, identified by LC/MS showing the relative abundance of each protein from larvae exposed to different thermal or physical treatment.

3.4a Assessment of binding ability of thermally and physically stressed larval haemolymph proteins to non-germinated *A.fumigatus* conidia

A. fumigatus is a fungal pathogen found within the environment and it accounts for a number of life threatening infections that affect immunocompromised individuals (Vartivarian *et al*., 1993; Walsh and Groll, 1999). The key role of the innate immune system is to identify and elicit adequate immune responses against this pathogen to eliminate or inhibit its progression. For this reason it is an important pathogen to understand and can be studied by using the *G. mellonella* infection model (Fallon *et al*., 2012).

A number of proteins bound to the conidial surface of *A.fumigatus* (Fig. 3.5) (Table 3.3). Of interest were arylphorin, (Band 2, Table 3.3) prophenoloxidase subunit 2 (Band 4, Table 3.3), beta-1, 3-glucan recognition protein precursor (βGBP) (Band 5, Table 3.3) and apoLp-III (Band 10, Table 3.3) which showed substantial changes in binding (Fig. 3.6). Arylphorin (Band 2, Table 3.3) showed an increased binding from haemolymph of larvae pre-incubated 37^oC (56k±6.6k), physically stressed (53k±6.6k) and incubated at 30^oC (48k±4.5k) when compared to the 15^oC incubated larvae (43k±7.5k). ProPO subunit 2 (Band 4, Table 3.3) demonstrated a greater binding to conidia among the 37^oC pre-incubated larvae (90k±11.2k), physically stressed (76k±16.0k) and the 30^oC incubated (81k±14.7k) larvae when compared to the 15^oC incubated larvae (70k± 12.0k). βGBP (Band 5, Table 3.3) is an important pattern recognition receptor for fungal cell wall component β-1, 3-glucan and the stimulation of phagocytosis (Johansson and Soderhall, 1996). Here βGBP demonstrated a higher binding affinity to conidia among the 37^oC pre-incubated larvae (105k± 11.8k), physically stressed (101k±14.1k) and the 30^oC incubated (91k± 15.6k) larvae when compared to the 15^oC incubated larvae (86k±16.5k).

ApoLp-III (Band 10, Table 3.3) had significantly higher amounts bound to *A.fumigatus* conidia in haemolymph from the physically stressed larvae (183k±36.4k, *p<0.001*) while the 30^oC (171k±37.3k) incubated larvae had a similar increase but this was not significant, unlike the 37^oC incubated larvae (159k±39.3k) which had less apoLp-III bound than the previous treatments but more than the 15^oC (135k±22.7k). ApoLp-III has important functions in pattern recognition of cell surfaces such as β-glucan in fungal cell walls (Whitten *et al*., 2004) as *A.fumigatus*. It also influences
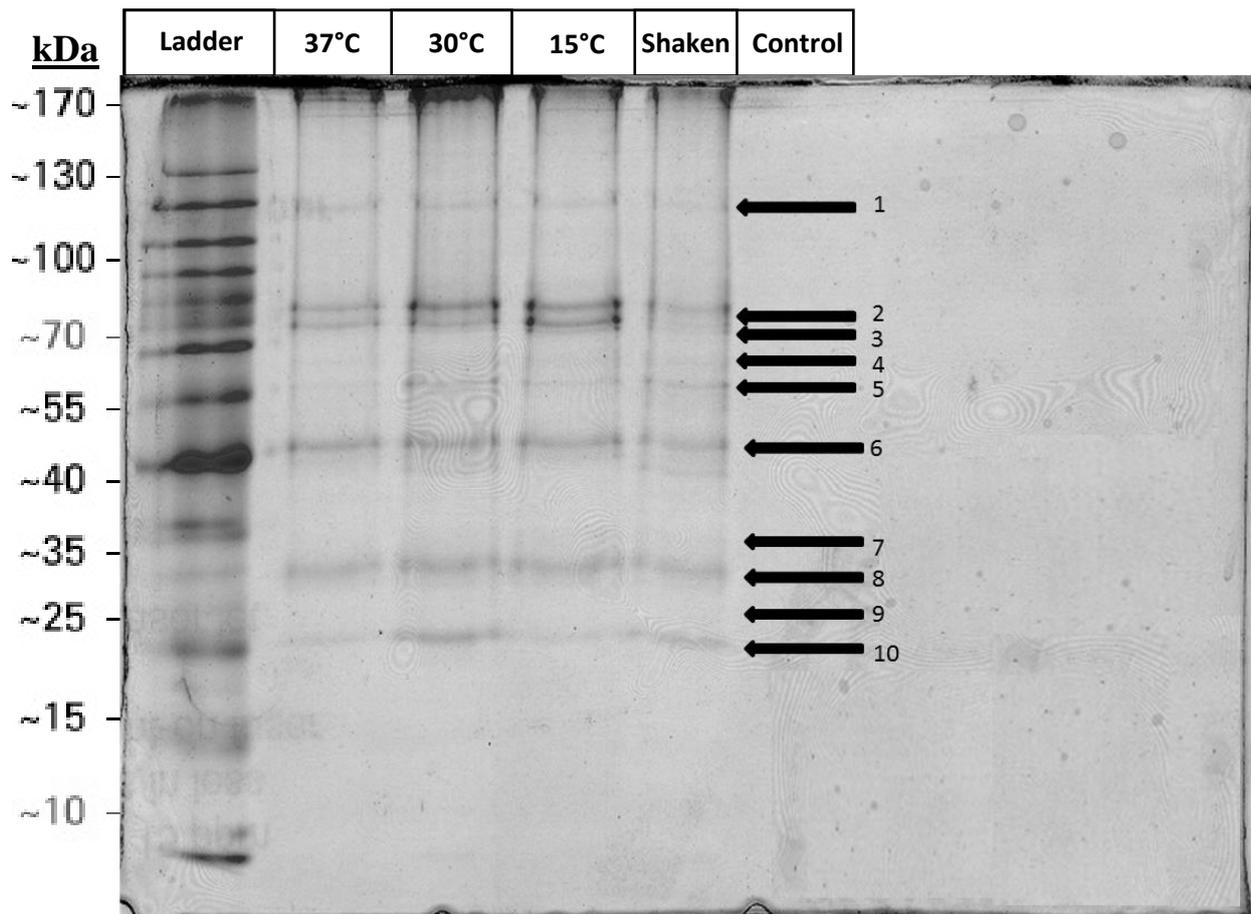
Fig. 3.5 1-Dimensional gel of separated *G.mellonella* larval haemolymph proteins bound to *A.fumigatus* conidia.

Lanes loaded with 20μl of haemolymph proteins separated from the fungal cell surface of *A.fumigatus* conidia by denaturing bound proteins. Samples were loaded from left to right 37°C, 30°C, 15°C, physically stressed larvae and negative control (IPS and non-germinated conidia), replicates (n=3). Proteins bands were cut and identified by LC/MS and identities numbered on the gel and listed in Table 3.3.

Band no.	Protein Identity	Organism	Mr	Score	Seq. coverage (%)	Accession no.	Protein function
1*	Hexamerin	*G. mellonella*	81807	400	13	AAA19801	Amino acid storage and immune functions.
2*	Arylphorin	*G. mellonella*	83651	87	2	AAA74229	Amino acid storage and immune functions.
3	Arylphorin	*G. mellonella*	83651	960	29	AAA74229	Amino acid storage and immune functions.
4*	Prophenoloxidase subunit 2	*G. mellonella*	80198	95	4	AAQ75026	Component of melanisation cascade.
5*	Beta-1,3-glucan recognition protein precursor	*G. mellonella*	55882	161	6	CAK22401	PRR for the fungal cell wall component β-1,3-glucan.
6*	Masquerade-like serine proteinase	*Pieris rapae*	46063	234	9	ACZ68116	Enzymes that cleave key components of the melanisation cascade.
7*	Heat shock protein 70	*Trichinella britovi*	71867	217	5	CAA73574	Role in maintaining unfolded precursor proteins in the cytosol and in transport.
8	NADH dehydrogenase subunit 2	*Orthetrum melania*	26549	84	12	BAD32642	Catalyzes the transfer of electrons from NADH to coenzyme Q.
9	Masquerade-like serine proteinase	*Pieris rapae* (cabbage white)	46063	113	5	ACZ68116	Enzymes that cleave key components of the melanisation cascade.
10*	Apolipophorin III	*G. mellonella*	20499	176	17	P80703	PRR of microbial components.

Table 3.3 Proteins identified by LC/MS from thermally and physically stressed *G.mellonella* larvae bound to *A.fumigatus* conidia.
Proteins 1-10 excised and identified by LC/MS. * representing protein with fold change represented in Fig. 3.6.
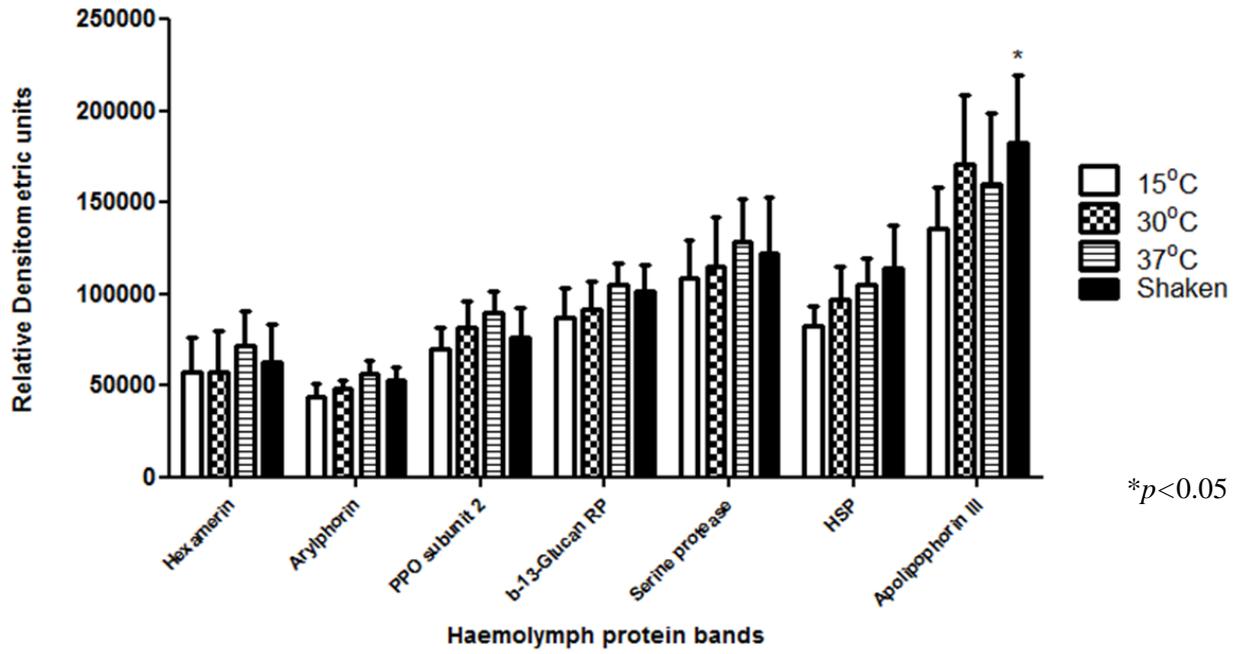
Fig. 3.6 Bound haemolymph proteins to *A.fumigatus* conidia, identified by LC/MS showing the relative abundance of each protein from larvae exposed to different thermal or physical treatment.

haemocytes spreading, recognition of foreign components and elimination of pathogens (Whitten *et al.*, 2004; Zakarian *et al.*, 2002).

Masquerade-like serine proteinase which is crucial to the activation of the proPO cascade and HSP 70 (Band 7, Table 3.3) which is important for regulation of protein structure, both proteins showed an increased abundance in haemolymph from larvae incubated at 30°C, 37°C and physically stressed when compared to the control (Fig. 3.6).

3.4b Assessment of binding ability of thermally and physically stressed larval haemolymph proteins to germinated *A.fumigatus* conidia

A.fumigatus conidia were germinated in AMM medium and incubated at 37°C for 7 h (Fig. 3.7) prior to use in the binding assay as previously described (Renwick *et al.*, 2006) (section 2.4.8). A number of proteins from haemolymph demonstrated binding to germinated *A.fumigatus* conidia (Fig. 3.8) with arylphorin (Band 3, Table 3.4) demonstrating a greater binding to the germinated *A.fumigatus* conidia particularly among the 37°C pre-incubated larvae (725k±55.5k, *p*<0.001) and the physically stressed larvae (371k±106.7k, *p*<0.05) compared to the 30°C (321k±57.6k) and the 15°C (267k±29.5k) control larvae (Fig. 3.9).

The binding of βGBP (Band 5, Table 3.4) was observed at its highest to germinated conidia from haemolymph of the 37°C incubated larvae (410k±125.0k, *p*<0.001) whereas the binding from physically stressed (169k±36.1k), 30°C (138k±41.1k) and control (83k± 1.2k) larvae was more constant. Apolipophorin (Band 1, Table 3.4) which recognises and binds to β-glucan demonstrated relatively similar amounts of binding overall in the germinated conidia when compared to the non-germinated conidia, with haemolymph of the 37°C and physically stressed larvae showing the highest binding at 131k±14.4k and 126k±20.6k respectively, compared to the control larvae (78k±4.5k). Hexamerin (Band 2, Table 3.4) binding was increased in haemolymph from the 30°C (90k±23.6k) and 37°C incubated larvae (92k±15.8k) but was greatest in the larvae physically stressed (115k±58.5k) compared to the control larvae (63k±3.1k).

Prophenoloxidase (Band 7, Table 3.4), serine proteinase-like protein 4 (Band 8, Table 3.4), masquerade-like serine proteinase (Band 10, Table 3.4) and Juvenile

87
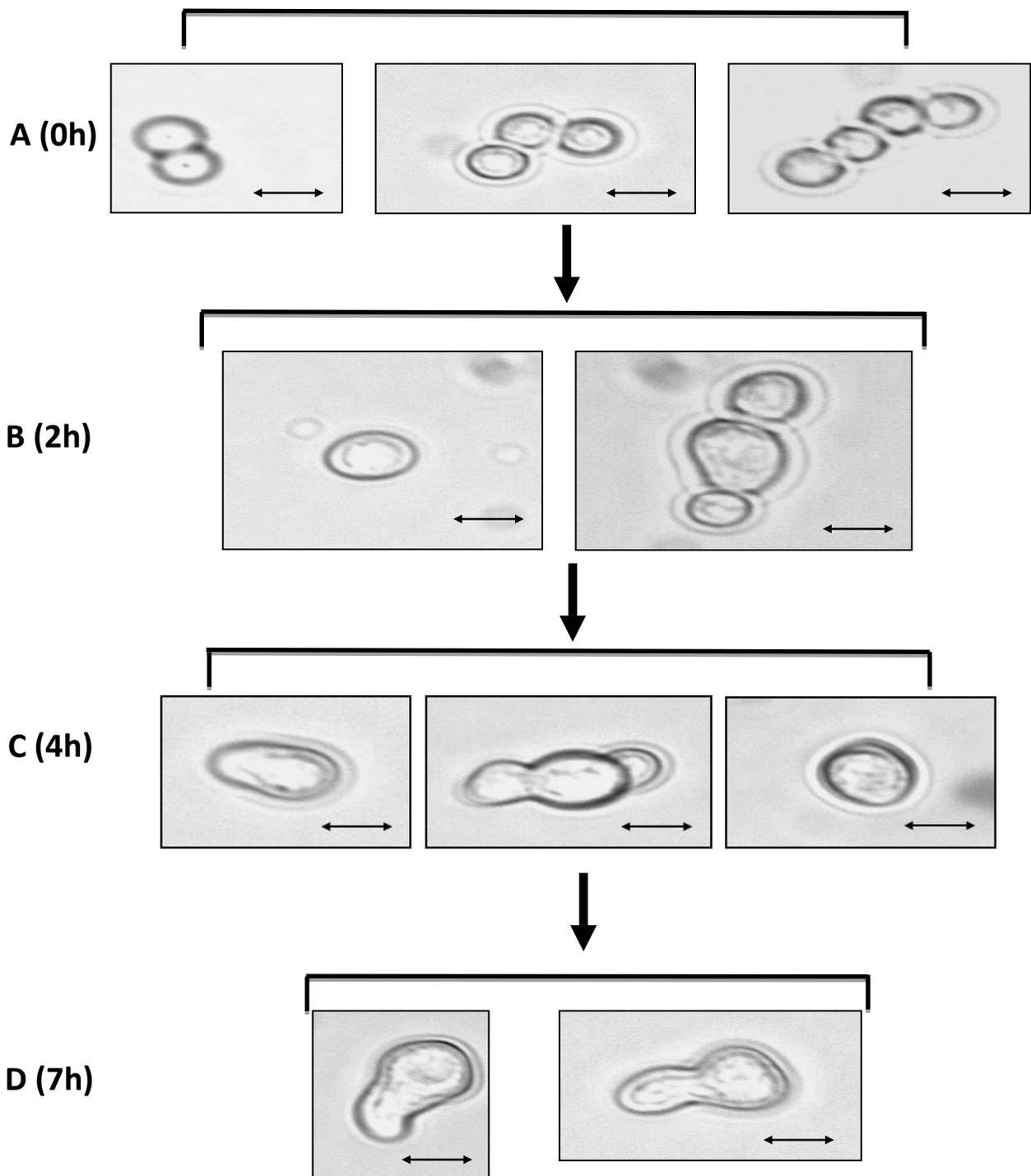
Fig. 3.7 Different stages of *A. fumigatus* conidia activation and germination.

A. fumigatus conidia ($1x10^{7}$/ml) were added to minimal essential media (MEM) supplemented with 5% feotal calf serum (FCS) and cultured at 200rpm at $37^{°}$C. Germination phases 0, 2, 4, and 7h. Image taken from (Renwick *et al*., 2006) The double ended arrow represents 3µm.
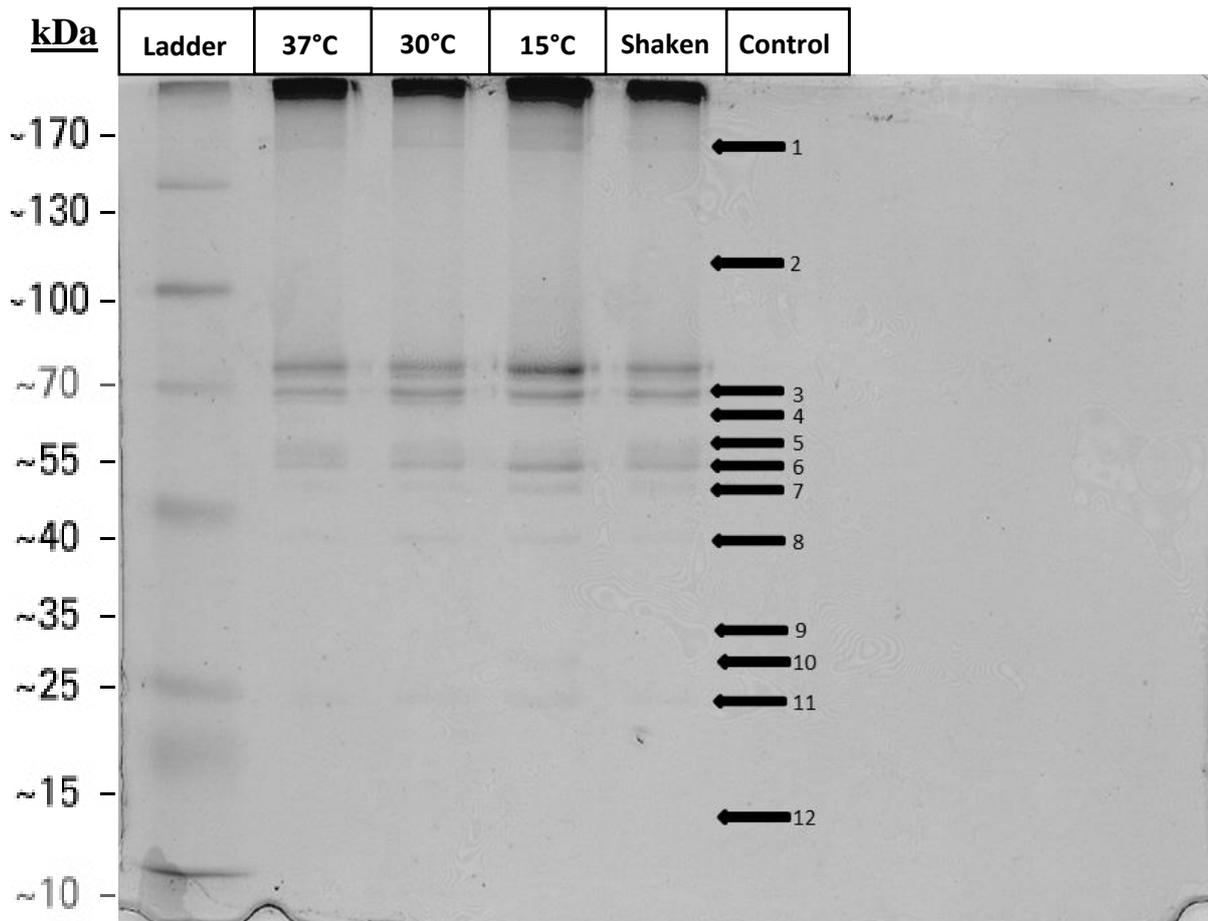
Fig.3.8 1-Dimensional gel of separated *G.mellonella* larval haemolymph proteins bound to germinated *A.fumigatus* conidia.

Lanes loaded with 20μl of haemolymph proteins separated from the fungal cell surface of germinated *A.fumigatus* conidia by denaturing bound proteins. Samples were loaded from left to right 37°C, 30°C, 15°C, physically stressed larvae and negative control (IPS and germinated *A.fumigatus* conidia), replicates (n=3). Proteins bands were cut and identified by LC/MS and identities numbered on the gel and listed in Table 3.4.

Band no.	Protein Identity	Organism	Mr	Score	Seq. coverage (%)	Accession no.	Protein function
1*	Apolipophorin	*G. mellonella*	168330	253	3	AAT76806	Precursor of apolipophorin-III PRR of microbial components.
2*	Hexamerin	*G. mellonella*	81807	400	13	AAA19801	Amino acid storage and immune functions.
3*	Arylphorin	*G. mellonella*	83651	960	29	AAA74229	Amino acid storage and immune functions.
4	Prophenoloxidase	*G. mellonella*	79076	280	12	AAK64363	Component of melanisation cascade.
5*	Beta-1,3-glucan recognition protein precursor	*G. mellonella*	55882	500	20	CAK22401	PRR for the fungal cell wall component β-1,3-glucan
6	Prophenoloxidase subunit 2	*G. mellonella*	80198	520	17	AAQ75026	Component of melanisation cascade.
7*	Prophenoloxidase	*G. mellonella*	79076	410	14	AAK64363	Component of melanisation cascade.
8*	Serine proteinase-like protein 4	*M. sexta*	44050	67	3	AAV91027	Enzymes that cleave key components of the melanisation cascade.
9	NADH dehydrogenase subunit 2	*Orthetrum melania*	26549	84	12	BAD32642	Catalyzes the transfer of electrons from NADH to coenzyme Q.
10*	Masquerade-like serine proteinase	*Pieris rapae* (cabbage white)	46063	113	5	ACZ68116	Enzymes that cleave key components of the melanisation cascade.
11*	Juvenile hormone binding protein precursor	*G. mellonella*	27460	123	16	AAS94224	Precursor of JHBP, regulates embryogenesis and reproduction; JHBP protects JH molecules from hydrolysis by non-specific esterases.
12	Actin 2	*Nilaparvata lugens*	42184	91	8	ABY48096	Nucleotide-Binding Domain of the sugar kinase/HSP70/actin superfamily.

Table 3.4 Proteins identified by LC/MS from thermally and physically stressed *G.mellonella* larvae bound to germinated *A.fumigatus* conidia.
Proteins 1-12 excised and identified by LC/MS. * representing protein with fold change represented in Fig. 3.9.
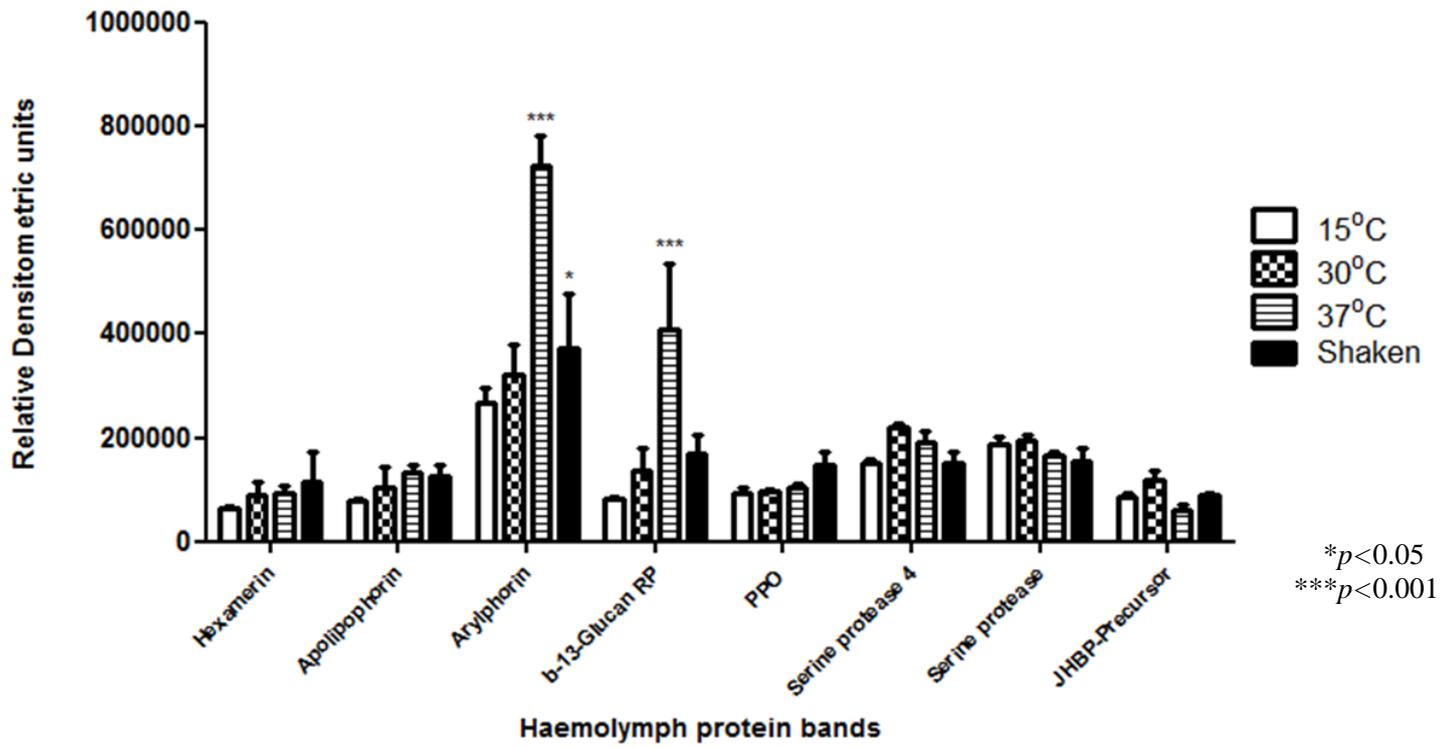
Fig. 3.9 Bound haemolymph proteins to germinated *A.fumigatus* conidia, identified by LC/MS showing the relative abundance of each protein from larvae exposed to different thermal or physical treatment.

hormone binding protein precursor (JHBP-precursor) (Band 11, Table 3.4) demonstrated small changes in binding to germinated conidia but remained relatively constant between the different treatments (Fig. 3.9). JHBP-precursor may interact with proteins that can bind to haemocyte cells and/microbes to enable suppression of haemocyte function as juvenile hormone has previously been implicated in immunosuppression (Hiruma and Riddiford, 1993).

3.5 Summary

The examination of *G.mellonella* larval haemolymph proteins binding to LPS demonstrated a number of known bacterial binding proteins. Haemolymph proteins from larvae also demonstrated binding ability to β-1, 3-Glucan although not as wide a range of bound proteins was observed as it was for the LPS binding assessment. The abundance of haemolymph proteins bound to either LPS or β-1, 3-Glucan was highest from thermally and physically stressed larval haemolymph. The binding of *G.mellonella* haemolymph proteins to *A.fumigatus* conidia demonstrated a difference in abundance with the 37°C incubated and the physically stressed larvae showing the largest amount of proteins bound. These proteins included PRRs βGBP, apoLp-III and proteins associated with antimicrobial signalling cascades proPO with the activation enzymes serine proteases.

It was also observed that the germinated conidia had a higher abundance of bound arylphorin and βGBP (Fig. 3.9) which were increased up to four times more than the non-germinated conidia (Fig. 3.6). These proteins were bound to germinated conidia in the highest concentration in haemolymph from the 37°C and physically stressed larvae. The germination of these conidia may expose β-1, 3-Glucan and other surfaces, therefore resulting in higher concentrations of haemolymph proteins binding particularly among the physically and thermally stressed larvae (Fig. 3.7).

3.6 Assessment of the binding ability of haemolymph proteins from thermally and physically stressed *G.mellonella* larvae to *C.albicans*

C. albicans cells (Fig. 1.9B) were washed and exposed to the haemolymph of larvae incubated at different temperatures and or physically stressed. It was observed that the PRR βGBP (Band 3, Table 3.5) was bound in high amounts in the haemolymph of the 37°C (143k± 49.8k) and 15°C control larvae (138k±42.7k) but was undetectable for the 30°C and physically stressed larvae (Fig. 3.11). This was also observed for the binding of proPO (Band 5, Table 3.5) to *C.albicans* from haemolymph of the 37°C (51k±10.0k) and 15°C treated larvae (59k±4.5k), and was at an undetectable level or absent in the 30°C and physically stressed larvae. Johansson and Soderhall, (1996) suggested βGBP is required for the subsequent binding of proPO and may explain the absence of binding protein (βGBP) potentially preventing the binding of other proteins (proPO) to *C.albicans* cell surfaces.

Arylphorin (Band 2, Table 3.5) remained relatively unchanged in abundance between the different treated larvae (Fig. 3.11) while hexamerin (Band 1, Table 3.5) demonstrated an increase in binding in the 15°C (69k±5.9k) incubated larvae when compared to the 30°C (59±13.5k), 37°C (58±6.4k) and the physically stressed (48k±0.6k) larvae.

3.7 Assessment of the binding ability of haemolymph proteins from thermally and physically stressed *G.mellonella* larvae to *S.aureus*

S.aureus is a common bacteria capable of causing infections in humans and capable of developing resistance to antibiotics (Heijer *et al*., 2013). *G.mellonella* larvae are a very useful tool to screen the pathogenicity of *S.aureus* strains and to assess the effectiveness of developmental or current antimicrobial drugs or compounds. The protein arlyphorin (Band 4, Table 3.6) was observed bound to *S.aureus* potentially through its proposed Gallysin-1 region (Phipps *et al*., 1994) (Fig. 3.13). Arylphorin is suggested to have cytotoxic activity that is similar to that observed among mammals and is triggered in the presence of LPS and *P. aeruginosa* vaccine (Beresford *et al*., 1997).
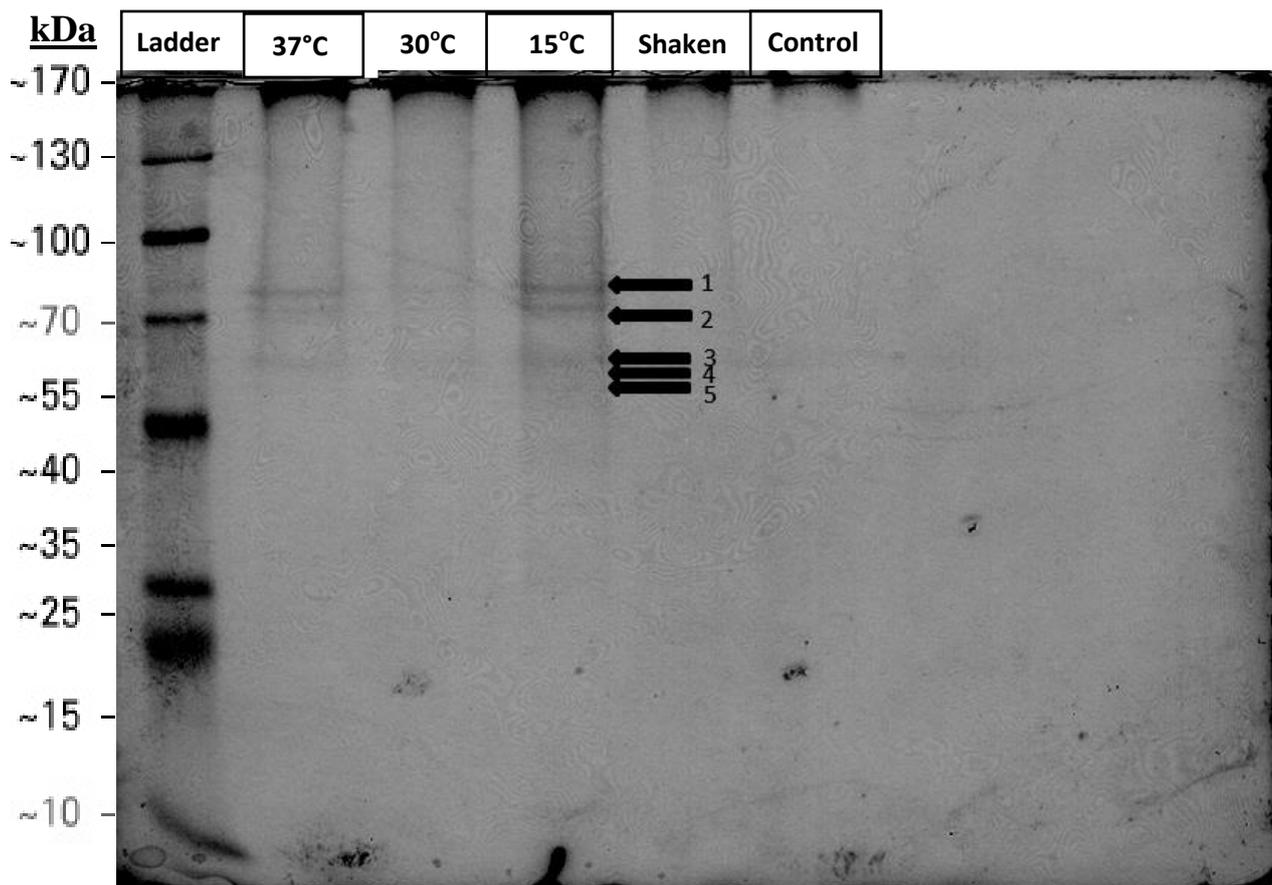
Fig. 3.10 1-Dimensional gel of separated *G.mellonella* larval haemolymph proteins bound to *C.albicans*.

Lanes loaded with 20μl of haemolymph proteins separated from the surface of *C.albicans* by denaturing bound proteins. Samples were loaded from left to right 37°C, 30°C, 15°C, physically stressed larvae and negative control (IPS and *C.albicans*), replicates (n=3). Proteins bands were cut and identified by LC/MS and identities numbered on the gel and listed in Table 3.5.

Band no.	Protein Identity	Organism	Mr	Score	Seq. coverage (%)	Accession no.	Protein function
1*	Hexamerin	*G. mellonella*	81807	392	14	AAA19801	Amino acid storage and immune functions.
2*	Arylphorin	*G. mellonella*	83651	788	31	AAA74229	Amino acid storage and immune functions.
3*	Beta-1,3-glucan recognition protein precursor	*G. mellonella*	55882	262	12	CAK22401	PRR for the fungal cell wall component β-1,3-glucan.
4	Prophenoloxidase subunit 2	*G. mellonella*	80198	233	8	AAQ75026	Component of melanisation cascade.
5*	Prophenoloxidase	*G. mellonella*	79076	157	9	AAK64363	Component of melanisation cascade.

Table 3.5 Proteins identified by LC/MS from thermally and physically stressed *G.mellonella* larvae bound to *C.albicans*.
Proteins 1-5 excised and identified by LC/MS. * representing protein with fold change represented in Fig. 3.11.
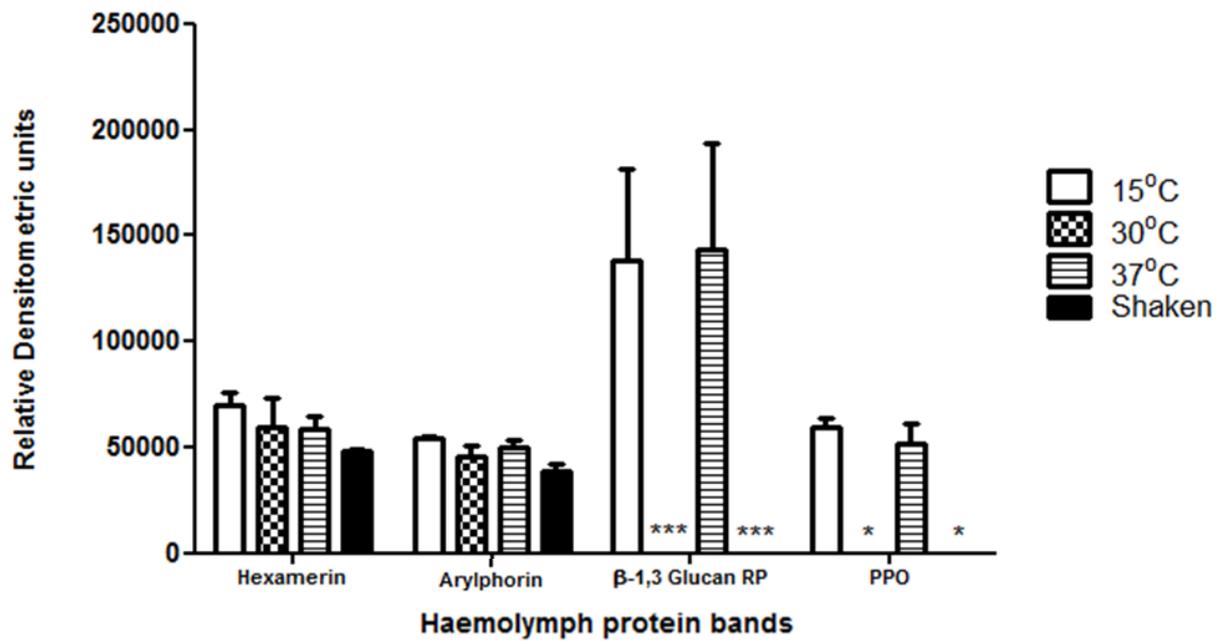
Fig. 3.11 Bound haemolymph proteins to *C.albicans*, identified by LC/MS showing the relative abundance of each protein from larvae exposed to different thermal or physical treatment.

Arylphorin was found bound in the highest abundance from haemolymph of the 37^oC ($98k\pm8.6k$) and the physically stressed ($107k\pm7.3k$) larvae but was present in lower amounts among the control larvae ($73k\pm 0.9k$) and was absent in haemolymph from larvae incubated at 30^oC. The binding of calreticulin (Band 6, Table 3.6) was higher from haemolymph of the 30^oC incubated larvae and the physically stressed larvae which showed $89k\pm23.1k$ and $87k\pm19.6k$ densitometric units, respectively, but binding was reduced in the 15^oC ($73k\pm0.9k$) and the 37^oC ($49k\pm17.7k$) incubated larvae (Fig. 3.13). Calreticulin has an important role in activation of superoxide anions within neutrophils which function to destroy internalised pathogens of neutrophils (Cho *et al.*, 1999) and similarly could function in *G.mellonella* larvae's highly conserved granular cells.

ProPO (Band 7, Table 3.6) showed the highest binding to *S.aureus* from the haemolymph of the 37^oC incubated larvae ($112k\pm101.7k$) when compared to the other larval treatments. ApoLp-III (Band 12, Table 3.6) demonstrated the greatest binding to *S.aureus* within the haemolymph of the physically stressed larvae ($202k\pm54.9k$, $p<0.05$), 37^oC ($175k\pm83.9k$) incubated larvae and to a lower extent in the 30^oC ($148k\pm70.8k$) incubated larvae when compared the control larvae ($70k\pm34.3k$). GAPDH (Band 10, Table. 3.6) also had increased binding or association to other proteins bound to *S.aureus* from haemolymph of the 30^oC ($81k\pm36.9k$), 37^oC ($98k\pm41.8k$) and physically stressed ($106k\pm28.2k$) larvae in comparison to that from the control larvae ($34\pm13.5k$).
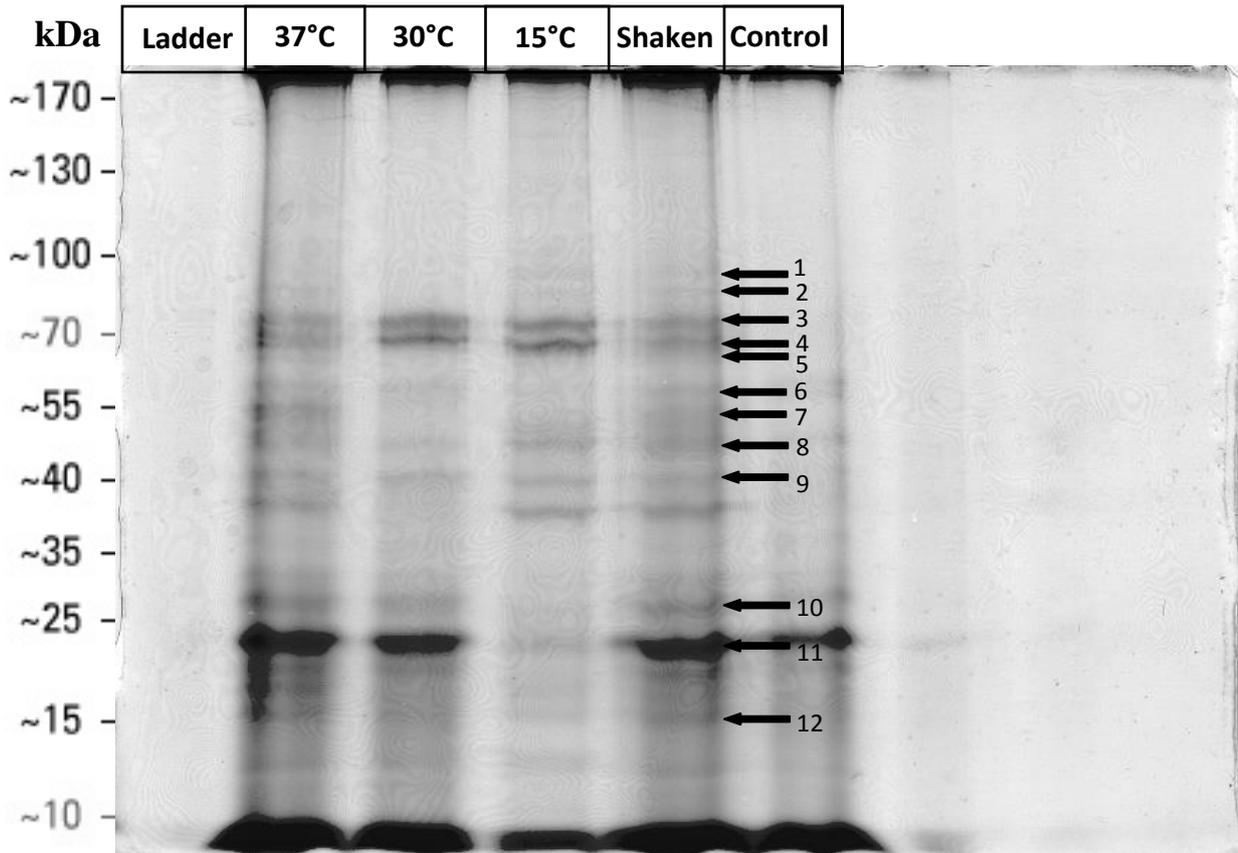
Fig. 3.12 1-Dimensional gel of separated *G.mellonella* larval haemolymph proteins bound to *S.aureus*.

Lanes loaded with 20µl of haemolymph proteins separated from the bacterial cell surface of *S.aureus* by denaturing bound proteins. Samples were loaded from left to right 37°C, 30°C, 15°C, physically stressed larvae and negative control (IPS and *S.aureus*), replicates (n=3). Proteins bands were cut and identified by LC/MS and identities numbered on the gel and listed in Table 3.13.

Band no.	Protein Identity	Organism	Mr	Score	Seq. coverage (%)	Accession no.	Protein function
1	Hexamerin	*G. mellonella*	81807	247	6	AAA19801	Amino acid storage and immune functions.
2	Heat shock protein 90	*Spodoptera exigua*	82935	289	12	ACL77779	Chaperone protein involved in protein folding and heat stress stability.
3	Hexamerin	*G. mellonella*	81807	300	12	AAA19801	Amino acid storage and immune functions.
4*	Arylphorin	*G. mellonella*	83651	822	25	AAA74229	Amino acid storage and immune functions.
5	Prophenoloxidase subunit 2	*G. mellonella*	80198	331	10	AAQ75026	Component of melanisation cascade.
6*	Calreticulin	*G. mellonella*	46343	77	4	BAB79277	Binding protein for anti-microbial peptides.
7*	Prophenoloxidase	*G. mellonella*	79076	528	27	AAK64363	Component of melanisation cascade.
8	Beta-tubulin	*B. mori*	50590	777	35	BAA32102	Component of microtubules of the cytoskeleton.
9	Hypothetical protein CAEBREN_28975 (ATP synthase subunit beta)	*Caenorhabditis brenneri*	57524	235	9	EGT45626	Synthesises ATP from ADP.
10	Glyceraldehyde-3-phosphate dehydrogenase	*B. mori*	35522	141	11	BAE96011	Enzyme of the glycolysis pathway.
11	Putative annexin IX-C	*M. sexta*	36231	155	17	CAI06088	Annexin (*lipocortin*), suppress phospholipase A2. Functions in membrane scaffolds, trafficking and vesicles.
12*	Apolipophorin-III	*Hyphantria cunea*	20733	69	7	Q6VU70	PRR of microbial components.

Table 3.6 Proteins identified by LC/MS from thermally and physically stressed *G.mellonella* larvae bound to *S.aurues*.
Proteins 1-12 excised and identified by LC/MS. * representing protein with fold change represented in Fig. 3.13.
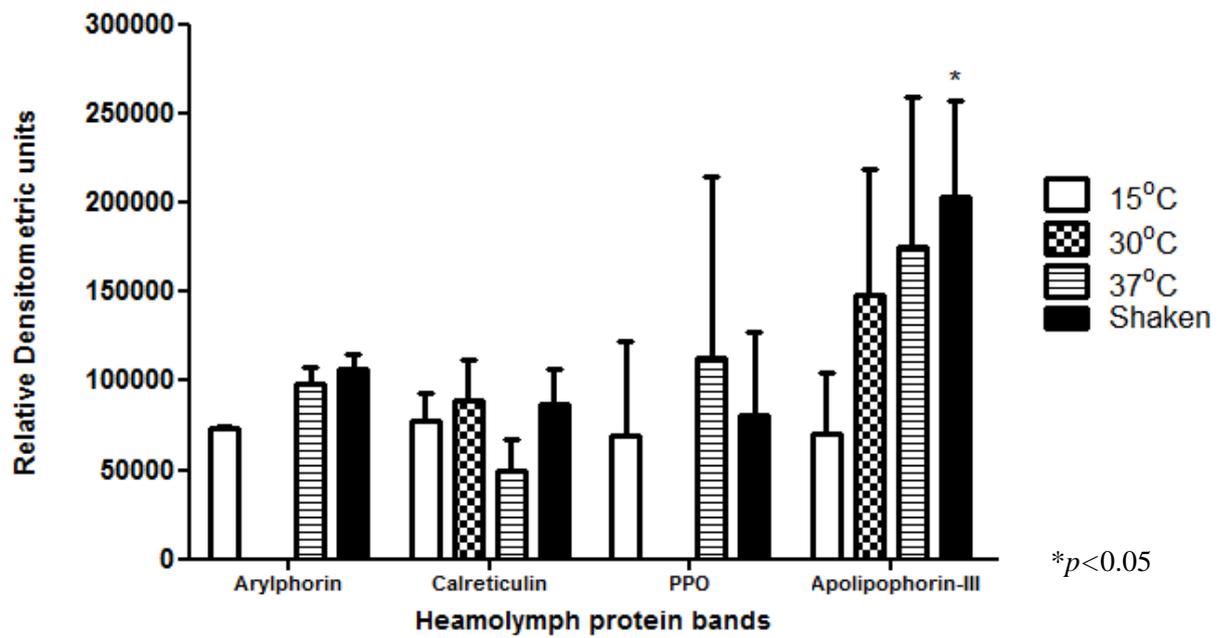
Fig. 3.13 Bound haemolymph proteins to *S.aureus*, identified by LC/MS showing the relative abundance of each protein from larvae exposed to different thermal or physical treatment.

3.8 Summary

It was observed that *G.mellonella* produce a wide range of pattern recognition receptors for fungal or bacterial recognition including βGBP, PRGP, calreticulin and apoLp-III, as well as activating antimicrobial defence pathways (proPO cascade) and other pathways induced by PRRs bound to microbial surfaces. The $37^{o}C$ incubated and the physically stressed larvae had significantly higher amounts of proteins bound to microbial surfaces and had a broader range of proteins bound compared to the $30^{o}C$ incubated larvae and the control larvae. The exception to this was within the binding assessment of haemolymph from thermally and physically stressed larvae to *C.albicans* surfaces which showed lower abundance of proteins bound from the $30^{o}C$ incubated treatments ($30^{o}C$ and physically stressed larvae).

It was observed among most binding assessments that apoLp-III and βGBP were found to commonly bind fungal cells through β-glucan. *S.aureus* and LPS demonstrated binding of apoLp-III, PGRP and calreticulin from larvae which bind to LPS, peptidoglycan and antimicrobial surfaces respectively. These PRRs and proPO cascade proteins were increased in haemolymph from the $37^{o}C$ incubated and the physically stressed larvae when compared to the $30^{o}C$ incubated larvae and the control larvae. The presence of HSPs bound to microbial surfaces was highest in abundance within larvae thermally and physically stressed.

