Schistosoma mansoni excretory-secretory products from miracidium to sporocyst stage and their effects on *Biomphalaria glabrata* defence cells

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Declaration of Degree

I hereby declare that whilst registered for a research degree at Kingston University, London. I have not been a registered candidate or enrolled student at any other academic or professional institution.

I declare that the materials contained in this thesis have not been used in any other submission for an academic award. All the sources of investigation have been duly acknowledged.

I have composed this thesis myself and all results presented in this thesis are from my own investigation.

Zahida Zahoor

May 2010

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Content

List of Abbreviations

ACTH	Adrenocorticotropin hormone
AGGP	Albumen gland gene product
APC	Antigen presenting cell
BiP	Immunoglobulin binding protein
BSA	Bovine serum albumin
CBP	Carbohydrate-binding protein
CBSS	Chernin's balanced saline solution
CREB	cAMP response element binding
DAF-FM	4-Amino-5-methylamino-2',7'-difluorofluorescein
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic cell
DEG	Differentially expressed gene
DEPC	Diethylpyrocarbonate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
ESPs	Excretory secretory products
EST	Expressed sequences tag
Equ	Equation
FAK	Focal adhesion kinase
Fig	Figure
FITC	Fluoroisothiocyanate
FREPs	Fibrinogen-related proteins
GO	Goblet (software)
GTP	Guanosine-5'-triphosphate
GTPase	Guanosine-5'-triphosphatase
h	Hours
H_2O_2	Hydrogen peroxide
HMDS	Hexamethyldisilazane
HSFs	Heat shock factors
HSPs	Heat shock proteins
IFN	Interferon
IL	Interleukin
JNK	c-Jun NH_2-terminal kinase
KEGG	Kyoto encyclopedia of genes and genomes (software)
LPS	Lipopolysaccharides
MAPK	Mitogen-activated protein kinase
MEK	Mitogen-activated protein kinase kinase
MICAL	Molecule interacting with CasL
MKP	Mitogen-activated protein kinase phosphatases
min	Minutes
MPO	Myeloperoxidase
mRNA	Messenger ribonucleic acid
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor-kappa B
NHM	Natural History Museum
NO	Nitric oxide
NOS	Nitric oxide synthase
NY	New York
ONOO-	Peroxynitrite
ORESTES	Open reading frame expressed sequence tags
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD98059	2'-amino-3'-methoxyflavone
Phosphor	Phosphorylation (p-)
PI-3K	Phosphoinositide 3-kinase

PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PMA	Phorbol myristate acetate
PTK	Protein tyrosine kinase
qPCR	Quantitative polymerase chain reaction
RAPD-PCR	Random amplified polymorphic DNA-PCR
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
ROS	Reactive oxygen species
RT	Room temperature
RTK	Receptor tyrosine kinase
s	Seconds
SAPK	Stress-activated protein kinase
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEA	Soluble egg antigen
SEM	Standard error of the mean
SOD	Superoxide dismutase
SSC	Sodium chloride-sodium citrate
SSH	Suppression subtractive hybridization
STAT	Signal transducer and activator of transcription
TEM	Transmission electron microscopy
TBS	Tris-buffered saline
Thr	Threonine
TLR	Toll-like receptor
TNF	Tumour necrosis factor
U0126	1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio] butadiene
UK	United Kingdom
USA	United States of America

Abstract

The processes through which parasites are able to survive in their susceptible host are complex and not fully understood. The platyhelminth parasite, *Schistosoma mansoni*, completes part of its life cycle in the snail *Biomphalaria glabrata*. During its intra-molluscan development, the parasite produces excretory-secretory products (ESPs), a cocktail of uncharacterised proteins and lipids. *Schistosoma mansoni* ESPs may play a role in modulating snail-host immune responses. Two laboratory strains of *B. glabrata* were used in this study; a schistosome-susceptible strain (NHM1742) and a schistosome-resistant strain (NHM 3017) refractory to infection. Snail haemocytes, the main type of circulating defence cells, which have similar characteristics to macrophages, were investigated. The extracellular signal-regulated kinase (ERK) signalling pathway is known to regulate defence reactions in cells. Activated ERK-like proteins were identified in *B. glabrata* haemocytes using phosphor-specific anti-ERK antibodies. The phosphorylation (or activation) of ERK was reduced by 60% in susceptible snail haemocytes following *S. mansoni* ESP exposure (20 µg/ml for 1 h). In contrast, resistant snail haemocytes did not exhibit any changes in phosphorylated ERK levels following ESP-challenge. Nitric oxide (NO), a reactive molecule, which plays a role in host defence mechanisms, increased 3.3 fold in resistant snail haemocytes when challenged with ESPs. ESP-challenged susceptible snail haemocytes did not show any significant modulation in NO levels. The use of scanning electron microscopy also highlighted an increase in haemocyte spreading (on glass coverslips) in ESP-challenged haemocytes from resistant snails only. Heat shock protein 70 (HSP70), an evolutionarily conserved protein with immune-modulation properties was investigated using western blotting and scanning confocal microscope using anti-HSP70 antibodies. HSP70 was significantly reduced in susceptible ($P \leq 0.01$) and resistant ($P \leq 0.05$) snail haemocytes following 1 h exposure to 20 µg/ml ESPs. Interestingly, the ERK cell signalling pathway was found to coordinate both NO and HSP70 levels, as the ERK inhibitor, U0126, significantly reduced levels NO output and HSP70 expression in *B. glabrata* haemocytes. Finally, haemocyte gene expression was analysed after ESP-challenge using a *B. glabrata* cDNA microarray. The microarray analysis highlighted differentially expressed genes (DEGs) between ESP exposed resistant and susceptible snail haemocytes. These DEGs transcribe proteins involved in protein degradation, protein transcription, cell-to-cell interaction and immune responses. This study has broadened our knowledge on the differences that exist between resistant and susceptible *B. glabrata* haemocytes and their differential response to *S. mansoni* ESPs; providing important insights into snail-schistosome interactions.

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List of Figures, Tables and Equations

Chapter 1: Introduction

The planorbid snail *Biomphalaria glabrata* (Gastropoda: Pulmonata) is an important intermediate host for the trematode *Schistosoma mansoni* which parasitizes humans. Various laboratory strains of *B. glabrata* exist, including one strain which is resistant to *S. mansoni* and thus refractory to infection, and many which are susceptible to *S. mansoni*, and can support high levels of infection (Paraense and Correa, 1963). The snail-schistosome interaction is complex and many factors may influence the course of the infection. Furthermore, infection rates may vary depending on the age of the snail, the size of the snail, the parasite miracidial dose on infection, the parasite strain and the geographical location in which the snail strains were obtained (Theron *et al.*, 1997; Lewis *et al.*, 2001). Nevertheless, schistosome-susceptible snails are generally considered disposed to schistosome infection and do not elicit an attack towards the parasite (Bayne and Yoshino, 1989). Despite potential limitations, such as limited genetic variability, laboratory snail strains of *B. glabrata* remain useful models to help understand the key factors contributing to defence against the parasite. The *B. glabrata—S. mansoni* interaction is particularly useful for genetic, molecular and immunological studies focused on aspects of snail/schistosome interplay. The introduction to this thesis outlines aspects of molluscan immunity and the role of snails as intermediate hosts for schistosomes.

1.1 Molluscan Defence Reactions

The innate immune response is an evolutionarily ancient form of defence against pathogens, found in vertebrates, invertebrates, plants and other multicellular organisms (Medzhitov and Janeway, 2002). The molluscan innate immune system employs a number of defence strategies; the use of pattern recognition receptors, anti-microbial peptides, phagocyte-like cells, and production of free radicals, all aim to limit infection (Loker *et al.*, 2004). A snail's body cavity is filled with haemolymph containing haemoproteins and blood cells called haemocytes (Fig. 1.1). In *B. glabrata* these cells are subdivided into hyalinocytes and granulocytes (Bayne, 2003). In principle, molluscan haemocytes are able to bind to foreign organisms *via* pattern recognition receptors, resulting in cell signalling and subsequently the release of compounds such as anti-microbial peptides and free radicals. These products interfere with the biological processes of the pathogen, leading to its destruction and subsequent death (Loker *et al.*, 2004). Molluscan haemocytes are also actively phagocytic, which means that they are able to recognise, bind and internalise small pathogens (Canesi *et al.*, 2002). The process of phagocytosis by molluscan haemocytes is not well characterised, but is likely to involve

internalising the pathogen in a primary phagosome, followed by fusion of a secondary phagosome containing lysosomal granules that kill and digest the pathogen (Kwiatkowska and Sobota, 1999; Sobota *et al.*, 2005). A range of factors may influence phagocytosis in bivalve haemocytes, including temperature, nutrition and gametogenesis (Canesi *et al.*, 2002). A number of immune defence mechanisms have been analysed in molluscs, including those involving carbohydrate-binding proteins (lectins), anti-microbial peptide and toxic metabolites, which either are produced by or interact with haemocytes.

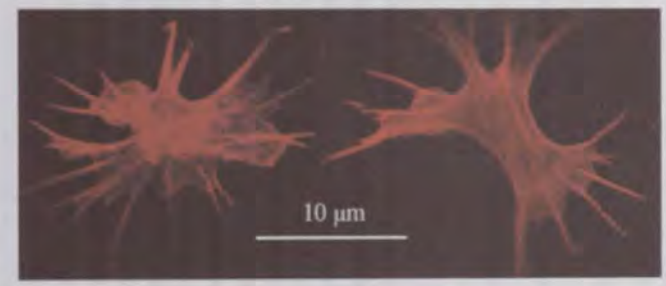

Fig. 1.1 Fluorescence images of two *B. glabrata* haemocytes
The cells were stained with rhodamine phalloidin (red), which binds to filamentous actin (F-actin) to visualise the cytoskeleton of the cells. The haemocytes are spread and display filapodia or projections. These cells are generally considered to be functionally similar to macrophages.

1.1.1 Innate defence reactions and lectins

Lectins are conserved receptor proteins present in a variety of organisms and are mainly considered as recognition molecules (a component of the humoral defence system). Lectins are capable of binding directly to foreign antigens and initiating the process of neutralisation and phagocytosis (Matsushita and Fujita, 1996; Kilpatrick, 2002). In mammals this process is referred to as the lectin pathway and is a recognised part of the complement system (Fig. 1.2). The lectin pathway is activated by microbial carbohydrate patterns, such as mannose-binding lectin (MBL) and ficolins (Endo *et al.*, 2006; Takahashi *et al.*, 2006). The activation of the complement system results in the cleavage of C3, a central component in the system (Fujita *et al.*, 2004; Fujita, Endo *et al.*, 2004). C3 has a thioester bond, which once activated, can form a covalent bond with microbial antigens (Takahashi *et al.*, 2006). This is followed by the assembly of a membrane attack complex (MAC or C5b-9), leading to pore formation in the phosholipid bilayer of the microbe, thus killing the microorganism. Other functions of the mammalian complement system include opsonising pathogens (to make pathogens more susceptible to phagocytes), attracting and activating leukocytes.

A number of lectin or lectin-like proteins have been identified in molluscan haemolymph by the use of cross-reactive antibodies (raised against mammalian lectins), affinity chromatography and the use of functional assays (Mansour, 1995; Johnston and Yoshino

1996; Yoshino *et al.*, 2001; Humphries and Yoshino, 2003). The haemolymph of *B. glabrata* and *Biomphalaria alexandrina* has been shown to contain lectin-like carbohydrate-binding proteins (CBP), synthesised and secreted by haemocytes and connective tissue cells (Monroy *et al.*, 1992; Mansour 1995; Walker, 2006). Interestingly, schistosome-resistant and susceptible strains of *B. glabrata* have been shown to possess different expression patterns of CBP following exposure to *S. mansoni* and the trematode *Echinostoma paraensei*; however, the biological significance of these findings is still currently unknown (Monroy *et al.*, 1992; Monroy and Loker, 1993).

Molluscan lectin-like molecules have been shown to recognise and precipitate parasite-derived molecules, as well as having different binding affinities towards fucosyllactose, a sugar present on the surface of *S. mansoni* miracidia (Mansour, 1995; Mansour *et al.*, 1995; Adema *et al.*, 1997). Sequence analysis of lectin-like molecules confirmed they belong to a group of proteins called fibrinogen-related proteins (FREPs), characterised by one or two immunoglobulin superfamily domains at the N-terminus and a fibrinogen domain at the C-terminus (Adema *et al.*, 1997; Zhang *et al.*, 2001). *Biomphalaria glabrata* snails infected with *E. paraensei* possess significantly increased levels of FREPs. Therefore, FREPs may bind to parasite-derived molecules and facilitate haemocytes in encapsulating and killing the parasite, a process which is similar to opsonisation in the mammalian immune system (Adema *et al.*, 1997). Snails susceptible to *E. paraensei* may elicit an insufficient FREPs response and are thus, unable to eradicate infection (Hertel *et al.*, 2005). Currently there are insufficient data to support the above hypothesis; the development of RNA interference (RNAi) based approaches to silence FREP expression in *B. glabrata* may reveal further information regarding the biological significance of increased FREP expression (Jiang *et al.*, 2006).

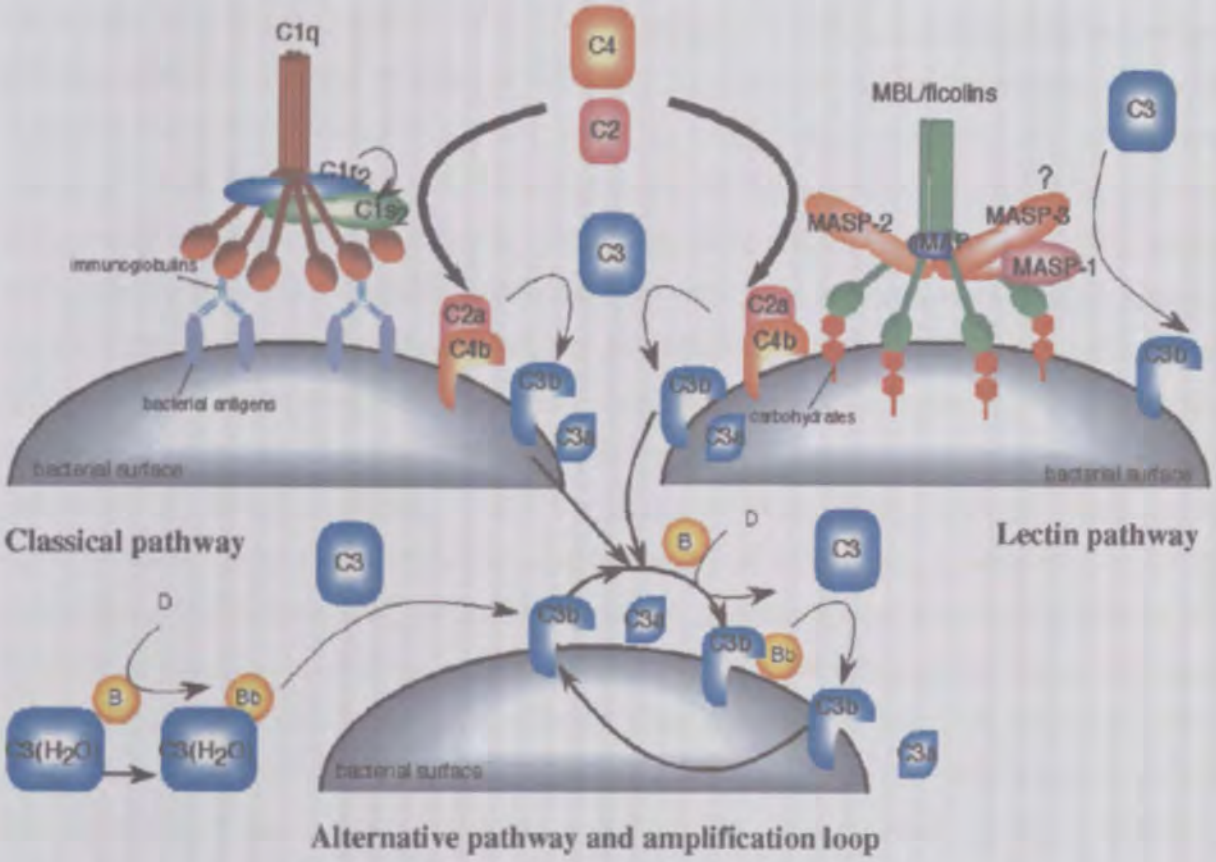

Fig. 1.2 The classical, alternative and lectin pathway in mammals
The classical pathway consists of the C1 complex (q, r and s) which binds to antibodies present on the surface of bacteria. The activated C1 complex cleaves C4 and C2, to form a C4bC2a enzyme complex, which subsequently cleaves C3. The lectin pathway is initiated by the binding of MBL or ficolin, which are associated with MBL-associated serine proteases (MASPs). Activated MASP-2 cleaves and activates C4 and C2, while activated MASP-1 cleaves C3 directly. The alternative pathway is initiated by the activation of hydrolysed C3 and activated factor B. Elements of the lectin pathway have been shown to exist in mollusc (adapted from Fujita *et al.*, 2004).

1.1.2 Innate defence reactions and toxic metabolites

An important part of cell-mediated killing is the production of reactive metabolites, free radicals such as reactive oxygen species (ROS) and nitric oxide (NO). The generation of ROS is mediated by the enzyme, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Roos *et al.*, 2003). This enzyme is highly regulated and is composed of a number of subunits; following cell activation these subunits are brought together, resulting in conformational changes and enzyme activation. In addition, oxidized halides are produced by the enzyme myeloperoxidase (MPO). Details on how precisely ROS and NO are involved in microbial killing have been loosely defined. Certain studies have highlighted that ROS and products of MPO are insufficient to kill microbes in phagocytic vacuoles (Segal, 2005). However, the activity of NADPH oxidase and MPO in cells can increase vacuolar pH leading to the release of cationic granule proteins such as elastase and cathepsin G, which are primarily responsible for microbe killing (Reeves *et al.*, 2002).

Molluscan haemocytes produce ROS in the presence of pathogens and parasite antigen (Bayne, 1990). A number of different ROS can be produced *via* the respiratory burst, for example the enzyme NADPH oxidase converts oxygen (O_2) into a number of reactive oxidants, while the enzyme, nitric oxide synthase (NOS), also utilises oxygen to produce nitric oxide (NO) a long-lived oxidant, and peroxynitrite ($ONOO^-$), a highly reactive, short-lived oxidant (Fig. 1.3). Carbohydrates such as galactose, mannose and fucose present on the surface of *S. mansoni* stimulate the production of ROS in host haemocytes (Hahn *et al.*, 2000). Schistosome-resistant and schistosome-susceptible strains of *B. glabrata* are equally capable of initiating and producing ROS following carbohydrate challenge (Hahn *et al.*, 2000). Inhibiting NADPH oxidase or NOS, (using enzyme inhibitors or oxidant scavengers) significantly reduces the ability of *B. glabrata* haemocytes to kill *S. mansoni* mother sporocysts, the first larval stage parasitic in the snail host (Hahn *et al.*, 2001b). At the molecular level, protein kinase C (PKC) and extracellular signal-regulated kinase (ERK) signalling pathways are known to regulate NO output by haemocytes of the gastropod snail *Lymnaea stagnalis*, following exposure to compounds, such as β-1,3-glucan laminarin and phorbol myristate acetate (PMA) (Wright *et al.*, 2006). In addition, hydrogen peroxide (H_2O_2) production is mainly under the control of PKC and mitogen-activated protein kinases (MAPK) signalling pathways in *L. stagnalis* and *B. glabrata* haemocytes, following stimulation with laminarin or PMA respectively (Lacchini *et al.*, 2006; Humphries and Yoshino, 2008).

Differences may exist between schistosome-susceptible and resistant *B. glabrata* strains in their oxygen-dependent killing mechanisms (Hahn *et al.*, 2000, Hahn *et al.*, 2001a, Hahn *et al.*, 2001b). For example the enzyme myeloperoxidase (MPO, which converts H_2O_2 into a more highly reactive molecule HOCl) may be present in different quantities in the two snail strains, with schistosome-susceptible snails expressing more MPO compared to resistant snails (Hahn *et al.*, 2001a; Bayne, 2003). Considering H_2O_2 is more toxic to *S. mansoni* mother sporocysts than HOCl, it is possible that MPO lowers the net quantity of H_2O_2 available for cytotoxic killing of the parasite (Hahn *et al.*, 2001a). A deficiency in MPO would result in accumulation of H_2O_2, resulting in a more vigorous attack on the parasite and hence resistance to infection (Hahn *et al.*, 2001a). This theory is supported by the fact that *B. glabrata* strains resistant to schistosome infection are capable of generating considerably more H_2O_2 compared to susceptible *B. glabrata* strains (Bender *et al.*, 2005).

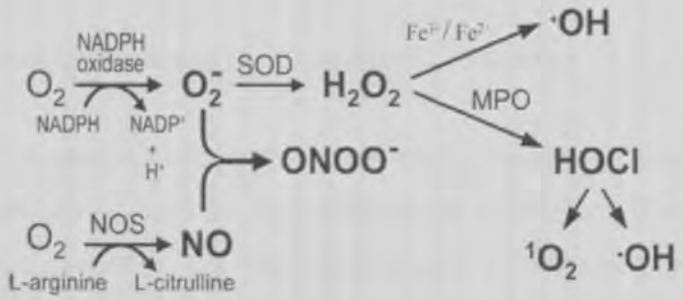

Fig. 1.3 The respiratory burst
NADPH oxidase complex converts O_2 into a reactive species O_2^- ; further oxidants are also created in the presence of MPO or iron. Oxygen can also be converted into NO via the enzyme nitric oxide synthase (NOS) (Hahn *et al.*, 2001b).

1.1.3 Innate defence reactions and other defence molecules

Molluscs also defend themselves against pathogens *via* the production of defence molecules and antimicrobial peptides. The molluscan defence molecule (MDM), characterised by five C2-like immunoglobulin (Ig) domains, has been isolated from *L. stagnalis* (Hoek *et al.*, 1996). The function of MDM is poorly understood, but it is capable of enhancing phagocytic activity of *L. stagnalis* haemocytes (de Jong-Brink *et al.*, 2001). Interestingly, the MDM gene is down-regulated in *L. stagnalis* snails infected with the avian schistosome *Trichobilharzia ocellata*, a possible strategy used by the parasite to evade detection in its snail host (Hoek *et al.*, 1996). Using mass spectrometric differential peptide profiling, Smith *et al.*, (2004) isolated and identified another molluscan defence molecule, granularin. Granularin is abundant in granular cells (cells which have direct contact with the open circulatory system); its function may involve promoting phagocytosis of haemocytes (Smit *et al.*, 2004).

The existence and diversity of antimicrobial peptides in molluscs has been the focus of various studies with much of the research done on bivalves. These antimicrobial peptides are cysteine-rich and have been classified into four main groups based on their structures: defensins, mytilins, myticins and mytimycin (Canesi *et al.*, 2002). Mytilins and myticins are found in haemocyte phagosomes where their function may involve antimicrobial activity (Mitta *et al.*, 2000), whereas the main function of defensins is to permeablise the cytoplasmic membrane of a bacterium (Charlet *et al.*, 1996). It would be interesting to determine whether these molecules also play a role in defending the mollusc host against parasitic infection.

1.2 Snails as Intermediate Hosts for Trematode Parasites

Schistosomiasis is a group of diseases and a global health problem which afflicts more than 200 million people in tropical and subtropical countries (Steinmann *et al.*, 2006). Human schistosome infections are the main causes of disease burden in children aged between 5 and 14 years old [World Health Organisation (WHO) 1999]. Morbidity is high and several thousands of deaths occur annually from schistosomiasis (King *et al.*, 2005). The medically important species of schistosomes are: *Schistosoma mansoni, Schistosoma haematobium* and *Schistosoma japonicum*; these species use different aquatic snail vectors as an intermediate host. *Schistosoma mansoni* infects snails of the genus *Biomphalaria,* mainly *Biomphalaria pfeifferi* in Africa and *Biomphalaria glabrata* in the Caribbean and South America. *Schistosoma haematobium* mainly infects *Bulinus truncatus* and *Bulinus globosus* respectively in Africa, while *Schistosoma japonicum* infects *Onchomelania hupensis* found in China.

1.2.1 Life cycle of schistosome parasites

Adult schistosomes live in mammalian blood vessels and feed on blood cells and plasma. *Schistosoma mansoni* and *S. japonicum* live in the mesenteric veins of the intestines, and *S. haematobium* inhabits the visceral veins of the bladder wall (Fig. 1.4). Unusually for trematodes, schistosomes have separate sexes, but the female lives inside the male's gynaecophoric groove. A female schistosome produces hundreds to thousands of eggs per day, depending on the species (Gryseels *et al.*, 2006). Some eggs may be expelled from the body in urine or faeces by reaching the lumen of the gut or the bladder, but most become trapped in surrounding tissues such as the liver leading to the formation of granulomatous lesions, fibrous extracellular matrix containing immune cells (Stadecker *et al.*, 2004; Gryseels *et al.*, 2006). Granulomas are known to be damaging to the host but the lesions can also serve a protective function by forming a barrier between the eggs and the host (Wilson and Coulson, 1998). Indeed, immunocompromised mice that are unable to mount a granulomatous response have a decreased survival rate compared to normal infected mice (Stadecker *et al.*, 2004). The formation of granulomas may allow the host to live with infection for many years rather than suffer early death.

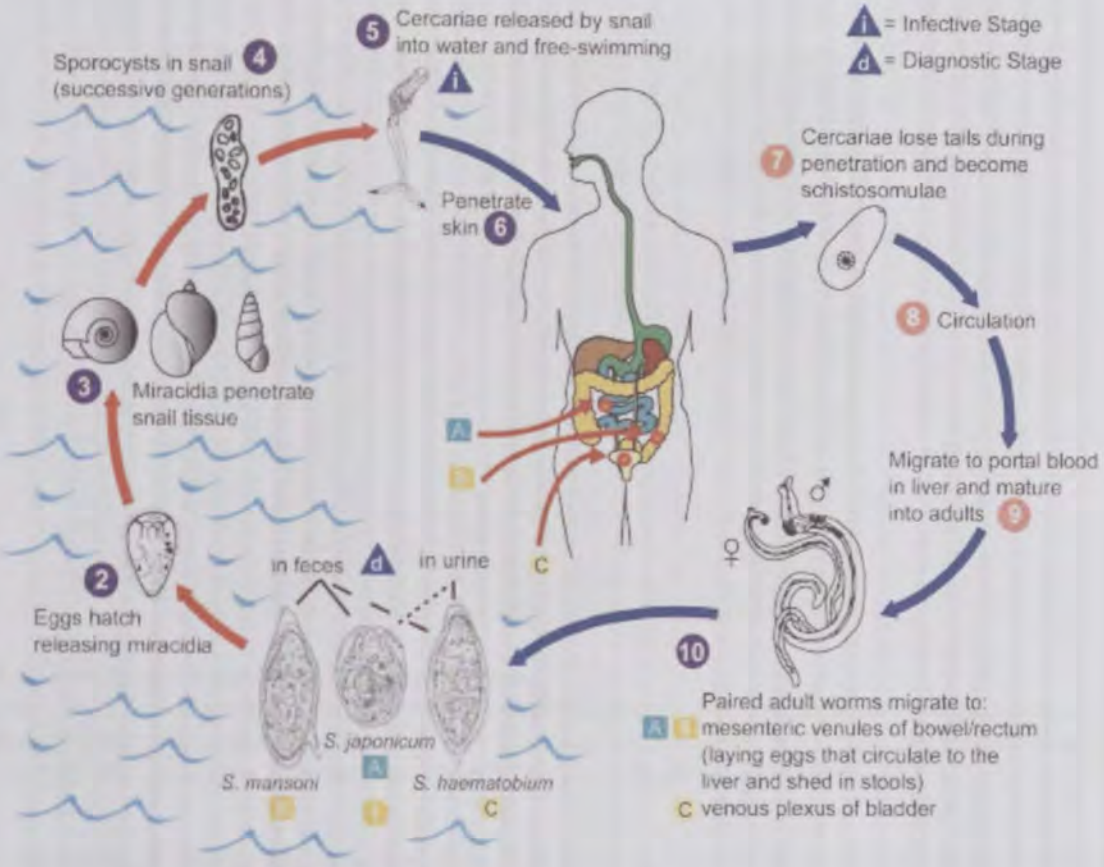

Fig. 1.4 A simplified life cycle for the three main species of schistosoma
Schistosoma japonicum (A) paired adults migrate to the mesenteric venules of the small intestine in the human host; *S. mansoni* (B) paired adults migrate to the mesenteric venules of the rectum while *S. haematobium* (C) paired adults migrate to mesenteric venules of the bladder. The trematodes then start to reproduce and a number of parasitic eggs are released into the faeces or urine. Following exposure to water, the eggs hatch releasing miracidia which can infect specific populations of snails. The parasites further develop in the snail host and are released into the water as free swimming cercariae, capable of infecting mammals, specifically humans (Source of image: Centre for Disease Control and Prevention, US).

Schistosome eggs are expelled from the body in urine or faeces and can remain viable for about two days. Once the eggs are in contact with fresh water they begin to hatch in daylight and release fully-developed ciliated miracidia that are capable of infecting amphibious or aquatic snails, guided by chemical stimuli such as hydrophilic organic molecules and amino acids produced by the snail (Haas *et al.,* 1995). Once a miracidium penetrates the snail, there is a rapid shedding of ciliary epidermal plates from the parasite's surface and a new tegument is created (Basch and DiConza, 1974; Basch and DiConza 1977). This process of "transformation" can take up to 24 h *in vitro* (Wu *et al.,* 2009). Inside the snail the miracidium develops into a post-miracidium mother sporocyst, then after 6-13 days it becomes a sac filled with daughter sporocysts in different developmental stages (Smith and Chernin, 1974). Yoshino *et al.,* (1993) identified significant proteinase activity in the culture medium of transforming sporocysts, which may be involved in establishing or maintaining infection in the snail. As the sporocysts develop they migrate to the digestive glands of the snail where they produce cercariae (Fig. 1.5).

Fig. 1.5 Living cercariae unstained in culture
Images 1, 2, 3 and 4 correspond to different developmental stages of schistosome cercaria; the cercaria develop from oval structures (1) into elongated rod shapes with characteristic bifurcated tails (4) that enable swimming, bars represent 100 µm (Basch and DiConza, 1977).

Fully developed cercariae emerge from the snail to become free-swimming forms that need to find a susceptible mammalian host to continue their life cycle. The cercaria use light, water turbulence, thermal gradient and mammalian skin chemicals, particularly free fatty acids, to locate their host (McKerrow and Salter, 2002; Gryseels *et al.*, 2006). Once each cercaria has found its mammalian host it attaches itself to the skin and begins migrating through the host's epidermis, a process which can take between 15 min and 20 h depending on the species (Fusco *et al.*, 1993; Haas *et al.*, 2002). The tail of the parasite is not lost during skin penetration and remains attached to the head of the parasite until it reaches the dermis (Fig. 1.6) (Whitfield *et al.*, 2003; Whitfield *et al.*, 2003b). The cercariae secretes proteolytic and immunomodulatory proteins as it migrates through the skin; these include the anti-inflammatory protein, Sm16, a metalloproteinase, SmPepM8, and a serine protease inhibitor, SmSerp_c (Curwen *et al.*, 2006). When the tail is shed the cercaria becomes a skin-stage schistosomulum.

The skin-stage schistosomulum finds a lymphatic vessel and is transported to the lungs (now referred to as the lung-stage schistosomulum) where it navigates its way to the liver (McKerrow and Salter, 2002). In the liver, the schistosomulum transforms into a liver-stage schistosomulum where the development to the adult stage can take 4-6 weeks (Gryseels *et al.*, 2006). In the portal veins the schistosomulum finds, and pairs, with an opposite sex partner; the pair migrate to the pervisceral or mesenteric venous plexus. An intimate association exist between the male and female worm, whereby the female

resides within the gynecophoric canal (ventral groove) of the male, a necessary process for female growth, reproductive development and egg production (LoVerde *et al.*, 2009).

Fig. 1.6 *Schistosoma mansoni* cercaria penetrating keratinocytes in culture
The head and tail of the parasite are in the tunnel formed by degrading cross-linked collagen, image taken with a scanning electron microscope. Bar represents 20 µm (Whitfield *et al.*, 2003)

1.2.2 The pathology of schistosomiasis

Schistosomiasis is the disease caused by infection with schistosomes. Initial symptoms may include itchy skin or rash, followed by fever, coughing and muscle aches. At later stages, symptoms may include abdominal pain, fever, diarrhoea, anaemia and malnutrition (symptoms may vary depending on species, strain and parasite burden). Acute schistosomiasis or Katayama fever occurs when granulomatous lesions start to establish. Most of the pathology of the disease is the direct or indirect result of host immune response to the parasite eggs (Gryseels *et al.*, 2006). The eggs of *S. haematobium* can induce inflammation and obstruction in the urinary system, leading to hyperplasia of the bladder, bladder cancer and secondary damage to the kidneys. The eggs of *S. mansoni* and *S. japonicum* can cause colonic obstruction and blood loss; the eggs can also be transported to the liver *via* the hepatic portal system, and cause liver enlargement, hypertension and liver fibrosis. In severe cases of schistosomiasis there is damage in the portal circulation and parasitic eggs are able to reach other organs including the heart and the lungs. Modern drug treatment against schistosomes usually involves oral administration of praziquantel, a widely used anti-helminitic drug that is currently active against all of the species (Lardans and Dissous, 1998; Doenhoff *et al.*, 2009). There is currently concern that the selection pressure exerted by this drug on schistsomes will eventually lead to drug-resistance (Doenhoff *et al.*, 2002; Doenhoff *et al.*, 2009).

Current research goals against human schistosomiasis include developing a vaccine against common schistosome species. Some individuals with schistosomiasis have severe and irreversible pathology that is diagnosed too late for chemotherapy to be beneficial. To date, a radiated-attenuated schistosome vaccine has been shown to protect rodents and primates against schistosomiasis (Hewitson *et al.*, 2005). However, the possibility of delivering a live (or attenuated) vaccine to a number of individuals, especially to children who are more at risk of the disease in a number of developing countries, is unrealistic (Wilson and Coulson, 1998). Therefore, greater focus has been placed on the development of a recombinant vaccine (use of bacteria or yeasts to produce mass quantities of a single parasite antigen). The surface of a schistosome (tegument) has been investigated for potential vaccine antigens, including tetraspanins, which are conserved scaffolding proteins that form dynamic networks of protein interactions. They play a role in a number of cellular functions including cell migration, regulating proteolytic activity, modulating cell signalling pathways and receptor expression (Charrin *et al.*, 2009). Mice vaccinated with schistosome tetraspanins develop some protection against the disease (Tran *et al.*, 2006; Loukas *et al.*, 2007). Another potential vaccine candidate is glutathione-S-transferase (GST), a 28 kDa enzyme that plays a key role in the detoxification system of the parasite, this enzyme currently in clinical trials (Capron *et al.*, 2002).

1.2.3 Mammalian immune response towards schistosomes

In schistosome-infected individuals the innate system is initially activated followed by the adaptive immune response which is required for the development of granulomas (Pearce, 2005). Schistosomulae stimulate macrophages *via* their Toll-like receptors (TLR), activating MyD88-dependent pathways (Jenkins *et al.*, 2005). This is followed by a T helper 1 (Th1) response (Th1 cells produce cytokines such as interferon-γ and interleukin-2, which promotes cellular killing by enhancing the killing efficiency of macrophages and activating the proliferation of cytotoxic killer $CD8^+$ T cells) (Stadecker and Hernandez, 1998). After five weeks infection there is an increase in granuloma formation and a subsequent change in the cytokine environment, resulting in a Th2 response (Th2 cells produce IL-4, IL-5, IL-10 and IL-13, which stimulates B cells to proliferate and to produce antibodies) (Stadecker *et al.*, 2004). By approximately ten weeks post-infection there is a down-regulation in the number of $CD4^+$ T cells *via* the production of IL-10 (produced by T-regulatory cells). The balance between Th1 and Th2 cytokine environments and how it is maintained is still under debate. Interestingly, C57BL/6 (a mouse model that exhibits a mild form of schistosomiasis when infected) immunised with schistosome egg antigens

produces a significant Th1 shift, resulting in enhanced immunopathology, with an increase in granuloma size and parenchymal inflammation (Rutitzky *et al.*, 2001). This phenomenon was not observed when wild-type mice or the high pathology CBA mice strain where immunised, implying that a Th1-dominant environment is linked to severe disease outcome and subsequent death (Rutitzky *et al.*, 2001).

Schistosome eggs are capable of influencing the host's immune response, for example glycoconjugates (derived from eggs) are able to induce a Th2 response in the presence of dendritic cells (DC) (Pearce, 2005). Soluble egg antigen (SEA) can exert an inhibitory effect on DC by suppressing maturation, thus suppressing up-regulation of co-stimulatory molecules and inhibiting IL-12 production (Kane *et al.*, 2004). This inhibitory effect could be achieved by modulating TLR responses (Segura *et al.*, 2007). Thus, egg antigens may be capable of suppressing the development of a general Th1 response.

1.2.4 Parasite-host interactions in the mammalian host

The host-parasite relationship is a combination of parasite survival strategies and host defence mechanisms. An adult worm is capable of surviving in the mammalian host for at least 10 years; it achieves this through three main mechanisms: antigenic variation, antigenic mimicry and immunomodulation (reviewed in Salzet *et al.*, 2000; Wilson *et al.*, 2007). In antigenic variation the parasite can alter the expressed antigens on its surface at a faster rate than the ability of the host to recognise and respond to the antigen, thus preventing an efficient host immune response. Antigenic mimicry involves the parasite exhibiting similar or identical epitopes to its host. The employment of molecular cloning in schistosomes has revealed a significant degree of similarity between the nucleotide sequences of parasites and those of mammalian genes. For example, *S. mansoni* encodes a homologue of the mammalian epidermal growth factor (EGF) receptor and a receptor serine-threonine kinase termed SmRK-1, which exhibits 58% similarity with its host (Shoemaker *et al.*, 1992; Davies *et al.*, 1998). SmRK-1 can potentially bind to growth factors present in the host plasma and facilitate the parasite's own development. Indeed, insulin from the host can regulate glucose uptake in the schistosome; two insulin receptors (SmIR-1 and SmIR-2) in *S. mansoni* have been characterised to date (Clemens and Basch, 1989; Khayath *et al.*, 2007). Schistosomes are further capable of host immunomodulation by inhibiting adhesion, migration and cytokine production by human polymorphonuclear cells. Schistosomes induce these responses *via* the production of adrenocorticotropin hormone (ACTH, directly acts on T-cells and decrease IFN-γ production), α-melanostimulating hormone (α-MSH, inhibits adhesion and cellular

movement), β-endorphin, and morphine-like and codeine-like substances (Duvaux-Miret *et al.*, 1992; Duvaux-Miret *et al.*, 1992; Salzet *et al.*, 2000).

Schistosome eggs and cercariae express glycans on their surface, such as mannose and fucose; these glycans can interact with TLR2 on DCs and induce tolerance (Jenkins and Mountford, 2005; Jenkins *et al.*, 2005). These tolerant DCs are further capable of activating IL-10-producing-T regulatory cells and thus damping down host immune responses against parasitic antigen (Caparros *et al.*, 2006; Cools *et al.*, 2007). The process of DC tolerance may involve the activation of C-type lectins on the surface of DCs, specifically DC-specific ICAM-3 grabbing non-integrin (DC-SIGN), leading to the activation of intracellular signalling pathways and the release of IL-10, an immunosuppressive cytokine (Meyer *et al.*, 2005; Caparros *et al.*, 2006).

At different stages of its life cycle a schistosome secretes compounds or sheds material from its surface, termed parasite excretory-secretory products (ESPs) (Lightowlers and Rickard, 1988). Early studies in the 1980s highlighted the function of ESPs in establishing and maintaining parasite infection by influencing the proliferation of schistosome-infected host peripheral blood mononuclear cells (PBMC) (Vieira *et al.*, 1986). Initial penetration of cercariae and subsequent generation of ESPs also affects immune cells located in skin tissue. The protein Sm16.8 (isolated from cercariae ESPs), was shown to invoke the production of interleukin-1 receptor antagonist (IL-1RA) in keratinocytes, suppressing lymphoproliferative responses (Ramaswamy *et al.*, 1995). As a cercaria develops into a schistosomulum ESPs are continually being released in the host environment; these ESPs have been shown to modulate transvascular permeability of endothelial cells (Trottein *et al.*, 1999).

1.2.5 Parasite-host interactions in the molluscan host

As in the case of parasite-host interactions in mammals, similar complex interactions exist between the schistosome larvae and its snail intermediate host. Antigenic variation, antigenic mimicry and immunomodulation are important strategies used by the transforming sporocyst to survive in its host (Bayne and Yoshino, 1989). For example, schistosome sporocysts can coat themselves with molluscan plasma proteins, mimicking snail epitopes and blocking the opsonisation and the encapsulation process in their susceptible host (Bayne *et al.*, 1986; Fryer *et al.*, 1989). Studies have shown schistosome sporocysts can produce (and secrete) glycosylated proteins that share characteristics with mucins (Roger *et al.*, 2008a). These proteins express a high degree of polymorphism, leading to antigenic variation and permitting evasion from host immune responses (Roger *et al.*, 2008a; Roger *et al.*, 2008b; Roger *et al.*, 2008c). Finally, schistosome sporocysts

and their ESPs may influence haemocyte behaviour by modulating key cell signalling pathways (Yoshino *et al.*, 2001; Humphries and Yoshino, 2003; Humphries and Yoshino, 2006) (Fig. 1.6).

Schistosome—snail interactions also consist of nutrients and other factors from the mollusc being taken up by the parasite and schistosome ESPs being released into the host's environment (Fig. 1.7) (Yoshino *et al.*, 2001). Schistosome ESPs are generally released from the penetration glands of the miracidium, or could be derived from ciliated plates that are shed when the miracidium transforms into a mother sporocyst (Bayne, 2009; Wu *et al.*, 2009). In addition, ESPs could be released through the excretory pore of the miracidium, or from the evolving tegument of the various intra-molluscan larval stages of the parasite (Yoshino and Lodes, 1988; Yoshino *et al.*, 2001). Lodes and Yoshino (1988) demonstrated that *S. mansoni* primary sporocysts cultured *in vitro* could synthesise and secrete a number of glycoproteins and polypeptides. To date, cysteine proteases, superoxide dismutase, glycolytic enzymes, antioxidant enzymes, heat shock proteins (HSPs), mucins, calcium and ion-binding proteins have been identified in *S. mansoni* larval stage ESPs (Lodes and Yoshino, 1989; Zelck and Von Janowsky, 2004; Guillou *et al.*, 2007; Roger *et al.*, 2008a; Roger *et al.*, 2008b; Wu *et al.*, 2009). Proteomic analysis has further shown that *S. mansoni* ESPs comprise mainly of non-secretory proteins (Wu *et al.*, 2009). Therefore, ESPs may be cytoplasmic proteins that leak out from the parasite as it penetrates its host (and loses its ciliated plates) or may be proteins that are released during sporocyst transformation (Wu *et al.*, 2009).

One of the functions of ESPs derived from transforming sporocysts (including *S. mansoni*, *E. paraensei* and *Echinostoma caproni*) appears to be altering molluscan defence responses. Haemocytes extracted from schistosome-susceptible snails and exposed to larval-stage ESPs generally have reduced motility, adhesion, phagocytosis, are less able to encapsulate sporocysts and produce reactive oxygen species (ROS) (Connors and Yoshino, 1990; Lodes and Yoshino, 1990; Connors *et al.*, 1991). In contrast, the effects of ESPs on haemocytes extracted from schistosome-resistant snails generally are limited; they may even have a positive effect, such as enhanced protein synthesis (Yoshino and Lodes, 1988; Connors and Yoshino, 1990; Lodes *et al.*, 1991). Thus, haemocytes from resistant snails may be more metabolically active in the presence of ESPs than those of susceptible snails (Bayne and Yoshino, 1989). Larval stage ESPs also have a protective function; ESPs contain scavenging superoxide anions that potentially protect the parasite from ROS produced by host snail haemocytes (Connors *et al.*, 1991; Humphries and Yoshino, 2007). Thus, schistosomes may have an antioxidant system that partially counteracts cytotoxic metabolites produced by haemocytes from susceptible snails. This

interplay between the reactive oxygen metabolites produced by the snail host, and the parasite's antioxidant system, may explain aspects of parasite-host compatibility (Connors and Yoshino, 1990; Connors *et al.*, 1991).

What specific components in *S. mansoni* ESPs modulate haemocyte behaviour and function is unknown, but they may include the protein Molecule Interacting with CasL (MICAL-like) and calreticulin (Guillou *et al.*, 2007). MICAL-like protein is a conserved signal transduction protein and plays a role in a number of cellular processes including interacting with the cytoskeleton (it is best characterised for its function in neuromuscular development), while calreticulin is a lectin-like chaperone and controls calcium homeostasis (Guillou *et al.*, 2007; Kolk and Pasterkamp, 2007). These ESP components may interact with molluscan plasma and haemocytes *via* carbohydrate-binding receptors (CBR), or lectin-like receptors (Johnston and Yoshino, 1996; Johnston and Yoshino, 2001). Following receptor-ligand interaction, cell signalling pathways may become activated or inhibited (Humphries and Yoshino 2003; Humphries and Yoshino, 2006). What specific gene transcripts are modulated in snail haemocytes following ESP-challenge is also currently unknown. Stimulation of Bge cell (*B. glabrata* embryonic cell line) with *S. mansoni* ESPs resulted in the differential expression of twenty three different genes, including genes that transcribed for proteins involved in energy production, metabolism, apoptosis, DNA silencing and regulating the activity of endogenous proteases (Coustau *et al.*, 2003).