3.9 An analysis of the similarities between insect and mammalian serum proteins binding to microbial surfaces

The second part of this Chapter focused on the similarities in binding proteins from human serum and insect haemolymph to microbial cell walls or their components. The experiments followed the procedure outlined in the first section with human serum obtained from more than three people which was pooled together to give a stock sample that was used for each experiment once quantified to match the concentrations of protein used for *G.mellonella* samples.

3.10 The assessment of the binding similarities between insect and mammalian serum proteins to LPS

It was observed that a number of proteins from human serum and insect haemolymph bound to LPS (Fig. 3.14). Chain A, Apo-Human Serum Transferrin (Non-Glycosylated) (Band 1, Table 3.7) was bound to LPS, this protein has functions in metal-binding for the transport of iron to cells and has bacteriostatic functions in a variety of biological fluids (Mason *et al*., 2004). Freely available iron can severely affect the mechanism of natural resistance to infection, leading to rapid bacterial or fungal growth in tissue fluids (Bullen *et al*., 2006). Ferritin and transferrin which are found in *G.mellonella* have similar important roles which include iron storage (Arosio *et al*., 2009), transport (Zhou *et al*., 2007) and have antioxidant functions (Strickler-Dinglasan *et al*., 2006). Transferrin and ferritins also function in preventing microbial growth in *G.mellonella* by limiting free iron in the haemolymph (Levy *et al*., 2004).

It is known that iron affects the ability of haemocytes to adhere to each other which is important for nodule formation , larval haemocytes also are known to reduce free iron during haemocyte lysis which limits bacterial growth (Dunphy *et al*., 2002). Here the presence of iron binding protein may be indirectly binding through attachment to another binding protein that has an iron domain.The binding of human apolipoproteins E (apoE) (Band 6, Table 3.7) and proapolipoprotein (Band 2 Table 3.7) to LPS was observed. Apolipoproteins are primarily thought to function in mammals as a reverse cholesterol transporter (Glomset, 1968; Tall, 1998) although mammalian apolipoproteins such as apoE are involved in LPS detoxification, phagocytosis, and possibly pattern recognition (Whitten *et al*., 2004). Apolipoproteins have interactions with neutrophils through binding sites and have important roles in

regulating neutrophil function and inflammatory responses (Blackburn *et al*., 1991). It has also been demonstrated that apolipoprotein possesses a binding region that can bind LPS and enhance the pro-inflammatory response toward LPS via a mechanism similar to lipid binding protein (LBP) which activates the TLR signalling pathway and TNFα response (Berbée *et al*., 2010).

Haptoglobin (Band 5, Table 3.7) from human serum was associated with LPS although haptoglobins main role is in binding iron from damaged haemoglobin enabling its removal, but it has also been suggested to have an antimicrobial role in binding free iron which can inhibit bacterial growth that would benefit from hemolysis of red blood cells (Eaton *et al*., 1982). Haptoglobin is known to bind to a number of immune surfaces including neutrophils blocking their responses (Dobryszycka, 1997). An alternative role for haptoglobin-haemoglobin complexes has been suggested to function within the phagolysosomes lining of phagocytic cells, generating ROS that are directly microbicidal (Wagner *et al*., 1996).

Ig kappa chain V-III (KAU cold agglutinin) (Band 7, Table 3.7) was found to bind to LPS, it is a key component of antibody with the variable region holding specificity for binding antigen such as LPS here and the kappa region representing the light chain of immunoglobulin. It was also observed that fibrin beta (Band 4, Table 3.7) and serum albumin (Band 3, Table 3.7) was present and are proteins that bind with apolipoprotein (Dergunov and Vorotnikova, 1993; Klose *et al*., 2000) such as apolipoprotein E or proapolipoprotein present here. Fibrin beta is important in clotting, cell adhesion and spreading (Laurens *et al*., 2006), and serum albumin is capable of binding to a number of steroids and metal ions (Peters Jr, 1985).

G. mellonella haemolymph proteins showed a number of bound or complex associated proteins including hexamerin (Band 8, Table 3.7), arylphorin (Band 10, Table 3.7) and JHBP (Band 13, Table 3.7). It was found that apoLp-III (Band 15, Table 3.7), which recognises LPS and can activate proPO cascade (Halwani *et al*., 2000; Park *et al*., 2005) was present alongside proPO subunit 2 (Band 11, Table 3.7) and the enzyme that converts proPO to PO, the masquerade-like serine proteinase (Band 14, Table 3.7). ApoLp-III similarly binds as human apolipoproteins, both sharing similar immune capabilities and detoxification abilities of LPS (Whitten *et al*., 2004).

Arlyphorin (Band 10, Table 3.7) from haemolymph was bound to LPS and has a proposed binding ability in its homologous Gallysin-1 region (Phipps *et al*., 1994)

and has potential cytotoxic activity that is similar to that observed among mammals which is triggered in the presence of LPS (Beresford *et al*., 1997).

3.11 The assessment of the similarities between insect and mammalian serum binding proteins to β-1, 3-Glucan

A number of proteins bound to β-1, 3-Glucan either directly or indirectly from human and *G.mellonella* serum (Fig. 3.15). Human serum showed Chain A, Apo-Human Serum Transferrin (Non-Glycosylated) (Band 1, Table 3.8) bound to β-glucan. It is known that transferrin binds to iron but it may be indirectly bound to an iron component associated with another binding protein and could be the reason that human haptoglobin (Band 5) binds here.

ApoE (Band 6, Table 3.8) and proapolipoprotein (Band 2, Table 3.8) from human serum were bound to β-glucan, both proteins are thought to possess functions in phagocytosis and pattern recognition (Whitten *et al*., 2004). ApoE and proapolipoprotein may function similarly to insect apoLp-III which can recognise and bind LPS and β-glucan. ApoLp-III (Band 15, Table 3.8) from haemolymph was bound to β-glucan and shares similar functions to apolipoproteins of humans including binding and detoxification of LPS (Dunphy and Halwani, 1997; Kato *et al*., 1994) and promoting phagocytosis (Wiesner *et al*., 1997).

Ig kappa chain V-III (KAU cold agglutinin) (Band 7, Table 3.8) may have specificity to β-glucan binding by adapting its variable binding region. It was similarly observed here with β-glucan as it was previously with LPS that apoLp-III, proPO subunit 2 and masquerade-like serine proteinase from haemolymph were bound, indicating the potential activation of the proPO cascade by recognition of β-glucan through apoLp-III.
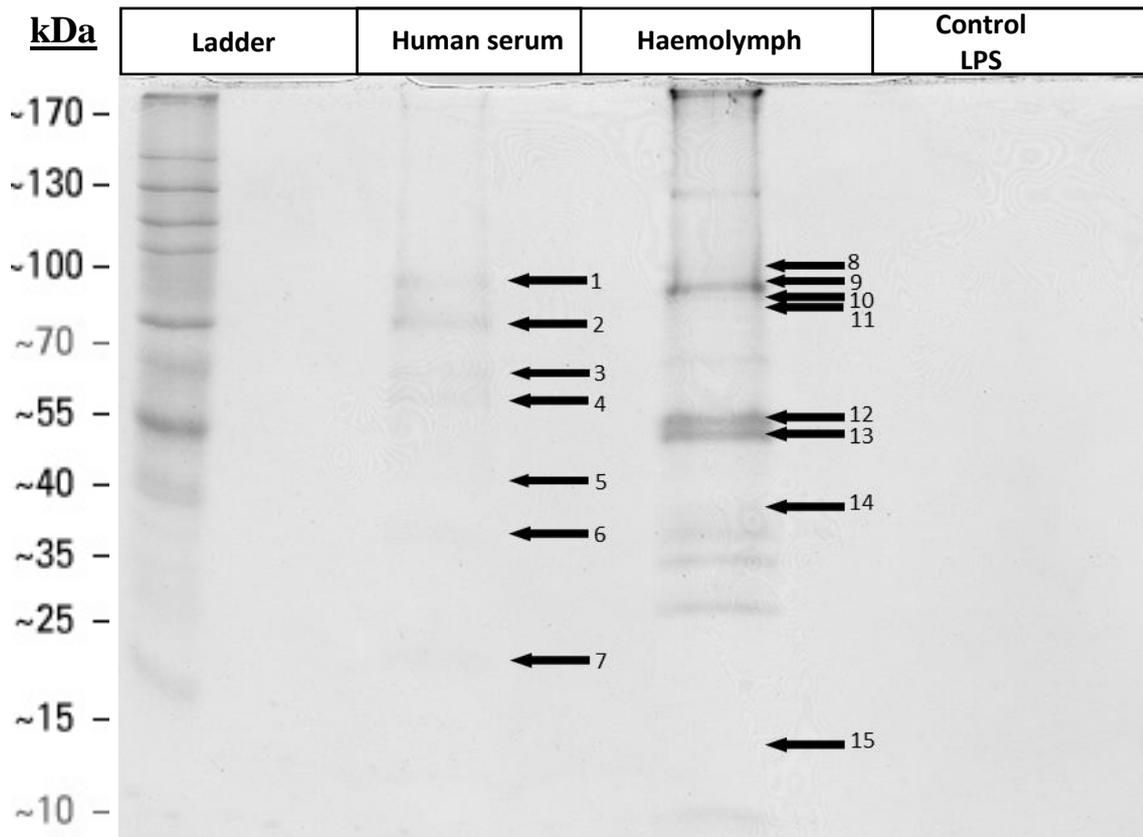
Fig. 3.14 1-Dimensional gel of separated *G.mellonella* larval haemolymph proteins and human serum proteins bound to LPS.

Lanes loaded with 20µl of haemolypmh/human serum proteins separated from the LPS by denaturing bound proteins. Lanes loaded from left to right, human serum, haemolymph and negative control (IPS and LPS), replicates (n=2). Proteins bands were cut and identified by LC/MS and identities numbered on the gel and listed in Table 3.7.

Human Sera

Band no.	Protein Identity	Organism	Mr	Score	Seq. coverage (%)	Accession no
1	Chain A, Apo-Human Serum Transferrin (Non-Glycosylated)	*H. sapiens*	76847	693	23	P02787
2	Proapolipoprotein	*H. sapiens*	28944	619	40	AAA51747
3	Serum albumin	*H. sapiens*	71127	971	24	BAG60658
4	Fibrin beta	*H. sapiens*	51369	295	19	0401173A
5	Haptoglobin	*H. sapiens*	38949	179	8	CAA25926
6	Apolipoprotein E	*H. sapiens*	36304	178	17	AAB59518
7	Ig kappa chain V-III (KAU cold agglutinin)	*H. sapiens*	23037	160	24	A23746

G. mellonella Sera

Band no.	Protein Identity	Organism	Mr	Score	Seq. coverage (%)	Accession no
8	Hexamerin	*G. mellonella*	81807	194	35	AAA19801
9	Prophenoloxidase subunit 2	*G. mellonella*	80198	125	5	AAQ75026
10	Arylphorin	*G. mellonella*	83651	384	15	AAA74229
11	Prophenoloxidase subunit 2	*G. mellonella*	80198	461	15	AAQ75026
12	Masquerade-like serine proteinase	*Pieris rapae*	46063	128	7	ACZ68116
13	Juvenile hormone binding protein precursor	*G. mellonella*	27460	221	25	AAS94224
14	Masquerade-like serine proteinase	*Pieris rapae*	46063	245	7	ACZ68116
15	Apolipophorin III	*G. mellonella*	20499	138	17	P80703

Table 3.7 1D-gel separated serum proteins from *G.mellonella* larvae or human serum bound to LPS with proteins 1-15 excised and identified by LC/MS.
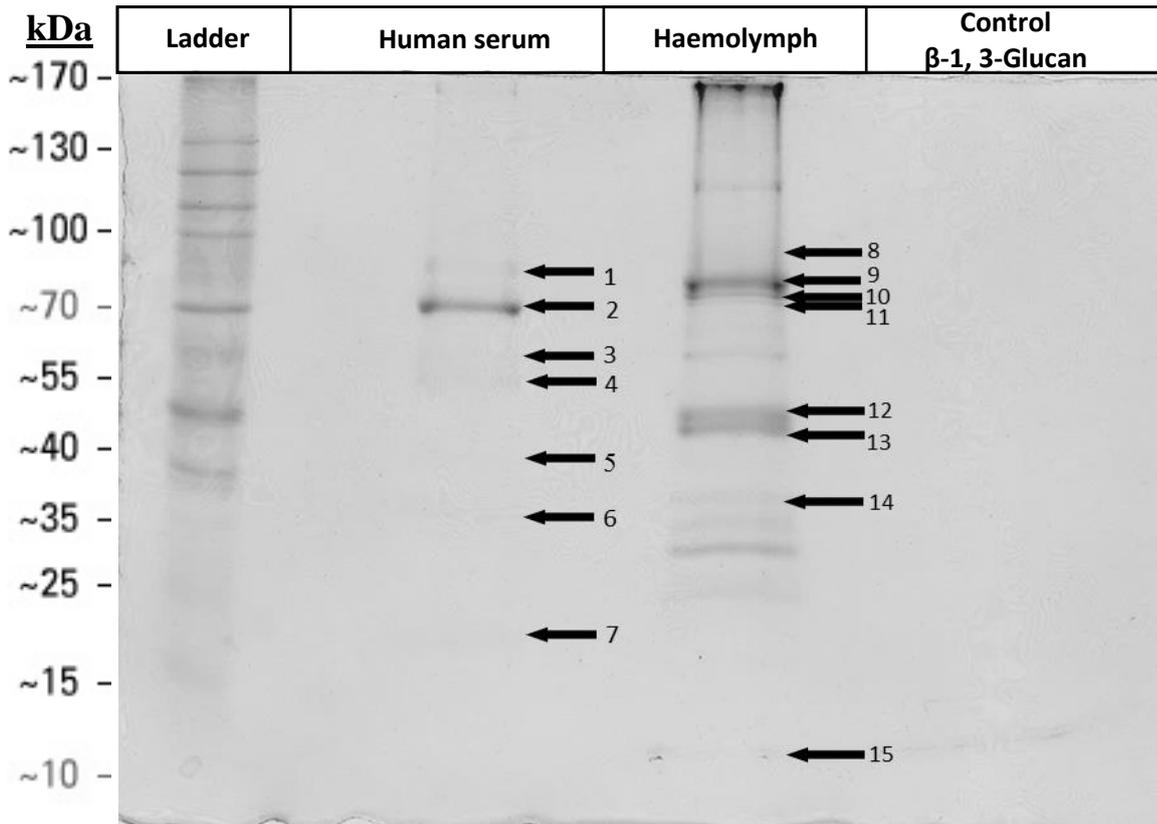
Fig. 3.15 1-Dimensional gel of separated *G.mellonella* larval haemolymph proteins and human serum proteins bound to β-1, 3-Glucan.

Lanes loaded with 20μl of haemolypmh/human serum proteins separated from the fungal cell surface β-1, 3-Glucan by denaturing bound proteins. Lanes loaded from left to right, human serum, haemolymph and negative control (IPS and β-1, 3-Glucan), replicates (n=2). Proteins bands were cut and identified by LC/MS and identities numbered on the gel and listed in Table 3.8.

Human Sera

Band no.	Protein Identity	Organism	Mr	Score	Seq. coverage (%)	Accession no.
1	Chain A, Apo-Human Serum Transferrin (Non-Glycosylated)	*H. sapiens*	76847	693	23	P02787
2	Proapolipoprotein	*H. sapiens*	28944	619	40	AAA51747
3	Serum albumin	*H. sapiens*	71127	971	24	BAG60658
4	Fibrin beta	*H. sapiens*	51369	295	19	0401173A
5	Haptoglobin	*H. sapiens*	38949	179	8	CAA25926
6	Apolipoprotein E	*H. sapiens*	36304	178	17	AAB59518
7	Ig kappa chain V-III (KAU cold agglutinin)	*H. sapiens*	23037	160	24	A23746

G. mellonella **Sera**

Band no.	Protein Identity	Organism	Mr	Score	Seq. coverage (%)	Accession no.
8	Hexamerin	*G. mellonella*	81807	194	35	AAA19801
9	Prophenoloxidase subunit 2	*G. mellonella*	80198	125	5	AAQ75026
10	Arylphorin	*G. mellonella*	83651	384	15	AAA74229
11	Prophenoloxidase subunit 2	*G. mellonella*	80198	461	15	AAQ75026
12	Masquerade-like serine proteinase	*Pieris rapae*	46063	128	7	ACZ68116
13	Juvenile hormone binding protein precursor	*G. mellonella*	27460	221	25	AAS94224
14	Masquerade-like serine proteinase	*Pieris rapae*	46063	245	7	ACZ68116
15	Apolipophorin III	*G. mellonella*	20499	138	17	P80703

Table 3.8 1D-gel separated serum proteins from *G.mellonella* larvae or human serum bound to β-1, 3-Glucan, with proteins 1-15 excised and identified by LC/MS.

3.12 The assessment of the similarities between insect and mammalian serum binding proteins to *A. fumigatus* conidia

A number of proteins from both the insect haemolymph and mammalian serum were observed bound to *A.fumigatus* conidia (Fig. 3.16). Human serum demonstrated the presence of complement component C3 (Band 1, Table 3.9) which functions as part of the complement cascade that mediates inflammation, phagocytosis, degranulation and cell lysis (Janssen *et al*., 2005). Importantly it has been shown that complement C3 binds to *A.fumigatus* conidial surfaces and enables the phagocytosis of conidia (Kozel *et al*., 1989) most likely through β-glucan (Thornton *et al*., 1996).

Gelsolin isoform b (Band 2, Table 3.9) from human serum is known to bind to bacteria, Gram negative LPS (Bucki *et al*., 2005) and Gram positive lipoteichoic acid (Bucki *et al*., 2008), gelsolin is not reported to bind to fungal cell wall components as it has here. Gelsolins are known to scavenge free F-actin released from damaged cells which can cause secondary tissue damage by inflammatory mediators (Rothenbach *et al*., 2004). The protein gelsolin is also found in *G.mellonella* with proposed function in forming clots complexed with proPO and lipoproteins (i.e. apoLp-III) and may share a similar function in humans (Karlsson *et al*., 2004).Chain A, Apo-Human Serum Transferrin (Non-Glycosylated) (Band 3, Table 3.9) and haptoglobin (Band 7, Table 3.9) bind to iron.

ApoE (Band 8, Table 3.9) and proapolipoprotein (Band 4, Table 3.9) both bound to conidia and may have bound through β-glucan, which can enable phagocytosis (Whitten *et al*., 2004). ApoE and proapolipoprotein may function similarly to apoLp-III recognition of β-glucan. ApoLp-III (Band 17, Table 3.9) from larval haemolymph bound to β-glucan and has a number of similar functions to apolipoproteins including binding and detoxification of LPS (Dunphy and Halwani, 1997; Kato *et al*., 1994) and promoting phagocytosis (Wiesner *et al*., 1997). It was observed that immunoglobulin light chain, Ig kappa chain V-III (KAU cold agglutinin) (Band 9, Table 3.9) was present but it may possess a specificity for *A.fumigatus* antigen such as β-glucan in its variable region.

The binding of *G.mellonella* haemolymph proteins to *A.fumigatus* conidia was demonstrated for a number of proteins including hexamerin (Band 10, Table 3.9), arylphorin (Band 12, Table 3.9) and JHBP-precusor (Band 15, Table 3.9). Of interest was the PRR apoLp-III (Band 17) and its known interaction with other bound proteins

that together activates the proPO cascade including proPO subunit 2 (Band 13, Table 3.9) and masquerade-like serine proteinase (Band 16, Table 3.9).

3.13 The assessment of the binding similarities between insect and mammalian serum proteins to *C.albicans* cells

Proteins were identified that bound to *C.albicans* surfaces from insect haemolymph and mammalian serum (Fig. 3.17). Human serum showed Chain A, Apo-Human Serum Transferrin (Non-Glycosylated) (Band 1, Table 3.10) bound to *C.albicans* and is known to bind through available iron ions on its surface (Han, 2014) inhibiting fungal growth. It could similarly be the reason haptoglobin (Band 5, Table 3.10) is seen binding as it binds iron also.

Apolipoproteins, apoE (Band 6, Table 3.10) and proapolipoprotein (Band 2, Table 3.10) from human serum were bound to *C.albicans*, potentially to surface antigens such as β-glucan that facilitates the phagocytosis process (Whitten *et al*., 2004). Ig kappa chain V-III (KAU cold agglutinin) (Band 7, Table 3.10) bound to *C.albicans* and may have specificity for *C.albicans* surface antigen such as β-glucan enabling it's binding through Ig's variable region.

The larval haemolymph demonstrated a reduced variety of proteins bound to *C.albicans*, for example apoLp-III not detected, proPO subunit 2 (Band 10, Table 3.10), putative serine protease-like protein 2 (Band 12, Table 3.10) and masquerade-like serine proteinase (Band 14, Table 3.10) were present which are key components of the proPO cascade.
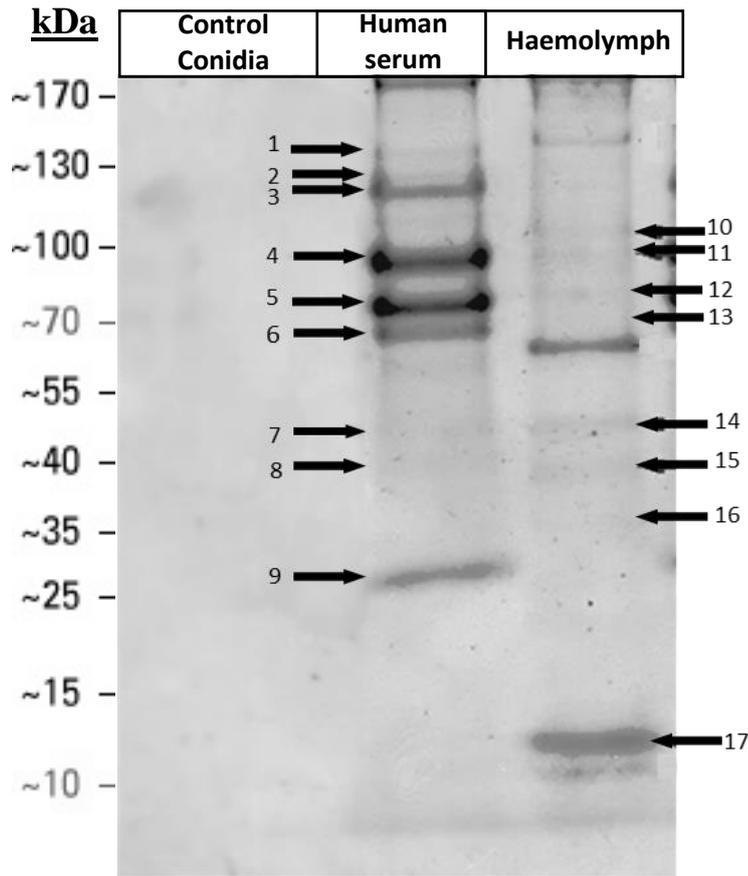
Fig. 3.16 1-Dimensional gel of separated *G.mellonella* larval haemolymph proteins and human serum proteins bound to *A.fumigatus* conidia.

Lanes loaded with 20μl of haemolypmh/human serum binding proteins separated from the surface of *A.fumigatus* conidia by denaturing bound proteins. Lanes loaded from left to right, human serum, haemolymph and negative control (IPS and *A.fumigatus* conidia), replicates (n=2). Proteins bands were cut and identified by LC/MS and identities numbered on the gel and listed in Table 3.9.

Human Sera

Band no.	Protein Identity	Organism	Mr	Score	Seq. coverage (%)	Accession no
1	Complement component C3	*H. sapiens*	188612	131	2	AAA85332
2	Gelsolin isoform b	*H. sapiens*	86050	285	9	NP_937895
3	Chain A, Apo-Human Serum Transferrin (Non-Glycosylated)	*H. sapiens*	76847	693	23	P02787
4	Proapolipoprotein	*H. sapiens*	28944	619	40	AAA51747
5	Serum albumin	*H. sapiens*	71127	971	24	BAG60658
6	Fibrin beta	*H. sapiens*	51369	295	19	0401173A
7	Haptoglobin	*H. sapiens*	38949	179	8	CAA25926
8	Apolipoprotein E	*H. sapiens*	36304	178	17	AAB59518
9	Ig kappa chain V-III (KAU cold agglutinin)	*H. sapiens*	23037	160	24	A23746

G. mellonella **Sera**

Band no.	Protein Identity	Organism	Mr	Score	Seq. coverage (%)	Accession no
10	Hexamerin	*G. mellonella*	81807	194	35	AAA19801
11	Prophenoloxidase subunit 2	*G. mellonella*	80198	125	5	AAQ75026
12	Arylphorin	*G. mellonella*	83651	384	15	AAA74229
13	Prophenoloxidase subunit 2	*G. mellonella*	80198	461	15	AAQ75026
14	Masquerade-like serine proteinase	*Pieris rapae*	46063	128	7	ACZ68116
15	Juvenile hormone binding protein precursor	*G. mellonella*	27460	221	25	AAS94224
16	Masquerade-like serine proteinase	*Pieris rapae*	46063	245	7	ACZ68116
17	Apolipophorin III	*G. mellonella*	20499	138	17	P80703

Table 3.9 1D-gel separated serum proteins from *G.mellonella* larvae or human serum bound to *A.fumigatus* conidia, with proteins 1-17 excised and identified by LC/MS.
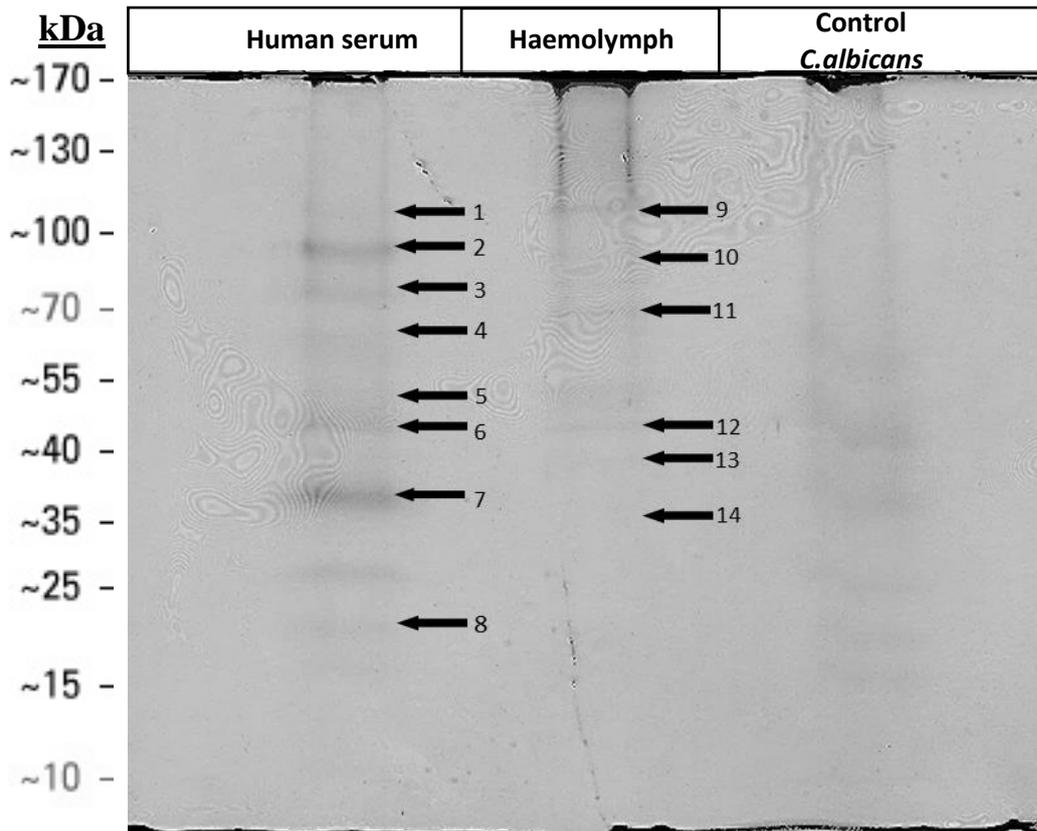
Fig. 3.17 1-Dimensional gel of separated *G.mellonella* larval haemolymph proteins and human serum proteins bound to *C.albicans*.

Lanes loaded with 20μl of haemolypmh/human serum proteins separated from the cell surface of *C.albicans* by denaturing bound proteins. Lanes loaded from left to right, human serum, haemolymph and negative control (IPS and *C.albicans*), replicates (n=3). Proteins bands were cut and identified by LC/MS and identities numbered on the gel and listed in Table 3.10.

Human Sera

Band no.	Protein Identity	Organism	Mr	Score	Seq. coverage (%)	Accession no
1	Chain A, Apo-Human Serum Transferrin (Non-Glycosylated)	*H. sapiens*	76847	693	23	P02787
2	Proapolipoprotein	*H. sapiens*	28944	619	40	AAA51747
3	Serum albumin	*H. sapiens*	71127	971	24	BAG60658
4	Fibrin beta	*H. sapiens*	51369	295	19	0401173A
5	Haptoglobin	*H. sapiens*	38949	179	8	CAA25926
6	Apolipoprotein E	*H. sapiens*	36304	178	17	AAB59518
7	Ig kappa chain V-III (KAU cold agglutinin)	*H. sapiens*	23037	160	24	A23746
8	Fibrin alpha C term fragment	*H. sapiens*	14443	275	15	0412237A

G. mellonella **Sera**

Band no.	Protein Identity	Organism	Mr	Score	Seq. coverage (%)	Accession no
9	Prophenoloxidase subunit 2	*G. mellonella*	80198	125	5	AAQ75026
10	Prophenoloxidase subunit 2	*G. mellonella*	80198	461	15	AAQ75026
11	Masquerade-like serine proteinase	*Pieris rapae*	46063	128	7	ACZ68116
12	Putative serine protease-like protein 2	*Danaus plexippus*	39613	78	8	EHJ69917
13	Juvenile hormone binding protein precursor	*G. mellonella*	27460	221	25	AAS94224
14	Masquerade-like serine proteinase	*Pieris rapae*	46063	245	7	ACZ68116

Table 3.10 1D-gel separated serum proteins from *G.mellonella* larvae or human serum bound to *C.albicans*, with proteins 1-14 excised and identified by LC/MS.

3.14 The assessment of the binding similarities between insect and mammalian serum proteins to *S.aureus* cells

A large number of proteins were identified from both human and *G.mellonella* serum that bound to *S.aureus* surfaces (Fig. 3.18). Human serum had albumin proteins (Band 2, 3 and 4, Table 3.11) bound to *S.aureus* which may indirectly associate with other bound proteins such as apolipoproteins (Dergunov and Vorotnikova, 1993). Ig mu chain C region (Band 4, Table 3.11) of humans was detected. Complement C4-A isoform 2 (Band 5, Table 3.11) is an essential component of the mammalian complement cascade. C4A forms amide bonds with immunoglobulin G (IgG) aggregates or protein antigens which enables solubilisation of antibody–antigen aggregates and there subsequent clearance through complement receptor (CR1) mediated phagocytosis (Blanchong *et al*., 2001).

The association of Chain A, Human Complement Component C3 (Band 6, Table 3.11) with *S.aureus* is a part of the complex of C4 and C5 that are required for the activation of the complement cascade and a key component of the humoral immune response (Blanchong *et al*., 2001). C1q B-chain precursor (Band 12, Table 3.11) or C1q is the target recognition protein of the classical complement pathway which is central to the clearance of pathogens and apoptotic cells (Kishore and Reid, 2000). C1q has a number of immune related roles including, phagocytosis, neutralisation, cell adhesion, dendritic cell modulation, B cells/fibroblasts and immune tolerance (Kishore *et al*., 2004).

Ig G1 H Nie (Band 8, Table 3.11) and Ig gamma 1 (Band 13, Table 3.11) were bound to *S.aureus* and these can associate with and activate the complement cascade via CR1 enabling the recognition of *S.aureus* and phagocytosis.

G.mellonella haemolymph also showed a number of proteins bound to *S.aureus* which included methionine-rich storage protein (Band 14, Table 3.11), a member of the hexamerins group of proteins which can bind to hormones as JH and have roles in detoxification of foreign xenobiotics (Burmester, 1999), immune protection and cytotoxic effector function (Phipps *et al*., 1994). Larval hemolymph protein (Band 16, Table 3.11) and JHBP-precursor (Band 19, Table 3.11), bound to *S.aureus* but these proteins are most likely associated to an other haemolymph protein as they have no known binding association to *S.aureus*.

A number of PRRs from haemolymph were bound to *S.aureus* including apoLp-III (Band 22, Table 3.11) and apoLp has been shown to bind lipoteichoic acids and cell surface components of *S.aureus* (Omae *et al*., 2013), PGRP-B (Band 23, Table 3.11), and PGRP-A (Band 24, Table 3.11) which bind to the peptidoglycan on the cell walls of Gram positive bacteria such as *S.aureus*. These three PRRs are individually capable of activating an appropriate immune response against *S.aureus* but combined can activate the Toll or IMD signal transduction pathways or proteolytic cascades (proPO) that generate antimicrobial effectors, and induce phagocytosis. The proPO cascade activating enzyme member masquerade-like serine proteinase (Band 20, Table 3.11) was bound which may be the initiation of the proPO cascade through PRR binding. Heat shock 70 kDa protein A, partial (Band 18, Table 3.11) from haemolymph was bound and is important in stabilising new proteins or proteins which may experience adverse conditions (Wojda and Jakubowicz, 2007).

3.15 Summary

The analysis of the type of human and *G.mellonella* binding proteins capable of binding to microbial pathogens demonstrated a number of similar PRRs that include the apolipoproteins of human serum and *G.mellonella* apoLp-III which are capable of binding to similar microbial surfaces and promoting phagocytosis. *G.mellonella* possess PGRP and βGBP which are PRRs also found in humans although these were not detected in human serum.

The potential activation of immune cascades was also seen including the mammalian complement cascade. Complement cascades enable activation of subsequent immune responses in a similar way to the proPO cascade of insects which is similarly activated by PRRs and triggers effector activation. Similar iron sequestering proteins exist in mammals such as Apo-Human Serum Transferrin and identified in later work in this thesis for *G.mellonella*. These similarities in insect and mammalian binding proteins show insects such as *G.mellonella* have structurally and functionally similar humoral immune systems to humans.
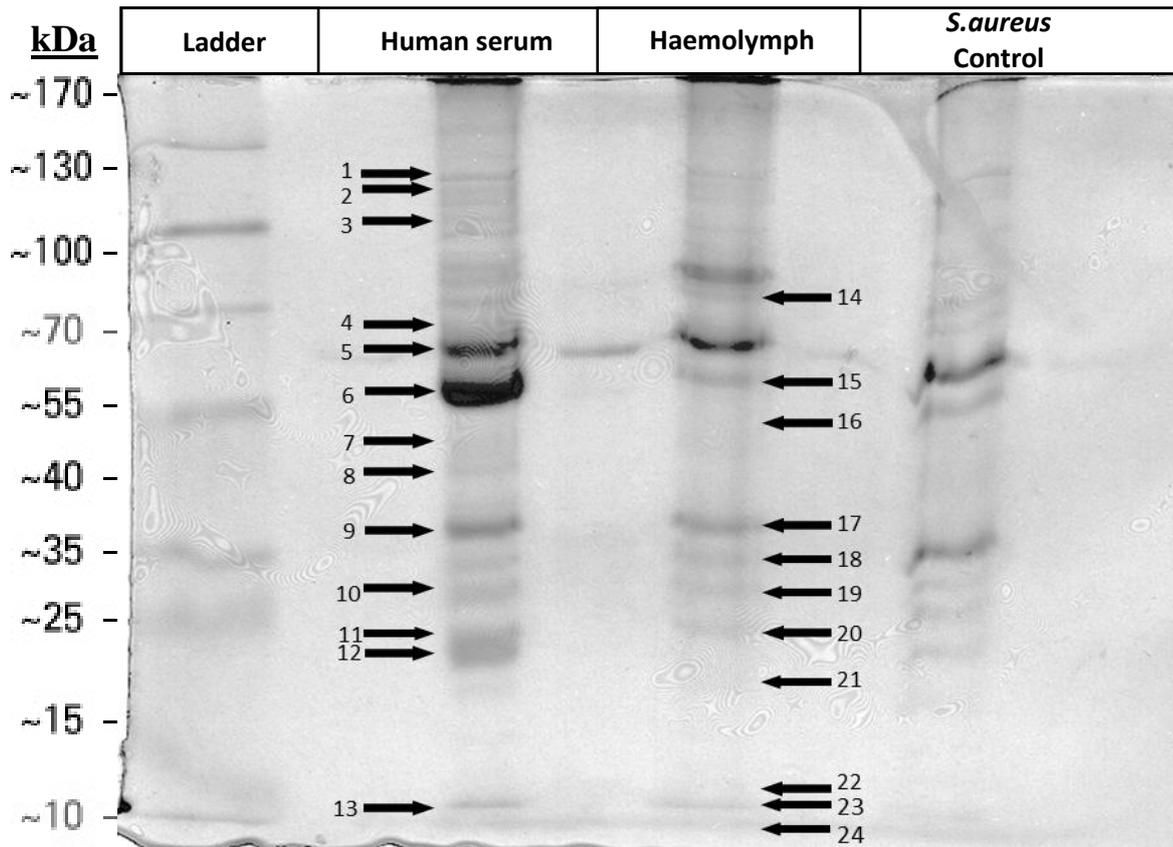
Fig. 3.18 1-Dimensional gel of separated *G.mellonella* larval haemolymph proteins and human serum proteins bound to *S.aureus*.

Lanes loaded with 20µl of haemolypmh/human serum proteins separated from the fungal cell surface of *S.aureus* by denaturing bound proteins. Lanes loaded from left to right, human serum, haemolymph and negative control (IPS and *S.aureus*), replicates (n=2). Proteins bands were cut and identified by LC/MS and identities numbered on the gel and listed in Table. 3.11.

Human Sera

Band no.	Protein Identity	Organism	Mr	Score	Seq. coverage (%)	Accession no.
1	Unnamed protein product	*H. sapiens*	71281	179	4	CAA23753
2	Albumin, isoform CRA_t	*H. sapiens*	60238	200	5	EAX05678
3	Albumin-like	*H. sapiens*	53440	181	8	AAA64922
4	Ig mu chain C region	*H. sapiens*	50135	419	28	S37768
5	PREDICTED: complement C4-A isoform 2	*H. sapiens*	189212	559	7	XP_003403644
6	Chain A, Human Complement Component C3	*H. sapiens*	71320	410	17	2A73_A
7	Unnamed protein product	*H. sapiens*	71281	185	3	CAA23753
8	Ig G1 H Nie	*H. sapiens*	49812	341	18	765044A
9	Unnamed protein product	*H. sapiens*	71378	172	3	BAG37325
10	Albumin, isoform CRA_t	*H. sapiens*	60238	156	5	EAX05678
11	Unnamed protein product	*H. sapiens*	58231	109	5	BAG37325
12	C1q B-chain precursor	*H. sapiens*	24158	113	16	CAA26880
13	Ig gamma1	*H. sapiens*	25562	149	27	1605217A

G. mellonella Sera

Band no.	Protein Identity	Organism	Mr	Score	Seq. coverage (%)	Accession no.
14	Methionine-rich storage protein	*Danaus plexippus*	88872	79	1	EHJ77141
15	Masquerade-like serine proteinase	*Pieris rapae*	46063	128	7	ACZ68116
16	Larval hemolymph protein	*G. mellonella*	13783	127	25	ABG91580
17	Arylphorin	*G. mellonella*	83651	282	8	AAA74229
18	Heat shock 70 kDa protein A, partial	*Ascaris suum*	45610	108	8	ADY43011
19	Juvenile hormone binding protein precursor	*G. mellonella*	27460	221	25	AAS94224
20	Masquerade-like serine proteinase	*Pieris rapae*	46063	245	7	ACZ68116
21	Similar to ORF	*Hydra magnipapillata*	127029	88	2	XP_002156961

		(hydrozoan)				
22	Apolipophorin III	*G. mellonella*	20499	176	17	P80703
23	Peptidoglycan recognition-like protein B	*G. mellonella*	16458	292	41	AAN15786
24	Peptidoglycan recognition-like protein A	*G. mellonella*	10451	229	41	AAN15782

Table 3.11 1D-gel separated serum proteins from *G.mellonella* larvae or human serum bound to *S.aureus*, with proteins 1-24 excised and identified by LC/MS.

3.16 Discussion

The aim of the first section of this Chapter was to determine if the thermal and physical treatment of larvae could influence the abundance and type of proteins that bound to microbial surfaces. The second section of this Chapter was interested in comparing similarities between the human and *G.mellonella* serum proteins that bound to the same microbial surfaces.

The thermal and physical treatment of larvae is an important consideration when designing an experiment as adequate controls must be in place to ensure that results are not adversely influenced by incubation at a given temperature or miss-handling. Thermal and physical treatment of larvae may alter or prime the larval immune response to infection or may make a developmental drug look promising when in fact it has no or little direct effect on increasing the survival of larvae following infection. It has been demonstrated by Mowlds and Kavanagh (2008) that pre-incubating the larvae at different temperatures had the effect of priming the immune system enabling the larvae to better survive infection from *C.albicans*. It was also shown that physical stress had a similar effect on priming the immune response and boosting the *G.mellonella* larval ability to overcome a microbial challenge (Mowlds *et al*., 2008). Wojda *et al*. (2009) showed the effect of heat shocking the larvae for a short exposure (15 min at 43°C or 30 min at 38°C) helped extend the life of larvae when infected with a pathogen.

Although the effect of temperature may boost the immune response it has not been assessed whether binding proteins of the humoral immune response may be affected as temperature can affect the protein conformation and therefore its ability to bind to microbial surfaces. The results observed here demonstrated that increased temperature and physical stress increased the abundance of *G.mellonella* haemolymph proteins bound to microbial surfaces and/or associated to the bound cell wall components.

The cell wall constituent LPS was exposed to haemolymph extracted from thermally and physically stressed larvae and showed elevated binding of haemolymph proteins from larvae incubated at 37°C and the physically stressed larvae. The physically stressed and 37°C incubated larvae had increased PRR binding such as apoLp-III when compared to the 30°C incubated larvae and control larvae, apoLp-III binds to LPS enabling its detoxification and phagocytosis (Halwani *et al*., 2000; Leon

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et al., 2006a; Leon *et al*., 2006b; Ma *et al*., 2006). PGRP-B which binds PG was bound to LPS, it is possible that trace amounts of PG may be present due to the LPS being synthesised in *E.coli* which contains PG and is not 100% pure LPS. It is known that only a (pg/l) of PG could be bound by PGRP as only pg/l of microbial cell wall components activate the proPO cascade (Sugumaran and Kanost, 1993; Söderhäll, 1982). Insect PRRs bind specifically and hydrolyze bacterial PG, activating the Toll or IMD signal transduction pathways or proteolytic cascades that produce antimicrobial effectors, and stimulate phagocytosis (Vogel *et al*., 2011).

Arylphorin from 37°C and physically stressed larval haemolymph demonstrated the highest level of binding to LPS. Arylphorin is known to have amino acid storage properties but has a conserved gallysin-1 region which has antimicrobial function in the presence of lysozyme enabling cytotoxic function (Phipps *et al*., 1994). Gallysin-1 is known to be induced by LPS and Gram negative bacteria, increasing the presence of gallysin-1 in haemolymph (Beresford *et al*., 1997). Calreticulin too was observed to bind to LPS in higher amounts from haemolymph where larvae were incubated at 37°C or physically stressed. This could elicit haemocyte activation via its G-protein linked signalling as observed in mammalian neutrophil activation enabling generation of superoxide anions (Yoshida *et al*., 1996). Calreticulin is important in cell adhesion, presentation of antigen, inflammation and phagocytosis (Llewellyn *et al*., 2000). Both arylphorin and calreticulin demonstrated the highest abundance of binding to LPS from haemolymph of larvae incubated at 37°C and physically stressed.

Interestingly components of the proPO cascade were bound to LPS and proPO was bound in most microbial binding assays including LPS. The proPO in the presence of serine proteases can convert from proPO to active PO, this can be activated by pg/l of microbial cell wall components (Sugumaran and Kanost, 1993; Söderhäll, 1982). These proPO associated proteins appeared to be bound in the highest amounts from haemolymph isolated from 37°C incubated larvae and physically stressed larvae. The thermally (30°C and 37°C) and physically stressed larvae demonstrated elevated HSP 70 binding relative to the control which may function to stabilise the conformation of proteins that are binding as well as non-binding proteins in response to the adverse conditions. These HSPs may act as a DAMP activating Toll like receptors 2 and 4 in humans (Asea *et al*., 2002) or may induce cross communication between the signalling pathways activated in response higher temperature and immunogens (Wojda and Jakubowicz, 2007).

A similar trend was observed when haemolymph from physically and thermally stressed larvae was exposed to the fungal cell wall component β-1, 3-Glucan. The proteins proPO subunit 2 of the proPO cascade and arylphorin both bound and are capable of binding or interacting with microbial surfaces. Proteins most likely to be indirectly associated to β-1, 3-Glucan included HSP 70 and GAPDH, the latter is suggested to sequentially phosphorylate signalling cascades which includes serine proteases (Sirover, 2011) that may function in the proPO cascade. Only arylphorin which was undetectable in haemolymph from larvae incubated at 37°C and physically stressed demonstrated a different trend to what was observed when these proteins bound to LPS. Interestingly βGBP and apoLp-III were not identified bound, both of which capable of binding β-glucan (Fabrick *et al*., 2003; Whitten *et al*., 2004).

The assessment of binding to germinated *A.fumigatus* conidia versus non-germinated *A.fumigatus* conidia demonstrated a distinct difference in both what could bind from thermally and physically stressed larvae as well as the difference overall in abundance of proteins bound to germinated versus non-germinated *A.fumigatus* conidia. The non-germinated conidia showed a number of haemolymph proteins that recognise the surface antigens present on conidia. These included the PRRs, apoLp-III and βGBP, with both PRRs capable of binding β-glucan on conidial surfaces and were bound in the highest amounts from the haemolymph of 37°C incubated larvae and the physically stressed larvae. Arylphorin had greater binding from haemolymph extracted from 37°C incubated and physically stressed larvae along with the binding of proPO cascade components proPO subunit 2 and masquerade-like serine proteinase.

In comparison the germinated *A.fumigatus* conidia had the same haemolymph proteins binding to the conidial surfaces as to the non-germinated conidia with the exception of the abundance of arylphorin and βGBP. This shows an increasing ability of arylphorin to bind to the germinated *A.fumigatus* conidia most likely attributed to the greater abundance of exposed fungal antigen as the conidia have swollen. It is also important to note the significant increase in the abundance of arylphorin binding to conidia from the 37°C incubated larval haemolymph (725k±55.5k, *p*<0.001), which was 2 fold greater than the abundance of arylphorin bound from 30°C and 15°C incubated larval haemolymph. While the physically stressed (371k±106.7k, *p*<0.05) larvae demonstrated a small increase in the abundance of bound arylphorin compared to the 30°C and 15°C incubated larvae. Germinated *A.fumigatus* conidia demonstrated

greater abundance of βGBP bound, compared to the abundance of βGBP bound to the non-germinated conidia. βGBP binding to germinated conidia in haemolymph from larvae incubated at 37°C (410k± 125.0k, $p<0.001$) demonstrated a significant increase over the other treatments (15°C, 30°C and physically stressed larvae) as seen with arylphorin binding.

It has not been fully elucidated how PRRs such as βGBP bind to cell surfaces or PAMPs that cause activation of a proteinase or proteinases involved in proPO activation. It has been suggested that βGRP binding to β-glucan or other PAMPs may trigger a conformational change that activates βGRP and facilitates its ability to interact with other plasma proteins that may mediate activation of immune responses (Fabrick *et al*., 2003), including proPO cascade and/or the synthesis of antimicrobial proteins (Yu *et al*., 2002).

It is well known that the morphological state of the conidia is important as swollen germinating conidia are more efficiently phagocytosed than resting conidia (Luther *et al*., 2007). Resting conidia possess a hydrophobin layer formed from RodA protein which limits access to β-glucan (Brakhage *et al*., 2010). Once this layer is shed from swelling and germinating conidia phagocytosing cells such as macrophages can recognise the conidia more efficiently through greater available antigen that PRRs can bind. Here a similar trend was observed as germinated conidia demonstrated increased binding of βGRP and arylphorin when compared to the same binding proteins in non-germinated conidia.

Examining the haemolymph proteins that bound to *C.albicans* did not show a similar trend, as the haemolymph from the 15°C incubated larvae demonstrated greater binding of hexamerin and arylphorin compared to the other treatments. The arylphorins, proPOs, and hexamerins share significantly similar sequences and are known to have evolved from a common ancestor (Beintema *et al*., 1994).

βGBP and proPO were both detected at similar binding abundance in the haemolymph extracted from larvae incubated at 15°C and 37°C but these proteins were undetectable in haemolymph extracted from 30°C and physically stressed larvae which both are larval treatments incubated at 30°C. The similar incubation temperature at 30°C may affect the abundance of haemolymph binding proteins available specifically to *C.albicans* when compared to the other two temperatures. Despite this, βGBP and proPO were observed to have bound to *A.fumigatus* conidia from haemolymph from all thermal and physical treatments and its abundance was

highest in the 30°C, 37°C and physically stressed larvae when compared to the control larvae.

The assessment of binding of proteins from haemolymph extracted from larvae thermally and physically stressed, demonstrated a variety of proteins capable of binding to *S.aureus*. A number of these proteins were PRRs such as apoLp-III which had significantly higher binding abundance in the physically stressed larvae and a non-significant increase in the 37°C and the 30°C incubated larvae when compared to the control larvae. ApoLp-III is well known to bind to lipoteichoic acids and cell surface components of *S.aureus* (Omae *et al*., 2013) and proPO which may be activated in response to apoLp-III binding. Arylphorin which possesses a conserved gallysin-1 region with antimicrobial function was shown binding to *S.aureus* in higher abundance in the haemolymph extracted from 37°C and physically stressed larvae but was not detected in the 30°C incubated larvae which may be bound at too low a concentration to be detected.