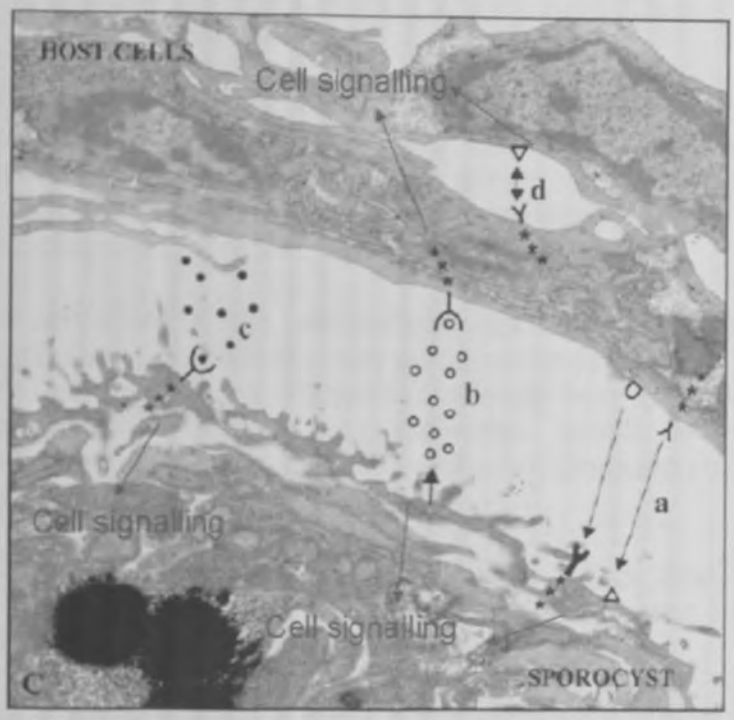

Fig. 1.7 Transmission electron microscopy (TEM) image of *S. mansoni* sporocyst and *B. glabrata* tissue
A TEM image labelled to indicate possible interactions between an *S. mansoni* sporocyst and *B. glabrata* tissue particularly those promoting intercellular signalling. (a) Direct ligand-receptor interaction between host and sporocyst (b) release of ESPs from the sporocyst which bind to host receptors triggering cell signalling (c) receptors on the sporocyst bind to and uptake host plasma factors (d) host cell-cell and cell-substrate interactions for normal processes. Blue lines highlight that cell signalling pathways may be activated or inhibited (modified from Yoshino *et al.*, 2001).

1.3 Statement of Aim and Objectives

This PhD project aimed to investigate the effects of *Schistosoma mansoni* ESPs on schistosome-susceptible and schistosome-resistant *Biomphalaria glabrata* haemocyte cell signalling and defence responses. The knowledge gained would enhance significantly our understanding of snail-schistosome interactions particularly in the context of host-phenotype. Specific objectives were to:

- Investigate activation of the extracellular signal-regulated kinase (ERK) cell signalling pathway in haemocytes from schistosome-susceptible and schistosome-resistant *B. glabrata* strains following challenge with *S. mansoni* ESPs. The experimental strategy was to use antibodies raised against evolutionarily-conserved regions in ERK to detect ERK activation (phosphorylation) in challenged haemocytes.

- Investigate morphological changes in haemocytes after *S. mansoni* ESP exposure by image analysis and calculating relative areas of spread haemocytes, using Visual Basic software.

- Determine any changes in nitric oxide (NO) production in *B. glabrata* haemocytes, in the presence and absence of *S. mansoni* ESPs, using a highly sensitive NO probe (DAF-FM diacetate) and to investigate the signalling pathways that regulate NO production in haemocytes.

- Elucidate gene expression patterns in *B. glabrata* haemocytes following *S. mansoni* ESP-challenge by using a *B. glabrata* cDNA microarray containing a range of oligonucleotide sequences and quantitative PCR (qPCR) to quantify specific gene expression levels.

- Determine expression of heat shock protein 70 (HSP70) protein in *B. glabrata* haemocytes following exposure to *S. mansoni* ESPs. The experimental strategy was to use antibodies raised against evolutionarily-conserved HSP70 to detect HSP70 protein in haemocytes.

Chapter 2: Materials and Methods

Information on commonly used reagents, equipment and their suppliers can also be found in the appendix.

2.1 Snail culture

A *Schistosoma mansoni*-resistant *Biomphalaria glabrata* strain with Natural History Museum (NHM) accession number 3017 originally derived from BS90 (a Brazilian field isolate) and an *S. mansoni*-susceptible *B. glabrata* strain with Natural History Museum (NHM) accession number 1742, were used in all experiments, infectivity rates for *S. mansoni* were determined for each snail strain, as discussed in section 2.2 (Paraense and Correa, 1963). Snails were housed in plastic boxes with filtered water (Brimak/carbon filtration unit purchased from Silverline Ltd, Winkleigh, UK) and marble chips (Fisher, Loughborough, UK). The boxes were stored in an incubator maintained at 26°C with a programmed 12 h: 12 h, light: dark cycle. The snails were fed fresh round lettuce regularly, and occasionally fish food to encourage reproduction.

2.2 Measuring *S. mansoni* infection rates for *B. glabrata* strains

Biomphalaria glabrata susceptible and resistant snails of 0.6-1.0 cm diameter were infected individually by exposing each snail to five *S. mansoni* miracidia (Belo Horizonte strain) for 12 h. Following infection, snails were placed into clean trays with fresh water and fed round lettuce weekly. On the 25th day post-infection snails were placed under artificial light for 2 h and checked for cercarial shedding using an inverted light microscope. The snails were then checked for cercarial release daily until 35 days post-infection. The snails were subsequently killed and their haemolymph removed for further use.

2.2.1 Infection rates of susceptible and resistant B. glabrata strains

Wild populations of *B. glabrata* vary in their susceptibility towards *S. mansoni* infection (Prugnolle *et al.*, 2006). Inbred laboratory snail strains, susceptible (NHM 1742) and resistant (NHM 3017) were re-tested (originally tested in 2002) at the NHM for their infection rates to confirm susceptibility/resistance towards *S. mansoni*. Table 2.1 shows cercarial counts for shedding snails; NHM 1742 strain were approximately 55% susceptible 35 days post-infection, while NHM 3017 were approximately 94% resistant (1 snail shedding only 1 cercaria) 35 days post-infection.

Infectivity rates are an approximation; a number of factors exist that can influence susceptibility/resistance including: the survival, age, size and sex of the individual snail (Theron *et al.*, 1998; Caillaud *et al.*, 2006; Prugnolle *et al.*, 2006). Susceptible laboratory *B. glabrata* strains maintain (through generations) an average of 50% susceptibility for a specific strain of *S. mansoni,* which corresponds with the finding here (Theron and Coustau, 2005). NHM 3017 laboratory *B. glabrata* strains were 94-100% resistant to *S. mansoni* infection; this variation in cercarial shedding has been previously noted (Jones *et al.*, 2001).

NHM 1742 *B. glabrata* strain (susceptible)		
Day	% of shedding snails	Cercarial count (in 25ml per snail)
22	40	10-50
28	50	10-50
36	55	†
NHM 3017 *B. glabrata* strain (resistant)		
22	0	0
28	0	0
36	6	1-10

Table 2.1 Infection rates for NHM 1742 and NHM 3017 snail strains
30 infected snails of each strain were monitored for cercarial shedding over 35 days post-infection in a total volume of 25 ml spring water, † data not available.

2.3 Collection of *S. mansoni* excretory secretory products

Schistosoma mansoni (Belo Horizonte strain) infected mice (CD1) were killed 40-50 days post-infection. The maintenance and the dissection of the mice were carried out at the Wolfson Wellcome Biomedical Laboratories, Department of Zoology, The NHM, London (under local ethical approval). Livers and spleens from the infected mice were removed and placed into physiological saline solution (0.85% (w/v) NaCl, Sigma, Poole, UK) for 10 min. Eggs were then collected by passing sections of liver and spleen tissue through a fine mesh (212 grade) and washed through with physiological saline. The suspension was transferred to a sedimentation flask and washed 5-6 times with 250 ml physiological saline to remove fats, blood, and other tissues from the preparation. Next, the eggs were left to hatch in 50 ml natural mineral water (Evian) in a Petri dish for 1.5 h under a fibre optic light source. Hatched miracidia were then collected, washed in mineral water and concentrated approximately 10x using a Millipore Stericup filter unit (0.45 µm pore size). On average 30-50,000 miracidia were obtained from 10-12 mice. The miracidia were cultured in 25 cm^2 culture flasks (Corning Costar, Schiphol-Rijk, Netherlands) containing Chernin's balanced saline solution [(CBSS: 48 mM NaCl, 2.0 mM KCl, 0.5 mM Na_2HPO_4,

1.8 mM $MgSO_4 \cdot 7H_2O$, 3.6 mM $CaCl_2 \cdot 2H_2O$, 0.6 mM $NaHCO_3$, 5.5 mM glucose and 3 mM threalose in 1 L of distilled water and sterilised through a 0.2 µm filter (Sminia, 1972) supplemented with 50 U/ml penicillin and streptomycin (Sigma)] for 36-40 h in the dark at 26°C.

Cultures of larvae were observed with an inverted microscope to ensure that the miracidia had transformed into sporocysts and were alive (i.e. sporocyst movement and/or flame cell movement was visible); the flasks were then shaken to remove any residual miracidial ciliated plates from the sporocysts' surface. Sporocysts were left to settle on the base of the flask before the supernatant (containing the ESPs) was collected and centrifuged for 10 min at 10,000 *g* to pellet any suspended miracidial plates. Next the ESPs were concentrated approximately 10-20x in a Vivapore concentrator at 4°C with a cut-off size of 7,500 MW (Vivascience, Sartorius, Epsom, UK). The protein concentration of the ESPs was then determined using a NanoOrange fluorescence-based protein assay kit (Molecular Probes, Leiden, Netherlands) following the manufacturer's instructions. The ESPs were diluted at different concentrations in distilled water, 1x NanoOrange reagent was then added and the solution was heated at 95°C for 5 min then cooled to room temperature (RT). Proteins present in the solution bind to the NanoOrange reagent and enhance its fluorescence (Fig. 2.1). NanoOrange reagent has an excitation peak of 470 nm and an emission peak of 570 nm which was measured using a fluorescence-based mirotitre plate reader (FluorStar Optima, BMG Labtech). The concentrated ESPs were finally stored in aliquots of 20-50 µl at -20°C until required.

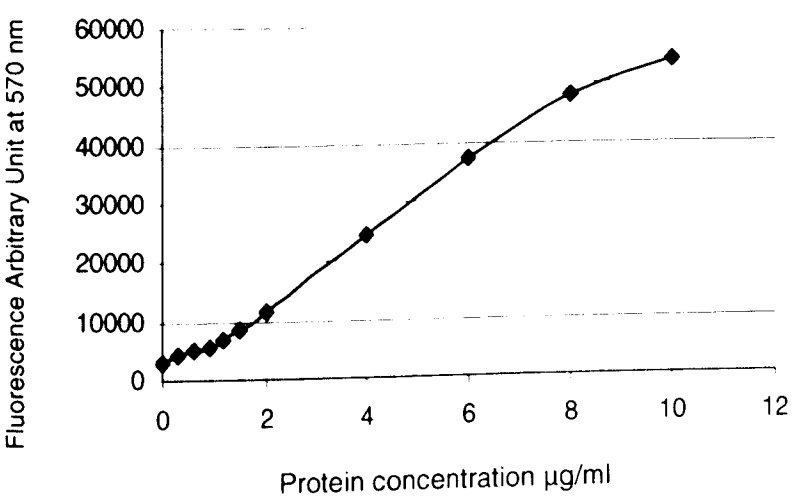

Fig. 2.1 BSA standard curve produced by the quantitative NanoOrange protein assay
ESP samples were diluted in triplicate 1:10, 1:50 and 1:100 in NanoOrange working solution and the mean fluorescence value was then obtained. The protein concentration of the ESP sample was then estimated from the BSA standard curve, as shown in the line graph.

2.4 Haemolymph extraction from *B. glabrata* and haemocyte treatments

Adult *B. glabrata* (approximately 1-1.5 cm in diameter) were washed with distilled water before haemolymph extraction using the head-foot retraction process (Sminia, 1972). Haemolymph from individual snails (of the same strain) was pooled together and diluted in CBSS while on ice (1 part haemolymph: 2 part CBSS). A monolayer of haemocytes (using either an equal volume of haemolymph or an equal number of haemocytes) was then created by allowing cells to adhere to individual wells of a 48 well culture plate (Costar, Corning, NY) for 30 min at RT. Cell numbers were estimated using trypan blue stain (Sigma) and disposable haemocytometers (Immune systems Ltd., Paignton, UK). Next, the haemocyte monlayers were washed three times with 250 µl CBSS before being exposed to either: *S. mansoni* ESPs, a mitogen-activated protein kinase (MAPK) kinase (MEK) inhibitor U0126 (New England BioLabs, Hitchin, UK), a proteasome inhibitor [carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG132) Merck Chemicals, Nottinghamshire, UK], a control containing vehicle 0.1% dimethyl sulfoxide (DMSO; Sigma), or CBSS only. The reagents were used at various concentrations for varying durations. Subsequently, medium was removed from each of the wells and 30 µl boiling 1x SDS-PAGE sample buffer [sample buffer: 64.2 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 2% (v/v) methanol, 5% (v/v) glycerol and 0.003% (v/v) bromophenol blue, chemicals were obtained from Sigma, while methanol was purchased from Fisher] was added to the cell monolayers to solubilise haemocyte proteins. Next, the samples were heated to 95°C for 1 min, and sonicated for 1-2 min. Finally, 1 µl of phosphatase inhibitor cocktail and proteinase inhibitor cocktail (both from Sigma) were added to the cooled mixture, to prevent phosphataes and proteinases from breaking down the phosphorylation and protein of interest. The samples were stored at -20°C overnight or electrophoresed immediately.

2.5 SDS-PAGE and western blotting of haemocyte protein

Protein samples and molecular weight markers (SDS-6H, Sigma) were loaded onto discontinuous SDS-PAGE gels. Resolving gels contained 10% acrylamide [375 mM Tris-HCl (pH 8.8), 30% (v/v) Protogel (National Diagnostics), 0.1% (w/v) sodium dodecyl sulphate (SDS), 0.25% (v/v) TEMED, 0.3% (w/v) ammonium persulfate (APS), reagents purchased from Sigma]. Stacking gels contained 21% acrylamide [125 mM Tris-HCl (pH 6.8), 6% (v/v) Protogel (from 30% stock), 0.1% (w/v) SDS, 0.25% (v/v) TEMED, 0.3% (w/v) APS]. Electrophoresis was carried out at 100-150 V constant for about 1-2 h (or until the bromophenol blue tracking dye had reached the base of the gels) in the presence of running buffer [0.3% (w/v) Tris, 1.45% (w/v) glycine, and 0.1% (w/v) SDS]. Gels were removed and placed in electrotransfer buffer [0.3% (w/v) Tris, 1.5% (w/v) glycine, and 20% (v/v) methanol] together with sized filter papers and Hybond nitrocellulose

membranes (0.45 µm pore size; GE Healthcare, Amersham, UK). Gels, filter papers and nitrocellulose membrane were then assembled and placed in a Bio-Rad semi-dry transfer apparatus and electrotransfer performed at 15 V at 300 mA for 90 min. Successful transfer of protein was confirmed by staining the membranes with Ponceau S (Sigma); the position of the molecular weight markers was marked with ink and the membranes washed with Tris-buffered saline [TBS; 150 mM Tris-HCl (pH 8), 1.5 M NaCl] containing 0.1% (v/v) Tween 20 (Sigma) to remove the Ponceau S stain. Membranes were then blocked for 1-2 h at RT with 5% (w/v) non-fat dried milk powder in TBS Tween. The membranes were incubated with primary antibodies diluted in TBS Tween and supplemented with 0.1% (w/v) bovine serum albumin (BSA; Sigma) at 4°C for 15 h then at RT for a further 2 h.

Table 2.2 summarises the antibodies and the dilutions used for the antibodies in the different experiments. Next, membranes were washed three times in TBS Tween prior to incubation with anti-rabbit (or anti-mouse) horseradish peroxidase (HRP)-conjugated secondary antibodies (Table 2.2) for at least 2 h at RT. Each membrane was again washed three times in TBS Tween before the signal was developed using SuperSignal West Pico chemiluminescent substrate (PerBioscience, Tatenhall, UK) and chemiluminescence hyperfilm (GE Healthcare) for 1-5 min depending on the intensity of the light signal. Following the purchase of GeneGnome Bio Imaging System (SynGene, Milton Keynes, UK), luminescent images were captured using Syngene software.

The membrane was usually stripped of antibodies, using Restore western blot stripping buffer (PerBioscience) for 3 h at RT and re-probed with anti-actin antibodies (Sigma). Stripping buffer removed both the secondary and primary antibodies (no signal was detected following subsequent exposure to chemiluminescent substrate). The intensity of the signal on individual blots was analysed using Kodak ID image analysis software or SynGene GeneTools software using the Bio Imaging System (similar results were obtained from the two separate software programmes). The data provided a semi-quantitative method of protein quantification and of phosphorylation of specific proteins.

Antibody	Dilution	Company purchased from
Anti-phosho-p44/42 MAPK (Thr202/Try204)	1:1000	Cell Signalling Technology
Anti-HSP70/HSC70 (BRM-22)	1:500	Santa Cruz (Heidelberg, Germany)
Anti-phosphor-MEK1/2 (Ser217/221)	1:2000	Cell Signalling Technology
Anti-actin	1:2500	Sigma
Mouse anti-rabbit secondary conjugated HRP	1:2500	Sigma
Goat anti-mouse secondary conjugated HRP	1:1000	Cell Signalling Technology

Table 2.2 Summary of antibodies and their dilutions used for western blotting.

2.6 Measuring nitric oxide (NO) production in *B. glabrata* haemocytes

Haemolymph was extracted from adult *B. glabrata* and diluted in CBSS (as described in section 2.4). Either 100 µl of diluted haemolymph or approximately $3x10^3$ cells per well from each snail strain were used to create haemocyte monolayers in individual wells of 96 well culture plates (Corning Costar). Haemocytes were left to adhere to the wells for 30 min at RT, the haemolymph was then removed and cell monolayers were washed twice with 250 µl CBSS. A fluorescent dye, 4-Amino-5-methylamino-2',7'-difluorofluorescein (DAF-FM) diacetate (Molecular Probes, Leiden, Netherlands) which is highly sensitive to intracellular NO (Fig. 2.2) was dissolved in DMSO and added to the cell monolayer at 5 µM [final DMSO concentration 0.1% (v/v)] for 1 h in the dark at RT. Control wells contained 0.1 % (v/v) DMSO alone. Next, cells were washed twice with 100 µl CBSS before being exposed to either: 10 µM phorbol myristate acetate (PMA; Cell signalling Technology), 10 µg/ml *Escherichia coli* lipopolysaccharides (LPS), 10 µg/ml zymosan A (from *Saccharomyces cerevisiae*), 10 mg/ml of the β-1,3-glucan laminarin (Sigma) or *S. mansoni* ESPs (at different concentrations). The extracellular signal-regulated kinase (ERK) inhibitor, U0126 (New England BioLabs) was dissolved in DMSO and used at a final concentration of 1 µM or 10 µM in CBSS for some experiments [final DMSO concentration 0.1% (v/v)]. Cell monolayers were incubated with U0126 for 20 min after removal of DAF-FM diacetate (as described above), but prior to ESP (20 µg/ml) challenge. The fluorescent signal was then measured in a Fluorstar Optima microplate reader at 485 nm excitation and 520 nm emission every 5 min.

Each assay was conducted on one day in triplicate or quadruplicate using the same pool of haemolymph and the same batch of ESPs. Assays were then repeated on different days using different pooled haemolymph samples to ensure consistency. The relative fluorescence for each sample over time was calculated by dividing the fluorescence values with background fluorescence (cells only with no DAF-FM diacetate). The data on different days was not combined for analysis as background fluorescence varied in each assay; for each experiment one typical graph was shown together with the statistical analysis.

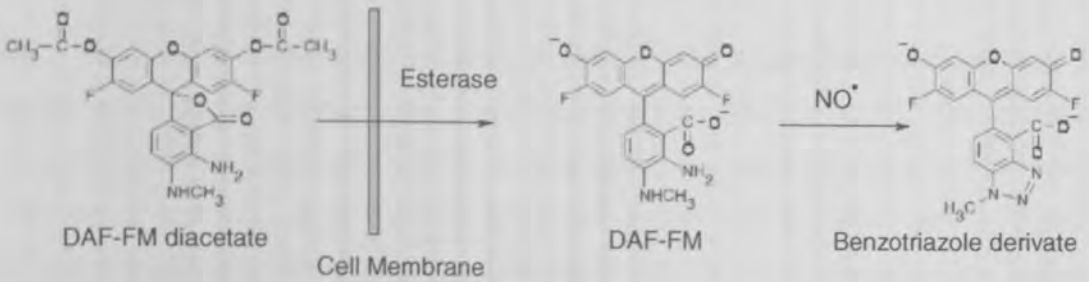

Fig. 2.2 Illustration of DAF-FM diacetate properties
DAF-FM diacetate is a non-fluorescent molecule and can passively diffuse across cell membranes where it is broken down by cellular esterases to become DAF-FM. This is a relatively weak fluorescent molecule but its fluorescence increases significantly after reacting with NO by approximately 160-fold (Molecular Probes, Nitric Oxide indicators product sheet 2001).

2.7 Measuring the viability of haemocytes over time

Haemocytes in the presence or absence of 20 µg/ml ESPs were assessed for their viability after 3–5 h *in vitro* culture in 96 well plates. Percentage cell death was calculated based on the uptake of 0.2% trypan blue (Sigma). A commercially available CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Southampton, UK) was also used to monitor cell viability; its chemical properties are outlined in Figure 2.3. This product has previously been used to examine the effects of molluscan haemolymph following bacterial challenge (Hooper *et al.*, 2007). Cell monolayers were incubated in either 100 µl CBSS, or 100 µl ESP (20 µg/ml) in CBSS; subsequently 20 µl of CellTiter 96 AQueous One Solution reagent was pipetted into each well and left for 1 h before the absorbance (at 490 nm) was measured over 5 h.

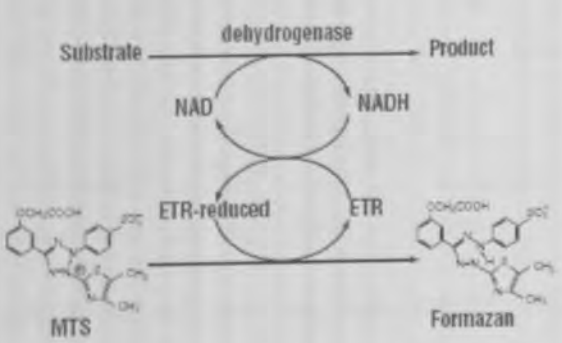

Fig. 2.3 Properties of CellTiter 96 AQueous One Solution Cell Proliferation Assay
MTS tetrazolium is bioreduced into formazan by NADH and NADPH *via* dehydrogenase enzymes that are present in viable cells only (Promega technical bulletin 2007).

2.8 Immunocytochemistry and confocal microscopy

Cells in the haemolymph (100 µl diluted in CBBS) from susceptible and resistant adult *B. glabrata* were left to adhere onto glass coverslips for 30 min at RT. The adhered cells were washed three times with CBSS, before being exposed to 20 µg/ml ESPs for 1 h at RT. Haemocytes were then washed once with phosphate buffered saline (PBS; Oxoid, UK) and immediately fixed and permeabilised with fixing/permeabilisation buffer [3.7% (v/v) formaldehyde (Sigma) and 0.18% (v/v) Triton X-100 (Sigma) in 10ml of PBS] for 12 min. Next, the cell monolayers were washed once with PBS, before being blocked in 1% BSA in PBS (blocking buffer) for a further 12 min at RT. Cells were then incubated in primary antibody diluted in blocking buffer for at least 3 h, followed by a further incubation for 30-90 min with a FITC-conjugated secondary antibody (the dilutions and the manufacturers of the antibodies are stated in Table 2.3). Cell monolayers were finally incubated with rhodamine phalloidin (0.1 µg/ml in blocking buffer for 15 min; Sigma) in order to visualise F-actin, a component of the cell cytoskeleton. In some experiments the cells were also incubated in 4',6-diamidino-2-phenylindole (DAPI, 0.1 µg/ml in blocking buffer for 5 min; Merck Chemicals, Nottingham, UK) a fluorescent dye that strongly binds to DNA. Three washes in PBS were carried out between incubations and all incubation steps were carried out at RT in humidified chambers. Coverslips were subsequently mounted onto the slides using Vectorshield anti-bleaching medium (Vector Laboratories) and sealed with clear nail varnish. The haemocytes were visualised using a Leica TCS SP2 AOBS laser scanning confocal microscope, using 63x immersion objective; the laser settings were kept constant for all observations. The images were captured using Leica Confocal software and were displayed in average pixel brightness mode.

Antibody	Dilution	Company purchased from
Anti-phosho-p44/42MAPK (Thr202/Try204)	1:100	Cell Signalling Technology
Anti-HSP70/HSC70 (BRM-22)	1:100	Santa Cruz (Heidelberg, Germany)
Rhodamine phalloidin	0.1 µg/ml	Sigma
DAPI	0.1 µg/ml	Merck
FITC Goat anti-Rabbit secondary Alexa Fluor 488	1:500	Invitogen (Paisley, UK)
FITC Goat anti-Mouse secondary Alexa Fluor 488	1:200	Sigma

Table 2.3 Summary of antibodies and other reagents and their dilutions for immunocytochemistry

2.9 Visualising *B. glabrata* haemocytes using a scanning electron microscope

Cell monolayers were created (using a total volume of 150 µl haemolymph diluted in CBBS) on glass coverslips by allowing pooled haemocytes from susceptible or resistant snails to adhere to the glass for 30 min at RT in a humidified chamber. Cell monolayers

were washed three times before challenge with ESPs (20 µg/ml) or CBSS alone for 1 h at RT; cells were then immediately fixed in 2.5 % (w/v) glutaraldehyde (Sigma) in 0.1 M phosphate buffer (pH 7.4) for 2 h at RT. Cells were then washed four times in 0.1 M Hepes buffer with 0.3 M sucrose (pH 7.2) and dehydrated through a graded series of ethanol; 50%, 70%, 80%, 90%, 95% for 20 min each, before being washed three times in 100% ethanol (protocol adapted from Aladaileh *et al.*, 2007). The samples were then left in hexamethyldisilazane (HMDS) for 1 h at RT and left to air dry overnight. The coverslips were finally mounted onto aluminium stubs and coated with gold, using a sputter coating machine (Polaron SC7640 coater). Samples were stored in a dry place before scanning. Haemocytes were visualised using a scanning electron microscope (Zeiss EVO 50), with the following parameters: 10 Kv beam intensity and approximately 100 pA spot size. Images of haemocytes were taken using SMARTSEM software. The relative cell surface area in contact with the glass coverslip (or the cell 'spread') was analysed using Microsoft Visual Basic (developed at Kingston University, IT department); the programme was calibrated using scale bars on each individual image.

2.10 HSP70 sandwich enzyme-linked immunosorbent assay (ELISA)

An HSP70 ELISA kit (Assay Designs, Stressgen, Michigan, USA) was used in an attempt to detect and quantify Heat Shock Protein 70 (HSP70) in haemocytes and in the cell medium. Briefly, CBSS-diluted haemolymph (from susceptible and resistant snails) was left to adhere to individual wells of a 48 well culture plate (Corning) for 30 min at RT. Monolayers were subsequently washed three times with CBSS before being challenged with 20 µg/ml ESPs or CBSS alone for 1 h at RT. Next, the medium was removed and the cells were lysed with 1x extraction buffer (reagents provided in the kit) containing protease inhibitors (while 5x extraction buffer was added to the medium); the samples were then sonicated for 1-2 min and centrifuged at 21,000 *g* for 10 min at 4°C. Supernatants were removed to fresh tubes and diluted in dilutent 2 (1 part lysate: 4 parts dilutent 2 solution); 100 µl (per well) of the diluted cell lysate or the cell medium was transferred to the ELISA plate and left for 2 h at RT. Wells were washed four times in 1x wash buffer before 100 µl of HSP70 antibody (provided in the kit) was added to each well for 1 h, followed by further four washes with 1x wash buffer before 100 µl HSP70 horseradish peroxidise conjugated anti-rabbit IgG was added for 1h. Finally, ELISA wells were given one more wash with 1x wash buffer before 100 µl stabilized tetramethylbenzidine (TMB) substrate was added to each well for 30 min in the dark. The reaction was stopped with 100 µl of stop solution 2 and the absorbance reading was measured at 450 nm using a microtitre plate reader in absorbance mode (FluorStar Optima, BMG Labtech).

2.11 Treatment of *B. glabrata* haemocytes and RNA extraction

Haemolymph from 35-40 adult susceptible or resistant snails was pooled and kept on ice in CBSS. A haemocyte monolayer was created by allowing cells to adhere to individual wells of a 12 well cell culture plate (Corning) for 30 min at RT (approximately 300 µl of diluted haemolymph per well). The monolayer was subsequently washed three times with 500 µl CBSS to remove the haemolymph and haemocytes were then challenged with 20 µg/ml ESPs or CBSS alone. After 1 h ESPs were removed from the monolayer and the RNA was extracted from the haemocytes using 1 ml of Trizol reagent (Invitogen, Paisley, UK) for 2-3 min. The mixture was then transferred to a 1.5 ml microcentrifuge tube and centrifuged for 10 min at 12,000 *g*. The supernatant was transferred to a clean tube and left to settle at RT for 5 min; 0.2 ml of chloroform (Sigma) was subsequently added and mixed vigorously before being left at RT for a further 2 min. Next the samples were centrifuged at 12,000 *g* for 15 min at 4°C. Only the aqueous upper phase was transferred to a new microcentrifuge tube and 0.5 ml of isopropanol (Sigma) was added and mixed by inversion; this was left at RT for 10 min, before being centrifuged at 12,000 *g* for 10 min at 4°C. The RNA could be seen as a pellet at the bottom of the tube. The supernatant was carefully discarded and the pellet was washed once with 0.25 ml 75% ethanol (Sigma), vortexed and centrifuged at 7,500 *g* for 5 min at 4°C. Finally, the ethanol was discarded and the RNA pellet was air dried. The pellet was re-suspended in 5-10 µl sterile diethylpyrocarbonate (DEPC)-treated water (Invitrogen, Paisley, UK) and left at RT until the RNA pellet had dissolved. Finally, the RNA samples were stored at -80°C in 100% ethanol until required.

The RNA extracts were DNAse treated prior to storage, to remove any DNA in the sample. The RNA sample was re-suspended in DNAse buffer (Promega), DNAse enzyme (1 U/µl; Promega) and sterile DEPC-treated water (Invitrogen) and incubated at 37°C for 1 h. Next, 100 µl phenol: chloroform: isoamyl alcohol (IAA; Sigma) was added to each sample and centrifuged for 15 min at 12,000 *g*. The supernatant was removed and the RNA was precipitated by adding three times the volume of ethanol and leaving the sample overnight at -80°C. Next, the DNA-free RNA was spun down at 13,000 *g* at 4°C for 30 min. The RNA sample could be seen as pellet at the bottom of the microcentrifuge tube. The RNA pellet was washed in 80% ethanol and left to air dry before being re-suspended in DNAse-free water (Invitogen). The amount of total RNA as well as its purity was estimated using a Nanodrop spectrophotometer (NanoDrop Technologies).

2.12 cDNA synthesis from *B. glabrata* haemocytes and DNA amplification

The RNA obtained from haemocytes extracted from susceptible and resistant snail strains was used to create first strand cDNA using the SMART PCR cDNA synthesis kit (Clontech-Takara, Saint-Germain-en-Laye, France). The kit produces high-quality cDNA from a small yield of total RNA (Fig. 2.4). The protocol was based on the manufacturer's guidelines; for each reaction 1 µg of total RNA, 1 µl 3' SMART CDS Primer IIA (sequence: AAGCAGTGGTATCAACGCAGAGTACT) at 10 µM and 1 µl of SMART IIA Oligonucleotide 5' (sequence: AAGCAGTGGTATCAACGCAGAGTACGCGGG) at 10 µM in a final volume of 5 µl in DNAse free water was incubated at 72°C for 2 min and cooled on ice for a further 2 min. Finally, 2 µl of first-strand buffer, 1 µl DTT (20 mM), 1 µl 50x dNTP (10 mM) and 1 µl PowerScript reverse transcript was added to the cooled sample and incubated at 42°C for 1 h in a PCR machine.

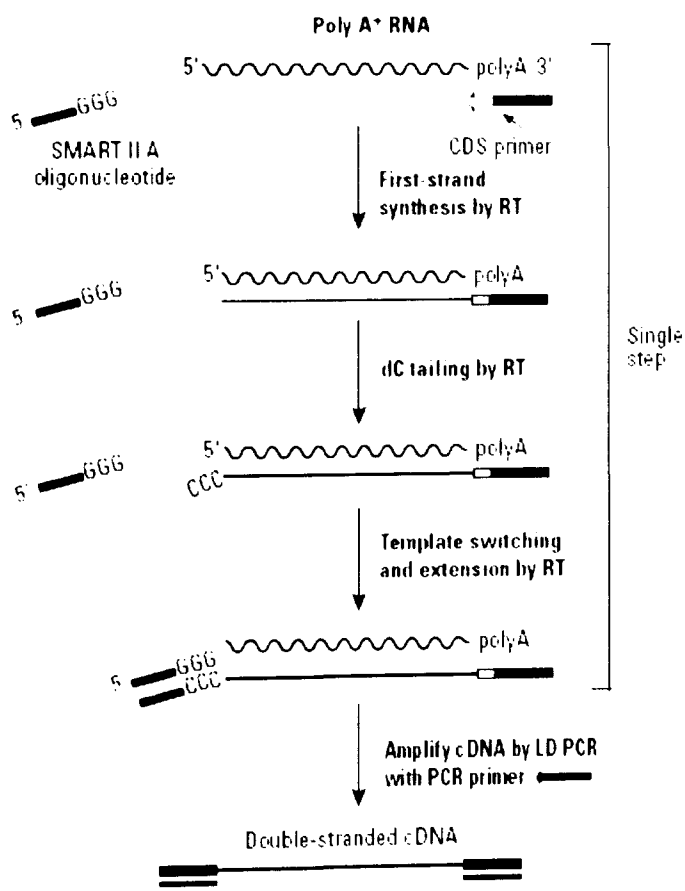

Fig. 2.4 Flow chart showing how a SMART PCR cDNA synthesis kit produces high-quality cDNA from total RNA
The oligo(dT) primer (SMART CDS primer II A) primes the first strand synthesis reaction at the 5 prime end of the RNA, the enzyme also adds a few additional nucleotides. The presence of an oligonucleotide (SMART II A Oligonucleotide) forms base-pairs with the additional nucleotide deoxycytidine sequence. This allows the reverse transcript enzyme to use the new template containing the additional nucleotides to produce a full length single stranded cDNA (image taken from Colontech SMART PCR cDNA synthesis kit user manual, 2007).

The cDNA was amplified using the same SMART PCR cDNA kit (Clontech). To the first-strand (2 µl) cDNA sample the following was added: 98 µl of master mix, 80 µl dH_2O, 10 µl

(10x) Advantage 2 PCR buffer, 2 µl (50x) dNTP (10 mM), 4 µl 5' PCR Primer IIA and 2 µl Advantage polymerase mix. The cDNA was subsequently amplified using the following themocycles: 95°C for 1 min, 95°C for 5 s, 65°C for 5 s and 68°C for 6 min for 16 cycles (16 cycles were recommended for 1 µg of RNA input, using the manufacturer's guidelines). A sample of the amplified double stranded cDNA products was examined by 1% agrose gel electrophoresis. The gel contained: high grade agrose (Sigma) dissolved in Tris-acetate-EDTA buffer [TAE buffer: 0.5 M EDTA pH 8.0, glacial acetic acid, Tris base (reagents from Sigma) and distilled H_2O_2] and 1 µl ethidium bromide (Sigma). Electrophoresis was carried out for 30 min at RT at 50-60 V constant in the presence of TAE buffer. The gel visualization demonstrated there was no contamination present in the PCR and the smear of PCR products, indicated the cDNA library had amplified (Fig. 2.5). The final amplified cDNA samples were stored at -20°C prior to the labelling reaction.

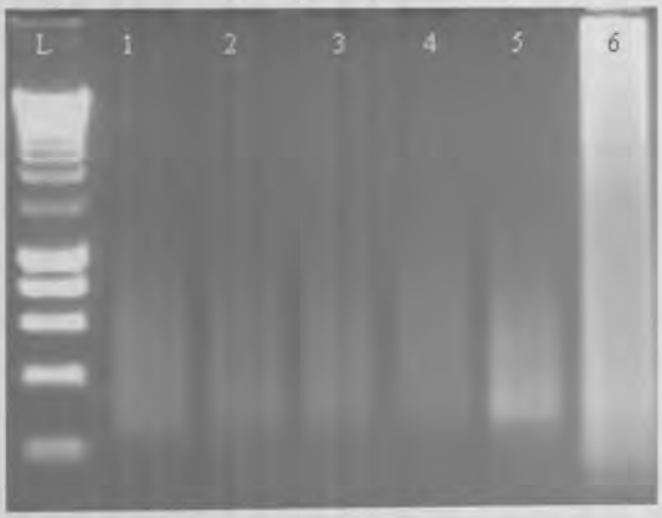

Fig. 2.5 Electrophoresis gel showing the amplified cDNA libraries for each biological sample The first three lanes (1-3) are cDNA products from haemocytes extracted from susceptible snails that had been challenged with 20 µg/ml ESP for 1 h, while the last lanes (4-6) are cDNA products from resistant snails that had been challenged with 20 µg/ml ESP. The letter L represents a 1 kb DNA ladder. More cDNA products were present in lanes 5 and 6 compared to the other lanes, therefore, less cDNA for samples 5 and 6 was used in the subsequent labelling reaction.

2.13 Labelling cDNA and the purification of labelled probes

The labelling reaction was carried out using a BioPrime DNA labelling system (Invitogen) and Cy fluorescent dyes (GE Healthcare) using the manufacturer's specification. Two different fluorophores (Cy3-dCTP and Cy5-dCTP) were used to label the amplified cDNA samples. The ratio of the fluorescence intensities between the two fluorophores provided a relative measure of the differences in gene copy number. The BioPrime DNA labelling system contains random primers that anneal to the denatured DNA template and are extended by Klenow fragment in the presence of a fluorescently labelled dCTP. The Klenow fragment is a mutated fragment of DNA polymerases (synthesises complementary

strands during DNA replication). The Klenow fragment has the polymerase activity but lacks exonuclease activity (the removal of single nucleotides from the end of a DNA or RNA chain) unlike the full length DNA polymerases enzyme.

Double-stranded DNA (15-21 µl) and 20 µl (2.5x) random primer reaction buffer (BioPrime DNA labelling Kit, Invitogen) was incubated at 95°C for 5 min and then cooled on ice. Next, the samples were labelled with 1 µl of Cy3-dCTP (2 resistant and 2 susceptible biological samples) and Cy5d-CTP (2 resistant and 2 susceptible biological samples). These labelling dyes were chosen due to their high sensitivity and their discrete spectral separation. Briefly, 5 µl of (10x) low-C dNTP mix, 1 µl of Cy3-dCTP or Cy5-dCTP (all purchased from GE Healthcare), 2 µl ultrapure water (Invitrogen) and 1 µl Klenow fragment (Invitrogen) were added to the pre-chilled DNA mixture. This mix was incubated at 37°C for 2 h and stopped by adding 5 µl stop buffer (GE Healthcare).

The labelled cDNA was purified using Auto-Seq G-50 columns (GE Healthcare) to ensure the removal of unincorporated fluorescent dye from the cycle reaction. The Auto-Seq G-50 column removes unbound dye from sequencing reactions by gel filtration, whereby small molecules that are likely to be impurities are retained in the gel matrix. Firstly, the Auto-Seq G-50 column was prepared by spinning the column for 1 min at 2,000 *g* then 20 µl of the labelled cDNA was pipetted onto the gel and spun again for 1 min at 2,000 *g*. The eluted solution was retained for further analysis. The amount of dye incorporated into the cDNA for each sample was quantified spectrophotometrically using a NanoDrop and the average incorporation efficiency for the samples was measured; values for individual samples are presented in figure 2.6. Purified Cy3-dCTP and Cy5-dCTP labelled products were pooled together. Finally, 10 µg of mouse Cot-1 DNA, which blocks repetitive DNA sequences (Invitrogen) and prevents non-specific hybridisation in microarray experiments, 3 M NaAc (pH 5.2) and 2.5x total volume of 100% ethanol was added to the labelled samples. The mixture was centrifuged for 10 min and the ethanol removed, the pellet was washed three times in 70% ethanol, air dried in the dark and was stored at -20°C.

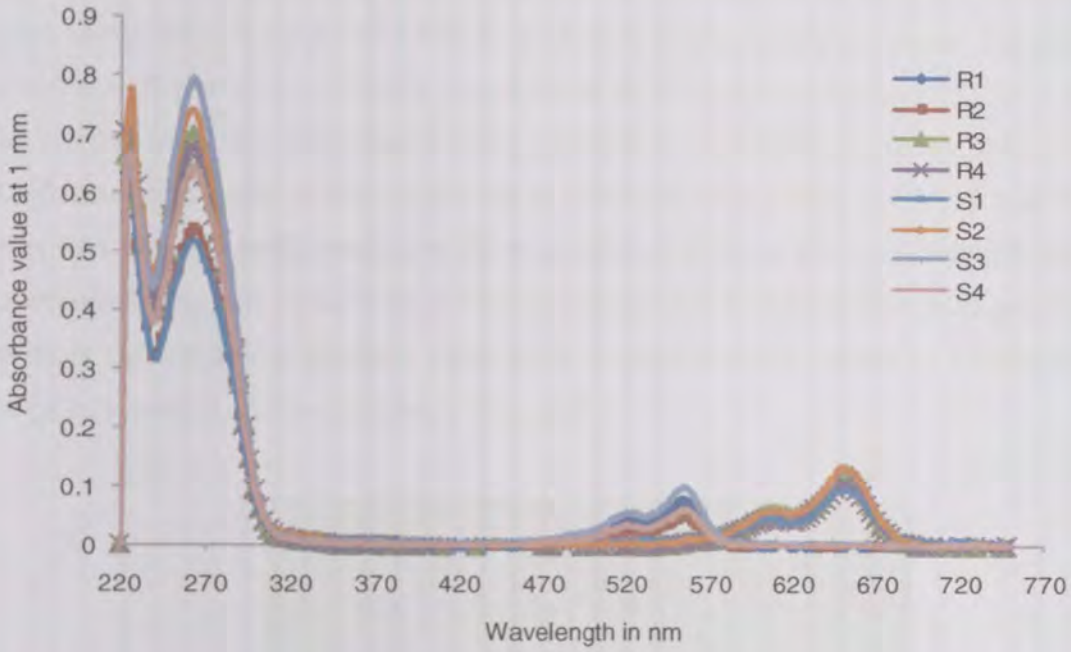

Fig. 2.6 The amount of fluorescent dye incorporated into each cDNA sample
The first large peak at 265 nm (optimum absorption wavelength of DNA) represents the total amount of cDNA per sample. The second peak of 550-570 nm corresponds to the absorption spectrum of Cy3 fluorescent dye, while the third peak of 650-670 nm corresponds to the absorption spectrum of Cy5 fluorescent dye. The cDNA samples (R1-R4: resistant and S1-S4: susceptible) had similar dye incorporation efficiency of 14.11 pmol/pg.

2.14 Microarray hybridisation

The cDNA microarray slides were printed by Cambridge University, Cambridge, UK. The array contains *B. glabrata* cDNA clones obtained from suppression subtractive hybridization (SSH) techniques and open reading frame expressed sequence tags (ORESTES) (Lockyer *et al.*, 2006; Bouchut *et al.*, 2007; Lockyer *et al.*, 2007; Lockyer *et al.*, 2007; Hanelt *et al.*, 2008). Each slide contained 5000+ cDNA clones spotted in duplicate, including control spots of yeast tRNA, *B. glabrata* genomic DNA (from the same snail strains used in the experiments), ribosomal 18S and cytochrome oxidase I genes, amplified from *S. mansoni*, and blank spots. The hybridisation protocol was adapted from Lockyer *et al.*, 2008. Firstly, the microarray slides were prepared by pipetting 37 µl of hybridization buffer [40% deionised formamide (v/v), 5x Denhardt's solution (Invitrogen), 5x sodium chloride-sodium citrate (SSC) buffer, 1 mM sodium pyrophosphate, 50 mM Tris-HCl (pH7.4), 0.1% (w/v) SDS and molecular grade water, (chemicals were purchased from VWR, Leicestershire, UK)] between the lifterslip and the microarray slide. The microarray was then incubated at 50°C for 1 h and subsequently washed for 5 min in 2x SSC and in DNA-free water before being dipped in isopropanol (Sigma) and air dried for approximately 15-30 min.

The labelled cDNA samples were prepared by re-suspending the DNA in hybridisation buffer along with: 1 µg/µl Cot DNA, 8 µg/µl Poly A and 4 µg/µl of yeast, *Saccharomyces cerevisiae* (Sigma), before being incubated at 95°C for 5 min, followed by 50°C for 5 min. The mixture was then centrifuged and pipetted between the lifter slip and microarray. The cDNA was left to bind to the microarray in a humidified chamber for 16-18 h at 50°C. The array was subsequently washed at RT in 2x SSC, then 0.1x SSC containing 0.1% SDS for 5 min and finally in 0.1x SSC for 5 min. The array was centrifuged at 1000 *g* and stored in the dark before being scanned (discussed in section 2.15). Below is a typical scanned image obtained from the microarray (Fig. 2.7).

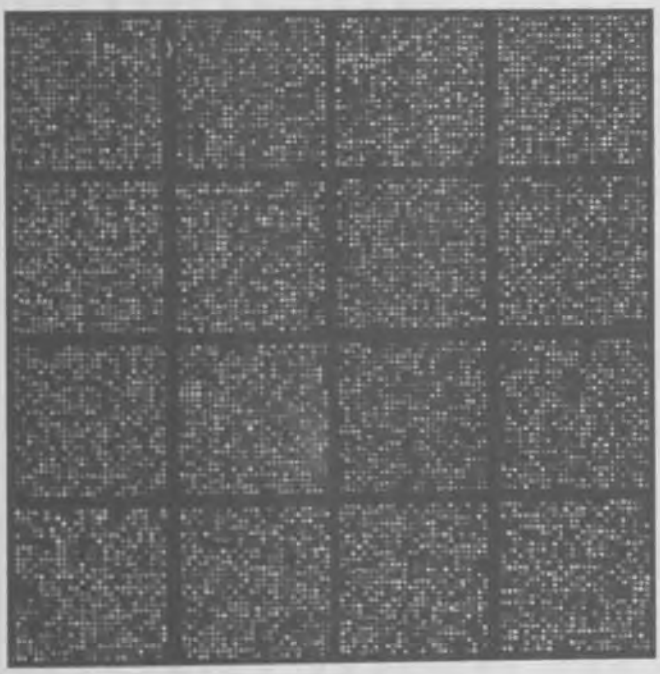

Fig. 2.7 Scanned image of a microarray
Each spot on the array represents the expression level of a specific gene; a total of 5000+ cDNA clones in duplicate was present on the *B. glabrata* array. A red spot represents an increased gene expression in the susceptible sample, a green spot represents an increased gene expression in resistant sample, a black spot means that the gene was not expressed in either sample, while a yellow spot indicates equal expression of the gene and a white spot indicates over exposure of fluorescent signal. The image is representative of four independent arrays.

2.15 Microarray analysis

The microarray slides were scanned using an Axon GenePix 4100A with a photomultiplier tube (PMT) range of 532-635 and a signal intensity ratio of one. Spot intensities were captured using GenePix Pro 6.1 image analysis software with saturation tolerance of 0.005%. For each microarray, individual spots were assessed as being 'good' for further analysis, or 'bad' and rejected. This was based on spot position, fluorescence intensity of the spot (above or below detection), or the presence of foreign particulates on the spot. Criteria were then used to remove spots of incorrect position or spots of incorrect size

(minimum surface area of spot was set as 60%). The average fluorescence intensity minus the background was obtained for each spot on the microarray.

In order to make the data comparable between the independent arrays and to correct for systematic technical biases; the data were normalised using Acuity 4.0 software. This was achieved by calculating the expression ratios (the log ratio of the fluorescent dyes). The median value for the data were normalised so all the arrays had the same median value; this was achieved by multiplying each data point with a normalisation factor (Equation 2.1). Next, a locally weighted scatter-plot smoothing (Loess) normalisation method was applied to the data sets. This method relies on two main assumptions; one that the proportion of differentially expressed genes are small and two, that there is symmetry in the expression values between up-regulated and down-regulated genes (Smyth and Speed, 2003). The Loess normalisation involves creating a Loess curve, calculated by linear regression from MA plots (MA represent mathematical equations; MA plots show the intensity-dependent ratio of the array data) and then subtracting individual data points from this curve to produce a linear set of results (Fig. 2.8). The Loess normalisation corrects for dye bias and the correction is intensity dependent (Yang *et al.*, 2002b).

$$N_{total} = \frac{\sum_{i=1}^{N_{array}} R_i}{\sum_{i=1}^{N_{array}} G_i}$$

Equation 2.1 used to normalise the median values for each microarray data
Where N(total) is the normalisation factor, R and G are the two fluorescent channels, *i* represents the element or gene of array and N(array) is the total genes on the array.

From the normalised data, differentially expressed genes (DEGs, the difference in gene expression between susceptible and a resistant ESP-exposed haemocytes) were evaluated. Values that were negative were classified as being differentially expressed in resistant snail samples, while positive values were differentially expressed in susceptible snail samples. Gene sequences that displayed significant differential expression ($P \leq 0.05$) in 3 out of the 4 microarrays were investigated further by using the following software programmes: SeqTools which was used for cluster analysis, Goblet which was used to analyse gene ontology functions based on BLASTX homologies (threshold of E value>0.5) and Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway analysis which was used for orthology assignment and pathway mapping.

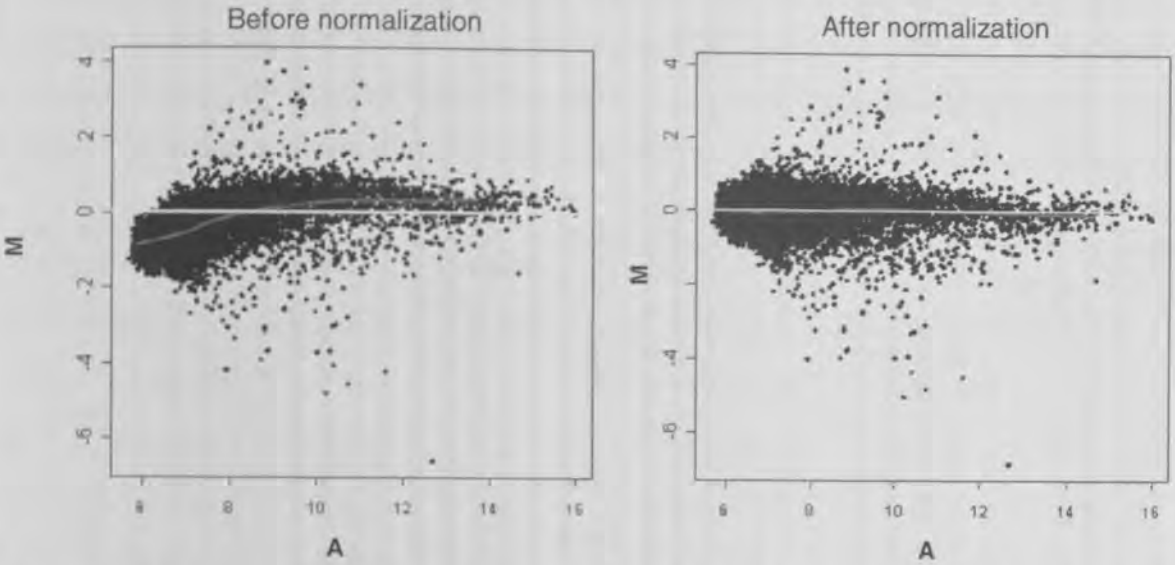

Fig. 2.8 MA plots showing microarray data before and after Loess normalisation
The red line indicates the Loess curve before and after normalisation; it can be seen that the data is less skewed once it has been normalised (adapted from http://www.ucl.ac.uk/oncology/MicroCore/HTML_resource/tut_frameset.htm).

2.16 Quantitative polymerase chain reaction (qPCR)

The mRNA from susceptible or resistant pooled haemocytes that had been either exposed or not exposed to 20 µg/ml ESPs was extracted using Trizol (as detailed in section 2.11). The quality and quantity of the RNA samples was assessed using a Nanodrop spectrophotometer. The mRNA was subsequently reverse transcribed into cDNA using an Advantage RT-for-PCR kit (Clontech-Takara, Saint-Germain-en-Laye, France), following the manufacturer's guidelines. 1 µg total RNA and 1 µl oligo(dT) primers were incubated at 70°C for 2 min, then cooled on ice. To the cooled solution, the following were added: 4 µl of 5x reaction buffer, 1 µl dNTP mix, 0.5 µl recombinant RNA inhibitor and 1 µl of Moloney Murine Leukemia Virus (MMLV) reverse transcriptase; these were left to incubate at 42°C for 1 h, followed by 94°C for 5 min to stop further synthesis and to destroy any DNase activity. The cDNA was diluted in sterile DEPC-treated water (Invitogen) to a final volume of 12.5 µl and stored at 4°C prior to further use.

The DNA-binding dye SYBR Green I was used in the qPCR reactions (GE Healthcare). This dye binds non-specifically to double-stranded DNA, and once bound its fluorescence intensity increases significantly. The qPCR reactions consisted of 10 pmol of specific sense and anti-sense primers (Table 2.4). These primers were designed using Primer Express software with the following criteria: melting temperature of 58-60°C, cytosine or guanine at 3' prime end, 18-20 base pair long, maximum 3 base pair repeats, cytosine and guanine content at 20-80% and no primer-dimer formation. The reaction also consisted of 1 µl of diluted cDNA and 1x Power SYBR Green Master Mix (Applied

Biosystems). The PCR cycling conditions were: 50°C for 2 min, 94°C for 10 min, then 35 cycles of 94°C for 30 s, 58°C for 30 s and 60°C for 1 min. The PCR products were detected using MiniOpticon Real-Time PCR detection system (Bio-Rad) and Ct values (or the threshold cycle) were determined using MiniOpticon Monitor software (BioRad).

DEG in R/S	Gene Bank Number	Function of Gene	DF value	Primer Sense	Primer Anti- sense	Efficiency (%)
R	N/A	NF-KB1*	-2.46	ACCAACATAGACAA GGCAGTAAA	ATCTCTGTTTTGTGGA AAGTAAGTG	60
R	CK656737	HSP70*	-2.66	GTATGGTGTCCGAT GCTGAG	AGCAGTAAGATTCAA GGGAGTTC	82
R	EW997087	Unknown	-5.27	AACCGACTACTTGA AGCGCC	GTCGCAGATCGAGAC GGG	115
R	CV548474	Unknown	-4.91	CCCTGCCAAACATC GAGTTC	CAAGCCTCACGATGT CTCTGAG	60
S	DW474845	HRAS-like suppressor-3	2.58	ACACTGAGGGTTG GTGACCG	CCCGAAACCAGCGTA AATTC	105
S	CN445888	Cathepsin D	2.28	TGTGGAGATGACG GATGTAAAGG	GCAGTCGGGTAAAAT ATACACTCC	97
C	CO870340	Unknown	0.206	ATGCAGGCGTGTC ACGAAG	GCCTTTCGCTCCAGT TCCA	83

Table 2.4 Differentially expressed genes (DEGs) chosen for qPCR analysis
Four resistant DEGs, two susceptible DEGs and one control gene (that showed no change in gene expression after ESP-challenge) were chosen for qPCR analysis. The primers were designed using Primer Express software and purchased from Sigma. R=differentially expressed gene in resistant samples, S= differentially expressed gene in susceptible samples, C= control (no change in gene expression) DF= differential value from microarray data. * Primers designed and purchased from PrimerDesign Ltd, Southampton, UK.

The qPCR was optimised using a serial dilution of a known concentration of cDNA in order to calculate primer efficiencies (indicated in Table 2.4). Furthermore, a dissociation curve was generated using the MiniOpticon Real-Time PCR detection system (Bio-Rad), to confirm that a single PCR product was being amplified. When the primers were sufficiently optimised, qPCR reactions were carried out (each with three technical replicates) on each cDNA sample. The data were then normalised to a reference gene (CO870340, unknown), a gene whose expression did not significantly change following ESP exposure. The normalised expression ratio of the target gene was calculated using equation 2.2, which takes into account different primer amplification efficiencies for the target and reference gene. The final data were presented as the mean normalised RNA expression (MNE; equation 2.3) using qGene software.

$$Ratio = \frac{(E_{target})^{\Delta Ct target}}{(E_{reference})^{\Delta Ct refrence}}$$

Equation 2.2 used to calculate the normalised expression ratio of a target gene compared to a reference gene
This equation is based on the efficiency-calibrated model, which takes primer efficiency into account, where E represents primer efficiency and ΔCt represents difference between the threshold cycle of a sample assay and the threshold cycle of the corresponding reference gene (Yuan *et al.*, 2006).

$$MNE = \frac{\frac{(E_{target})^{\Delta Ct\ target}}{(E_{reference})^{\Delta Ct\ refrence}} + \frac{(E_{target})^{\Delta Ct\ target}}{(E_{reference})^{\Delta Ct\ refrence}} + \frac{(E_{target})^{\Delta Ct\ target}}{(E_{reference})^{\Delta Ct\ refrence}}}{3}$$

Equation 2.3 used to calculate MNE for technical qPCR replicates
The MNE was calculated by averaging three independently calculated normalized expression values (using equation 2.2) (Muller *et al.*, 2002).

2.17 General statistical analysis

Data were analysed in Microsoft Excel (2007) or SPSS (version 15.0.1) and the mean, standard deviation (SD) or the standard error of the mean (SEM) calculated. Where appropriate, statistical significance was calculated using paired Student's t-test, or analysis of variance (ANOVA) using Tukey's pair-wise comparisons when a number of subsets where analysed.*$P \leq 0.05$ was considered significant and **$P \leq 0.01$ was considered highly significant.

Chapter 3: ERK signalling in *Biomphalaria glabrata* haemocytes

3.1 Introduction

The evolutionarily conserved extracellular signal-regulated kinase (ERK) cell signalling pathway was studied in haemocytes from *Biomphalaria glabrata* susceptible and resistant strains, especially when challenged with *Schistosoma mansoni* ESPs. This was to investigate whether *S. mansoni* ESPs had the potential to modulate key cell signalling pathways in host defence cells and whether susceptible and resistant *B. glabrata* strains responded differentially.

3.1.1 An overview of the ERK signalling pathway

Mitogen activated protein kinase (MAPK) signalling pathways are involved in a number of essential mammalian physiological functions including: embryogenesis, cell differentiation, immunity, cell proliferation and cell death. There are three MAPK pathways: ERK, stress-activated protein kinase/c-Jun NH2-terminal kinase (SAPK/JNK) and p38 MAPK; all of these belong to a family of protein-serine/threonine kinases (Pearson *et al.*, 2001). Each MAPK family consists of three evolutionarily conserved kinases MAPK, MAK kinase (MAPKK) and MAPK kinase kinase (MAPKKK). A stimulus activates MAPKKK, which becomes phosphorylated and activates MAPKK, which in turn activates MAPK *via* its threonine and tyrosine residues. The MAPKs are able to mediate a range of physiological functions in mammals by phosphorylating phospholipases, cytoskeletal proteins, MAPK-activated protein kinases (MK) as well as activating various transcription factors (reviewed in Roux and Blenis, 2004).

In mammals, ERK is a key regulator of cell proliferation, differentiation and growth, while abnormal signalling through the ERK pathway is known be a characteristic of cancerous cells (Roux and Blenis, 2004; Sridhar *et al.*, 2005; Panka *et al.*, 2006). Interestingly, prolonged ERK activity results in pro-apoptotic cellular conditions, while a transient ERK activation leads to promotion of cell survival (Zhuang and Schnellmann, 2006). Experiments employing ERK mutants have highlighted the importance of this signalling protein in cell spreading, migration and integrin expression (Lai *et al.*, 2001). Specific studies using mammalian immune cells have shown that ERK is involved in leukocyte chemotaxis as well as influencing the differentiation of CD^+ T cells (Jorritsma *et al.*, 2003; Luo *et al.*, 2006).

Exactly how ERK is capable of coordinating a number of cellular functions is still not fully understood. The complexity of the ERK signalling pathway is highlighted in figure 3.1.

Generally the ERK signalling pathway is activated by growth factors, hormones, or cytokines, which bind to receptor tyrosine kinase (RTK) or G protein-coupled receptors (Roux and Blenis, 2004). Activated growth factor receptors undergo dimerization and autophosphorylation, and become a platform for the recruitment of specific adapter proteins. A sequence of events leads to the phosphorylation (activation) of Raf, which subsequently activates MAPK kinase (MEK) isoforms (Sridhar *et al.,* 2005). The Raf protein is present in low abundance, but a high concentration of MEK allows for amplification of signal (Pearson *et al.,* 2001). MEK subsequently activates ERK isoforms; ERK1 (p44 MAPK) and ERK2 (p42 MAPK), differing on their tyrosine and threonine phosphorylation site residues (Thr202/Tyr204 for ERK1 and Thr185/Tyr187 for ERK2). Phosphorylated ERK interacts with scaffolding proteins that guide it to the nucleus where it targets a number of transcription factors, such as, Elk-1, c-Fos and c-Myc (Fig. 3.1). Integrins (protein receptors that mediate cell attachment to the surrounding tissue) are involved in controlling the ERK signalling cascade by enhancing the efficiency of the pathway, and trafficking activated ERK (Boudreau and Jones, 1999; Luo *et al.,* 2007; Ulrich and Heisenberg, 2009). MAPK phosphatases (MKPs) specifically regulate ERK by de-phosphorylating both sites of the activated protein (Theodosiou and Ashworth, 2002). The ERK signalling pathway communicates and interacts with other signalling molecules such as protein kinase C (PKC) and PKA, with most of the interaction being up-stream to Raf (Wellbrock *et al.,* 2004; Katz *et al.*, 2007).

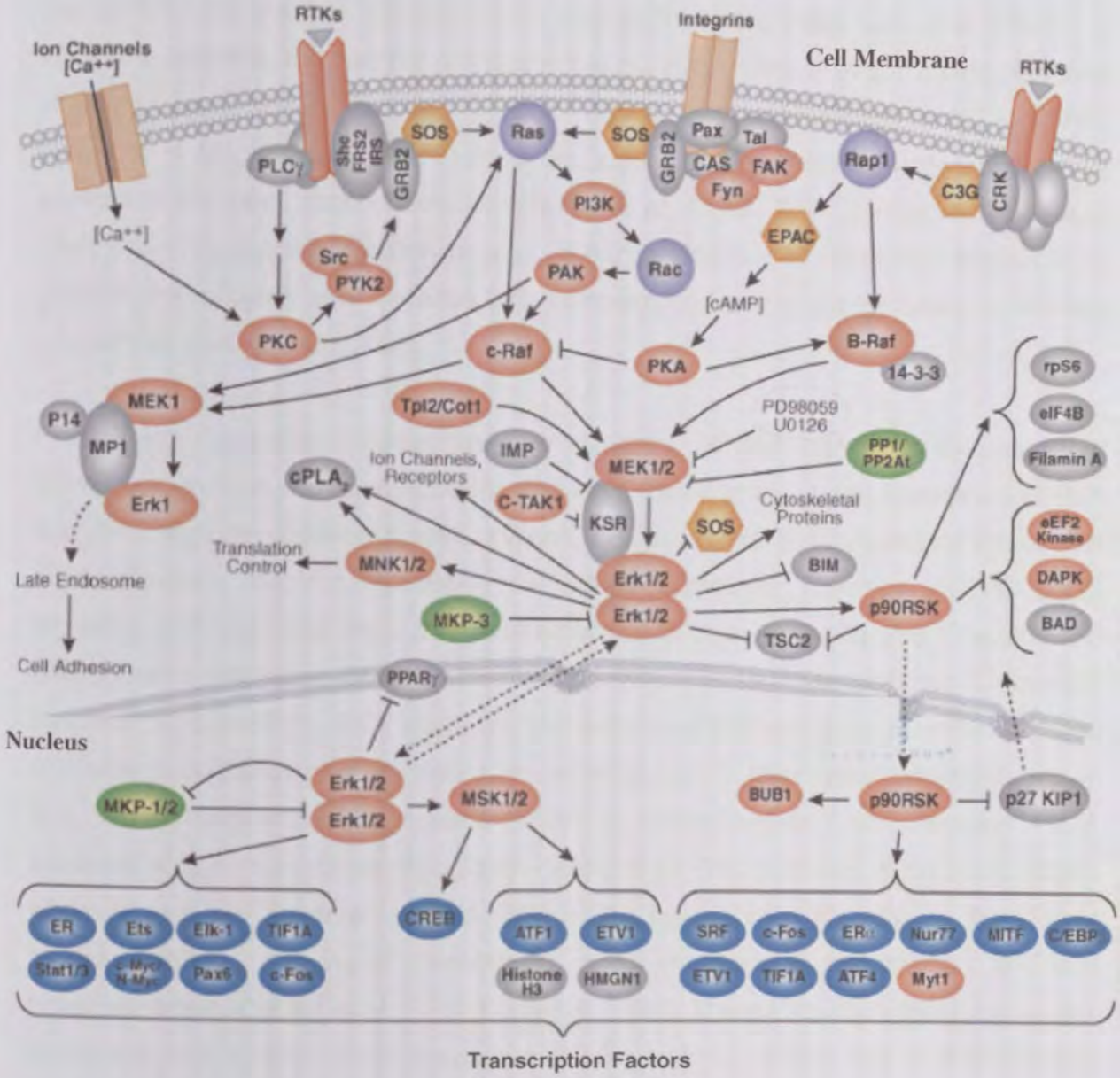

Fig. 3.1 The activation and regulation of the mammalian ERK signalling pathway
The ERK signalling pathway in mammals is activated by G protein-coupled receptors, integrins and RTK. The signalling pathway can be modulated by a range of proteins including PKA, PKC, Type 1 protein phosphatase (PP1) and artificial MEK inhibitors, PD98059 and U0126. Activated ERK is able to translocate into the nucleus where it phosphorylates a number of transcription factors that regulate gene expression such as Elk and cAMP response element binding (CREB). Red circles are kinases, green circles are phosphatases and blue circles are transcription factors. Solid arrows indicate direct stimulatory modification, dotted arrows indicate translocation of the protein while a lined arrow indicates direct inhibition (adapted from cellsignal.com).

3.1.2 Signal transduction in molluscan haemocytes

Genomic and proteomic studies from a range of organisms have identified evolutionarily conserved signalling pathways, particularly in the innate immune system (Belvin and Anderson, 1996; Ausubel, 2005). However, limited information exists on molluscan cell signalling pathways, of which the bivalve *Mytilus galloprovincialis* has been mainly studied (Walker, 2006). Canesi *et al.,* (2002, 2005) showed that p38 MAPK and PKC was activated in haemocytes from *M. galloprovincialis* following bacterial challenge. When the

p38 MAPK pathway was inhibited (using SB203580) haemocytes were less efficient at killing the bacteria, *Escherichia coli* (Canesi *et al.*, 2002; Canesi *et al.*, 2002b). Different strains of bacteria also reduced levels of MAPK (ERK, p38 MAPK and SAPK/JNK) activation in *M. galloprovincialis* haemocytes, implying a potential mechanism of bacterial evasion of the host immune defence (Canesi *et al.*, 2005). DNA cloning and sequence analysis led to the identification of a gene transcribing a p38 MAPK-like protein in *B. glabrata* haemocytes, which shared 68% homology to humans (at the amino acid level) (Humphries and Yoshino, 2006).

Challenging molluscan haemocytes with a range of stimulants has furthered our knowledge on molluscan cell signalling pathways. Sassi *et al.*, (1998) identified that PKA and PKC signalling pathways were involved in inducing *M. galloprovincialis* haemocyte shape changes, following stimulation with adrenocorticotropin hormone (ACTH). These signalling pathways and the subsequent activation of transcription factors (Fos, Jun and SMAD) were delineated using pharmacological inhibitors such as calphostin C (for PKC inhibition) and H-89 (for PKA inhibition) and antibodies that recognise mammalian forms of the kinases (Ottaviani *et al.*, 1999; Ottaviani *et al.*, 2001). Mammalian cytokines such as IL-2 have been shown to increase levels of adrenaline and noradrenaline in *M. galloprovincialis* haemocytes *via* calcium-independent PKC pathways (Cao *et al.*, 2004), while mammalian interferon-gamma (IFN-γ) has been shown to enhance haemocyte bacterial killing *via* signal transducers and activator of transcription (STAT1) phosphorylation (Canesi *et al.*, 2003; Cao *et al.*, 2004). These data imply that cytokine-like molecules may exist in some species of mollusc and may play a role in their immune responses.

Plows *et al.*, (2004, 2006) provided evidence that inhibiting specific signalling pathways in haemocytes of a gastropod mollusc leads to an altered cellular response. Inhibition of ERK, PKC and phosphatidylinositol 3-kinase (PI3-K) significantly reduced phagocytosis of *Escherichia coli* bioparticles by *Lymnaea stagnalis* haemocytes (Plows *et al.*, 2004; Plows *et al.*, 2006). Further work from this laboratory showed PKC to be involved in regulating nitric oxide (NO) and hydrogen peroxide (H_2O_2) production in *L. stagnalis* haemocytes following exposure to phorbol myristate acetate (PMA), bacterial lipopolysaccharides (LPS) or the β1-3 glucan, laminarin (Lacchini *et al.*, 2006; Wright *et al.*, 2006). The inhibition of PI-3K, ERK, SAPK/JNK or p38 MAPK in *B. glabrata* haemocytes also resulted in a reduction of cell spreading, phagocytosis, encapsulation of sporocysts, and H_2O_2 production (Zelck *et al.*, 2007). Even though cell signalling pathways are highly conversed among different species, it is important to note that the identification of cell signalling molecules using selective inhibitors or antibodies that have been developed originally

against mammalian proteins could potentially target unknown signalling molecules in addition to those intended.

Interestingly, in the context of snail-schistosome interactions, carbohydrates such as fucose and galactose, present on the surface of schistosomes, are capable of down-regulating ERK signalling in *L. stagnalis* haemocytes (in the absence of plasma) (Cummings and Nyame, 1999; Hahn *et al.*, 2000; Plows *et al.*, 2005). This implies that specific carbohydrates found on the surface of *S. mansoni* can potentially modulate the activities of cell signalling pathways in its host. Hence, a study was carried out to assess whether products secreted/excreted by *S. mansoni* into culture medium (ESPs) would result in a similar down-regulation of ERK signalling in *B. glabrata* haemocytes. It is known that *S. mansoni* ESPs affect *B. glabrata* haemocyte behaviour; however, what cell signalling pathways ESPs affect in haemocytes is still largely unknown (Lodes and Yoshino, 1990; Lodes *et al.*, 1991; Humphries and Yoshino, 2003).

In this section, schistosome-susceptible (NHM1742) and schistosome-resistant (NHM1307) *B. glabrata* strains were used, thus snail strains will be referred to as susceptible or resistant for clarity. The detection of ERK-like protein was achieved using western blotting performed on total *B. glabrata* haemocyte protein extracts (as mentioned in Chapter 2). The use of phosphor-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) primary antibody, revealed the presence of a phosphorylated ERK-like molecule under unchallenged conditions. This antibody identified one single band, with a molecular weight of approximately 45 kDa (Fig. 3.2). The phosphor-ERK antibody that was used in this study has been previously used to detect phosphorylated (activated) ERK1/2 like molecules in a range of invertebrates, including molluscs (Humphries *et al.*, 2001; Plows *et al.*, 2004; Plows *et al.*, 2005; Lacchini *et al.*, 2006; Wright *et al.*, 2006).

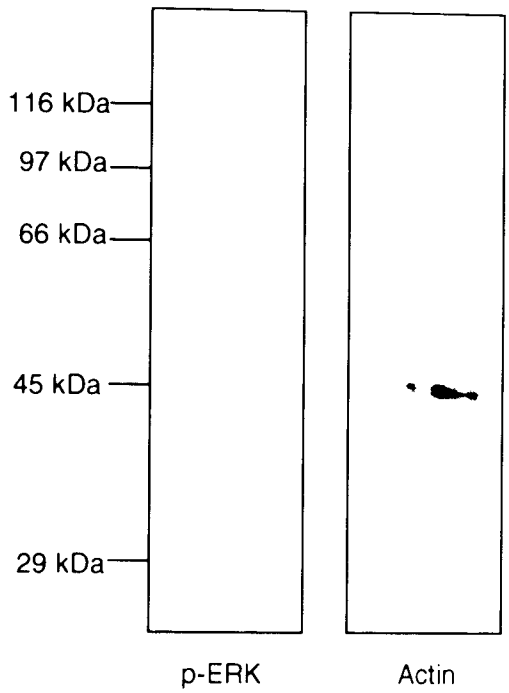

Fig. 3.2 Detection of phosphor-ERK and actin in unchallenged schistosome-susceptible *B. glabrata*
The anti phosphor-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody (New England BioLabs) was used to detect phosphorylated (active) ERK 1/2 homologues in haemocyte extracts from unchallenged susceptible snails. Only one band at 45 kDa was consistently detected. Blots were stripped using Restore Western blot stripping buffer (PerBioscience) for 3 h at RT (no bands were detected following incubation with stripping buffer), and incubated in anti-actin polyclonal rabbit antibody to confirm equal loading onto the gel (n=2).

3.2 Results

3.2.1 Phenotypic differences between susceptible and resistant B. glabrata with respect to haemocyte numbers

Phenotypic differences exist between susceptible and resistant snails of similar size and age in respect of the numbers of haemocytes per volume of haemolymph (Table 3.1). The number of haemocytes (per ml^3) from uninfected resistant snails was significantly greater ($P \leq 0.05$) compared to uninfected susceptible snails; resistant snails have nearly twice as many haemocytes (Table 3.1). In order to investigate basal levels of phosphorylated ERK (phosphor-ERK) in *B. glabrata* strains an equal number of haemocytes from each of the two strains was lysed following centrifugation without being challenged with ESPs. Western blotting revealed that basal levels of phosphor-ERK were present in approximately equal amounts in susceptible and resistant snails when variation in cell numbers is taken into account (Fig. 3.3). In subsequent experiments equal amounts of haemolymph from resistant and susceptible snails were used and the numbers of haemocytes were not adjusted; this was to compare results in a physiologically relevant context.

Snails	N° of haemocytes per ml^3 from: Resistant snail strain (x10^3)	Susceptible snail strain (x10^3)
1	18.8	1.2
2	7.2	2.4
3	16	2.8
4	7.2	8.4
5	4.4	9.6
6	5.2	3.6
7	10.8	6
8	5.6	3.6
9	14	9.6
10	2.4	8
Average	9.16	5.52

Table 3.1 Total number of haemocytes in individual susceptible and resistant snails per volume of haemolymph
The table shows haemocyte counts from 10 individual samples extracted from susceptible and resistant *B. glabrata* strains; snails were selected based on similar size and age.

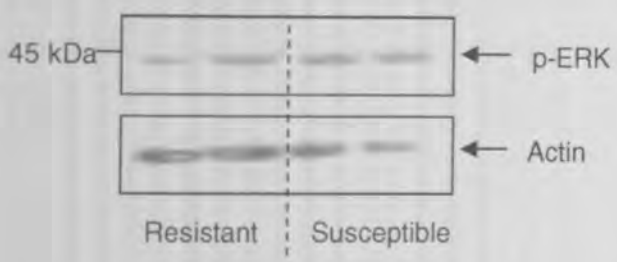

Fig. 3.3 Western blot showing that similar amounts of phosphor-ERK are present in haemocytes from the two snail strains
The upper blot shows phosphor-ERK present in approximately equal amounts in susceptible and resistant snails, using equal numbers of haemocytes in each sample. An equal amount of protein was loaded into each lane (lower blot); the blot is representative of two independent experiments.

3.2.2 The effect of different concentrations of ESPs and duration of challenge on ERK cell signalling in haemocytes

A temporal response assay was carried out to determine levels of phosphorylated ERK in haemocytes from susceptible and resistant *B. glabrata* strains following exposure to *S. mansoni* ESPs (20 µg/ml) for increased durations (0-90 min). For all experiments haemocytes were pooled from several snails of the same strain; thus, any individual variation in susceptibility/resistance to schistosomes was likely to be compensated for between experiments. Western blotting revealed that haemocytes extracted from susceptible snails had decreased phosphor-ERK levels following ESP (20 µg/ml) challenge after 60 min, with little or no phosphorylation after 90 min (Fig. 3.4). However, phosphor-ERK levels were unaffected in unchallenged control haemocytes over the same time period, indicating that the modulation of phosphor-ERK activity was due to the presence of ESPs (Fig. 3.4A). In contrast, haemocytes extracted from resistant snails and exposed to ESPs (20 µg/ml) over 90 min did not display changes in phosphor-ERK levels,

compared to controls (Fig. 3.4B). Different protein concentrations of ESPs (0-20 µg/ml) were subsequently used to challenge haemocytes from susceptible and resistant snails for 1 h. Western blotting, image analysis and ANOVA revealed that an ESP concentration of 10-20 µg/ml led to a significant ($P \leq 0.01$) down-regulation of phosphor-ERK by approximately 60% in haemocytes from susceptible snails; lower doses had little effect (Fig. 3.5A). On the other hand, haemocytes from resistant snails showed no significant down-regulation of phosphor-ERK following ESP exposure (Fig. 3.5B).

To investigate whether the modulation of ERK phosphorylation in haemocytes from susceptible snails was due to ESPs and not cell death, cell viability assays were carried out. Extracted haemocytes (in the presence of 0-20 µg/ml ESPs) were assessed for their viability after 1 h *in vitro* culture in 48 well plates. Percentage cell death was calculated based on the uptake of 0.2% trypan blue. Results showed that ESPs were non toxic to the cells, even at 20 µg/ml concentrations, compared to controls. Therefore, the modulation of ERK found in haemocytes from susceptible snails is likely to be due to ESPs rather then cell death.

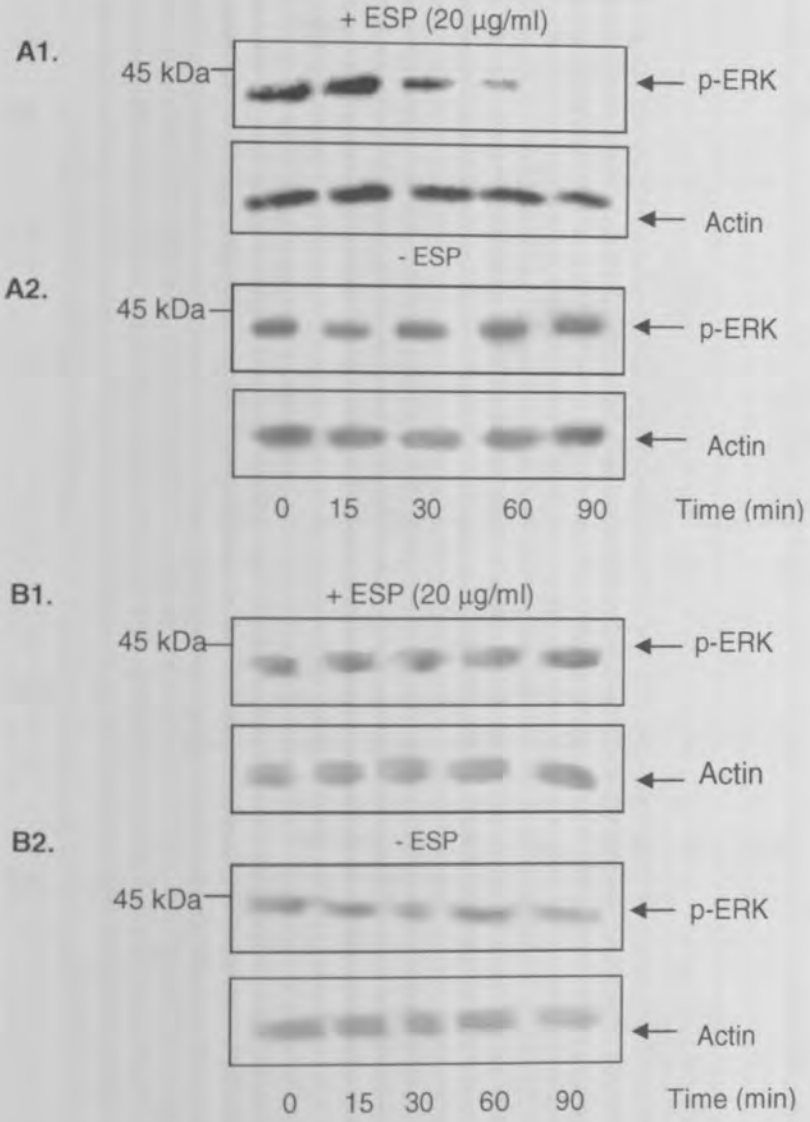

Fig. 3.4 Western blots illustrating phosphor-ERK levels in haemocytes from susceptible and resistant snails over time in the presence and absence of ESPs
(A1) Shows a typical western blot of haemocytes extracted from susceptible snails and challenged with 20 µg/ml ESPs; the blot illustrates down-regulation of phosphorylated ERK (p-ERK) levels in susceptible snail haemocytes after 60-90 min exposure. (A2) This effect is ESP-dependent as no challenge (-ESP) does not result in decreased phosphor-ERK levels. (B1) Haemocytes extracted from resistant snails do not show altered phosphor-ERK levels in the presence (upper panel) or absence (lower panel) of 20 µg/ml ESPs challenge at any time point studied. (B2) Western blot showing p-ERK levels in resistant snail haemocytes unchallenged (-ESP) over time. To confirm that equal amount of protein was loaded into each well, each membrane was washed and stripped of antibodies and re-probed with anti-actin antibodies (lower panels). All blots are representative of two individual experiments.

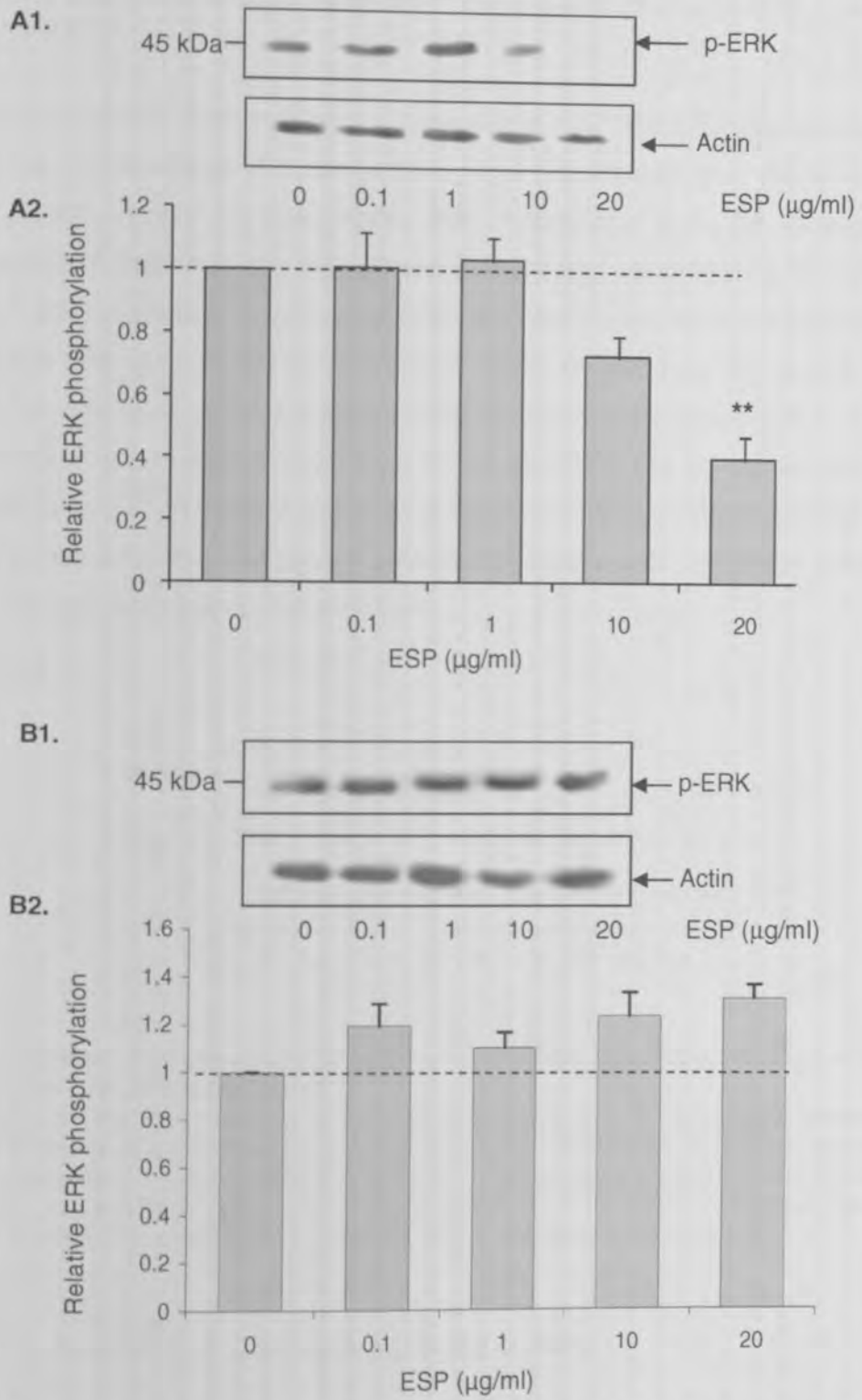

Fig. 3.5 Western blots and bar graphs showing effects of ESP dose on phosphor-ERK levels in haemocytes from susceptible and resistant snail strains

(A1) Western blot showing phosphor-ERK levels in haemocytes from susceptible snails that had been exposed for 60 min with ESPs at different concentrations. (A2) Bar graph showing western blot analysis from four independent experiments; a decline in relative phosphor-ERK levels was observed at 10 µg/ml ESP concentration, while at 20 µg/ml ESP concentration there was a significant decrease compared to controls (**$P \leq 0.01$). (B1) Western blot showing p-ERK levels in haemocytes from resistant snails that had been exposed for 60 min with ESPs at different concentrations. (B2) Bar graph showing western blot analysis from four independent experiments, no significant modulation in relative phosphor-ERK levels at any ESP dose was observed. Four individual membranes were probed with anti-phospho ERK1/2 (1:1000) antibodies, stripped and probed with anti-actin antibodies. The intensity of each band was analysed and normalised against actin values; the mean relative phosphor-ERK levels were then determined and are presented as bar graphs. The SEM is also shown (error bars), along with representative western blots.

3.2.3 The stability of different frozen ESP preparations: effects on ERK signalling pathway in haemocytes

Some experiments were carried out using stored (-20°C) ESP preparations that were 2-3 years old. When effects of these samples were investigated, they did not down-modulate ERK phosphorylation in haemocytes from susceptible snails as expected (Fig. 3.6). Different ESP batches were then pooled together and concentrated. The effects of these pooled ESP samples on phosphor-ERK in haemocytes from susceptible snails were assessed (Fig. 3.6). Fresh preparations of ESPs (a few days old, stored at -4°C) were found to attenuate ERK phosphorylation in susceptible haemocytes, whereas older preparations (more than 2 years old, stored at -20°C) did not induce this response as strongly (Fig. 3.6). A fresh preparation of ESPs that were concentrated and kept at -20°C for less then 6 months was used in subsequent experiments and freeze thaw cycles of the ESP samples were kept to the minimum.

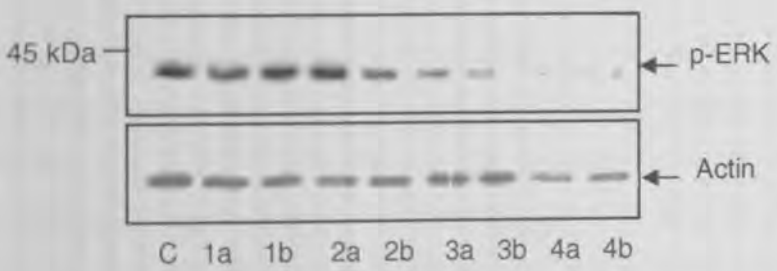

Fig. 3.6 Western blot showing ESP stability on storage, determined through effects on haemocyte ERK phosphorylation
The upper panel shows phosphor-ERK levels in susceptible snail haemocytes following challenge with: CBSS only (C), 3 year old pooled ESP (20 µg/ml) samples (lanes 1a, 1b), two separate preparations each three years old (lanes 2a, 2b), ESP samples 2 years old pooled prior to use (lanes 3a, 3b) and freshly prepared ESPs (lanes 4a, 4b). The lower panel shows the amount of protein in each lane determined through the use of anti-actin antibodies.

3.2.4 The modulation of ERK signalling is ESP specific

To determine whether the down modulation of phosphor-ERK was due to *S. mansoni* ESPs or interference from residual mouse liver extracts present in the sporocyst culture medium, a control preparation was produced. A control medium lacking ESPs was prepared by removing miracidia immediately after the wash step; the same volume of CBSS was used as for the ESP preparation, and the resultant solutions were filtered and concentrated similarly (as stated in Chapter 2). Thus, the preparation was identical to the normal protocol, except for the presence of ESPs. Haemocytes challenged with 20 µg/ml ESPs reduced ERK phosphorylation in susceptible snail, whereas the control medium that lacked ESPs did not, when similar volumes were used (Fig. 3.7). Therefore, ESPs

produced from *S. mansoni* appear to alter ERK cell signalling in the susceptible snail haemocytes and not any by-products of the parasite collection process.

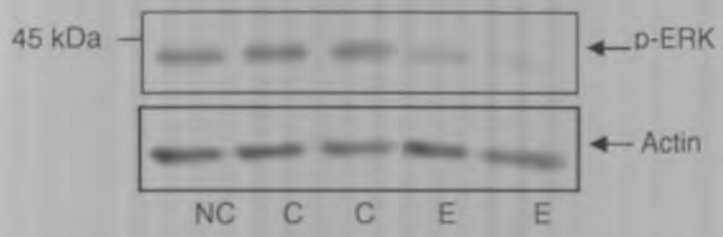

Fig. 3.7 Western blot showing phosphor-ERK levels in haemocytes from susceptible snails challenged with ESP (20 µg/ml) or control extracts
Phosphorylated ERK levels in non-challenged cells (NC), cells exposed to control medium (C) and ESPs (20 µg/ml) (E) were investigated after 1 h exposure. The lower blot shows an equal amount of protein was loaded into each lane. The blot shown is representative of two independent experiments.

3.2.5 The effect of ESPs on ERK signalling in individual haemocytes

Laser scanning confocal microscopy was employed to investigate the cellular location of phosphor-ERK and to ascertain levels of phosphor-ERK in the presence and absence of 20 µg/ml ESPs for 1 h at RT. Unchallenged haemocytes from both susceptible and resistant snails exhibited basal phosphorylation (activation) of ERK, which is agreement with western blotting data (Fig. 3.3 and 3.4). However, individual cells showed variation in fluorescent intensity; although susceptible snail haemocytes exposed to ESPs often appeared to result in a reduction in phosphor-ERK fluorescence (Fig. 3.8). Confocal microscopy also revealed that phosphorylated ERK was usually distributed throughout the cytoplasm of haemocytes, with some clustering possibly occurring between phosphorylated ERK and filamentous actin (Fig. 3.8, merged images). No obvious relocation of phosphorylated ERK within haemocytes from either snail strain was observed following treatment with ESPs.

Haemocytes were left to adhere onto glass coverslips with CBBS; most haemocytes from both snails strains appeared round, with some cells appearing more spread (Fig. 3.8). *Biomphalaria glabrata* have two haemocyte populations, small granular hyalinocytes, which appear round in shape and larger granulocytes, which are more spread (Barracco *et al.*, 1993; Bayne, 2003; Humphries and Yoshino, 2003). Confocal fluorescent images were captured of both round and spread cells. Interestingly, spread haemocytes from susceptible snails appeared to show generally the most reduction in phosphor-ERK levels.