The abundance of apoLp-III, proPO and arylphorin from haemolymph extracted from 37°C and the physically stressed larvae appeared similar to that observed in the LPS binding assessment as it was to the *S.aureus* binding assessment. A similar range of binding proteins were used from larval haemolymph to detect bacteria either by directly or indirectly binding to LPS and *S.aureus,* these binding proteins included calreticulin, proPO, apoLp-III and arylphorin.

In contrast to the bacterial binding assays a common trend was observed in the binding of proteins to fungal cell surfaces by the βGBP, proPO, arylphorin and hexamerin to germinated *A.fumigatus* conidia, non-germinated *A.fumigatus* conidia and to *C.albicans*. But apoLp-III which binds β-glucan was not identified bound to *C.albicans* or β-1, 3-glucan, this may be as this peptide was not identified on the LC/MS or the level of binding was too low in abundance to be detected. It was seen again that for the majority of these proteins that bound to fungal surfaces the highest amounts of bound proteins was from the haemolymph of the 37°C incubated and physically stressed larvae. The germination of *A.fumigatus* conidia demonstrated a greater abundance of proteins capable of binding from haemolymph to conidia surfaces, potentially as more antigen became exposed on the germinated conidia. Some bound proteins could have a role in stabilising binding protein structures such as heat shock proteins, or have unknown or broad range binding ability such as arylphorin and hexamerin.

The second half of this Chapter focused on the similarities between what bound to microbial pathogens from *G.mellonella* haemolymph and human serum. It was demonstrated that the Gram negative cell wall component LPS had apolipoproteins, proapolipoprotein and apoE from human serum bound and apoLp-III from *G.mellonella* larvae which are proteins structurally and functionally homologous in insects and mammals (Cole *et al*., 1987). In mammals apolipoproteins such as apoE are capable of detoxifying LPS preventing endotoxic shock (Feingold *et al*., 1995) and are also capable of stimulating immune reactions including phagocytosis (Carvalho *et al*., 2000) and nitric oxide (NO) release from platelets (Riddell *et al*., 1997). Apolipoproteins have also been shown to interact with neutrophils binding sites and have roles in regulating neutrophil function and inflammatory responses (Blackburn *et al*., 1991). ApoLp-III similarly can bind and detoxify LPS (Kato *et al*., 1994), promote phagocytosis (Wiesner *et al*., 1997), stimulate antibacterial activity (Niere *et al*., 2001) and superoxide generation by haemocytes (Dettloff and Wiesner, 1999). ApoLp-III can bind to a number of surfaces including Gram positive bacteria lipoteichoic acid and in this case LPS (Kato *et al*., 1994) or β-glucan from conidia (Whitten *et al*., 2004).

It was also observed that Chain A, Apo-Human Serum Transferrin (Non-Glycosylated) and haptoglobin which both have similar functions to insect transferrin by sequestering iron within the haemolymph impeding microbial growth (Levy *et al*., 2004) was associated with LPS. Insect ferritin and transferrin found in *G.mellonella* have similar roles to mammalian transferrins which function in iron storage (Arosio *et al*., 2009), transport (Zhou *et al*., 2007) and antioxidant response (Strickler-Dinglasan *et al*., 2006). Transferrins in humans have been important in limiting systemic spread of *C.albicans* in leukemia patients (Caroline *et al*., 1969). Binding of transferrin is most likely associated with iron found on proteins bound to the surface of LPS.

Ig kappa chain V-III (KAU cold agglutinin) the light chain of immunoglobulin was found to interact with LPS, a key component of antibody with the variable region enabling specificity for binding antigen such as LPS. Arylphorin is induced by LPS and Gram negative bacteria increasing the presence of gallysin-1 in haemolymph (Beresford *et al*., 1997) and was found associated to LPS. ProPO subunit 2 and the enzyme that can convert proPO to PO the masquerade-like serine proteinase was present along with apoLp-III which is known to recognise LPS and subsequently activate proPO cascade (Halwani *et al*., 2000; Park *et al*., 2005). The complement

pathway is a central component of the innate immune response in the clearance of pathogens and apoptotic cells in mammals (Kishore and Reid, 2000) and it functions in a similar way to the proPO cascade of insects.

β-1, 3-Glucan showed a number of proteins that recognised and bind to its surface including the apoLp-III of larvae and the apolipoproteins of the human serum. It is known that apoLp-III binds β-glucan but it is not established if apolipoproteins of mammals do although they are highly similar to insects apolipoproteins (Whitten *et al*., 2004). The presence of both proPO and masquerade-like serine proteinase would enable proPO activation through the apoLp-III (Halwani *et al*., 2000; Park *et al*., 2005). Chain A, Apo-Human Serum Transferrin (Non-Glycosylated) binding to β-glucan may indirectly bind to iron from another binding protein. Ig kappa chain V-III (KAU cold agglutinin) was bound with potential specificity to the β-glucan. Interestingly neither complement of the human serum or βGBP from *G.mellonella* larvae were found to bind β-1, 3-Glucan.

A.fumigatus conidia was bound by mammalian complement C3 which functions as a key part of the complement cascade that mediate inflammation, phagocytosis, degranulation and cell lysis (Janssen *et al*., 2005). It was also shown previously that Complement C3 binds to *A.fumigatus* conidial surfaces and enables the phagocytosis of conidia (Kozel *et al*., 1989) most likely through β-glucan (Thornton *et al*., 1996). ProPO was also shown to have been bound to the surface of conidia and has functions similar to complement with the ability to be activated by apoLp-III which can recognise β-glucan (Sugumaran and Kanost, 1993; Söderhäll, 1982). ApoLp-III and the mammalian apolipoprotiens were present and may also have similar interactions with conidial surfaces although this has not been shown to date, both mammalian and insect apolipoproteins possess a lot of conserved structures and functions (Whitten *et al*., 2004).

The protein gelsolin isoform b from human serum was shown to be bound to the surfaces of conidia although it is only known to bind to bacteria, Gram negative LPS (Bucki *et al*., 2005) and Gram positive lipoteichoic acid (Bucki *et al*., 2008). Gelsolins interact by scavenging free F-actin from damaged cells which can cause secondary tissue damage due to the action of inflammatory mediators. Gelsolin is also found in *G.mellonella* and has been suggested to have a function in forming clots complexed with proPO and lipoproteins (i.e. apoLp-III) with potentially similar function in humans (Karlsson *et al*., 2004). Mammalian haptoglobin and Apo-Human

Serum Transferrin (Non-Glycosylated) were observed bound to *A.fumigatus* conidia with their association possibly through the interaction with iron on proteins or on the surface of the conidia.

The binding of human or insect proteins to *C.albicans* including human apolipoproteins (apoE and proapolipoproteins) and insect apoLp-III are important in facilitating the phagocytosis of pathogens, although only apoLp is known to bind β-glucan (Whitten *et al*., 2004). Immunocompromised patients with acute leukaemia are often accompanied by high iron content in blood and patients are surprisingly susceptible to *C. albicans* infection. In a study of 34 patients who had acute myeloid leukaemia, 21 had high serum iron and 17 had fully saturated transferrin with two of these patients having systemic candidiasis (Caroline *et al*., 1969). Such iron binding proteins may be important in this role of limiting free iron to impede the growth of pathogens.

It was also observed that proPO, putative serine protease-like protein 2 and masquerade-like serine proteinase were bound and may function in activating the proPO cascade activating effectors and initiating phagocytosis (Söderhäll, 1982) in a similar way to the mammalian complement pathway (Janssen *et al*., 2005).

Interesting Complement C4-A isoform 2 was bound to *S.aureus* and functions as an essential component of the complement cascade, C4A forms amide bonds with immunoglobulin G (IgG) immune aggregates or protein antigens and is important in the solubilisation of antibody–antigen aggregates that are cleared through CR1 binding and phagocytosis (Blanchong *et al*., 2001). The target recognition protein C1q of the classical complement pathway is critical to clearance of pathogens and apoptotic cells while facilitating roles in phagocytosis, neutralisation, cell adhesion, modulation of dendritic cell, B cells and fibroblasts and immune tolerance (Kishore *et al*., 2004).

Ig G1 H Nie and Ig gamma1 were bound to *S.aureus* antigen which may have an association with the complement cascade via CR1 enabling the recognition of *S.aureus* and its phagocytosis. In *G. mellonella* the proPO cascade activating enzyme member masquerade-like serine proteinase was present which may be activated by PRRs. The thiol ester-like motif GCGEQNMI present in the complement components; C3, C4 and a2-macroglobulins (De Bruijn and Fey, 1985; Sottrup-Jensen *et al*., 1984; Tertia Belt *et al*., 1984) was also identified in crustacean proPO GCGWPQHM (Aspan *et al*., 1995; Sritunyalucksana *et al*., 1999). It is interesting that here both

pathways of the proPO and the complement cascade were implicated in insect and mammalian innate immune responses to the same pathogen despite over 500 million years of evolutionary divergence (Kavanagh and Reeves, 2004).

Larval haemolymph had a number of PRRs such as apoLp-III which has been shown to bind lipoteichoic acids and cell surface components of *S.aureus* (Omae *et al*., 2013), PGRP-B and PGRP-A which bind to the peptidoglycan on the cell walls of Gram positive bacteria such as *S.aureus*. These three PRRs are individually capable of activating an appropriate immune response against *S.aureus* but combined can activate the Toll or IMD signal transduction pathways or proteolytic cascades (proPO) that generate antimicrobial effectors, and induce phagocytosis. Much of these pathogen surface antigens are used by the mammalian innate immune response to detect pathogens such as *S.aureus*.

Thermal and physical stress on larvae showed a consistent trend in the abundance and type of proteins bound to microbial surfaces, with the exception of *C.albicans*. The greatest increase in binding or associated binding proteins appeared in larvae that were incubated at 37°C or physically stressed prior to extraction of haemolymph and exposure to microbial surfaces. The greater abundance of humoral binding proteins would enable a better response to pathogens and therefore a better survival which is what has been demonstrated previously when larvae were exposed to thermal stress and microbially challenged (Mowlds and Kavanagh, 2008; Wojda *et al*., 2009) or following physical stress and microbial challenge (Mowlds *et al*., 2008).

When examining the similarities between insect and mammalian humoral binding proteins to a range of pathogens, a number of similarities and differences were observed. The binding of apolipoproteins was seen from human serum to microbial surfaces as was the apoLp-III of *G.mellonella* both sharing similar structures and functions (Whitten *et al*., 2004). The innate immune cascades of the mammalian complement and insect proPO have both conserved regions and function in activating effectors and phagocytosis (Söderhäll and Cerenius, 1998). Transferrin from human serum was found bound or associated with microbial surfaces which is a protein common in *G.mellonella* with near identical roles. Transferrin and ferritin proteins which similarly act to sequester free iron and in the process have bacteriostatic functions both in humans (Mason *et al*., 2004) and insects (Dunphy *et al*., 2002)

Although PRRs were found commonly in insects, the human serum contained immunoglobulins which are capable of recognition of microbial antigen but is associated with the adaptive immune response that insects lack. As a result similar PRRs present in insect haemolymph observed to bind to microbial surfaces were not observed binding from human serum to microbial surfaces. It is important to also note that in both the assessment of the thermal and physical effects and in the comparison of the *G.mellonella* with human serum binding, that *G.mellonella* larvae were capable of employing PRRs specifically to the pathogen they were exposed to. LPS was bound by apoLp-III, PGRP that recognise PG and calreticulin that binds microbial surfaces which in combination with other receptors may make a response more tailored to the invading pathogen.

It was also observed that fungal infections could be detected by apoLp-III and βGBP both capable of recognising β-glucan antigen and activating an appropriate response. This ability of more than one of these proteins to recognise more than one microbial polysaccharide enables great redundancy in their ligand binding specificity (Yu *et al*., 2002). It may be that the immune responses to these microbial pathogens is shaped specifically or broadly determined by what number of pathogenic surfaces are detected and therefore the pathways that activate a given set of effectors for a bacterial or fungal infection.

It is known that a number of similar PRRs do exist between insects and mammals as PGRP (Liu *et al*., 2000) and the apolipoproteins of mammals which show conserved functions and structures to the insect apolipophorins as apoLp-III (Whitten *et al*., 2004). Despite the differences observed here there is a vast amount of conserved functions and structures that make *G.mellonella* larvae a useful model to use in preliminary screening of both pathogens and new antimicrobial drugs or compounds.

Chapter 4

Assessment of the effects of Thermal and Physical stress on the cellular and humoral immune response of *G.mellonella* larvae

4.0 Thermal and physical stress effects on the humoral and cellular immune response of *G.mellonella* larvae

G.mellonella larvae may encounter a number of factors in their environment that could influence their immune response including injury, infection, thermal and physical stress. The experiments described in this Chapter were designed to examine how thermal and physical stress affects the immune response of larvae and their capacity to overcome a microbial challenge.

Insects lack the adaptive immune response present in vertebrates, yet despite this insects possess an effective immune response composed of both the cellular and humoral immune systems. The insects cellular immune response is mediated by haemocytes which can engulf, encapsulate or neutralize pathogens (Pech and Strand, 1996; Ratcliffe, 1993). Circulating immune haemocytes in the haemocoel are the first to the site of infection and their numbers can be supplemented by the release of haemocytes bound to internal organs such as the fat body (Kavanagh and Reeves, 2004). The humoral immune response of insects is mediated by a number of mechanisms including the melanisation cascade, haemolymph clotting (Kavanagh and Reeves, 2004) and anti-microbial peptides, (Hoffmann, 2003; Janeway, 1989; Ratcliffe, 1985) that impede the growth and development of the pathogen within the host.

Insects have been shown to boost ('prime') their immune response when exposed to a sub-lethal infection and survive a subsequent challenge with a potentially lethal infection.

The aim of the work described in this Chapter was to establish whether an immune priming effect in *G. mellonella* larvae, induced as a result of exposure to thermal or mild physical stresses, altered the cellular and humoral responses and for what duration it lasted. A better understanding of the insect responses to changes in the environment may influence its ability to be used to assess virulence of a pathogen or to determine the antimicrobial effects of a drug or compound.

4.1 The effect of thermal and physical stress on larval survival following a microbial challenge

Larvae of *G. mellonella* were physically or thermally stressed as described (Section 2.7.3) prior to infection with *S. aureus*, *A. fumigatus* or *C.albicans* after 24 h. Larvae thermally (30 or 37°C) or physically challenged showed greater survival (96.7±3.3%) 24 hours after infection with *S. aureus* compared to the control (15°C) larvae (73.3±6.7%) (*p*<0.01) (Fig. 4.1). Larvae pre-incubated at 37°C or physically stressed demonstrated enhanced survival following *S.aureus* infection (23.3±8.8%, *p*<0.01 or 6.7±3.3%, *p*<0.05) respectively when compared to the control at 72 hours (0% survival).

Larvae incubated for 24h at their respective stress temperatures followed by 48h at 30°C (72h long term stress) were infected with *S.aureus* to determine the duration of the physical and thermal stresses. The larvae incubated at 37°C had reduced survival 48h after infection (66.7±3.3%) while control larvae had survival at 78±6.5%. The control larvae 72h post infection had 68.9±2.8% survival compared to the 37°C pre-incubated larvae (56.7±8.8%) demonstrating a reduction in the 37°C long term stressed larval survivals (Fig. 4.2). The physically stressed larvae showed significantly reduced survivals from 24h to 72h after infection when compared to the control while the 30°C long term stressed larvae demonstrated a significant decrease in survival at 48h after infection (50±5.8%, *p*<0.05) when compared to the controls (78±6.5%) at the same time point.

Those larvae stressed for a short term (24h) prior to infection with *A. fumigatus* demonstrated increased survival among the 37°C pre-incubated larvae and physically stressed larvae when compared to the controls (Fig. 4.3). Forty eight hours after infection physically stressed larvae showed 86.6±3.3% survival compared to 70±5.8% survival in the control and subsequently at 72h after infection the physically stressed larvae showed 73.3±3.3% survival compared to 36.7±8.8% survival in control larvae (*p*<0.001). Larvae pre-incubated at 37°C and 30°C had a survival rate of 66.7±3.3%, (*p*<0.001) and 40±5.8%, (*p*<0.05) respectively at 72h when compared to the control (16.7±8.8%). However, larvae that were physically or thermally stressed 72 hours prior to infection with *A. fumigatus* showed reduced survival when compared to the control (Fig. 4.4). The control larvae demonstrated greater survival overall with 83.3±8.8% survival at 48h point from infection compared to the 37°C pre-incubated

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larvae which demonstrated 27.3±13.8% survival ($p<0.001$). At 72h from infection in the long term stressed larvae there was a continued reduction of survival by 77.5% within the 37°C pre-incubated larvae (12±7.2%) when compared to the control larvae's survival (53.3±8.8%) ($p<0.01$) (Fig. 4.4). A similar trend of reduced survival was observed among the 30°C long term stressed larvae and the physically stressed larvae when compared to the control larvae.

Larvae thermally (37°C) and physically stressed for 24h before inoculation with a lethal dose of *C.albicans* ($1x10^{6}$ cells/ml) demonstrated a greater survival 93.3±6.7% and 93.3±6.7% respectively 24h post infection (Fig. 4.5). The larvae followed a similar trend of survival at 48h with the highest survival observed in the 37°C (63.3±8.82%) pre-incubated and the physically stressed (66.7±6.7%) larvae while the 30°C incubated larvae had a 60.0±20.8% compared to the control (43.3±16.7%) larvae. Thermally and physically stressed larvae demonstraeted greater survival over the control larvae (16.7±3.33%) at 72h post infection with physically stressed larvae demonstrating significantly higher survival (40.0±11.54% $p<0.05$).

4.2 Summary

Larvae thermally (30 and 37°C) or physically stressed for a total of 24h (short term) prior to inoculation with *A.fumigatus*, *C.albicans* or *S.aureus* demonstrated greater survival to microbial challenge compared to the control. Larvae thermally or physically stressed for a total of 72h (long term) before inoculation with a microbial infection of either *A.fumigatus* or *S.aureus* showed decreased survival when compared to the controls. This suggests the priming effects of the higher temperatures and physical stresses wear off in the long term stressed larvae possibly due to its costly nature to maintain.
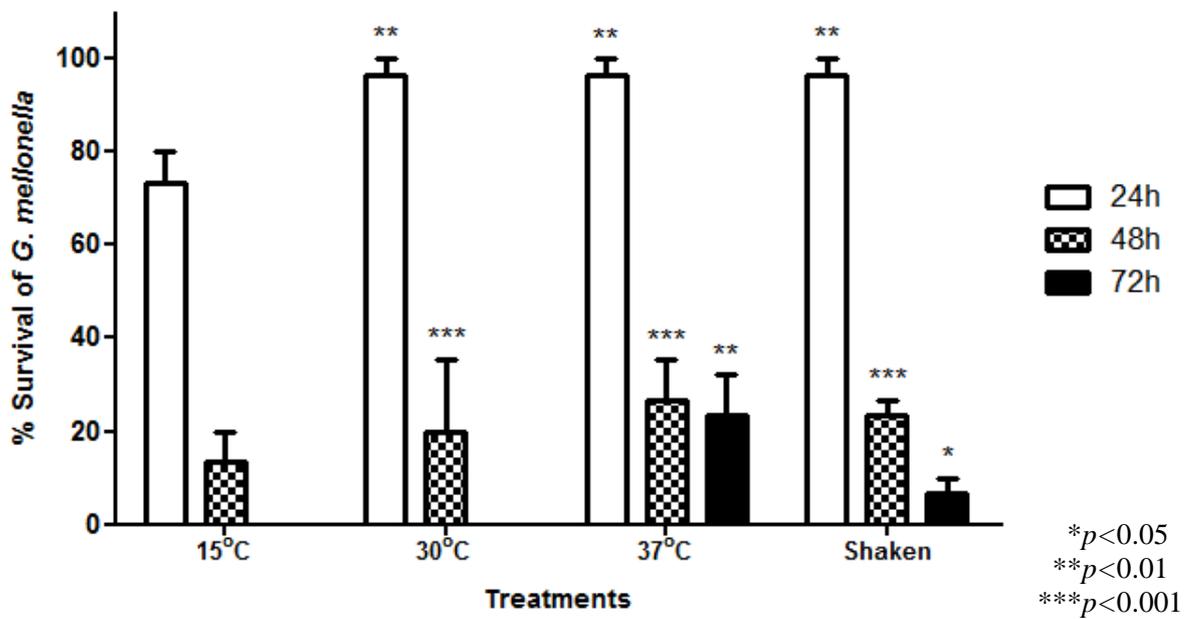
Fig. 4.1 Survival (%) of larvae thermally or physically stressed 24h prior to infection with *S.aureus*.
Statistical significance of *Galleria* survival was analysed with log rank (Mantel-Cox) method using GraphPad Prism version 5.00.
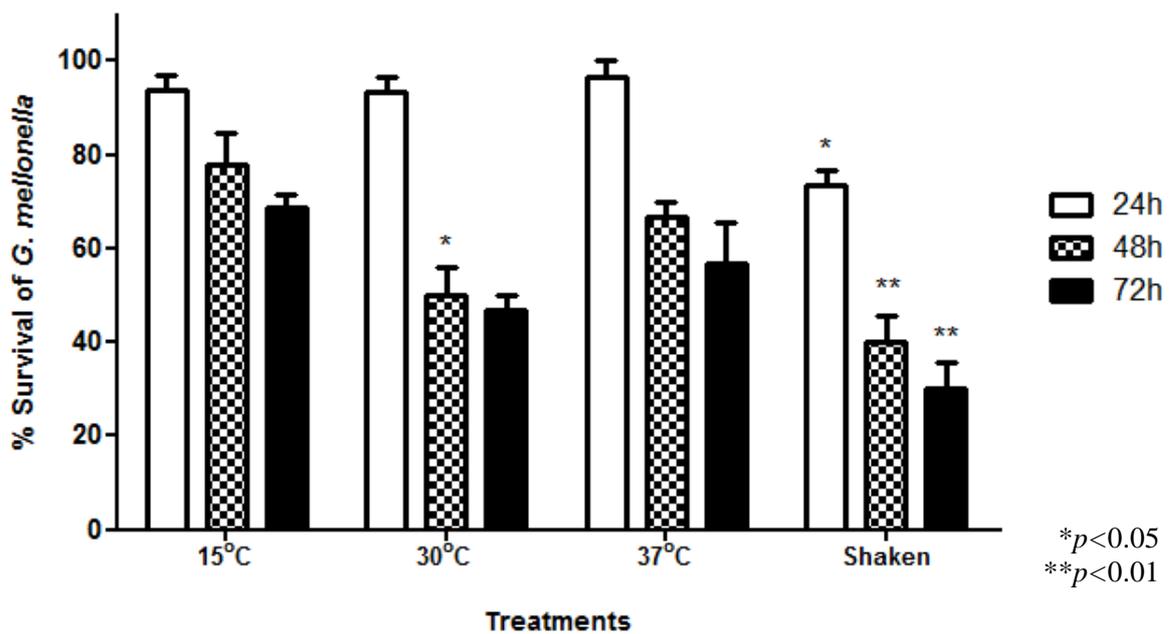
Fig. 4.2 Survival (%) of larvae thermally or physically stressed 72h prior to infection with *S.aureus*.
Statistical significance of *Galleria* survival was analysed with log rank (Mantel-Cox) method using GraphPad Prism version 5.00.
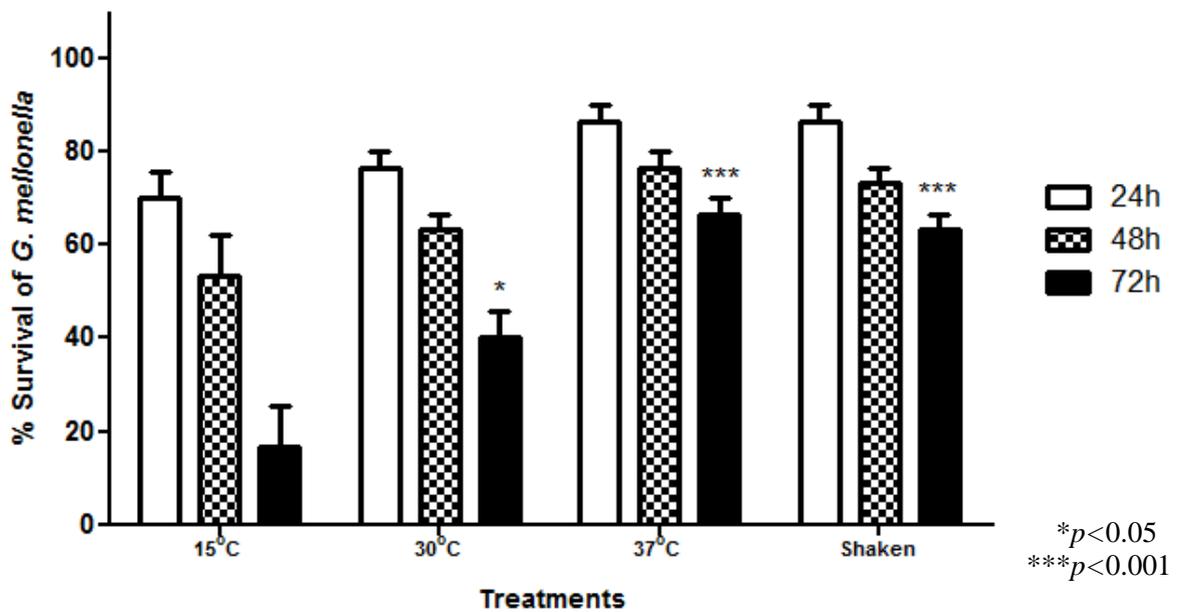
Fig. 4.3 Survival (%) of larvae thermally or physically stressed 24h prior to infection with *A.fumigatus* conidia.
Statistical significance of *Galleria* survival was analysed with log rank (Mantel-Cox) method using GraphPad Prism version 5.00.
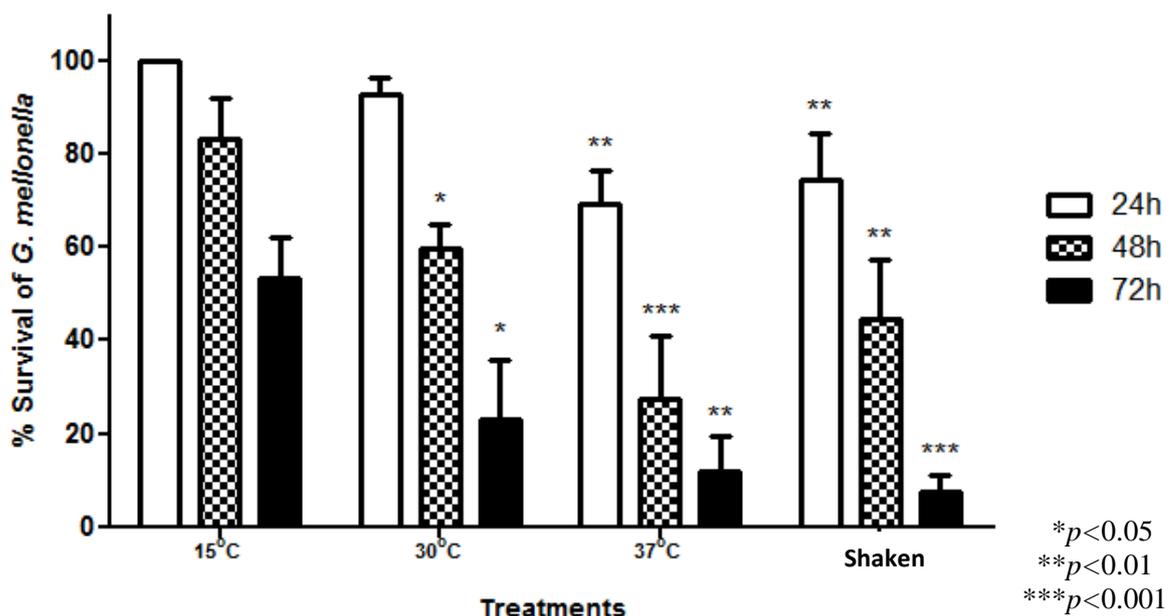
Fig. 4.4 Survival (%) of larvae thermally or physically stressed 72h prior to infection with with *A.fumigatus* conidia.
Statistical significance of *Galleria* survival was analysed with log rank (Mantel-Cox) method using GraphPad Prism version 5.00.
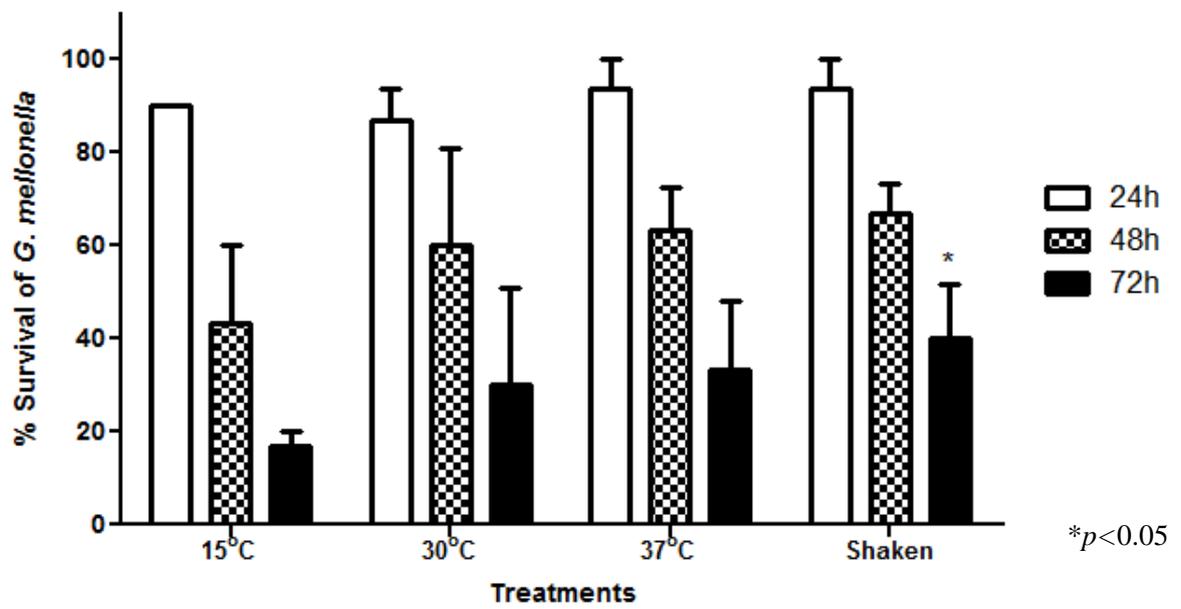
Fig. 4.5 Survival (%) of larvae thermally or physically stressed 24h prior to infection with *C.albicans*.
Statistical significance of *Galleria* survival was analysed with log rank (Mantel-Cox) method using GraphPad Prism version 5.00.

4.3 Proteomic analysis of larval haemolymph from thermally or physically stressed larvae

Larvae were thermally or physically stressed as described previously and incubated for 24, 48 or 72 h at 15, 30 or 37°C as appropriate. Protein was extracted from larvae and resolved by 1-D SDS-PAGE as described (Section 2.11.1). Analysis of the alterations in the intensity of apolipophorin, prophenoloxidase and arylphorin (Fig. 4.6), revealed an increased intensity of all three proteins in haemolymph from larvae incubated at 37°C or physically stressed, at 24 h (Fig. 4.6 A, B, C). However relative intensity of these three proteins was reduced at 48 and 72 h in the thermally and physically stressed larvae.

For better interpretation of how proteins changed in expression following a thermal stress, haemolymph was extracted from larvae incubated at 37°C for 24, 48 or 72 h and resolved by 2D SDS-PAGE (Fig. 4.7). A number of proteins with immune functions were shown to be increased in intensity at 24 h but declined thereafter (Table 4.1). The 26kDa (Spot 4) and 32kDa ferritin (spot 5) proteins are important in maintaining the homeostasis of iron in the haemocoel and have important roles in the immune response (Levy *et al*., 2004). Both proteins show a 1.34 – 1.4 fold increase in intensity at 24 h but this declined at 48 and 72 h (Table 4.1). Transferrin (spot 1) showed a 2.27 fold increase in expression at 24h compared to the control, however the intensity declined to 1.62 and 1.25 fold at 48 and 72 h, respectively. ApoLp-III (spot 3) has several functions including lipid transport and a function in the innate immune response (Gupta *et al*., 2010), here apoLp-III showed a 1.82 fold increase in intensity at 24h but this declined to 0.95 and 0.7 at 48h and 72h respectively. Arylphorin (spot 9) showed an increase in expression of 1.9 fold at 24 h but the intensity reduced to 0.53 and 0.59 fold at 48 and 72 h, respectively. Arylphorin is a storage protein for amino acids and aids in the immune defence of insects (Beresford *et al*., 1997). Serpin 1(Spot 6) is important in the inhibition of proteolytic cascades such as the proPO cascade (Aspán *et al*., 1990), here it demonstrated a 1.46 fold increase in expression at 24 h but its relative expression decreased thereafter. The expression of juvenile growth hormone precursor (spot 8) was maintained at a relatively constant level across the time points and may be considered as a loading control (Banville *et al*., 2012).

Fig. 4.6A

Apolipophorin

Fig. 4.6B

Arylphorin

Fig. 4.6C

Prophenoloxidase

Fig. 4.6D

*p<0.05
**p<0.01
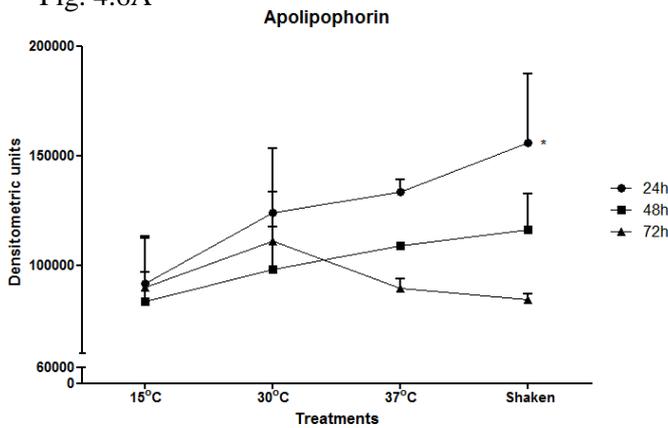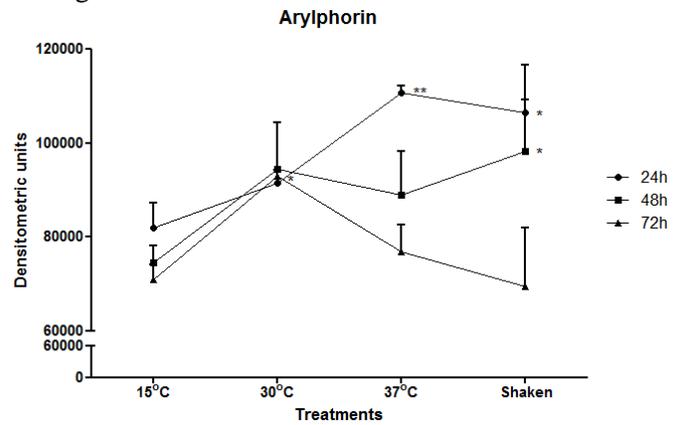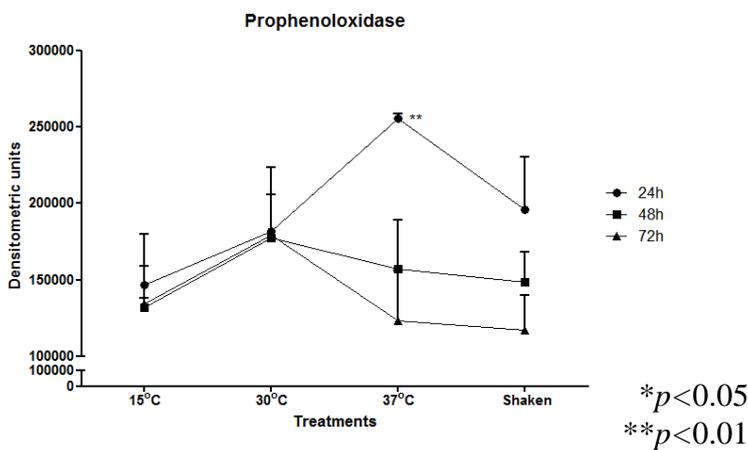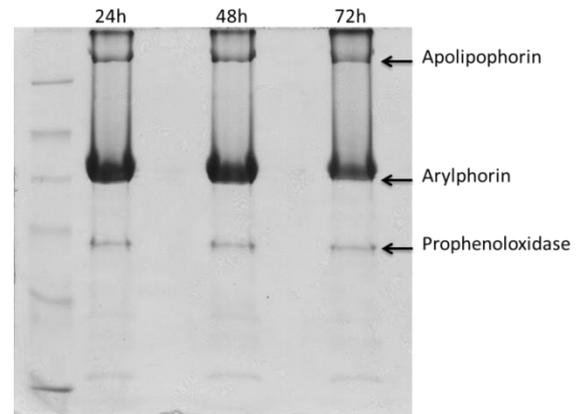
Fig. 4.6 Changes in protein expression from thermally and physically stressed larvae over 24, 48 and 72h analysed by 1D-SDS PAGE.

Protein expression; Apolipophorin (Fig. 4.6A), Arylphorin (Fig. 4.6B) and Prophenoloxidase (Fig. 4.6C). Gel representation of separated haemplymph protein on 12.5% SDS-PAGE, replicates (n=3). Proteins of interest excised and identified by LC/MS (Fig. 4.6D).
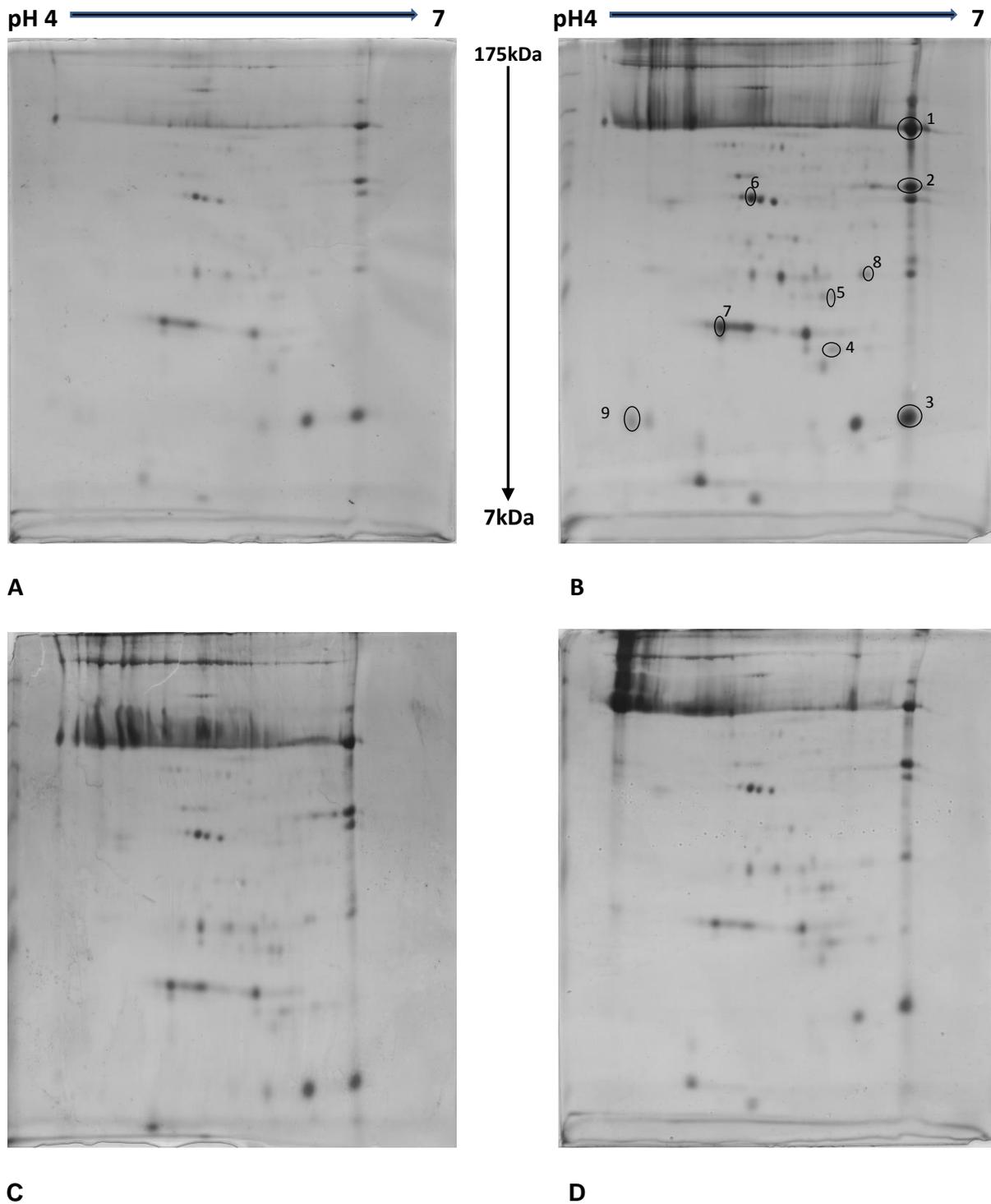
Fig. 4.7 Haemolymph proteins resolved by 2-D SDS PAGE from larvae pre-incubated at 37°C for 0, 24, 48 and 72h.
Gels at 0h (A), 24h (B), 48h (C) and 72h (D), replicates (n=3). Proteins of interest were excised and identified via LC/MS (1-9).

Spot no.	Protein Identity	Organism	Score	Seq. coverage (%)	Accession no.	0h Control	24h	48h	72h	Protein function
						Relative Expression				
1*	Transferrin precursor	*G. mellonella*	340	8	AAQ63970	1	1.93 *	1.33	1.17	Involved in iron storage, transport and immune system.
2	Imaginal disc growth factor-like protein	*Mamestra brassicae*	158	13	ABC79625	1	1.77	1.18	1.18	Important in regulating cell proliferation/ growth.
3*	Apolipophorin-III	*G. mellonella*	604	66	P80703	1	1.72	1.07	1.04	PRR of microbial components.
4*	26kDa ferritin subunit	*G. mellonella*	504	44	AAG41120	1	1.46	1.23	1.13	Involved in iron storage, transport and immune system.
5*	32 kDa ferritin subunit	*G. mellonella*	469	35	AAL47694	1	2.03 ***	1.13	0.97	Involved in iron storage, transport and immune system.
6*	Serpin 1	*Danaus plexippus*	86	6	EHJ75277	1	1.59	1.13	1.34	Serpins inhibit chymotrypsin-like serine proteases.
7	27 kDa glycoprotein precursor	*B. mori*	93	7	Q8T113	1	1.99 *	1.36	0.99	Unknown function
8*	Juvenile hormone binding protein precursor	*G. mellonella*	262	24	AAS94224	1	1.21	1.11	0.99	Precursor of JHBP, regulates embryogenesis and reproduction; JHBP protects JH molecules from hydrolysis by non-specific esterases.
9*	Arylphorin	*G. mellonella*	168	5	AAA74229	1	1.91 *	0.90	0.95	Amino acid storage and immune functions.

Table 4.1 Identities of peptides from haemolymph of *G.mellonella* larvae exposed to 37°C for 0, 24, 48 or 72h with proteins 1-9 excised and identified by LC/MS.

Protein expression measured as a fold change relative to 0h control gel. *$p<0.05$ and ***$p<0.00.1$. Spot no. with (*) representing protein with fold change represented in graph (Fig. 4.8)
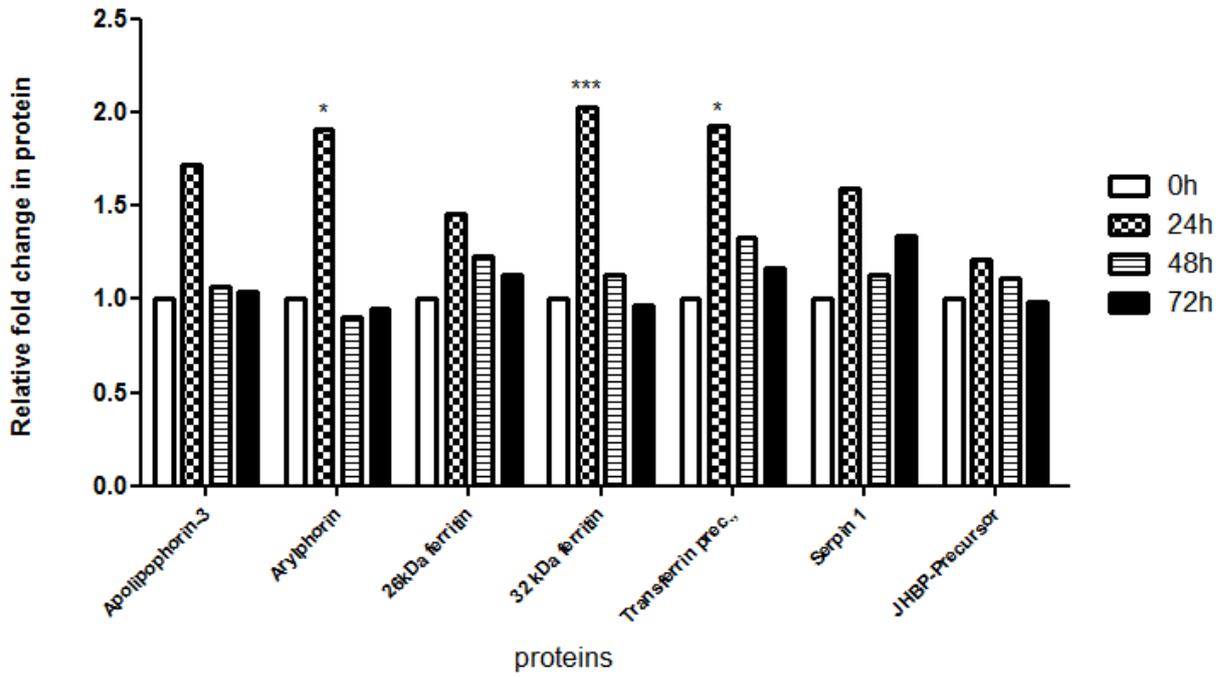
Fig. 4.8 Changes in haemolymph protein abundance from larvae pre-incubated at 37°C for 0, 24, 48 and 72h.
Proteins abundance measured as a fold change relative to 0h control gel. (*) = $p<0.05$ and (***) = $p<0.001$.

4.4 Real Time PCR analysis of antimicrobial gene expression of larvae thermally or physically stressed for 1, 4, 12, 24, 48 and 72h

Larvae thermally and physically treated were incubated for 1, 4, 12, 24, 48 and 72h prior to RNA extraction and assessment of relative gene expression. All genes (*Galiomicin*, *Gallerimycin, IMPI* and *Transferrin*) were normalised against the expression of *S7e* reference (housekeeping) gene and larval treatments were compared to the 15°C control larvae.

Galiomicin, which encodes for a defensin 1 antimicrobial peptide (Mukherjee *et al*., 2010), demonstrated a general reduction in expression among thermally and physically stressed larvae when compared to that in the 15°C control larvae (Fig. 4.9). It was observed at the 1h that the 30°C incubated larvae (0.34±0.25, p <0.01) had a significant reduction in expression when compared to the control larvae, while the 37°C incubated larvae (0.91±0.21) and the physically stressed (0.93±0.25) larvae were comparable to the controls. The expression of *Galiomicin* in the 30°C incubated larvae rose from 1h to 12h while the expression in the 37°C incubated larvae (0.37 ±0.18, *p*<0.01) and the physically stressed larvae (0.31±0.23, *p*<0.01) demonstrated a reduction at 12h which extended up to 24h. Although the 30°C, 37°C incubated and physically stressed larvae demonstrated an increase in expression of *Galiomicin* from 24h to 48h, the expression of *Galiomicin* decreased at 72h by ~50% less than the control larval expression. The expression of *Galiomicin* reduced within the thermally and physically stressed larvae compared to the controls.

Gallerimycin codes for a cysteine-rich antifungal peptide important in the innate immune response (Schuhmann *et al*., 2003). *Gallerimycin* expression was shown to be significantly reduced at 1h among the 30°C, 37°C and physically stressed larvae relative to the control but at 4h expression rose within the 30°C incubated (1.05±0.34) and physically stressed (1.21±0.14) larvae compared to the control (1.0±0.4) and the 37°C incubated larvae (0.44±0.23) (Fig. 4.10). The expression of *Gallerimyicin* from thermally and physically stressed larvae decreased at 24h before rising at 48h and surpassing the control at 72h for the 30°C incubated (1.56±0.17, *p*<0.05) and physically stressed (3.80±0.15, *p*<0.001) larvae while the 37°C (0.99±0.15) incubated larvae had an expression level equivalent to the control.

The gene *IMPI* (inducible metallo-proteinase inhibitor) (Clermont *et al*., 2004) encodes two inhibitors one of which inhibits microbial metallo-proteinases essential to the immune response of *G. mellonella* larvae and the other inhibitor regulates endogenous MMPs (matrix metallo-proteinases) during the metamorphosis of larvae (Wedde *et al*., 2007). *IMPI* expression remained constant between all pre-incubation conditions from the control, 30°C, 37°C and physically stressed larvae up to 24h (Fig. 4.11). At 48h the expression of *IMPI* rose significantly in physically stressed larvae (2.44 ± 0.47, $p<0.001$) and a non-significant rise was observed in the 30°C (1.22 ± 0.27) and the 37°C (1.11 ± 0.26) incubated larvae when compared to the control larvae (1.0 ± 0.47). At the 72h the largest expression change in *IMPI* was observed among the 30°C (2.67 ± 0.24, $p<0.001$) and the physically stressed larvae which peaked at 48h but remained high at 72h (2.28 ± 0.14, $p<0.001$). The 72h expression of *IMPI* in the 37°C (1.53 ± 0.21, $p<0.05$) incubated larvae also demonstrated a significant rise compared to that in the control larvae (1.0 ± 0.13).