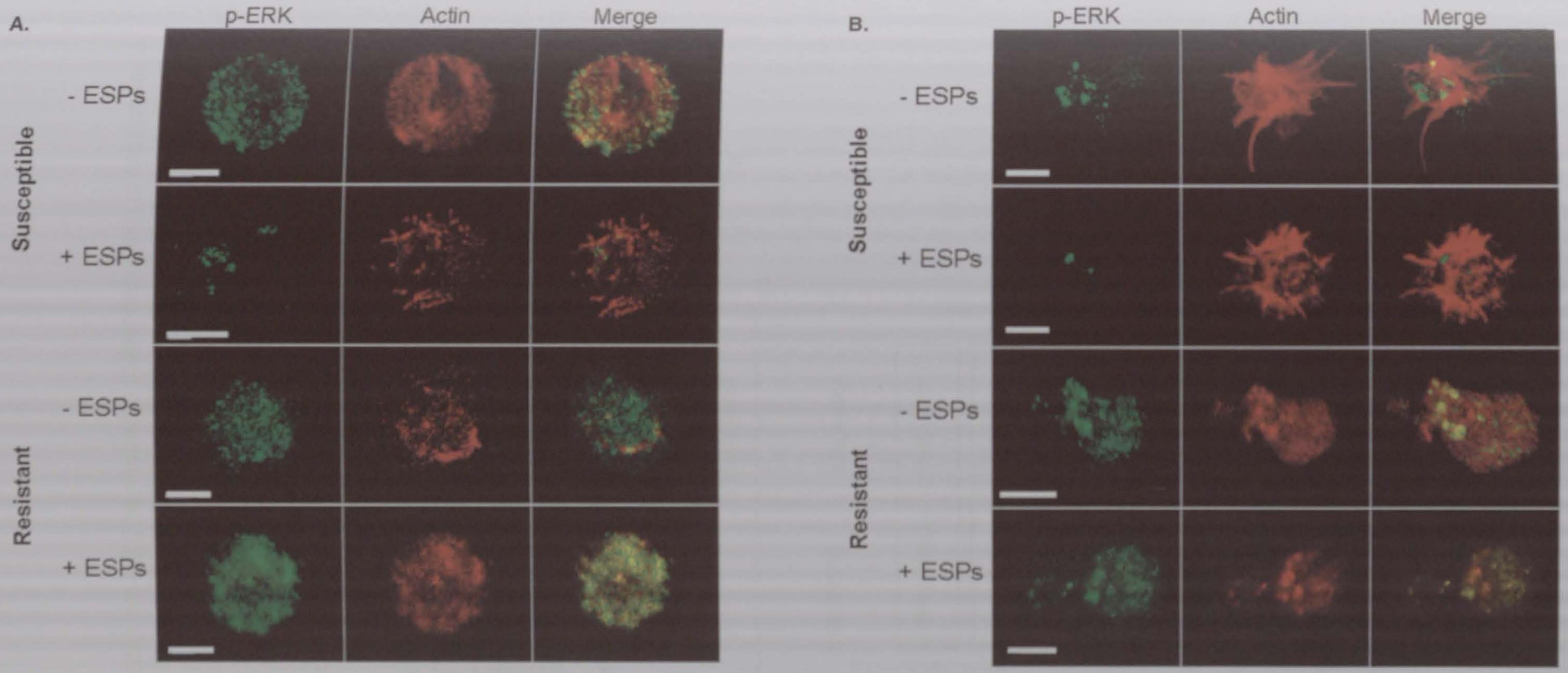

Fig. 3.8 Confocal images of haemocytes from susceptible and resistant *B. glabrata* strains challenged with and without ESPs
Panel (A) shows round *B. glabrata* haemocytes that had adhered to coverslips and were challenged with (+ESP) and without (-ESP) 20 µg/ml ESP for 1 h. Panel (B) shows more spread cells challenged with and without ESPs. The colour green represents phosphor-ERK revealed using FITC secondary antibodies, while red represents F-actin (revealed using rhodamine phalloidin stain). Individual haemocytes were observed with a Leica laser scanning confocal microscope and images show z-axis projections of round haemocytes and haemocytes exhibiting a degree of cell spread. Representative images of cells are displayed in average pixel brightness mode; results are representative of four independent experiments. Scale bars correspond to 10 µm in length.

3.2.6 Investigating ERK signalling in the presence of snail plasma

Snail plasma is capable of "mopping up" glycoconjugates and thus preventing modulation of ERK cell signalling in *L. stagnalis* haemocytes (Plows *et al.*, 2005). Therefore, the effects of ESPs on haemocyte ERK phosphorylation were tested in the presence of plasma. In haemocytes from snails, phosphor-ERK levels remained constant in the presence and absence of snail plasma after 20 µg/ml ESP-challenge for 1 h (Fig. 3.9 and Fig. 3.5). In contrast, ESPs continued to suppress ERK phosphorylation in haemocytes from susceptible snails by approximately 60% ($P \leq 0.01$) even in the presence of snail plasma (Fig. 3.9).

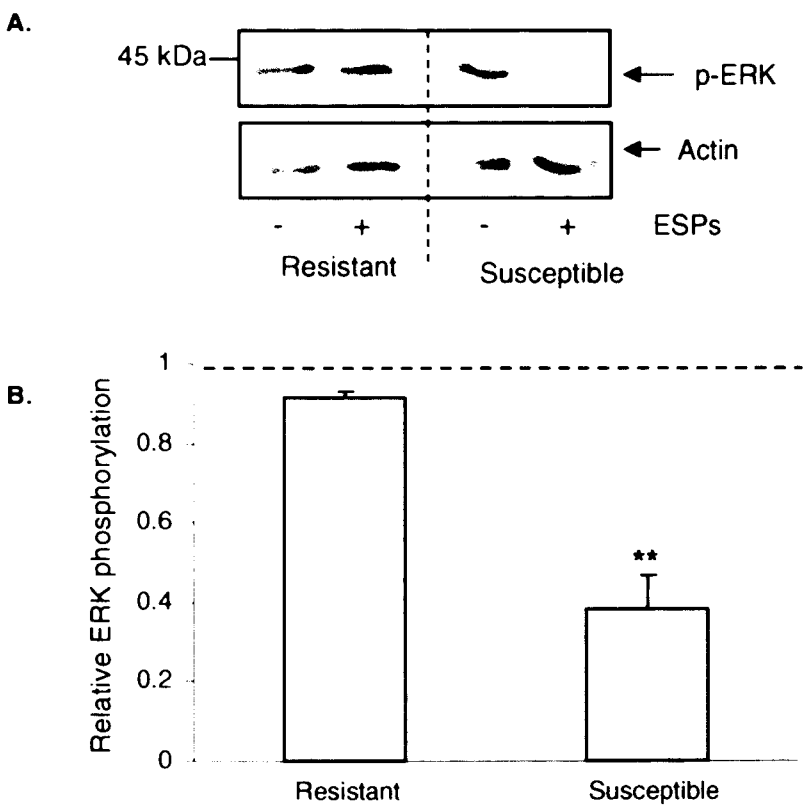

Fig. 3.9 Relative phosphor-ERK levels in haemocytes from resistant and susceptible strains after ESP-challenge in the presence of snail plasma
Haemocytes were exposed to 20 µg/ml ESPs (+) for 1 h or left unchallenged (-) in the presence of plasma. (A) An individual western blot showing phosphor-ERK levels in the samples. (B) Shows the mean relative change in phosphor-ERK levels in susceptible and resistant ESP-challenged haemocytes (in the presence of snail plasma). ** $P \leq 0.01$ when compared to haemocytes not exposed to ESPs (shown as the dotted line and having a relative value of 1). The result are representative of four independent experiments, SEM are shown by the error bars.

3.2.7 Investigating MEK activity in haemocytes challenged with ESPs

Phosphorylation (activation) of MEK, the upstream kinase that phosphorylates ERK, was investigated to ascertain the effects of ESP on this regulatory enzyme in susceptible snail haemocytes. Western blots revealed a 45 kDa phosphor-MEK-like protein (activated MEK) in unchallenged, non-adherent *B. glabrata* haemocytes (Fig. 3.10A). To confirm the antibody was indeed detecting phosphor-MEK, haemocytes were challenged for 20 min with a phosphor-MEK inhibitor; U0126 (10 µM); image analysis of duplicate blots revealed an approximate 60% reduction in phosphor-MEK levels in the presence of this inhibitor (Fig. 3.10B). A high basal level of phosphor-MEK was detected in unchallenged haemocytes from susceptible snails which could be reduced following ESP (20 µg/ml) exposure for 1 h (Fig. 3.11). Image analysis revealed an approximately 40% reduction in phosphor-MEK levels after ESP-challenge ($P \leq 0.05$), compared to unexposed haemocytes from susceptible snails (Fig. 3.11). This implies that ESPs are, at least in part, deactivating ERK through the reduction of MEK phosphorylation (activation).

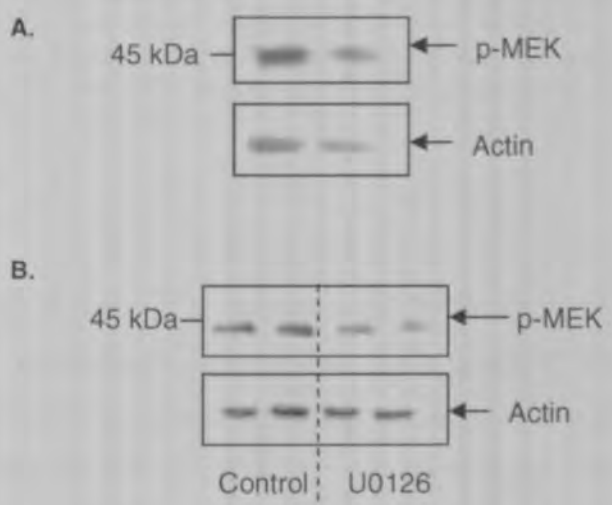

Fig. 3.10 Western blots showing basal levels of phosphor-MEK in susceptible *B. glabrata* haemocytes (A) and following challenge with a MEK inhibitor, U0126 (B)
(A) Haemocytes were pooled from susceptible snails, unchallenged and kept on ice, basal levels of phosphor-MEK were subsequently assessed. (B) Haemocytes from susceptible snails were left to adhere in individual wells of cell culture plates before being exposed to the MEK inhibitor, U0126 (10 µM) for 20 min, control cells were exposed with 0.1% DMSO (vehicle for U0126) prior to extraction. Individual lanes show duplicate experiments.

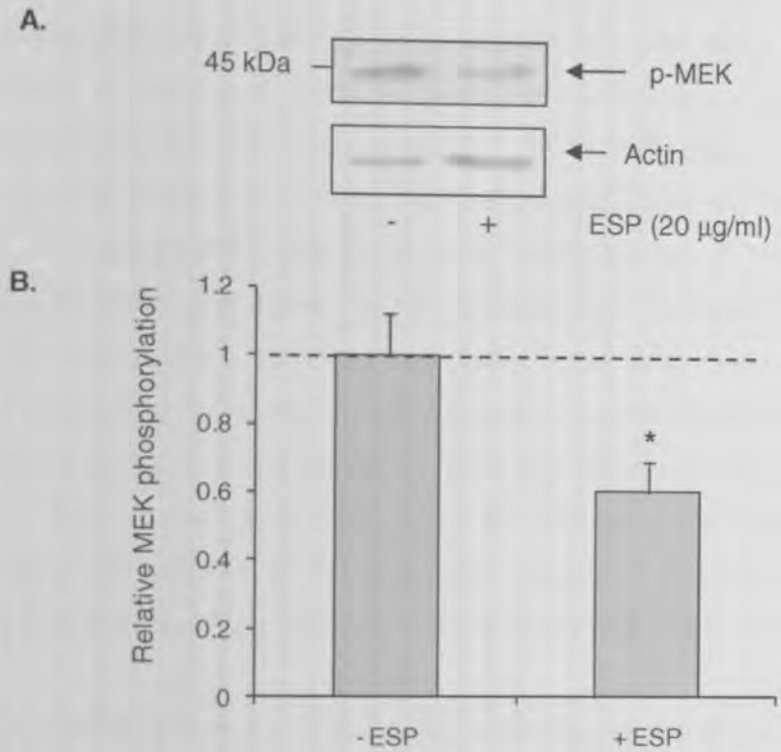

Fig. 3.11 Western blot and bar graph showing relative change in phosphor-MEK levels in haemocytes from susceptible snails following 1 h ESP (20 µg/ml) challenge
(A) Shows typical western blot of phosphor-MEK levels in haemocytes exposed to 20 µg/ml ESP (+ESP) compared to control (-ESP). The bar graph (B) shows a significant decline in relative phosphor-MEK levels after ESP exposure (*$P \leq 0.05$, where n=6), SEM are shown by the error bars.

3.3 Discussion

In summary, the effects of *S. mansoni* ESPs on ERK phosphorylation in haemocytes extracted from schistosome-susceptible and resistant snails were investigated. This approach enabled comparative analysis of the effects of schistosome components on host defence signalling in the context of host phenotype. There was a significant down-modulation of phosphor-ERK in haemocytes from susceptible snails; the extent of the down-modulation was dependent on ESP concentration and the duration of challenge, with significant differences observed between 10-20 µg/ml for 60-90 min. This response was ESP specific, as control extracts did not attenuate phosphor-ERK levels. Furthermore, phosphor-ERK levels in resistant haemocytes were unaffected by ESPs, even at high ESP concentrations. In both snail strains basal phosphor-ERK levels were constant over 0-90 min; this implies that a pool of phosphorylated ERK exists in *B. glabrata* haemocytes and may be a consequence of the vigilant nature of these cells.

Phenotypic differences exist between susceptible and resistant snail strains, in relation to haemocyte numbers; uninfected resistant snails possess on average almost twice as many haemocytes per volume of haemolymph compared to uninfected susceptible snails

(Walker and Rollinson, 2008). Interestingly, previous studies demonstrated that *L. stagnalis* can produce a higher number of haemocytes following infection with *T. ocellata.* Furthermore, circulating haemocytes from *Trichobilharzia ocellata* infected *L. stagnalis* were noted to be larger compared to those of uninfected snails with a greater surface area and an increased number of branched pseudopods (van der Knaap *et al.,* 1987). The number of haemocytes present in the haemolymph of the snail, *Potamopyrgus antipodarum,* were also shown to be relatively high in areas where infection with the trematode, *Microphallus sp.* were common (Osnas and Lively, 2006). Infection with a parasite may lead to a significant increase in haemocyte numbers, a strategy (in combination with other mechanisms) that the molluscan host may use to overcome infection. The reason why uninfected *B. glabrata* resistant snails possess more haemocytes per volume of haemolymph compared to uninfected susceptible snails remains unknown, but may involve genetic variations between the two strains.

The ERK signalling pathway coordinates a number of defence mechanisms. In molluscs, the ERK signalling pathway is known to regulate the production of cytotoxic molecules such as NO and H_2O_2, as well as playing a significant role in haemocyte spreading, phagocytosis and encapsulation processes (Hahn *et al.,* 2000; Hahn, Bender and Bayne 2001a; Wright *et al.,* 2006; Humphries and Yoshino, 2007; Zelck *et al.,* 2007). The finding here that *S. mansoni* ESPs can disrupt ERK signalling in haemocytes from susceptible snails implies that the parasite may be using this strategy to evade detection or blunt host immune responses. Interestingly, whole transformed *S. mansoni* sporocysts also attenuate phosphorylated ERK levels in haemocytes from susceptible snails only; haemocytes exposed to approximately 1000 fixed sporocysts over 60 min showed significantly reduced phosphorylated ERK levels (Walker A.J published in Zahoor *et al.,* 2008). These results imply that a molecule that ultimately leads to the downregulation of phosphor-ERK may be present on the surface of sporocyst as well as in ESPs. This study was combined with this chapter and was published (Zahoor *et al.,* 2008).

From the data it can be noted that there is high basal activation of ERK in freshly extracted haemocytes from both snail strains even after a 90 min equilibration period, a similar finding has been observed previously in *L. stagnalis* haemocytes (Plows *et al.,* 2005; Lacchini et *al.,* 2006; Wright *et al.,* 2006). Why this occurs is unknown, but these cells may have a high state of immuno-vigilance, due to constant exposure to pathogens. Secondly, from the data it can be inferred that ESPs do not down-regulate ERK signalling fully; approximately 40% of activated ERK (and 60% activated MEK) was still present in ESP-challenged haemocytes extracted from susceptible snails when compared to unexposed controls. Total inhibition of the ERK pathway is difficult to achieve without using high concentrations of inhibitors. For example MEK inhibitors such as U0126 (1 μM)

and PD98059 (10 μM) failed to suppress fully the phosphorylation of ERK in *L. stagnalis* haemocytes prior to being stimulated with LPS (Plows *et al.*, 2004). Nevertheless, these MEK inhibitors (at the concentrations mentioned) do significantly impair haemocyte spreading and NO output by approximately 50% and 80% respectively (Wright *et al.*, 2006, Humphries and Yoshino, 2008). The magnitude of ERK de-phosphorylation observed in this study may have an associated inhibitory effect on haemocyte defence processes that are regulated by ERK signalling.

To date, there have been two main reports studying the effects of parasite ESPs on snail cell signalling processes (Humphries and Yoshino 2006; Iakovleva *et al.*, 2006). Humphries *et al.*, (2006) did not uncover any modulation of p38 MAPK activity in *B. glabrata* haemocytes following 5 min exposure to *S. mansoni* ESPs (10 μg/ml). Iakovleva *et al.*, (2006) also did not demonstrate any modulation of the MAPK pathway in *Littorina litteorea* haemocytes following 45 min exposure to the trematode, *Himasthla elongata* ESPs (5 μg/ml). In our experiments we studied *S. mansoni* ESPs at concentrations of 0-20 μg/ml over 0-90 min exposure time, demonstrating that it is important to use different ESP doses over longer durations. Although it is impossible to ascertain what concentrations ESPs attain *in vivo* following infection it is reasonable to suggest that high ESP concentrations could be reached in the immediate area surrounding the miracidia or the developing sporocyst stages.

Mammalian models of infection have also shown a reduction in MAPK signalling in immune cells following parasite ESP exposure. Dirgahayu *et al.*, (2002) identified a reduction in ERK and p38 MAPK signalling in LPS stimulated macrophages that were pre-incubated for 24 h with ESP (5 μg/ml) from the tapeworm, *Spirometra erinaceieuropaei.* Furthermore, *S. erinaceieuropaei* ESPs were able to reduce mRNA expression levels of tumor necrosis factor (TNF-α) in these macrophages; this cytokine plays an important role in regulating immune cells *via* the MAPK pathway (Miura *et al.*, 2001). In contrast, soluble egg antigen (SEA) from *S. mansoni* (0.1-10 μg/ml) was found to increase ERK cell signalling in endothelial cells activating cell proliferation and migration (Kanse *et al.*, 2005). The secreted protein ES-62, from *Acanthocheilonema viteae*, a filarial parasite, was found to activate murine B cells at concentrations of 25-50 μg/ml, but at low concentrations of 0.2-2 μg/ml ES-62 could inhibit B cell proliferation by modulating PKC, PI-3K and ERK pathways (Harnett and Harnett, 1993; Goodridge *et al.*, 2005). This implies that the effects of parasite ESPs on cell signalling may vary depending on the type of defence cell involved and the particular stage in the parasites' life cycle the ESPs were collected.

The presence of snail serum can play an important role in the process of non-self recognition (Adema *et al.*, 1997; Zelck, 1999). Lectin-like molecules, such as fibrinogen-related proteins (FREPs), glycosidases and molluscan-like cytokines are present in snail serum and may play a role in combating schistosome infection (Granath *et al.*, 1994; Zelck, 1999; Zhang *et al.*, 2001). In our investigation, the presence of serum did not influence the ability of *S. mansoni* ESPs to suppress ERK cell signalling in haemocytes from susceptible snails. This implies that serum from schistosome-susceptible snails cannot neutralise schistosome ESPs; therefore, ESPs may be binding directly to haemocytes and regulating cell signalling. Potentially, *S. mansoni* ESPs could be down-regulating ERK cell signalling by activating MAPK phosphatases (MKPs). These phosphatases are conserved proteins, but a MKP homologue has yet to be found in *B. glabrata*.

In summary, *S. mansoni* ESPs are likely to be binding to carbohydrate or protein cell surface receptors on haemocytes leading to the activation of MKP like molecules or other unknown molluscan specific enzymes, resulting in attenuation of phosphorylated ERK. Equally likely, *S. mansoni* ESPs may block the activity of other signal transduction pathways that are involved in maintaining basal ERK phosphorylation levels under normal physiological conditions. Why this response is specific for haemocytes from susceptible snail strains is unknown. Haemocytes from resistant snails may not express an ESP binding receptor, or the receptor may be mutated, or the activation of MKPs may differ in haemocytes of the two snail strains. Identifying and sequencing the receptor to which ESPs may bind to, as well as identifying specific ESP components that are involved in regulating cell signalling, would further aid research in this area.

Chapter 4: Haemocyte spreading

4.1 Introduction

Molluscan haemocytes are involved in defence mechanisms and are capable of chemotaxis, antigen recognition, phagocytosis and the production of anti-microbial products, as well as playing an important role in physiological responses including nutrient transport and shell repair (van der Knaap *et al.*, 1987; Fryer and Bayne, 1989; Schneeweiss and Renwrantz, 1993; Hooper *et al.*, 2007; Martin *et al.*, 2007; Travers *et al.*, 2008). *Biomphalaria glabrata* generally has two main haemocyte populations, hyalinocytes and granulocytes; they have distinct characteristics. Hyalinocytes are relatively small cells that spread poorly on glass, they are rich in ribosomes, non-phagocytic, do not express the BGH1 cell surface marker and usually account for 10% of the haemocyte population (Yoshino and Granath, 1985; Barracco *et al.*, 1993). Granulocytes, on the other hand, are larger cells that readily spread on glass, tend to be phagocytic and express the *B. glabrata* haemocyte marker (BGH1) (Yoshino and Granath, 1985; Barracco *et al.*, 1993).

DeGaffe and Loker (1998) observed that *B. glabrata* haemocytes challenged with *Echinostoma paraensei* ESPs for 30 min increased in number of round (unspread) cells by approximately 30-40%. Correlation was found between the ability of *E. paraensei* to infect *B. glabrata* and the capacity of the parasite to produce ESPs that could inhibit haemocyte spreading (DeGaffe and Loker, 1998); this study did not differentiate between hyalinocytes and granulocytes. The effect of ESPs on the morphology of different haemocyte populations was later investigated by Hertel *et al.*, (2000) who studied calcium dynamics. Rounding of cells involved cytoskeletal changes induced by the activation of a number of cell signalling pathways, which could be measured by calcium fluctuations. Hertel *et al.*, (2000) also showed that ESPs from *E. paraensei* induced haemocyte rounding by approximately 30%. Furthermore, these cells exhibited more calcium fluctuations compared to controls, indicating that *E. paraensei* ESPs are actively changing haemocyte shape (Hertel *et al.*, 2000). In the present study, the general cell spread of *B. glabrata* haemocytes (on glass coverslips) in the presence and absence of *Schistosoma mansoni* ESPs was investigated.

4.2 Results

Haemocytes were extracted from schistosome-susceptible and schistosome-resistant snail strains and challenged with 20 µg/ml ESPs for 1 h at RT on glass coverslips (a total

volume of 250 µl haemolymph was used in each experiment as mentioned in the Materials and Methods Chapter). The cells were subsequently fixed and processed before being viewed using scanning electron microscopy (images were viewed from above; the microscope platform was not tilted). At least 30 micrographs of individual haemocytes (taken at random) were collected and categorised visually into round or spread cells (Fig. 4.1). The relative cell surface area (or the spread of the haemocyte on the glass coverslip) was calculated by measuring cell parameters, using a Visual Basic software programme (calibrated using scale bars present on the scanning electron micrographs), developed at Kingston University IT department.

Figure 4.1 shows images of the two types of haemocytes (spread and round) that were observed typically in both susceptible and resistant *B. glabrata* strains. Membrane ruffles (or blebs) were often observed on the surface of the cells (Fig. 4.1 and 4.2). The general shape and structure of haemocytes extracted from either susceptible or resistant snails (of similar age and size) were alike, even after 1 h challenge with 20 µg/ml ESPs (Fig. 4.2). Only the surface area of spread cells was investigated, as the area of attachment to the surface of the coverslip in rounded cells could not be measured. Nevertheless, image analysis highlighted that spread (120 μm^2 mean; 299-25μm^2 range), unchallenged, resistant snail haemocytes had a significantly greater mean relative cell surface area ($P \leq 0.01$) compared to spread, unchallenged, susceptible snail haemocytes (65.75 μm^2 mean; 130-21 μm^2 range).

Spread cells from resistant snails exhibited an increase in cell surface area by approximately 25% in the presence of ESPs (not statistically significant). ESP-challenged, resistant, snail haemocytes were the most spread cells, with a mean relative cell surface area of 150.8 μm^2 (32-302 μm^2 in range), compared to other samples (Fig. 4.3). In contrast, haemocytes from susceptible snails maintained the same relative surface area following ESP stimulation, with spread cells maintaining a mean relative surface area of 65.8 μm^2 (29-94 μm^2 in range) (Fig. 4.3).

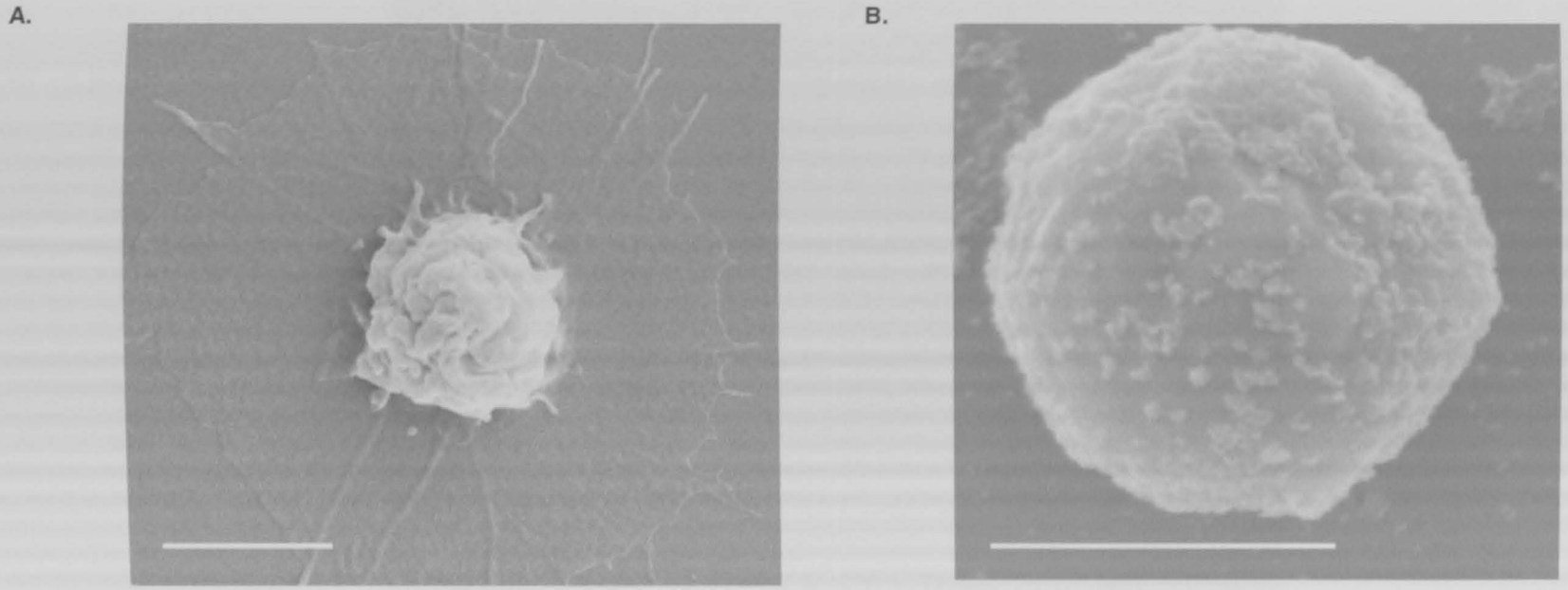

Fig. 4.1 Scanning electron micrographs of unchallenged haemocytes extracted from *B. glabrata* strains
Images of spread (A) and round (B) haemocytes on glass cover slips; cells were left to attach to coverslips for 30 min prior to fixing and processing for scanning electron microscopy. Images are a representative of those collected from two independent experiments and were typical of haemocytes extracted from both susceptible and resistant snail strains. Bars represent 10 μm in length.

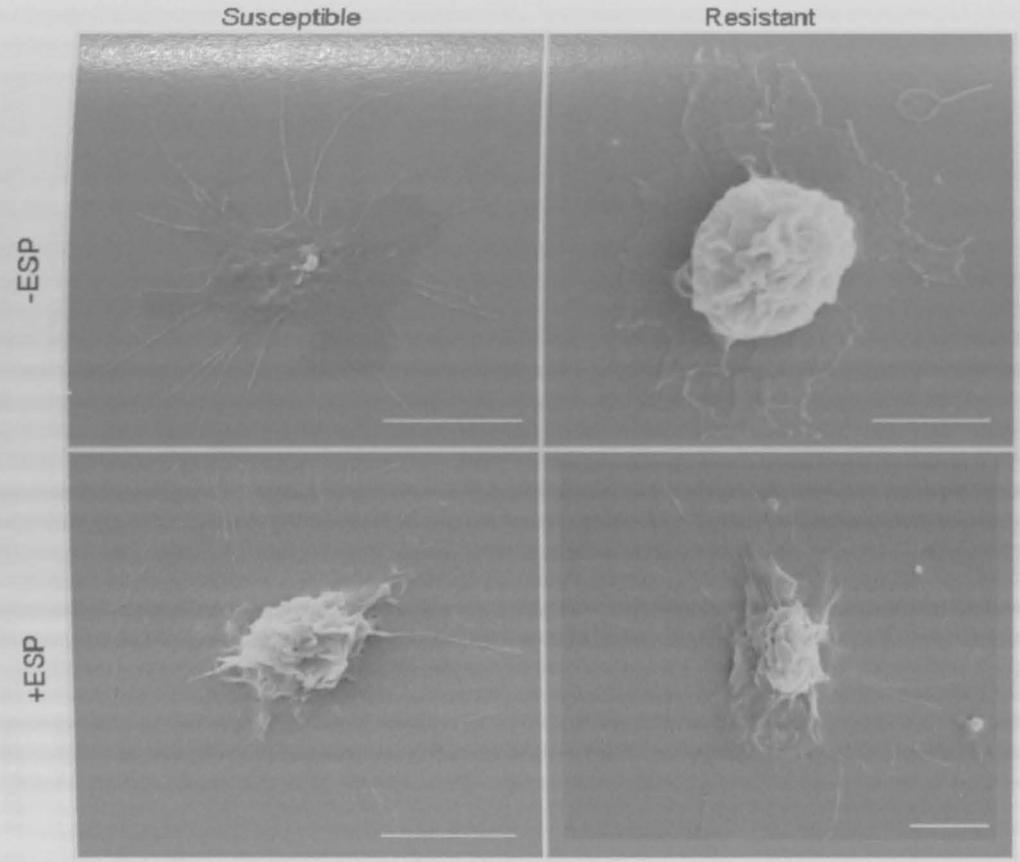

Fig. 4.2 Scanning electron micrographs of haemocytes extracted from susceptible and resistant snails and placed onto glass coverslips in the absence and presence of *S. mansoni* ESPs

Cells were left to attach to coverslips for 30 min and then incubated in either ESP (20 µg/ml) (bottom panel) or CBSS (top panel) for 1 h prior to fixing and processing for scanning electron microscopy. Images are a representative of those collected from two independent experiments and were typical of spread haemocytes extracted from both susceptible and resistant snail strains. Bars represent 10 µm in length.

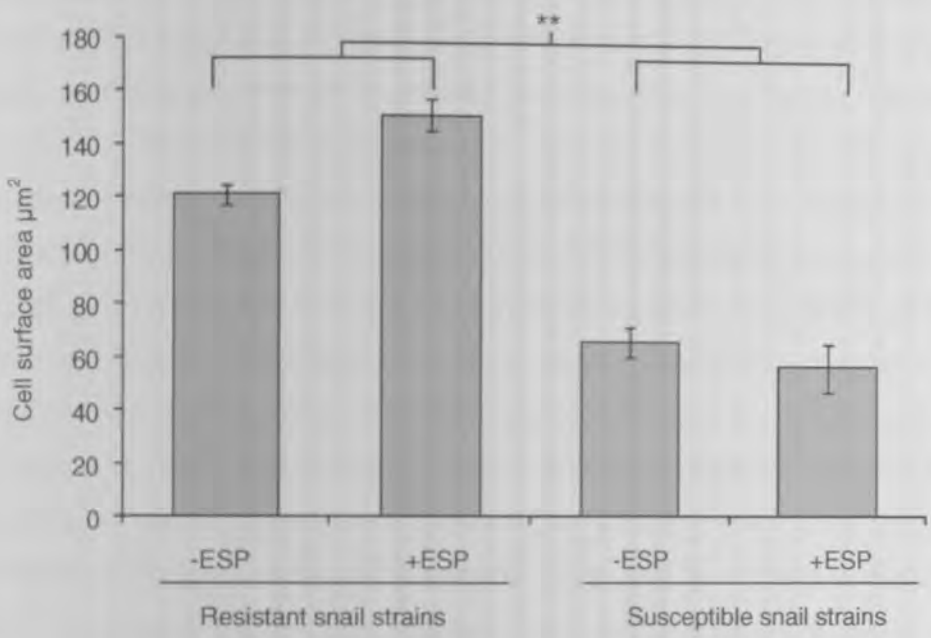

Fig. 4.3 Mean relative cell surface area of haemocytes extracted from susceptible and resistant snails challenged with *S. mansoni* ESPs
Scanning electron micrographs of 30 individual haemocytes (pooled from approximately 4-5 individual snails) from susceptible and resistant snails exposed (+ESP) and unexposed (-ESP) to 20 µg/ml ESP for 1 h were captured. The relative surface area of each haemocyte was calculated using Microsoft Visual Basic software. The results are from two independent experiments are shown, error bars indicate SEM values, (**$P \leq 0.01$, when samples from different strains were compared).

4.3 Discussion

To date there is no uniform or sound classification system for molluscan haemocytes. The application of electron and light microscopy, differential centrifugation, flow cytometry, lectin binding affinity assays and functional analyses to the study of haemocyte biology has still left the classification of haemocytes in much debate (Sminia, 1972; Yoshino and Granath, 1985; Noda and Loker, 1989; Adamowicz and Bolaczek, 2003; Martin *et al.*, 2007; Martins-Souza *et al.*, 2009). Most researchers agree that two main types of haemocytes exist, round and spread, but disagree on subtypes and lineage. Earlier work, using light microscopy, identified three different types of *B. glabrata* haemocytes but these were categorised according to the degree of cell spread (unspread, partially or fully spread); recent studies using flow cytometry also identified three groups of *B. glabrata* haemocytes, small, medium and large based on granularity and size (Noda and Loker, 1989; Noda and Sato, 1990; Martins-Souza *et al.*, 2009). Whether these cells are different haemocytes or represent different maturation stages (including stem cells) is unknown.

In the current study, spread and round haemocytes were identified using scanning electron microscopy, and whether these cells were hyalinocytes or granulocytes is

unknown; individual cells were not characterised or cell sorted. However, round haemocytes were not analysed as the relative cell surface area could not be measured from scanning electron micrographs (the area of attachment to the surface of the glass coverslip). Susceptible and resistant snail haemocytes did not show any significant change in relative cell spread following ESP-challenge. This was an unexpected finding; a reduction in relative cell surface area in ESP-challenged haemocytes from susceptible snails and no change in cell spread in haemocytes from resistant snails were predicted. This is because other studies in this thesis have shown that *S. mansoni* ESPs can attenuate phosphorylation of ERK in haemocytes from susceptible but not resistant snails (Chapter 3). ERK cell signalling is also known to influence the ability of haemocytes to spread on glass; exposure to ERK inhibitors have shown a decrease in cell spreading behaviour in Bge cell lines (Humphries *et al.*, 2001). In this analysis it was that found ESP-challenged haemocytes (spread) from susceptible snails had a similar relative cell surface area compared to controls. This may imply that cell spread may be regulated *via* an ERK independent pathway in *B. glabrata* haemocytes.

There are a number of limitations in this study including: no clear distinction between haemocyte populations; difficulty in addressing whether the cells are in an active state of spreading or are granulocytes; and difficulty in estimating the number of circulating hyalinocytes and granulocytes in a snail. However, the BGH1 marker could be used to sort cells into potential hyalinocytes and granulocytes, and intracellular calcium dynamics could also be used to distinguish actively spreading cells and granulocytes (Yoshino and Granath, 1985; Hertel *et al.*, 2000). Indeed, pre-treating the coverslip to enhance cell adhesion may provide an insight into whether bound spread cells can increase their potential to spread following ESP-challenge. Nevertheless, this study demonstrates that schistosome susceptible and resistant *B. glabrata* haemocytes have different capacities to spread on glass.

Cell spreading is an important physiological process involved in haemocyte killing mechanisms and encapsulation processes. Encapsulation of *S. mansoni* would involve haemocytes migrating towards the parasite larva, projecting cytoplasmic extensions around the parasite and immobilising it, in concert with other haemocytes. This process requires circulating haemocytes to convert into more adhesive cells capable of binding to the target (Pech and Strand, 1996; Lavine and Strand, 2002; Araque *et al.*, 2003). In theory, haemocytes from susceptible snails may be incapable of migrating towards the schistosome larvae and thus, incapable of killing the parasite (Bayne, 1990; Hahn *et al.*, 2001b). However, according to Sapp *et al.*, (2000) the presence of *S. mansoni* miracidia does not affect the adhesion of haemocytes or haemocyte spreading behaviour (Sapp and

Loker, 2000). In this study, it was found that *S. mansoni* ESPs may have differential effects on susceptible and resistant *B. glabrata* cell surface spread. Interestingly, studies have shown that the number of round and partially-spread *B. glabrata* haemocytes increase following *S. mansoni* infection, while larger cells (or more spread) decrease, due to the encapsulation process removing them from the circulation (Noda and Loker, 1989; Noda 1992; Martins-Souza *et al.*, 2009). These findings indicate that haemocytes from resistant snails may be in a more active physiological state compared to haemocytes from susceptible snails and *S. mansoni* ESPs may enhance their activity (Fryer and Bayne, 1990; Hahn *et al.*, 2001a; Hahn *et al.*, e 2001b; Bender *et al.*, 2005; Humphries and Yoshino, 2008). Nevertheless, future investigations may involve sorting the cells into BGH1 positive and negative cells (using primary antibodies and flow cytometry) and subsequently exposing the different cell types to *S. mansoni* ESPs and analysing relative cell surface area. This may address whether the two cell types behave differently to parasite components. Investigating intracellular calcium fluctuations in *B. glabrata* haemocytes following *S. mansoni* ESP-challenge using confocal microscopy may also highlight whether resistant snail haemocytes are in a more active state compared to susceptible snail haemocytes.

Chapter 5: Nitric oxide production in *Biomphalaria glabrata* haemocytes

5.1 Introduction

Nitric oxide (NO) is a highly reactive molecule produced in vertebrate, invertebrate and plant cells. In mammals, NO functions as a neuronal messenger molecule, a vasodilator of smooth muscle, a regulator of cell proliferation and apoptosis, and a defence molecule (Franchini *et al.*, 1995; Nagy *et al.*, 2007). How NO regulates a number of cellular functions is unclear, but probably it involves influencing the activities of key cell signalling pathways (Hancock *et al.*, 2001). In mammals, NO is capable of activating GTPase and Ras which in turn activate phosphoinositide 3-kinases (PI3-K) and the mitogen-activated protein kinase (MAPK) signalling pathways (Raines *et al.*, 2007). Endogenous NO can also inhibit Ras, blocking calcium-induced activation of extracellular extracellular signal-regulated kinase (ERK) (Raines *et al.*, 2007). Thus, depending on cellular conditions, and presumably cell type, NO can activate or inactivate Ras.

NO is synthesised by the oxidation of L-arginine to L-citrulline, catalysed by the enzyme NO synthase (NOS). NO can react with oxygen species to form stable products and peroxynitrite ($OONO^-$), a toxic free-radical (Fig. 5.1). In mammals, three isoforms of NOS have been characterised, neuronal NOS (nNOS) and endothelial NOS (eNOS), which are both constitutively expressed, and inducible NOS (iNOS), which is expressed in response to inflammation and proinflammatory cytokines (Nagy *et al.*, 2007). NOS is an evolutionarily-conserved enzyme which displays a great degree of sequence similarity between invertebrates and vertebrates (Fig. 5.2) (Matsuo *et al.*, 2008). In molluscs, NOS-like activity has been identified in *Mytilus galloprovincialis*, *Lymnaea stagnalis*, *Limax*, *Aplysia* and *Biomphalaria glabrata* haemocytes; while nNOS has been found in *L. stagnalis* and *Aplysia californica* neurons (Elofsson *et al.*, 1993; Moroz *et al.*, 1994; Korneev *et al.*, 1998; Novas *et al.*, 2004; Bodnarova *et al.*, 2005).

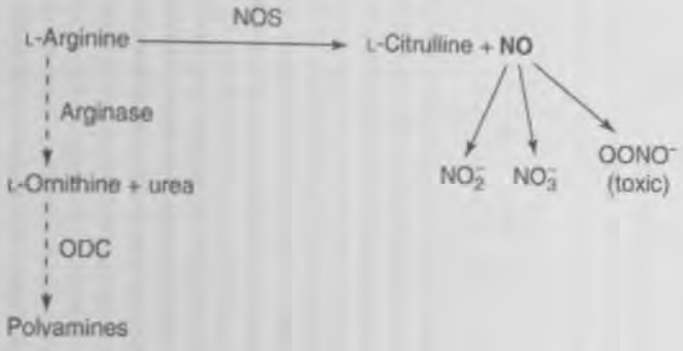

Fig. 5.1 The NO synthesis pathway
L-arginine is converted to L-citrulline and NO by the enzyme NOS. The molecule NO is highly unstable and forms stable molecules such as nitrites (NO^-_2) and nitrates (NO^-_3), as well as peroxynitrite ($OONO^-$) a toxic radical. The enzyme arginase can further covert L-arginine into polyamines (Rivero, 2006).

Reactive oxygen species (ROS), NO and its reactive intermediates can damage enzymes and DNA and are produced by host cells to protect themselves against a range of pathogens (Rivero, 2006). However, uncontrolled production of reactive species by the host may cause self-damage. Therefore, a number of mechanisms exist to limit the effects of these toxic intermediates, for example the expression of superoxide dismutase (SOD) in host cells which converts superoxide ions into hygrogen peroxide (H_2O_2), the latter being converted into water and oxygen by the enzyme peroxidase. Intriguingly, parasites have also evolved mechanisms to protect themselves against free radicals produced by their host upon infection; these include manipulating the host's arginine levels, colonising certain host tissue that are rich in scavenging molecules, producing anti-oxidants and inhibiting host ROS production (Nowak and Loker, 2005).

Resistant *B. glabrata* haemocytes can generate sufficient quantities of NO and H_2O_2 to kill *Schistosoma mansoni* sporocysts *in vitro* (Hahn *et al.*, 2001a). As a counterattack, *S. mansoni* ESPs can reduce superoxide production in host haemocytes (more significantly in susceptible snail haemocytes compared to those from resistant snails) by producing a 108 kDa polypeptide which remains uncharacterised (Connors and Yoshino, 1990; Connors *et al.*, 1991). In the present study it was important to address whether intracellular NO output by resistant and susceptible *B. glabrata* haemocytes was affected by *S. mansoni* ESPs. Furthermore, the signalling mechanism(s) which regulate NO production in *B. glabrata* haemocytes are currently unknown. Previous studies have shown that the ERK signalling pathway plays an important role in controlling NO and H_2O_2 output in *L. stagnalis* haemocytes (Lacchini *et al.*, 2006; Wright *et al.*, 2006). In addition, *S. mansoni* ESPs (10-20 µg/ml) were found to inhibit ERK signalling in haemocytes from susceptible, but not resistant, *B. glabrata* (Chapter 3). Thus, the effect of ERK inhibition on NO output in haemocytes from these *B. glabrata* strains was also investigated.

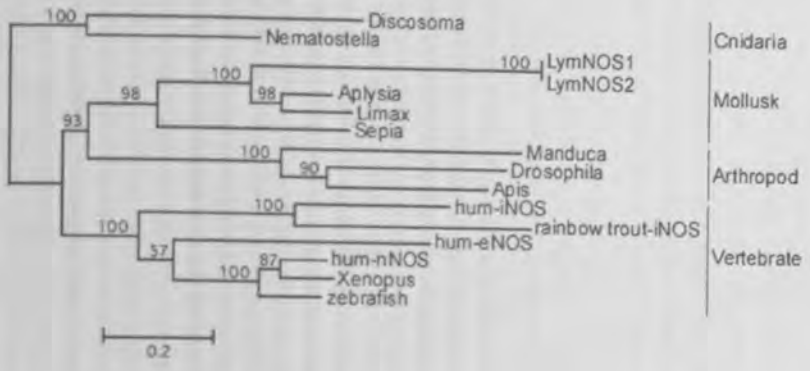

Fig. 5.2 A phylogenetic tree for NOS
A multiple alignment of the full amino acid sequences of NOS from various species including the gastropods *Lymnaea* (Lym), *Limax*, and *Aplysia*. The numbers on the bars are bootstrap scores (%), the scale bar indicates the number of amino acid substitutions per site (Matsuo *et al.*, 2008).

5.2 Results

5.2.1 Nitric oxide output in haemocytes from susceptible B. glabrata

Intracellular NO levels of pooled haemocytes from susceptible or resistant snails were measured using the fluorescent dye 4-amino-5-methylamino-2',7'-difluorofluorescein (DAF-FM) diacetate. Each assay was conducted on one day in triplicate or quadruplicate using samples from the same pool of haemolymph. Assays were then repeated on different days using different pooled haemolymph samples to ensure the observed effects were consistent. Because background fluorescence varied in each assay, data from separate assays could not be combined; therefore, for each experiment one typical graph is shown together with the statistical analysis.

Initially, haemocytes were exposed to a range of compounds including PMA, LPS, zymosan A and laminarin in an attempt to induce an increase in NO output. These compounds have been shown previously to induce NO production in haemocytes of the snail *L. stagnalis* (Wright *et al.*, 2006). In *B. glabrata*, susceptible snail haemocytes significantly increased NO output following exposure to 10 µM PMA compared to controls ($P \leq 0.01$ at 3 h; Fig. 5.3). NO output did not significantly increase following exposure to LPS (10 µg/ml), zymosan A (10 µg/ml), or laminarin (10 mg/ml); nevertheless, mean relative NO levels were observed consistently to be greater than those of unexposed controls.