Transferrin (an iron-binding protein, (Yoshiga *et al*., 1997)) is a fundamental component of the insect immune response in *G.mellonella* as it enables the removal of free iron from haemolymph that can be acquired by infecting pathogens (Levy *et al*., 2004). The expression of *Transferrin* was assessed and demonstrated initially an unchanged expression from the larval treatments at 1h, but the 30°C incubated larvae showed a significant decrease at 4h compared to the 37°C incubated and physically stressed larvae which maintained a similar expression to the control (Fig. 4.12). At the 12h the 30°C incubated (0.31 ± 0.0, $p<0.01$), 37°C incubated (0.30 ± 0.19, $p<0.01$) and the physically stressed (0.42 ± 0.12, $p<0.01$) larvae demonstrated significantly lower expression of *Transferrin* compared to the control (1.0 ± 0.14, $p<0.01$). The expression of *Transferrin* followed a similar trend to *IMPI* from 12h among 30°C incubated and physically stressed larvae. At 48h there was a rise in expression of 30°C incubated (0.79 ± 0.12) and physically stressed (1.36 ± 0.10) larvae, followed by further increase in *Transferrin* expression at 72h for the 30°C incubated (1.30 ± 0.33) and the physically stressed (2.56 ± 0.16, $p<0.001$) larvae when compared to the control. Interestingly the 37°C incubated larvae showed a significantly lower expression of *Transferrin* at 72h (0.29 ± 0.30, $p<0.01$).
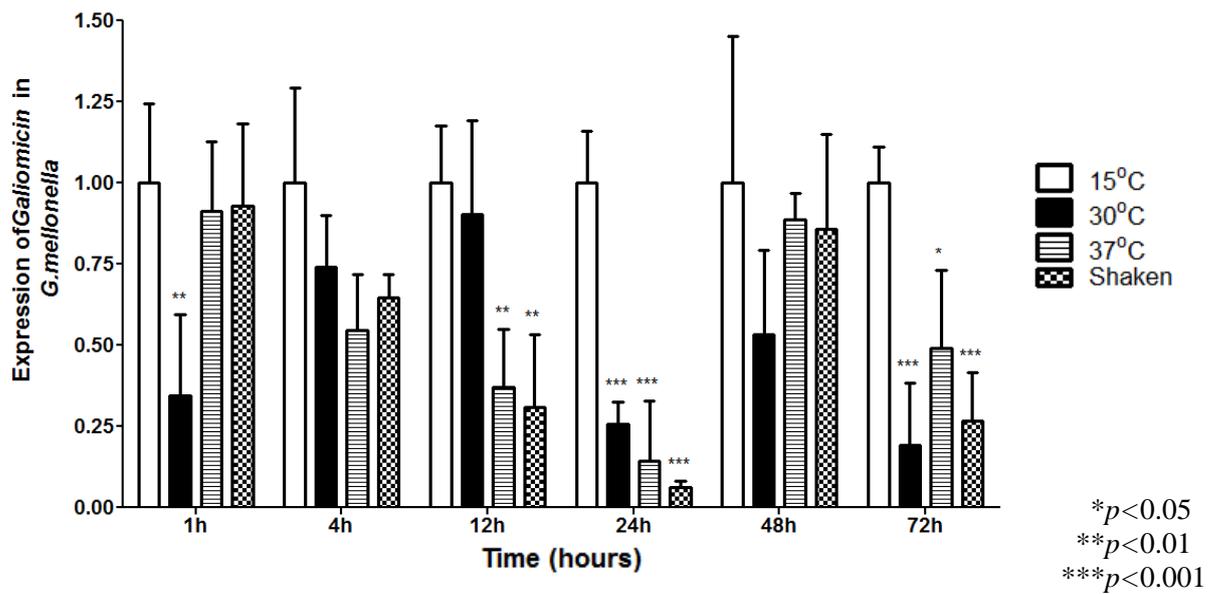
Fig. 4.9 qPCR analysis of *Galiomicin* gene expression in larvae thermally and physically stressed for 1, 4, 12, 24, 48 and 72h.
Larvae were normalised against S7e housekeeping gene. Two-Way Anova performed comparing treatments of 30°C, 37°C and physically stressed larvae against 15°C control larvae at each time point.
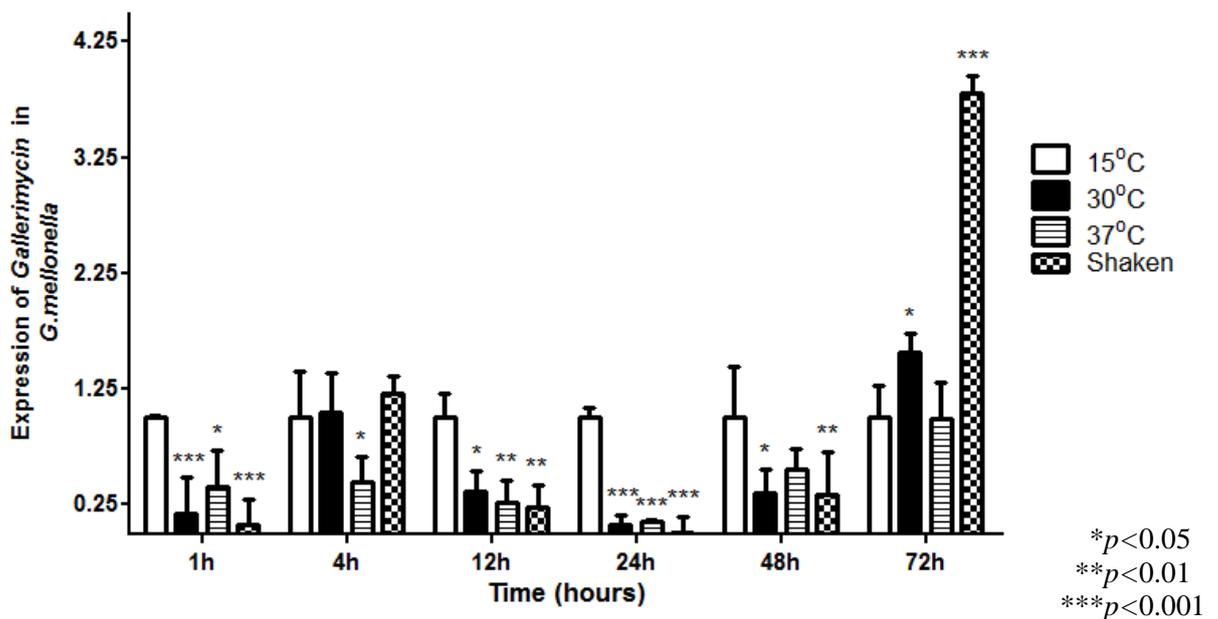
Fig. 4.10 qPCR analysis of *Gallerimyicin* gene expression in larvae thermally and physically stressed for 1, 4, 12, 24, 48 and 72h.
Larvae were normalised against S7e housekeeping gene. Two-Way Anova performed comparing treatments of 30°C, 37°C and physically stressed larvae against 15°C control larvae at each time point.
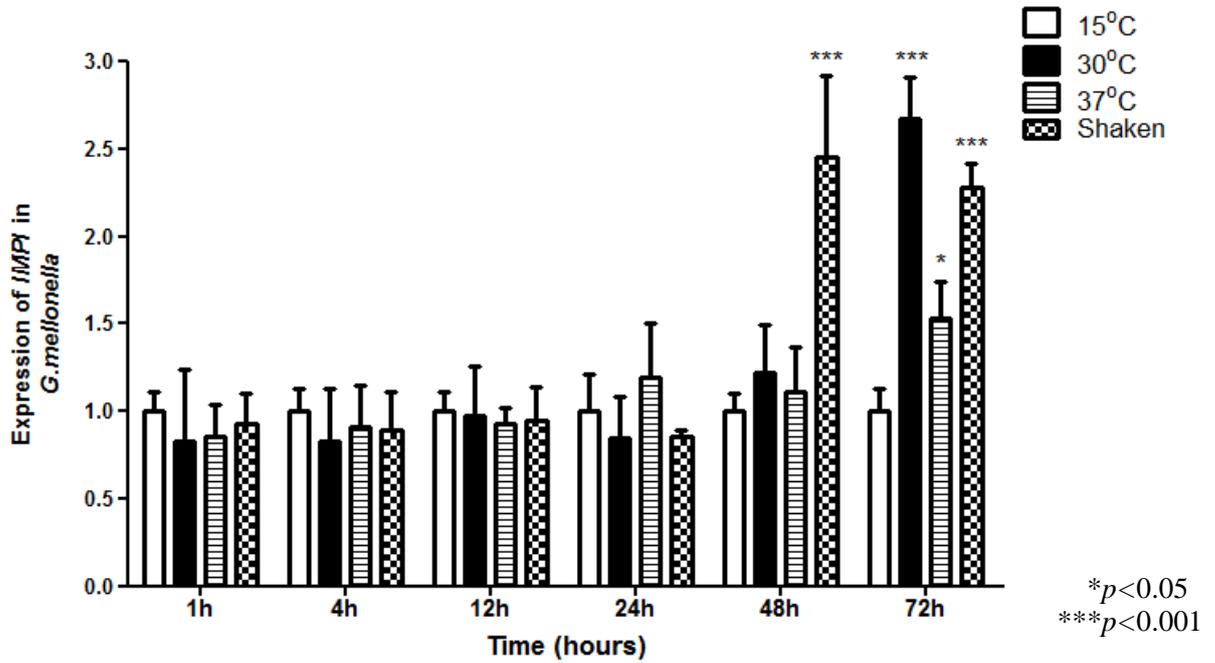
Fig. 4.11 qPCR analysis of *IMPI* gene expression in larvae thermally and physically stressed for 1, 4, 12, 24, 48 and 72h.
Larvae were normalised against S7e housekeeping gene. Two-Way Anova performed comparing treatments of 30°C, 37°C and physically stressed larvae against 15°C control larvae at each time point.
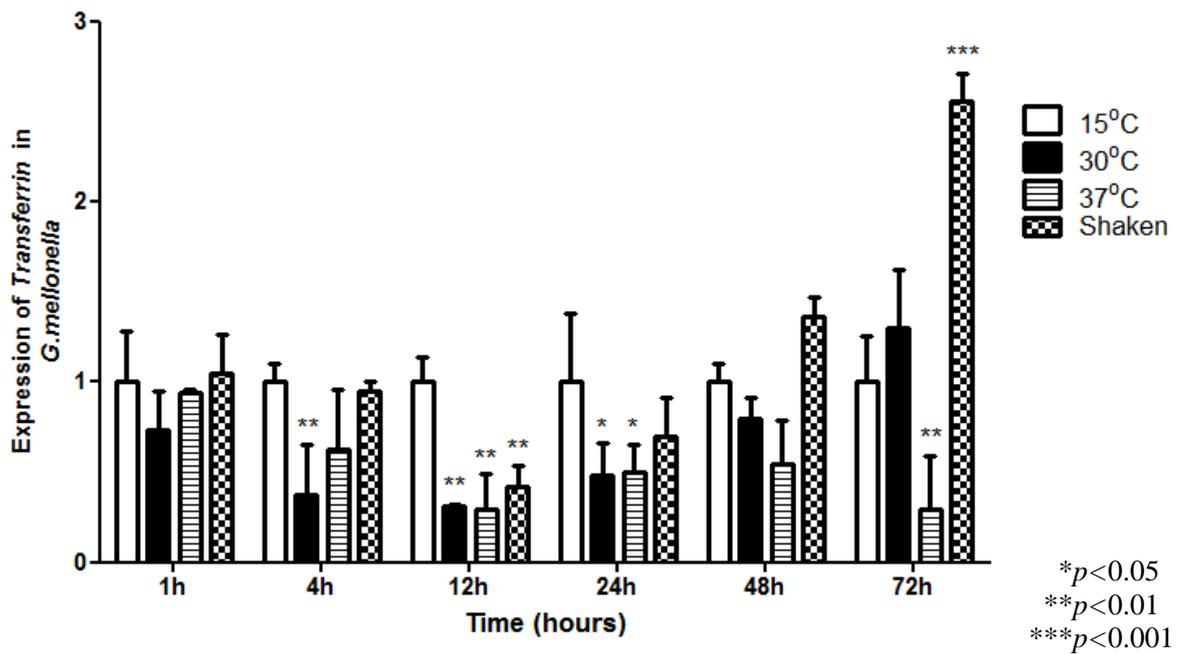
Fig. 4.12 qPCR analysis of *Transferrin* gene expression in larvae thermally and physically stressed for 1, 4, 12, 24, 48 and 72h.
Larvae were normalised against S7e housekeeping gene. Two-Way Anova performed comparing treatments of 30°C, 37°C and physically stressed larvae against 15°C control larvae at each time point.

4.5 Summary

The 1-D SDS PAGE analysis demonstrated an increase in expression of prophenoloxidase, arylphorin and apolipophorin at 24 h in those larvae that had been thermally (30°C and 37°C) or physically stressed but the expression of these proteins declined in larvae incubated for 48 and 72 h. 2D-SDS PAGE demonstrated an increased abundance of a number of immune related proteins in the 37°C pre-incubated larvae for 24h but declined thereafter.

The abundance of immune related proteins from the haemolymph of thermally and physically stressed larvae increased at 24h and declined at 48h and 72h to levels comparable to the control. It was observed that the expression of antimicrobial genes in response to thermal and physical stress was similar to the control at 1h with exception of *Gallerimycin,* while a drop in fold expression was seen in all genes up to 12h in the 30°C, 37°C and physically stressed larvae when compared to the control. *IMPI* expression remained relatively constant in all larval treatments up to 12h unlike *Galiomicin, Gallerimycin* and *Transferrin*, but the expression of all genes at 24h within the 30, 37°C and physically stressed larvae began to rise thereafter. *Galiomicin* demonstrated an increase in expression among 30°C, 37°C and physically stressed larvae after 24h but dropped after 48h and never surpassed the control larvae at any time point.

4.6 Effect of physical and thermal stress on haemocytes density

Exposing larvae to physical or thermal stresses lead to an increase in the density of circulating haemocytes in the haemolymph 24 h later (Fig. 4.13). In particular larvae pre-exposed to 37°C for 24 h showed 6.23 ± 0.04 x 10^{7} haemocytes per ml of haemolymph compared to 5.97 ± 0.04 x 10^{7} per ml in larvae incubated at 15°C ($p < 0.001$). Those larvae that were physically stressed showed a similar increase in haemocyte density at 24 h i.e. (6.23 ± 0.05 x10^{7}/ml). Interestingly the haemocyte density in larvae pre-incubated at 37°C or physically stressed declined at 48 and 72 h possibly suggesting a return to pre-stress levels. Larvae incubated at 15°C showed no significant alteration in haemocyte density over the 72 h.
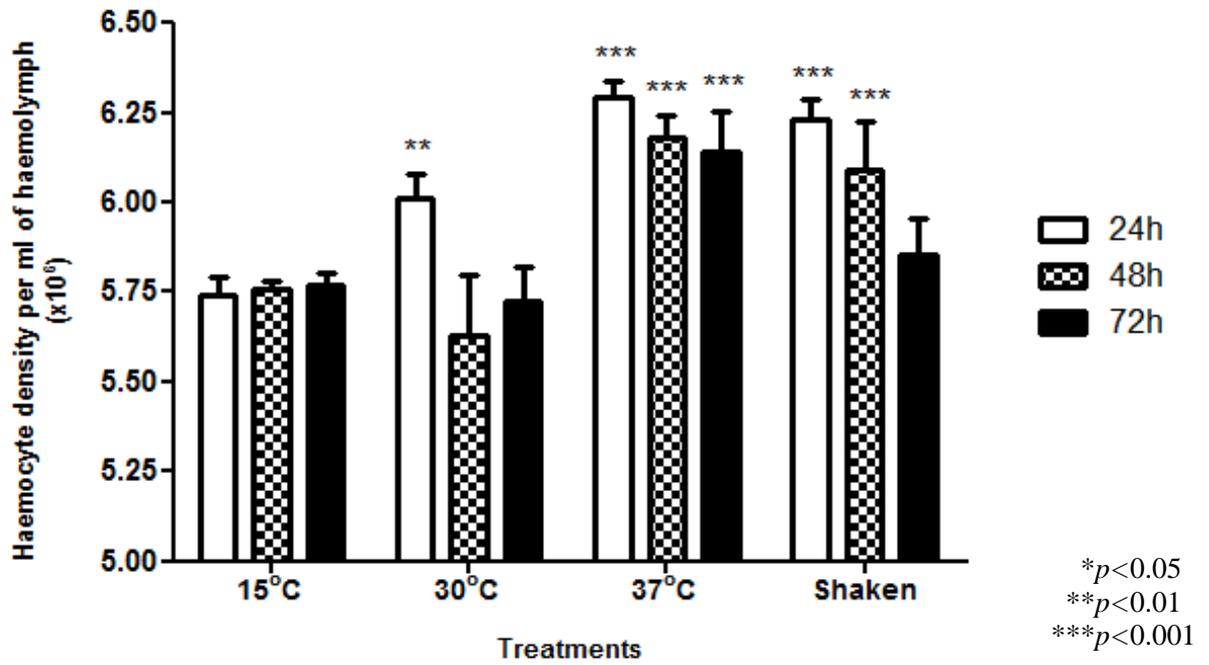
Fig. 4.13 Haemocyte density of pre-incubated larvae thermally stressed (15°C, 30°C and 37°C) or physically stressed for 24, 48 or 72h.

4.7 FACS analysis and identification of haemocyte populations by immunofluorescence microscopy

The sorting of the cell populations by FACS ARIA allowed the isolation of haemocytes based on morphology depicted on the scatter plot which identified populations P1, P2, P3, P5 and P7. The P1 population on the scatter plot was identified as a small cell with a low abundance of granular inclusions (Fig. 4.14). Based on the scatter plot location, appearance and apparent change in the P1 population over time in thermally and physically stressed larvae it is most likely a prohaemocyte (Kavanagh and Reeves, 2004; Tanada and Kaya, 1993). P2 granular like haemocytes were viewed with rhodamine stain showing a large nucleus similar to the size of the P7 granular cell. This P2 haemocyte may be an immature granular cell that can develop into a large granular cell under the correct stimuli including metamorphosis (Zhai and Zhao, 2012) and in this case stress stimuli triggering physiological change. The P3 haemocyte population was not recovered in sufficient numbers for identification purposes.

The P5 haemocytes, the small-medium cells with low granularity, appeared under the microscope as a haemocyte with a large nucleus and some small globular like inclusions, which is very similar to the description of an adipohaemocyte based on the literature (Kavanagh and Reeves, 2004; Tanada and Kaya, 1993). P7, the large granular cells, resembled a granular like haemocyte or granulocyte that function in insects to phagocytose pathogens along with other granular cell functions (Kavanagh and Reeves, 2004; Tanada and Kaya, 1993).

4.8 Effects of thermal and physical stress on haemocyte populations distributions as assessed using FACS ARIA

Larvae were exposed to thermal and physical stresses for 24, 48 and 72h before haemocytes were extracted, quantified and fixed prior to running through the FACS ARIA to assess the relative proportions of each population. A number of haemocyte populations were identified based on their morphology which included the P1, a population of small cells with a low abundance of granular inclusions, P2 the granular like cells, P3 the large cells with low granularity, P5 the small-medium cells with low granularity and the P7 the large granular cells.
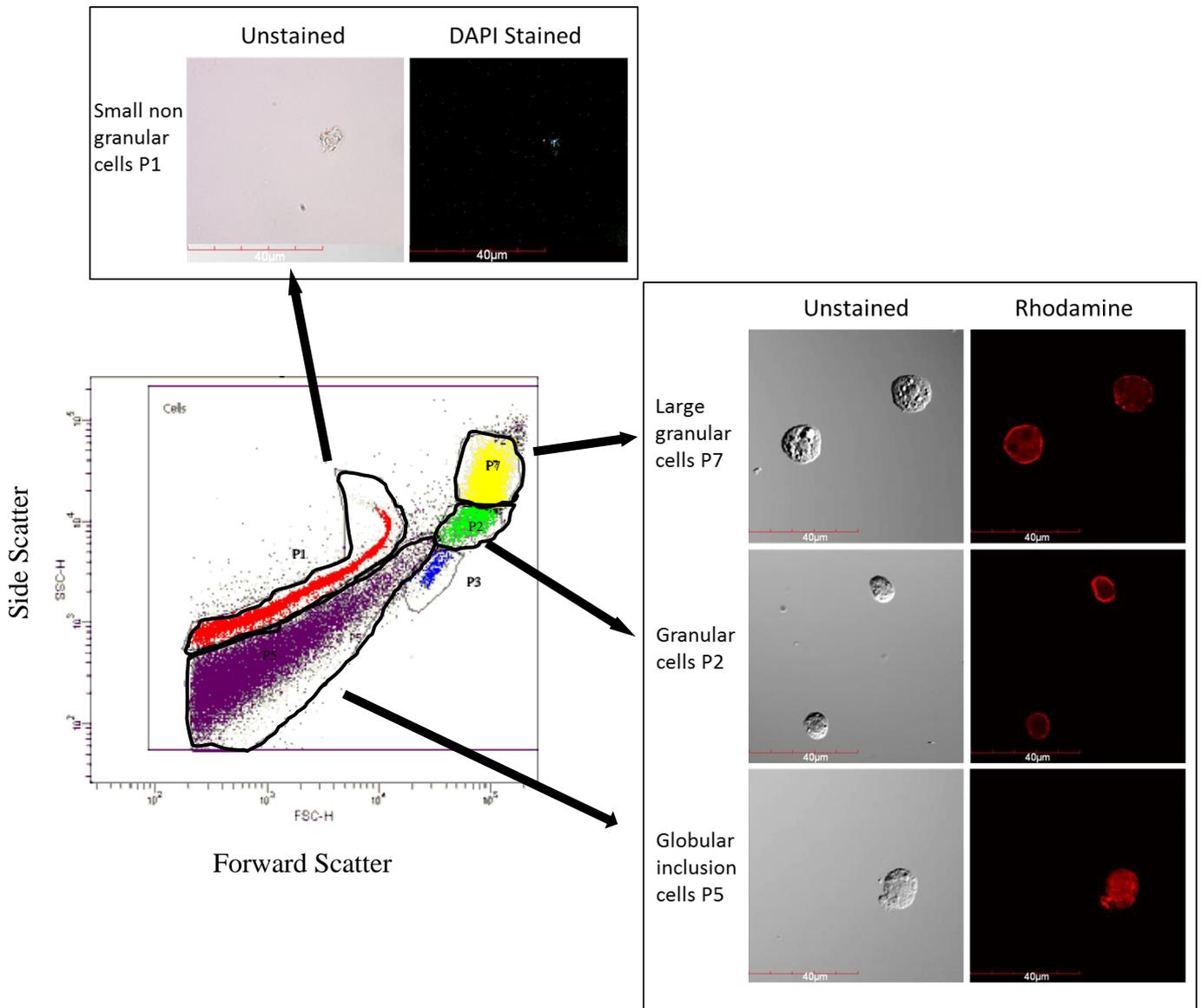
Fig. 4.14 FACS scatter plot image of cell populations (left) and identified populations viewed on an Olympus confocal immunofluorescence microscope.
(Right) unstained and rhodamine stained; P7 Large granular cells, P2 small granular cells and P5 Globular inclusion cells. (Top) unstained and DAPI stained P1, small non granular cells.

A change in the proportions of haemocyte populations was observed particularly at 48 and 72h as the P1 population was increased among the thermally and physically stressed larvae at 24, 48 and 72h compared to the control. The P1 population was significantly higher within the 30°C and physically stressed larvae at all-time points but the 37°C incubated larvae, which had a higher proportion of P1 haemocytes than the control, still had a lower proportion than the 30°C incubated and physically stressed larvae (Fig. 4.15A). The similar changes in haemocytes observed between the 30°C and physically stressed larvae is likely from their similar incubation temperature of 30°C whereas the 37°C incubation of larvae may impair the production of these P1 cells. It is also interesting to note the alteration or development of the P1 population as seen on the scatter plot which shows a sigmoidal shape develop at the upper region of the P1 population but this was not observed for the 15°C control larvae potentially because the effect only occurs at temperatures above 15°C (Appendix, Fig. 9.1).

It was observed that the P3 population was reduced in 30°C ($1.07\pm0.0\%$, $p<0.001$), 37°C ($1.45\pm0.05\%$, $p<0.001$) and physically stressed ($1.25\pm0.05\%$, $p<0.001$) larvae at 24h when compared to the control larvae ($2.25\pm0.05\%$) (Fig. 4.15B). This trend followed through to the 48 and 72h although the decrease was not as significant and the difference appears to reduce between the control and the thermally and physically stressed larvae with time.

The P5 population (Fig. 4.17A) demonstrated a significant rise at 24h in the 37°C ($53.15\pm1.05\%$, $p<0.05$) and the physically stressed ($54.35\pm2.85\%$, $p<0.01$) larvae but a significant decline in the 30°C ($46.25\pm1.55\%$, $p<0.05$) incubated larvae when compared to the control ($49.65\pm0.05\%$). The 37°C incubated larvae demonstrated a significantly higher presence of the P5 haemocytes at 48h ($66.80\pm1.10\%$, $p<0.001$) and 72h ($61.85\pm0.15\%$, $p<0.001$) over the control at the same time points ($55.85\pm0.05\%$) and ($44.35\pm0.05\%$) unlike the 30°C and physically stressed larvae which showed significantly reduced levels of these haemocytes at the same time points.

The combined granular cell population of P2 and P7 at 24h showed the overall population was significantly reduced among the larvae incubated at 30°C, 37°C or physically stressed when compared to the control larval haemocyte proportions (Fig. 4.16A). At 48h these two granular cell populations appeared different and were grouped either as P2 smaller granular haemocytes (Fig. 4.16B) or P7 large granular haemocytes (Fig. 4.16C). The P2 smaller granular haemocytes demonstrated a significant ($p<0.001$) reduction in the proportion of haemocytes among the 30°C, 37°C and physically stressed larvae at 48h and 72h when compared to the control larvae. It was previously demonstrated by García-García, (2009) that a population of haemocytes in the same location as these P7 haemocytes were large granular phagocytosing haemocytes.

The P7 population (large granular haemocytes) demonstrated a significantly higher proportion among larvae incubated at 30°C or physically stressed at 48h (20.45±2.05%, $p<0.001$ and 17.75±0.77%, $p<0.001$) and at 72h (22.55±1.62%, $p<0.001$ and 23.90±1.13%, $p<0.001$) respectively when compared to the control larvae at 48h (5.95±0.07%) and at 72h (2.35±0.07%). The 37°C incubated larvae demonstrated a similar proportion of P7 haemocytes to the control at 48h and 72h.

4.9 Summary

Physically and thermally stressed larvae demonstrated the highest abundance of circulating haemoctyes at 24h, but these haemocytes declined at 48 and 72h. A rise in the proportion of P1 haemocytes was demonstrated by FACS in the thermally and physically stressed larvae compared to the control larvae. The P3 population showed a general reduction in abundance among the thermally and physically stressed larvae over time compared to the control larvae. The P5 population demonstrated the highest proportion of these haemocytes in the 37°C incubated larvae at 48h and 72h. The 30°C incubated and physically stressed larvae both had significantly higher proportions of P7 haemocytes at 48 and 72h compared to the control and the 37°C incubated larvae. The P2 haemocytes remained low in the thermally and physically stressed larvae compared to the control larvae.

Fig. 4.15A

Fig. 4.15B

P1

P3
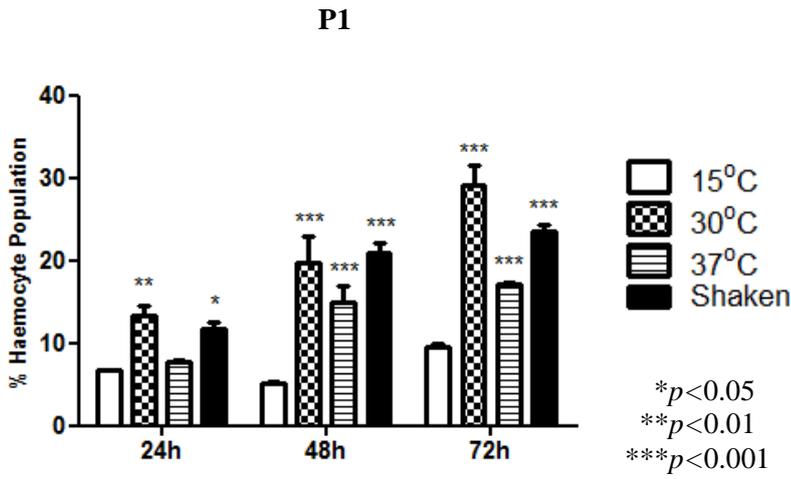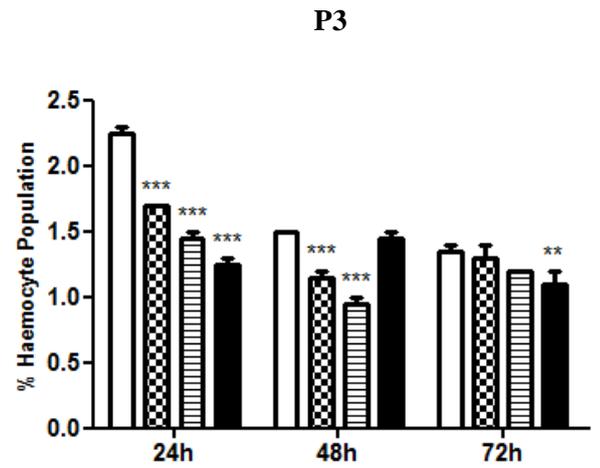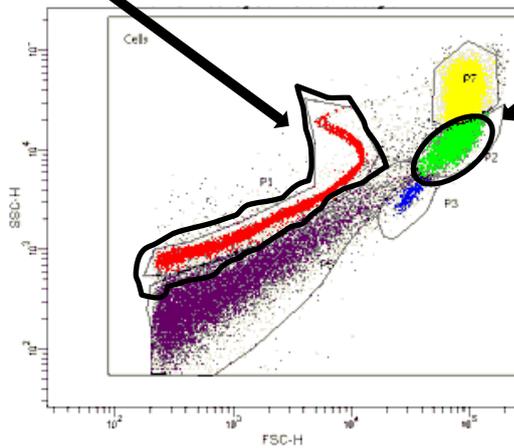
Fig. 4.15C

Fig. 4.15 Proportion haemocyte populations P1 (Fig. 4.15A) and P3 (Fig. 4.15B) isolated from thermally (15^oC, 30^oC and 37^oC) and physically stressed larvae. Scatter plot image below (Fig. 4.15C) highlighting the haemocyte populations of 1 and 3, 15^oC = Control group.

Fig. 4.16 Proportion of haemocyte populations P2 and P7 isolated from thermally (15°C, 30°C and 37°C) and physically stressed larvae.

P2 and 7 of 24h combined (Fig. 4.16A) and individually separated sub-populations P2 (Fig. 4.16B) and P7 (Fig. 4.16C) for 48 and 72h time points. Scatter plot image below highlighting the haemocyte populations of 2 and 7 combined (Fig. 4.16D) at 24h and individually right sub-populations 2 and 7 (Fig.4.16E). 15°C = Control group.

Fig. 4.17A   Fig. 4.17B

P5

*p<0.05
**p<0.01
***p<0.001
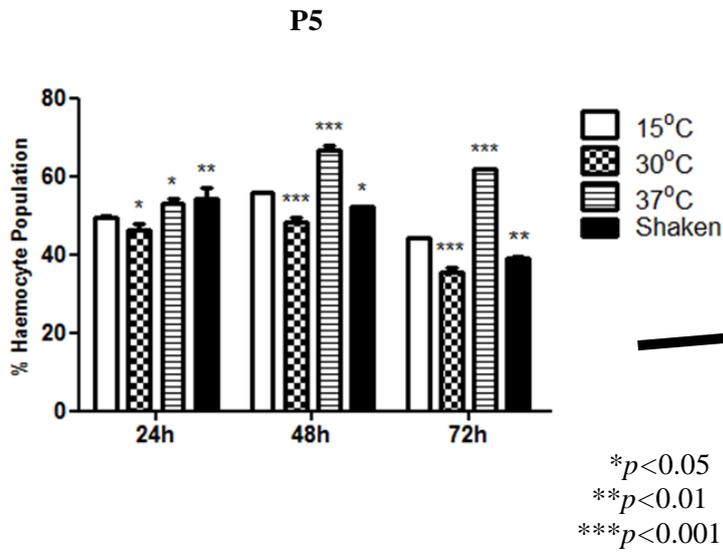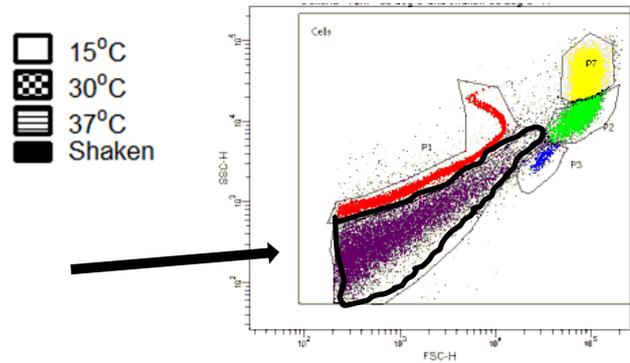
Fig. 4.17 Proportion of haemocytes in population P5 isolated from thermally (15°C, 30°C and 37°C) and physically stressed larvae (Fig. 4.17A).
Scatter plot image highlighting P5 haemocyte population (Fig. 4.17B). 15°C = Control group.

4.10 2D SDS PAGE analysis of haemocyte cell lysates from thermally and physically stressed larvae incubated for up to 72h

Larvae were thermally or physically stressed as described previously and incubated for 24, 48 or 72 h at 15, 30 or 37$^{\text{o}}$C as appropriate. Haemolymph was extracted from ~50 larvae per treatment and haemocytes were lysed and adjusted to 400μg of protein before separating by 2D-SDS PAGE (Fig. 4.18). Resolved gels demonstrated a number of changes in protein abundance such as the microbial binding protein calreticulin (spot 4 and 7) which demonstrated a large fold increase in abundance among haemocytes extracted from the 30$^{\text{o}}$C, 37$^{\text{o}}$C incubated and physically stressed larvae particularly at 24h but its abundance appeared to decrease there after (Fig. 4.19A). Calreticulin is known to partially localise on the surface of neutrophils and anti-microbial bound calreticulin signals to cells via a G-protein activating neutrophils that subsequently can generate superoxide anions (Yoshida *et al*., 1996). Previously it has been shown in *G. mellonnella* to have a role in the early phase encapsulation response and therefore an important function in the cellular immune response of invertebrates (Choi *et al*., 2002).

ApoLp-III (spot 35) is capable of binding to microbial pathogen surfaces (Whitten *et al*., 2004) which was observed in haemocytes to be low in abundance among the thermally and physically stressed larvae at 24h but remained at a similar level to the control larvae at 48h and 72h (Fig. 4.19C). ApoLp-III has a number of important functions including lipid transport and in the innate immune response (Gupta *et al*., 2010). Translationally-controlled tumor protein homolog (TCTP) (spot 27) demonstrated the highest fold abundance in haemocytes at 24h in the 37$^{\text{o}}$C (2.77) while the 30$^{\text{o}}$C and physically stressed larvae had a similar fold change of 1.59 and 1.98 compared to the control but the levels returned to an abundance resembling the control at 48h and 72h (Fig. 4.19B). TCTP has been shown to bind to pathogens, opsonise and promote phagocytosis of pathogens (Wang *et al*., 2013). It can also induce the production of anti-microbial peptides via signalling pathways and TCTP may act as a dual-functional protein involved in both the cellular and the humoral immune response of *B.mori* (Wang *et al*., 2013).

FK506-binding protein (spot 26) which has been reported to protect the cells against oxidative damage (Gallo *et al*., 2011) was observed to be higher in abundance

among the thermally and physically stressed larvae (Fig. 4.19D). The oxidative stress proteins glutathione-S-transferase-like protein (GST) (spot 30) and superoxide dismutase [Cu-Zn] (SOD) (spot 34) showed no obvious change in abundance within haemocytes over time (Appendix, Table 9.1)

The 14-3-3 epsilon protein (spot 23) which has links to receptor tyrosine kinase signalling including RAS related GTPases (Finlin and Andres, 1999) as well as regulating apoptotic cell death, and cell cycle control (Fu *et al*., 2000) demonstrated higher levels from haemocytes of the thermally and physically stressed larvae when compared to the control particularly at the 24h but declined at 48 and 72h. Interestingly the proteasome 35kD subunit (spot 20) found in haemocytes had a 3.45 fold increase at 48h within the 37°C incubated larvae compared to the smaller increases within larval haemocytes from the 30°C (1.25) and physically stressed (1.73) larvae at the same time point. An increase in these heat shock related proteins would be expected particularly at the elevated temperature of 37°C and it was previously shown that increased temperature (38°C) resulted in higher abundance of HSP 90 in *G.mellonella* which also aided in better survival following microbial challenge compared to larvae incubated at 28°C (Wojda and Jakubowicz, 2007). In comparison the abundance of heat shock protein hsp21.4 (spot 24) decreased initially within the physically and thermally stressed larval haemocytes at the 24h time point but at 48h and 72h it was similar to the control.

Some metabolic functioning proteins were shown to be altered in haemocytes with exception of putative enolase protein, partial (spot 13) functioning as an enzyme of the glycolysis pathway important in energy generation (Pancholi, 2001), here it was observed to have very little change through out the stress conditions and time. Pyruvate dehydrogenase E1 component subunit beta, mitochondrial (spot 22) important in linking the glycolysis and tricarboxylic cycle (TCA) together (Zhou *et al*., 2001) was observed to be substantially higher within the 30°C (2.09), 37°C (2.83) and physically stressed (1.95) larval haemocytes at 24h but dropping back to normal levels comparable to the control at the 72h. This increase may result from larval haemocytes undergoing changes in response to higher temperatures which favour physiological activities. Aldehyde dehydrogenase (spot 12), known to metabolise a wide range of endogenous and exogenous aldehydes including ethanol (Lindahl, 1992; Vasiliou *et al*., 2000), was observed to have initially low levels of expression in the thermally and physically stressed larval haemocytes at 24h but this dramatically rose within the

37°C larvae (3.11) at 48h and in the 30°C (3.32), 37°C (3.99) and physically stressed larvae (5.71) at 72h when compared to the control (Fig. 4.19E). This change may be attributed to the higher metabolic demands of the larvae with time and greater aldehydes being generated from oxidative lipid degradation known as lipid peroxidation that must be detoxified (Shin *et al*., 2009).

GAPDH (spot 17) demonstrated a small fold increase within haemocytes from the 30°C (1.75) and physically stressed (1.48) larvae at 48h but remained relatively constant throughout the stresses and time points of 24h and 48h. Signalling protein Ras-related GTP binding protein Rab11 (Rab) (spot 28) is a member of GTPases that recruit specific sets of effector proteins onto membranes regulating vesicle formation, actin- and tubulin-dependent vesicle movement and membrane fusion (Stenmark and Olkkonen, 2001). These Rab functions includes vesicle transport required specifically for phagocytosis described in *D.melanogaster* (Shim *et al*., 2010), here a higher abundance of Rab was observed within the haemocytes extracted from larvae incubated at 30°C (1.58), 37°C (2.96) or physically stressed (1.27) at 24h but dropped thereafter at 48h and 72h to a level similar to the control (Fig. 4.19F).

4.11 Summary

Haemocyte proteins capable of pathogen recognition (calreticulin and TCTP) or activation of haemocyte phagocytosis (Rab) demonstrated elevated abundance at 24h within the thermally and physically stressed larvae but declined to normal levels at 72h. ApoLp-III was initially low within the thermally and physically stressed larval haemocytes at 24h before returning to a level similar to the control at 48h and 72h. Low apoLp-III abundance within haemocytes at 24h coincided with higher apoLp-III observed within the haemolymph at 24h analysed by 1D and 2D SDS PAGE from stressed larvae (Section 4.3). The relative abundance of oxidative stress protein FK506-binding protein, thermal stress protein proteasome 35kD subunit and metabolic proteins demonstrated an increase within haemocytes from thermally and physically stressed larvae.
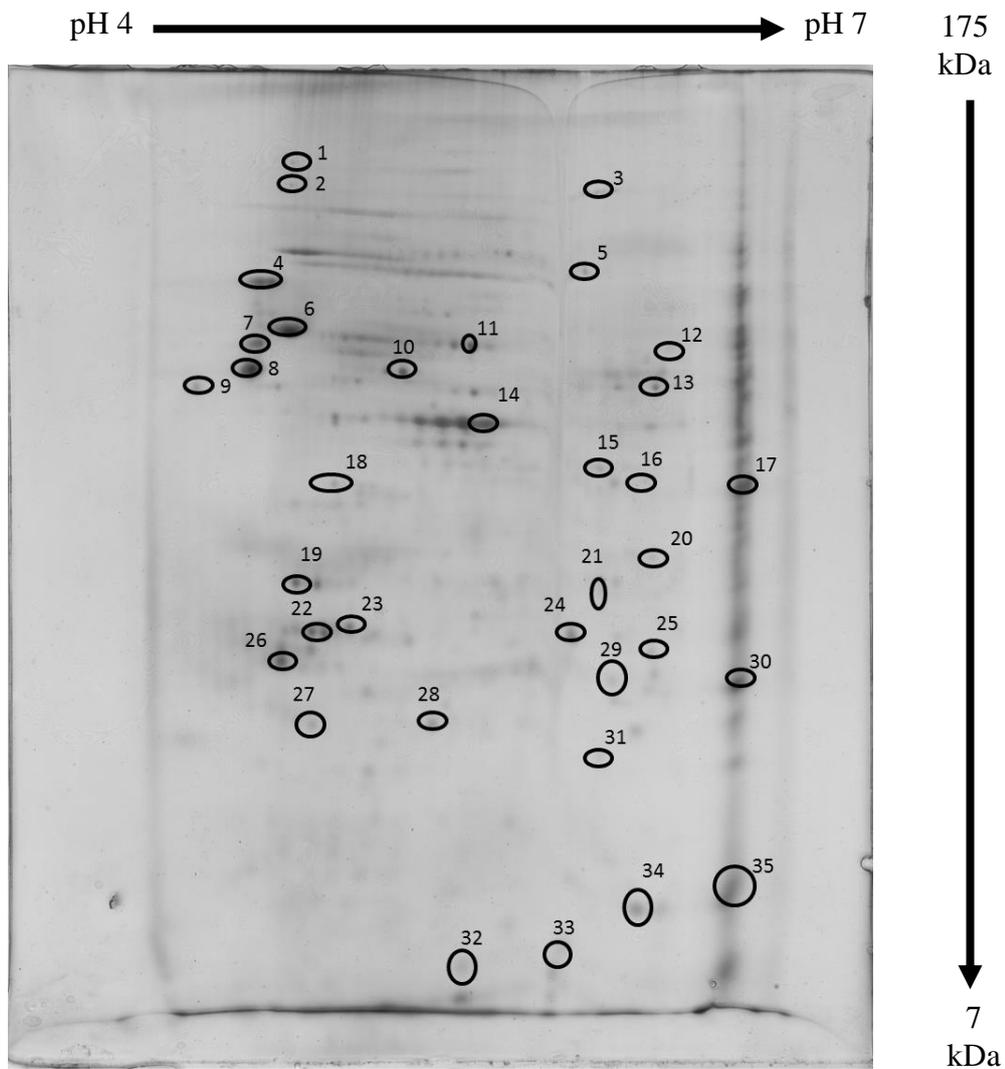
Fig. 4.18 2D-SDS PAGE representative of haemocyte proteins excised for trypsin digestion and identified by LC/MS. Replicates (n=3).

Spot no.	Protein Identity	Organism	Mr	pI	Score	Seq. coverage (%)	Accession no.	Protein Function
1	Arylphorin	*G. mellonella*	83651	5.23	143	6	AAA74229	Amino acid storage and immune functions.
2	Control protein HCTL033	*Heliconius erato*	52215	4.75	99	5	AEU11802	Catalyzing the disulfide bonds of new polypeptides in the ER.
3	Hypothetical protein KGM_05737	*Danaus plexippus*	106217	5.80	102	1	EHJ73077	Catalyzing the disulfide bonds of new polypeptides in the ER.
4*	Calreticulin	*G. mellonella*	46343	4.52	631	34	BAB79277	Binding protein for anti-microbial peptides.
5	Putative dipeptidyl peptidase iii	*Danaus plexippus*	69623	5.86	267	9	EHJ68135	Zinc-dependent enzymes with specific cleaving of N terminus from different length peptides.
6	Protein disulfide isomerase	*Papilio xuthus*	55531	4.62	261	12	BAM17793	Catalyzing the disulfide bonds of new polypeptides in the ER.
7	Calreticulin	*G. mellonella*	46343	4.52	424	27	BAB79277	Binding protein for anti-microbial peptides.
8	DNA supercoiling factor	*Danaus plexippus*	37823	4.56	198	37823	EHJ69265	Calcium sensors and signal modulators.
10	H+ transporting ATP synthase beta subunit isoform 2	*Danaus plexippus*	54882	5.20	1031	56	EHJ64315	ATP synthase that uses a proton gradient to drive ATP synthesis.
11	Protein disulfide-isomerase like protein ERp57	*Danaus plexippus*	55108	5.36	376	14	EHJ76798	Catalyzing the disulfide bonds of new polypeptides in the ER.
12*	GH13324 (aldehyde dehydrogenase)	*D. grimshawi*	57255	6.57	99	4	XP_001993018	Roles in acetaldehyde oxidation.
13	Putative enolase protein, partial	*G. mellonella*	40844	5.58	831	58	AGB91349	Enolase, is a metalloenzyme involved in Glycolosis.
14	Actin	*D. melanogaster*	42203	5.30	694	49	AAA28314	Nucleotide-Binding Domain.
15	Arginine kinase	*Scolytus scolytus*	30621	5.96	313	26	AEB32417	Enzyme converts (ATP and L-arginine) to (ADP + Nomega-phospho-L-

							arginine).
16	ADP/ATP tranlocase	*Danaus plexippus*	33036	9.80	484	29	EHJ74594	Mitochondrial carrier protein
17	Glyceraldehyde-3-phosphate dehydrogenase	*B. mori*	35522	7.70	431	34	BAE96011	Enzyme of the glycolysis pathway.
19	14-3-3 epsilon protein	*B. mori*	29769	4.66	728	45	NP_001091764	Regulator of cell survival in response to oxidative stress and other apoptotic signals.
20	Proteasome 35kD subunit	*Papilio xuthus*	30462	6.60	433	34	BAM18671	Multisubunit proteolytic complex involved in protein degradation.
21	32 kDa ferritin subunit	*G. mellonella*	26731	5.69	309	28	AAL47694	Involved in iron storage, transport and immune system.
22	Pyruvate dehydrogenase E1 subunit beta, mitochondrial	*Apis mellifera*	38657	5.83	465	38	NP_001229442	Catalyses pyruvate to acetyl-CoA in the bridging step between glycolysis and the TCA cycle.
23	14-3-3 protein zeta	*B. mori*	28195	4.90	787	60	Q2F637	Acts as a molecular adaptor enabling a range of cellular functions.
24	Heat shock protein hsp21.4	*B. mori*	21391	5.79	357	47	NP_001036985	Small HSP functioning in oligomers as an ATP independent chaperone.
25	GJ20789	*D. virilise*	24708	5.89	61	3	XP_0020 49371	Unknown function.
26*	FK506-binding protein	*Plutella xylostella*	23931	4.71	272	27	AGA84515	FK506-BP an immunophilin protein, with roles in immunoregulation.
27*	Translationally-controlled tumor protein homolog	*B.mori*	19905	4.66	357	42	NP_001037572	TCTP capable of pathogen recognition and promoting phagocytosis.
28*	Ras-related GTP-binding protein Rab11	*Danaus plexippus*	24107	5.37	681	59	EHJ63705	Rab GTPases, roles in vesicle formation, motility and fusion.
29	Proteasome 25 kDa subunit	*Danaus plexippus*	25940	6.07	310	19	EHJ74121	Proteasome, proteolytic protein involved in protein degradation.

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30	Glutathione-S-transferase-like protein	*G. mellonella*	24358	6.90	683	78	AAK6436 2	Function in detoxification of toxins,drugs and ROS.
31	19.9 kDa small heat shock protein	*Choristoneura fumiferana*	19960	6.44	101	13	AAZ14791	Small HSP functioning in oligomers as an ATP independent chaperone.
32	Cellular retinoic acid binding protein	*B. mori*	14965	5.66	194	26	NP_00103 7364	Lipocalins, transporters for small lipids, steroid hormones, bilins, and retinoids.
33	Cellular retinoic acid binding protein	*Danaus plexippus*	14891	5.63	204	42	EHJ79039	Lipocalins, transporters for small lipids, steroid hormones, bilins, and retinoids.
34	Superoxide dismutase [Cu-Zn]	*B. mori*	16006	5.78	139	19	NP_00103 7084	Superoxide dismutases catalyse the conversion of superoxide radicals to molecular oxygen
35*	Apolipophorin-III	*G. mellonella*	20499	8.59	414	64	P80703	Apolipophorin-III PRR of microbial components.

Table 4.2 *G.mellonella* haemocyte proteins (1-35) excised from a 2D-gel and identified by LC/MS.
* representing protein with fold change represented in graph (Fig. 4.19)

Fig 4.19A

Calreticulin

Fig 4.19B

TCTP

Fig 4.19C

ApoLp-III

Fig 4.19D

FKBP

Fig 4.19E

Aldehyde DH

Fig 4.19F

Rab
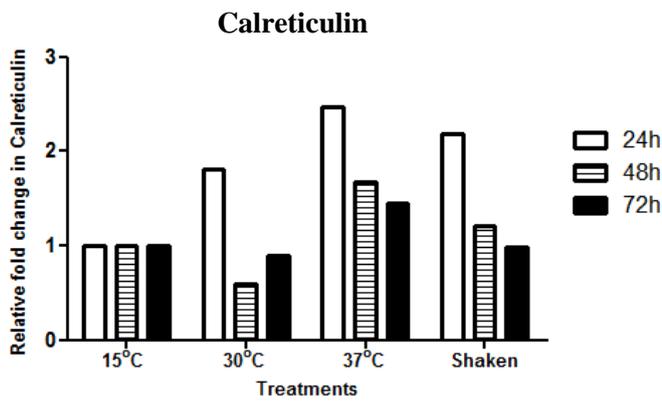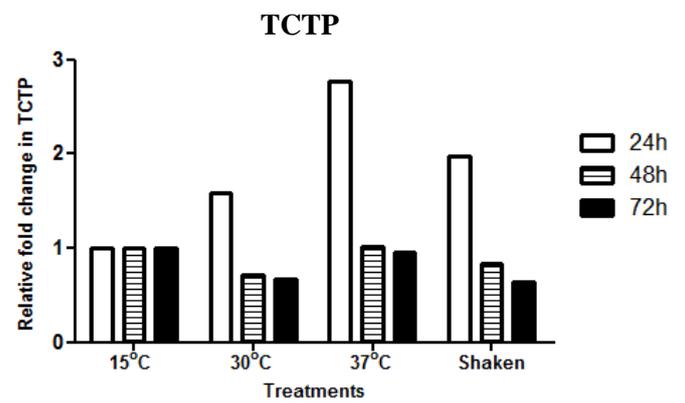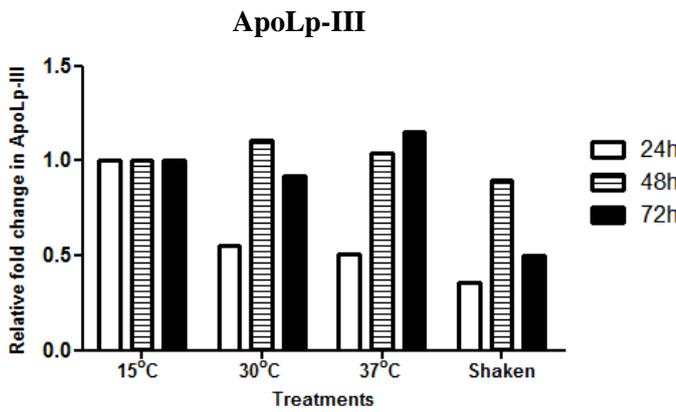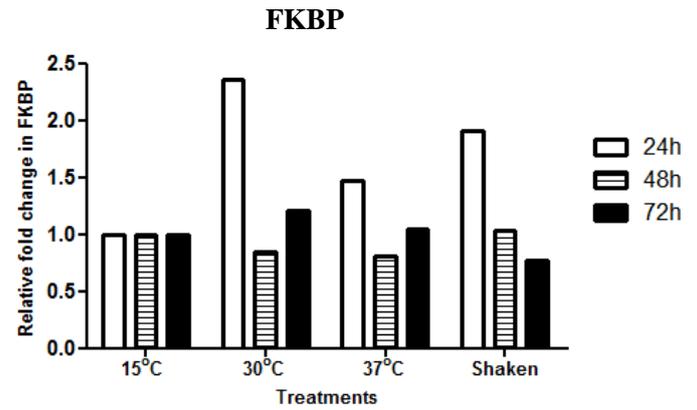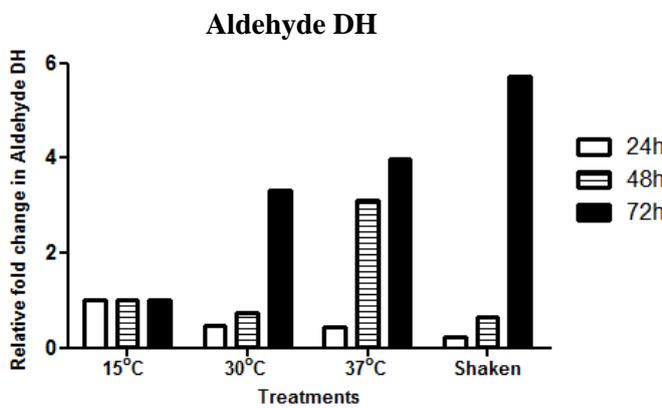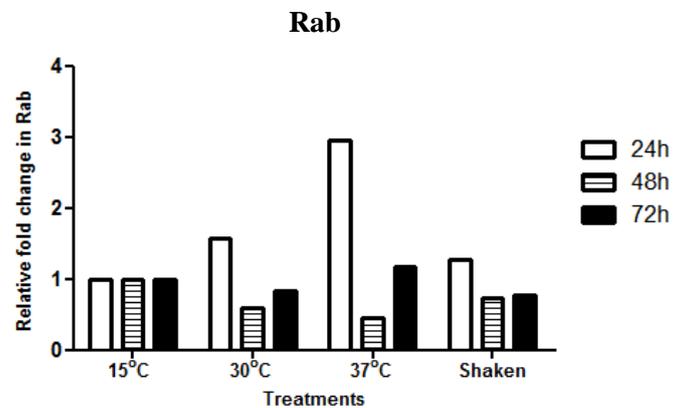
Fig. 4.19 Relative abundance of haemocyte proteins from thermally and physically stressed larvae over 24, 48 and 72h.
Protein fold changes with respect to the control larvae (15°C) for calreticulin (Fig 4.19A), TCTP (Fig. 4.19B), apoLp-III (Fig. 4.19C), FKBP (Fig.4.19D), aldehyde dehydrogenase (Fig. 4.19E) and Rab (Fig. 4.19F) Replicates (n=3). Table below with protein identities and protein fold expression changes (Table 4.3).

4.12 Analysis of the proteome of *G.mellonella* larval haemocytes and a comparison to human neutrophils

This section assessed the phosphorylation of NADPH^{phox} oxidase, and the similarities in signalling proteins and enzymes activated in response to artificial stimulants PMA and fMLP detected by 2-D SDS PAGE using a phosphorylation stain (Pro-Q Diamond). 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). PMA activates the NADPH oxidase cascade through Protein Kinase C and is widely used for neutrophil activation (Fallon *et al*., 2010) and previously used in haemocyte assays (Bergin *et al*., 2005). fMLP functions in mammals as a strong enhancer of phagocytosis (Ogle *et al*., 1990) and a chemoattractant (Heit *et al*., 2008).

4.13 Phosphorylated proteins identified from stimulated and un-stimulated haemocytes using Pro-Q stain and 2D SDS PAGE

The detection of foreign components within the insect haemocoel leads to phosphorylation processes within the immune response (Gillespie and *et al*., 1997), activating the prophenoloxidase cascade and protein kinases responsible for the activation of the NADPH oxidase complex (Renwick *et al*., 2007). It is also suggested that phosphorylation may mediate the encapsulation process within haemocytes (Lapointe *et al*., 2012). The phosphorylation of proteins is crucial to the activation and translocation of NADPH oxidase components (El Benna *et al*., 1994; Renwick *et al*., 2007).

A number of proteins demonstrated increased phosphorylation within the PMA stimulated haemocytes and these included, malate dehydrogenase (Spot 4), 26S protease regulatory subunit 8-like (Spot 5), transitional endoplasmic reticulum ATPase TER94 (Spot 18), hydroxyacyl-coenzyme A dehydrogenase (Spot 26), vacuolar ATPase B subunit (Spot 43) and 60 kDa heat shock protein, mitochondrial-like (Spot 47) (Fig. 4.23) (Table 4.3). Malate dehydrogenase has an important role in the TCA cycle for energy metabolism (Musrati *et al*., 1998) and demonstrated a 1.57 fold increase in phosphorylation relative to the control/unstimulated haemocytes. Malate dehydrogenase had an identical fold change in phosphorylation from fMLP stimulated haemocytes and PMA stimulated haemocytes. Previously Costa *et al.* (1995)

demonstrated that PMA stimulated the activation of NADP+ dependent malate dehydrogenase in macrophages which appears similarly activated for both PMA and fMLP stimulated haemocytes. Although the total abundance of malate dehydrogenase protein was higher in the PMA (1.25) and fMLP (1.15) stimulated haemocytes compared to the control, phosphorylation was substantially higher in the stimulated haemocytes (Table 4.4) (Fig. 4.20).

The enzyme hydroxyacyl-coenzyme A dehydrogenase functions in fat metabolism but here PMA stimulated haemocytes had raised phosphorylation of this protein by 35% compared to the control. It has previously been shown that PMA stimulation can involve metabolism in fat cells of rats and this may occur in insect cells (Cherqui *et al*., 1986). It was also demonstrated that PMA can activate macrophages and increase lipid peroxidation which has implications for protein kinase C in cellular lipid metabolism (Carter *et al*., 1996). Hydroxyacyl-coenzyme A dehydrogenase was observed to have greater phosphorylation within fMLP stimulated haemocytes (1.44) when compared to the control haemocytes. But the total abundance of hydroxyacyl-coenzyme A dehydrogenase was highest in the fMLP stimulated haemocytes at 1.78, making the proportion of phosphorylation low overall when compared to the control and a similar trend in protein abundance was observed within the PMA stimulated haemocytes (1.21).

The TER94 protein when in the phosphorylated state is known to prevent the apoptosis of cells in insects and here it was observed that the PMA stimulated larvae had a 1.38 fold increase in phosphorylation state compared to the control. fMLP stimulated haemocytes demonstrated a 3 fold increase in phosphorylation of TER94. The abundance of TER94 protein measured by colloidal staining demonstrated no large change in the control (1.0), fMLP (0.94) or PMA (1.17) stimulated haemocytes (Table 4.4) (Fig. 4.20).

The phosphorylation of vacuolar proton pump within the PMA stimulated haemocytes demonstrated the highest phosphorylation at 1.25 compared to the control (1.0) and fMLP stimulated haemocytes at 0.54. It has been suggested that ER interactions with the phagocytic organelles appeared to be regulated by vacuolar proton pumps V-ATPase with roles also in the fusion properties of vesicles in forming the phagosome (Gagnon *et al*., 2002). The abundance of the vacuolar proton pump protein within the PMA stimulated haemocytes was lowest at 0.79 when compared to

the control and fMLP (1.04) which when taken into account makes the phosphorylation state appear higher in PMA stimulated haemocytes.

Actin, (cytoskeletal 3A) (Spot 33), Actin 5 (Spot 35) both demonstrated higher phosphorylation levels within the PMA stimulated haemocytes which is known to coincide with actin reassembly within activated phagocytosing haemocytes, enabling the processes of phagocytosis and degranulation to occur (Fallon *et al*., 2011a; Grogan *et al*., 1997). The colloidal staining analysis of protein abundance for actin, cytoskeletal 3A and actin 5 was lower at 0.42 and 0.82, respectively, within the PMA stimulated haemocytes, compared to the control, making these proteins phosphorylation state more substantial relative to abundance of protein (Fig. 4.21). Haemocytes stimulated with fMLP had increased phosphorylation of tubulin alpha chain (Spot 46) which is rearranged during the phagocytosis and degranulation events within the haemocyte but the relative abundance of the protein was higher within fMLP stimulated haemocytes at 1.56 compared to the control reducing its relative phosphorylation state (Table 4.4) (Fig. 4.21).

The heat shock related proteins of 26S protease regulatory subunit 8-like (Spot 5) and 60 kDa heat shock protein (Spot 47), mitochondrial-like, demonstrated the highest level of phosphorylation from PMA stimulated haemocytes at 1.24 and 1.68 fold expression respectively. Although 26S protease regulatory subunit had higher protein abundance within the PMA stimulated haemocytes therefore reducing the impact of the proteins phosphorylation state (Table 4.4) (Fig. 4.20). HSP 60 had reduced abundance within the PMA stimulated haemocytes (0.85) (Table 4.4) but a greater phosphorylation state was observed when compared to the control (Table 4.3) (Fig. 4.20). HSP 60 is associated with the activation of phagocytes such as macrophages within mammals (Kol *et al*., 2000). HSP 60 may be first released in response to PMA stimulation enabling activation of further phagocytes in response to HSP 60. It is also well established that HSPs play important roles upon activation of phagocytes such as macrophages by functioning as molecular chaperones protecting the macrophage from auto-oxidation damage during the respiratory burst (Teshima *et al*., 1996). The PMA stimulated haemocytes demonstrated higher level of phosphorylation in HSP 60 and may result in NADPH oxidase complex activation leading to greater production of ROS and therefore a need for prevention of auto-oxidation.

fMLP and PMA stimulated haemocytes demonstrated increased phosphorylation of a number of proteins including, malate dehydrogenase (Spot 4), transitional endoplasmic reticulum ATPase TER94 (Spot 18), hydroxyacyl-coenzyme A dehydrogenase (Spot 26) and tubulin alpha chain (Spot 46) (Fig. 4.23) (Table 4.3). fMLP stimulated haemocytes possessed a number of proteins with increased phosphorylation that were not observed within PMA stimulated haemocytes and these included phosphorylated peptidyl-prolyl cis-trans isomerase f, (PPIF) (Spot 7), nucleoside-diphosphate kinase NBR-A (Spot 8), H+ transporting ATP synthase beta subunit isoform 1(Spot 38) (Fig. 4.21).

The peptidyl-prolyl cis-trans isomerase are known to function in neutrophils as a key enzyme in the activation of the NADPH oxidase activity which occurs by a prolyl isomerase that specifically recognizes phosphorylated serine or threonine residues located immediately at the N-terminal of a proline, and then isomerizes the peptide bond (Wulf *et al*., 2005; Yaffe *et al*., 1997). Peptidyl-prolyl isomerase was lower in abundance compared to the control (Table 4.4) which enhances the level of phosphorylation present within fMLP stimulated haemocytes (Table 4.3) (Fig. 4.21). Nucleoside-diphosphate kinase NBR-A (NDP kinase) was demonstrated to be only activated by fMLP in neutrophils and not by PMA (Guignard and Markert, 1996) which was similarly observed here as fMLP stimulated haemocytes had greater phosphorylated NDP kinase (1.54) than the PMA stimulated haemocytes at 1.11 similar to the control (Table 4.3). It was also observed that the abundance of NDP kinase protein did not change between treatments (Table 4.4) (Fig. 4.21). NDP kinase binds to GTP-binding proteins in combination with formyl-peptide receptor has been suggested to activate superoxide anions through G-proteins which are central to ROS generation in phagocytic cells (Rossi, 1986). NDP kinase was suggested to regulate NADPH oxidase activity in neutrophils (Guignard and Markert, 1996; Wieland and Jakobs, 1992), this may similarly occur in phagocytosing haemocytes.

H+ transporting ATP synthase beta subunit isoform 1 which functions to synthesis ATP was observed to have higher phosphorylation (1.46 fold) when compared to the control, although fMLP stimulated haemocytes had a greater abundance of protein compared to the control therefore reducing fMLP stimulated haemocytes overall phophorylation level. H+ transporting ATP synthase beta subunit 2 demonstrated higher phosphorylation state and lower abundance of the protein within PMA and fMLP stimulated haemocytes when compared to the control

highlighting this pumps greater activation in PMA and fMLP stimulated haemocytes (Fig. 4.21). The PMA stimulated haemocytes also had higher phosphorylated vacuolar ATPase. It is well established that activated neutrophils generate an abundance of H+ during the large oxidative bursts generated through ATPase ion pumps (Merzendorfer *et al*., 1997). These proton pumps are also crucial for generating the low pH of the lysosmes and the phagosomes in phagocytes which enables their degradative function against pathogens (Kurashima *et al*., 1996; Sun-Wada *et al*., 2003).
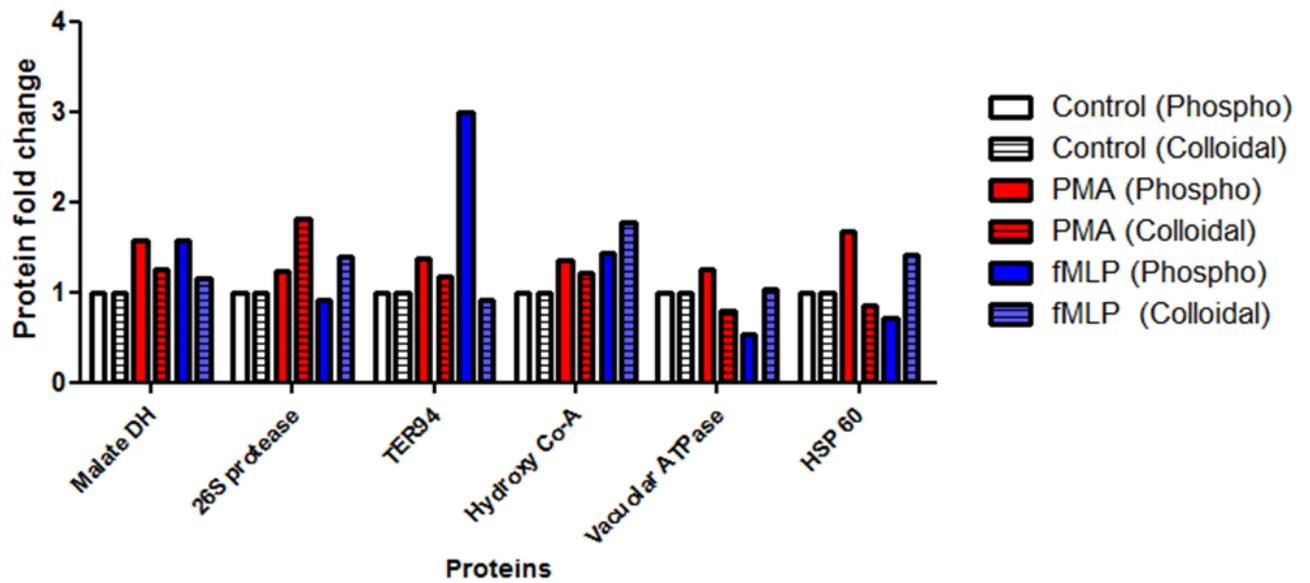
Fig. 4.20 Change in haemocyte protein abundance and phosphorylation state in response to PMA or fMLP.
Control, PMA and fMLP stimulated haemocytes, protein abundance (Colloidal) or phosphorylation abundance (Phospho), replicates (n=2).
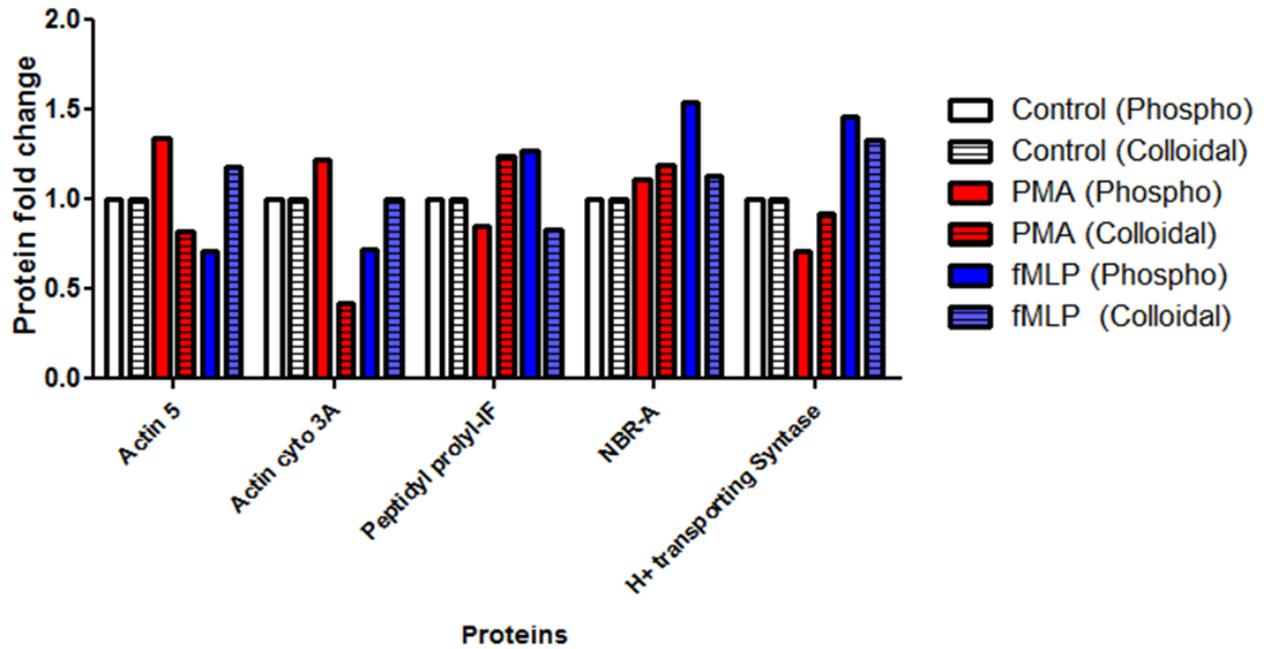
Fig. 4.21 Change in haemocyte protein abundance and phosphorylation state in response to PMA or fMLP.

Control, PMA and fMLP stimulated haemocytes, protein abundance (Colloidal) or phosphorylation abundance (Phospho), replicates (n=2).
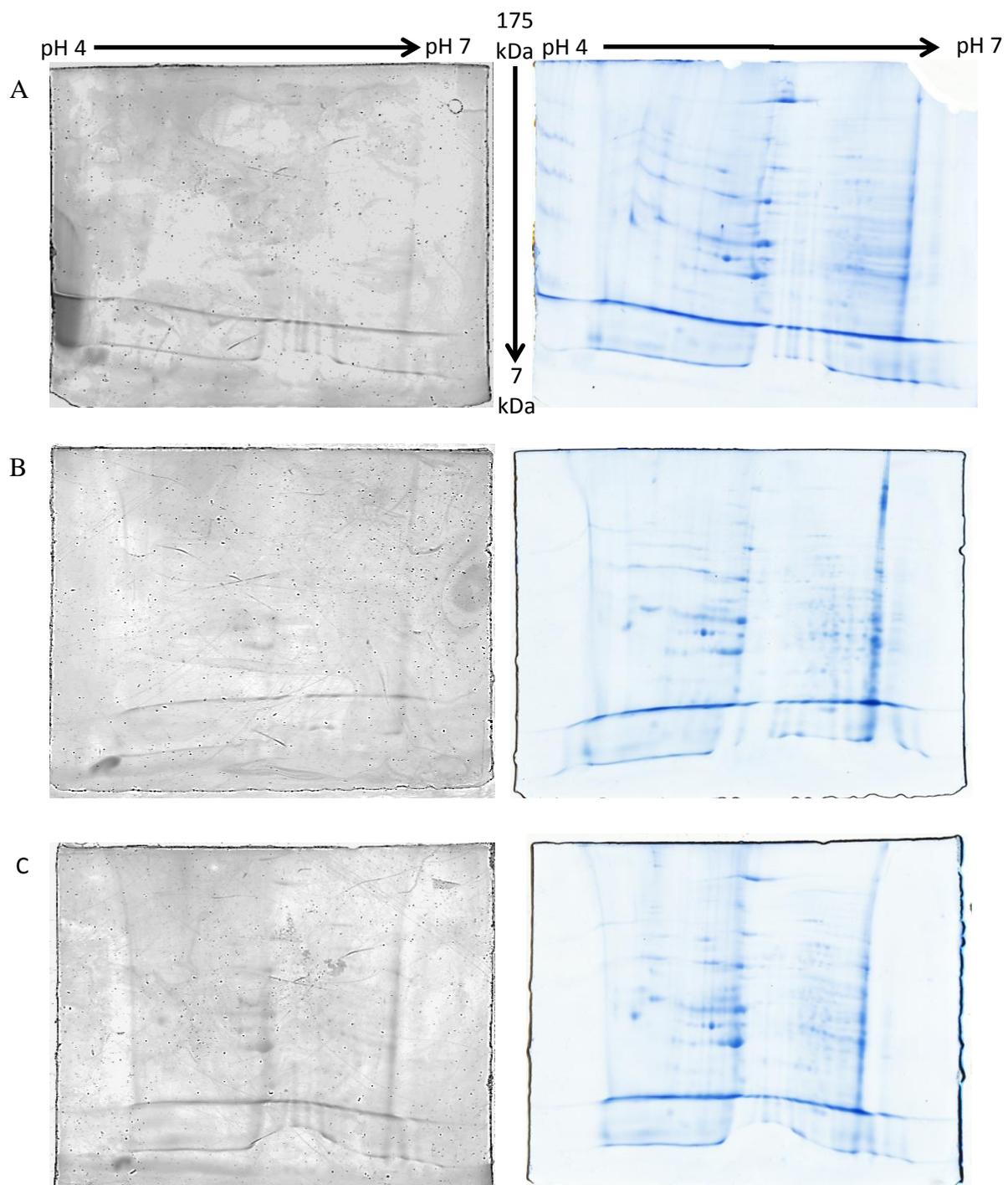
Fig. 4.22 Haemocyte cell proteins separated by 2D-SDS PAGE, stained with Pro-Q diamond phospho-stain and Colloidal Coomassie stain.
Left side stained in ProQ diamond phospho-stain (Phosphorylated proteins), Right side Colliodal coomassie stained (Complete gel protein visualisation), replicates (n=2). Haemocytes were incubated at 37°C with either; unstimulated (Fig. 4.22 A), Phorbol 12-myristate 13-acetate (PMA) (Fig. 4.22 B) or formyl-methionyl-leucyl-phenylalanine (fMLP) (Fig. 4.22 C).
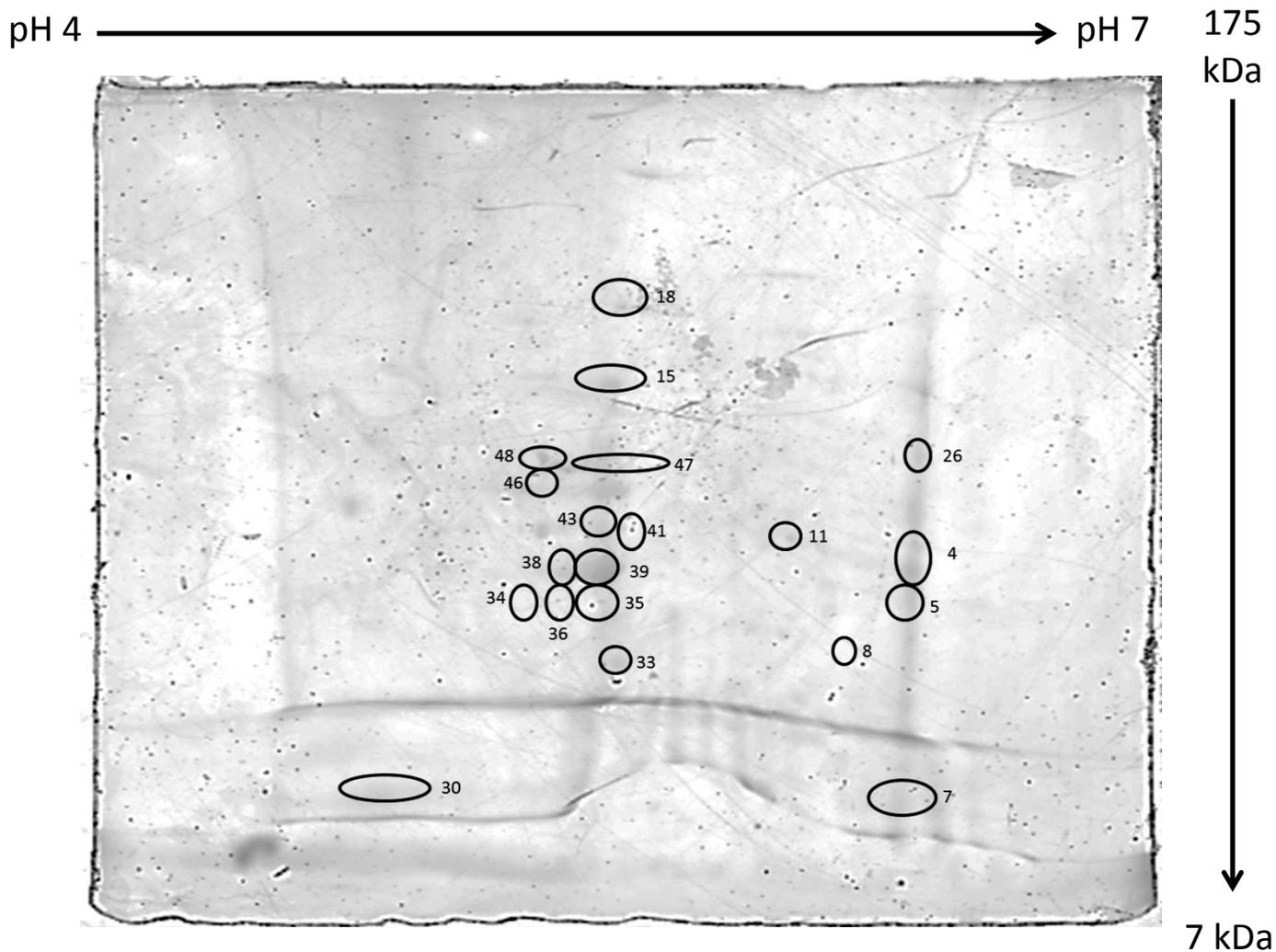
Fig. 4.23 Gel image representative of haemocyte cell proteins identified from mini 2-Dimensional gel electrophoresis and stained with ProQ diamond.

Table of protein identities and protein phosphorylated fold changes shown below (Table 4.3).

Spot no.	Protein Identity	Organism	Mr	pI	Score	Seq. coverage (%)	Accession no.	Phosphorylated protein change			Protein Function
								Con	PMA	fMLP	
4	Malate dehydrogenase	*B. mori*	67969	6.12	149	7	NP_001093280	1	1.57	1.58	TCA cycle enzyme that converts malate to oxaloacetate (using NAD+).
5	PREDICTED: 26S protease regulatory subunit 8-like	*B. mori*	45596	8.51	324	15	XP_004926152	1	1.24	0.92	Protein involved in targeted protein degradation by the ubiquitin (Ub) pathway.
7	Peptidyl-prolyl cis-trans isomerase f, ppif	*Papilio polytes*	17986	8.36	275	38	BAM19227	1	0.85	1.27	An enzyme which accelerates protein folding by catalyzing the cis-trans of peptide bonds.
8	Nucleoside-diphosphate kinase NBR-A	*Papilio xuthus*	17169	6.75	222	40	BAM17911	1	1.11	1.54	Synthesis of nucleoside triphosphates other than ATP
15	Heat shock 70 kDa cognate protein	*Ostrinia furnacalis*	71614	5.32	562	24	ADR00357	1	0.80	1.11	Role in maintaining unfolded precursor proteins in the cytosol and in transport.
18	Transitional endoplasmic reticulum ATPase TER94	*B. mori*	89792	5.30	1164	29	NP_001037003	1	1.38	3.0	Cell division protein 48 (CDC48), ATPase and N-ethylmaleimide sensitive fusion protein.
26	Hydroxyacyl-coenzyme A dehydrogenase	*B. mori*	82554	9.38	277	7	NP_001040132	1	1.35	1.44	Important function in fatty acid metabolism.
30	Histone H2B	*Chironomus thummi thummi*	13851	10.37	99	12	P21897	1	1.27	0.72	Histone proteins involved in the structure of chromatin.
33	Actin, cytoskeletal 3A	*Strongylocentrotus purpuratus*	42028	5.46	277	17	NP_999694	1	1.22	0.79	Nucleotide-Binding Domain of the sugar kinase/HSP70/actin superfamily.
34	Beta actin	*Xestia cnigrum*	42119	5.48	753	57	ABW03226	1	0.37	0.61	Involved in cell motility, structure and integrity.
35	Actin 5	*Aedes aegypti*	42201	5.30	960	54	AAY81972	1	1.34	0.71	Nucleotide-Binding Domain of the sugar kinase/HSP70/actin superfamily.
36	Beta actin (Actin 5)	Xestia cnigrum	42119	5.48	1186	67	ABW03226	1	0.36	0.36	Nucleotide-Binding Domain

		(moths)								of the sugar kinase/HSP70/actin superfamily.
38	H+ transporting ATP synthase beta subunit isoform 1	*B. mori*	55012	5.26	893	46	NP_001040450	1	0.79	1.46	ATP synthase that uses a proton gradient to drive ATP synthesis and hydrolyzes ATP to build the proton gradient.
39	H+ transporting ATP synthase beta subunit isoform 2	*Danaus plexippus*	54882	5.20	1393	69	EHJ64315	1	1.12	1.16	ATP synthase that uses a proton gradient to drive ATP synthesis and hydrolyzes ATP to build the proton gradient.
43	Vacuolar ATPase B subunit	*B. mori*	55161	5.23	627	28	ACE78271	1	1.25	0.54	V/A-type ATP synthase subunit B. ATPases couple ATP hydrolysis to the build up of a H+ gradient.
41	Protein disulfide-isomerase like protein ERp57	*Danaus plexippus*	55108	5.36	425	15	EHJ76798	1	0.96	0.74	Involved in oxidative protein folding in the ER by its isomerase and chaperone activities.
46	Tubulin alpha chain	*Aedes aegypti*	50573	5.01	742	52	XP_001652144	1	.93	1.87	A microtubule forms cellular structures such as the mitotic spindle and the interphase network.
47	60 kDa heat shock protein, mitochondrial-like	*B. mori*	61196	5.51	684	26	XP_004923957	1	1.68	0.71	Chaperonin involved in productive folding of proteins
48	Hypothetical protein CAPTEDRAFT_149167	*Capitella teleta*	50760	4.94	397	19	ELT89394	1	0.89	0.60	A microtubule forms cellular structures such as the mitotic spindle and the interphase network.

Table 4.3 Phosphorylated proteins identified from stimulated haemocyte separated by 2D SDS PAGE. Haemocytes stimulated with either PMA or fMLP and control haemocytes unstimulated. Relative fold changes in expression of phosphorylated protein from 2D-Gels stained with ProQ Diamond phosphorylation stain analysed with progenesis, and protein spots of interest excised and identified by LC/MS.
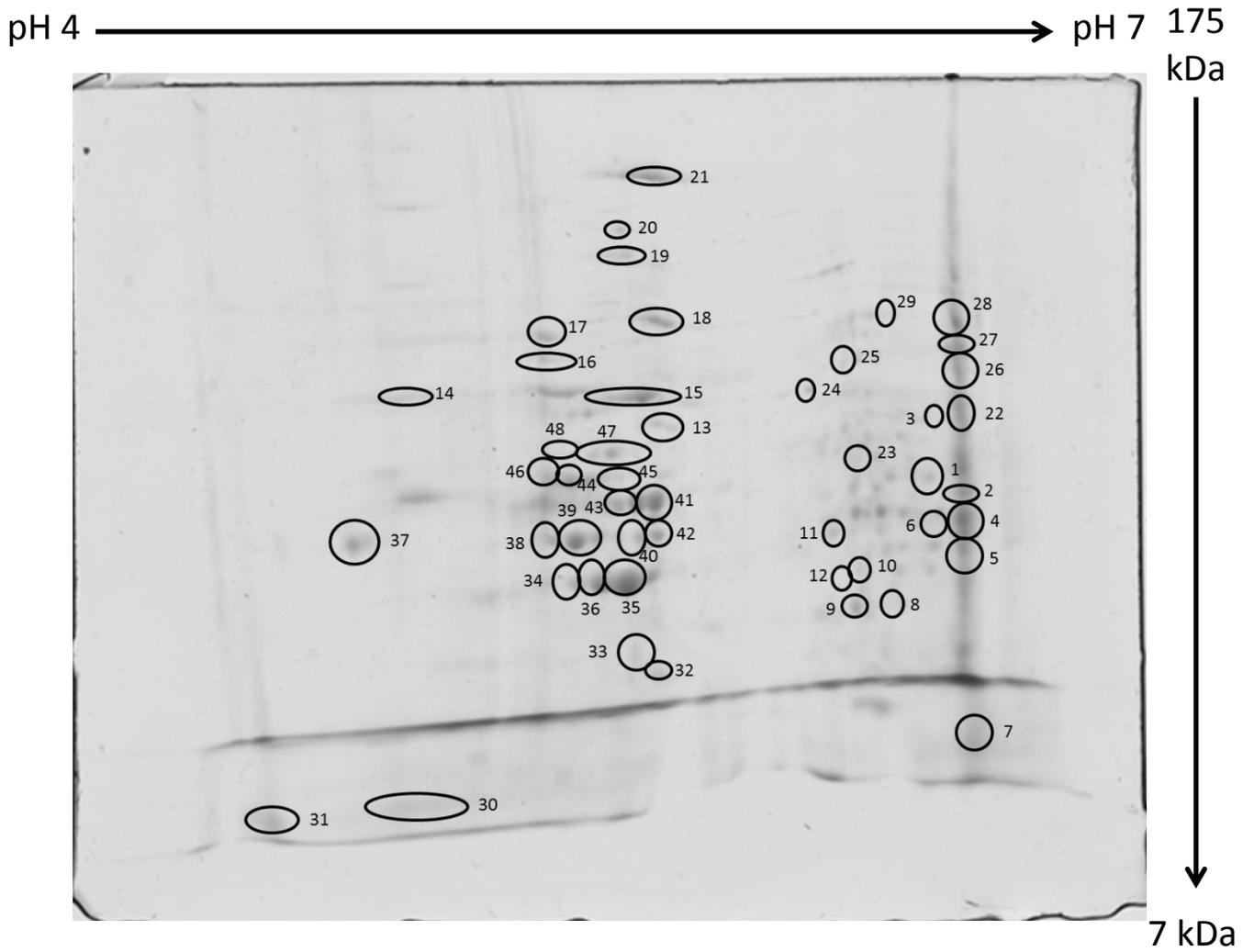
Fig. 4.24 Gel image representative of haemocyte cell proteins identified from mini 2-Dimensional gel electrophoresis and stained with Colloidal coomassie.

Table of protein identities and protein fold changes shown below (Table 4.4).

Spot no.	Protein Identity	Organism	Mr	pI	Score	Seq. coverage (%)	Accession no.	Protein Fold Change			Protein Function
								Con	PMA	fMLP	
1	Mitochondrial aldehyde dehydrogenase	*B. mori*	53134	5.57	180	8	NP_001040198	1	3.19	1.50	Roles in acetaldehyde oxidation; in response to UV light exposure and during ethanol metabolism.
2	ATP synthase	*B. mori*	59795	9.21	532	20	NP_001040233	1	2.17	1.60	Synthesises ATP from ADP.
3	Chaperonin	Aedes aegypti	60214	6.17	185	6	XP_001660595	1	1.99	0.92	Protein complex that assists the folding of a subset of newly-synthesized proteins in an ATP-dependent manner.
4	Malate dehydrogenase	*B. mori*	67969	6.12	149	7	NP_001093280	1	1.25	1.15	TCA cycle enzyme that converts malate to oxaloacetate (using NAD+).
5	PREDICTED: 26S protease regulatory subunit 8-like	*B. mori*	45596	8.51	324	15	XP_004926152	1	1.81	1.40	Protein involved in targeted protein degradation by the ubiquitin (Ub) pathway.
6	Putative enolase protein, partial	*G.mellonella*	40844	5.58	1181	56	AGB91349	1	1.61	0.67	Enolase, is a metalloenzyme responsible for converting 2-phosphoglycerate (2-PG) to phosphoenol-pyruvate (PEP) in Glycolosis.
7	Peptidyl-prolyl cis-trans isomerase f, ppif	*Papilio polytes*	17986	8.36	275	38	BAM19227	1	1.24	0.83	An enzyme which accelerates protein folding by catalyzing the cis-trans of peptide bonds.
8	Nucleoside-diphosphate kinase NBR-A	*Papilio xuthus*	17169	6.75	222	40	BAM17911	1	1.19	1.13	Synthesis of nucleoside triphosphates other than ATP.
9	Arginine kinase	*Crossotarsus minusculus*	30604	6.03	513	45	AEB32406	1	0.45	0.54	Enzyme converts (ATP and L-arginine) to (ADP + Nomega-phospho-L-arginine).
10	Hexamerin	*G. mellonella*	81807	6.03	90	1	AAA19801	1	0.13	0.16	Amino acid storage and immune functions
11	Mitochondrial processing peptidase beta subunit	*Papilio xuthus*	52033	5.93	367	15	BAM18267	1	1.92	2.24	Metallopeptidases, zinc peptidases comprises a pair of homologous

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											domains that form two halves of a "clam-shell" surrounding the active site.
12	Actin	*Spodoptera exigua*	42339	5.60	268	15	AEJ38216	1	0.16	0.24	Nucleotide-Binding Domain of the sugar kinase/HSP70/actin superfamily
13	Kinesin-related protein 1	*Crassostrea gigas*	111667	6.04	60	3 (matched peptides 2)	EKC32320	1	1.53	1.52	Phophopeptide binding site. Drive myosin and kinesin mediated processes.
14	Heat shock 70 kD protein cognate precursor	*B. mori*	73168	5.13	583	23	NP_001036837	1	0.78	0.67	Role in maintaining unfolded precursor proteins in the cytosol and in transport.
15	Heat shock 70 kDa cognate protein	*Ostrinia furnacalis*	71614	5.32	562	24	ADR00357	1	0.82	0.77	Role in maintaining unfolded precursor proteins in the cytosol and in transport.
17	Endoplasmin-like precursor	*B. mori*	91675	4.93	407	9	NP_001266403	1	1.30	1.94	Molecular chaperone HSP90 that functions in the processing and transport of secreted proteins. Histidine kinase-like ATPase activity
18	Transitional endoplasmic reticulum ATPase TER94	*B. mori*	89792	5.30	1164	29	NP_001037003	1	1.17	0.91	Cell division protein 48 (CDC48), ATPase and N-ethylmaleimide sensitive fusion protein.
19	Hypoxia up-regulated protein 1-like	*B. mori*	102644	5.10	132	3	XP_004928340	1	0.95	1.26	Cytoprotective cellular mechanisms triggered by oxygen deprivation. May play a role as a molecular chaperone.
20	Actin	*Heliothis virescens*	41956	5.21	247	28	AAK52066	1	0.36	0.73	Nucleotide-Binding Domain of the sugar kinase/HSP70/actin superfamily
21	Histidine-tRNA ligase, cytoplasmic-like isoform X1	*Apis dorsata*	69259	6.15	61	3 (matched peptides 2)	XP_006613565	1	0.48	0.69	Ankyrin repeat,functions to mediate protein-protein interactions.
22	Chaperonin subunit 6a zeta	*B. mori*	58115	6.59	109	3	NP_001040108	1	1.75	1.34	T-complex protein 1 subunit

											zeta is a protein chaperone.
23	T-complex protein 1 subunit alpha-like	*B. mori*	59945	5.89	190	7	XP_004923560	1	0.26	0.41	T-complex protein 1 subunit zeta is a protein chaperone.
24	Putative dipeptidyl peptidase iii	*Danaus plexippus* (monarch butterfly)	69623	5.86	100	4	EHJ68135	1	1.48	1.69	Zinc-dependent enzymes with specific cleaving of N terminus from different length peptides.
25	Prophenoloxidase subunit 2	*G. mellonella*	80198	5.95	161	4	AAQ75026	1	0.42	0.67	Active component of melanisation cascade key to insect Innate immune defence
26	Hydroxyacyl-coenzyme A dehydrogenase	*B. mori*	82554	9.38	277	7	NP_001040132	1	1.22	1.78	Important function in fatty acid metabolism.
28	Hypothetical protein KGM_16016	*Danaus plexippus*	55327	7.17	215	12	EHJ72366	1	1.09	1.51	Unknown
30	Histone H2B	*Chironomus thummi thummi*	13851	10.37	99	12	P21897	1	1.15	1.83	Histone proteins involved in the structure of chromatin.
31	Calmodulin	*D.melanogaster*	16800	4.09	353	51	NP_523710	–	–	–	Proteins interact reversibly with Ca^2+ to form a protein . Ca^2+ complex, whose activity is regulated by cellular Ca^2+ flux.
32	Annexin B	*Heliothis virescens*	36602	5.54	405	23	ACR78451	–	–	–	Annexin (*lipocortin*), suppress phospholipase A2. Functions in membrane scaffolds, trafficking and vesicles.
33	Actin, cytoskeletal 3A	*Strongylocentrotus purpuratus*	42028	5.46	277	17	NP_999694	1	0.42	1	Nucleotide-Binding Domain of the sugar kinase/HSP70/actin superfamily.
34	Beta actin	*Xestia cnigrum*	42119	5.48	753	57	ABW03226	1	1.20	1.79	Are involved in cell motility, structure and integrity.
35	Actin 5	*Aedes aegypti*	42201	5.30	960	54	AAY81972	1	0.82	1.18	Nucleotide-Binding Domain of the sugar kinase/HSP70/actin superfamily.
36	Beta actin	*Xestia cnigrum*	42119	5.48	1186	67	ABW03226	1	0.62	0.98	Are involved in cell motility, structure and integrity.
37	DNA	*Danaus*	37823	4.56	350	17	EHJ69265	1	0.57	0.83	Calcium sensors and calcium

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	supercoiling factor	*plexippus*									signal modulators; most l have 2 active canonical EF hands.
38	H+ transporting ATP synthase beta subunit isoform 1	*B. mori*	55012	5.26	893	46	NP_001040450	1	0.92	1.33	ATP synthase that uses a proton gradient to drive ATP synthesis and hydrolyzes ATP to build the proton gradient.
39	H+ transporting ATP synthase beta subunit isoform 2	*Danaus plexippus*	54882	5.20	1393	69	EHJ64315	1	0.68	0.65	ATP synthase that uses a proton gradient to drive ATP synthesis and hydrolyzes ATP to build the proton gradient.
41	Protein disulfide-isomerase like protein ERp57	*Danaus plexippus*	55108	5.36	425	15	EHJ76798	1	0.89	1.66	Involved in oxidative protein folding in the ER by its isomerase and chaperone activities.
42	Protein disulfide isomerase	*Helicoverpa armigera*	45416	5.21	251	12	AEB26317	1	0.94	0.83	Involved in oxidative protein folding in the ER by its isomerase and chaperone activities.
43	Vacuolar ATPase B subunit	*B. mori*	55161	5.23	627	28	ACE78271	1	0.79	1.04	V/A-type ATP synthase subunit B. ATPases couple ATP hydrolysis to the build up of a H+ gradient.
45	Alpha-tubulin	*Papilio xuthus*	50615	5.01	1174	66	BAM17695	1	0.27	0.34	A microtubule occuring as single tubes and form cellular structures such as the mitotic spindle and the interphase network.
46	Tubulin alpha chain	*Aedes aegypti*	50573	5.01	742	52	XP_001652144	1	1.44	1.56	A microtubule forms cellular structures such as the mitotic spindle and the interphase network.
47	60 kDa heat shock protein, mitochondrial-like	*B. mori*	61196	5.51	684	26	XP_004923957	1	0.85	1.41	Chaperonin involved in productive folding of proteins
48	Hypothetical protein CAPTEDRAFT_149167	*Capitella teleta*	50760	4.94	397	19	ELT89394	1	0.21	0.28	A microtubule forms cellular structures such as the mitotic spindle and the interphase network.

Table 4.4 Proteins identified from stimulated and non-stimulated haemocyte separated by 2D SDS PAGE. Haemocytes stimulated with either PMA or fMLP and control haemocytes unstimulated. Relative fold changes in abundance of protein from 2D-Gels stained with Colloidal coomassie, analysed with progenesis, and protein spots of interest excised and identified by LC/MS. (-) = to no density measurement.

4.14 Discussion

The application of thermal and physical stress to larvae resulted in a number of changes to the cellular and humoral immune responses. The results here indicate that thermal and/or mild physical stress can increase the resistance of *G. mellonella* larvae to infection by *A. fumigatus*, *C. albicans* or *S. aureus* if larvae are inoculated 24 hours after the initiation of the stress event. In contrast, those larvae inoculated 72 hours after the initiation of the thermal or physical stress events demonstrated increased susceptibility to infection from *A. fumigatus* and *S. aureus*. Previously it was shown that a (24h) thermal or physical stress enabled better survival by larvae to a subsequent infection (Mowlds *et al*., 2008; Mowlds and Kavanagh, 2008).

The expression of prophenoloxidase, arylphorin and apolipophorin was increased at 24 hours in those larvae that had been thermally or physically stressed but the expression of these proteins declined in larvae incubated for 48 and 72 hours. The 2D SDS PAGE analysis of the 37°C pre-incubated larvae over 24, 48 and 72h demonstrated a similar peak in a number of immune related proteins (26 and 32kDa ferritin, apoLp-III and arylphorin) at 24h which may contribute to the higher survival rate observed in larvae thermally stressed. Ferritin has a number of important roles including limiting free iron within the haemolymph that could be used by microbial pathogens to spread (Levy *et al*., 2004). ApoLp has important functions in lipid transport and in the innate immune response as a PRR (Whitten *et al*., 2004) while arylphorin has antimicrobial activity (Beresford *et al*., 1997). ApoLp and arylphorin demonstrated a similar increase in abundance within the thermally and physically stressed larvae from 1D-SDS PAGE analysis and within the 37°C thermally stressed larvae assessed by 2D-SDS PAGE analysis.

The 1D SDS PAGE analysis demonstrated increased proPO within the thermally and physically stressed larvae at 24h. This high presence of proPO may function as a reserve which in response to a pathogen threat can respond faster by activation of the proPO cascade through the cleavage of proPO to PO. This reserve of proPO must be controlled to prevent its activation to a non-threat and is potentially why serpin 1 which inhibits the enzyme cleavage of proPO to PO was increased in 2D SDS PAGE gels within the 37°C incubated larvae at 24h, preventing PO activation until required. This proPO reserve may enhance the survival of thermally and physically stressed larvae to a microbial infection.

Galiomicin, *Gallerimycin*, *IMPI* and *Transferrin* demonstrated differential expression in response to thermal and physical stress. *Galiomicin* demonstrated its highest expression among thermally and physically stressed larvae at the 24h but never surpassed the control. The expression of the antifungal *Gallerimycin* was lower among stressed larvae compared to the control larvae until the 48 and 72h where its expression rose above the control significantly within the 30°C incubated and the physically stressed larvae but not within the 37°C incubated larvae which matched the expression level of the control.