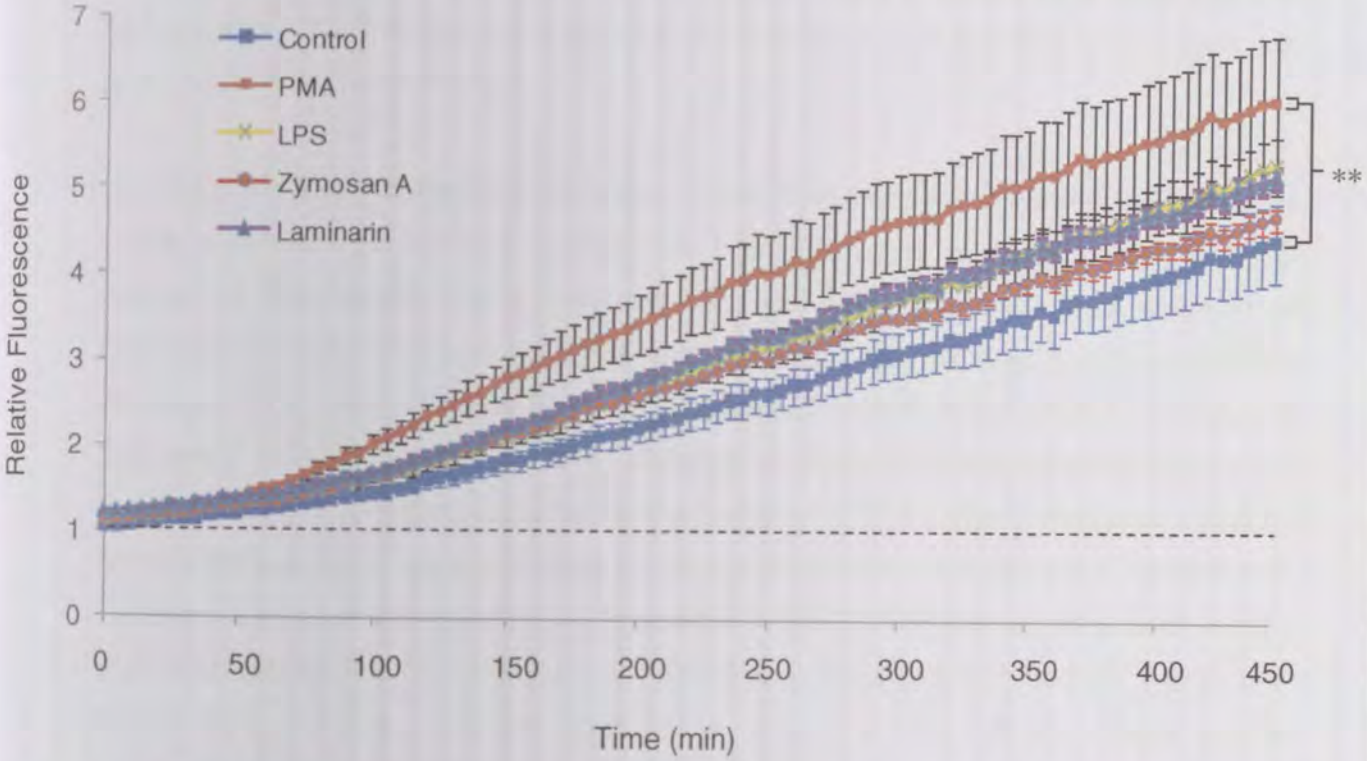

Fig. 5.3 Relative NO output by haemocytes from susceptible snails that were challenged with PMA (10 µM), LPS (10 µg/ml), zymosan A (10 µg/ml) or laminarin (10 mg/ml)
Pooled haemocytes from several individual susceptible snails were exposed to the compounds in 96 well plates (approximately $3x10^3$ cells per well) and the relative NO output was measured over time. Only PMA-exposed haemocytes produced significantly more NO, compared to controls. Mean relative fluorescence (±SEM, n=3) is shown for each time point as a proportion of the background fluorescence (no DAF-FM diacetate, indicated by the dotted line). The data is from one assay, representative of two different assays conducted on separate days. $^{**}P \leq 0.01$ when compared to mean control values at 3 h.

5.2.2 Nitric oxide output in B. glabrata haemocytes following ESP stimulation

The relative amounts of NO produced by haemocytes from susceptible and resistant snail strains in the presence and absence of 20 µg/ml ESPs over 5 h was investigated using an equal amount of haemolymph (100 µl per well) or an equal number of haemocytes ($3x10^3$ cells per well). ESP-challenged haemocytes from resistant snails exhibited significantly increased NO levels compared to CBSS-only controls ($P \leq 0.05$, at 5 h; Fig 5.4). After 5 h ESP-challenge, these haemocytes produced 3 times more relative NO than control haemocytes (mean relative increases of 3.420 and 1.045, respectively; Fig 5.4A). In contrast, mean NO output by haemocytes from susceptible snails decreased slightly when challenged with ESPs; although this difference was not statistically significant, a decrease was consistently observed from four biological replicates (data from four separate assays, conducted on separate days). *Schistosoma mansoni* ESPs (20 µg/ml) gave little or no auto-fluorescence over time; thus the presence of ESPs in the assay did not affect the fluorescence values. Interestingly, there was also a difference in the basal NO outputs

produced over time between unchallenged haemocytes from resistant and susceptible snails. Unchallenged haemocytes from susceptible snails produced 62% more NO than haemocytes from resistant snails after 5 h of incubation in CBSS (mean relative increases of 2.73 and 1.04, respectively).

Resistant snails generally have twice the number of haemocytes compared to susceptible snails of similar age and size (section 3.2.1). In order to compensate for this, an equal number of haemocytes ($3x10^4$ cell per well) was used from each snail strain in all subsequent experiments. Haemocytes from resistant snails exposed to 20 µg/ml ESPs produced 38% more NO over 5 h than unexposed controls (mean relative increases of 3.96 and 2.42, respectively; Fig. 5.4B). Relative NO levels in haemocytes from susceptible snails were not significantly affected by the presence of ESPs. Even when accounting for haemocyte numbers, basal NO levels in susceptible snail haemocytes were higher than in resistant snail haemocytes after 5 h incubation in CBBS. Haemocytes were viable after 5 h *in vitro* incubation (in CBSS-only or CBSS with 20 µg/ml ESPs); both trypan blue alive/dead cell estimations and CellTiter 96 AQueous One Solution Assay showed haemocytes remained 100% viable even after 5 h (Fig. 5.5).

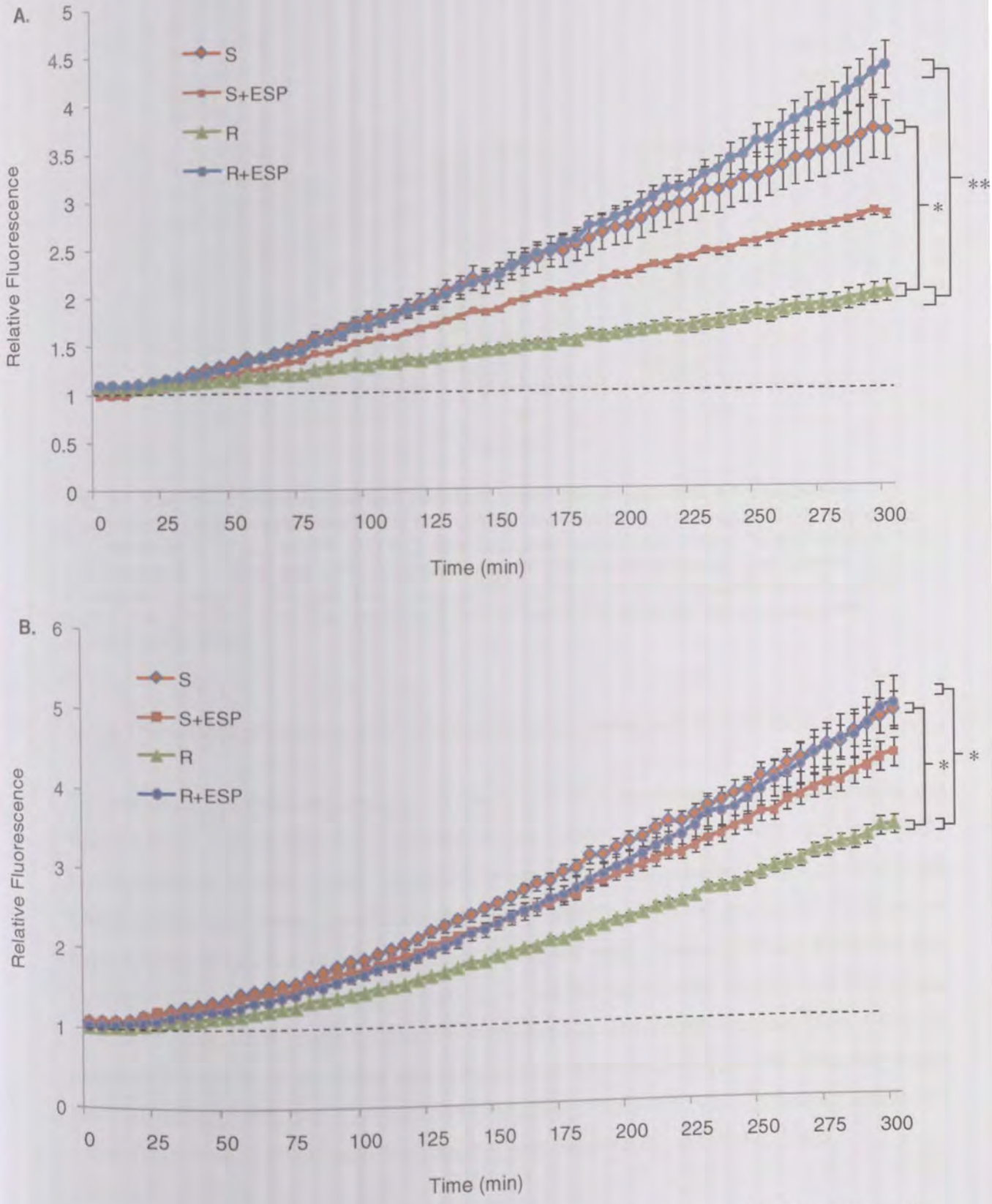

Fig. 5.4 Relative NO production in haemocytes from susceptible (S) and resistant (R) snail strains challenged with 20 µg/ml ESPs

Line graphs showing NO produced in haemocytes from susceptible (S) and resistant (R) snail strains in the presence and absence of 20 µg/ml ESP over time. Equal volumes (100 µl) of diluted haemolymph (A), or an equal number of haemocytes ($3x10^3$) (B) were used per well. Mean relative fluorescence is shown for each time point (±SEM, n=4) as a proportion of background fluorescence (no DAF-FM diacetate) indicated by the dotted line. The data shown is from one assay and is representative of three different assays carried out on separate days. *$P \leq 0.05$, and **$P \leq 0.01$ when compared to differences in mean fluorescent values between treatments at 2 h.

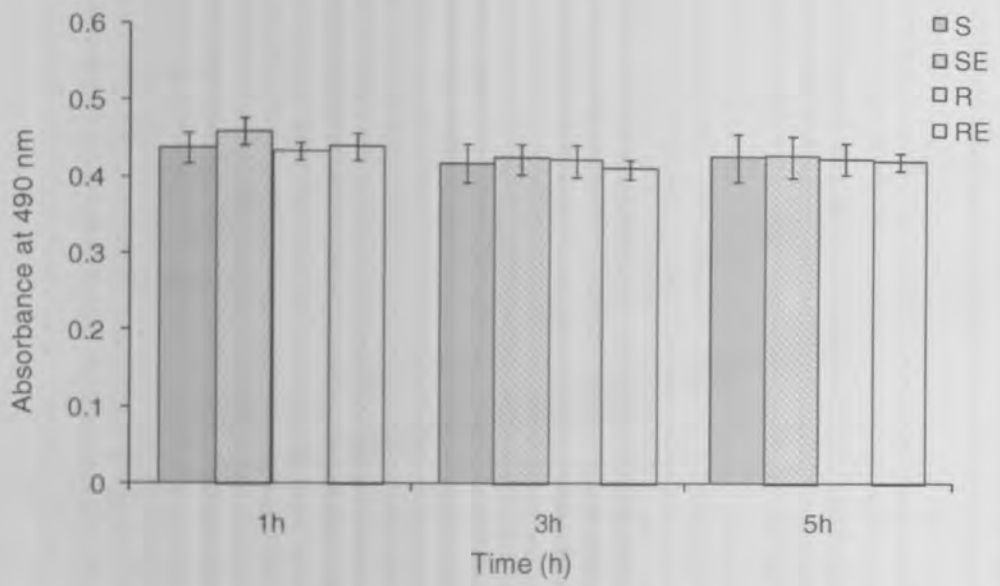

Fig. 5.5 Viability of susceptible and resistant snail haemocytes over 5 h incubation
Haemocytes ($3x10^3$ cell per well) from susceptible (S) or resistant (R) snails in CBSS only and in the presence of 20 µg/ml ESP (SE/ RE) were incubated in individual wells of 96 well plates for 5 h; the viability of the cells was then monitored using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega). This assay contains tetrazolium which becomes bio-reduced in active cells into formazan, the absorbance (at 490 nm) of this molecule was measured after 1, 2 and 5 h (±SD, n=6).

5.2.3 The effects of different ESP concentrations on haemocyte NO production

To determine the dose-dependency of haemocyte NO responses to ESPs, cells extracted from either susceptible or resistant snails were challenged with different ESP concentrations (0.1-20 µg/ml). As a general linear relationship between NO generation by haemocytes, and time, was observed during the first 2 h of incubation in previous experiments (Figs. 5.3 and 5.4), relative NO levels were measured every 5 min for this duration. ESP concentrations of 0.1-20 µg/ml did not significantly affect mean NO output in haemocytes from susceptible snails compared to unexposed controls (Fig. 5.6A). In addition, a clear dose-response was not observed when haemocytes from resistant snails were exposed to different ESP concentrations; only 20 µg/ml ESPs significantly enhanced mean relative NO output when compared to controls ($P \leq 0.05$, at 2 h; Fig 5.6B).

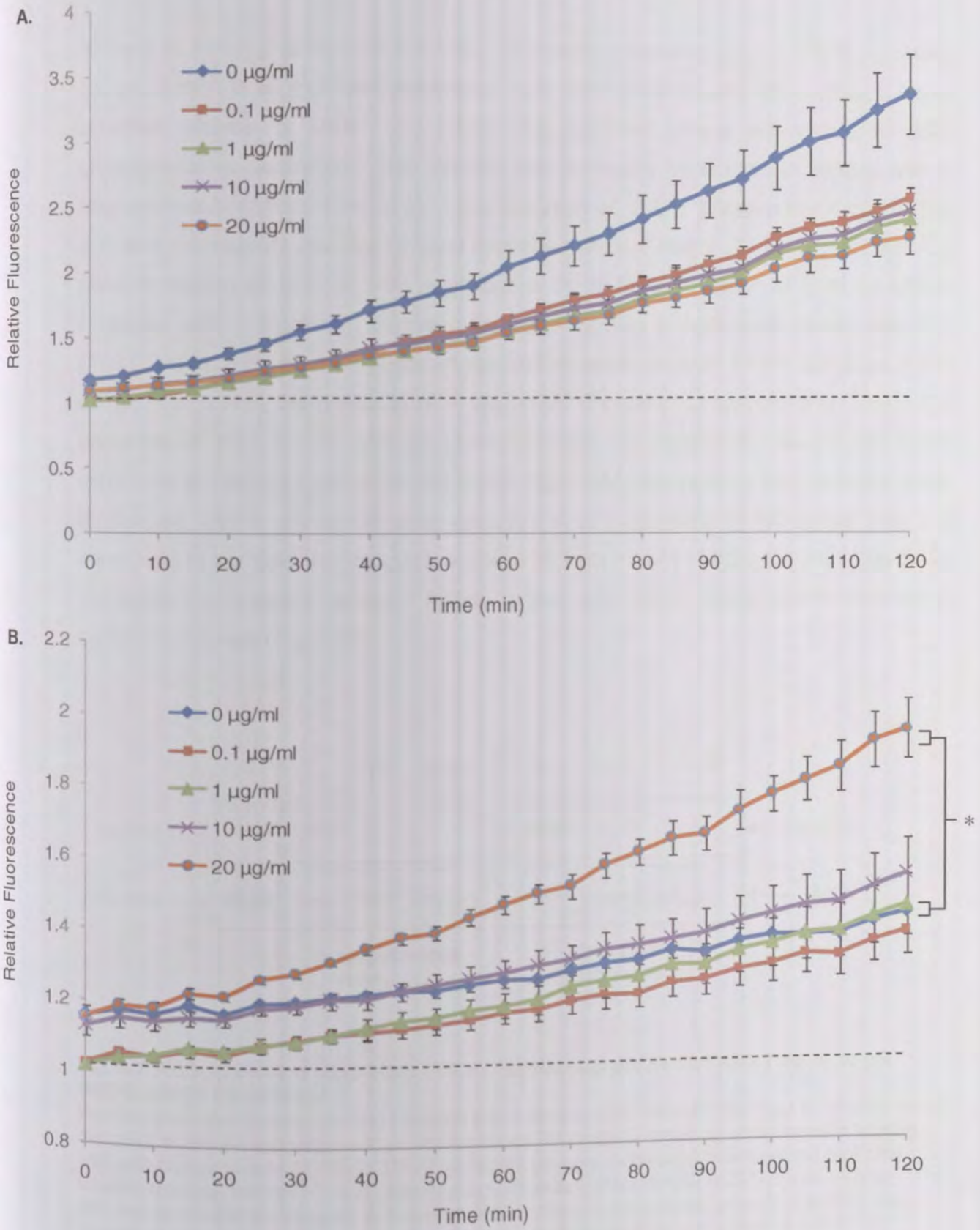

Fig. 5.6 Relative NO production in haemocytes following challenge with various ESP concentrations

An equal number of haemocytes ($3x10^3$ cells per well) were used from susceptible (A) and resistant snails (B) in each experiment. Haemocytes were challenged with different ESP concentrations (0.1-20 µg/ml) or in CBSS alone (0 µg/ml). Mean relative fluorescence is shown for each time point (±SEM, n=3) as a proportion of background fluorescence (no DAF-FM diacetate) indicated by the dotted line. The data is from one assay and is representative three different assays carried out on separate days.*$P \leq 0.05$, when compared to control mean values at 2 h.

5.2.4 The effects of ERK inhibition on haemocyte NO production

In order to investigate whether the ERK cell signalling pathway plays a role in regulating NO production in *B. glabrata* haemocytes, cells were pre-incubated with U0126, a highly selective inhibitor of MEK1 and MEK2, the upstream kinase responsible for ERK phosphorylation (activation). This inhibitor has previously been used in studies with *L. stagnalis* haemocytes (Plows *et al.*, 2004; Wright *et al.*, 2006). Western blot analysis (Fig. 5.7) and subsequent analysis of band intensities showed that 1 µM U0126 inhibited the phosphorylation of ERK in both snail strains by 80-85%. Inhibition of ERK by U0126 coincided with a significant decrease in NO production in both snail strains over 2 h ($P \leq 0.05$) (Fig. 5.8). Haemocytes from susceptible snails showed a 64% decrease in NO output over 2 h in the presence of 1 µM U0126 compared to controls (Fig. 5.8A). The presence of both U0126 and 20 µg/ml ESP did not significantly reduce NO levels compared to U0126 (1 µM or 10 µM) alone (Fig. 5.8A). Haemocytes from resistant snails challenged with 1 µM U0126 also exhibited a 60% decrease in NO levels over 2 h compared to controls. Haemocytes exposed to both 10 µM U0126 and 20 µg/ml ESPs displayed the greatest reduction in NO output, with mean values almost reduced to background levels (Fig. 5.8B).

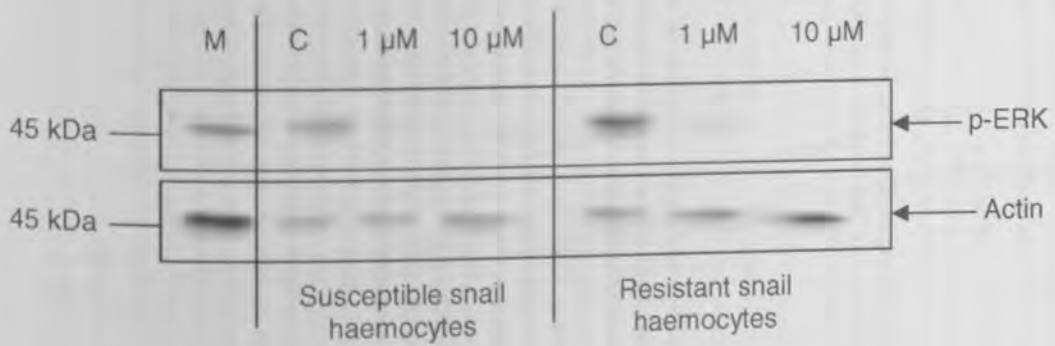

Fig. 5.7 Phosphorylated (activated) ERK in haemocytes pre-treated with 1 µM or 10 µM U0126, a MEK1/2 inhibitor
Haemocytes from susceptible and resistant snails were treated with U0126 (1 µM or 10 µM), for 20 min prior to protein extraction and phosphorylatedERK (p-ERK) detection using western blotting with anti-phosphor-p44/42 (Thr202/Tyr204) MAPK primary antibodies (New England BioLabs). Unexposed haemocytes (C) and unexposed mammalian (M) cells (line HC60) are also shown. The blot was subsequently stripped and re-probed for actin to confirm equal amount of protein was present in each haemocyte sample. The blot is representative of results obtained in two independent experiments.

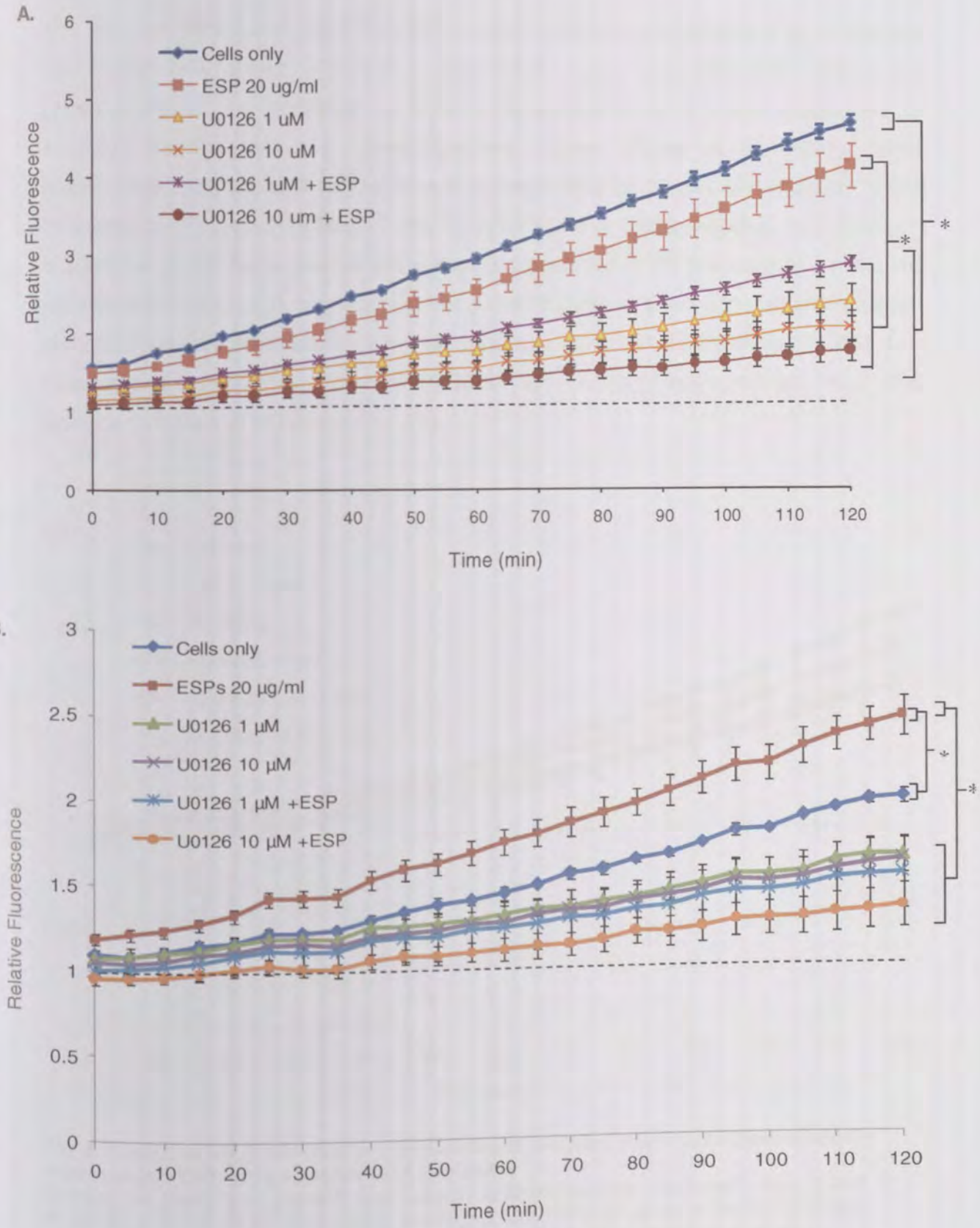

Fig. 5.8 Relative NO production in susceptible and resistant snail haemocytes following exposure to U0126, a MEK1/2 inhibitor

An equal number of haemocytes ($3x10^3$ cell per well) from susceptible (A) and resistant snails (B) were pre-incubated for 20 min with U0126 (1 µM or 10µM) before being exposed to ESPs (20 µg/ml). Mean relative fluorescence is shown for each time point (±SEM, n=3) as a proportion of background fluorescence (no DAF-FM probe) indicated by a dotted line. The data shown is from one assay and is representative of three different assays carried out on separate days.* $P \leq 0.05$ when compared to difference in mean fluorescence values between treatments at 2 h.

The selective MEK1 inhibitor, PD98059, was also investigated for its effects on *B. glabrata* haemocyte NO output. Previous studies have shown that PD98059 inhibits the phosphorylation and activation of ERK in lipopolysaccharides (LPS) challenged *L. stagnalis* haemocytes in a dose-dependent manner (Plows *et al.*, 2004). Here, haemocytes from susceptible snails were pre-incubated for 20 min with 1 µM or 10 µM PD98059 before being challenged with 20 µg/ml ESPs or CBSS-only (Fig. 5.9). Although a decrease in the mean relative NO output was observed in the presence of 1 µM or 10 µM PD98059 (1.1 to 0.6, respectively) compared to controls, the change was not deemed statistically significant (data from two different assays conducted on different days). Co-treatment of haemocytes with both PD98059 and 20 µg/ml ESPs did not result in a significant decline in NO production (Fig. 5.9).

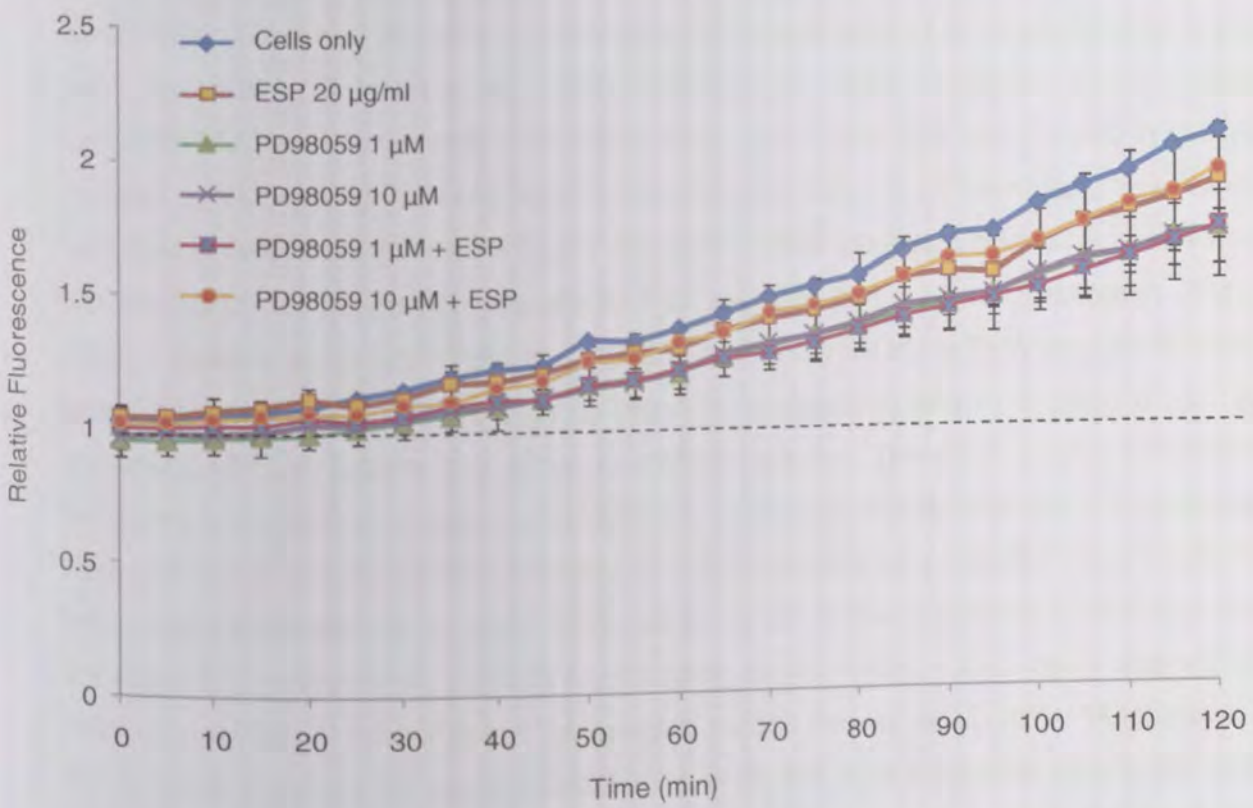

Fig. 5.9 Relative NO production in susceptible and resistant snail haemocytes following exposure to PD98059, a selective MEK 1 inhibitor
Haemocytes ($3x10^3$ cells per well) from susceptible snails were pre-incubated for 20 min with PD98059 (1 µM or 10µM) before being challenged with ESPs (20 µg/ml) or left in CBSS alone. Mean relative fluorescence is shown for each time (±SEM, n=3) point as a proportion of background fluorescence (no DAF-FM probe) indicated by a dotted line. The data shown is from one assay and is representative of two different assays carried out on separate days.

5.3 Discussion

The effect of *S. mansoni* ESPs on *B. glabrata* haemocyte NO production was found to be strain specific; haemocytes from resistant snails displayed significantly increased NO output following ESP-challenge, while haemocytes from susceptible snails expressed a slight reduction in NO levels. Furthermore, U0126, an inhibitor of ERK signalling reduced NO output in both susceptible and resistant snail haemocytes, demonstrating that NO production is partly under the control of ERK signalling, which is consistent with earlier work on *L. stagnalis* haemocytes (Wright *et al.*, 2006).

A highly sensitive NO probe (DAF-FM diacetate), which detects intracellular NO, was used in this study. This probe has been previously used in other invertebrate models including: the squid, *Euprymna scolopes*, the moth, *Manduca sexta,* the sea squirt, *Ciona intestinalis* and the snail *L. stagnalis* (Collmann *et al.*, 2004; Davidson *et al.,* 2004, Wright *et al.*, 2006; Comes *et al.,* 2007). In molluscs, NO levels have been mainly studied in the nervous system (Elofsson *et al.*, 1993; Moroz *et al.*, 1994). Franchini *et al.,* (1995) identified NOS activity in molluscan haemocytes and found that this enzyme could be induced by stimulating the cells with *Escherichia coli.* Other NOS stimulators have been identified including the potent PKC activator, PMA, which induces a significant increase in NO production in *M. galloprovincialis* and *L. stagnalis* haemocytes (Arumugam *et al.,* 2000; Tafalla *et al.*, 2002; Wright *et al.*, 2006). In the current study PMA also significantly increased NO levels in *B. glabrata* haemocytes, suggesting that PKC plays a part in controlling NO production in a range of mollusc species. The β1,-3 glucan, laminarin, increased NO output in *M. galloprovincialis* and *L. stagnalis* haemocytes but in the present work did not affect *B. glabrata* haemocyte NO generation when similar concentrations were used (Arumugam *et al.*, 2000; Wright *et al.*, 2006). In this current study, LPS also did not affect NO output in *B. glabrata* haemocytes; similar findings have been reported in haemocytes from *L. stagnalis* and *M. galloprovincialis* (Novas *et al.,* 2004; Wright *et al.,* 2006). Nevertheless, LPS can increase NO levels in the clam *Ruditapes decussates* and increase NOS activity in the mollusc, *Viviparus ater* (Franchini *et al.,* 1995; Tafalla *et al.,* 2003; Novas *et al.,* 2004; Wright *et al.*, 2006). Zymosan A, a protein-carbohydrate complex, isolated from the yeast *Saccharomyces cerevisiae*, did not affect NO levels in *B. glabrata* haemocytes, in this study, but has been previously shown to increase NO output in *L. stagnalis* haemocytes by 2-fold (Wright *et al.*, 2006). These findings imply that haemocytes may recognise PMA, laminarin, LPS and zymosan differentially and that NOS responses to them may be species specific.

Oxygen-dependent killing mechanisms in *B. glabrata* haemocytes play a crucial role in killing schistosome sporocysts (Hahn *et al.*, 2000; Hahn *et al.*, 2001a; Hahn *et al.*, 2001b; Bayne, 2003). It has been hypothesised that differences exist between susceptible and resistant *B. glabrata* strains in their oxygen-dependent killing mechanisms (Bayne, 1990). In this study we have highlighted that NO production is differentially affected in the two *B. glabrata* strains in the presence and absence of *S. mansoni* ESPs. Haemocytes from resistant snails increased NO output following 5 h ESP-challenge compared to controls, while haemocytes from susceptible snails generally displayed a slight decrease in relative NO levels. *Schistosoma mansoni* ESPs contain H_2O_2 scavenging activity and NOS inhibitors as well as other unknown compounds that affect or counteract molluscan haemocyte functions (Wu *et al.*, 2009). The balance between the host's ability to defend itself against parasitic infection and the schistosomes' counteraction mechanisms may explain the existence of susceptibility *vs.* resistance (Bayne and Yoshino, 1989).

Basal levels of NO were significantly different between the two snail strains, with haemocytes from susceptible snails producing relatively more NO over time. The reason for the basal NOS activity in extracted haemocytes is unknown; similar basal activities were also observed previously in extracted *L. stagnalis* haemocytes (Wright *et al.*, 2006). Earlier studies have shown haemocytes from *B. glabrata* resistant strains maintain higher levels of intracellular superoxide and H_2O_2 when stimulated with *S. mansoni* ESPs and PMA respectively, compared to a susceptible strain (Connors and Yoshino, 1990; Bender *et al.*, 2005). Interestingly, Hahn *et al.*, (2000) reported no differences in the relative production of ROS in haemocytes from susceptible and resistant snail strains following stimulation with carbohydrates known to be present on the schistosome surface *(Hahn et al.*, 2000). A recent study by Humphries *et al.*, (2008) reported no modulation of H_2O_2 in resistant haemocytes exposed to *S. mansoni* ESPs (Humphries and Yoshino, 2008).

Human recombinant interleukin-2 (IL-2), a known NO stimulant for mammalian macrophages, was found to considerably enhance NO production in *M. galloprovincialis* haemocytes by approximately 13-fold, an effect which was reduced in the presence of a PKA inhibitor (Novas *et al.*, 2004). Therefore, a cAMP-dependent protein kinase and PKC, which is an important NO regulator in *L. stagnalis* haemocytes may play an important role in NO generation in molluscs (Novas *et al.*, 2004; Wright *et al.*, 2006). In this study, a MEK inhibitor, U0126, significantly reduced ERK phosphorylation in haemocytes and subsequently NO output in susceptible and resistant *B. glabrata* haemocytes. This is consistent with ERK signalling playing a role in NO output through NOS regulation, similar to that reported in *L. stagnalis* haemocytes (Wright *et al.*, 2006). However, a selective inhibitor for MEK1, PD98059, that has previously been shown to influence *L. stagnalis*

haemocytes (Plows *et al.*, 2004) did not significantly down regulate NO production in susceptible *B. glabrata* haemocytes. This indicates that MEK2-like proteins may be more important than MEK-1-like proteins in NO regulation in these cells. It is also possible that PD98059 may not fully recognise molluscan MEK; further investigation is required to clarify the involvement of different MEK isoforms in NO regulation, and gene silencing may aid research on this topic.

Haemocytes from susceptible *B. glabrata* challenged with ESPs displayed significantly reduced ERK phosphorylation (refer to Chapter 3). In the current investigation, haemocytes from the susceptible snails generally had slightly reduced mean NO levels following ESP exposure, a finding that is consistent with the inhibitory effect of ESPs on ERK signalling in haemocytes from susceptible snails. The reduced NO output in the presence of U0126 could possibly be the result of effects of U0126 on ERK-like protein(s) not recognised by the anti-phosphorylated ERK antibody; indeed only one ERK like protein was detected by this antibody in *B. glabrata* haemocytes extracts, whereas two ERK isoforms were sometimes detected in *L. stagnalis* haemocytes (Wright *et al.*, 2006). The increased NO output observed in resistant snail haemocytes following ESP exposure might be a consequence of the sustained ERK phosphorylation previously seen in these cells under ESP-challenge (Chapter 3). In addition, ESPs may also be influencing the activities of other cell signalling pathways, such as those involving PKC or PKA which may modulate intracellular NO production; such interactions are worthy of further investigations.

Given the cytotoxic effects of NO, long-term effects of ESPs on NO production in haemocytes from both snail strains may influence survival of invading schistosomes *in vivo*. In the present *in vitro* study, 5 h assays revealed that susceptible snail haemocytes produce more intracellular NO under basal conditions than those of resistant snails. Why this phenomenon exists is currently unknown, but may involve the haemocytes maintaining different intracellular NO equilibria. Intracellular ROS equilibria are known to play an important role in allowing long-term survival of the host and parasite; in the case of malaria, increased oxidative stress in the host's erythrocytes can lead to exacerbated disease progression, while partial inhibition of macrophage NO production by *Toxoplasma gondii* enhances the parasite's survival rate (Luder *et al.*, 2003; Becker *et al.*, 2004). Furthermore, an intracellular NO equilibrium may be physiologically important; mammalian models have shown intracellular NO and H_2O_2 have the capacity to either promote cell death or cell survival (Fig. 5.10). Thus, depending on the environment of the cell and the type of cell, NO and H_2O_2 can regulate a number of important physiological responses,

including cell survival, by modulating certain cell signalling pathways or by regulating post-translational modification of proteins (Nagy *et al.*, 2007; Veal *et al.*, 2007).

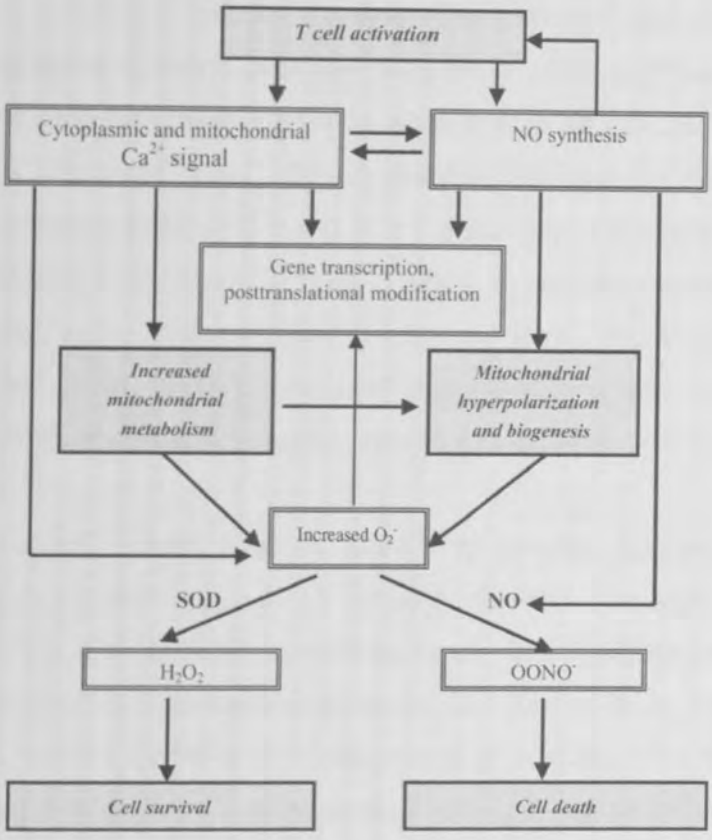

Fig. 5.10 A flow of events that occurs in an activated mammalian T cell
The quantity of H_2O_2 and $OONO^-$ produced in the cell dictates whether the cell will survive or undergo cell death. Increased levels of O_2^- levels are converted into H_2O_2 by the enzyme superoxide dismutase (SOD) (Nagy *et al.*, 2007).

Chapter 6: *Biomphalaria glabrata* cDNA microarray analysis

6.1 Introduction

Variation in schistosome-susceptibility between *Biomphalaria glabrata* strains, isolated from different geographic locations, was first reported in 1949, by Flies and Cram. It was hypothesised at the time that these variations were due to differences in genetic material between the snails (Richards, 1987). Cross breeding between schistosome-susceptible and schistosome-resistant snail strains led to the discovery of a single dominant genetic factor that could induce resistance (Richards, 1970). A number of phenotypes that give rise to susceptibility were later identified (Richards, 1975). More recent experiments carried out by Jones *et al.*, (2001) concluded that resistance was variable, with snails exhibiting a wide range of cercarial shedding counts (Jones *et al.*, 2001).

Current research is still aimed towards identifying genetic markers associated with schistosome-resistance (Lewis *et al.*, 2001; Jones *et al.*, 2001). To date, random amplified polymorphic DNA-PCR (RAPD-PCR) techniques have identified DNA fragments of 1.2 kb and 1 kb in size, present in a resistant snail strain only (Knight *et al.*, 1999). On the other hand, Spada *et al.*, (2002) identified DNA fragments of 4.06 kb, 1.75 bp, 1.1 bp and 1.1 kb, present only in a susceptible *B. glabrata* strain using the same technique but different arbitrary primers. Further information on these DNA fragments cannot yet be obtained due to limited *B. glabrata* sequence information. The development of suppression subtractive hybridization (SSH) techniques has partly overcome this problem; this technique does not rely on prior sequence knowledge or sequencing data. In simple terms, SSH involves hybridising cDNA samples from resistant and susceptible snails, the non-hybridised cDNAs are then cloned and investigated further (Lockyer *et al.*, 2006; Bouchut *et al.*, 2007; Lockyer *et al.*, 2007). This technique was used to identify a novel *B. glabrata* albumen gland gene product (AGGP) found to be elevated in resistant snails infected with *Schistosoma mansoni* miracidia (Miller *et al.*, 1996). Studies using differential display techniques have identified heat shock protein 70 (HSP70) and a cytochrome p450-like molecule to be down-regulated in schistosome-infected resistant snails (Laursen *et al.*, 1997; Jones *et al.*, 2001).

The use of haemocyte cDNA libraries from schistosome-infected resistant snails has lead to the identification of a genetic transcript similar to that for *Escherichia coli* transposase Tn5 which is associated with creating instability in the genome (Zhou and Reznikoff, 1997; Goryshin and Reznikoff, 1998). Later investigations using cDNA libraries from haemocytes extracted from infected and non-infected susceptible and resistant *B.*

glabrata lead to the discovery of genes with homology to fibrinogen-related proteins (FREPs), ferritin, and serine protease HtrA2 (Lockyer *et al.*, 2007). These genes are known to play a role in host defence responses and were down-modulated following *S. mansoni* infection (Lockyer *et al.*, 2007; Adema *et al.*, 1997; Hertel *et al.*, 2005).

Knight *et al.*, (1998) created a number of expressed sequence tags (ESTs) from genomic DNA isolated from *B. glabrata* and *S. mansoni* to identify closely related genes between the snail and the parasite (Knight *et al.*, 1998). ESTs are expressed genes which are sequenced to provide short stretches of nucleotides (200-800 bp) that can be used to generate a database of cDNA libraries (Nagaraj *et al.*, 2007). Using similar methods, Raghavan *et al.*, (2003) identified a number of novel genes expressed in haemocytes extracted from schistosome-infected resistant snails; including an enzyme with homology to reverse transcriptases (Raghavan *et al.*, 2003). ESTs have a number of limitations including under-representation of rare transcripts, redundancy of transcripts, only being able to account for ~60% of an organism's genome and being prone to a high sequence error, especially if the 3 prime or 5 prime end of the RNA is sequenced (Nagaraj *et al.*, 2007) The development of open reading frame ESTs (ORESTES), which involves sequencing the middle region of the cDNA, rather than the ends, has lead to less un-translated and better quality *B. glabrata* sequences (Lockyer *et al.*, 2007). This technique has been used to create ORESTES libraries from different snail tissues including the head-foot region, brain, ovotestis, haemocytes and haemopoietic organ from susceptible and resistant snail strains, either exposed or not exposed to *S. mansoni* for 2-24 h. A total of 41 ORESTES libraries have been created with 1843 non-redundant sequences ranging from 80-1068 bp (Lockyer *et al.*, 2007).

The growing number of *B. glabrata* libraries: ORESTES, ESTs differential display and SSH has lead to the development of a novel *B. glabrata* cDNA microarray (Lockyer *et al.*, 2008). The array currently contains a total of 5000 (plus controls) cDNA clones spotted in duplicate from different schistosome-susceptible and schistosome-resistant *B. glabrata* tissues, either exposed or not exposed to *S. mansoni* over 2, 4, 6, 8, and 24 h *(Lockyer et al.*, 2008). This custom-built array (GAPSII, Corning, printed at the Microarray Facility at Department of Pathology, Cambridge University, UK) was used in the following experiments that aimed to establish the effects of *S. mansoni* ESPs on gene expression in haemocytes drived from either susceptible or resistant *B. glabrata.*

6.2 Results

6.2.1 Differential gene expression in schistosome-susceptible and schistosome-resistant snails following ESP-challenge

Haemocytes pooled from either schistosome-susceptible (1742 strain) or schistosome-resistant (3017 strain) snails were challenged with 20 µg/ml *S. mansoni* ESPs for 1 h prior to mRNA extraction, amplification (by creating cDNA) and microarray hybridisation (as described in Materials and Methods, section 2.11-2.14). Differentially expressed genes (DEGs), between the phenotypes, were then identified from the microarray analysis using a significance level of $P \leq 0.05$ (n=4). A total of 58 DEGs were identified in resistant snail haemocytes and 40 DEGs in susceptible snail haemocytes, with 61% having unknown identity or BLASTX sequence alignments (Table 6.1; Fig 6.1). Certain genes were present in duplicate on the array: HSP70 was identified by two ORESTES sequences (CK656737, CK656707), elongation factor was identified by two SSH sequences (EW997555, EW997067), whereas matrilin (CK656770, CK988725) and paramyosin (CO870407, EW996929, CO870250 and CK656849) were identified by both SSH and ORESTES sequences.