IMPI showed a constant level of expression within all treatments up to 48 and 72h where it significantly increased in the thermally and physically stressed larvae. The highest expression of *IMPI* was observed in the 30°C incubated and physically stressed larvae although the physically stressed larvae peaked a 48h possibly from the extra physical stress exerted compared to the 30°C incubated larvae. *Transferrin* expression followed a very similar trend to that observed with *Gallerimycin* with fold increases higher than the control larvae for the 30°C incubated larvae and significantly ($p<0.001$) for the physically stressed larvae at 72h although remaining low in the 37°C incubated larvae potentially due to the higher temperature as this trend was seen for all genes at 37°C. The physically stressed larvae and 30°C incubated larvae were both incubated at the same temperature potentially favouring the expression of genes seen to peak at 48 and 72h, unlike the 37°C incubated larvae which had a delayed increase in gene expression potentially in response to the higher temperature. Previously it was demonstrated that physically stressed larvae had increased expression of *IMPI* after 24h compared to the control (Mowlds *et al*., 2008) which was also observed here. Wojda *et al*. (2009) previously showed the induction of antimicrobial genes in response to heat shock peaked at 72h as was similarly observed here.

The analysis of the cellular component of the immune response demonstrated higher haemocyte densities in larvae incubated at 37°C or physically stressed at 24 hours but declining at 48 and 72 hours which may promote better survival early on to infection. It was shown previously that thermal and physical stress resulted in higher abundance of haemocytes at 24h and a decline at 48h among the 37°C incubated larvae (Mowlds and Kavanagh, 2008) and physically stressed larvae (Mowlds *et al*., 2008). A number of changes were observed when examining the proportions of haemocytes using FACS analysis. This included the 30°C incubated and physically

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stressed larvae which demonstrated a significantly higher proportion of P7 haemocytes at 48 and 72h, identified as granular haemocytes, which may contribute to better phagocytosis and ability to overcome infections (Kavanagh and Reeves, 2004; Tanada and Kaya, 1993). It has been demonstrated that the process of metamorphosis causes morphological changes in cell size within granular haemocytes where they change into larger macrogranular or macrogranulocytes (Zhai and Zhao, 2012), this may be partially what is observed here with the increase in abundance of larger granular cells particularly among 30^oC incubated treatments when compared to the smaller P2 granular cell population. It was also demonstrated that little change occurs to the P2 small granular haemocytes and P7 the large granular haemocytes populations within the 37^oC and 15^oC incubated larvae. It was also reported by Zhai and Zhoa, (2012) that during the metamorphosis process along with the increases in the size of phagocytosing cells there was a greater degradation of adipohaemocytes which were seen to be reduced within the 30^oC and physically stressed larvae when compared to the 37^oC incubated larvae.

The 37^oC incubated larvae had the highest proportion of P5 haemocytes which had reduced globular inclusions and resemble adipohaemocytes (Kavanagh and Reeves, 2004; Tanada and Kaya, 1993). This P5 population in combination with elevated calreticulin recognition receptor and heat shock proteins among 37^oC incubated larvae may activate a danger response from DAMPs such as heat shock proteins (Asea *et al*., 2002) contributing to a primed immune response to infection and therefore elevated resistance to microbial challenge. The P1 haemocytes were identified to be most like prohaemocytes, as they appear to be differentiating with time (Nardi *et al*., 2003) as well as increasing in proportion within the 30^oC, 37^oC and physically stressed larvae. It has been identified in *M.sexta* that the hematopoietic organs give rise to the generation of haemocyte cells which include immune related phagocytosing cells (Nardi *et al*., 2003). Here changes were observed in haemocyte cell populations that may stem from such hematopoietic organs particularly the P1 haemocytes. It has been suggested by Rodrigues *et al*. (2010) that mosquito's differentiation and proliferation of a subset of haemocytes (phagocytic cells) is responsible for the improved survival and decreased *Plasmodium oocyst* numbers encysted in the gut epithelium upon secondary challenge.

The analysis of the haemocyte proteome over the same time periods as the FACS analysis demonstrated an increased abundance of haemocyte proteins from

thermally and physically stressed larvae that can bind to pathogens and that can promote phagocytosis (e.g. Rab signalling protein) (Shim *et al*., 2010). Oxidative stress protein FK506-binding protein (spot 26) and heat stress protein proteasome 35kDa subunit (spot 20) increased in abundance as did metabolic enzymes potentially in response to greater physiological activity and stress within the thermally and physically stressed larvae (Mowlds *et al*., 2008; Wojda and Jakubowicz, 2007). The abundance of proteins involved with immune and protein folding functions were previously seen to be elevated in physically stressed larval haemolymph (Mowlds *et al*., 2008). It was also established recently by Wang *et al*. (2014) that constant high temperature could induce heat oxidative stress in conjunction with the heat shock protein. It is also known that ROS contribute to the oxidative damage of cellular constituents during environmental stress (Monaghan *et al*., 2009).

An interesting trend within this analysis was seen in the similarities between the 30°C incubated and physically stressed larvae with both incubated at 30°C and appearing different to the changes observed within the 37°C incubated larvae. The two 30°C incubated treatments demonstrated a similar rise in P7 haemocytes and in antimicrobial gene expression at 48 and 72h while showing similar proteomic abundances, which is likely attributed to the similar temperature the two stress treatments are incubated at.

On the other hand the 37°C incubated larvae possessed a similar proportion of P7 haemocytes to the control larvae but demonstrated higher abundance of heat shock associated and immune binding proteins from haemocytes as well as a higher proportion of P5 haemocytes compared to the 30°C stress treatments. The equally high survival of 37°C incubated larvae following infection as seen with physically stressed larvae may result from having a tailored response to cope with the higher temperature in terms of heat shock proteins and PRRs that enable better recognition and survival following microbial challenge. Whereas the 30°C incubated larvae and physically stressed larvae (both incubated at 30°C) may favour haemocyte mounted responses as the conditions may more suit cell mediated defence unlike the 37°C incubated larvae. But it is important to note that the 37°C and physically stressed larvae had the highest abundance of haemocytes compared to the control and 30°C which may have increased their survival following microbial challenge.

The greater haemocyte density and expression of antimicrobial peptides in larvae thermally or physically stressed for 24 hours prior to infection contributed to

increased survival. This highlights the potential immune priming effects which are well characterised in insects, serving to protect the insect from a potentially lethal infection by raising immune defences following exposure to a sub-lethal infection. It was previously established that non-lethal physical and thermal stress can lead to increased resistance of *G. mellonella* to infection with the yeast *C. albicans* (Mowlds *et al.*, 2008; Mowlds and Kavanagh, 2008). This work extends these observations and demonstrates that the priming effect in *G. mellonella* larvae is a short term event that declines after 24 hours.

Immune priming in insects has the advantage of giving protection from a subsequent potentially lethal infection but is costly to maintain and can result in death if compensatory feeding is unavailable (Moret and Schmid-Hempel, 2000). Immune priming can also be affected by the social interaction and behaviour of challenged bees (Richard *et al.*, 2008). Immune priming in *Formica selysi* workers following challenge with *Beauveria bassiana* is short term (Reber and Chapuisat, 2012) and raises the possibility that colony living preclude the necessity of requiring a prolonged immune priming effect as other compensatory mechanisms may be operating in the colony. For example, in honey bee colonies an elevated nest temperature is generated as a colony-level response to prevent chalk brood (Starks *et al.*, 2000). Social immunity in insects has been suggested in a number of colony based communities and it has been demonstrated that ants which are infected within a colony can regurgitate droplets which were capable of transferring immune factors to other uninfected ants (Hamilton *et al.*, 2011). A potential role for 'behavioural fever', where insects alter their temperature through thermoregulatory behaviour, leading to an increased ability to withstand infection has been suggested (Blanford *et al.*, 1998; Elliot *et al.*, 2002; Watson *et al.*, 1993).

The examination of PMA and fMLP activation of phagocytosing haemocytes was assessed by measuring the phosphorylation of proteins important to the NADPH oxidase function. Neutrophils and haemocytes phagocytosing capacity is activated by the phosphorylation of a number of proteins (Bergin *et al.*, 2005). It was evident that although a number of proteins were phosphorylated in both fMLP and PMA stimulated haemocytes the two stimulants activated different pathways or sets of proteins to activate phagocytes (Guignard and Markert, 1996). This included peptidyl-prolyl cis-trans isomerase and NDP kinase that had increased phosphorylation within fMLP stimulated haemocytes unlike PMA stimulated haemocytes.

It was also observed that PMA stimulated haemocytes had a greater abundance of phosphorylated HSP 60. These proteins may have a protective function against auto-oxidation during the activation of NADPH oxidase which generates ROS for the destruction of internalised pathogens within phagocytes (Teshima *et al*., 1996). HSPs have also been implicated in modulating the phagocytosis process and in particular the NADPH oxidase activity (Chen *et al*., 2011; Vega and De Maio, 2005). Heat shock proteins are also essential for the activation of actin polymerisation within neutrophils, which enables phagocytosis, and this event requires HSPs to be phosphorylated (Howard and Oresajo, 1985; Vega and De Maio, 2005).

This work highlights a number of similariteis in the activation of neutrophils and other phagocytosing cells of mammals to that of the *G. mellonella* haemocytes. Here similar hallmarks of NADPH oxidase activation were observed within haemocytes through the phosphorylation of a number of proteins associated with the activation of haemocyte phagocytosing capacity. The proteins that were phosphorylated were observed at higher levels within the PMA and fMLP stimulated haemocytes and suggests that these artificial stimulants have similar activation effects on phagocytosing haemocytes as they do on mammalian neutrophils (García-García *et al*., 2009). The PMA and fMLP activated haemocytes also demonstrated some similar proteins that were phosphorylated (Spot 4, 18 and 26) and some differences within phosphorylated proteins present in PMA (Spot 5 and 47) or fMLP (Spot 7 and 8) stimulated haemocytes (Table 4.3).

This Chapter highlights how thermal and physical stress on larvae enhances immune protection afforded by the priming events described here in *G. mellonella* larvae which reached a peak 24 hours after the initiation of the stress event and then declined. The relatively short duration of the immune priming effect in *G.mellonella* may be due to the fact that in its natural habitat *G. mellonella* live in bee colonies where the high temperature may offer some degree of protection against pathogens therefore not requiring a long term, heightened immune response that may be costly to maintain. The observation of a short term (24 hours approximately) immune priming effect in *G. mellonella* larvae is of interest in that it may have evolved as a response to life within the colony of another insect. This raises the possibility of the immune response of one insect (i.e. *G. mellonella*) being modulated by living in the colony of another (i.e. *Apis mellifera*).

Chapter 5

Analysis of the effect of pre-incubation on the susceptibility of *Galleria mellonella* larvae to infection

5.0 An analysis of the effect of pre-incubation on the ability of *G. mellonella* larvae to tolerate fungal and bacterial infection

While insects are now popular and effective models, no standardised procedures for their use have been developed (Cook and McArthur, 2013). Larvae of *G. mellonella* are commonly stored at 15°C or room temperature and may be maintained under these conditions for 1 to 3 weeks in advance of inoculation (Cook and McArthur, 2013). It has previously been established that variations in incubation temperature (Mowlds and Kavanagh, 2008), physical stress (Mowlds *et al*., 2008) and the access to nutrients (Banville *et al*., 2012) significantly alters the response of *G. mellonella* larvae to infection. It was also established that prior exposure of larvae to yeast cells (Bergin *et al*., 2006) or glucan (Mowlds *et al*., 2010) stimulated their immune response and lead to reduced sensitivity to infection. This priming effect was mediated by an elevated density of circulating haemocytes and increased abundance of immune related and antimicrobial proteins in the haemolymph.

The effect of age on *G.mellonella* larvae response to infection has not previously been assessed. Aging is generally characterized by the increasing potential for death to occur, and effects of aging on mammals include a greater susceptibility to disease due to a weakening immune system, it is also observed that weight declines as muscle is replaced with fat and bones lose minerals and density (Pletcher *et al*., 2002). The process of aging has been explored to a small extent in insects using *D.melanogaster* (Augustin and Partridge, 2009; Mackenzie *et al*., 2011; Pletcher *et al*., 2002), although for most studies the introduction of simulated age by altering genes to shorten life span could also alter normal functions, such as the ability to fight infection, or interfere with healthy metabolism (Pletcher *et al*., 2002).

The aim of the work presented here was to establish whether incubating *G. mellonella* larvae for extended periods at 15°C in advance of infection affected their susceptibility to infection and if so to determine how this was mediated. Identification of the role of pre-incubation in altering the larval immune response would be useful for those using larvae as an *in vivo* model system. In addition understanding how pre-incubation affects the susceptibility of larvae to infection might give insights into how aging affects the vertebrate innate immune system.

5.1 Effect of pre-incubation at 15°C on susceptibility of *G. mellonella* larvae to infection

Larvae were stored in the dark at 15°C for 1, 3, 6 or 10 weeks prior to being inoculated through the last left proleg with *C. albicans* or *S. aureus* as described (Section 2.7.4). Subsequent to infection larvae were placed at 30°C and survival was monitored over 24h. The results demonstrated that those larvae that were incubated at 15°C for 3, 6 or 10 weeks were the most sensitive to infection with *C. albicans* with survival at 24 hours post-infection being 43.3±13.3% ($p<0.01$), 46.7±6.6% and 30.0±10.0% ($p<0.05$) respectively, while larvae infected after 1 week pre-incubation (control larvae) showed 73.3±3.3% survival at the same time point (Fig. 5.1). Similarly, larvae pre-incubated for 3, 6 or 10 weeks and infected with *S. aureus* showed significantly reduced survivals 24h after inoculation, i.e. 24.1±6.6% (p < 0.01), 20.7±5.7% ($p<0.001$) or 13.3±3.3% ($p<0.001$) respectively, compared to larvae incubated for 1 week at 15°C in advance of infection which showed 65.5±3.3% survival at the same time point post-infection (Fig. 5.2).

5.2 Pre-incubation leads to alteration in the haemocyte population of *G. mellonella* larvae

Elevated density of circulating haemocytes following physical (Mowlds *et al.*, 2008) or thermal (Mowlds and Kavanagh, 2008) stress was associated with increased resistance to infection. Haemocytes were extracted from larvae incubated at 15°C for up to 10 weeks and enumerated. The results demonstrated a decline ($p<0.05$) in the haemocyte density of larvae incubated for 3, 6 or 10 weeks compared to the density in larvae incubated at 15°C for 1 week (Fig. 5.3).

FACS analysis was employed to establish if there was a change in the relative proportion of each haemocyte sub-population in larvae pre-incubated for 1, 3, 6 or 10 weeks. Haemocyte populations were differentiated on the basis of size and granularity and at least five distinct sub-populations, labeled P1, P2, P3, P5 and P7, were identified (Fig. 5.4). The results (Fig. 5.5) demonstrated an increase in the relative abundance of P2 haemocytes (granular cells) in the total haemocyte population over time i.e. week 1; 23.8±2.1%, week 3; 44.0±9.4 % ($p<0.001$), week 6; 30.3±1.3 % and week 10; 43.55±1.25 % ($p<0.001$) while the proportion of P5 haemocytes (cells with
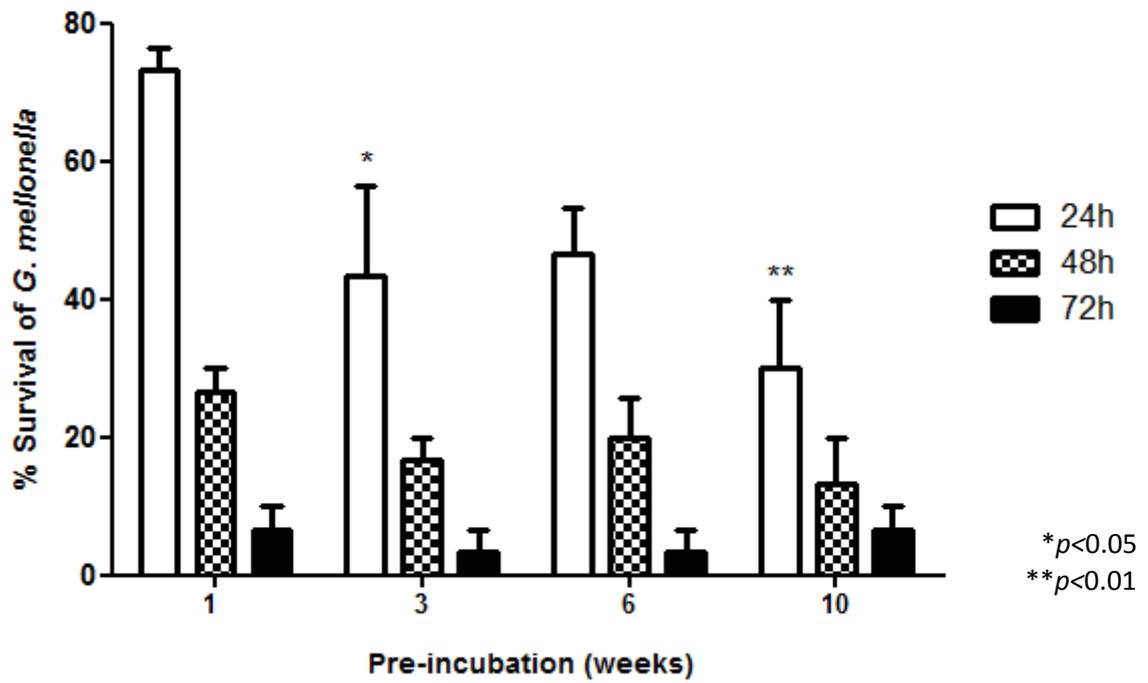
Fig. 5.1 Survival (%) of larvae pre-incubated for 1, 3, 6 and 10 weeks following infection with *C.albicans*.

Statistical significance of *Galleria* survival was analysed with log rank (Mantel-Cox) method using GraphPad Prism version 5.00.
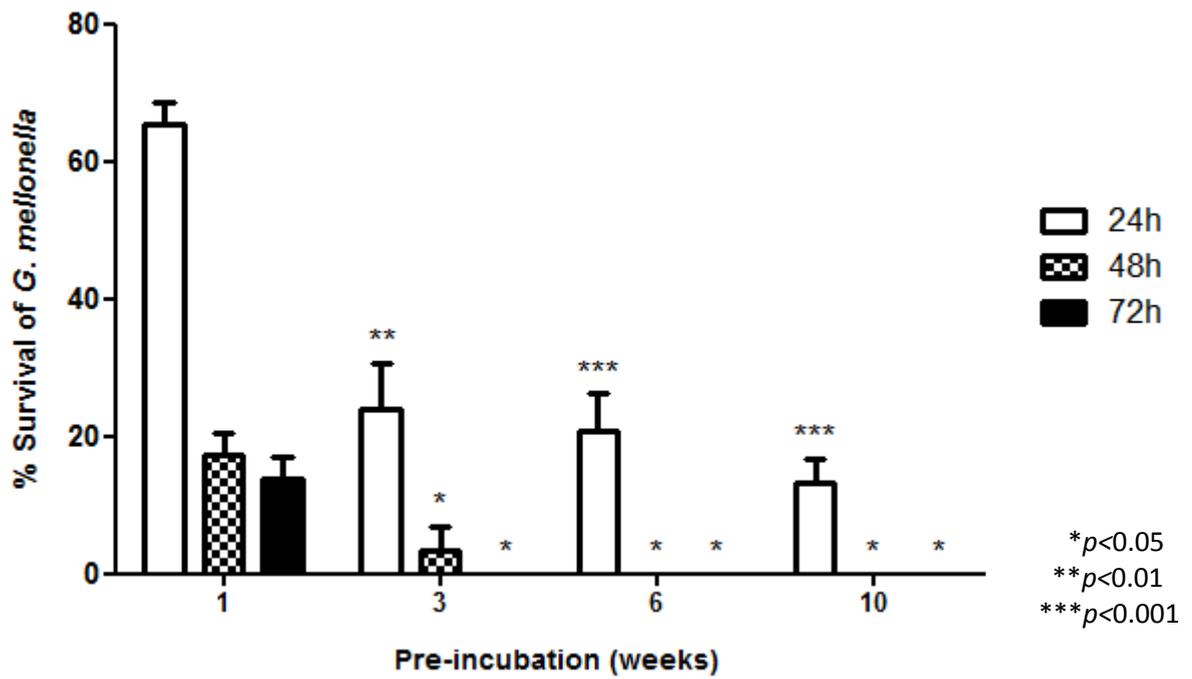
Fig. 5.2 Survival (%) of larvae pre-incubated for 1, 3, 6 and 10 weeks following infection with *S.aureus*.

Statistical significance of *Galleria* survival was analysed with log rank (Mantel-Cox) method using GraphPad Prism version 5.00.

*p<0.05
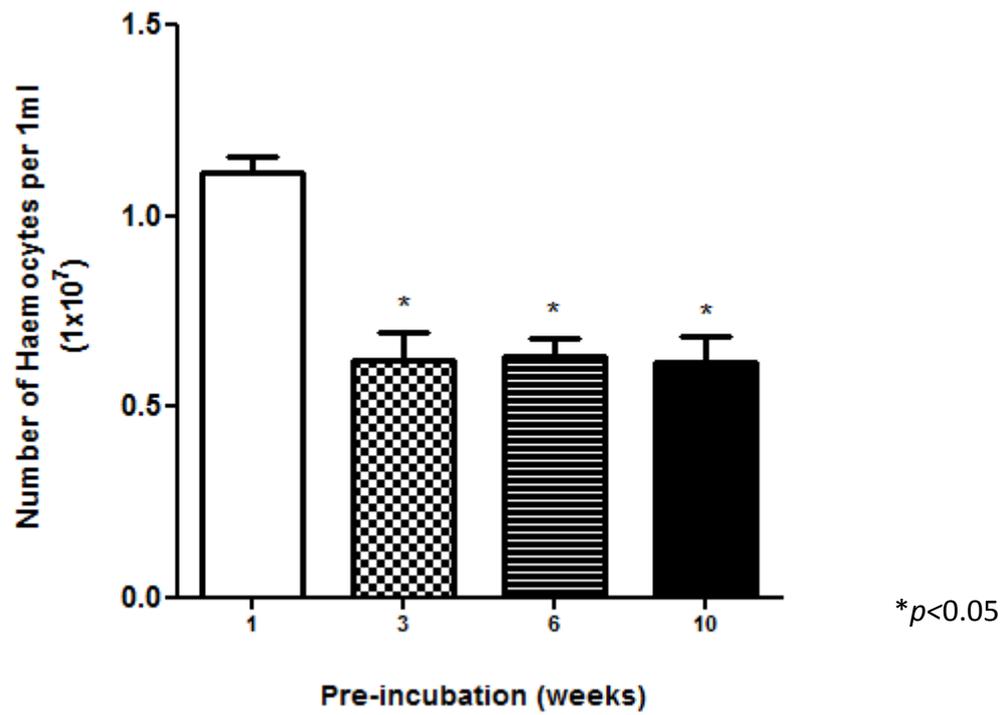
Fig. 5.3 Haemocyte densities enumerated from larvae of ages 1, 3, 6 and 10 weeks on a haemocytometer.
Statistical significance of *Galleria* haemocyte densities was analysed with two-way Anova method using GraphPad Prism version 5.00.
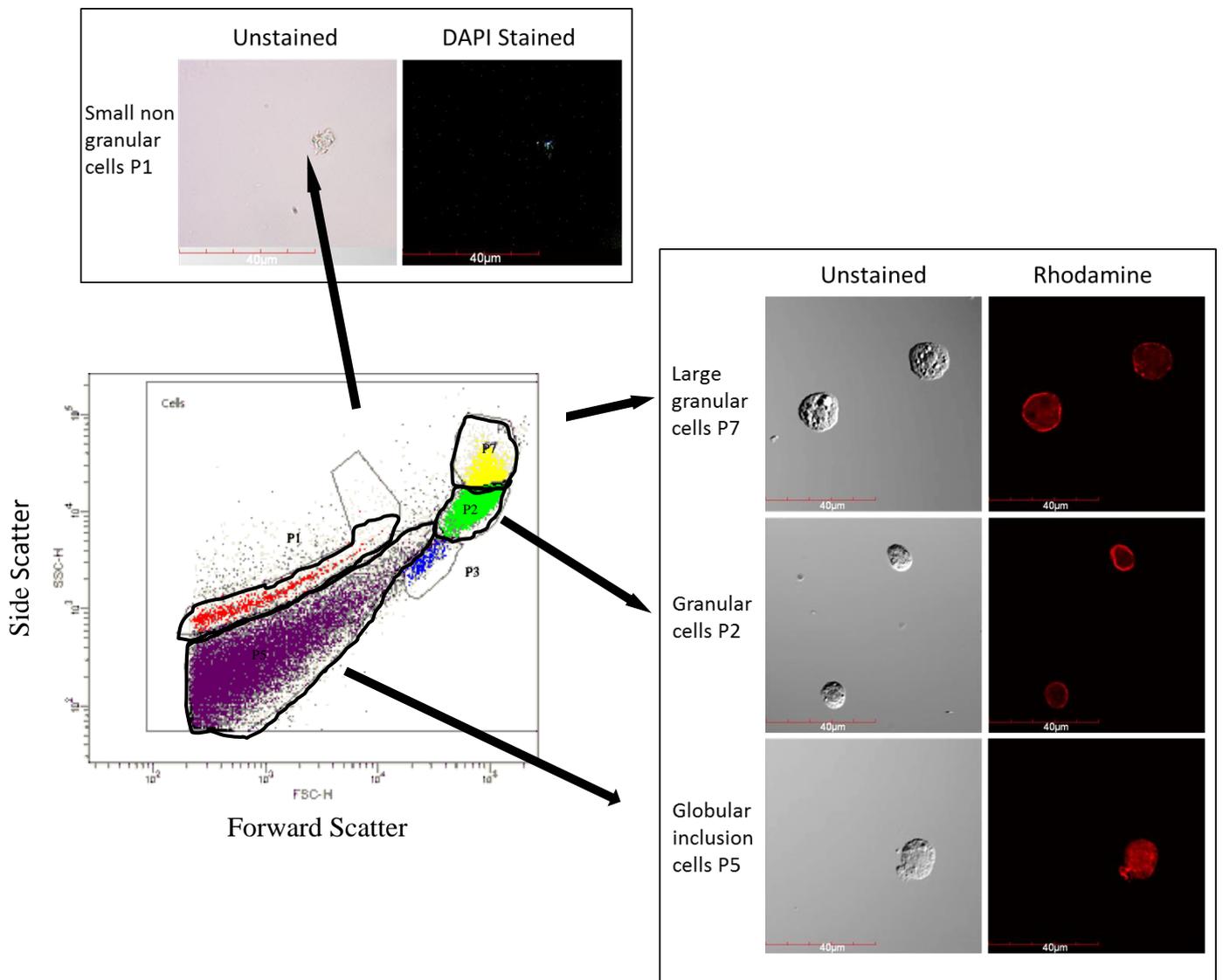
Fig. 5.4 FACs scatter plot image of haemocyte populations and their identities visualised by an Olympus confocal immunofluorescence microscope (right).
Scatter plot image (left), images of haemocyte population P1 (top left), P7 (top right), P2 (middle right) and P5 (bottom right).
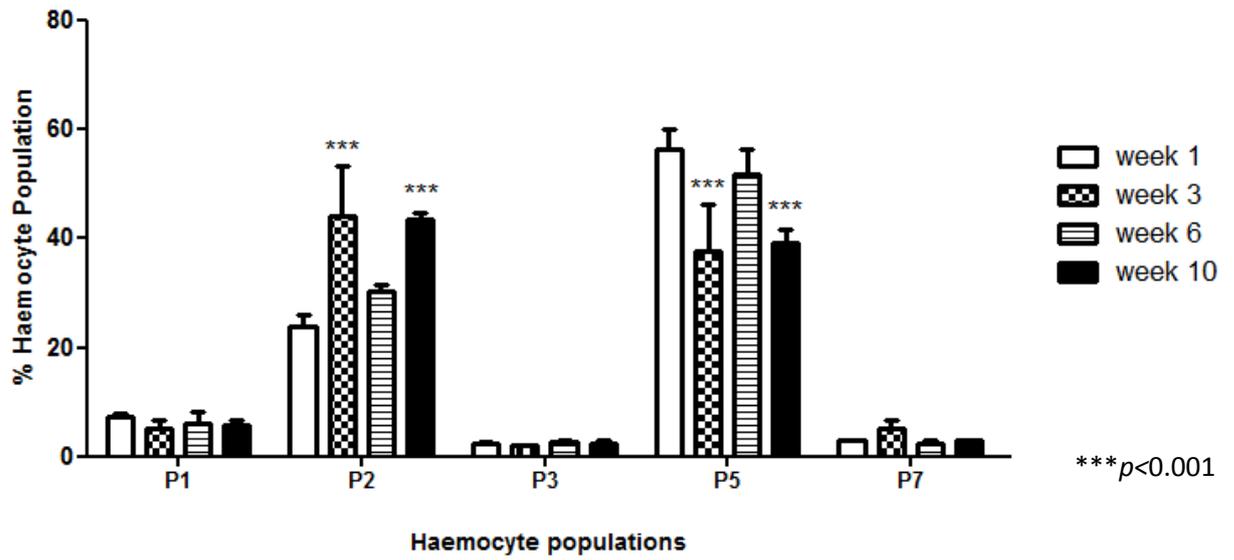
Fig. 5.5 Graph representation of haemocyte proportions from sub-population P1, P2, P3, P5 and P7 isolated from larval ages of 1, 3, 6 and 10 weeks old.

globular inclusions) in the population decreased over time, i.e. week 1; 56.4±3.8 %, week 3; 37.65±8.75 (*p*<0.001), week 6; 51.7± 4.8 % and week 10; 43.55±1.25 % (p < 0.001) (Fig. 5.5). The abundance of the other haemocyte populations (P1, P3 and P7) remained relatively constant in larvae incubated for 1, 3, 6 and 10 weeks (Fig. 5.5).

5.3 Summary

Pre-incubation at 15°C for 1, 3, 6 or 10 weeks demonstrated significant differences in the susceptibility of larvae to infection with *C.albicans* and *S.aureus*. Analysis of the haemocyte density in larvae pre-incubated for 3 - 10 weeks showed a significant reduction in circulating haemocytes over time. But when assessing the proportions of what made up the haemocyte populations there was an increase in the population of P2 granular haemocytes with increased duration of incubation and a reduction in P5 haemocytes with time compared to the 1 week pre-incubated as determined by FACS analysis.

5.4 Analysis of the proteome from larvae pre-incubated at 15°C for up to 10 weeks

Proteomic analysis by label free quantitation using the Q-Exactive Mass Spectrometer (Thermo Scientific^{TM}), demonstrated a significantly altered abundance of proteins between 1 week pre-incubated larvae and 10 week incubated larvae (Fig. 5.6). It was observed that 26kDa and 32kDa ferritin subunits were significantly increased within the 10 week old larvae compared to the control, this was previously shown to occur in older *Drosophila* (Robinson *et al*., 2010) and in insects in response to colder temperatures (Carrasco *et al*., 2011). It is known that ferritins have roles which include iron storage (Arosio *et al*., 2009), transport (Zhou *et al*., 2007) and as antioxidants (Strickler-Dinglasan *et al*., 2006). Transferrin and ferritins also function in preventing microbial growth in *G.mellonella* by limiting free iron in the haemolymph (Levy *et al*., 2004) (Fig. 5.6) (Table 5.1D). In contrast the abundance of arylphorin declined significantly in 10 week pre-incubated larvae compared to the 1 week pre-incubated larvae and it is a protein known to have immune functions (Beresford *et al*., 1997).

The abundance of proPO associated proteins such as proPO was reduced in 10 week pre-incubated larvae but serpins were significantly higher in abundance in 10 week pre-incubated larvae (Active Serpin K) or were present (protease inhibitor 3) in 10 week but absent in 1 week pre-incubated larvae (Table. 5.1C). Proteins associated with metabolic functions such as isocitrate dehydrogenase which functions in TCA metabolism was absent in 10 week pre-incubated larvae and triacylglycerol lipase, important in degradation of diacylglycerol stores from adipohaemocytes, was absent in 1 week old larvae but present in 10 week pre-incubated larvae (Table. 5.1B). The hormone protein 3-dehydroxyecdysone was absent in 10 week pre-incubated larvae unlike the 1 week pre-incubated larvae, this protein has functions in molting including roles which are also crucial to insect growth, development and reproduction (Yang *et al*., 2011).

In contrast, the relative abundance of immune related proteins (apolipophorin and apolipophorin-II) was higher in abundance in 1 week pre-incubated larvae while scavenger receptor was detected in 1 week pre-incubated larval haemolymph but was absent in 10 week pre-incubated larvae. The 10 week pre-incubated larvae demonstrated increased abundance of immune proteins (hemolin and spodptericin), with other immune proteins detected in 10 week pre-incubated larval haemolymph (seroin and putitive defence proteins) but absent in 1 week pre-incubated larvae (Table. 5.1A).

In order to confirm the label free analysis results, 2D SDS PAGE analysis was carried out on haemolymph from larvae incubated at 15°C for 1, 3, 6 or 10 weeks as described. In total, 14 peptide spots were shown to be altered in abundance in larvae (Fig. 5.7). Proteins showing alteration in abundance were excised, digested and identified by LC/MS (Table 5.2) as described. The results demonstrated a similar trend to the results obtained by label free analysis with proteins associated with the proPO cascade (e.g. masquerade-like serine proteinase, protease serine 1 precursor and proPO subunit-2) showing a gradual decrease in abundance over time (Fig. 5.8). Proteins with functions in metabolic pathways (e.g. malate dehydrogenase, fructose-1,6-bisphosphatase and aliphatic nitrilase) (Fig. 5.8) also showed a decrease in abundance in those larvae incubated at 15°C for 6 or 10 weeks. Label free analysis showed the absence of the isocitrate dehydrogenase in 10 week pre-incubated larvae

while the 2D SDS PAGE analysis demonstrated the reduced abundance of malate dehydrogenase in 10 week pre-incubated larvae both proteins components of the TCA cycle.

The relative abundance of selected immune proteins (e.g. beta-1, 3-glucan recognition protein precursor) important in fungal recognition (Johansson and Soderhall, 1996), increased over the course of the incubation period (Fig. 5.8) whereas the label free results demonstrated some immune proteins to have increased abundance in 10 week pre-incubated or dropped in abundance at 10 weeks compared to the control. It was also observed that the abundance of 26kDa and 32kDa ferritin demonstrated an increase in abundance in larvae incubated at $15^{o}C$ for 10 weeks (Fig. 5.8) and this was similar to the label free analysis result. The abundance of arylphorin declined with increased incubation of larvae measured by 2D SDS PAGE analysis which was similar to that found with the label free analysis.

5.5 The effect of pre-incubation on gene expression in *G.mellonella* larvae

The expression of genes coding for *Galiomicin*, (a defensin (Lee *et al*., 2004)), *Gallerimycin* (a cysteine-rich antifungal peptide (Schuhmann *et al*., 2003)), IMPI (inducible metallo-proteinase inhibitor (Clermont *et al*., 2004)) and *Transferrin* (an iron-binding protein (Yoshiga *et al*., 1997)) was assessed in larvae following pre-incubation at $15^{o}C$ for 1, 3, 6 or 10 weeks and normalised against the expression of *S7e* (Fig. 5.9). *Galiomicin* demonstrated a slight and non-significant decrease in expression in larvae following 3 (0.94±0.38), 6 (0.74±0.35) and 10 (0.83±0.08) weeks incubation relative to the expression in larvae incubated at that temperature for 1 week (1.0±0.21). A similar decrease was observed in the expression of *Gallerimycin* which was significantly decreased in week 10 larvae (0.221±0.36, $p < 0.05$) compared to that in larvae incubated at $15^{o}C$ for 1 week (1.0±0.45). The expression of *Transferrin* and *IMPI* remained relatively constant over the course of the experiment.

195
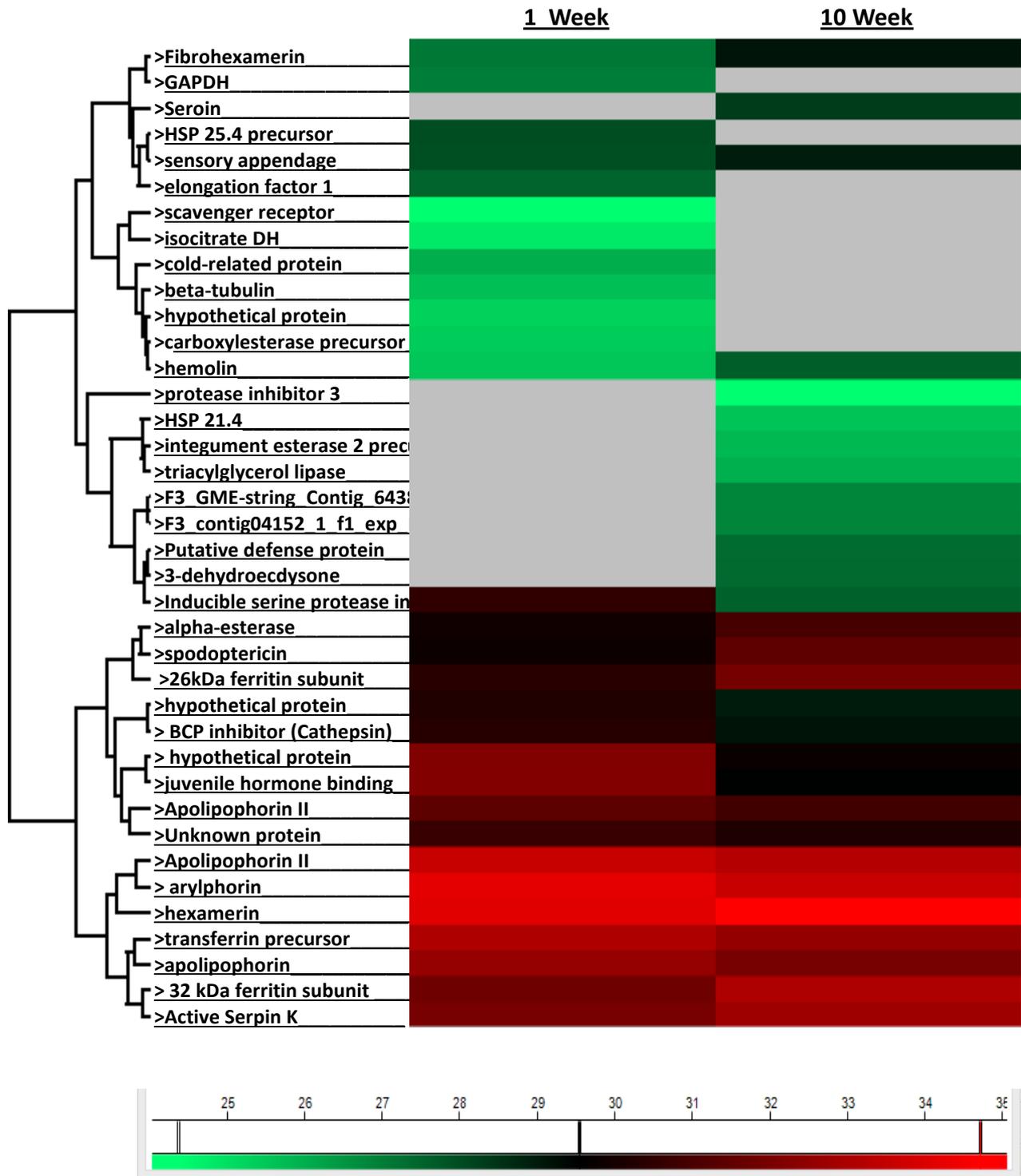
Fig. 5.6 Heat map of identified proteins by label free analysis on 1 and 10 week pre-incubated larvae.

Protein identities (left), relative abundance represented by colours (right) for 1 and 10 week pre-incubated larvae. Colour legend (bottom) with increasing protein expression from left to right, green (left) representing the lowest expression and red (right) highest expression. Grey colour representing absence of protein. Only significant fold changes above ($p<0.05$) were taken or proteins that were absent in one treatment over the other. Replicates (n=4)

A

Immune related proteins					
Protein	**Week 1** (Protein intensity)	**Week 10** (Protein intensity)	**Fold change relative to 1 week**	**Significance** ($p<0.05$)	**Protein function**
Seroin	Absent	28.3	-	N/A	Antiviral and antibacterial activity
Scavenger receptor	24.0	Absent	-	N/A	Binds Gram-negative bacteria and LPS.
Hemolin	25.5	27.6	-4.3	+	Binds to bacteria and LPS.
Putative defense protein	Absent	27.4	-	N/A	Proteins that function as PRRs, antimicrobials or as protease inhibitors.
Spodoptericin	29.8	31.5	-3.3	+	Defensin-like gene.
Apolipophorin II	31.4	30.9	1.1	+	Apolipophorin-III PRR of microbial components.
Apolipophorin II	33.5	33.2	0.75	+	
Apolipophorin	32.6	32.0	1.2	+	

B

Metabolic proteins					
Protein	**Week 1** (Protein intensity)	**Week 10** (Protein intensity)	**Fold change relative to 1 week**	**Significance** ($p<0.05$)	**Protein function**
GAPDH	27.0	Absent	-	N/A	Involved in glycolysis and can sequentially phosphorylate signalling cascades.
Isocitrate DH	24.8	Absent	-	N/A	Component important in the TCA cycle.
Triacylglycerol lipase	Absent	26.0	-	N/A	Metabolises triglycerides.
3-dehydroxyecdysone	Absent	27.4	-	N/A	Crucial to insect growth, development, reproduction

C

Prophenoloxidase pathway proteins					
Protein	**Week 1** (Protein intensity)	**Week 10** (Protein intensity)	**Fold change relative to 1 week**	**Significance** ($p<0.05$)	**Protein Function**
Protease inhibitor 3	Absent	24.4	-	N/A	Serpins inhibit chymotrypsin-like serine proteases. (e.g. proPO cascade)
Inducible serine protease inhibitor 2	30.5	27.6	5.9	+	
Active Serpin K	31.9	32.8	-1.6	+	
Prophenoloxidase	28.3	27.8	0.9	-	Component of melanisation cascade.

D

Dual function proteins					
Protein	**Week 1** (Protein intensity)	**Week 10** (Protein intensity)	**Fold change relative to 1 week**	**Significance** ($p<0.05$)	**Protein Function**
32 kDa ferritin	31.8	33.1	-2.5	+	Involved in iron storage, transport and immune system.
26kDa ferritin	30.4	31.9	-3.0	+	
Transferrin precursor	33.1	32.5	1.2	+	
Arylphorin	34.2	33.6	1.2	+	Amino acid storage and immune functions.

Table 5.1 Change in fold abundance of proteins identified by label free analysis from 1 and 10 week pre-incubated larvae.

Protein intensity changes in expression represented as a decrease (-1.0) or an increase (1.0) relative to the 1 week pre-incubated larvae. Significant protein fold changes (+) or not applicable (N/A), non significant (-) and (Absent) representing proteins not detected. Tables showing immune related proteins (A), metabolic proteins (B), prophenoloxidase pathway proteins (C) and dual function proteins (D), replicates (n=4).
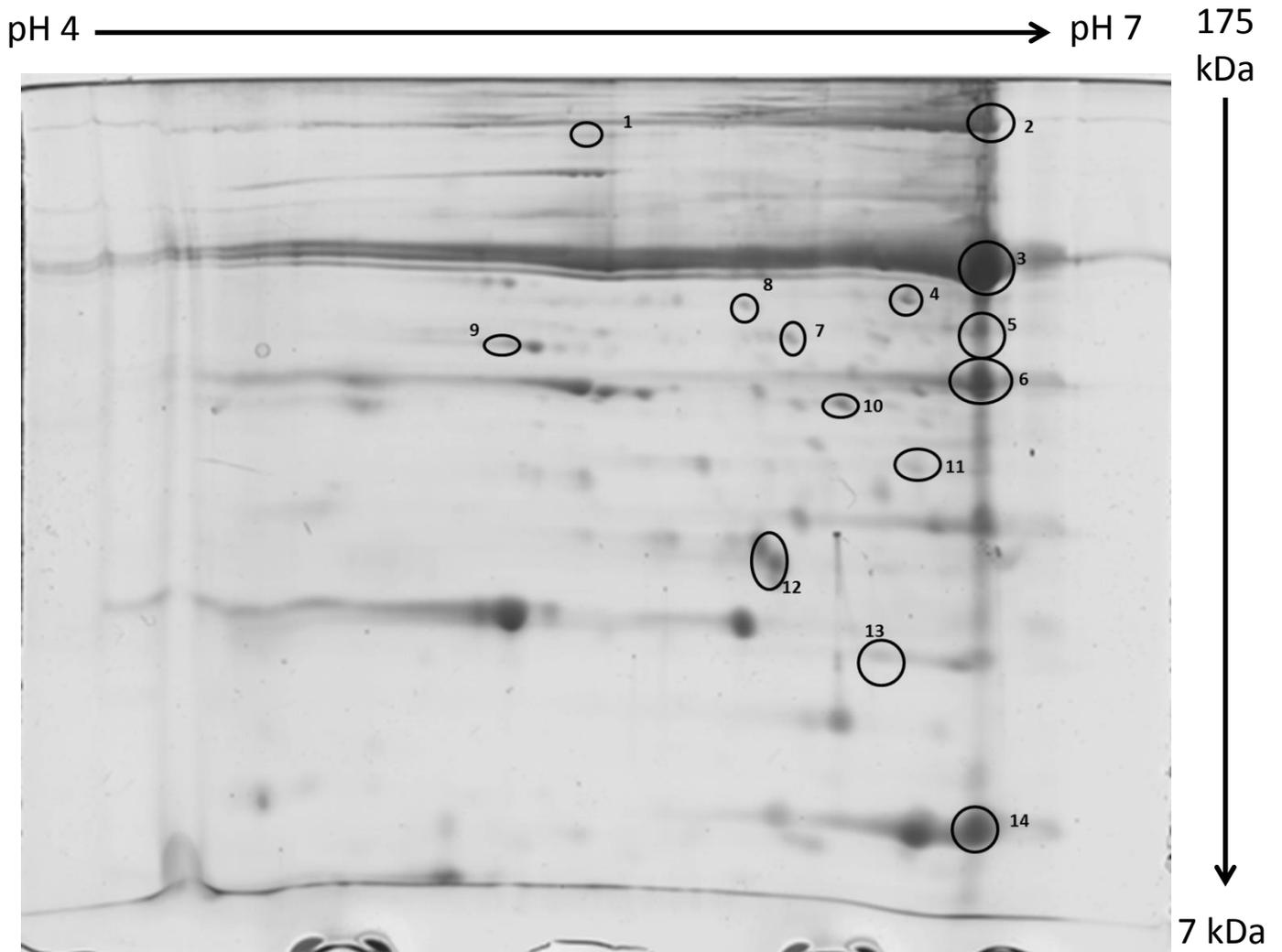
Fig. 5.7 2D SDS PAGE reference gel of haemolymph from 15°C pre-incubated *G. mellonella* larvae. Haemolymph was extracted from larvae and protein was separated on 12.5% acrylamide gels as described, replicates (n=3). Proteins showing alterations in abundance over the course of the incubation period were identified (1 – 14), excised, digested and analysed by LC/MS.

Spot No.	Protein Identity	Organism	Mr	pI	Score	Seq. coverage (%)	Relative Expression			
							Week 1	Week 3	Week 6	Week 10
1	Arylphorin	*G. mellonella*	83651	5.23	371	14	1	0.79	0.60	0.53
2	Prophenoloxidase subunit 2	*G. mellonella*	80198	5.95	428	11	1	1.10	0.73	0.55
3	Transferrin precursor	*G. mellonella*	77238	6.76	362	13	1	0.80	0.58	1.04
4	Malate dehydrogenase	*B. mori*	67969	6.12	149	7	1	0.93	0.77	0.29
5	Apolipophorin	*G. mellonella*	168330	6.25	1673	28	1	1.10	1.04	0.84
6	Hemolin	*G. mellonella*	47408	6.85	137	9	1	0.99	0.74	0.39
7	Protease, serine, 1 precursor	*M. musculus*	26814	4.75	75	8	1	1.34	1.00	0.81
8	Beta-1,3-glucan recognition protein precursor	*G. mellonella*	55882	5.65	289	14	1	0.84	0.99	1.81
9	Masquerade-like serine proteinase	*P. rapae*	46063	5.54	147	7	1	1.23	0.92	0.71
10	Aliphatic nitrilase	*B. mori*	44742	6.16	150	8	1	0.72	0.74	0.72
11	Fructose-1,6-bisphosphatase	*B. mori*	36896	8.40	107	9	1	0.71	0.54	0.17
12	32 kDa ferritin subunit	*G. mellonella*	26731	5.69	615	39	1	0.97	1.23	2.09
13	26kDa ferritin subunit	*G. mellonella*	23936	6.22	228	27	1	1.28	1.49	1.62
14	Apolipophorin-III	*G. mellonella*	20499	8.59	361	35	1	1.51	1.09	1.60

Table 5.2 Protein spot identities from larval haemolymph.
Protein identities from excised and trypsin digested spots 1-14 were identified by LC/MS. The relative fold changes in proteins abundance was determined from 1, 3, 6 and 10 week old larvae using Progenesis SameSpot Software.
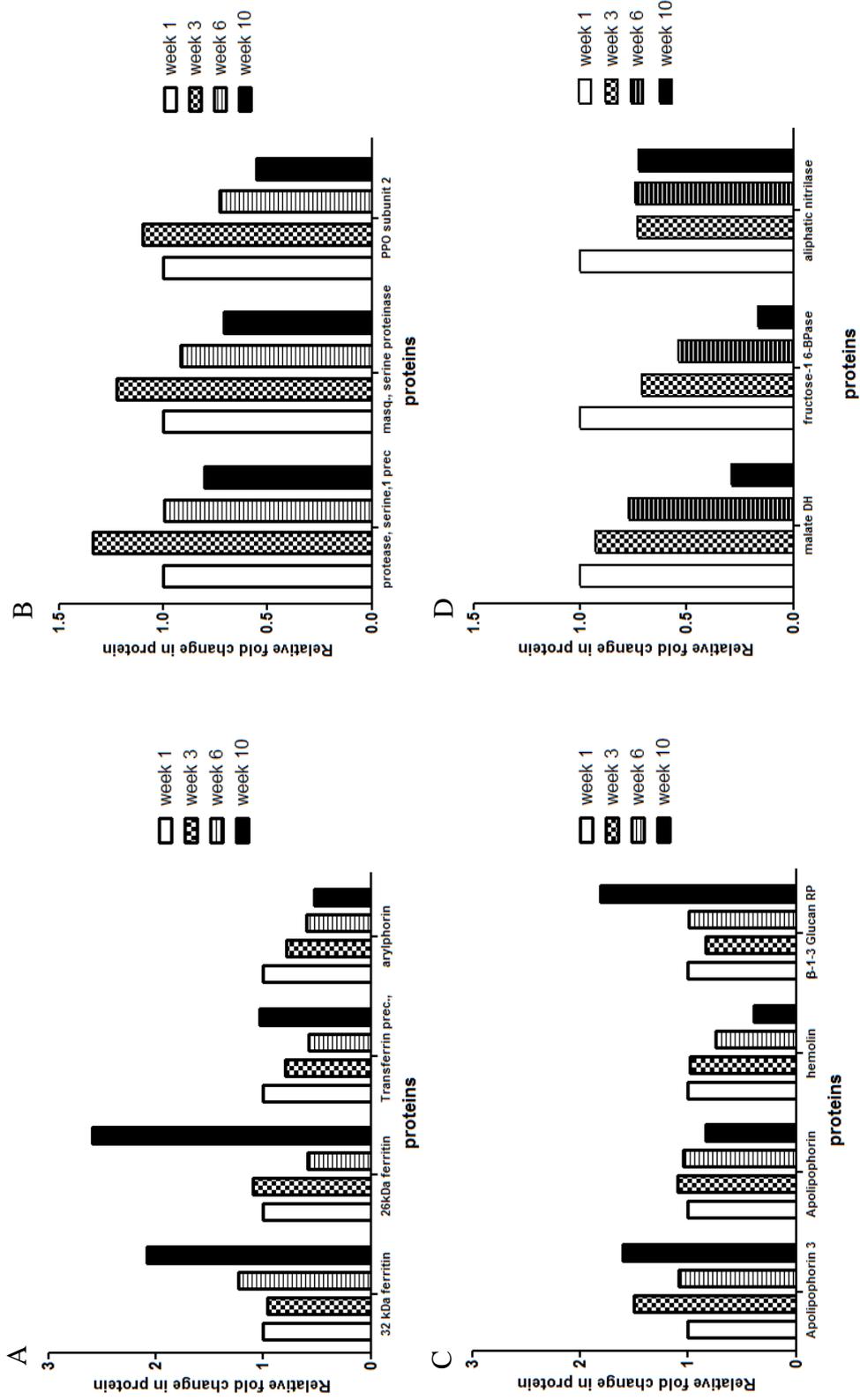
Fig. 5.8 Relative fold change in protein abundance of larvae incubated at 15°C for 1, 3, 6 and 10 weeks as determined using Progenesis SameSpot Software.

The relative abundance of proteins was assessed and proteins were classified by function; Dual function proteins (A), Prophenoloxidase pathway proteins (B), Immune related proteins (C) and Metabolic proteins (D).
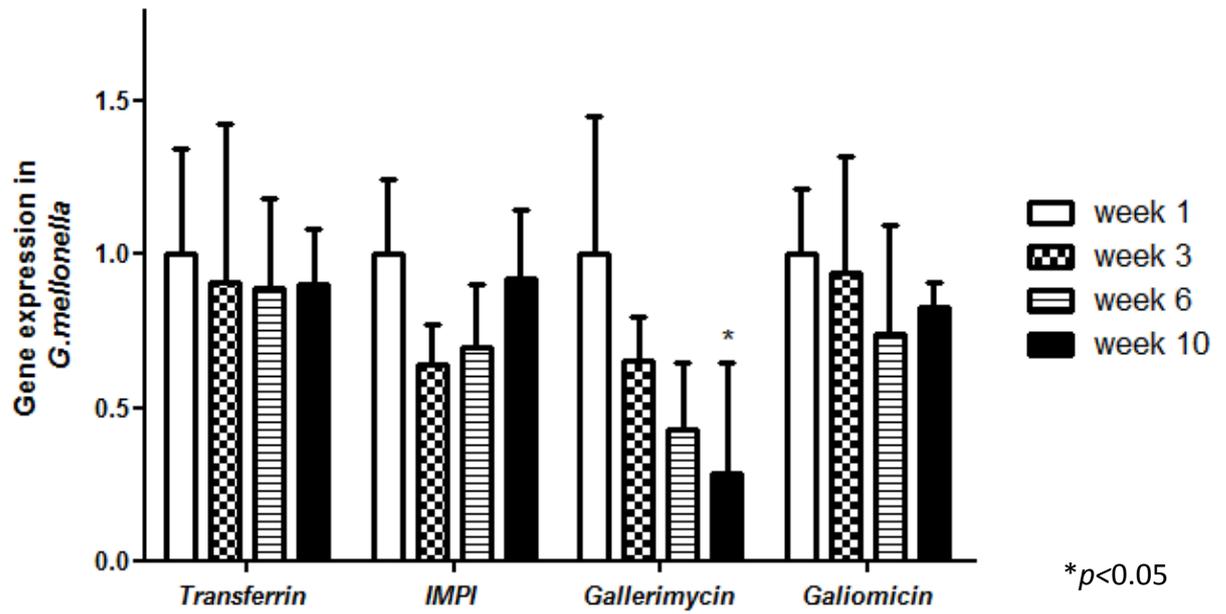
Fig. 5.9 qPCR analysis of the relative expression of *G.mellonella* larval genes *Transferrin, IMPI, Gallerimycin* and *Galiomicin* in 1, 3, 6 and 10 week pre-incubated larvae.
Relative fold changes were assessed from RNA extracted and cDNA synthesised from *G.mellonella* from 1, 3, 6 and 10 week old larvae, replicates (n=3).

5.6 Analysis of the proteome of larvae starved for 7 day

The differences in larval survival to infection when pre-incubated for varied durations prompted the assessment into the effects of food deprivation on larvae as researchers use larvae in the presence or absence of food. The haemolymph of larvae that were deprived of nutrients for 0 or 7 d was extracted and prepared for 2D SDS-PAGE, as described (Section 2.11.1). The resolved gels demonstrated a down regulation in the abundance of proteins in the serum from starved larvae (Fig. 5.10). A number of proteins with known immune function were reduced in abundance (Table 5.3). Apolipophorin (Spot 3), which functions in the haemolymph to transport lipids and aids in the activation of innate immune responses, (Gupta *et al*., 2010) demonstrated an approximately 50% reduction in abundance in starved larval haemolymph. Arylphorin (Spot 4) was down regulated by 37% in the haemolymph of starved larvae. Arylphorin functions in the storage of the aromatic amino acids but it also plays a central role in insect immunity (Beresford *et al*., 1997). Spot 5 showed homology to lipocalin which functions in the immune response by binding to toxins and by complexing invading pathogens (Pandian *et al*., 2010) and its expression was reduced by approximately 14% in starved larvae.

Ferritin (Spot 1) and transferrin precursor (Spot 6) were reduced in expression by approximately 50% in food-deprived larvae. Ferritin is important in iron binding and storage and maintaining cellular iron homeostasis along with playing a role in the immune response (Choi *et al*., 2006; Levy *et al*., 2004). Transferrin can sequester iron ions essential to invading pathogens and impede microbial colonisation of the insect hemocoel (Seitz *et al*., 2003). Imaginal disc growth factor-like protein (Spot 2) had reduced expression of 40% in starved larvae. The expression of juvenile hormone binding protein (Spot 7) remained relatively constant at approximately 90% in starved larvae and as such may be considered as a loading control. The expression of this protein was demonstrated to be maintained at a constant level when *G. mellonella* larvae were challenged with β-glucan (Mowlds *et al*., 2010). Analysis of the proteome of starved larvae indicates a decrease in the abundance of a range of proteins associated with the immune response. The humoral immune response is important in defending insects from infection, the decrease observed in the abundance of immune related proteins could make larvae susceptible to infection.
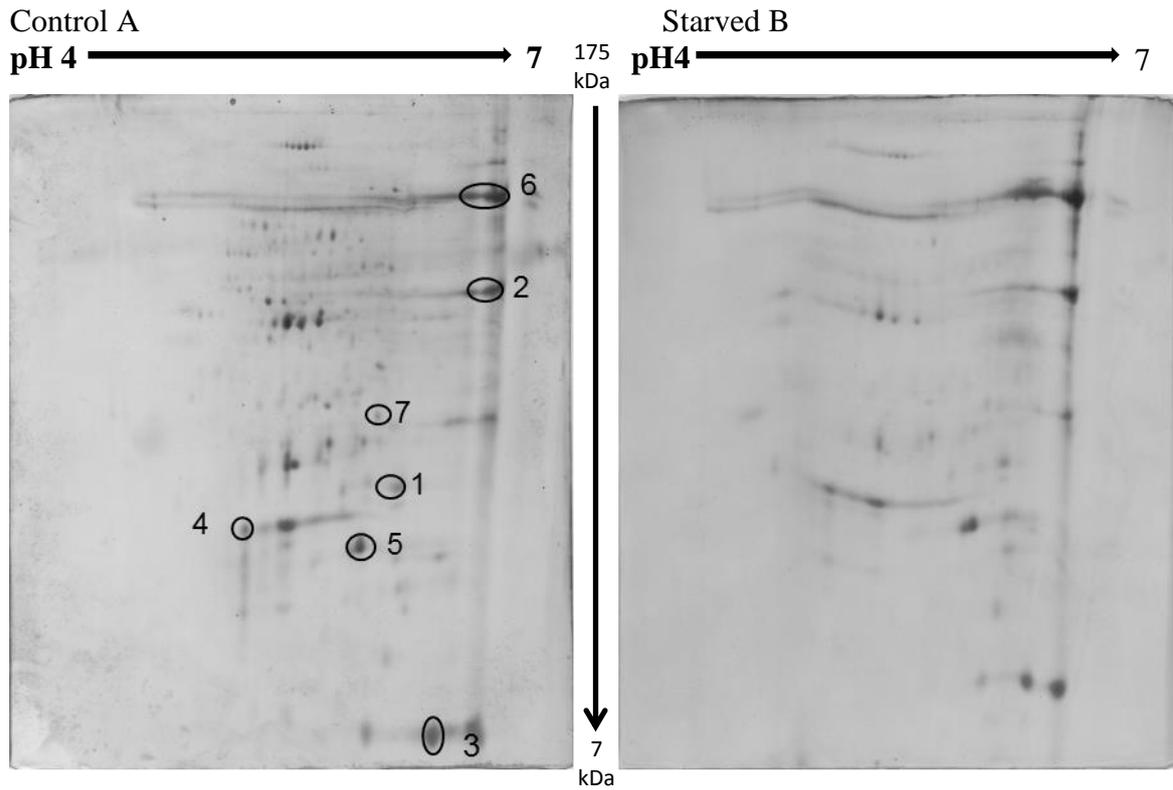
Fig. 5.10 Proteomic profiles from haemolymph of unstarved larvae (A) and larvae starved for 7 d (B). Protein was extracted from larval haemolymph as described and resolved by 2D SDS-PAGE, replicates (n=3). Protein spots showing alterations in expression were extracted and identified.

Spot no.	Protein Identity	Organism	Mr	pI	Score	Seq. coverage (%)	Accession no.	Protein fold change		Protein Function
								Con	Starved	
1	32 kDa ferritin subunit	*G. mellonella*	26731	5.69	173	18	AAL47694	1.0	0.53	Involved in iron storage, transport and immune system.
2	Imaginal disc growth factor-like protein	*Mamestra brassicae*	48030	7.08	111	6	ABC79625	1.0	0.61	Important in regulating cell proliferation/ growth.
3	Apolipophorin-III	*G. mellonella*	20499	8.59	254	37	P80703	1.0	0.53	PRR of microbial components.
4	Arylphorin	*G. mellonella*	83651	5.23	78	3	AAA74229	1.0	0.639	Amino acid storage and immune functions.
5	Lopap (Lipocalin)	*Danaus plexippus*	25036	5.46	83	11	EHJ63999	1.0	0.87	Functions in the immune response by binding to toxins and pathogens
6	Transferrin precursor	*G. mellonella*	77238	6.76	111	6	AAQ63970	1.0	0.46	Involved in iron storage, transport and immune system.
7	Juvenile hormone binding protein precursor	*G. mellonella*	27460	6.84	163	13	AAS94224	1.0	0.91	Precursor of JHBP, regulates embryogenesis and reproduction; JHBP protects JH molecules from hydrolysis by non-specific esterases.

Table 5.3 Mass spectrometry identities of peptide matched to gel spots (1-7) from unstarved larvae on Gel (A). The fold decrease indicated is relative to Gel (A), (Con) = control.

5.7 Summary

Proteomic analysis revealed decreased abundance of proteins associated with the prophenoloxidase pathway and metabolic pathways. Label free analysis demonstrated a change in the abundance of immune related proteins between 1 week and 10 week pre-incubated larvae. The abundance of a number of proteins analysed by label free and 2D SDS PAGE methods correlated well for the 26 and 32 kDa ferritin subunits which increased within 10 week pre-incubated larvae while metabolic proteins and arylphorin decreased in 10 week pre-incubated larvae relative to 1 week pre-incubated larvae. The effect of pre-incubation on larval gene expression demonstrated a reduced expression of *Gallerimycin and Galiomicin* over time while *IMPI* (inducible metallo-proteinase inhibitor) and *Transferrin* expression remained relatively constant.

Food restriction on larvae for a 7 d period resulted in reduced abundance of humoral immune proteins (apoLp, ferritin and arylphorin). Such effects on the immune response whether food restriction or age can have implications on the larvae's ability to launch an effective immune response against a pathogen.

5.8 Discussion

Insects have become popular and useful alternatives to the use of mammals for assessing the virulence of microbial pathogens and for determining the *in vivo* efficacy of antimicrobial agents (Fallon *et al*., 2012; Fuchs and Mylonakis, 2006; Kavanagh and Reeves, 2004; Lionakis *et al*., 2005). Despite their widespread use standardised procedures for their incubation and infection have not yet been developed (Cook and McArthur, 2013). *G. mellonella* larvae are commonly incubated at 15°C or room temperature for various periods of time prior to infection.

Here it was observed that larvae that were incubated for extended periods at 15°C have greatly reduced survivals when infected. Larvae incubated for 3 weeks or more demonstrated reduced survival to *S.aureus* infection after 24h when compared to the 1 week pre-incubated larvae. Larvae pre-incubated for longer periods also demonstrated reduced survival to infection with *C.albicans* at 24h. These survival assessments following microbial challenge provide evidence that incubation of larvae

at 15°C for up to 10 weeks prior to infection leads to a decrease in the ability of *G. mellonella* larvae to withstand bacterial and fungal infection.

Larvae incubated at 15°C for 3 weeks or more showed a lower density of haemocytes and the composition of the haemocyte population was changed compared to that in larvae incubated for 1 week. Previous work demonstrated a reduction of up to 31.8% in the number of circulating haemocytes in 4 week old *Drosophila* relative to the 1 week old flies. The same study also observed that the phagocytosing capacity of cells reduced from 24.3%±1.15% in 1 week old flies to 16.7%±0.99% in four week old flies (Mackenzie *et al*., 2011).

While the overall density of haemocytes decreased there was an increase in the proportion of P2 haemocytes but a corresponding decrease in the proportion of P5 haemocytes in larvae incubated for up to 10 weeks. P5 haemocytes have reduced globular inclusions and resemble adipohaemocytes (Kavanagh and Reeves, 2004; Tanada and Kaya, 1993) which store energy in the form of lipids and glycogen (Araújo *et al*., 2008). The reduction in P5 cells in the haemocyte population of larvae incubated for up to 10 weeks may be an indication that energy reserves in the form of lipids are being utilised by the larvae during the prolonged incubation stage. The P2 haemocytes are granular cells which function in phagocytosing pathogens (Kavanagh and Reeves, 2004; Tanada and Kaya, 1993). It has been recently described during the metamorphosis process of lepidoteran that small granular haemocytes like the P2 population increase in size to large granular haemocytes such as the P7 population and coincided with a reduction in adipohaemocytes (Zhai and Zhao, 2012). Although it was observed that the P5 population which resemble adipohaemocytes declined in larvae with increased incubation, these older larvae did not demonstrate an increased proportion of large granular cells (P7) suggesting this is not associated with a morphological change in larvae but may be age related depletion of energy reserves within adipohaemocytes.

The proteomic method of label free analysis is a highly sensitive method capable of delivering a large dynamic range of protein identifications compared to traditional gel based proteomic analysis. Here analysis of proteomic changes from larvae incubated at 15°C for up to 10 weeks indicated decreased abundance of a number of proteins involved in metabolism. Malate dehydrogenase, an enzyme associated with the TCA cycle, demonstrated a decrease in abundance within the 2D SDS PAGE analysis (Fig. 5.8D). The label free analysis demonstrated the absence of

TCA enzyme isocitrate dehydrogenase in 10 week pre-incubated larvae unlike the 1 week pre-incubated larvae. Aconitase, an enzyme in the TCA cycle, and adenine nucleotide translocator which regulates the intramitochondrial ADP/ATP ratio, were also shown to decline by approximately 50% in abundance during aging in insects (Das *et al*., 2001; Yan and Sohal, 1998).

The abundance of proteins with homology to prophenoloxidase subunit-2, masquerade-like serine proteinase and protease serine 1 precursor identified by 2D SDS PAGE analysis were decreased in larvae pre-incubated for 10 weeks compared to the control larvae (Table 5.2). It was previously shown by Pletcher *et al.*, (2002) that serine type proteases were down-regulated at the gene transcript level within aged *Drosophila*. Label free analysis showed a reduction in the abundance of proPO within 10 week pre-incubated larvae when compared to 1 week pre-incubated larvae which was similar to the 2D SDS PAGE analysis. The label free analysis also demonstrated the absence of protease inhibitor 3 and a lower abundance of Active Serpin K in 1 week pre-incubated larvae which may contribute to a reduced inhibition of the melanisation cascade and therefore a greater ability of 1 week pre-incubated larvae to overcome a microbial challenge.

In contrast the abundance of 26kDa ferritin and 32kDa ferritin increased over time particularly in the 10 week pre-incubated larvae when analysed using 2D SDS PAGE and label free proteomic analyses. Ferritins function in iron storage (Arosio *et al*., 2009), transport (Zhou *et al*., 2007), and have antioxidant properties (Strickler-Dinglasan *et al*., 2006). Increased expression of ferritin may improve resistance to oxidative stress (Missirlis *et al*., 2006). Alternatively the higher abundance of ferritin may act as a compensatory immune function for the reduced population of haemocytes in larvae, through ferritin's ability to impede microbial growth. Triacylglycerol lipase which was absent in 1 week but present in 10 week pre-incubated larvae is important in degradation of diacylglycerol stores from adipohaemocytes. The adipohaemocytes were identified to most likely be the P5 population and were significantly decreased within older larvae potentially as these glycerol reserves are depleted.

Label free analysis demonstrated a number of changes in the abundance of immune related proteins. This included the absence of PRR scavenger receptor and the reduced abundance of (apolipophorin and apolipophorin-II) in 10 week pre-incubated larvae when compared to the 1 week pre-incubated larvae. The 10 week

pre-incubated larvae demonstrated increased abundance of immune proteins (hemolin and spodoptericin homologue) and the presence of immune proteins (seroin and putitive defence proteins) when compared to the 1 week pre-incubated larvae. The 2D SDS PAGE analysis demonstrated the abundance of a number of pattern recognition proteins to have remained constant (e.g. apolipophorin, apolipophorin-III) between pre-incubated larvae. While the abundance of PRR β-1, 3-Glucan recognition protein precursor, increased by 81% in larvae pre-incubated for 10 weeks relative to the abundance of those larvae incubated for 1 week. This PRR and other immune proteins increased in 10 week pre-incubated larvae may be up-regulated as a result of decreased phagocytosis ability and melanisation which has been observed in *Drosophila* (Zerofsky *et al*., 2005). Arylphorin was increased in abundance within the 1 week pre-incubated larvae for both the label free and 2D Gel analyses. Overall the 2D SDS PAGE and the label free analyses of the larval proteome demonstrated some correlations but it is important to note that label free analysis is a far more sensitive analytical method than 2D SDS PAGE.

Analysis of the expression of genes associated with the immune response to pathogens revealed decreased expression of *Gallerimycin* over the course of the experiment which was deemed significant in 10 week pre-incubated larvae when compared to the 1 week pre-incubated larvae. A small but non-significant reduction was observed in the expression of *Galiomicin* with increased larval incubation time, but the expression of *IMPI* and *Transferrin* remained relatively constant. This possibly indicates that certain processes are being maintained in the larvae while others (e.g. immune defence and metabolism) are reduced.

The increased susceptibility of larvae to infection observed here may be due to a weakening of the immune response due to the prolonged incubation at 15°C. The alteration in the relative populations of haemocytes and in the abundance of selected proteins may indicate a change in the physiology of larvae to adapt to the prolonged incubation state. These findings were similarly observed within *Drosophila* which demonstrated reduced survival to infection with age (Ramsden *et al*., 2008) and coincided with a decrease in abundance of circulating haemocytes (Mackenzie *et al*., 2011). It was also observed that age was associated with increased pathogen susceptibility and reduced phenoloxidase levels in the cricket *Gryllus texensis* (Adamo *et al*., 2001). This trend also followed for older mosquitoes (*Aedes aegypti*) which had reduced survival following infection with *E.coli*, a lower ability to

clear the infection, and reduced phagocytic haemocytes compared with younger mosquitoes (Hillyer *et al*., 2005). Whereas the scorpion fly (*Panorpa vulgaris*), demonstrated no change in haemocyte abundance during ageing although activity of phagocytosing cells declined (Kurtz, 2002). These findings have implications for those utilising *G. mellonella* larvae and this effect may contribute to some of the inter-experimental variability that can be encountered in using larvae (Cook and McArthur, 2013). Using larvae that had been incubated for different periods of time prior to infection with a pathogen of interest will give variable results, consequently steps should be taken to ensure that larvae of the equivalent age are used and preferably those that have been stored for less than 3 weeks. However, this finding may be exploited if studying pathogens of relatively low virulence (Bergin *et al*., 2003) where larvae pre-incubated for extended periods (3 − 10 weeks) and with a weaker immune response may be more susceptible to infection compared to those pre-incubated for only a short period of time.

Larvae deprived of food over 7d demonstrated an altered proteome as indicated by decreased expression of a number of proteins with known anti-microbial action (e.g., transferrin) and immune function (e.g., arylphorin). It is known that food deprivation leads to impaired learning in honey bees (*Apis mellifera*) demonstrating a clear link between feeding and neurological function (Toth *et al*., 2005). Analysis of the changes in the reduction of mass-specific metabolism in *D. melanogaster* (Djawdan *et al*., 1997) may indicate that stress has implications on lipid transport. The effects of incubating larvae in the presence or absence of a nutrition source will significantly affect the ability of the larvae to mount an immune response. Consequently, researchers utilising *G. mellonella* larvae for studying the virulence of fungal or bacterial pathogens should specify whether feeding is provided for larvae and the type of material supplied since this may affect experimental outcomes and influence inter-laboratory comparisons.

Using larvae of a standardised age or pre-incubation period within experiments (i.e. less than 3 weeks) would reduce the potential for inter and intra laboratory variability when studying the virulence of pathogens and/or the effectiveness of antimicrobial drugs or compounds. It is also possible to use larvae which are more susceptible to infection specifically to study weaker pathogens which

would not be possible within less susceptible younger larvae (i.e. 1 week pre-incubated larvae) which would overcome weaker pathogens such as *C.glabrata*.

Starvation of larvae also altered the physiological state of the larval haemolymph by reducing the abundance of immune related proteins within starved larvae. The results demonstrate the need for larvae to receive adequate food during experiments to ensure the larval immune response is not compromised as immune defence is energetically costly and may be limited in the absence of compensatory food.

These changes induced in larvae by prolonged pre-incubation or starvation potentially could reduce their ability to mount an effective immune response to overcome a microbial challenge. This highlights the requirement to set a standard time frame to use larvae within (< 3 weeks) and the need to ensure larvae are supplied with an adequate food supply when carrying out experiments so to reduce experimental variability.

Chapter 6

**Assessment of efficacy of
G.mellonella larvae for evaluating
antimicrobial activity of 1, 3-
dibenzyl-4,5-diphenyl-imidazol-2-
ylidene silver(I) acetate (SBC3)**

6.0 Assessment of *in vivo* antimicrobial activity of the carbine silver(I) acetate derivative SBC3 using *G.mellonella* larvae

The aim of the work described in this Chapter was to assess the use of *G.mellonella* larvae as an *in vivo* model for screening of novel drugs as it is widely used to assess the virulence of fungal (Cotter *et al*., 2000; Mylonakis *et al*., 2005) and bacterial (Mukherjee *et al*., 2010; Senior *et al*., 2011) pathogens and has been used to assess the activity of novel antimicrobial compounds against pathogens (Kelly and Kavanagh, 2011; Rowan *et al*., 2009). Here the compound 1,3-dibenzyl-4,5-diphenyl-imidazol-2-ylidene silver(I) acetate SBC3 which is a member of the carbene-silver acetate derived compounds was assessed for its antimicrobial activity *in vivo*.

Extensive use and misuse of the available antibiotic arsenal and the shortage of new drugs reaching the market have made antimicrobial resistance a major threat to healthcare worldwide (Maple *et al*., 1989). This has resulted in an increasing need for new antimicrobial compounds as conventional antibiotics become ineffective against pathogens. The continuous development of novel resistance-disrupting antimicrobial drugs should therefore be a prime activity of industry and academia (Rex, 2014). There is significant unexplored space for antimicrobial silver-based drugs (Mijnendonckx *et al*., 2013; Oehninger *et al*., 2013) and it has been suggested that carbene-silver acetates derived from methylated caffeine may have the stability and antibiotic activity to become drug candidates (Hindi *et al*., 2008; Kascatan-Nebioglu *et al*., 2006). Silver(I) ion is known for its anti-bacterial and anti-fungal (Coyle *et al*., 2003; Rowan *et al*., 2006) properties for a number of years which has led to the development of a number of silver based medical devices such as silver imbedded catheters (Bechert *et al*., 1999), wound dressing (Thomas and McCubbin, 2003) and topical creams for burn treatments (Honari *et al*., 2001). Silver imbedded catheters have been shown to reduce bacterial adherence by up to 40% (Bechert *et al*., 1999), while silver containing wound dressing have demonstrated potent activity against antibiotic resistant strains of *S.aureus* and *P. aeruginosa* (Olson *et al*., 2000; Ulkur *et al*., 2005). The application of topical creams such as silvadene has also been used to treat skin infections demonstrating effective activity against *P. aeruginosa* while also reducing post-burn destruction of skin (Fox, 1968).

This concept led to the development of more lipophilic benzyl-substituted imidazole- and benzimidazole-derived carbene-silver complexes showing activity against Gram-positive and Gram-negative bacteria (Hackenberg and Tacke, 2014; Patil *et al*., 2011b). So far, the most promising derivative is 1,3-dibenzyl-4,5-diphenyl-imidazol-2-ylidene silver(I) acetate (SBC3) (Patil *et al*., 2011a) (Fig. 6.1). SBC3 shows MIC values ranging from 20 to 3.13 µg/ml against Methicillin-sensitive and -resistant *S.aureus* as well as *Salmonella*, *E.coli* and *P.aeruginosa* (Sharkey *et al*., 2012).

Evaluating the *in vivo* efficacy and stability of novel antimicrobial compounds such as SBC3 is possible using insects such as *G.mellonella* larvae. The insect immune system shows many similarities to the innate immune system of mammals (Kavanagh and Reeves, 2004; Müller *et al*., 2008) and, as a consequence, insects have been used as models to measure the virulence of microbial pathogens (Fuchs and Mylonakis, 2006) and to evaluate the potency of antimicrobial drugs (Hamamoto *et al*., 2004; Lionakis and Kontoyiannis, 2005; Lionakis *et al*., 2005; Rowan *et al*., 2009) and give results consistent with those that can be obtained using mammals (Brennan *et al*., 2002; Jander *et al*., 2000). A number of insect species can be employed for evaluating the *in vivo* activity of novel antimicrobial drugs (Kavanagh and Fallon, 2010) and larvae of the Greater Wax Moth, *G.mellonella* are now widely used in this capacity (Desbois and Coote, 2012; Kelly and Kavanagh, 2011; Rowan *et al*., 2009). *G.mellonella* larvae provide a rapid turnover of results when screening promising compounds which can cut down the pool of compounds to be assessed when examining their effects in more expensive mammalian models (Fallon *et al*., 2012).

The aim of this Chapter was to establish the antimicrobial effects of a novel compound (SBC3) against the bacterium *S.aureus* and the yeast *C.albicans in vitro* and to demonstrate the *in vivo* activity and toxicity of SBC3 against these two pathogens in *G. mellonella* larvae.
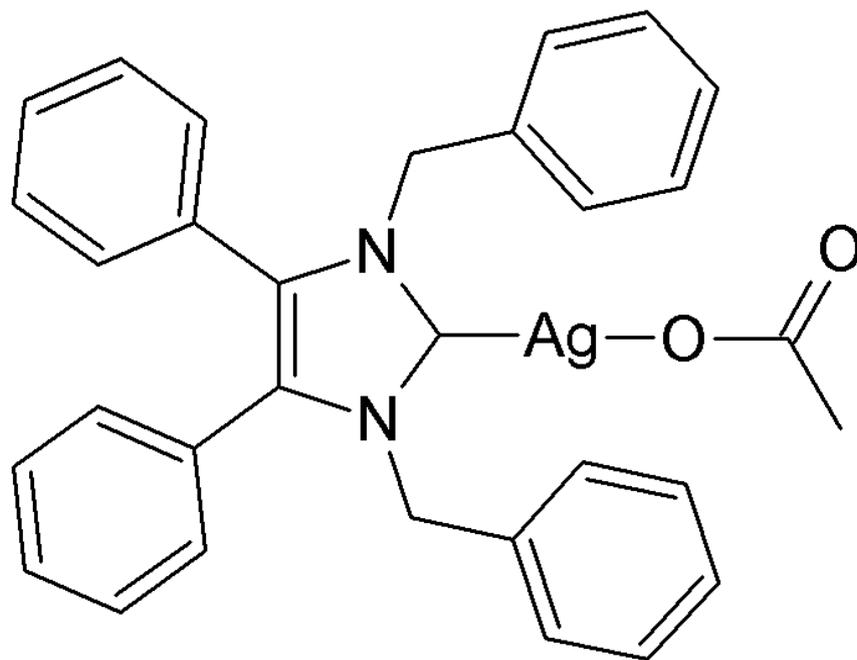
Fig. 6.1 Molecular structure of 1,3-dibenzyl-4,5-diphenyl-imidazol-2-ylidene silver(I) acetate SBC3. Supplied by (Patil *et al*., 2011b)

6.1 Determination of *in vitro* efficacy of SBC3 against *S. aureus* and *C. albicans*

The effect of SBC3 on the growth of *S. aureus* and *C. albicans* was measured as described (2.18.2). In the case of *S. aureus* a concentration of 3.12µg/ml inhibited growth by 19.7±2.28% and increasing inhibition was observed at 6.25µg/ml (40.4±0.81%) and 12.5µg/ml (54.5±0.81%, $p<0.001$). A concentration of 25 µg/ml resulted in 71.2±1.28% ($p<0.001$) inhibition of growth while 50 and 100 µg/ml demonstrated similarly high inhibition of *S.aureus* growth at 76.0±0.16% and 75.1±0.21%, respectively (Fig. 6.2).

The inhibition of *C.albicans* began at a concentration of 12.5µg/ml which resulted in 42.6±8.69% ($p<0.05$) growth inhibition, while concentrations of 25, 50 and 100µg/ml resulted in 86.2±1.42% ($p<0.001$), 93.1±0.62% ($p<0.001$) and 94.4±0.38% ($p<0.001$) inhibition of *C. albicans* growth respectively (Fig. 6.2). Both microbes demonstrated susceptibility to SBC3 but there appears to be greater inhibition of the growth of *C. albicans* than *S. aureus* at the higher concentrations (>25 µg/ml).

6.2 Determination of effect of SBC3 on larval viability

The effect of SBC3 on the viability of *G. mellonella* larvae was assessed as described (2.18.3). The results (Fig. 6.3a, Fig. 6.3b) indicated no toxic effect up to a concentration of 250 µg/ml however larvae inoculated with a dose of 500 µg/ml showed a 40.0 ± 11.54% ($p< 0.01$) reduction in viability after 24 h while the 1000µg/ml induced 100% larval death at 24 h. The larval deaths that occurred from a concentration of 500 µg/ml and 1000µg/ml may be attributed to the higher concentration of DMSO used to dissolve the compound which was marked by larvae becoming soft, flattened and immobile. Larvae inoculated with SBC3 (10, 100, 250 µg/ml) did not show signs of cuticular darkening (melanisation) which would be an indicator of acute toxicity (Fig. 6.3b).
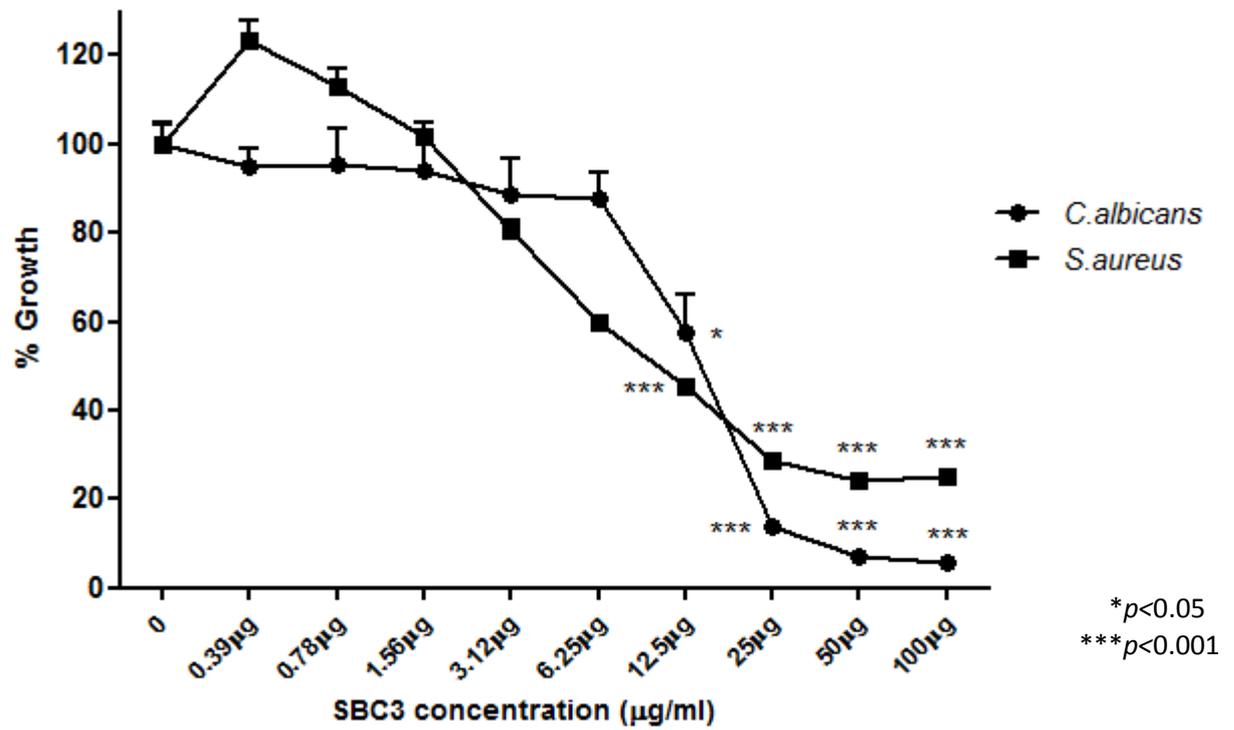
Fig. 6.2 *In vitro* assessment of SBC3 toxicity on *C.albicans* and *S.aureus* growth.
SBC3 toxicity to *C.albicans* ($1x10^6$/ml) or *S.aureus* ($4 x10^7$ cells/ml) at concentration from 100µg to 0.39µg/ml with controls (pathogen only).
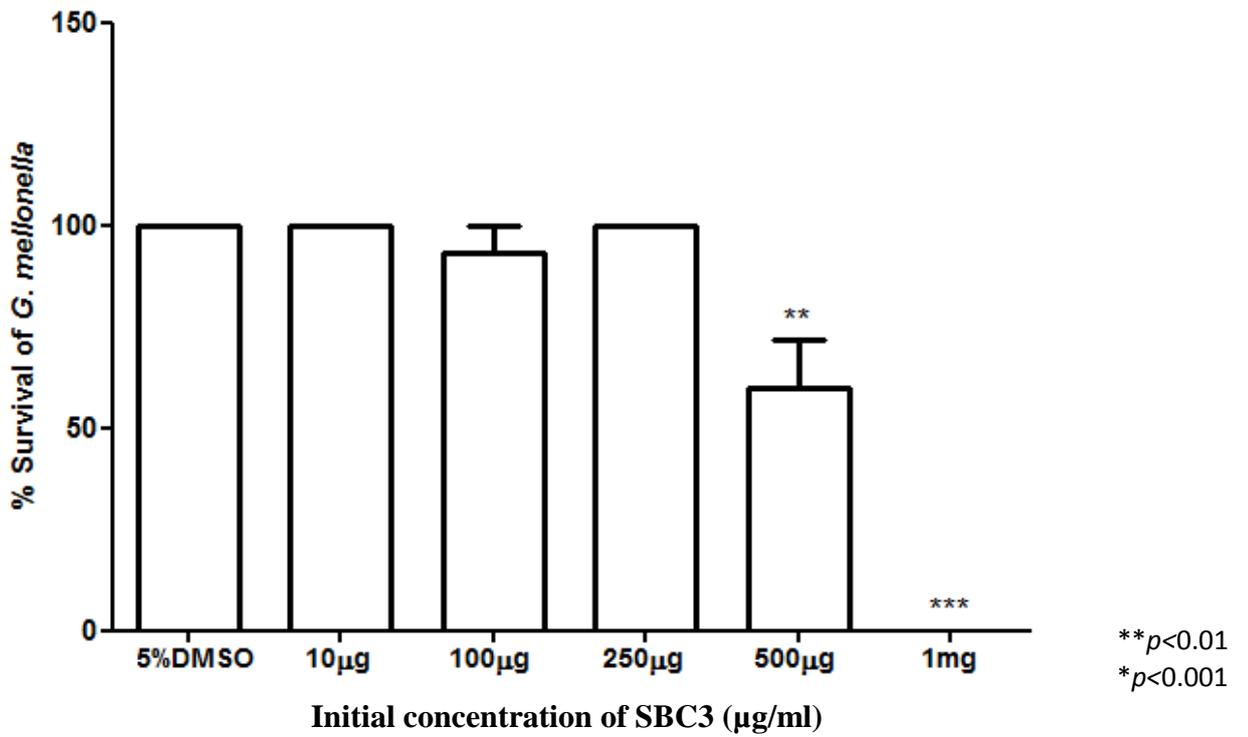
Fig. 6.3a Larval survival at 24h following administration of SBC3.
Larval survivals assessed 24h after injected with control solution of 5% DMSO or compound (10, 100, 250, 500, 1000µg).

5%DMSO   10µg of SBC3

100µg of SBC3   250µg of SBC3

500µg of SBC3   1000µg of SBC3
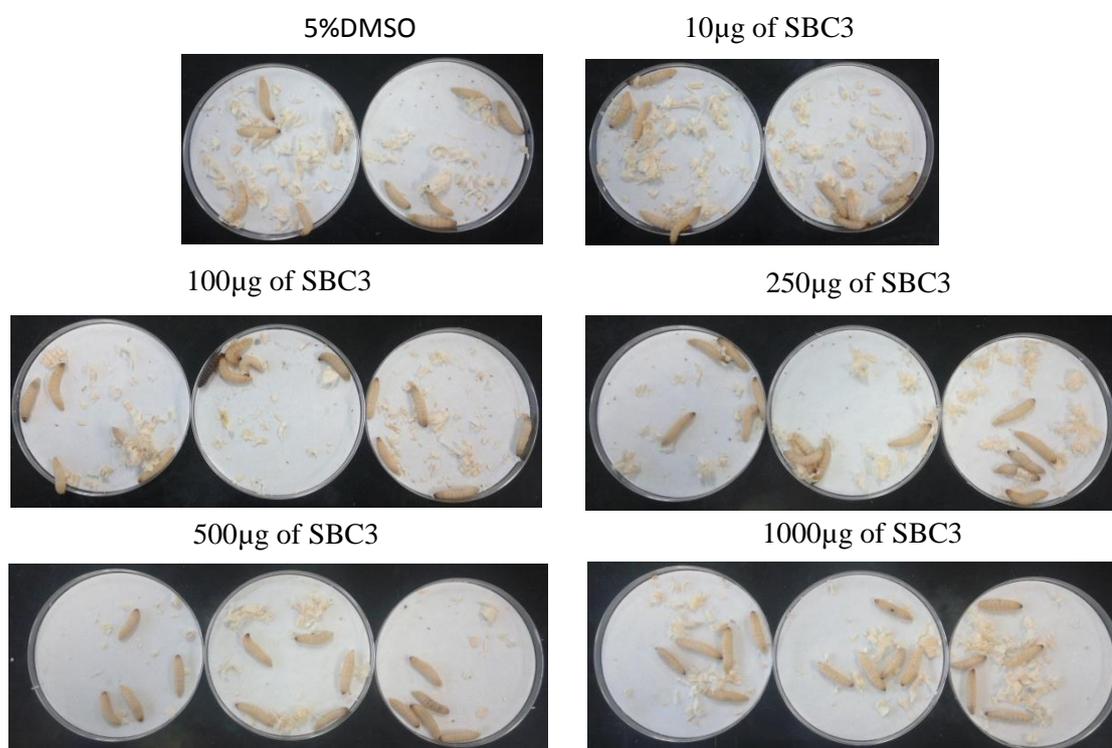
Fig. 6.3b Images of larvae at 24h after receiving SBC3. Larval survivals assessed 24h after injected initial concentrations of the control solution of 5% DMSO or compound (10, 100, 250, 500, 1000µg).

6.3 Effect of SBC3 on survival of larvae infected with *S. aureus* or *C. albicans*

In order to ascertain the *in vivo* activity of SBC3, larvae were infected with each pathogen as described and subsequently administered 20 µl of SBC3 (10, 100 or 250 µg/ml) 4 hours post infection. Larvae inoculated with *S.aureus* demonstrated greater survival to infection when they received a 250 µg/ml dose of SBC3, with survival at 93.3±3.33% when compared to the 100 µg/ml (70±10.0%), 10 µg/ml (70±11.5%) and control (80±10.0%) larvae at 24h. Those larvae infected with *S. aureus* showed 15±5.0% survival at 72 h however the larvae administered a dose of 10, 100 or 250 µg/ml SBC3 showed 46.7 ± 12.01% ($p<0.05$), 36.7 ± 3.33% and 40.0 ± 5.77% survival, respectively, at the same time point (Fig. 6.4a Fig. 6.4b).

Larvae infected with *C.albicans* demonstrated reduced survival at 24h (23.3±10.8%) when compared to larvae that received 10, 100 and 250 µg/ml doses of SBC3 which had significantly higher survivals ($p<0.001$), equal to or greater than 66.6±8.8% (Fig. 6.5a Fig. 6.5b). Larvae inoculated with *C. albicans* alone showed 100% mortality at 48 h. However those larvae inoculated with a dose of 10, 100 or 250 µg/ml SBC3 subsequent to infection showed 30±5.77% ($p<0.01$), 16.7±6.66% ($p<0.05$) and 13.3 ± 3.33% ($p<0.05$) survival at the same time point, respectively (Fig. 6.5a). The greatest survival following *C.albicans* infection at 72h appeared in the larvae that received a 10 µg/ml dose of SBC3 which was similarly observed for the *S.aureus* infected larvae at 72h

6.4 Assessment of the effect of SBC3 on the immune response of *G. mellonella* larvae

The cellular immune response of insects is mediated by immune cells (haemocytes) which phagocytose and kill invading pathogens (Lavine and Strand, 2002) and by the production of antimicrobial peptides (Boman and Hultmark, 1987). Previous work has demonstrated that introduction of selected agents (e.g. silver nitrate, caspofungin, β-glucan) into the haemocoel of *G. mellonella* larvae can prime the immune response and lead to an increase in the density of circulating haemocytes and in the expression of antimicrobial and immune related proteins (Kelly and Kavanagh, 2011; Mowlds *et al.*, 2008; Rowan *et al.*, 2009). In order to exclude the possibility
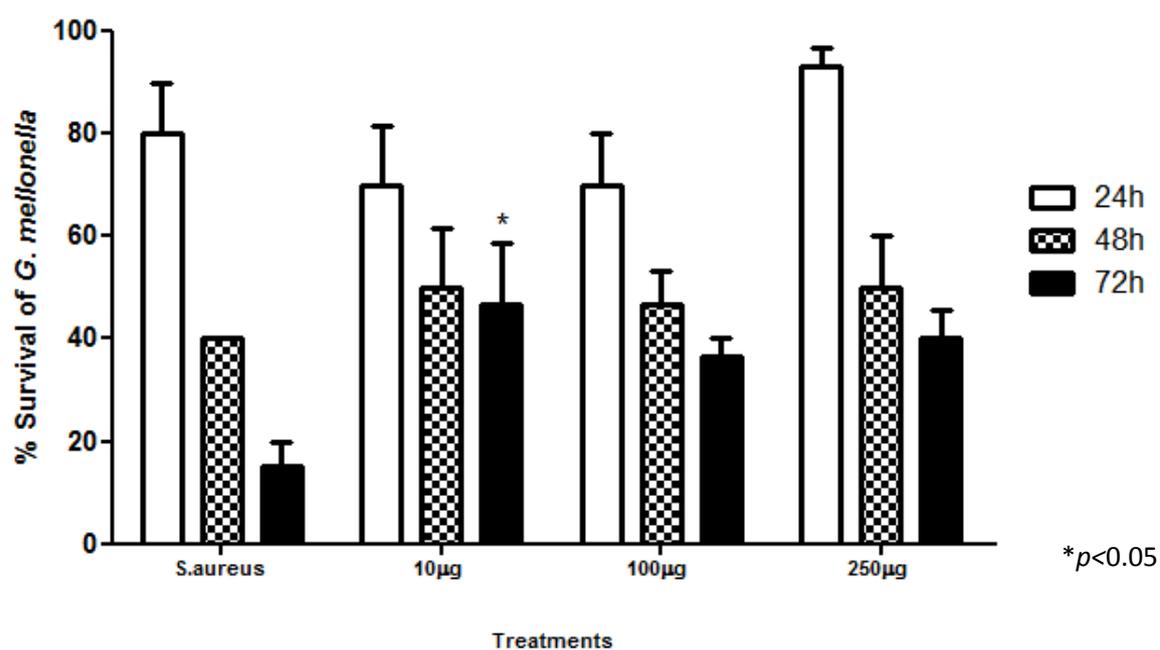
Fig. 6.4a (%) Survivals of *S.aureus* infected larvae following SBC3 inoculation.
Larvae received either *S.aureus* alone (Control) or SBC3 compound (10, 100 or 250μg)
injected 4h after infection, with larval survival assessed at 24, 48 and 72h.

Fig. 6.4b Images of *S.aureus* infected larvae following SBC3 inoculation. Larvae received either *S.aureus* alone (Control) or SBC3 compound (10, 100 or 250μg) injected 4h after infection, with larval survival assessed at 24, 48 and 72h. Larvae colour change from yellow (alive), brown (melainising) and black (dead or dying).

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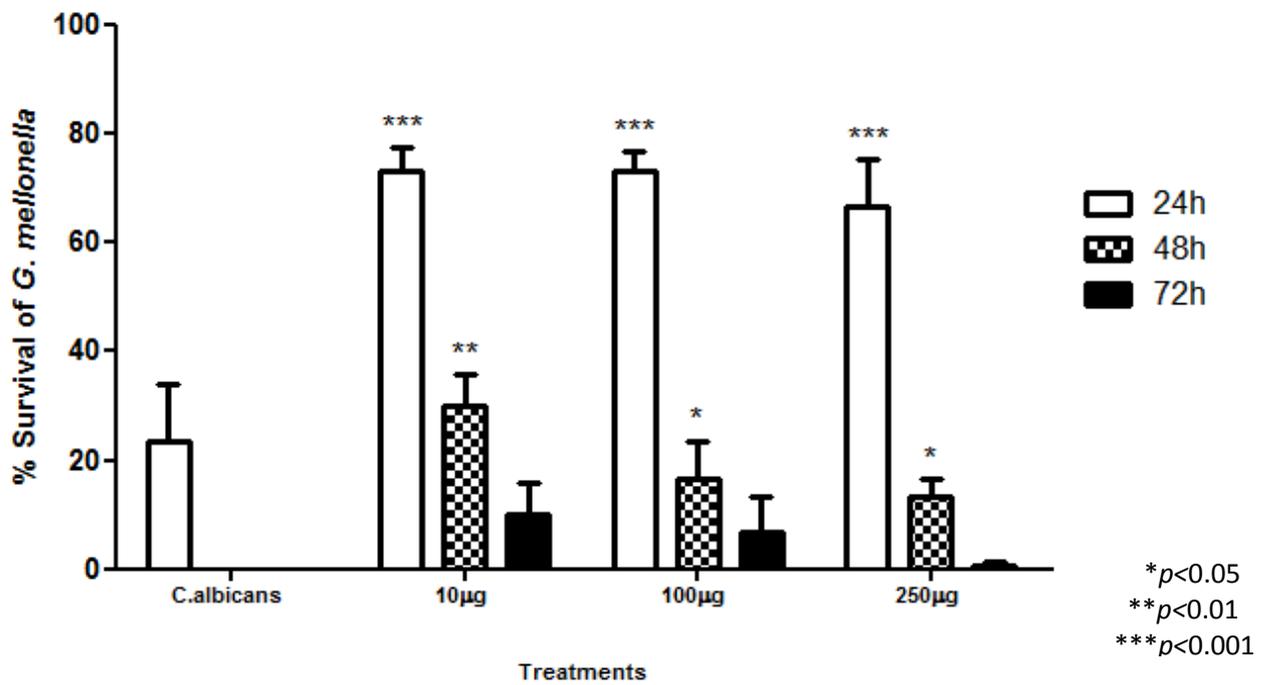
Fig. 6.5a (%) Survivals of *C.albicans* infected larvae following SBC3 inoculation.
Larvae received either *C.albicans* alone (Control) or SBC3 compound (10, 100 or 250µg)
injected 4h after infection, with larval survival assessed at 24, 48 and 72h.