Genes that exhibit similar sequences were detected using SEQtools clustering software analysis; this software analyzes similarity between gene sequences using BLASTX score values. Table 6.2 shows a number of known and unknown genes that formed clusters. Genes with homology to haemagglutinin and dermatopontin clustered together as well as cathepsin D and cathepsin L, while theromacin and matrilin clustered with unknown DEGs. A large cluster of 6 unknown DEGs was also identified from the analysis (25 genes was identified in 10 different clusters).

The function of identified DEGs was investigated using Goblet (GO) software analysis; this software annotates gene products from different model organisms, it uses vocabulary that can be applied to all eukaryotes and groups products according to, biological process, molecular function and cellular component (Williams and Andersen, 2003). From the analysis the resistant snail haemocytes expressed more genes with associated gene ontologies compared to susceptible haemocytes (Fig. 6.2). The largest GO categories represented by identified genes from the resistant haemocytes were cellular processes and physiological processes with 13 and 14 gene probes associated with these ontologies, respectively. Furthermore, resistant snail haemocyte DEGs had nucleic, ion-, protein- and nucleotide-binding properties, while susceptible snail haemocyte DEGs had ion and nucleotide-binding properties. Gene ontologies only associated with DEGs in haemocytes from susceptible snails were for metabolism and protein complex formation.

While gene ontologies found for DEGs only in the resistant snail haemocyte sample were associated with cell stress, cell adhesion, translation regulatory activity, ligase and transferase activity (Fig. 6.2).

Using BLASTx alignment, 20 DEGs were identified as being homologous to other known proteins in the resistant sample and 12 DEGs in the susceptible snail sample (Fig. 6.1). Genes associated with immune mechanisms, such as NF-κB and HSP70 were differentially expressed in resistant snail haemocytes by approximately 3-fold and 2.4-fold (average for 4 arrays), respectively. Theromacin was differentially expressed 3.7 fold in susceptible snail haemocytes. Genes associated with protein transcription and degradation including elongation factors and cathepsin D were identified in both susceptible and resistant snail haemocytes. Finally, genes associated with cell-to-cell interactions and cell communication were identified mainly in the susceptible haemocyte sample, these genes include dystrophin, matrilin and actin (possessing differential expression values of 2-2.5).

The Kyoto Encyclopaedia of Genes and Genomes (KEGG) database and associated software was used to investigate the function of DEGs using genome information. Specifically the KEGG pathway database reconstructs protein interaction networks that are responsible for various cellular processes, including networks of enzymes and gene regulatory networks (Kanehisa *et al.*, 2004). Analysis identified three DEGs for enzymes present in the susceptible snail haemocyte sample, whose function may be involved in metabolism and cellular processes including cellular communication and the immune system (Table 6.3). In contrast, seven enzymes were identified in the sample from resistant snail haemocytes that may be involved in genetic information processing (protein degradation, sorting and folding) as well as enzymes involved in cell communication, cell motility and immunity (Table 6.3).

Gene Bank Accession Number	SSH/ORESTES/EST	Mean Value (n=4)	SD of arrays (n=4)	BLASTX alignments	N° of Clones	E value	Organism
EW997105	SSH	-4.9935	0.411754	Streptavidin v2 precursor	1	9.00E-14	*Burkholderia thailandensis*
EW997087	SSH	-5.273	0.415436	Unknown	1		
CO870321	ORESTES	-3.8115	0.58644	Unknown	1		
EW997485	SSH	-2.959	0.808079	Unknown	1		
CV548474	ORESTES	-4.91467	1.007972	Unknown	1		
CK149390	ORESTES	-3.1425	0.565712	Unknown	1		
DY523254	SSH	-2.93725	0.75957	Unknown	1		
EW996998	SSH	-3.0665	0.603358	Unknown	1		
CO870356	ORESTES	-2.62275	0.77555	Unknown	1		
DY523255	SSH	-2.56525	0.660635	Unknown	1		
EW997096	SSH	-3.58	0.37775	Unknown	1		
CK656646	ORESTES	-2.49	0.500734	Unknown	1		
EW997397	SSH	-2.76075	0.257719	Unknown	1		
CV548501	ORESTES	-2.51825	0.921622	Peritrophic matrix insect intestinal mucin	1	7.00E-07	*Plutella xylostella*
EW997226	SSH	-2.5315	0.170541	Unknown	1		
EW996827	SSH	-3.07067	0.450283	Transmembrane protease serine 2	1	8.00E-23	*Homo sapiens*
EW997449	SSH	-3.138	0.707339	Unknown	1		
EW997199	SSH	-3.115	0.655468	Unknown	1		
EW996825	SSH	-3.05975	0.744176	Unknown	1		
EW997445	SSH	-2.566	0.251597	Unknown	1		
EW997106	SSH	-3.01175	0.798864	Unknown	1		
CK989609	EST	-1.98025	0.484119	unknown	1		
Unknown (ZB9620)	ORESTES	-2.45775	0.286745	Nuclear factor NF-kB1	1	3.00E-19	*Saccoglossus kowalevskii*

Table 6.1 List of resistant snail haemocyte and susceptible snail haemocyte DEGs following ESP-challenge
DEGs ($P \leq 0.05$, n=4) identified from the microarray analyses are listed; the normalised expression ratios and the standard deviation (±SD) of the genes are presented. A negative value represents differential expression in resistant snail haemocytes, while a positive value represents differential expression in susceptible snail haemocytes. The number of clones identified and whether it was ORESTES, SSH, or ESTs is also indicated. The gene sequences of DEGs were aligned using BLASTX software (significance E value of <0.5) with unknowns listed as those considered to not have significant alignments. Gene alignments not identified have been previously characterised (Raghavan *et al.*, 2003; G. Mitta *et al.*, 2005; Lockyer *et al.*, 2008). † Alignments were provided by Mitta G. or Adema CM

Gene Bank Accession Number	SSH/ORESTES/EST	Mean Value (n=4)	SD of arrays (n=4)	BLASTX alignments	N° of Clones	E value	Organism
CK656893	ORESTES	-2.31975	0.257711	Unknown	1		
EW997345	SSH	-2.48075	0.367973	Unknown	1		
CK656737	ORESTES	-2.432	0.838419	Heat shock protein 70	2	7.00E-68	*Metapenaeus ensis*
EW997555	SSH	-2.5485	0.933284	Elongation factor-2	2	7.00E-32	*Mus musculus*
EW997560	SSH	-2.724	0.914381	Nas-14 protein	1	9.00E-11	*Caenorhabditis elegans*
CK149567	ORESTES	-2.10575	0.205058	Hypothetical protein PH0216.1n	1	1.00E-27	*Magnetospirillum gryphiswaldense*
CV548449	ORESTES	-2.09667	0.208205	COG0457: FOG: TPR repeat	1	3.00E-16	*Philodina roseola*
CK988686	EST	-2.30825	0.347312	Hemagglutinin/amebocyte aggr (also corresponds to dermatopontin 2)	1	1.00E-54	*Biomphalaria glabrata*
CV548443	ORESTES	-3.047	1.332662	Unknown	1		
CK656890	ORESTES	-2.04475	0.183669	Unknown	1		
CK656733	ORESTES	-2.5595	0.697224	Ubiquitin-conjugating enzyme E2D 2	1	8.00E-18	*Homo sapiens*
EW997053	SSH	-2.65375	1.012909	Elongation factor 1α	1	5.00E-12	*Neosartorya fischeri*
CK656770	ORESTES	-2.59225	0.894643	Matrilin	2	1.00E-27	*Biomphalaria glabrata*
CK989655	EST	-2.0535	0.646143	Dystrophin	1		†
EW997561	SSH	-2.334	0.742165	Uncharacterized conserved	1	1.00E-17	*Spirosoma linguale*
CV548492	ORESTES	-2.9755	1.408958	Unknown	1		
CK988910	EST	-2.10175	0.284914	unknown	1		
EW997432	SSH	-2.311	0.909836	Unknown	1		
EW997374	SSH	-2.10825	0.560787	Unknown	1		
EW997424	SSH	-2.4835	0.968646	Unknown	1		
CV548469	ORESTES	-2.25875	1.059946	Unknown	1		
CK988741	EST	-2.17925	0.378944	Dermatopontin 2	1	1.00E-40	*Biomphalaria glabrata*
DY523259	SSH	-2.08675	0.614187	Unknown	1		
CK656903	ORESTES	-2.05125	0.67108	Unknown	1		
EW997342	SSH	-2.28675	1.035171	Actin	1	1.00E-48	*Brachionus plicatilis*

Table 6.1 Continued

Gene Bank Accession Number	SSH/ORESTES/EST	Mean Value (n=4)	SD of arrays (n=4)	BLASTX alignments	N° of Clones	E value	Organism
EW997546	SSH	-1.951	0.439292	Unknown	1		
EW997618	SSH	-2.6805	1.311945	Unknown	1		
CV548156	ORESTES	-1.9675	0.4474	Neural and ectodermal dev factor	1	8.00E-11	*Drosophila melanogaster*
EW997073	SSH	-2.80075	1.388963	Unknown	1		
EW997064	SSH	-2.4985	1.250547	40S ribosomal protein S9	1	5.00E-48	*Gryllus rubens*
CV042579	EST	-2.05175	0.810563	Unknown	1		
CV548460	ORESTES	-2.955	1.865209	Unknown	1		
DW474845	EST	2.58375	1.109871	HRAS-like suppressor 3	1	1.00E-17	*Perca flavescens*
CK386795	EST	2.31625	0.74807	Unknown	1		
EE723175	EST	2.316	0.659444	Unknown	1		
CN445888	EST	2.27625	0.628247	Cathepsin D	1	3.00E-33	*Todarodes pacificus*
DW474412	EST	2.328	0.5876	Angiotensin-converting enzyme	1		†
EE723025	EST	2.21125	0.439949	Unknown	1		
EE723156	EST	2.56925	0.763537	Unknown	1		
CO870292	ORESTES	2.6185	1.149096	Unknown	1		
DW473937	EST	2.594	0.791807	Phospholipase B	1		†
DW474522	EST	2.24425	0.334588	Unknown	1		
CO635865	EST	2.3905	0.522666	Cathepsin L	1		†
CK989051	EST	1.96825	0.262707	Hypothetical protein	1	7.00E-08	*Xenopus (Silurana) tropicalis*
CK989407	EST	2.376667	0.219101	Hypothetical protein	1		†
CO635883	EST	2.876	0.550452	Unknown	1		
CV548309	ORESTES	2.53475	0.431158	Unknown	1		
CV548643	ORESTES	2.87025	0.279098	Unknown	1		
DW474665	EST	2.49525	0.173127	Unknown	1		
DW474511	EST	2.795	0.397936	Unknown	1		
DW474747	EST	2.3275	0.239884	Unknown	1		

Table 6.1 Continued

Gene Bank Accession Number	SSH/ORESTES/EST	Mean Value (n=4)	SD of arrays (n=4)	BLASTX alignments	N° of Clones	E value	Organism
EW996804	SSH	2.6805	0.263844	Unknown	1		
CK149267	ORESTES	2.247	0.570942	Unknown	1		
CK988781	EST	2.59475	0.47925	unknown	1		
EW996739	SSH	2.66825	0.23647	ATP synthase-like protein	1	2.00E-07	*Choristoneura parallela*
CK327222	EST	3.399	0.567906	Unknown	1		
CK656849	ORESTES	3.188333	0.485923	Paramyosin	4	2.00E-05	*Mytilus galloprovincialis*
CK800787	EST	2.964	0.14239	Unknown	1		
CN013302	EST	2.9525	0.141611	Unknown	1		
EW996791	SSH	2.85375	0.313893	Unknown	1		
DW473910	EST	3.1875	0.299939	Unknown	1		
CV548134	ORESTES	2.74375	0.510712	Unknown	1		
CK989034	EST	2.992	0.400223	Cytidin deaminase	1	9.00E-76	*Biomphalaria glabrata*
CV548292	ORESTES	4.43525	0.766136	Unknown	1		
CK989169	EST	2.61125	1.883563	Unknown	1		
CK989160	EST	3.25375	0.578687	Intrinsic factor precursor	1	0.002	*Monodelphis domestica*
CK989482	EST	3.5885	0.405136	Unknown	1		
CK989131	EST	3.65725	0.521235	Theromacin precursor	1	3.00E-16	*Aplysia californica*
CO654043	EST	4.967333	0.330778	Unknown	1		
CK988968	EST	4.191	0.571586	Unknown	1		

Table 6.1 Continued

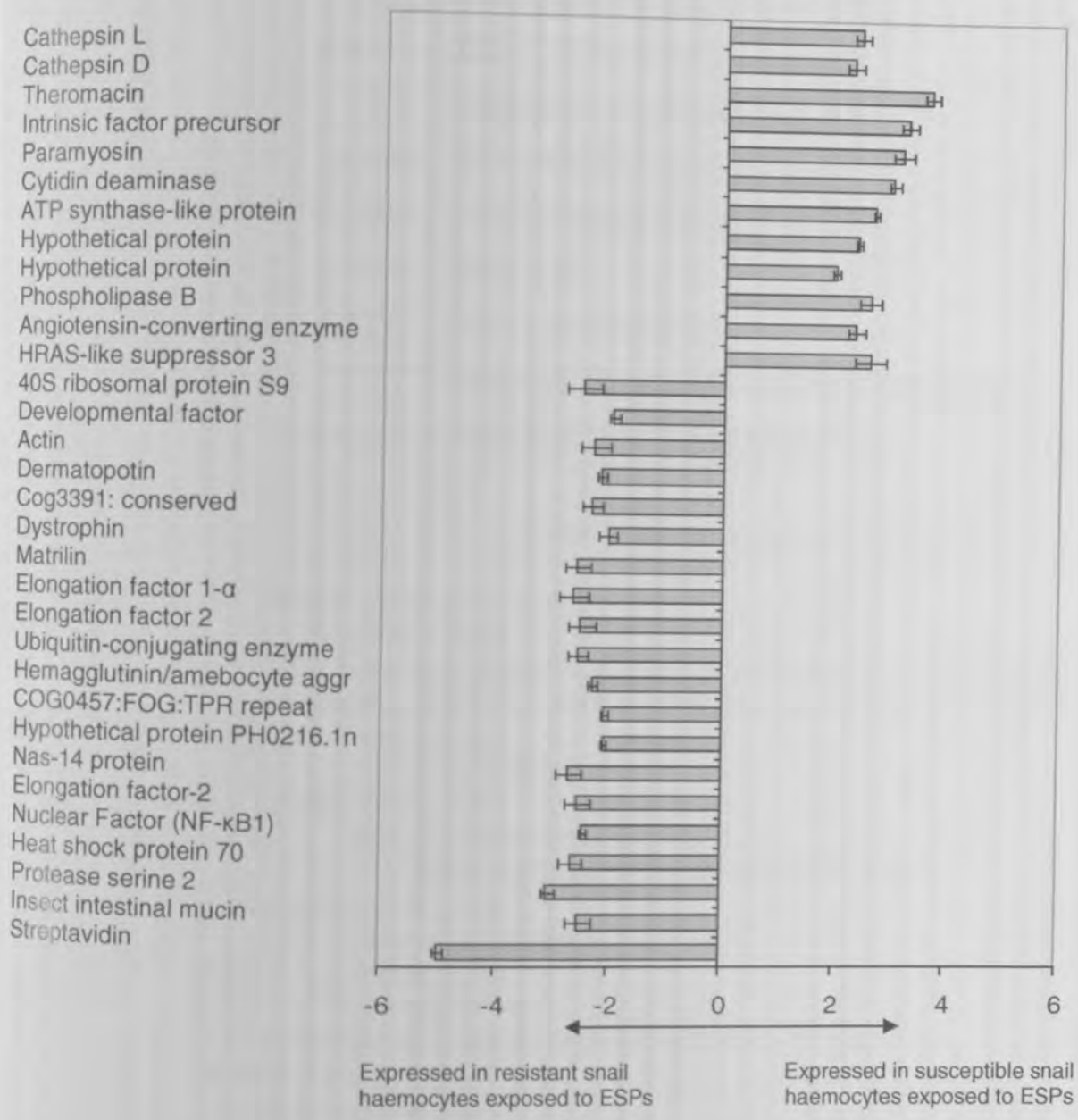

Fig. 6.1 Identified DEGs and their fold difference in expression between resistant and susceptible snail haemocytes following ESP-challenge
Susceptible or resistant *B. glabrata* haemocytes were exposed to 20 µg/ml *S. mansoni* ESPs for 1 h prior to mRNA extraction, amplification by converting the mRNA into cDNA, and microarray hybridisation. Microarray analysis was carried out using GenePix Pro 6.1 image analysis and the arrays were normalised using Acuity 4.0 software. Each bar represents an identified DEG ($P \leq 0.05$, n=4); a positive value indicates the gene is expressed differentially in susceptible snail haemocytes, whereas a negative value indicates the gene is expressed differentially in resistant snail haemocytes. The value 0 indicates constant gene expression in both samples. The SEM is represented as an error bar for each DEG.

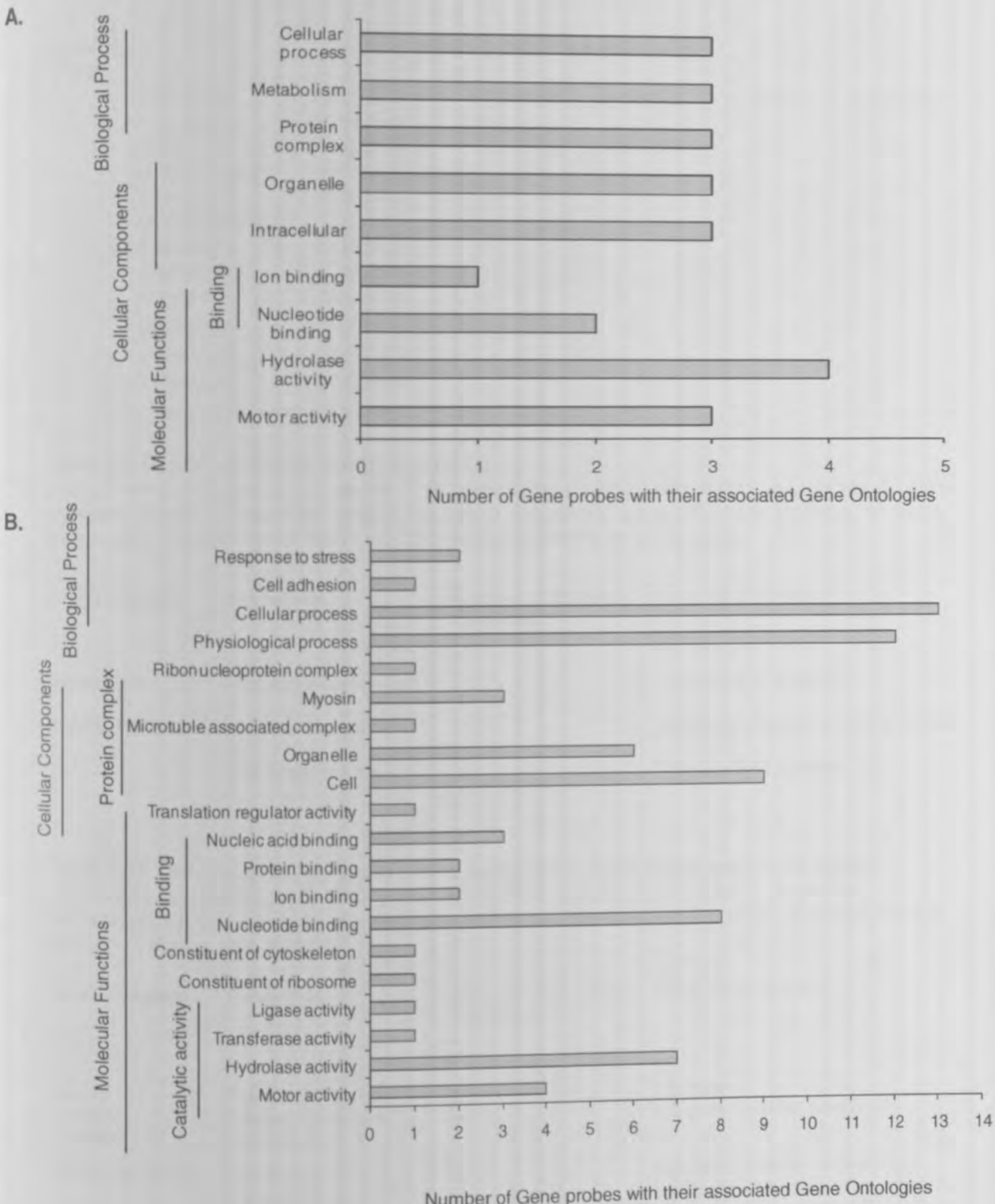

Fig. 6.2 Genes with their associated gene ontologies following Goblet software analysis

Genes identified as being significantly differentially expressed from the microarray analysis were further investigated using GO software. The gene products were characterised according to GO ontologies: molecular function, cellular components and biological process. The graphs show functional annotation of DEGs and the number of gene probes with their associated gene ontology in DEGs from (A) susceptible and (B) resistant snail haemocytes.

Cluster Number	Gene Bank Accession number					
6	CK149390	CO870321	DY523254	DY523255	EW996998	EW997485
3	EW996825	EW997106	EW997449			
2	CK988686 (Hemagglutinin)	CK988741 (Dermatopontin)				
2	CK989131 (Theromacin)	CK989482				
2	CN445888 (Cathepsin D)	CO635865 (Cathepsin L)				
2	CV548443	CV548492				
2	CK800787	DW474511				
2	DY523259	EW997374				
2	CV548309	EW996804				
2	CK656770 (Matrilin)	CK988725				

Table 6.2 Sequence cluster analysis of DEGs
DEGs from susceptible and resistant snail haemocytes identified from the microarray results were clustered together based on similar nucleotide sequences using SEQtools software. If similar genes were identified more than once, they were removed from the analysis.

KEGG category	Processes	Number of Enzymes Identified	Function of Enzyme
Metabolism	Nucleotide Metabolism	1	Pyrimidine metabolism
Cellular Processes	Immune System	1	Antigen processing and presentation
	Cell Communication	1	Tight junction formation

Table 6.3A Pathway analysis using KEGG software for susceptible haemocyte genes
DEGs were categorised according to their biochemical pathways using KEGG categories: metabolism, genetic information and cellular processes. The number of enzymes identified and the function of the enzyme is listed.

KEGG category	Processes	Number of Enzymes Identified	Function of Enzyme
Genetic Information Processing	Translation	1	Ribosome
	Folding, Sorting and Degradation	1	Ubiquitin mediated proteolysis
Cellular Processes	Cell Motility	1	Regulation of actin cytoskeleton
	Immune System	1	Leukocyte transendothelial migration
	Cell Communication	3	Focal adhesion Adherent junctions Tight junction

Table 6.3B Pathway analysis using KEGG software for resistant haemocyte genes DEGs were categorised according to their biochemical pathways using KEGG categories: metabolism, genetic information and cellular processes. The number of enzymes identified and the function of the enzyme are listed.

Using the same cDNA microarray, Lockyer *et al.*, (2008) recently investigated gene expression in haemocytes obtained from susceptible and resistant *B. glabrata* infected with 10 *S. mansoni* for 2-24 h. In collaboration with Lockyer, DEGs that were found in each study were compared using a sequence clustering analysis method (susceptible and resistant DEGs were combined). A total of 27 gene clusters were identified from this analysis, indicating that similar genes may be differential expressed in haemocytes exposed to ESPs compared to haemocytes extracted from infected snails (Table 6.4). Genes with known homology that were identified from the analysis included: HSP70, elongation factor 2, elongation factor 1α, ubiquitin-conjugated enzyme, serine transmembrane protease and a streptavidin precursor; these genes were specific to resistant snail strains.

Cluster Number	Gene Bank Accession number and Cluster Group					
6	CK149390	CO870321	DY523254	DY523255	EW996998	EW997485
5	EW996825	EW996825	EW997106	EW997449	EW997449	
5	CK656734	CO870200	CO870223	CO870221	EW997004	
4	CK656737	CK656707	CK656707	CK656737 (HSP70)		
4	EW99755	EW997067	EW997067	EW997555 (Elongation factor 2)		
4	CK656849	CO870250	CO870407	EW996929		
3	EW997424	CK327222	EW997424			
2	CN445888	CO635865				
2	CK656733	CK656733 (Ubiquitin conjugated enzyme)				
2	CV548443	CV548492				
2	CO870193	CV548460				
2	CK800787	DW474511				
2	DY523259	EW997374				
2	CV548309	EW996804				
2	CK656770	CK988725				
2	EW996827	EW996827 (Serine 2 transmembrane protease)				
2	EW99705	EW997053 (Elongation factor 1α)				
2	CK988686	CK988741				
2	EW997087	EW997087				
2	EW997105	EW997105 (Streptavidin precursor)				
2	EW997432	EW997432				
2	CK656739	CK656711				
2	CK989131	CK989482				
2	1742HAEMEX14F8 (DY523267, ZB9365*)	EW997405				
2	EW997170	EW996972				
2	EW997092	EW997118				
2	EW997099	ZBA4304 (EW997194)				

Table 6.4 Sequence cluster analysis of DEGs using data obtained *in vitro* and *in vivo*
DEGs (susceptible and resistant) that were identified from the microarray analysis reported here (highlighted in red) were compared to microarray results obtained from *in vivo S. mansoni* infected snail haemocytes (Lockyer *et al.*, 2008). Genes were clustered together based on similar nucleotide sequences using SEQtools software programme. *Gene bank accession number not found

6.2.2 Investigating the profile of specific DEGs using qPCR

In order to investigate RNA expression levels, quantitative PCR (qPCR) was carried out on specific genes of interest (Table 6.4). The following susceptible snail haemocyte DEGs: DW474845 (HRAS-like suppressor 3), CN445888 (cathepsin D), CK386795 and the following resistant snail haemocyte DEGs: NF-κB, HSP70, CV548474 and EW997087 were chosen for further analysis using qPCR. These genes were chosen based on their high differential expression values obtained from the microarray. A number of other genes

of interest were not investigated due to the limited efficiencies of primers and limited template availability (30-40 snails was used for each biological sample). Pooled haemocytes from schistosome-susceptible or resistant snails were exposed to 20 µg/ml ESP for 1 h prior to mRNA extraction and cDNA synthesis (to form a stable product), three biological replicates and three technical replicates were performed and analysed.

Considerable variation in normalised gene expression (normalised to control gene CO870340, a gene that was not differentially expressed in susceptible or resistant samples) was observed between biological replicates, this was mainly due to biological variation between the samples (Table 6.5). The mean normalised expression levels varied in some biological samples by a factor of 12,000 therefore no statistically significant differences could be found between samples. General trends in gene expression levels indicated an up-regulation of HSP70 gene expression in ESP-challenged resistant and susceptible snail haemocytes compared to controls. Gene expression for NF-κB was generally similar for haemocytes of both snail strains, while CV548474 (unknown) showed a general down-regulation in resistant samples, which is inconsistent with the microarray results. The susceptible snail DEG, HRAS-like suppressor 3 also showed inconsistent results compared to the microarray data, with an increase in gene expression in resistant samples. While the susceptible DEGs Cathepsin D and CK386795 were generally expressed in ESP-challenged susceptible snail haemocytes (Table 6.5).

DEGs	Mean normalised gene expression levels	Range of values
Resistant DEGs		
Gene: HSP70		
R	0.08	0.08-0.09
RE	0.31	0.6-0.17
S	0.47	0.35-0.69
SE	0.69	0.47-1.06
Gene: NF-κB		
R	84.46	71.9-105.3
RE	92.50	98.09-105.3
S	57.43	38.9-106
SE	69.90	39.22-74.4
Gene: EW997087 (Unknown)		
R	0.00	0.0005-0.002
RE	0.00	†
S	0.01	0.001-0.015
SE	0.00	0.0007-0.0013
Gene: CV548474 (Unknown)		
R	62777.81	123626-12929.12
RE	217.80	†
S	99159.83	12.21-297332
SE	115349.79	27.99-345943.65
Susceptible DEGs		
Gene: DW474845 (HRAS-like suppressor 3)		
R	0.01	0.002-0.01
RE	2.37	4.7-0.02
S	0.02	0.0065-0.01
SE	0.02	0.01-0.03
Gene: CN445888 (Cathepsin D)		
R	0.63	0.01-1.24
RE	0.01	0.001-0.2
S	0.01	0.004-0.016
SE	0.01	0.002-0.01
Gene: CK386795 (Unknown)		
R	25.00	0.7-73
RE	1.19	0.53-73
S	290.05	0.56-867
SE	114.17	1.06-339.7

able 6.5 mRNA expression levels in haemocytes either challenged or not challenged with SPs

ranscribed RNA was isolated from pooled haemocytes from susceptible (S) or resistant (R) snails hat had been exposed to ESPs (20 µg/ml) for 1 h (E). Gene expression was analysed with gene-specific primers using qPCR and values normalised to those for the control gene CO870340. The mean normalised expression levels and the range of values obtained for three biological replicates and three technical replicates (n=6) is presented, † indicates that biological replicates failed to amplify in the qPCR reaction.

6.3 Discussion

This study aimed to compare schistosome-susceptible and resistant haemocyte gene expression following *S. mansoni* ESP-challenge. By comparing transcribed RNA from ESP-exposed haemocytes from these snail strains, DEGs were identified using two-colour cDNA microarray analyses. The *B. glabrata* genome is still to be completed; therefore, DEGs were identified using known gene homologies (using BLASTX searches against the non-redundant sequences in GenBank). Nevertheless, novel genes have been highlighted

this study as well as genes that have been previously identified in genomic studies focusing on *B. glabrata—S. mansoni* interactions. DEGs were categorised according to their potential anti-parasitic properties, whether they were involved in protein transcription or degradation, and if they were associated with cell-cell interactions; DEGs that did not fit in any of these categories were catagorised miscellaneous.

Two highly-significant DEGs were identified in ESP-exposed haemocytes from resistant snails; streptavidin precursor (EW997105) and EW997087 (unknown) were identified from the microarray analysis, with both genes exhibiting a 5-fold change in expression compared to susceptible snail ESP-exposed haemocytes. Why a streptavidin precursor was differentially expressed is unclear, haemocytes may express a protein similar to streptavidin. Interestingly, EW997105 and EW997087 were also identified as differentially expressed in haemocytes from *S. mansoni* infected snails (gene cluster analysis).

Unfortunately the qPCR results presented here are highly variable and inconclusive. Other qPCR data using mRNA from molluscs have also shown to be highly variable. Indeed only one biological replicate was used to present mRNA expression levels of schistosomin in *B. glabrata* (Zhang *et al.*, 2009; personal communication; University of New Mexico, Albuquerque). HSP70 mRNA expression levels were also found to be significantly variable in the bivalves, *Laternula elliptica* and *Nacella concinna* (Clark *et al.*, 2008a). What tissue samples were used, how the mRNA was extracted and how the qPCR was analysed can make qPCR data difficult to compare between experiments. For example Lockyer *et al.*, (2008) presented normalised gene expression levels against actin; in the current microarray investigation, actin was found to be differentially expressed. However, such differential expression of mRNA did not seem to affect actin protein levels as levels appeared stable in all experiments conducted (see Chapters 3, 4, and 7).

6.3.1 DEGs with anti-parasitic properties

A highly DEG with homology to type II transmembrane serine protease was identified in resistant snail haemocytes from the microarray analysis. This enzyme is a cell-surface membrane-anchored protein that has proteolytic activity and is capable of interacting with other cell surface proteins, soluble proteins or matrix components (Ha and Kim, 2008). The N-terminal region of this serine protease also plays an important role in intracellular signal transduction (Hooper *et al.*, 2001; Szabo *et al.*, 2008). In mammalian studies, type II transmembrane serine protease plays a crucial role in a number of physiological and developmental processes including food digestion, regulation of blood pressure and blood coagulation (Page *et al.*, 2008). Little is known about type II transmembrane serine

proteases in invertebrates; however, a serine protease zymogen factor G, has been identified in the arthropod horseshoe crab that can activate the haemolymph clotting cascade (Muta *et al.*, 1995). Previous studies have highlighted the role of proteases and protease inhibitors as anti-parasitic components in the snail host (Mitta *et al.*, 2005; Guillou *et al.*, 2007).

The DEG, CK656737, present in resistant snail haemocytes transcribes a homolog of HSP70. Heat shock proteins are involved in post-translational modifications of proteins as well as providing thermotolerance to cells following stress (discussed further in Chapter 7). Lockyer *et al.*, (2004) previously showed that HSP70 gene expression was up-regulated in resistant *B. glabrata* mantle tissue following 6 h *in vivo S. mansoni* infection using differential display (Lockyer *et al.*, 2004). In contrast, Ittiprasert *et al.*, (2009) observed that HSP70 gene expression was increased in haemocytes from *S. mansoni* infected juvenile susceptible snails (Ittiprasert *et al.*, 2009). In this study we found *S. mansoni* ESPs were capable of differentially affecting HSP70 gene expression in resistant snail haemocytes (using the microarray data analysis). Considering that HSP70 protein is a stimulator of the innate immune system in mammalian models, a similar role for HSP70 may exist in molluscs (Wang *et al.*, 2006). HSP70 protein expression levels in ESP-challenged *B. glabrata* haemocytes was subsequently investigated and is the focus of Chapter 7.

In conjunction with HSPs, several other proteins are usually synthesised following and during cellular stress, including the protein ubiquitin (Mager and De Kruijff, 1995; Mathew *et al.*, 1998). The DEG, CK656733, with homology to ubiquitin-conjugating enzyme was differentially expressed in resistant snail haemocyte samples. Ubiquitin is involved in post-transcription of proteins as well as targeting abnormal and short-lived proteins for degradation (Mathew *et al.*, 1998). Ubiquitin or polyubiquitin gene expression has been found previously to be abundant in *S. mansoni*-exposed and non-exposed resistant and susceptible snail tissue, including haemocytes (Raghavan *et al.*, 2003; Lockyer *et al.*, 2007; Lockyer *et al.*, 2008).

The transcription factor, nuclear factor-kappa B (NF-κB) plays a vital role in immune mechanisms and cellular stress. The microarray demonstrated a significant increase in NF-κB gene expression in ESP-exposed resistant snail haemocytes. A general increase in NF-κB expression may indicate a heightened state of immune alertness in resistant snails compared to susceptible snails. This is consistent with studies in mammalian models, whereby NF-κB is chronically active in certain inflammatory diseases (Li and Verma, 2002).

A novel antimicrobial peptide, theromacin, was identified as a DEG in susceptible snail haemocytes; this peptide was, originally isolated from *Theromyzon tessulatum* (Tasiemski *et al.*, 2004). Theromacin is a cationic peptide containing 10 cysteine residues with no similarities with other known antibacterial peptides (Tasiemski *et al.*, 2004). Theromacin was also found differentially expressed in *B. glabrata* haemocytes following *E. caproni* challenge (Mitta *et al.*, 2005), suggesting that it might be a response to helminth infection in general. An amoebocyte aggregation factor was identified as a DEG in resistant snail haemocytes from the microarray analysis. This protein might be involved in the encapsulation process, the coordinated killing of large foreign intruders by haemocytes. Amoebocyte aggregation factor was differentially expressed in *B. glabrata* haemocytes following *in vitro* challenge with *S. mansoni* miracidia (Raghavan *et al.*, 2003). Interestingly, *in vivo* studies have shown ameobocte aggregates can be induced in the ventricular heart cavity of *B. glabrata* by *S. mansoni* sporocysts (Lie and Heyneman, 1976).

6.3.2 DEGs involved with protein transcription and degradation

Cathepsin D is an aspartic protease with a primary function of breaking down proteins targeted to lysosomes for degradation, in addition to playing a role in apoptosis, antigen processing and hormone production (Fusek and Vetvicka, 2005; Zaidi *et al.*, 2008). Cathepsin L is known to be important for general protein degradation. Cathespin-like proteases are conserved through evolution, cathespin-like enzymes have been characterised in the squid *Todarodes pacificus* and in the slug *Deroceras reticulatum* (Walker *et al.*, 1999; Komai *et al.*, 2004). Lockyer *et al.*, (2008) showed cathepsin-like-molecules were expressed in resistant strain-specific *B. glabrata* haemocytes following *S. mansoni* infection (Lockyer *et al.*, 2008). In contrast, this study showed cathepsins D and L were differentially expressed in ESP-challenged haemocytes from susceptible snails.

Elongation factors are conserved proteins that control protein translation. Elongation factor-2 and 1α were differentially expressed in resistant haemocytes exposed to ESPs. Elongation factor-1α interacts with the cytoskeleton and binds amnio-tRNAs to 80S ribosomes (Condeelis, 1995). Elongation factor-2 catalyzes the ribosomal translocation reaction and it is regulated by elongation factor-2 kinase (Ryazanov and Spirin, 1990). Studies have shown that cellular stress causes abnormal physiology of ribosomes in molluscs reducing the capacity of cells to initiate protein synthesis (Kalpaxis *et al.*, 2003). Exposing *B. glabrata* haemocytes to *S. mansoni* ESPs has been shown to affect protein

metabolism which is consistent with this study (Yoshino and Lodes, 1988). Furthermore, elongation factor has previously been identified in resistant *B. glabrata*, and *L. stagnalis* tissue (Davison and Blaxter, 2005; Lockyer *et al.*, 2007; Lockyer *et al.*, 2008).

Cytidine deaminase which catalyzes the hydrolytic deamination of cytidine to form uridine was differentially expressed in haemocytes from susceptible *B. glabrata*. Previous studies have highlighted that cytidine deaminase is expressed in resistant *B. glabrata* following *in vivo* infections with *E. caproni* (Roger *et al.*, 2006; Bouchut *et al.*, 2007). Considering that a humoral defence system exists in molluscs, parasitic components may influence cytidine deaminase, but whether this is specific to susceptible or resistant snails is currently unclear (van der Knaap *et al.*, 1981; Bayne 2003; Pruzzo *et al.*, 2005).

6.3.3 DEGs involved with cell-cell interactions

Adhesion related genes or genes involved in cellular interactions play an important role in host-parasite interactions, especially if host haemocytes are inhibited from migrating towards or binding to the parasite (Bouchut *et al.*, 2006, Bouchut *et al.*, 2007). Dystrophin is a rod-shaped cytoplasmic protein that connects the cytoskeleton of a muscle fibre to the surrounding extracellular matrix (ECM), while dermatopontin is an ECM glycoprotein involved in cell-matrix interactions and matrix assembly (Royuela *et al.*, 1999); both proteins were differentially expressed in resistant snail haemocytes. Dermatopontin mRNA is highly up-regulated in resistant *B. glabrata* haemocytes following infection with *E. carponi* (Mitta *et al.*, 2005). Dermatopontin has sequence similarities with amebocyte aggregation factor and may be involved in the encapsulation process (Mitta *et al.*, 2005). The cytoskeletal protein actin, also differentially expressed in resistant snail haemocytes in the present study, participates in a number of physiological processes, including cellular movement. To date, actin genes have been identified and characterised in the molluscs, *Haliotis iris*, *B. tenagophila* and *B. glabrata* (Bryant *et al.*, 2006; Jannotti-Passos *et al.*, 2008).

Matrillin, differentially expressed in resistant snails following ESP-challenge, mediates interactions between collagen-containing fibrils and other matrix constituents. In mammals, the matrilin family contains epidermal growth factor-like domains and can be found mainly in the cartilage (Deak *et al.*, 1999; Wagener *et al.*, 2005). Matrilin has been found to be differentially expressed in haemocytes extracted from the zebra mussel, *Dreissena polymorpha*, following challenge with a mixture of LPS, peptidoglycan and zymogan (Xu and Faisal, 2007). This protein was found to be up-regulated in resistant *B.*

glabrata haemocytes following infection with *E. caparoni* and was later identified in *B. tenagophila* resistant snails that had been exposed to *S. mansoni* (Mitta *et al.*, 2005; Mannotti-Passos *et al.*, 2008).

Genes that have adhesion properties may influence parasite-host compatibility; *B. glabrata* that are susceptibile/resistant to *E. caparoni* showed differential expression of certain genes with homology to dermatopontin, matrilin and cadherin (Bouchut *et al.*, 2006). A highly adhesive and potent coagulation system in susceptible snail haemolymph may prevent haemocytes migrating towards the parasite and thus, reducing the snails ability to kill the pathogen (Bouchut *et al.*, 2006).

6.3.4 Miscellaneous DEGs

Angiotensin-converting enzyme is a carboxypeptidase that plays an important role in vasodilatation in mammals. Angiotensin can influence a number of cellular functions and is known to contribute to the pathogenesis of inflammatory diseases (Suzuki *et al.*, 2002). Angiotensin-like molecules are evolutionarily conserved and have been isolated from the mollusc, *Mytilus edulis* (Laurent *et al.*, 1997; Laurent *et al.*, 1998). The angiotensin system is connected to immune modulation in invertebrates, for example leech immune cells pre-incubated with mammalian IL-2 can produce significant amounts of NO following stimulation with angiotensin II (Salzet and Verger-Bocquet, 2001).

The resistant snail haemocyte DEG, nematode astacin protease (NAS-14) has zinc binding activity. It is involved in a number of physiological functions such as food digestion, general peptide processing, hatching and morphogenesis (Mohrlen *et al.*, 2003). Intestinal mucin is an extracellular envelope that lines the digestive tract (mainly the mid-gut tissue) of most insects, protecting the insect from pathogens (Wang and Granados, 1997). A unique mucin transcript was found to be abundant in *B. glabrata* haemocytes following *S. mansoni* miracidia infection (Raghavan *et al.*, 2003). Neural and ectodermal factor is a secreted immunoglobulin, implicated in *Drosophila* development. Previous work has shown neural proteins are predominately expressed in unchallenged *B. glabrata* haemocytes (Raghavan *et al.*, 2003). Neural proteins are evolutionarily-conserved and may have homology to immune related proteins in the mollusc (Bayne, 2003).

Proteins involved in energy metabolism may be an important factor in maintaining host-parasite interactions (Lockyer *et al.*, 2007). The ATP synthase-like protein identified in this study as differentially expressed in susceptible snails is involved in energy

metabolism. Metabolic proteins have been isolated from *S. mansoni* cercarial secretions and have been found to be highly expressed in *B. glabrata* following exposure to *E. caproni* (Knudsen *et al.*, 2005; Bouchut *et al.*, 2007). ATP synthase has further been identified in *B. tenagophila* snails (Jannotti-Passos *et al.*, 2008). The susceptible haemocyte DEG, paramysoin, is a structural component in mammalian muscle tissue and a major component in *S. mansoni* cercarial secretion (Knudsen *et al.*, 2005). The protein Sm39 isolated from *S. mansoni*, and Bg39 isolated from *B. glabrata* have significant homology to one another, and correspond to tropomyosin, a structural protein (Dissous *et al.*, 1990). Therefore, certain DEGs with homology to host counterparts could be produced by the parasite to facilitate its survival.

In summary, a range of DEGs were identified from the microarray analysis that were classified broadly into genes that possess anti-parasitic properties, and are involved in protein transcription, protein degradation or cell-cell interactions. A large proportion of DEGs were unknown and their function could not be assigned. DNA microarrays have been developed for *S. mansoni* (Hoffmann *et al.*, 2002, Vermeire *et al.*, 2006) *S. japonicum* (Moertel *et al.*, 2006) and *B. glabrata* (Lockyer *et al.*, 2008) with the genome only recently available for *S. japonicum* (Liu *et al.*, 2009) and *S. mansoni* (Berriman *et al.*, 2009). With the *B. glabrata* genome project still in progress the microarray data provide important but in some cases limited information; as the genome data becomes more complete greater insight into host-parasite compatibility will follow.

Chapter 7: HSP70 protein expression

7.1 Introduction

7.1.1 Heat shock proteins in mammals

Environmental stress factors such as infection, toxins, metals and temperature changes (increase or decrease) can lead to elevated synthesis of intracellular heat shock proteins (HSPs) also referred to as stress proteins. The production of HSPs ensures cell survival under such conditions, protecting the cell from irreversible damage and death (Mager and De Kruijff, 1995). Under normal physiological conditions intracellular HSPs are essential in cell growth processes, including DNA replication, and the transcription, folding and transportation of protein; HSPs are often referred to as having molecular chaperone functions (Morimoto, 1993; Mager and De Kruijff, 1995). Heat shock proteins are usually categorised according to their molecular mass: HSP100, HSP90, HSP70, HSP60, P30, HSP26 and HSP12; several of these HSPs are induced following stress (Mager and Ferreira, 1993; Morimoto, 1993; Yura *et al.*, 1993; Mager and De Kruijff, 1995). Figure 7.1 shows a crystal structure of a highly conserved small HSP.

The rapid synthesis of HSPs usually involves the activation of heat shock factors (HSFs). In a non-stressed cell, HSFs are maintained in a monmeric, non-DNA binding form *via* their interactions with HSPs. Following stress, HSPs disassociate from HSFs and assemble into trimers allowing HSFs to bind to their gene promoters and to become phosphorylated (or activated) by the GTPases, Rac and Ras (Morimoto, 1993). Suppression of HSF transcriptional activity requires the phosphorylation of ERK in a Ras-dependent manner (Bornfeldt, 2000).

Amongst the HSP family, HSP70s are the most evolutionarily conserved, generally localised in the cytoplasm, endoplasmic reticulum, mitochondria and plastids of cells; they have an intracellular chaperone function and an extracellular immunoregulatory function (Boorstein *et al.*, 1994). HSP70 can be actively secreted from a cell or released during necrosis. Active section of HSP70 occurs independent of the common secretory pathway as inhibitors of the classical transport pathway do not block HSP70 release (Lancaster and Febbraio, 2005). HSP70 protein can also be transported out of cells in vesicles by blebbing or *via* endolysosomes that fuse with the cell surface membrane (Mambula *et al.*, 2007). Outside the cell, HSP70 can interact with neuronal cells, antigen presenting cells (APC) or blood vessels (Calderwood *et al.*, 2007a). Furthermore, HSP70 can be recognised by target cells via a range of receptors including: oxidized low density lipoprotein (LDL) binding protein, CD91, CD40, TLRs (Toll-like receptors), the chemokine

receptor, CCR5, and scavenger receptors such as low-density lipoprotein receptor (LOX-1) and a scavenger receptor expressed by endothelial cell-I (SREC-1) (Calderwood *et al.*, 2007a, Calderwood *et al.*, 2007b).