Fig. 6.5b Images of *C.albicans* infected larvae following SBC3 inoculation. Larvae received either *C.albicans* alone (Control) or SBC3 compound (10, 100 or 250μg) injected 4h after infection, with larval survival assessed at 24, 48 and 72h. Larvae colour change from yellow (alive), brown (melainising) and black (dead or dying).

that the observed *in vivo* antimicrobial activity of SBC3 was due to the increased density of immune cells in the larvae, the density of circulating haemocytes in larvae was assessed. The results indicate that there was no significant change in the haemocyte density in those larvae that received doses of 10 and 100 µg/ml SBC3 (Fig. 6.6). Those larvae that received 20 µl doses of 500 or 1000 µg/ml demonstrated a 76.7±1.0% or 55.9±4.0% reduction, respectively, in haemocyte density relative to the control.

The humoral immune response of insects is mediated by a series of antimicrobial peptides and immune related proteins (Kavanagh and Reeves, 2004). The proteomic profile of larvae that had received SBC3 was analysed in order to determine whether exposure to the compound increased this element of the larval immune response (Fig. 6.7). The results indicate that the abundance of a number of proteins is reduced in larvae that received SBC3 (Table 6.1). Proteins such as larval haemolymph protein (Spot 2), 27 kDa haemolymph protein (Spot 7) and apoLp-III (Spot 8) were decreased in intensity. The 27 kDa haemolymph protein is a glycoprotein with signalling functions (Kelly and Kavanagh, 2011) and apoLp-III has functions in the immune response and in lipid transport (Gupta *et al*., 2010). The abundance of arylphorin (Spot 1) was increased by 2-fold in SBC3 injected larvae which has functions in the immune response and amino acid storage (Beresford *et al*., 1997). The abundance of masquerade-like serine proteinase (Spot 4) demonstrated a reduction of 32% within the larvae which received SBC3, this protein has important functions within the activation of the melanisation cascade (Söderhäll, 1982). Carboxylesterase (Spot 3) an enzyme with multiple roles including immune defence, detoxification and development (Taylor and Radic, 1994; Vogt *et al*., 1985) demonstrated a 22% reduced in abundance within the SBC3 injected larvae.
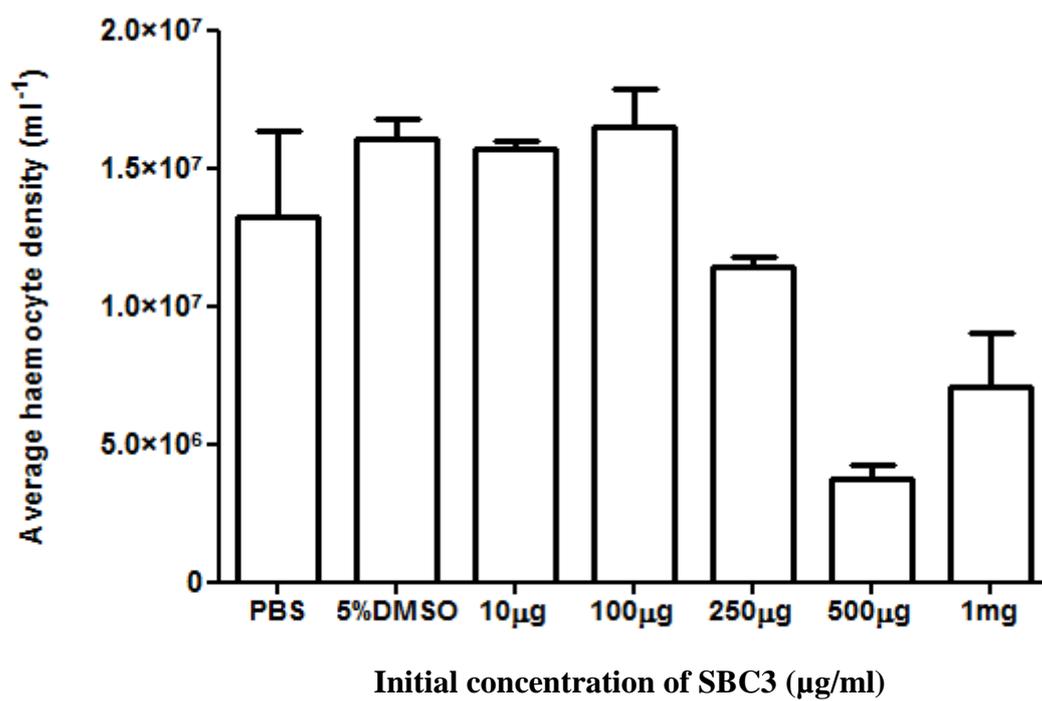
Fig. 6.6 Haemocyte density of *G.mellonella* larvae administered SBC3 compound (10, 100, 250, 500, 1000µg) or control solutions (PBS and 5%DMSO) measured after 24 h.
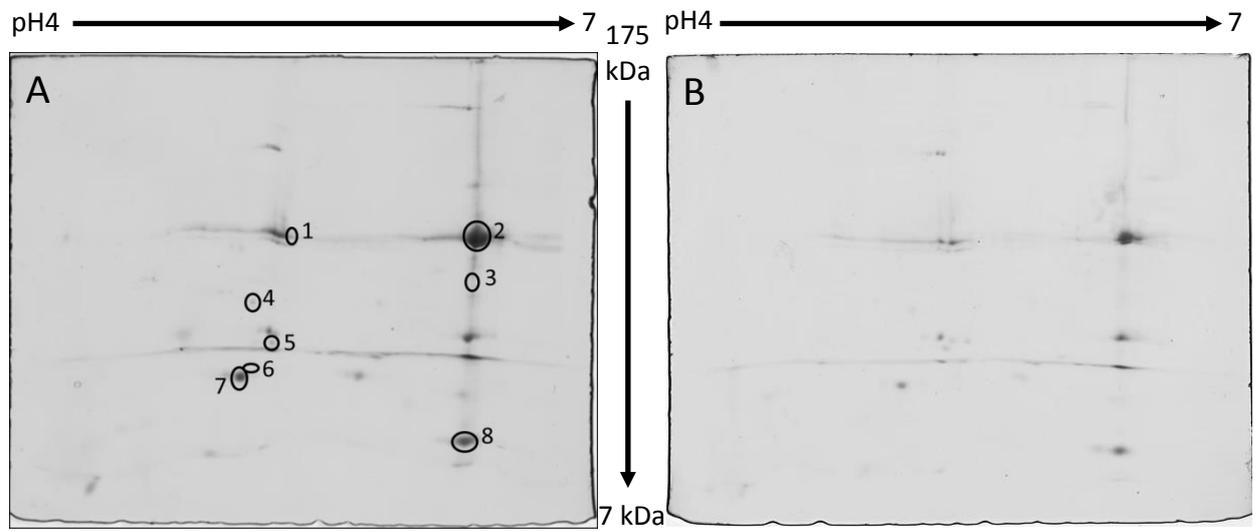
Fig. 6.7 2D SDS-PAGE of separated haemolymph proteins from *G.mellonella* treated with 5% DMSO (A), or SBC3 100µg (B). Proteins of interest excised and identified via LC/MS (1-8).

Spot no.	Protein Identity	Organism	Mr	pI	Score	Seq. coverage (%)	Accession no.	Relative Expression		Protein function
								DMSO Control	SBC3 (100μg)	
1	Arylphorin	*G. mellonella*	83651	5.23	1280	40	AAA74229	1	2.01	Amino acid storage and immune functions.
2	Larval hemolymph protein	*G. mellonella*	13783	9.08	74	10	ABG91580	1	0.80	Insect larval storage protein.
3	Carboxyl-esterase	*G. mellonella*	58669	8.34	85	15	*	1	0.68	Functions in hormone and pheromones metabolism, detoxification, defence and behaviour.
4	Masquerade-like serine proteinase	*P.rapae*	46063	5.54	173	7	ACZ68116	1	0.58	Enzymes that cleave key components of the melanisation cascade.
5	Actin-4	*B. mori*	42200	5.47	560	48	AGR44814	1	0.85	Nucleotide-Binding Domain of the sugar kinase/HSP70/actin superfamily.
6	Hypothetical protein YQE_03765, partial.	*D. ponderosae*	54682	5.44	79	3	ENN79708	1	1.27	Death Domain, involved in cell apoptosis.
7	27 kDa hemolymph protein	*G. mellonella*	26387	5.17	597	57	P83632	1	0.57	Protein in the outer cell membrane, secreted into the haemolymph as signal molecule.
8	Apolipophorin-III	*G. mellonella*	20499	8.59	659	54	P80703	1	0.79	PRR of microbial components.

Table 6.1 Identity of proteins showing alteration in abundance in larvae that received a 20 μl dose of SBC3 (100μg/ml). (*) Spectrum Mill MS Proteomics analysis.

6.5 Discussion

Due to the many similarities between the immune system of insects and the innate immune system of mammals, insects have become popular choices for measuring the virulence of microbial pathogens and give results comparable to those that can be obtained using mammals (Brennan *et al*., 2002; Jander *et al*., 2000). For this reason insects have become a popular choice in screening pathogens and in testing the effectiveness of novel drug compounds such as SBC3 against pathogens. Insects have the benefit of being inexpensive to purchase and house, and can give results in 24 − 48 h. Insects are widely used to evaluate the *in vivo* activity of antimicrobial drugs (Hamamoto *et al*., 2004; Lionakis *et al*., 2005) but caution must be exercised in their use as the introduction of foreign material into the insect haemoceol can provoke a non-specific immune response (Kelly and Kavanagh, 2011; Mowlds *et al*., 2010).

The comparable innate immune system of insects and mammals makes *G.mellonella* larvae ideal for assessing how silver derived drugs may interfere with the functions of the immune system in terms of cellular and humoral immune response. The insect has a similar range of PRRs to mammals capable of activating the melanisation cascade which is comparable in structure and function to mammalian complement cascade (Kavanagh and Reeves, 2004). A high level of conserved signal pathways exist within the innate immune response of insects and mammals and include the Toll like pathway (Lemaitre *et al*., 1996) and the insect IMD pathway with the mammalian TNFα pathway (Khush *et al*., 2001). Metabolic functions are highly conserved between mammals and insects (Bharucha, 2009; Gilbert, 2008; Kühnlein, 2010) which enables larval metabolic functions to be assessed for any interference of a compound with normal functions.

The assessment of silver based compounds efficacy such as SBC3, is attributed to the known antimicrobial activity of silver (Percival *et al*., 2005) and for this reason silver has been incorporated into many medical products (e.g. catheters, plasters) where it can limit the growth of bacteria and the development of biofilms (Adams *et al*., 1999). While the mode of action of silver is not fully elucidated it has been shown to inactivate NADH and succinate dehydrogenase and so interfere with electron transfer in respiration (Park *et al*., 2009). Exposure to silver may also lead to the generation of ROS which induces protein and DNA degradation (Atiyeh *et al*.,

2007; Castellano *et al*., 2007; Park *et al*., 2009). Previous work demonstrated the induction of an oxidative stress response in *C. albicans* when exposed to Ag(I) ions (Rowan *et al*., 2010).

The experimental drug SBC3 was demonstrated to be of low toxicity to *G. mellonella* larvae but at the higher doses (500, 1000µg/ml) larvae demonstrated 40% to 100% mortality respectively. This is likely in response to the high concentration of the drug and the solvent DMSO which is required to dissolve higher concentrations of the compound. Despite this, results presented here indicated that larvae infected with *C. albicans* or *S. aureus* and subsequently administered SBC3 demonstrated increased survival. The *in vitro* analysis did demonstrate the effectiveness of the compound to inhibit growth significantly at 12.5µg/ml against both *S.aureus* and *C.albicans*, although 25µg/ml and above demonstrated greater inhibition in *C.albicans* than *S.aureus* growth. This was previously demonstrated by Rowan *et al*. (2006) as a number of silver derived compounds inhibited *C.albicans* growth greater than *S.aureus* and could be attributed to different mechanisms of entry and removal from cells as well as cell size and membrane permeability. The *in vitro* analysis demonstrated a small peak in growth of *S.aureus* following exposure to SBC3 between 0.39-1.56µg/ml which is commonly observed in response to low doses of antibiotics as increased membrane permeability can improve nutrient flow and therefore growth commonly known as hormesis model (Stebbing, 1982).

SBC3 does not provoke an enhanced immune response as indicated by the lack of an increase in the haemocyte density and the expression of antimicrobial and immune related proteins. A number of proteins were shown to decrease in abundance in larvae that received SBC3 and these included carboxylesterase (Spot 3, Table. 6.1) which has physiological functions in neurotransmitter degradation, hormone and pheromones specific metabolism, detoxification, defence and behaviour (Taylor and Radic, 1994; Vogt *et al*., 1985). The 27 kDa haemolymph protein (Spot 2) which is secreted into the haemolymph as a signal molecule (Kelly and Kavanagh, 2011) and apoLp-III (Spot 8) which functions in the innate immune response (Gupta *et al*., 2010) also decreased in abundance in larvae that received SBC3. Spot 6, hypothetical protein YQE_03765 partial, was increased in abundance in larvae that received SBC3 and functions as a death domain protein with roles in apoptosis, immune defence, and immune signalling (Lahm *et al*., 2003). The abundance of arylphorin (Spot 1), which

is involved in storage of amino acids and in aiding the immune defence of insects (Beresford *et al*., 1997), was increased in abundance by 2-fold.

In contrast, administration of silver nitrate to *G. mellonella* larvae stimulated the immune response by increasing the density of circulating haemocytes (Rowan *et al*., 2009). One possibility for this difference in the response of *G. mellonella* larvae to silver in silver nitrate and in SBC3, is that silver nitrate releases ionic $Ag+$ and $NO3−$ in biological media, whereas SBC3 is covalently bonded L-Ag-OAc (L=NHC) and no free $Ag+$ is formed. The lipid nature of SBC3 may not provoke an immune response as it may be better tolerated by the insect's immune system. As a consequence it can be concluded that the increased survival of larvae that received SBC3 is due to the anti-microbial properties of the compound and not to a non-specific immune response induced by the introduction of the compound.

This is the first demonstration of the *in vivo* activity of SBC3 against *S. aureus* and *C. albicans*. Future studies of SBC3 could assess the mode of action of the compound in insect larvae and mice followed by survival as well as pharmacokinetic/pharmacodynamic (PK/PD) studies in rodents. It is hoped that SBC3 could be developed into an intravenous antibiotic for use against pathogenic bacteria and fungi that show resistance to conventional antimicrobial drugs. This assessment highlights the utility of insect models such as *G.mellonella* larvae as it was possible to measure the effectiveness of the drug SBC3 on larval survival to infection by using a range of parameters. It was also seen that the *in vitro* analysis of SBC3 against fungal and bacterial pathogens correlated well to how the drug performed *in vivo* in *G.mellonella* larvae against the pathogens. This work and previous work (Rowan *et al*., 2009) has demonstrated the validity of using insects as a preliminary screening model offering academia and industry an ideal model to tackle the demand to develop new antimicrobial drugs in the fight against microbial drug resistance.

Chapter 7

General Discussion

7.0 General discussion

The aim of this project was to establish how the immune response of *G.mellonella* larvae was affected by environmental stresses. It also sought to establish standard practice for the use of these larvae so that inter-laboratory comparisons could be carried out. This work aimed to examine parallels between the insect and mammalian humoral and cellular components of the innate immune response to further develop this insect model as a screening tool and to demonstrate the larvae's screening capacity by testing the efficacy of a novel antimicrobial agent (SBC3). The rational for this work was driven by the fact that *G.mellonella* larvae are widely used as a screening model in both industry and academia but the rapid uptake of this model has not kept pace with a standardisation of the use of these larvae. The uptake of *G.mellonella* as an *in vivo* screening model in the absence of a standardised use of the model between laboratory groups, has limited the inter-laboratory comparisons and therefore highlighted the need to create a standard procedure for the larvae's use.

Much to the larvae's success and utility is due to the numerous advantages it offers as a preliminary screening model as it has a highly conserved innate immune response similar to that of mammals. Larvae are a cheap alternative to mammal screening models, deliver a fast turnover of results (24-48h) and they have no ethical or legal requirements (Kavanagh and Fallon, 2010). These elements of the larvae's use has led to their increased employment in assessing the virulence of fungal (Bergin *et al*., 2003; Bergin *et al*., 2006; Mylonakis *et al*., 2005; Wojda *et al*., 2009) and bacterial (Mukherjee *et al*., 2010) pathogens as well as in the assessment of the efficacy of antimicrobial compounds (Rowan *et al*., 2009). Research using *G.mellonella* has also branched out into using the model for the assessment of *Listeria* infections of the brain which shows a similar pathology to that observed in mammals (Mukherjee *et al*., 2013). *G. mellonella* larvae are commonly stored at 15$^{\text{o}}$C or room temperature for 1 to 3 weeks in advance of inoculation (Cook and McArthur, 2013). It has previously been established that variations in incubation temperature (Mowlds and Kavanagh, 2008; Wojda and Jakubowicz, 2007), physical stress (Mowlds *et al*., 2008) and the access to nutrients (Banville *et al*., 2012) significantly altered the response of *G. mellonella* larvae to infection. It was also established that prior exposure of larvae to yeast cells (Bergin *et al*., 2006) or glucan (Mowlds *et al*., 2010) stimulated their immune response and lead to reduced sensitivity to infection.

Researchers using the larvae demonstrated a number of variations in food, incubation temperature, handling and duration of incubation (Cook and McArthur, 2013) which has led to these variable conditions being assessed on larvae here.

In the first part of Chapter 3 the nature of the larval haemolymph binding proteins was assessed against microbial pathogens after larvae were thermally or physically treated. It was demonstrated that larvae that were thermally or physically stressed possessed a higher abundance of binding proteins that could bind to microbial surfaces and a greater range of binding proteins compared to the control larvae when assessed by 1-D SDS PAGE analysis. Binding of haemolymph proteins from thermally and physically stressed larvae demonstrated a higher abundance of proteins bound to LPS than the control e.g. apoLp-III, arylphorin, calreticulin, proPO and PGRP-B. It was previously demonstrated that haemolymph from *G.mellonella* larvae that was thermal stressed possessed a greater abundance of AMPs which enabled better survival following infection (Mowlds and Kavanagh, 2008; Wojda and Jakubowicz, 2007).

This was similarly observed with the binding of larval haemolymph proteins to β-1,3-glucan as a greater number of proteins were bound from thermally and physically stressed larvae compared to the control larvae and these proteins had specificity to the fungal cell wall constituent i.e. hexamerin and proPO. It is known that proPO interacts with microbial surfaces through PRRs (Sugumaran and Kanost, 1993; Söderhäll, 1982) and was previously demonstrated to bind to *A.fumigatus* conidia (Fallon *et al*., 2011b). When examining the binding of larval haemolymph proteins to germinated *A.fumigatus* conidia versus non-germinated *A.fumigatus* conidia it was obvious that there was a greater abundance of bound proteins from the thermally and physically stressed larvae compared to the control larvae. But it was also observed that the larvae had a higher abundance of bound proteins and range of binding proteins to the germinated *A.fumigatus* conidia when compared to the non-germinated *A.fumigatus* conidia potentially due to the exposure of more antigen binding sites accessible on the germinated conidia. The morphological state of *A.fumigatus* is important to its detection and phagocytosis by the immune system (Brakhage *et al*., 2010; Luther *et al*., 2007). Fallon *et al*. (2011b) also demonstrated the ability of similar proteins from *G.mellonella* larval haemolymph to bind to *A.fumigatus* conidia.

Haemolymph proteins from thermally and physically stressed larvae possessed a similar range of proteins that bound to *C.albicans* as observed with *A.fumigatus* conidia, although the abundance was lowest in the 30°C and physically stressed larvae. A similar range of binding proteins and abundance of proteins was observed from thermally and physically stressed larval haemolymph to *S.aureus* cells as was observed binding to LPS. Larvae used similar proteins to detect bacterial LPS and *S.aureus* including calreticulin, proPO, apoLp-III and arylphorin. The ability of more than one PRR (e.g. apoLp-III, calreticulin, arylphorin and hemolin) to bind to one or more microbial polysaccharides enables great redundancy in their ligand binding specificity (Yu *et al*., 2002) and is possibly what is occurring here. In contrast larval haemolymph proteins demonstrated binding affinity to fungal cell wall antigen via βGBP, proPO, arylphorin and hexamerin to germinated, non-germinated *A.fumigatus* conidia and to *C.albicans*. Although β-1, 3-glucan did not demonstrate the same range of proteins bound potentially as it is only a single antigen type.

In the second part of Chapter 3 the similarities in binding proteins to microbial surfaces from insect and mammalian serum was assessed. It was observed that a number of similarly functioning proteins bound to microbial surfaces from both insect and human serum including the apolipoproteins, apolipoprotein E and proapolipoprotein from human serum and apoLp-III from insect haemolymph (Whitten *et al*., 2004). Both mammalian and insect immune responses employ similar immune cascades which are highly conserved including the mammalian complement and insect proPO pathways. ProPO and complement were both identified bound to microbial surfaces here. This is unsurprising as complement and proPO pathways have conserved regions within their structure and share similar functions in the activation of immune effectors and induction of phagocytosis (Söderhäll and Cerenius, 1998).

The mammalian serum possessed the protein transferrin that bound to microbial surfaces and is a protein also found in *G.mellonella* larvae. Both species transferrins function to reduce free iron within the serum by sequestering iron which functions to limit microbial growth in human (Mason *et al*., 2004) or insect (Dunphy *et al*., 2002) haemolymph. The PRR calreticulin was also observed bound from insect haemolymph and is known to bind to bacterial surfaces and functions similarly to mammalian calreticulin (Cho *et al*., 1999; Choi *et al*., 2002). In both insect haemolymph and human serum a similar range of PRRs, immune related proteins and

immune cascades were capable of recognising microbial surfaces and potentially activating appropriate responses (Medzhitov *et al*., 1997). These findings demonstrate the highly similar innate immune recognition and effector activation processes shared between the insect and mammalian models despite 500 million years of evolutionary divergence.

Immune priming in insects is characterised by the insect elevating its immune response following a sub-lethal infection which prepares the insect to withstand a subsequent and potentially lethal infection (Bergin *et al*., 2006). Insect immune priming has been demonstrated in response to abiotic factors including temperature (Mowlds and Kavanagh, 2008), physical injury (Mowlds *et al*., 2008) or availability of food (Pletcher *et al*., 2002). Larvae were first challenged with a fungal or bacterial inoculum, 24 or 72h after thermal or physical stress treatment to determine the duration of the immune priming event. Larvae that were thermally or physically stressed 24h prior to infection demonstrated greater survival when compared with larvae kept at 15°C (control). The larvae that were thermally and physically stressed 72h prior to infection with either *A.fumigatus* or *S.aureus* demonstrated significantly reduced survival when compared to the control. This demonstrated that the immune priming effect which appeared to increase the survival of thermally and physically stressed larvae 24h after the stress event was short term as larvae stressed 72h prior to infection had reduced survival. Immune priming following thermal or physical stress was previously assessed 24h after the priming event (Mowlds *et al*., 2008; Mowlds and Kavanagh, 2008), this work extends these observations and demonstrates that the priming effect in *G. mellonella* larvae is a short term event that declines after 24 hours.

The proteome of the larval haemolymph also demonstrated some changes within the thermally and physically stressed larvae which had an elevated expression of immune related proteins (arylphorin, apoLp-III and proPO) that peaked at 24h before declining to levels comparable to the control at 72h. The expression of *Gallerimycin*, *IMPI* and *Transferrin* demonstrated significant increases in expression at 72h which may have contributed to an attenuated survival of thermally and physically stressed larvae as other immune proteins declined in abundance at 48 and 72h.

Further analysis of the cellular immune response of larvae demonstrated the density of circulating haemocytes were significantly higher in abundance within

thermally and physically stressed larvae. Similarly significant alterations in AMP expression, protein abundance and haemocyte density was observed following thermal (Mowlds and Kavanagh, 2008; Wojda and Jakubowicz, 2007) and physical stress (Mowlds *et al*., 2008) within larvae. When the relative populations of haemocytes were assessed by FACS analysis there was a higher proportion of the P7 population (granular haemocytes) in the physically shaken and 30°C pre-incubated larvae (Lavine and Strand, 2002). The P1 haemocyte population which appeared to be prohaemocytes was significantly higher in abundance among the 30°C, 37°C and physically stressed larvae compared to the control larvae. Prohaemocytes are known to generate haemocytes such as granular haemocytes (Rodrigues *et al*., 2010) which may enhance the stressed larva's resistance to infection.

The proteome of haemocytes extracted from thermally and physically stressed larvae demonstrated a higher abundance of proteins associated with pathogen recognition, (e.g. calreticulin and apoLp-III) and with phagocytosing immune cells (Rab signalling protein, proteasome 35kDa subunit and FK506-binding protein) when compared to the control larvae. These proteins had increased abundance in thermally and physically stressed haemocytes and are proteins known to be associated with cell stress and immune function (Gallo *et al*., 2011; Shim *et al*., 2010; Wojda and Jakubowicz, 2007).

The greater abundance of circulating haemocytes, phagocytosing haemocytes and amount of antimicrobial peptides, possibly contributed to greater survival within thermally and physically stressed larvae compared to the control larvae, although the immune priming response peaked at 24h before decreasing to levels similar to the control at 72h. This demonstrated the immune priming response was short lived potentially due to the costly nature of maintaining a heightened immune response (Moret and Schmid-Hempel, 2000).

The mechanisms of phagocytosis and degranulation have been elucidated in both insects and mammals (Kavanagh and Reeves, 2004; Renwick *et al*., 2007). The comparison of larval haemocytes stimulated with fMLP and PMA and mammalian phagocytes demonstrated a number of similar proteins activated in haemocytes by phosphorylation. These proteins were demonstrated to contribute to the activation of the NADPH oxidase and are known to similarly function in neutrophils activation highlighting another similarity shared between both insect and mammalian immune cells.

Age has a detrimental impact on the capacity to launch an effective immune response. Age effects are known to result in a deterioration of physiological components including immune function within mammals thus increasing their potential for death (Mackenzie *et al*., 2011; Pletcher *et al*., 2002). Aging in insects has shown similar features such as muscle wastage (Augustin and Partridge, 2009), reduced immune competence and ability to overcome infection in *Drosophila* (Mackenzie *et al*., 2011; Pletcher *et al*., 2002). It was not previously demonstrated how pre-incubation of *G.mellonella* larvae affects their ability to survive a subsequent infection. The focus of Chapter 5 was to explore how pre-incubation affected larval response to infection when larvae were incubated at 15°C for 1, 3, 6 or 10 weeks prior to infection. Larvae demonstrated reduced survival to microbial challenge when they were incubated for 3 weeks or more. This reduced survival coincided with a reduced abundance of circulating haemocytes in 3, 6 and 10 week pre-incubated larvae when compared to the 1 week pre-incubated larvae. Ramsden *et al*. (2008) demonstrated a similar reduction in *Drosophila* survival to infection with age. When the proportion of these haemocytes were assessed by FACS analysis it was observed that the number of small granular haemocytes increased in proportion with increased incubation period, but the P5 haemocyte population (adipohaemocyte) had reduced in proportion with increased incubation. The adipohaemocytes population may be depleted with age as it serves as a food/energy reserve (Tanada and Kaya, 1993). Previously it was demonstrated that *Drosophila* older than 3 weeks had a 30% reduction in circulating haemocytes which was similar to that observed here (Mackenzie *et al*., 2011). A reduced ability to survive infection and eliminate pathogens by phagocytosis was also observed in older mosquitoes (Hillyer *et al*., 2005).

Larval proteome changes were observed as a number of proteins with immune function had decreased abundance while others increased in abundance with duration of larval incubation. Metabolic proteins demonstrated reduced abundance with increased larval incubation time and other dual function proteins such as ferritins increased in abundance with incubation. It was also observed that the expression of *Galiomicin* significantly decreased with increased duration of incubation. Observations by Pletcher *et al*. (2002) in *Drosophila* showed significant alterations in gene expression in response to age. The findings of this Chapter demonstrated the reduced survival of larvae pre-incubated for 3 weeks or more as these larvae demonstrated reduced abundance of circulating haemocytes and a reduced expression

of metabolic proteins which together may reduce the speed and potency of an immune response to infection.

It was previously demonstrated that nutrient deprived larvae had reduced number of circulating haemocytes and ultimately reduced survival following infection when compared to larvae with an unrestricted diet (Banville *et al*., 2012). Further analysis was carried out here on the proteome of starved larvae and indicated a decreased abundance of immune related proteins which may have contributed to reduced survival. The effect of age on larvae and lack of access to food demonstrated the importance of these variables in experiments and how such features could compromise the results of experiments (Cook and McArthur, 2013).

Chapter 6 focused on demonstrating the use of *G.mellonella* larvae to screen antimicrobials as a proof of concept and as a means to assess the efficacy of a novel antimicrobial, SBC3. Previously *G.mellonella* larvae were used to assess the antimicrobial activity of novel silver based complexes (Rowan *et al*., 2009). The compound SBC3 demonstrated significant *in vitro* potency at 12.5μg/ml against *S.aureus* and *C.albicans* (54.5% and 42.6% growth inhibition respectively), common pathogens of immunocompromised patients (Blumberg *et al*., 2001; Heijer *et al*., 2013). Previous *in vivo* assessment of the efficacy of caspofugin in *G.mellonella* larvae demonstrated priming of the immune response (Kelly and Kavanagh, 2011), but SBC3 demonstrated little or no evidence of priming of the cellular or proteomic immune responses. The compound also showed no toxic effects on larvae across the therapeutic dose range highlighting the compounds *in vivo* tolerance by larvae. SBC3 demonstrated strong activity against infection within larvae which was not due to the compound initiating any immune priming response within the larvae. Immune priming is an important distinguishing factor when assessing such compounds activity (Kelly and Kavanagh, 2011). The lack of any priming response is due to the compound's design which incorporates a lipophilic shell around the antimicrobial Ag+ ion, potentially reducing the immune response and therefore making the compound better tolerated. The assessment of SBC3 demonstrated the efficacy of the compound as a potent antimicrobial as well as validating the use of *G.mellonella* larvae as a useful screening model.

The work carried out in this thesis demonstrated how abiotic factors such as temperature, incubation period or starvation dramatically impacted on the larva's ability to overcome an infection. This has therefore highlighted how inter-laboratory

variation of the above factors can affect the validity of experimental outcomes and their comparison with other laboratory studies using *G.mellonella* larvae (Cook and McArthur, 2013). It was shown that *G.mellonella* larvae possessed a high level of conserved innate immune responses from both the cellular and humoral immune components to that of mammals, validating *G.mellonella* as an alternative preliminary screening model for infection and antimicrobial drug studies.

Future studies using these larvae may incorporate greater proteomic analysis of the larval innate immune system using label free techniques to compare their responses and mammalian responses to infection enabling greater parallels to be drawn between the two immune responses. The label free proteomic analysis enables the identification of AMPs which are usually too small to be captured by gel analysis, therefore reducing the validity of qPCR assessments which can only focus on a limited and biased number of AMPs unlike the global unbiased identification of AMPs by label free analysis. It is also possible to detect post translational modifications that occur on proteins potentially highlighting the activation state of a protein involved in a signalling pathway. This may offer a better insight into the level of conserved functions and structures between invertebrate and vertebrates since their evolutionary divergence 500 million years ago.

The use of FACS analysis will become a much more accepted means of identifying haemocyte sub-populations within insects with potential ability to clarify the discrepancies introduced to invertebrate haemocyte characterisation prior to the advent of the FACS analysis. Due to the differences apparent within different insects it may be possible to identify similar haemocytes between insect groups using a combination of specific fluorescent labels, morphological differences and phagocytosing abilities.

The ability to use larvae to study the pathology of *Listeria* infection of the brain in a comparable infection pattern to that observed in humans offers the potential to conduct assessments of other human infections in larvae to establish if an infection follows a similar pathology to that observed in humans. This opens the potential future applications of the larvae in the study of human infections and would further strengthen the similarities between insect and mammalian infection models.

Chapter 8

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9.0 Appendices

15°C

30°C

37°C

Shaken
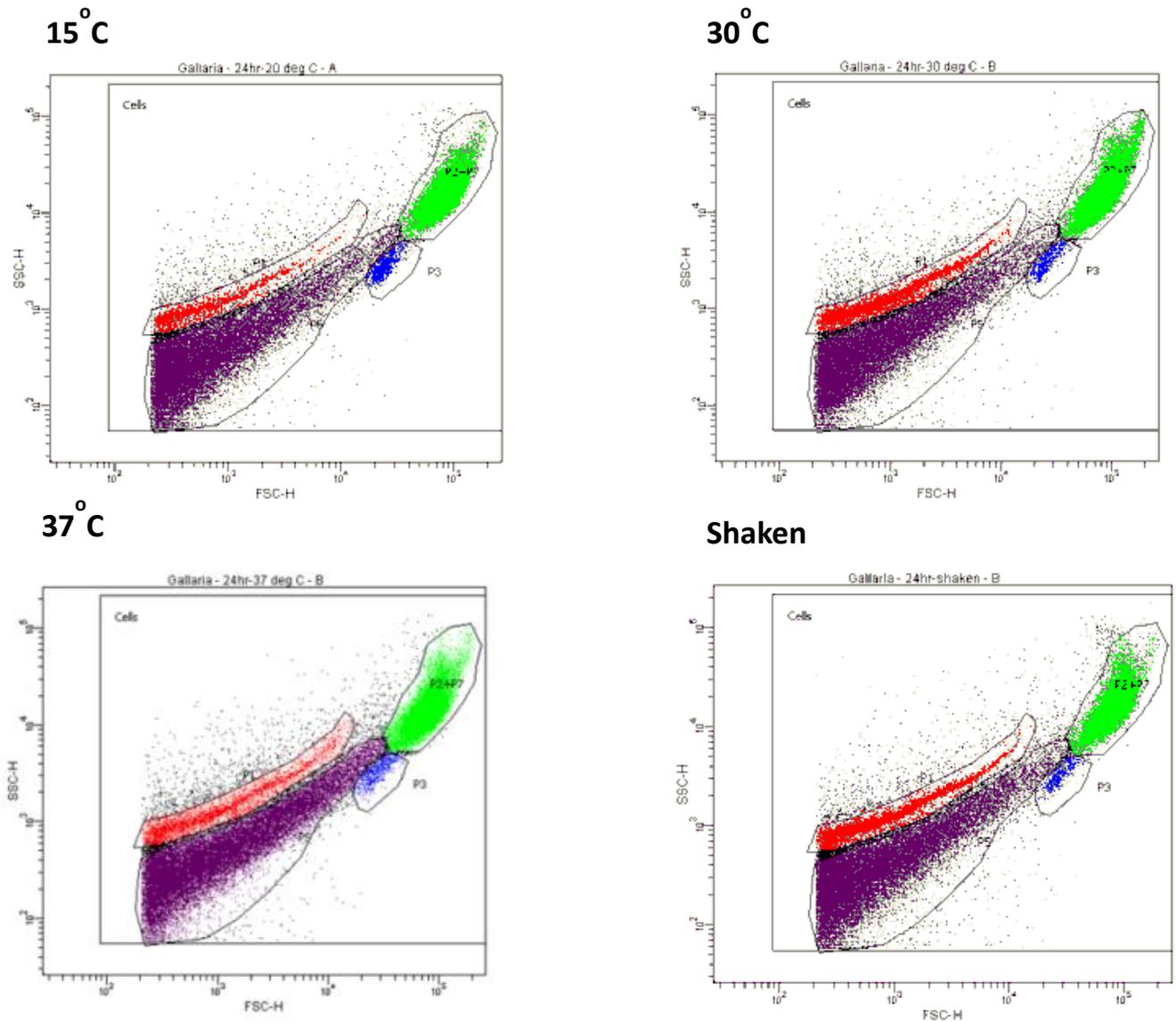
Fig. 9.1 Haemocytes sorted into four sub-populations using FACS Aria Flow
Cytometer, BD ® from larvae incubated for 24h.

15°C

30°C

37°C

Shaken
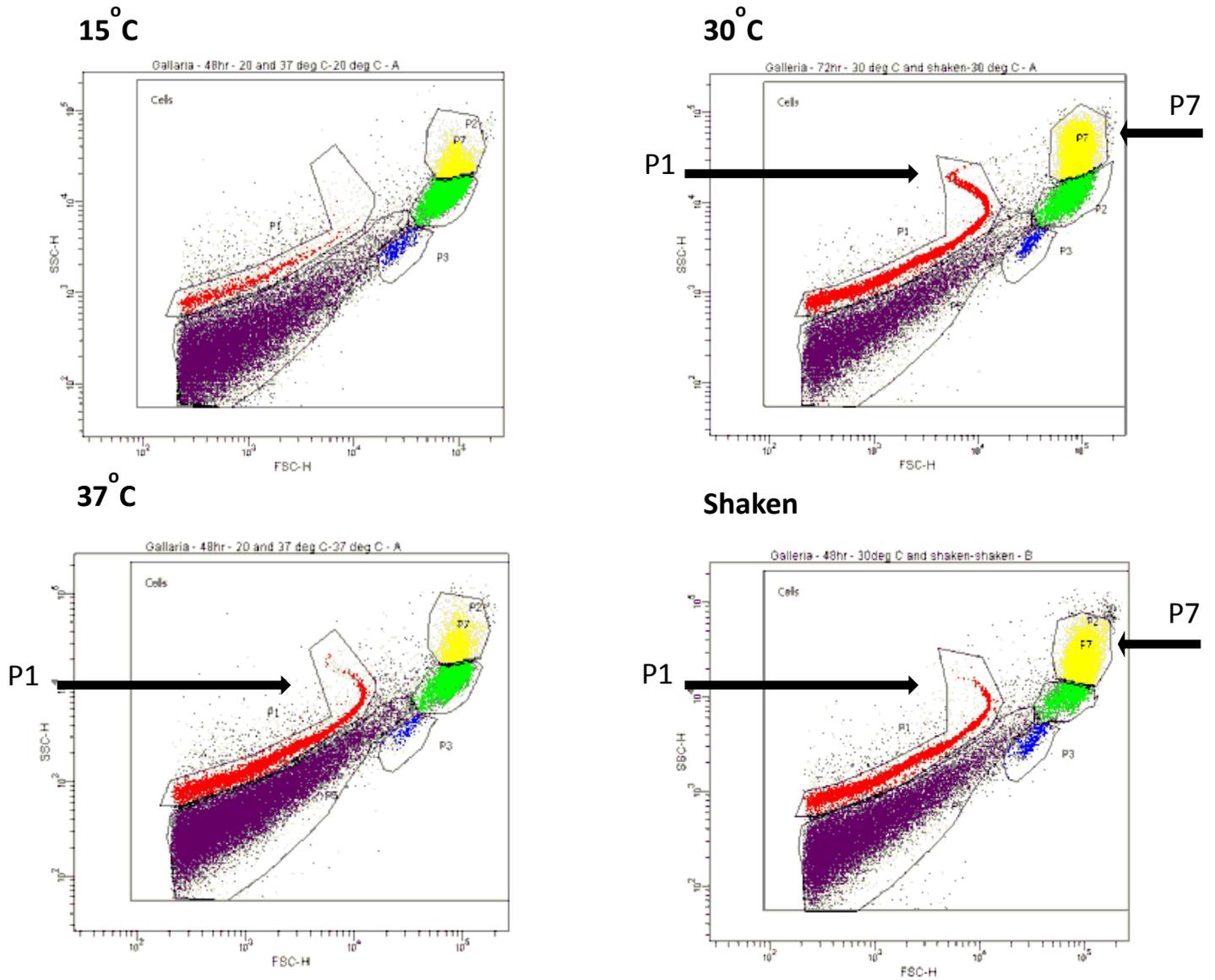
Fig. 9.2 Haemocytes sorted into five sub-populations using FACS Aria Flow Cytometer, BD ® from larvae incubated for 48h.
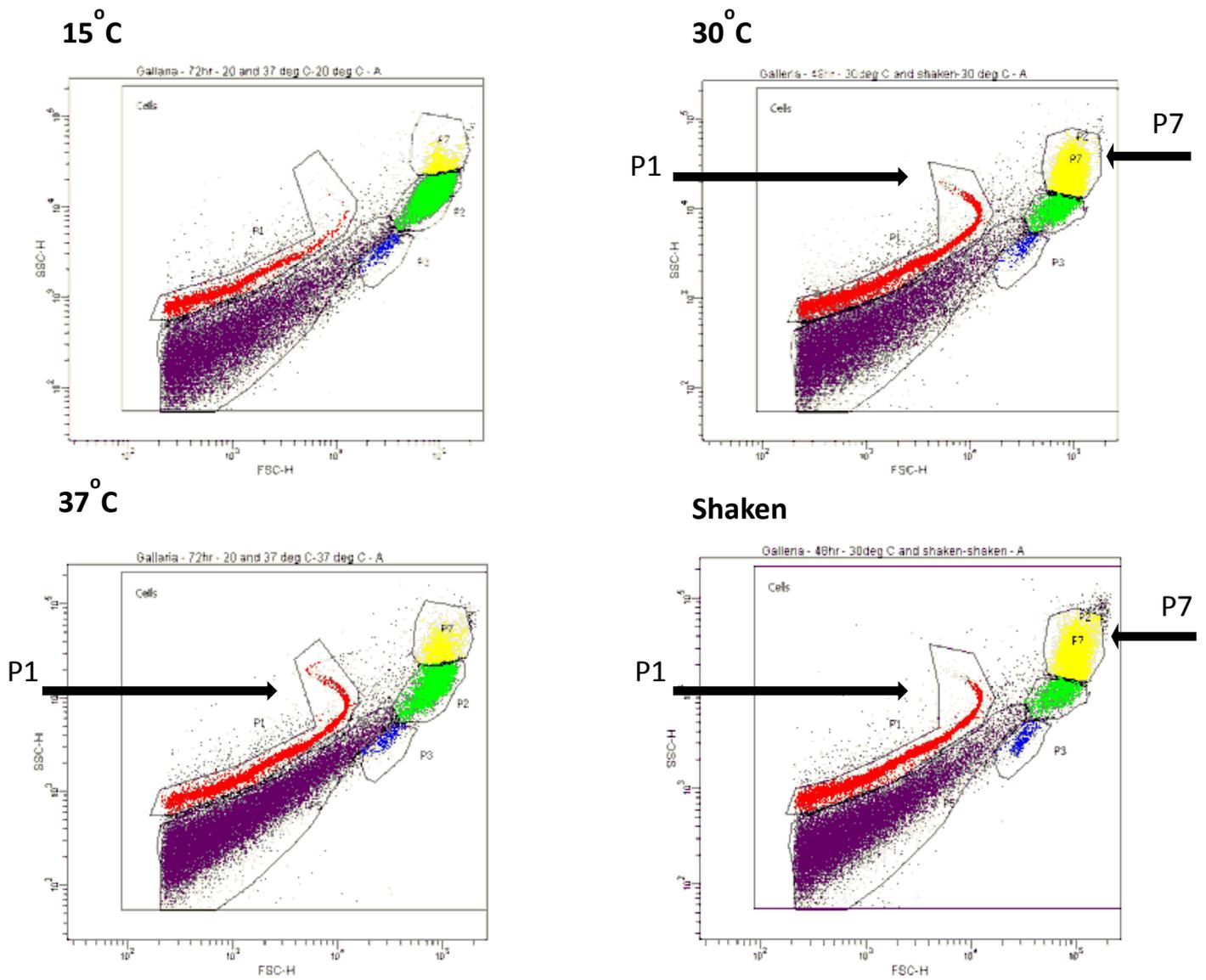
Fig. 9.3 Haemocytes sorted into five sub-populations using FACS Aria Flow
Cytometer, BD ® from larvae incubated for 72h.

Spot no.	Protein	Treatment	24h	48h	72h
4*	Calreticulin	15°C	1	1	1
		30°C	1.81	0.59	0.9
		37°C	2.47	1.67	1.45
		Shaken	2.18	1.21	0.98
6	Protein disulfide isomerase	15°C	1	1	1
		30°C	1.49	0.86	1.09
		37°C	1.93	0.81	1.02
		Shaken	2.38	0.96	1.27
7	Calreticulin	15°C	1.00	1.00	1.00
		30°C	0.43	0.57	0.86
		37°C	1.78	0.68	1.22
		Shaken	1.03	0.61	1.08
8	8 DNA supercoiling factor	15°C	1.00	1.00	1.00
		30°C	0.82	0.96	0.67
		37°C	1.39	1.71	0.95
		Shaken	1.13	1.10	0.64
10	H+ transporting ATP synthase beta subunit isoform 2	15°C	1.00	1.00	1.00
		30°C	0.88	0.94	0.67
		37°C	1.12	0.93	0.95
		Shaken	1.09	1.06	0.64
11	Protein disulfide-isomerase like protein ERp57	15°C	1.00	1.00	1.00
		30°C	1.48	1.03	0.98
		37°C	1.28	0.78	1.03
		Shaken	1.65	1.13	0.96
12*	GH13324 (aldehyde dehydrogenase)	15°C	1.00	1.00	1.00
		30°C	0.47	0.75	3.32
		37°C	0.45	3.11	3.99
		Shaken	0.23	0.65	5.71
13	Putative enolase protein, partial	15°C	1	1	1
		30°C	0.78	0.64	0.68
		37°C	0.88	0.79	0.51
		Shaken	0.53	0.78	0.49
14	Actin	15°C	1	1	1
		30°C	1.25	1.43	0.87
		37°C	0.94	1.22	0.89
		Shaken	1.13	1.68	0.79
15	Arginine kinase	15°C	1	1	1
		30°C	1.41	0.94	1.15
		37°C	1.60	0.94	0.78
		Shaken	0.99	1.00	0.69
16	ADP/ATP tranlocase	15°C	1	1	1
		30°C	1.55	0.62	1.67
		37°C	1.55	1.06	1.79

		Shaken	1.39	0.73	1.11
17	Glyceraldehyde-3 phosphate dehydrogenase	15°C	1	1	1
		30°C	0.86	1.75	0
		37°C	0.74	0.91	0
		Shaken	0.81	1.48	0
19	14-3-3 epsilon protein	15°C	1	1	1
		30°C	2.71	0.99	0.78
		37°C	2.31	0.99	0.75
		Shaken	1.56	1.50	0.51
20	Proteasome 35kD subunit	15°C	1	1	1
		30°C	0.70	1.25	0.00
		37°C	1.00	3.45	0.00
		Shaken	0.87	1.73	0.00
22	Pyruvate dehydrogenase E1 component subunit beta, mitochondrial	15°C	1	1	1
		30°C	2.09	1.10	0.76
		37°C	2.83	1.71	1.11
		Shaken	1.95	1.58	0.56
23	14-3-3 protein zeta	15°C	1.00	1.00	1.00
		30°C	2.71	0.99	0.69
		37°C	2.31	1.26	0.77
		Shaken	1.56	1.70	0.61
24	Heat shock protein hsp21.4	15°C	1.00	1.00	1.00
		30°C	0.36	0.94	0.67
		37°C	0.40	1.05	0.49
		Shaken	0.34	0.94	0.47
26*	FK506-binding protein	15°C	1	1	1
		30°C	2.36	0.85	1.21
		37°C	1.47	0.81	1.04
		Shaken	1.91	1.03	0.77
27*	Translationally-controlled tumor protein homolog	15°C	1.00	1.00	1.00
		30°C	1.59	0.71	0.67
		37°C	2.77	1.01	0.95
		Shaken	1.98	0.84	0.64
28*	Ras-related GTP-binding protein Rab11	15°C	1.00	1.00	1.00
		30°C	1.58	0.59	0.84
		37°C	2.96	0.46	1.17
		Shaken	1.27	0.73	0.77
30	Glutathione-S-transferase-like	15°C	1.00	1.00	1.00
		30°C	0.77	1.18	0.96

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	protein	37°C	0.91	0.87	0.80
		Shaken	0.66	1.00	0.81
32	Cellular retinoic acid binding protein	15°C	1.00	1.00	1.00
		30°C	0.00	0.45	0.78
		37°C	0.00	0.83	0.82
		Shaken	0.00	0.80	1.34
33	Cellular retinoic acid binding protein	15°C	1.00	1.00	1.00
		30°C	0.83	1.04	0.00
		37°C	0.54	1.19	0.00
		Shaken	0.27	1.15	0.00
34	Superoxide dismutase [Cu-Zn]	15°C	1.00	1.00	1.00
		30°C	0.00	0.91	0.66
		37°C	0.00	0.87	0.59
		Shaken	0.00	0.67	0.56
35*	Apolipophorin-III	15°C	1.00	1.00	1.00
		30°C	0.55	1.11	0.92
		37°C	0.51	1.04	1.15
		Shaken	0.36	0.90	0.50

Table 9.1 Fold change in haemocyte proteins extracted from thermally and physically stressed larvae incubated for 24, 48 and 72h. * representing protein with fold change represented in graph (Fig. 4.19)