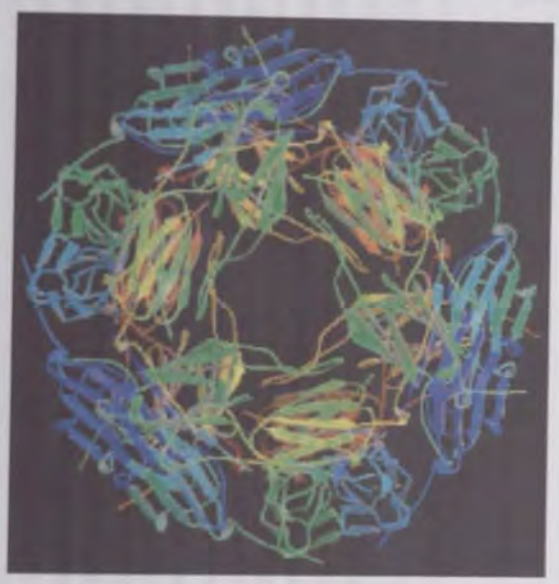

Fig. 7.1 A crystal structure of a HSP from the microbe *Methanococcus jannaschii*
Small HSPs are 12-43 kDa in size and have conserved amino acid sequences among organisms. The crystal structure of the protein contains twenty-four monomers that form a hollow spherical complex of octahedral symmetry (Kim *et al.*, 1998).

7.1.2 HSPs and the heat shock response in molluscs

Aquatic organisms have been shown to exhibit variations in HSP expression when exposed to range of stressful conditions including seasonal acclimatization, climate change (increase and decrease in temperature), competition for space, food availability, wave exposure, heavy metal exposure, and laboratory adaptation (Buckley *et al.*, 2001; Helmuth and Hofmann, 2001; Helmuth *et al.*, 2002, Tomanek and Sanford, 2003; Franzellitti and Fabbri, 2005; Kefaloyianni *et al.*, 2005; Lund *et al.*, 2006; Todgham *et al.*, 2007). Specific HSP-like molecules have been identified in the sea slug, *Aplysia californica*, the oysters, *Crassostrea virginica*, *Ostrea edulis*, the mussel, *Crassostrea gigas*, and the clams *Tapes philippinarum* and *Scapharca inaequivalvis* (Kuhl *et al.*, 1992, Tirard *et al.*, 1995; Piano *et al.*, 2002b). Furthermore, HSP70 genes have been cloned and characterised in *Biomphalaria glabrata* embryonic (Bge) cells and in *M. galloprovincialis* (Laursen *et al.*, 1997; Kourtidis *et al.*, 2006; Kourtidis *et al.*, 2006b).

Information on the regulation and transcription of HSPs in gastropod molluscs is limited. However, haemocytes extracted from the mollusc, *Haliotis tuberculata* and the oyster *C. gigas* exposed to noradrenaline or the α-adrenoceptor agonist, phenylephrine, expressed increased levels of the inducible HSP70 protein, likely promoted *via* phospholipase C

(PLC), protein kinase C (PKC) or phosphoinositide kinase-3 (PI-3K) signalling pathways (Lacoste *et al.*, 2001, Lacoste, Malham *et al.*, 2001). Noradrenaline accumulation can occur in heat-treated oyster haemocytes in addition to HSPs (Lacoste *et al.*, 2001). Similar to mammals, HSP70 protein can be secreted out of mollusc cells; using HSP70-immunogold labelling, Martynova *et al.*, (2007) observed granules of HSP70 being exocytosed from cardiac cells from the snail *Achatina fulica* (Martynova *et al.*, 2007).

Microarray analysis (Chapter 6) and previous gene-based studies identified HSP70 as a gene that might be modulated in schistosome-infected *B. glabrata* snail strains (Lockyer *et al.*, 2000; Jones *et al.*, 2001; Lockyer *et al.*, 2004). The aim of the present study was to investigate whether *Schistosoma mansoni* ESPs affected HSP70 protein levels in *B. glabrata* haemocytes and to investigate whether HSP70 responses were dependent upon the ERK signalling pathway.

7.2 Results

7.2.1 Detecting HSP70 protein in B. glabrata haemocytes

Western blotting with anti-HSP70 monoclonal antibodies (clone: BRM-22) that detect HSP70/HSC70, identified two discrete immunoreactive bands of approximately 70-75 kDa in unchallenged *B. glabrata* haemocytes; immunoreactive bands of similar size were also detected in mammalian control cells (HC60 cell line; Fig. 7.2A). This anti-HSP70 antibody has previously been used to detect HSP70/HSC70 in mammals and *Xenopus*, as well as homologues in invertebrates including the fruit fly, *Drosophila melanogaster*, and the zebra mussel, *Dreissena polymorpha* (Clayton *et al.*, 2000; G. B. Smith *et al.*, 200; Lilja *et al.*, 2007).

For clarity, the immunoreactive proteins are referred to here as HSP70s; they could comprise heat shock cognate protein (HSCP) or other HSP forms. Unchallenged schistosome-susceptible and schistosome-resistant snail haemocytes expressed HSP70 protein in approximately equal amounts (relative to actin) when an equal number of haemocytes was used for each snail strain (Fig. 7.3). Basal levels of HSP70/HSC70 protein in tissue not exposed to stress have also been noted in a number of organisms including the oyster, *Ostrea edulis*, and the sea urchin, *Paracentrotus lividus*, indicating a possible housekeeping role (Matranga *et al.*, 2000; Piano *et al.*, 2002a).

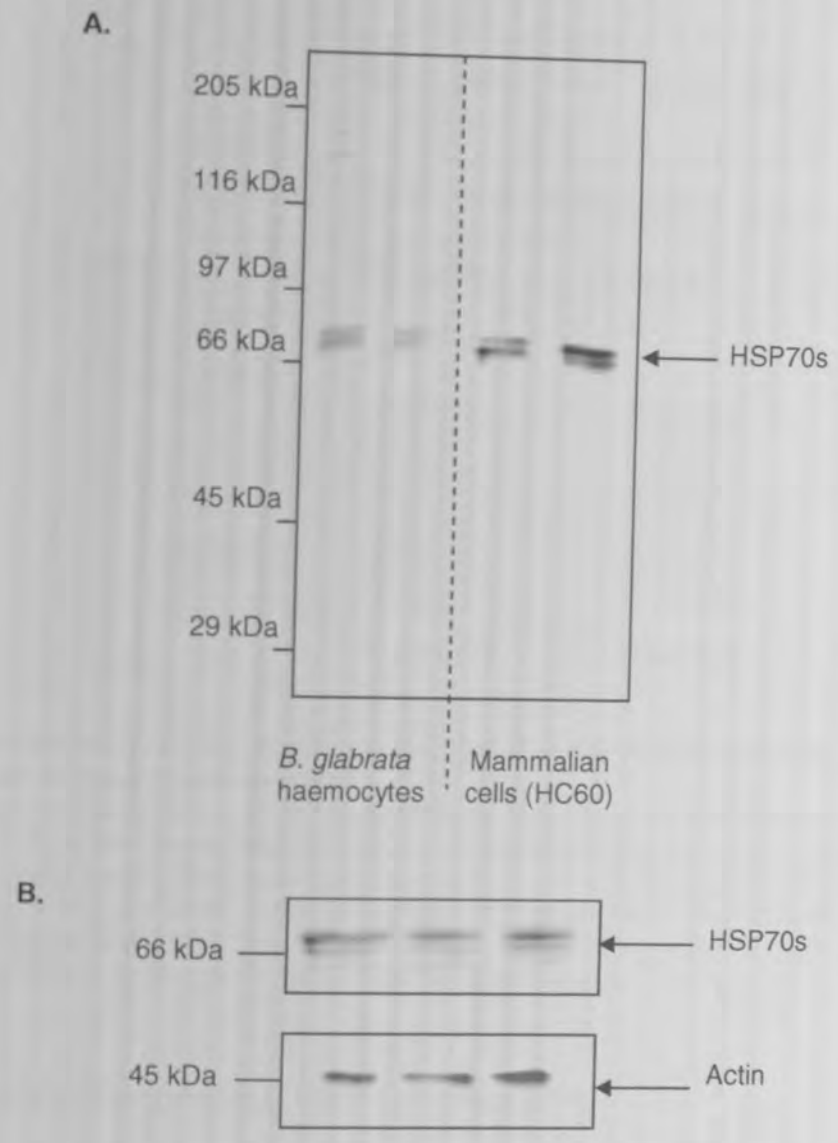

Fig. 7.2 Detecting HSP70 protein in unchallenged haemocytes
(A)Typical western blot showing immunoreacive HSP70 protein in unchallenged susceptible snail haemocytes and mammalian HC60 cells. Haemocytes were extracted from snails and kept on ice prior to cell lysis and western blotting. (B) Western blot showing HSP70 protein in unchallenged haemocytes from susceptible snails, a third band was occasionally detected but was often faint.

In order to investigate basal levels of HSP70 protein in haemocytes from susceptible and resistant snails, an equal number of haemocytes were isolated from the two snail strains. Two immunoreactive bands of approximately 70 kDa in size were indentified in both snail strains (Fig. 7.3). Schistosome-susceptible and schistosome-resistant snail haemocytes expressed HSP70 protein in approximately equal amounts when equal number of haemocytes were used (Fig. 7.3).

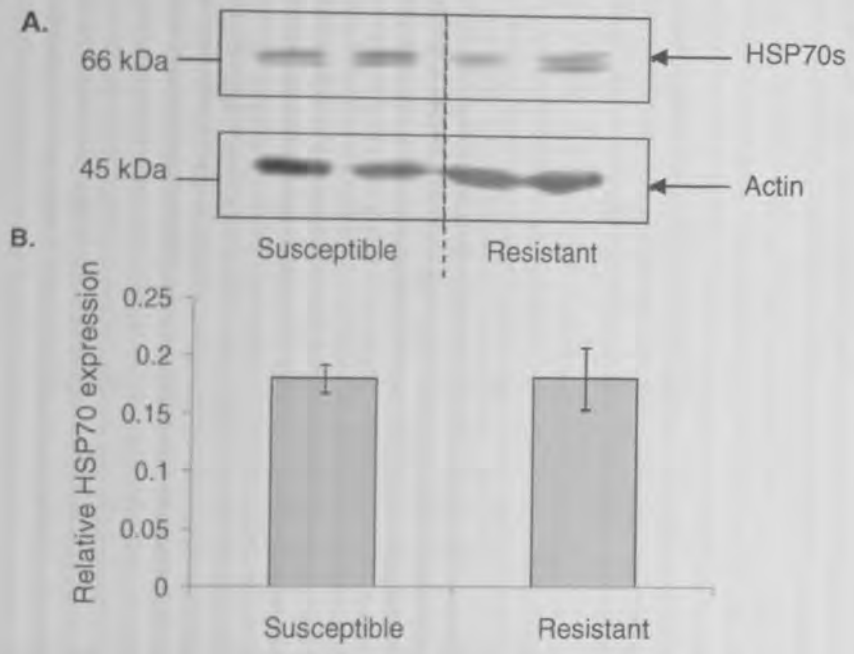

Fig. 7.3 Western blot and bar graph showing basal levels of HSP70 protein in susceptible and resistant *B. glabrata* haemocytes
(A) An equal number of haemocytes (approximately $1x10^5$ cells) were extracted from snails and kept on ice before being lysed and the proteins processed for western blotting with anti-HSP70 antibodies (upper panel). Blots were re-probed with an anti-actin antibodies (lower panel), to confirm equal loading of protein between samples. (B) Quantitative band analysis was used to calculate mean (±SEM) HSP70 protein levels in each snail strain relative to actin expression; n=4 for each snail strain, performed in two independent experiments.

7.2.2 Heat shock response in B. glabrata *haemocytes*

To assess if HSP70 protein expression could be increased in *B. glabrata* haemocytes following heat stress, individual snails were incubated at 37°C for 1 h and then allowed to recover for 3 h at RT, followed by extraction of haemocyte proteins. Relative haemocyte HSP70 protein levels were variable following heat stress in both snail strains, with no significant changes observed (Fig. 7.4). Snails were subsequently exposed to heat shock at 40°C for 1 h followed by a 1 h or 4 h recovery period at RT, prior to haemocyte extraction. Again variable results were observed; generally, susceptible snail haemocytes exhibited lower HSP70 levels compared to resistant snail haemocytes after 1 h recovery and resistant snail haemocytes generally exhibited lower HSP70 protein levels after 4 h recovery (Fig. 7.5). When the temperature was increased to 45°C, susceptible snail haemocytes showed a marked increase in HSP70 levels (heat shocked for 1 h followed by 4 h recovery; Fig. 7.6).

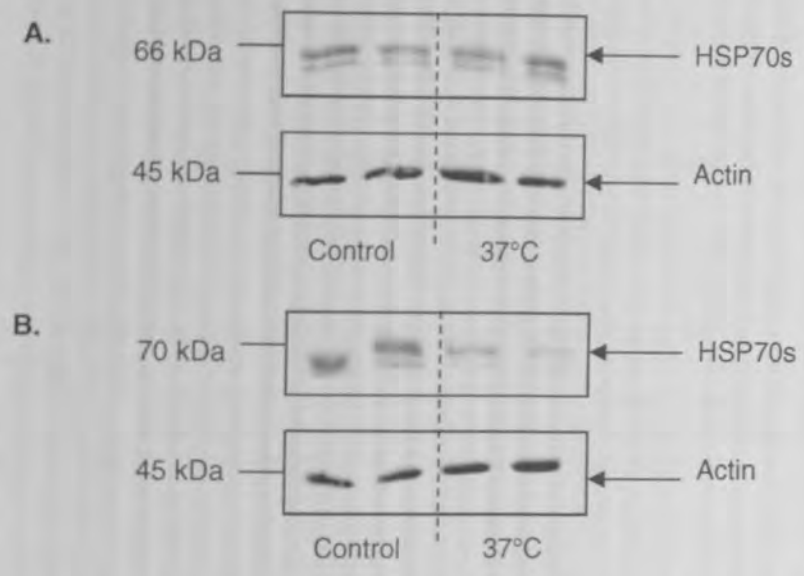

Fig. 7.4 Heat shock response of haemocytes in *B. glabrata* at 37°C
Western blots showing intracellular HSP70 protein levels in susceptible (A) and resistant (B) snail haemocytes following heat shock of snails at 37°C for 1 h and recovery at RT for 3 h (upper panels). Control samples were incubated at RT for 4 h. Blots were then re-probed with anti-actin antibodies to confirm equal loading of proteins between samples. Blots are representative of at least two independent experiments.

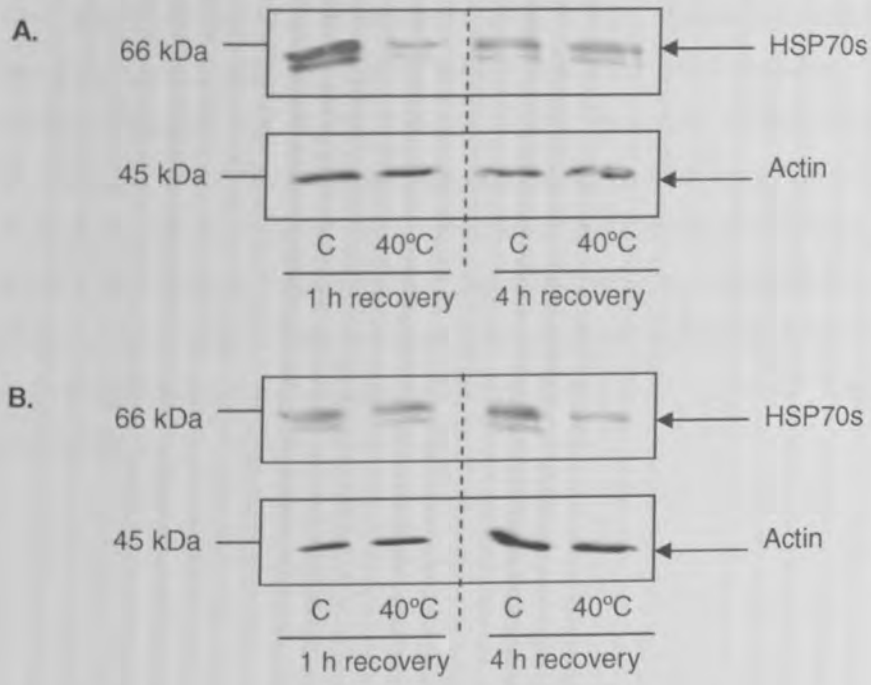

Fig. 7.5 Heat shock response of haemocytes in *B. glabrata* at 40°C
Western blots showing intracellular HSP70 protein levels in susceptible (A) and resistant (B) snail haemocytes following heat shock of snails at 40°C for 1 h and then left to recover for 1 h or 4 h at RT (upper panel). Control samples (represented by the letter C) were incubated at RT only. Blots are representative of at least two independent experiments.

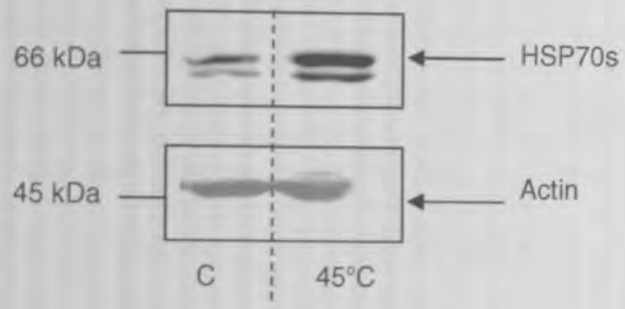

Fig. 7.6 Heat shock response of haemocytes in *B. glabrata* at 45°C
Western blot showing HSP70 protein levels in susceptible haemocytes following heat shock of snails at 45°C for 1 h and allowed to recover at RT for 4 h (upper panel). The letter C, represent control samples incubated at RT only. Lower blot shows actin levels in each blot to confirm each protein loading. The blot is representative of two independent blots.

7.2.3 HSP70 protein levels in haemocytes following S. mansoni *ESP-challenge*

Pooled haemocytes from either susceptible or resistant *B. glabrata* strains were exposed to 20 µg/ml ESP for 1 h prior to protein extraction and HSP70 detection by western blotting. Analysis of relative band intensities revealed that mean HSP70 protein levels were significantly reduced ($P \leq 0.01$) in susceptible snail haemocytes following ESP exposure when compared to controls (Fig. 7.7A). Resistant snail haemocytes also exhibited attenuated relative HSP70 levels following ESP-challenge, but the effect was less marked ($P \leq 0.05$; Fig. 7.7B). Overall a 50% decrease in HSP70 was observed (Fig. 7.7) for resistant snail haemocytes, compared to an 80% reduction in haemocytes from the susceptible strain; thus a 60% difference in HSP70 levels was observed in ESP-challenged haemocytes between the two snail strains. Interestingly, haemocytes from susceptible snails would often express only one immunoreactive band (approximately 70 KDa in size) following ESP exposure, while haemocyte resistant snails often expressed two bands (Fig. 7.7).

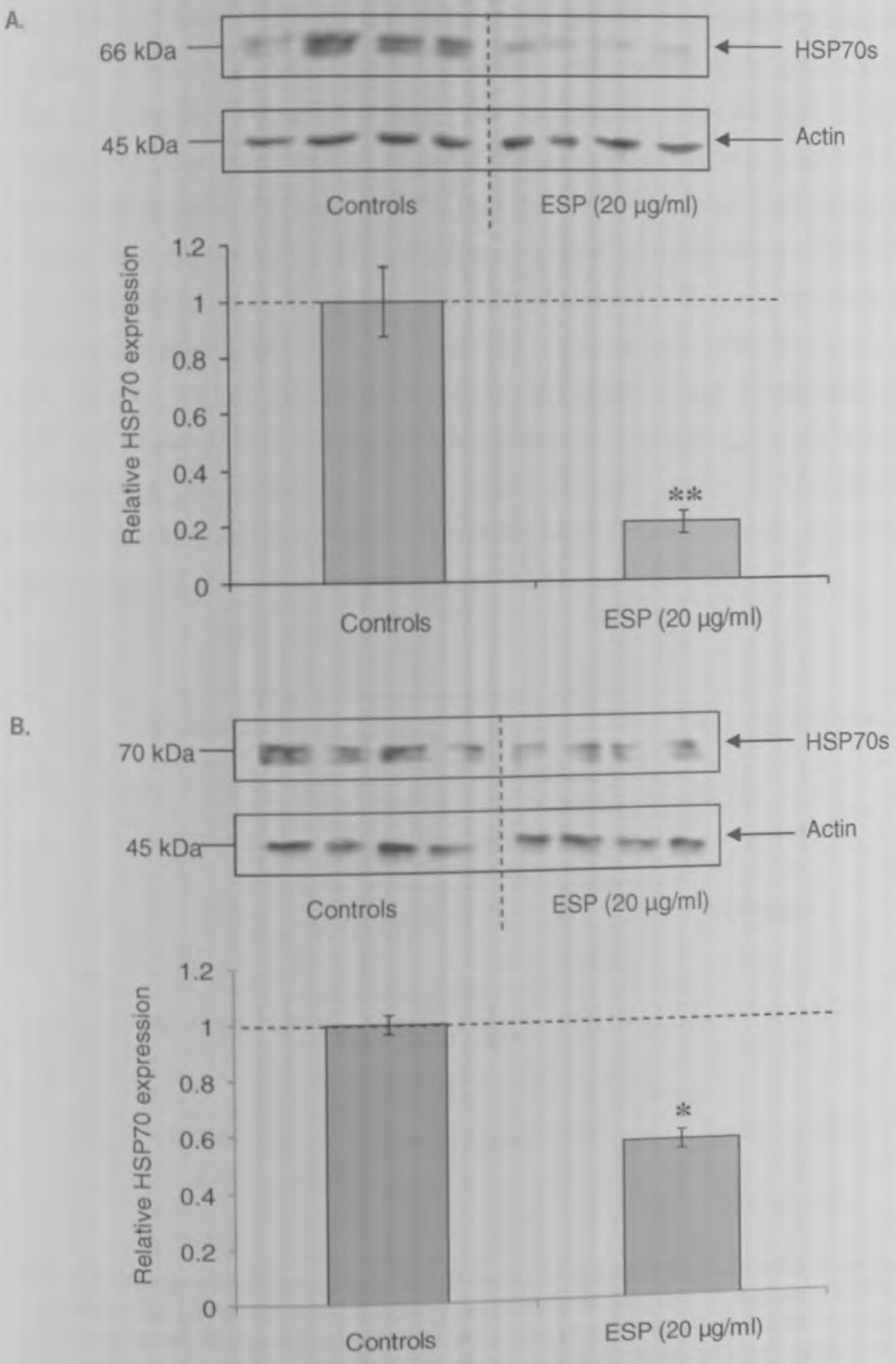

Fig. 7.7 Western blots and bar graphs showing relative HSP70 levels in haemocytes from susceptible (A) and resistant (B) *B. glabrata* exposed to *S. mansoni* ESPs
Haemocytes were exposed to 20 µg/ml ESPs for 1 h prior to cell lysis and western blotting with an anti-HSP70 antibody (upper panels); the blots were re-probed for actin (lower panels) to confirm equal loading of protein in each lane (control haemocytes were kept in CBBS only). The relative intensity for each band was analysed and HSP70 levels calculated relative to actin. The values were then normalised to control values, represented by the dotted line and having a vale of 1. $^{**}P \leq 0.01$, $^{*}P \leq 0.05$, when compared to control values (n=4, ±SEM).

Haemocytes extracted from susceptible or resistant snails were challenged with different ESP concentrations (0.1–20 µg/ml) for 1 h in order to determine the response of HSP70 protein levels to increasing ESP exposure. Susceptible snail haemocytes showed a general reduction in HSP70 protein when exposed to 1 µg/ml or greater ESPs (Fig. 7.8). Analysis of blots revealed that 10 µg/ml ESPs decreased HSP70 protein by approximately 80% compared to controls, a 90% reduction was observed with 20 µg/ml ESPs (Fig. 7.8A). Haemocytes from resistant snails consistently had decreased HSP70 protein levels following exposure to 10 µg/ml ESPs, but showed the greatest reduction at the highest dose by approximately 50% (20 µg/ml; Fig. 7.8B). In both snail strains 0.1 µg/ml ESP did not affect HSP70 protein levels. To assess the effect of heat denaturation on the ability of ESPs to modulate haemocyte HSP70 expression levels, 20 µg/ml ESPs were boiled for 5 min and subsequently cooled to RT before they were added to haemocyte monolayers. When haemocytes from either snail strain were challenged with these boiled ESPs no attenuation in HSP expression levels was observed (Fig. 7.9).

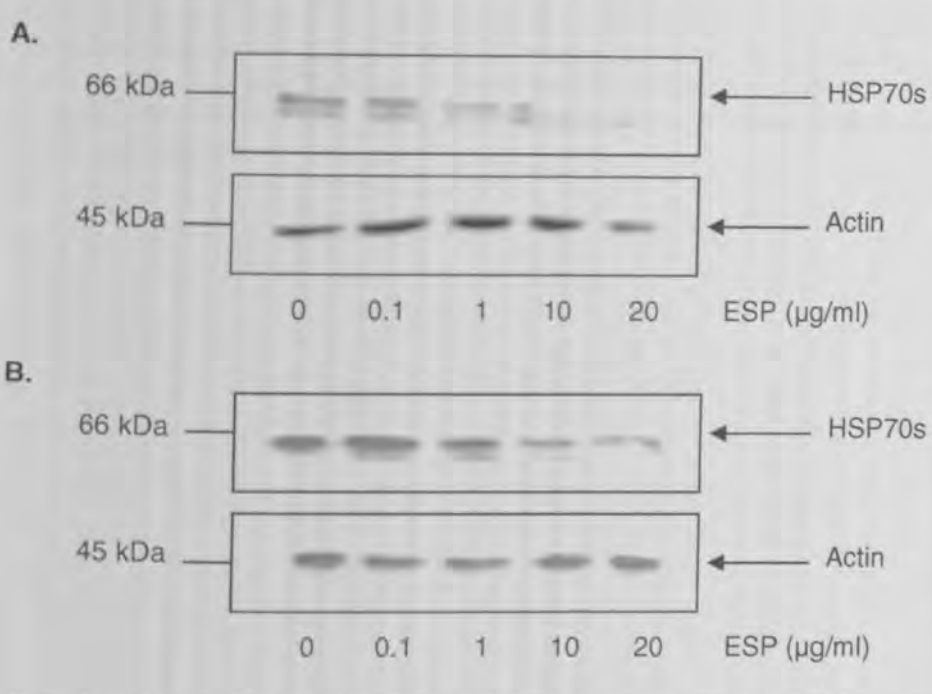

Fig. 7.8 Western blots showing HSP70 levels in haemocytes extracted from susceptible (A) and resistant (B) *B. glabrata* following exposure to different *S. mansoni* ESP concentrations
Haemocytes were challenged with different doses of ESPs (0-20 µg/ml) for 1 h prior to cell lysis and subsequent HSP70 detection by western blotting; blots were further washed and probed with anti-actin to confirm equal loading of protein between samples (lower panels). The western blots shown are representative of those obtained in two independent experiments.

Fluorescence confocal microscopy was used to visualise the general distribution of HSP70 protein in haemocytes in the presence and absence of 20 µg/ml ESPs for 1 h (Fig. 7.10). Individual haemocytes exhibited varying degrees of HSP70 expression. However, haemocytes from susceptible snails consistently showed a general decrease in HSP70

levels following ESP-challenge, while changes in HSP70 expression in haemocytes from the resistant strain were less marked (Fig. 7.10). Image analysis of haemocyte z-sections indicated that HSP70 may be located in or near the cell nucleus (Fig. 7.10C and D). Furthermore, HSP70 was often observed in close association with actin often forming a partial ring around the nucleus (Fig. 7.10C and D).

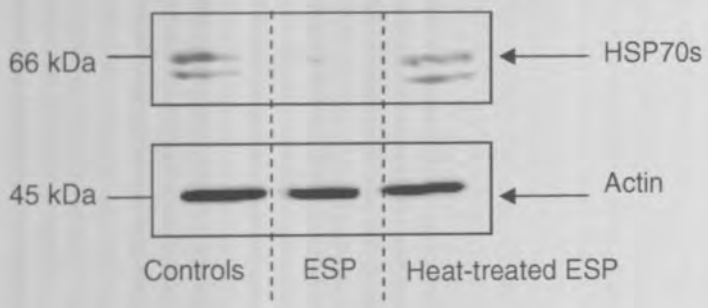

Fig. 7.9 Western blot showing levels of HSP70 protein in *B. glabrata* haemocytes exposed to heat-treated S. *mansoni* ESPs
Susceptible snail haemocytes were challenged for 1 h with either 20 µg/ml ESPs (in CBBS), 20 µg/ml ESPs that had been boiled for 5 min and then cooled to RT, or CBSS only (controls). Protein extracts were then processed for western blotting with anti-HSP70 antibodies (upper panel) and anti-actin antibodies (lower panel).The blot is representative of those obtained from two independent experiments.

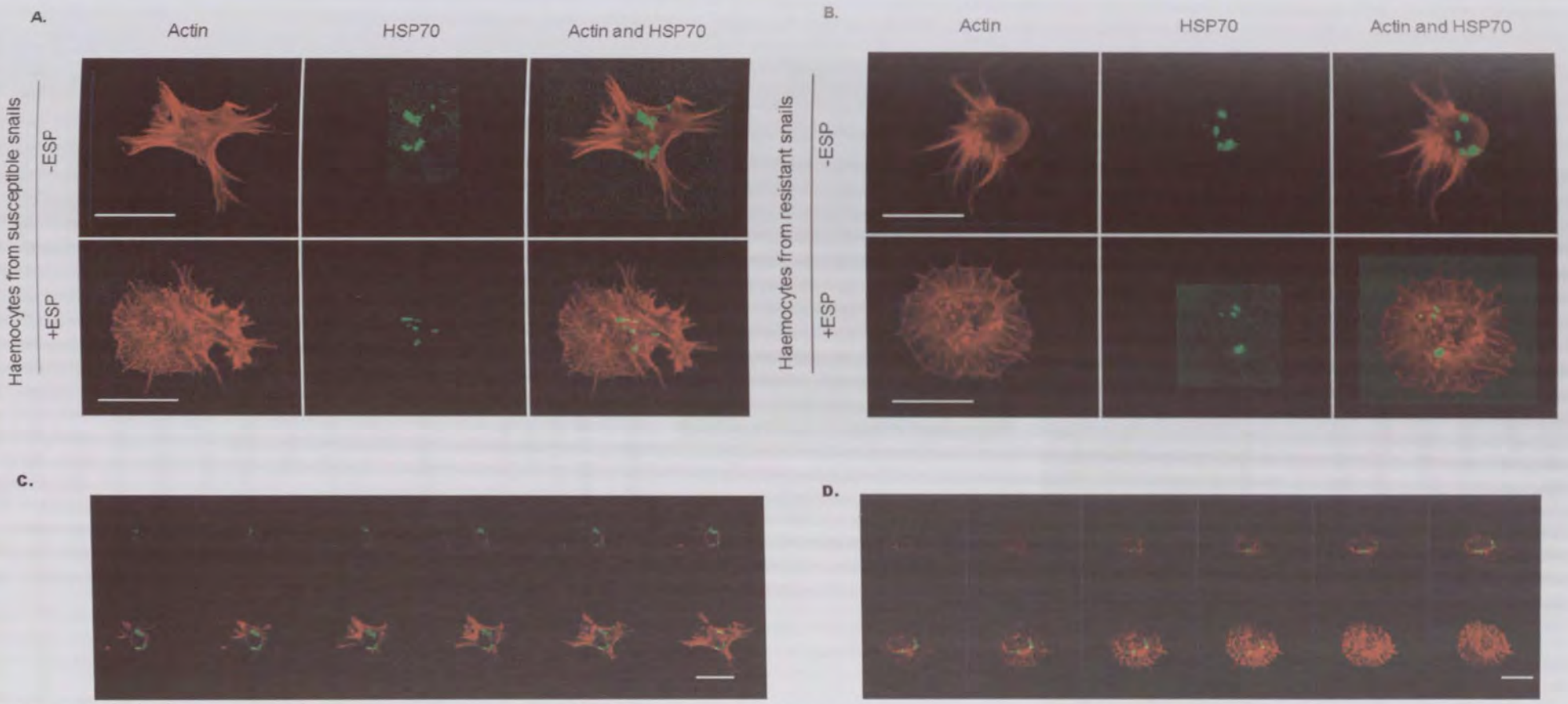

Fig. 7.10 Images from confocal microscopy showing HSP70 levels in *B. glabrata* haemocytes challenged with *S. mansoni* ESPs
Haemocytes from susceptible (A) and resistant (B) snails that had been exposed (+) or not exposed (-) to ESP (20 µg/ml) for 1 h on glass coverslips were fixed and incubated with a primary HSP70 antibody and a secondary FITC-conjugated antibody (green), the cells were then washed and incubated with rhodamine phalloidin (red), which binds to filamentous-actin to allow the visualisation of the cell cytoskeleton. Z-sections of individual haemocytes from susceptible (C) and resistant (D) snail strains were captured using average pixel brightness mode; the images are representative of those obtained in two independent experiments (scale bars represent 10 µm).

In order to broadly identify the cellular location of HSP70, haemocytes were stained with 4'-6-Diamidino-2-phenylindole (DAPI), a DNA binding fluorescent probe and rhodamine phalloidin. Microscopy revealed that HSP70 protein was consistently located close to the nucleus, but not within, and was often identified in the cytoplasm of the haemocyte (Fig. 7.11 and Fig. 7.10). Whether HSP70 was present in the cytosol and/or the endoplasmic reticulum or Golgi could not be ascertained.

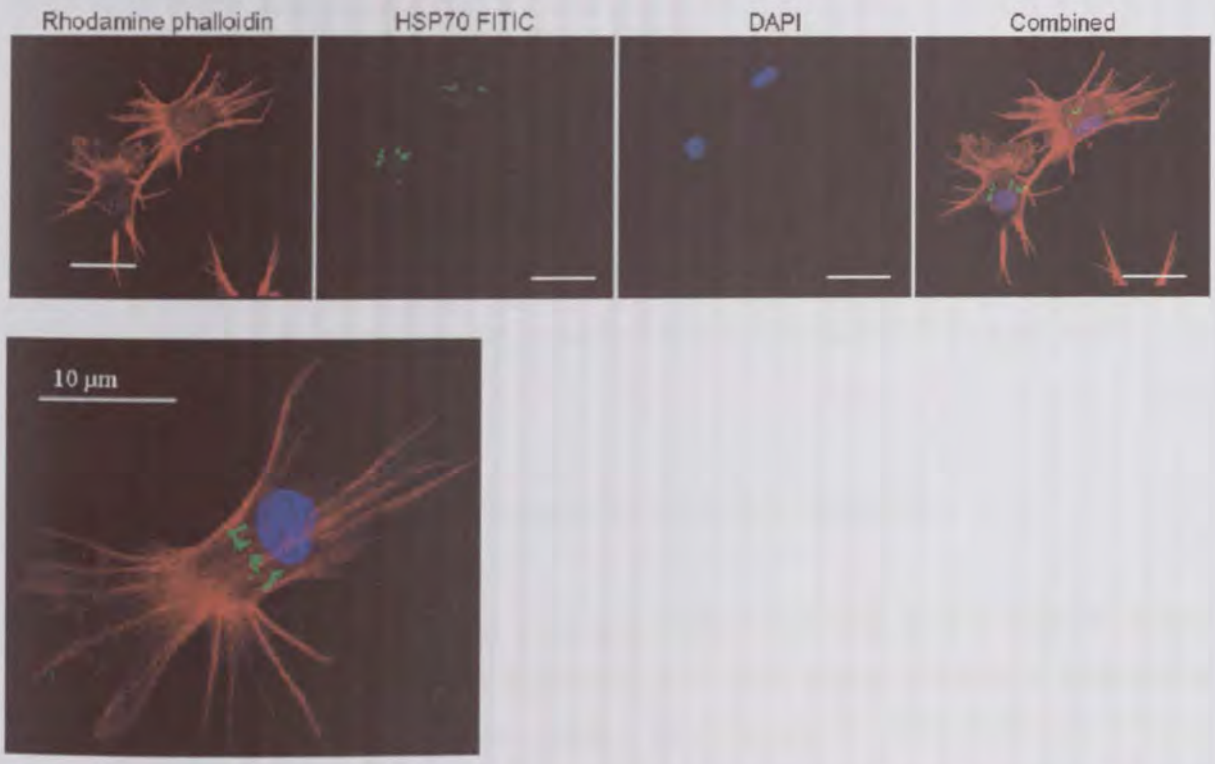

Fig. 7.11 Confocal microscopy micrographs showing the location of HSP70 in susceptible snail haemocytes
Haemocytes not exposed to ESPs were fixed incubated with HSP70 and FITC-conjugated antibodies (green), rhodamine phalloidin (red) and DAPI (blue) on glass coverslips. The average pixel brightness mode was used to capture each image (images are representative of those obtained in at least two independent experiments); scale bars=10 µm.

7.2.4 HSP70 protein levels in haemocytes from S. mansoni infected snails

The effects of *S. mansoni* infection on *B. glabrata* haemocyte HSP70 protein levels were determined *in vivo*. Susceptible snails were individually exposed to 5 miracidia, 35 days post-exposure, snails that shed significant numbers of cercariae (between 10 and 50 at any one observation) were killed and haemolymph from three snails were pooled. A similar volume of haemolymph was also collected from three adult (control) snails that had not been exposed to miracidia. Successful infection, development and reproduction of the parasite resulted in considerably reduced HSP70 protein expression in host haemocytes (Fig. 7.12). Image analysis of blots from duplicate experiments revealed that HSP70 levels were attenuated by approximately 70% in *S. mansoni*-infected snails compared to

uninfected susceptible controls. In contrast, haemocytes from infected resistant snails seemed to maintain similar HSP70 protein levels in compared to uninfected controls.

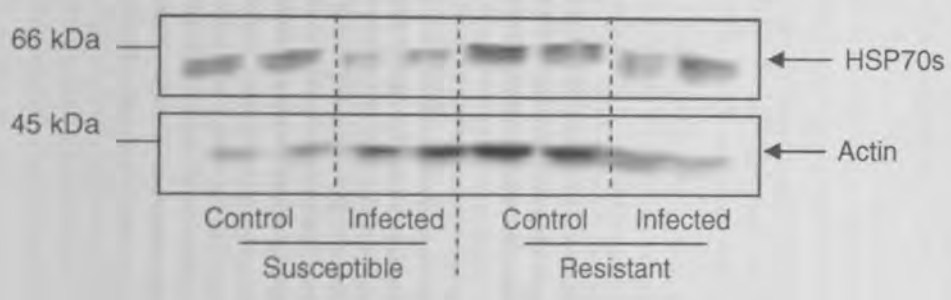

Fig. 7.12 Western blot showing relative HSP70 protein levels in haemocytes extracted from *S. mansoni* infected and uninfected susceptible and resistant *B. glabrata* strains
Snails were exposed to *S. mansoni* miracidia and haemolymph containing haemocytes was extracted and pooled from three infected snails 35 days post-exposure; an equal volume of pooled haemolymph was also obtained from three unexposed snails (control). Haemocytes were then recovered, lysed and proteins processed for western blotting with anti-HSP70 and anti-actin antibodies.

7.2.5 HSP70 protein levels in haemocytes after 5 h in vitro incubation

The level of protein in a cell can alter over time depending on the rate of protein degradation *vs.* the rate of protein synthesis. Thus, the relative amount of intracellular HSP70 expression over 5 h was studied in response to ESP-challenge. Pooled haemocytes were ESP-challenged (20 µg/ml) for 1 h, ESPs were subsequently removed and the haemocytes left to recover at RT for 5 h before cell lysis and HSP70 detection by western blotting. Haemocytes have previously been show to remain viable in culture plates for at least 5 h at RT in the presence and absence of *S. mansoni* ESPs (see Sections 2.7 and Chapter 5). Resistant snail haemocytes exposed to ESPs still exhibited significantly reduced HSP70 protein levels after the 5 h recovery period ($P \leq 0.05$; Fig. 7.13) with a reduction (50%) similar to that observed after 1 h exposure (Fig. 7.7B) . In contrast, ESP-challenged haemocytes from susceptible snails consistently showed a general increase in HSP70 levels (Fig. 7.13), although this was not statistically significant. Nevertheless, a significant difference in mean haemocyte HSP70 expression was observed between the two snail strains following ESP-challenge and 5 h recovery ($P \leq 0.01$; Fig. 7.13).

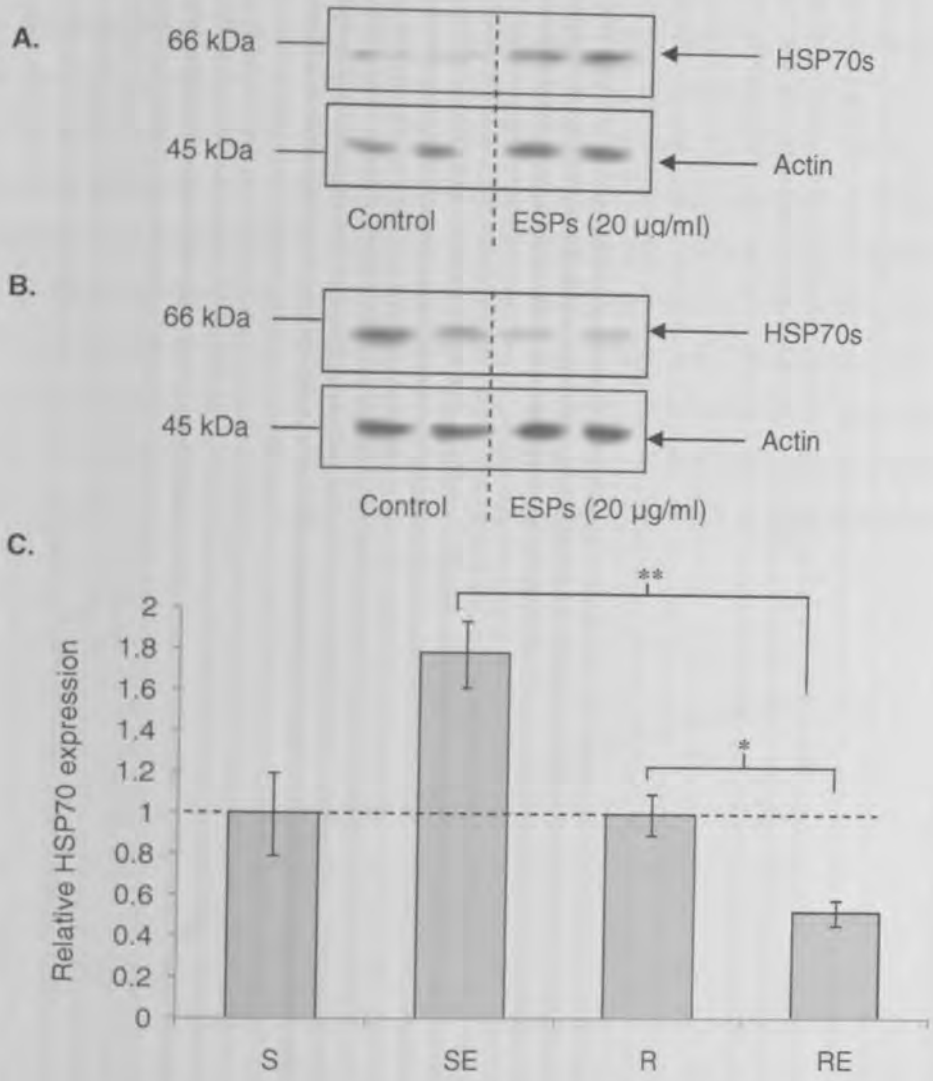

Fig. 7.13 HSP70 levels in haemocytes from susceptible (A) and resistant (B) *B. glabrata* exposed to *S. mansoni* ESPs (20 µg/ml) for 1 h followed by a 5 h recovery period
Haemocytes from susceptible (A) and resistant (B) snails were exposed to 20 µg/ml ESPs in CBBS, or CBBS alone (control) for 1 h followed by a 5 h recovery period at RT before haemocyte proteins were extracted and processed for western blotting. Blots were probed with HSP70 antibodies (upper panels); blots were re-probed for actin to confirm equal loading of protein in each lane (lower panels). (C) Immuno-reactive bands on blots were analysed for their relative intensities and mean change in HSP70 expression calculated, relative to control values having a relative value of 1 (and represented by the dotted line).** $P \leq 0.01$, * $P \leq 0.05$ when compared to controls Three independent experiments were analysed, (n=6, ±SEM). S=susceptible, SE=susceptible snail haemocytes exposed to ESPs, R=resistant, RE= resistant snail haemocytes exposed ESPs.

7.2.6 Detecting HSP70 cellular secretion and HSP70 protein degradation

The observed reduction of HSP70 protein in haemocytes following ESP exposure raised the question as to what was happening to the protein following exposure. The protein could be either transported out of the cell or degraded intracellularly (Lancaster and Febbraio, 2005; Mambula *et al.*, 2007). Initial experiments focused on the active secretion of HSP70 from haemocytes by removing the medium from ESP-challenged or unchallenged haemocyte monolayers and processing it for gel electrophoresis and

western blotting. However, the HSP70 protein could not be detected in the cell medium using this method (data not shown). The protein may have fallen below the detection limit given the larger volume of medium. Subsequently, secretion of HSP70 was investigated using a HSP70 ELISA assay (StressXpress, Assay designs, USA; the kit contains antibodies with similar properties to the antibody used for western blotting). HSP70 could not be detected in the cell medium or cell lysate using this assay. Figure 7.14 shows a typical standard curve that was produced using the ELISA kit (using the manufacturers HSP70 standard protein). HSP70 could be confidently detected in the concentration range of 1.5-12.5 ng/ml; however, experimental samples produced average absorbance values below the detection range of the assay (0.2-0.4 average absorbance value), even when higher number of haemocytes per well were used (250-350 µl total haemolymph volume).

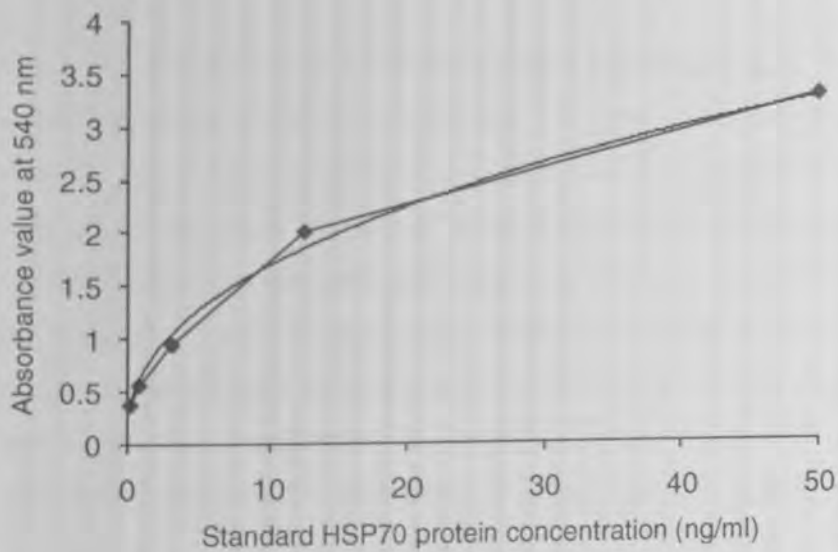

Fig. 7.14 Line graph showing standard curve for HSP70 obtained from an ELISA assay (StressXpress, Assay design, USA)
The average net absorbance value for each HSP70 standard (from kit) is shown. The graph is representative of two independent experiments (black line represents power trend line).

In order to investigate if HSP70 was being degraded intracellularly, haemocytes were exposed to the proteasome inhibitor, Z-Leu-Leu-Leu-aldehyde (MG132) which is a selective 26S proteasome-specific inhibitor that inhibits general intracellular protein degradation (Kim *et al.*, 1999). Haemocytes from susceptible and resistant snails were exposed to 50 µM MG132 for 1 h. The dose and duration of MG132 challenge used in this study have been previously shown to increase HSP70 protein levels in mammalian cell lines (Kim *et al.*, 1999). In the present study, susceptible snail haemocytes exposed to 50 µM MG132 maintained similar levels of HSP70 protein compared to non-exposed controls (Fig. 7.15).

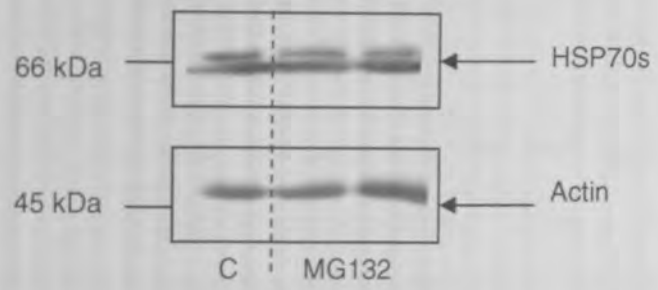

Fig. 7.15 Western blot showing susceptible *B. glabrata* haemocytes exposed to the proteasome inhibitor, MG132 (50 µM for 1h), prior to HSP70 detection
An equal volume of haemolymph from susceptible snails was plated into 48 well plates for 30 min the cell monolayer was then washed with CBBS and 50 µM MG132 in CBSS was subsequently added for 1 h. Proteins were extracted from the cells and analysed using western blotting. The western blot was probed for HSP70 and to confirm equal loading between samples the membrane was washed and re-probed for actin. Control haemocytes (C) were incubated in CBBS only.

Susceptible and resistant snail haemocytes were challenged with 50 µM MG132 in the presence and absence of 20 µg/ml ESPs for 1 h prior to protein extraction and HSP70 protein detection by western blotting (Fig. 7.16). In the presence of both MG132 and ESP, relative HSP70 levels were sustained, when compared to haemocytes exposed to the inhibitor alone in susceptible and resistant snail haemocytes. This contrasts with the significant reduction in HSP70 expression observed in haemocytes treated with ESPs in the absence of the inhibitor ($P \leq 0.05$; Fig. 7.16). MG132 alone, slightly reduced mean HSP70 protein levels in haemocytes when compared to levels in haemocytes exposed to DMSO (control); however, the difference observed was not statistically significant (Fig. 7.16).

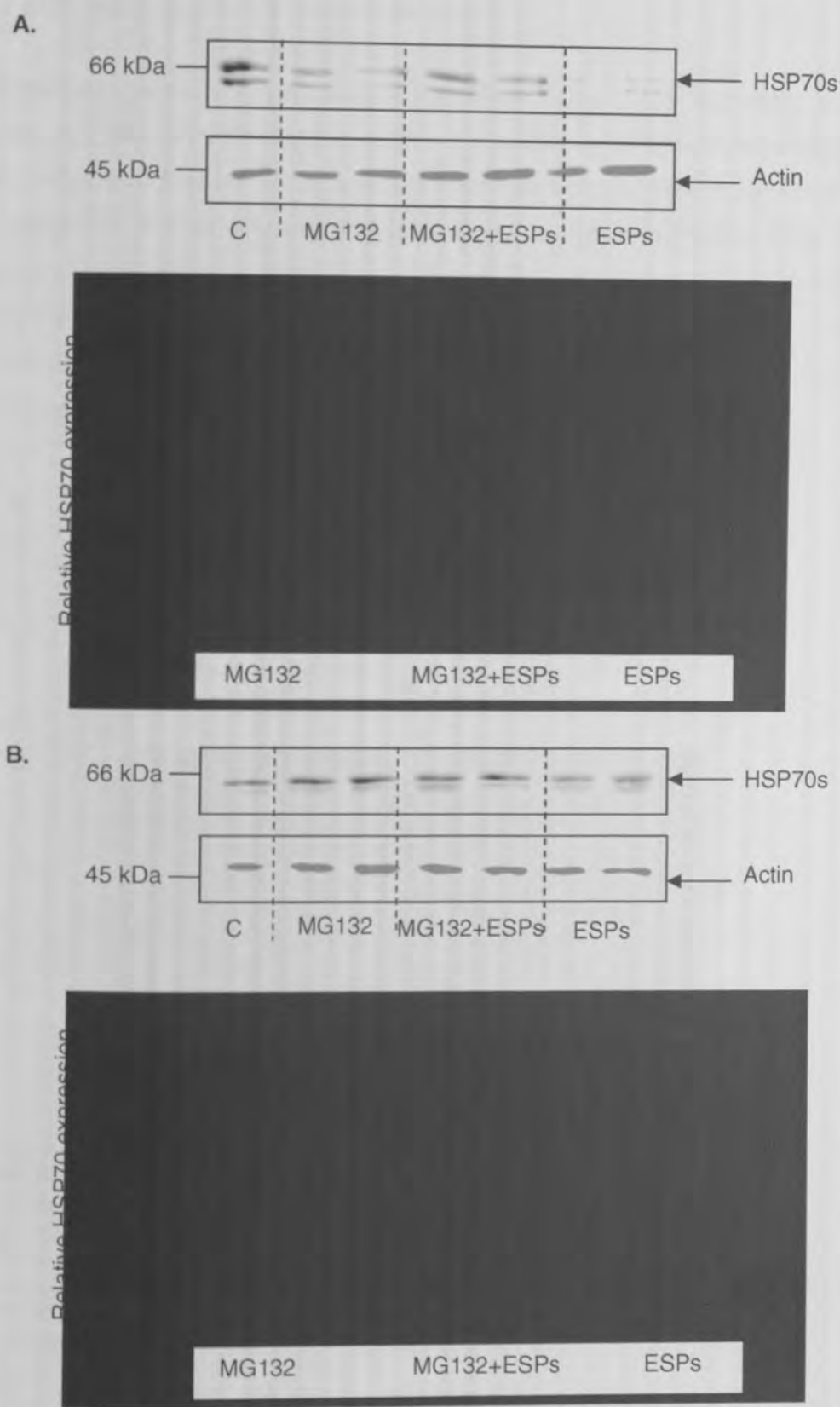

Fig. 7.16 The effect of MG132 and *S. mansoni* ESPs on HSP70 expression in susceptible and resistant *B. glabrata* haemocytes

Western blot and bar graph showing relative HSP70 levels in haemocytes from (A) susceptible and (B) resistant snails treated with and without ESPs 20 µg/ml for 1 h in CBBS containing MG132 (50 µM), or with either ESPs alone, or DMSO (vehicle control, C). Blots were probed with anti-HSP70 antibodies (upper panel) and re-probed with anti-actin antibodies (lower panel). Immuno-reactive bands on blots were analysed for their relative intensities and mean change in HSP70 expression calculated (graphs), relative to DMSO control values having a relative value of 1 (and represented by the dotted line). Blots are characteristic of those from at least three independent experiments. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ when compared to unchallenged DMSO controls (n=4, ±SEM).

7.2.7 ERK regulated HSP70 protein expression

Schistosoma mansoni larval ESPs differentially affect ERK signalling in *B. glabrata* haemocytes with ERK attenuation occurring in haemocytes from schistosome susceptible snails only (see Chapter 3). Susceptible snail haemocytes were exposed to 1 µM or 10 µM U0126 for 1 h at RT and then processed for western blotting (Fig. 7.17). Image analysis of replicate blots demonstrated that HSP70 levels were suppressed by 60% at 10 µM U0126, when compared to DMSO controls ($P \leq 0.01$; Fig. 7.17B). A lower concentration of U0126 (1 µM) was less effective at reducing HSP70 protein levels (not statistically significant).

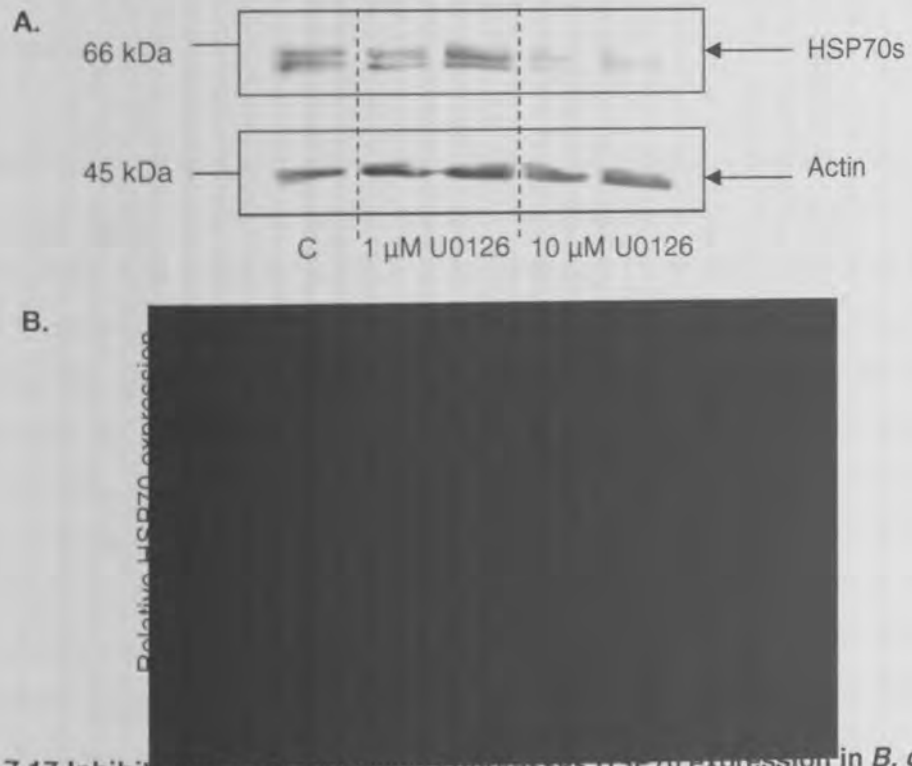

Fig. 7.17 Inhibition of ERK signalling suppresses HSP70 expression in *B. glabrata* haemocytes
Haemocytes were exposed to U0126 (1 µM or 10 µM), or DMSO vehicle (C, control) for 1 h at RT before protein extraction. (A) Western blot were probed with ant-HSP70 antibodies (upper panel) and ant-actin antibodies (lower panel).The blots are representative of at least three independent experiments. (B) Densitometric analysis of the blots was used to calculate relative HSP70 levels (relative to actin), values were normalised to controls (indicated by the dotted line at 1). $^{**}P \leq 0.01$, when compared to DMSO controls (n=6, ±SEM).

In order to investigate the effect of U0126 over a longer duration, susceptible snail haemocytes were exposed to 10 µM U0126 for 1 h, then allowed to recover for 4 h at RT (Fig. 7.18). Even after 4 h, U0126 suppressed levels of HSP70 protein compared to controls. Densitometric analysis revealed an approximately 40% reduction in HSP70 levels in U0126 challenged cells compared to DMSO controls, indicating that ERK inhibition may regulate HSP70 protein levels over a longer period of time (Fig. 7.18).

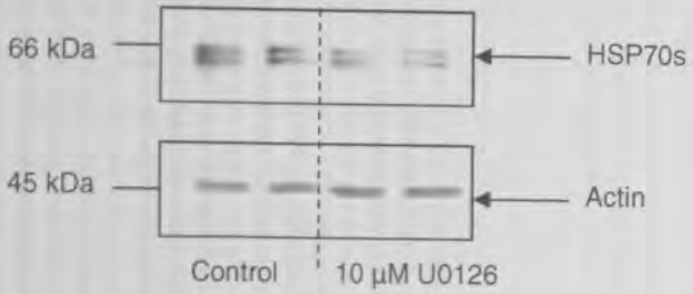

Fig. 7.18 HSP70 protein levels in susceptible *B. glabrata* haemocytes following 1 h U0126 exposure and 4 h recovery
Susceptible snail haemocytes were exposed to 10 µM U0126 for 1 h at RT, washed with CBBS and then left to recover for a further 4 h at RT (in CBBS only) prior to cell lysis and HSP70 (upper panel) and actin (lower panel) detection by western blotting. The western blot shown is representative of those obtained in at least two independent experiments.

7.3 Discussion

Biomphalaria glabrata haemocytes possess variable intracellular HSP70 protein levels following heat shock of whole snails. At temperatures of 37-40°C haemocytes from susceptible and resistant snail strains generally have similar or slightly lower levels of HSP70 protein compared to control snails. At 45°C, HSP70 protein levels do increase in susceptible snail haemocytes, following a 4 h recovery period. The classic heat shock response in most mammals and invertebrates involves the up-regulation of HSP70 protein; yet, a number of organisms do not produce a classic heat shock response including the hydra, *Hydra oligactis*; the protozoa, *Euplotes focardii* and some species of notothenioid fish (Bosch *et al.*, 1988; Buckley *et al.*, 2004; Clark *et al.*, 2008b). These organisms permanently express HSP70 and lack the ability to induce HSP70, a potential adaptation to their environmental conditions (Clark *et al.*, 2008b). The mollusc, *Ostrea edulis*, also exhibited variable HSP expression patterns following different thermal conditions, with little or no change in HSP72 and HSP77 protein expression in response to increasing temperature stress (Piano *et al.*, 2002a). Nevertheless, *B. glabrata* haemocytes seem to require high temperatures (e.g. 45°C) to induce HSP70 protein expression.

Particularly important is the finding that *B. glabrata* haemocytes exposed to *S. mansoni* ESPs showed a consistent decline in intracellular HSP70 protein. Susceptible snail haemocytes showed the greatest reduction, but after 5 h, HSP70 protein levels seemed to increase. In contrast, intracellular HSP70 levels in resistant snail haemocytes remained suppressed even after 5 h. Why *S. mansoni* ESPs failed to suppress HSP70 protein levels over a longer period of time in susceptible snail haemocytes is unknown; it is possible that continued attenuation in haemocytes from this snail strain relies upon the continual presence of ESPs. Although extracellular HSP70 could not be detected in our system

after 1 h ESP exposure, HSP70 could potentially be secreted from haemocytes, as occurs in other immune cell types independent of the secretory pathway (Lancaster and Febbraio, 2005). If this is the case, resistant snail haemocytes may actively secrete HSP70 in response to ESPs over the duration of the assay, whereas susceptible snail haemocytes may retain *de novo* synthesised HSP70 intracellularly.

Indeed, it is important to consider the trade-off between stress responses and the energy required to maintain these responses. Continued high HSP production has deleterious long term consequences for the fruit fly, *Drosophila*, including lower reproduction rates and reduced survival rates (Silbermann and Tatar, 2000; Vermeulen and Loeschcke, 2007). In the context of snail–schistosome interactions, shorter-term effects might be more relevant to the success of infection as the parasite transforms over several hours to the mother sporocyst stage in susceptible snails, but is usually killed relatively quickly in resistant snails. Nevertheless, in susceptible snails infected with *S. mansoni* for 35 days, haemocyte HSP70 protein expression was approximately 70% less than that in haemocytes from snails not exposed to the parasite. Thus it appears that *S. mansoni* infection, development and reproduction leads to a general down-regulation of haemocyte HSP70 *in vivo*.

Studies with mammals have shown that HSPs are essential in protein folding and protein transportation; therefore, a decrease in intracellular HSP70 protein levels may correlate with a general decrease in protein synthesis (Tavaria *et al.*, 1996). An observation previously noted in ESP-challenged *B. glabrata* haemocytes (Yoshino and Lodes, 1988). It has also been shown that HSP70 protein can be reduced in cells by three different mechanisms: active intracellular degradation, secretion (independent of the common secretory pathway) or released during necrosis (Lancaster and Febbraio, 2005; Mambula *et al.*, 2007). These mechanisms may also exist in molluscs as HSP70 has been shown to exocytose from cardiac cells in the snail, *Achatina fulica* (Martynova *et al.*, 2007). In this current study we have found that HSP70 is mainly intracellularly degraded after *S. mansoni* ESP-challenge. Protein degradation was inhibited in *B. glabrata* haemocytes *via* the mammalian proteasome inhibitor, MG132 which can increase heat shock factor (HSF1) activity in mammalian cells, as well as increase temperature resistance in the yeast, *Saccharomyces cerevisiae* (Lee and Goldberg, 1998; Kim *et al.*, 1999). This inhibitor did not fully block HSP70 down-modulation in ESPs exposed haemocytes, indicating that HSP70 may also be actively secreted, or indeed the inhibitor may failing to fully suppress protein degradation in *B. glabrata* haemocytes. However, this inhibitor does not appear to induce HSF1-mediated *de novo* synthesis of HSP70 protein in *B. glabrata*

haemocytes, as HSP70 expression in inhibitor-treated cells was similar to that in untreated controls.

HSP70 protein is usually localised in the cytoplasm, endoplasmic reticulum, mitochondria or the plastids of cells (Boorstein *et al.*, 1994). Confocal microscopy not only showed a general decrease in HSP70 protein in haemocytes following challenge with *S. mansoni* ESPs, but also highlighted the localisation of HSP70 near the cell nucleus. Interestingly, HSP70 protein was often noted in association with actin forming a partial ring around the nucleus. From mammalian studies it is known that actin plays a role in nuclear activities including transcription, chromatin remodelling and nucleocytoplasmic trafficking (Bettinger *et al.*, 2004).

Studies using zebrafish fibroblast cells demonstrated a correlation between an increase in HSP70 mRNA transcription after heat shock treatment and the activation of the ERK cell signalling pathway (Keller *et al.*, 2008). Results of the present study are consistent with the ERK cell signalling pathway playing a role in regulating HSP70 production in *B. glabrata* haemocytes, as inhibition of the ERK pathway with U0126 resulted in a significant reduction in HSP70 protein. The ERK pathway seems to be required to maintain basal levels of HSP70 protein. Thus, modulation of ERK by *S. mansoni* ESPs, particularly in susceptible snails, might be relevant to the outcome of infection. In addition, *S. mansoni* ESPs may activate other cell signalling pathways that induce HSP70 degradation or secretion.

Lockyer *et al.*, (2004) identified that the production of HSP70 mRNA was up-regulated in resistant *B. glabrata* mantle and brain tissue after *in vivo S. mansoni* infection using fluorescent-based differential display. The same researchers used a *B. glabrata* cDNA microarray to confirm that HSP70 mRNA expression was differentially up-regulated within 24 h in resistant *S. mansoni*-infected snails when compared to the susceptible strain (Lockyer *et al.*, 2008). In contrast, Ittiprasert *et al.*, (2009) demonstrated that juvenile susceptible *B. glabrata* exposed to *S. mansoni* miracidia for 24 h had increased mRNA HSP70 expression levels, but after 48 h expression levels decreased. Furthermore, resistant and non-susceptible snails presented low or slightly attenuated levels of HSP70 mRNA when challenged with irradiated or non-irradiated *S. mansoni* miracidia (Ittiprasert *et al.*, 2009). These two studies use different techniques, different snail tissues and different developmental stages of *B. glabrata* to investigate HSP70 gene expression levels. Importantly, measurements of mRNA levels only suggest possible changes in protein levels or function. In a biological context, characterisation of protein levels is required to fully understand the data (Valasek and Repa, 2005). Here we have shown that

resistant and susceptible snail haemocytes both have reduced HSP70 protein levels following *S. mansoni* larval ESP exposure for 1 h, but after 5 h HSP70 levels begin to increase in ESP-challenged susceptible snail haemocytes. Moreover, analysis of haemocytes from susceptible or resistant snails infected *in vivo* by *S. mansoni* miracidia for 35 days revealed that parasitism also resulted in suppression of haemocyte HSP70 levels. Clearly, it is difficult to unravel fully the interaction between the parasite (intact, irradiated, or parasite ESPs) and the haemocytes in respect of HSP70 expression without further study.

A number of studies have investigated HSP70 protein levels in gastropods mainly after thermal stress or in response to heavy metal pollutants (Tirard *et al.*, 1995; Piano *et al.*, 2004; Kefaloyianni *et al.*, 2005). In addition, a few studies have highlighted that HSP70 gene expression may be modulated in molluscs following *in vivo* infection with miracidia (Lockyer *et al.*, 2008; Ittiprasert *et al.*, 2009). In the present work it has been shown that *S. mansoni* ESPs significantly influence HSP70 protein expression in *B. glabrata* haemocytes and that this effect can vary over time and between susceptible and resistant snail strains. Furthermore, the inhibition of ERK signalling also influences HSP70 protein levels in *B. glabrata* haemocytes. HSP70 levels in haemocytes from resistant snails are affected less than in susceptible snails which may be due to the differential effects of ESPs on ERK signalling. It is also likely that HSP70 expression is modulated through additional cell signalling pathways or through complex interactions of cell signalling pathways yet to be characterised. Finally, addressing the important question of whether HSP70 is actively being secreted from haemocytes during schistosome infection, and whether such secretion can stimulate haemocyte defence activity will provide important insights into snail–schistosome interactions and possibly the outcome of infection. Whether or not the parasite has evolved further strategies to manipulate intracellular HSP70 protein levels in haemocytes from both snail strains also requires further investigation.

Chapter 8: Conclusion

Schistosome-susceptible and schistosome-resistant *Biomphalaria glabrata* haemocytes were used in this study to understand the interaction of these defence cells with *Schistosoma mansoni* components in the context of host phenotype. Under basal conditions, haemocyte numbers varied between the two snail strains, with resistant snails possessing nearly twice as many haemocytes compared to susceptible snails (per unit volume). Resistant snail haemocytes also seemed to be able to spread better on glass coverslips more than susceptible snail haemocytes. Furthermore, intracellular NO levels varied between the two snail strains, with basal NO levels being higher in susceptible snail haemocytes (even when the difference in haemocyte numbers was taken into account). Differences in schistosome susceptibility are thought to be genetically determined, as previous studies have shown differential gene expression in the two snail strains under both basal and parasite-stressed conditions (Richards, 1973; Richards, 1975; Rollinson *et al.*, 1998; Lewis *et al.*, 2001; Miller *et al.*, 2001; Spada *et al.*, 2002; Lockyer *et al.*, 2004; Mitta *et al.*, 2005; Lockyer *et al.*, 2006; Bouchut *et al.*, 2007; Guillou *et al.*, 2007; Lockyer *et al.*, 2007; Lockyer *et al.*, 2008). Differing characteristics between susceptible and resistant snail haemocytes or haemolymph may explain their differing capacities to kill *S. mansoni* miracidia and sporocysts. In the broadest sense, a greater number of haemocytes in resistant snails which have greater cell spread may allow these haemocytes to target and encapsulate sporocysts more efficiently compared to susceptible snail haemocytes.

In this project, the effect of *S. mansoni* ESPs on susceptible and resistant snail haemocytes was investigated in order to gain a further understanding of host—parasite interactions. In summary, susceptible snail haemocytes exposed to *S. mansoni* ESPs showed a slight reduction in intracellular NO levels but a substantial attenuation in ERK activity. In contrast, ESP-exposed resistant snail haemocytes exhibited a significant increase in intracellular NO levels, but no modulation of ERK signalling. Haemocytes from both snail strains also displayed significantly reduced intracellular HSP70 levels following ESP-challenge. *Schistosoma mansoni* ESPs may bind to haemocyte cell surface receptors and influence key cell signalling pathways including those involving ERK, potentially *via* phosphatase activity. This may result in altered levels of intracellular NO production, HSP70 degradation or excretion and gene transcription. Investigating haemocyte ESP-binding-receptors may provide further insight into strain specific differences in terms of downstream signalling events (Johnston and Yoshino, 2001; Humphries and Yoshino, 2003). The current study indicated that *S. mansoni* ESPs can

manipulate molluscan immune responses through interference of ERK cell signalling. Future experiments may involve investigating other signalling pathways such as those involving PKC or PI3K to decipher the extent ESPs can dampen-down the hosts' immune defences.

There are some limitations to the above findings; the effects of ESPs on snail haemocytes were investigated mainly *in vitro,* and whether or not haemocytes are exposed to similar ESP doses (0-20 µg/ml) *in vivo* needs to be explored. The anti-phospho ERK antibodies used in this study have been validated for use in invertebrates such as *Drosophila melanogaster,* in the yeast *Saccharomyces cerevisiae*, and vertebrates such as *Xenopus* and zebra fish, and appear to behave as expected in molluscs such as *B. glabrata* (Plows *et al.*, 2005; Cell signalling Technology product information). However, gene cloning of signalling molecules, such as ERK, in molluscs will help clarify how homologous molluscan proteins are to their mammalian counterparts. The completion of the *B. glabrata* genome project and improved methodologies, gene silencing (or RNA interference; RNAi) will help to clarify the function of certain mollusc signalling molecules. Such techniques have successfully been applied to knock-down fibrinogen-related protein 2 (FREP2) in *B. glabrata* (Jiang *et al.*, 2006). In the context of the present work, it would be interesting to suppress ERK expression by RNAi in resistant *B. glabrata* to see if phenotype switching and thus parasite survival can be achieved in this otherwise refractory host.

Genetic analysis of *S. mansoni* ESP-challenged haemocytes from susceptible and resistant snails using microarray analysis in this study provided a catalogue of DEGs that are involved in immune defence responses, protein transcription, protein degradation and cell-to-cell interactions. It is important to remember the limitations of using cDNA microarrays: gene expression cannot always be easily quantified, data can be lost due to a high fluorescent signals and a very low fluorescent signals may lead to false negative results (Wang *et al.*, 2002; Yang *et al.*, 2002a, Smyth and Speed, 2003). Although the current data appears robust, the microarray was limited to those oligonucleotides present on the array; without the full *B. glabrata* genome represented on the array differential expression of important transcripts involved in the immune response might be missed. Furthermore, quantification of mRNA levels does not necessarily mean changed protein levels or cellular function (Valasek and Repa, 2005). In future studies proteomics may represent a valuable tool to analyse changes in protein levels in haemocytes following schistosome exposure. Moreover, adenoviral transfections with haemocytes could be used to up-regulate the expression of specific proteins to investigate their effect on

haemocyte behaviour and physiology; this technique has previously been applied to the Bge cell line (Yoshino *et al.*, 1998).

Understanding how the ERK signalling pathway integrates with other evolutionarily conserved signalling molecules in *B. glabrata* haemocytes will further explain how ERK can influence a range of cellular processes, including NO production and HSP70 degradation or excretion. Employment of comparative approaches may help to identify other cell signalling pathways that may be critical for certain snail defences, such as encapsulation. Separating *S. mansoni* ESP components into protein, fats, and carbohydrates and exposing them to haemocytes will also provide important insights into which ESP components are most effective at modulating haemocyte function. This could be achieved using chromatographic techniques, such as high-performance liquid chromatography (HPLC).

Overall, this study has provided valuable data on the molecular mechanisms controlling certain aspects of haemocyte defence reactions in two *B. glabrata* strains and how these two strains differ in their response to *S. mansoni* ESPs. These data will facilitate further research into *B. glabrata*—*S. mansoni* interactions enabling researchers to decipher the cause of susceptibility *vs.* resistance in snail hosts following parasite infection.

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Cell Signalling Company website: http://www.cellsignal.com/pathways/map-kinase.jsp

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Natural History Museum, London website:
http://www.nhm.ac.uk/ and http://www.nhm.ac.uk/research-curation/projects/schistosomes/

PDB Protein database website: http://www.rcsb.org/pdb/home/home.do

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Chapter 10: Appendix

10.1 Appendix: Reagents, equipment and their suppliers

Reagent	Cat N°†	Supplier
1,4-Diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene (U0126)	9903	New England Biolabs, Hitchen, UK
2´-deoxynucleoside 5´-triphosphates (dNTP) mix	18427088	Invitrogen, Paisley, UK
4'-6-Diamidine-2-phenylindole (DAPI) (Dihydrochloride)	268298	Merck Chemicals, Nottingham, UK
4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM diacetate)	D-23844	Invitrogen, Paisley, UK
Acrylamide 30% (v/v)	EC-890	National Diagnostics, Hull, UK
Advantage RT-for-PCR kit	639505	Clontech-Takara Bio Europe Saint-Germain-en-Laye, France
Agarose	A9539	Sigma Aldrich, Poole, UK
Albumin from bovine serum (BSA)	A3294	Sigma Aldrich, Poole, UK
Anti-actin antibody	A5060	Sigma Aldrich, Poole, UK
Anti-Phospho MEK1/2 (Ser217/221)	9121	New England Biolabs, Hitchen, UK
Anti-Phospho p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	9101	New England Biolabs, Hitchen, UK
Bromophenol blue	B5525	Sigma Aldrich, Poole, UK
Carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG132)	474790	Merck Chemicals, Nottinghamshire,
CellTiter 96 AQueous one solution cell Proliferation Assay	G3582	Promega, Southampton, UK
Chloroform	C2432	Sigma Aldrich, Poole, UK
Cy3-dCTP/Cy5-dCTP	PA53021	GE Healthcare, Amersham, UK
Dimethyl sulphoxide (DMSO)	D8418	Sigma Aldrich, Poole, UK
FITC-conjugated goat anti rabbit secondary antibody Alexa Fluor 488	A11008	Invitrogen, Paisley, UK
FITC-conjugated goat anti mouse secondary antibody Alexa Fluor 488	F-6257	Sigma Aldrich, Poole, UK
Fluorstar Optima microplate spectrofluorometer		BMG Labtech, Aylesbury, UK
Formaldehyde (37 % wt in water)	252549	Sigma Aldrich, Poole, UK
GeneGnome chemiluminescence imaging system	-	Syngene, Cambridge,UK
Glycerol	G7893	Sigma Aldrich, Poole, UK
Glycine	G7126	Sigma Aldrich, Poole, UK
Goat anti mouse secondary conjugated HRP	sc-2005	Santa Cruz, Heidelberg, Germany

†Catalogue Number; according to 2009

Hexamethyldisilazane (HMDS)	H4875	Sigma
Anti-HSP70/HSC70 (sc-59572)	BRM-22	Santa Cruz, Heidelberg, Germany
HSP70 ELISA	EKS-700B	Assay Designs, Stressgen, Michigan, USA
Hydrochloride 32% (v/v)	H1050	Fisher Scientific Ltd, Loughborough, UK
Hyperfilm	RPN2103K	GE Healthcare, Amersham, UK
Hyperladder I DNA molecular weight marker	BIO-33025	Bioline Ltd., London, UK
Isopropanol	I9516	Sigma Aldrich, Poole, UK
Kodak GBX developer	P7042	Sigma Aldrich, Poole, UK
Kodak GBX fixer	P7167	Sigma Aldrich, Poole, UK
Laser Scanning Spectral Confocal Microscope	-	Spectral confocal & multiphoton system Leica TCS SP2
MiniOpticon Real-Time PCR detection system	-	Bio-Rad, Hemel, Hempstead, UK
Mouse anti-Rabbit secondary conjugated HRP	A9044	Sigma Aldrich, Poole, UK
Nail varnish (clear)	-	Rimmel London, UK
Nitrocellulose membrane (0.45 µm)	RPN303D	GE Healthcare, Amersham, UK
N'N'N'N'-Tetramethylethylenediamine (TEMED)	T9281	Sigma Aldrich, Poole, UK
Oligonucleotide DNA microarray	-	Cambridge University, Cambridge, UK.
Penicillin-Streptomycin	15140-148	GIBCO, Paisley, UK
Phorbol 12-myristate 13-acetate (PMA)	9905	New England Biolabs, Hitchen, UK
Phosphatase inhibitor cocktail 2	P5726	Sigma Aldrich, Poole, UK
Ponseau S stain	P7170	Sigma Aldrich, Poole, UK
Restore western blot stripping buffer	21059	Thermo Scientific, Loughborough, UK
RNase/DNase free sterile water	BPE561-1	Fisher Scientific Ltd, Loughborough, UK
Scanning electron microscope	-	Zeiss EVO 50
Semi skimmed milk (powdered)	-	Sainsbury's PLC
Sensimix DT with SYBR green	QT6T3	Quantace Ltd., London, UK
SMART PCR cDNA synthesis kit	634902	Clontech-Takara, Saint-Germain-en-Laye, France
Sodium chloride	S9888	Sigma Aldrich, Poole, UK
Sodium dodecyl sulphate (SDS)	436143	Sigma Aldrich, Poole, UK
Standard mixture for molecular weights 30,000-200,000 for SDS PAGE	SDS6H2	Sigma Aldrich, Poole, UK
Superscript II reverse transcriptase kit	18064	Invitrogen, Paisley, UK
Supersignal west dura extended duration substrate	34075	Thermo Scientific, Loughborough, UK
Supersignal west pico chemiluminescent substrate	34080	Thermo Scientific, Loughborough, UK
SYBR green I master mix	64309155	GE Healthcare, Amersham, UK
Synthetic primers	-	Primer Design Ltd, Southampton, UK
Synthetic primers	-	Sigma Aldrich, Poole, UK
TriReagent	T9424	Sigma Aldrich, Poole, UK
Tris(hydroxymethyl)aminomethane	25 285-9	Sigma Aldrich, Poole, UK

Triton x-100	T9284	Sigma Aldrich, Poole, UK
Trypan Blue 0.4%	T8154	Sigma Aldrich, Poole, UK
Trypsin, 0.05% (1x) with EDTA 4Na	25300-054	GIBCO, Paisley, UK
Tween 20	P1379	Sigma Aldrich, Poole, UK
Vectasheild mounting medium	H-1000	Vecta Laboratories Ltd, Peterborough, UK

10.2 Appendix: External and Internal Conference Abstracts

The Role of Signalling Pathways in Cell Migration and Parasite Encapsulation by Molluscan Defence Cells[1]

Zahida Zahoor, Angela J. Davies, Ruth S. Kirk, David Rollinson, Anthony J. Walker

The snail *Biomphalaria glabrata* is an important intermediate host for the transmission of *Schistosoma mansoni*, a parasite that can cause chronic disease in the human host. There is a lack of knowledge concerning molluscan immunity and how the parasite is able to survive and reproduce in the snail. One possibility is that parasite components such as Excretory and Secretory products (ESPs) might interact with the snail's immune system to modulate its behaviour. It is currently known that ESPs can modulate snail immune responses, but specifically what signalling molecules are involved is unknown (Yoshino *et al*, 1995). We hypothesise that ESPs may affect snail immune cell migration and may modulate the activity of key signalling pathways. We will characterise how ESPs affect PKC and MAPK signalling molecules as these signalling proteins have already been reported to play a role in snail immune responses (Walker *et al*, 2003). Other signalling molecules that will be investigated include PI3-K and GTPase. By using inhibitors against signalling protein, we can determine the relevance of certain pathways upon ESPs exposure. Furthermore, the functional role of these pathways will be studied including how ESPs affect defence cell movement and migration. Thus, in this project I want to clarify whether ESPs play a key role in down-modulating the snail's immune system in order for the parasite to survive. I also want to clarify the roles of certain signalling molecules in molluscan immune cells and their involvement in physiological processes.

Walker A. J. and Plows L. D. (2003) Bacterial lipopolysaccharide modulates Protein Kinase C signalling in *Lymnaea stagnalis* haemocytes. *Biology of the cell*, 95: 527-533

Yoshino P. T. and Davids J. B. (1995) *Schistosoma mansoni*: Excretory-Secretory polypeptides exhibit selective binding to plasma components of the snail *Biomphalaria glabrata*. *Experimental Parasitology*, 81: 292-301

The Effect of Schistosome Components on Cell Signalling and Downstream Gene Expression in Molluscan Defence Cells[2]

Zahida Zahoor, Angela J. Davies, Ruth S. Kirk, David Rollinson, Anthony J. Walker

The snail *Biomphalaria glabrata* is an important intermediate host for *Schistosoma mansoni*, a parasite that causes chronic disease in the human host. There is currently a lack of knowledge concerning molluscan immunity and how schistosomes survive and replicate in the snail. One possibility is that parasite components such as excretory/secretory products (ESPs) interact with the snail's defence cells (haemocytes)

[1] Kingston University Life Science, Postgraduate Research Seminar (June 2006)
[2] Wellcome Trust course on Microarray: gene expression and regulation (April 2007) Hixton Campus, Cambridge, UK

and modulate their behaviour to blunt the defence response (Walker A.J., Trends Parasitol. 22:154-159).

We have two strains of *B. glabrata*, one resistant to schistosome infection and one susceptible (based on parasite load). Our work aims to compare gene expression in haemocytes from these different snail strains when challenged with *S. mansoni* ESPs and to link gene expression patterns to upstream cell signalling events. We have extensive experience of working on cell signalling in molluscs and have recently obtained results that suggest that ESPs modulate the activities of key signalling proteins in haemocytes from susceptible snails; thus we are now in an unrivalled position to link modulation of signalling through to haemocyte gene expression following exposure. To facilitate this, a custom-built DNA microarray, developed on a Wellcome Trust funded project (to D. Rollinson et al., Natural History Museum (NHM), London), will be used and further developed as appropriate. The work will bring us closer to understanding how schistosomes evade the immune system of their intermediate hosts at the molecular level, knowledge that will be of significant value to the international scientific community.

The microarray work is due to commence in summer 2007 in the Zoology Department of the NHM. By attending the Wellcome Trust course, I will obtain vital knowledge in microarray technology and gene expression which I will immediately be able to apply to my work; thus attendance would be extremely valuable to my forthcoming research.

The Effect of Schistosome Components on Cell Signalling and Downstream Gene Expression in Molluscan Defence Cells[3]

Zahida Zahoor, Angela J. Davies, Ruth S. Kirk, David Rollinson and Anthony J. Walker

The snail *Biomphalaria glabrata* is an important intermediate host for the human parasite *Schistosoma mansoni*. This snail is useful for studying host-parasite interactions as two laboratory strains exist, one which is resistant to the parasite and thus refractory to infection, and one which is susceptible to the parasite and can support a high parasite load. Excretory-secretory products (ESPs), which are released into the snail host by the schistosome, are able to interfere with snail defence responses. It has been shown that ESPs are able to inhibit phagocytosis of antigens and suppress the production of superoxides by defence cells (haemocytes) derived from both snail strains (Connors and Yoshino, 1990). The present study is aimed to establish which cell signalling pathways are modulated when *B. glabrata* haemocytes come into contact with *S. mansoni* ESPs. Western blotting and fluorescence confocal microscopy using anti-phosphospecific antibodies has revealed that the phosphorylation (activation) status of mitogen-activated protein kinase (MAPK) is significantly down-regulated ($P \leq 0.01$, n=4) in haemocytes of susceptible, but not in resistant, *B. glabrata*. Interestingly, other researchers recently showed that another MAPK pathway (p38 MAPK) was affected by ESPs in cultured embryonic *B. glabrata* (Bge) cells, but no modulation of p38 MAPK signalling was observed in haemocytes from either of the snail strains (Humphries and Yoshino, 2006). In order to further understand differences in defence responses between susceptible and resistant *B. glabrata*, and the effects of ESP-challenge, work is currently focusing on cDNA microarray experiments at the Natural History Museum, London.

Connors V. A. and Yoshino T. P. (1990) *In vitro* effect of larval *Schistosoma mansoni* excretory-secretory products on phagocytosis-stimulated superoxide production in haemocytes from *Biomphalaria glabrata*. *Journal of Parasitology* 76:895-902.

Humphries J. E. and Yoshino T. P. (2006) *Schistosoma mansoni* excretory-secretory products stimulate a p38 signalling pathway in *Biomphalaria glabrata* embryonic cells. *International Journal for Parasitology* 36:37-46.

[3] Kingston University Life Science, Postgraduate Research Seminar (July 2007)

Schistosoma mansoni disrupts defence-cell signalling in schistosome-susceptible *Biomphalaria glabrata*[4],[5]

Zahida Zahoor, Angela J. Davies, Ruth S. Kirk, David Rollinson and Anthony J. Walker

The snail *Biomphalaria glabrata* is an important intermediate host for *Schistosoma mansoni*, the cause of human intestinal schistosomiasis. *Biomphalaria glabrata* can be used to study snail-schistosome interactions as susceptible and resistant snail strains exist. During the transformation of *S. mansoni* miracidia to mother sporocysts, Excretory-Secretory Products (ESPs) are produced; which interfere with snail defence responses. Our research has shown a cell signalling pathway in haemocytes from schistosome-susceptible *B. glabrata* that is modulated by *S. mansoni* ESPs. Western blotting and fluorescence confocal microscopy with anti-phosphospecific antibodies have revealed that the phosphorylation (activation) status of mitogen-activated protein kinase (MAPK) was significantly down-regulated in haemocytes of susceptible, but not in resistant, *B. glabrata* snails. This study is the first to demonstrate an effect of *S. mansoni* ESPs on signalling process in *B. glabrata* haemocytes, furthering our understanding of molecular interplay between schistosomes and their hosts.

Investigating gene expression in *Biomphalaria glabrata* defence cells when challenged with excretory-secretory products from *Schistosoma mansoni*[6]

Zahida Zahoor[†], Anne E. Lockyer[†], Aidan Emery[†], Angela J. Davies, Ruth S. Kirk, David Rollinson[†], Catherine S. Jones[‡], Leslie R. Noble[‡] and Anthony J. Walker

School of Life Sciences, Kingston University, Penrhyn Road, Kingston upon Thames, Surrey, KT1 2EE, UK. [†]Wolfson Wellcome Biomedical Laboratories, The Natural History Museum, Cromwell Road, London, SW7 5BD, UK. [‡]School of Biological Sciences, University of Aberdeen, Aberdeen, AB24 2TZ. UK

A custom-built cDNA microarray of *Biomphalaria glabrata* genes has recently been developed to investigate potential genes involved in snail-schistosome interactions. The present study investigates the interaction between snail defence cells called haemocytes and excretory-secretory products (ESPs) produced by *Schistosoma mansoni* following transformation from miracidia to sporocysts. The microarray has been used to study the effect of these ESPs on the gene expression profile of haemocytes from both a susceptible and a resistant *B. glabrata* strain. Haemocytes from both strains were exposed to *S. mansoni* ESPs (20 µg/ml) for 60 min and the RNA extracted. Microarray analysis revealed that a number of genes were differentially expressed between the two snail strains following ESP-challenge ($P \leq 0.05$, n=4). Such genes included those involved in energy production, intracellular protein degradation, protein-protein interactions, cell motility and regulation of cell shape. Candidate genes displaying significant differential expression are currently being further analysed by quantitative PCR (qPCR), using gene specific primers. The possible importance of such genes to snail-schistosome interactions and outcome of infection will be discussed.

[4] British Society for Parasitology conference, Glasgow (March 2008)
[5] The Natural History Museum Annual Student Conference, London (2008)
[6] Kingston University Life Science, Postgraduate Research Seminar (July 2008)

Exploring the effects of *Schistosoma mansoni* excretory-secretory products on gene expression in *Biomphalaria glabrata* haemocytes, using a cDNA microarray[7]

Zahida Zahoor[†], Anne E. Lockyer[†], Aidan Emery[†], Angela J. Davies, Ruth S. Kirk, David Rollinson[†], Catherine S. Jones[‡], Leslie R. Noble[‡] and Anthony J. Walker

School of Life Sciences, Kingston University, Penrhyn Road, Kingston upon Thames, Surrey, KT1 2EE, UK. [†]Wolfson Wellcome Biomedical Laboratories, The Natural History Museum, Cromwell Road, London, SW7 5BD, UK. [‡]School of Biological Sciences, University of Aberdeen, Aberdeen, AB24 2TZ. UK

A cDNA microarray of *Biomphalaria glabrata* genes has recently been developed in order to investigate potential genes involved in snail-schistosome interactions. The microarray has been used here to study the effect of *Schistosoma mansoni* excretory-secretory products (ESPs) on the gene expression profile of *B. glabrata* defence cells (haemocytes). Haemocytes extracted from schistosome-susceptible and schistosome-resistant *B. glabrata* strains were exposed to *S. mansoni* ESPs (20 µg/ml) for 60 min. Microarray analysis revealed that a range of genes were differentially expressed between the two snail strains following ESP-challenge ($P \leq 0.05$ where n=4). Such genes included those involved in energy production, intracellular protein degradation, protein-protein interaction, cell motility and regulation of cell shape. Candidate genes displaying differential expression have been further analysed by quantitative PCR (qPCR), using gene specific primers. The possible importance of such genes to snail-schistosome interactions and outcome of infection will be discussed.

Interaction between *Schistosoma mansoni* and *Biomphalaria glabrata*: host-parasite compatibility[8]

Zahida Zahoor, Ruth S. Kirk, Angela J. Davies, David Rollinson, Anthony J. Walker

The platyhelminth parasite, *Schistosoma mansoni,* resides in the snail *Biomphalaria glabrata* during its life cycle. Two laboratory strains of *B. glabrata* exist, one which is resistant to *S. mansoni* infection and one which is susceptible to infection. How the parasite survives in its susceptible snail host is currently not fully understood. During intra-molluscan development the parasite produces excretory-secretory products (ESPs), a cocktail of unknown proteins and lipids. One function of ESPs is to influence the behaviour of susceptible snail-host defence cells (haemocytes), reducing their potential to move, spread and synthesise protein. The current project has shown that ESPs also influence intracellular nitric oxide (NO) production in host haemocytes (Zahoor *et al.,* 2009); NO is a cytotoxic molecule capable of killing the parasite. *Schistosoma mansoni* ESPs may be inducing these responses by binding to haemocyte cell surface receptors and down-regulating the activities of key cell signalling pathways, such as extracellular signal-regulated kinase (ERK) (Zahoor *et al.,* 2008) and potentially altering gene expression for a range of proteins including heat shock proteins (HSPs).

Zahoor Z., Davies A.J., Kirk, R.S., Rollinson D. and Walker A.J. (2008). Disruption of ERK signalling in *Biomphalaria glabrata* defence cells by *Schistosoma mansoni:* implications for parasite survival in the snail host. Developmental and Comparative Immunology, 32: 1561-1571.

Zahoor Z., Davies A.J., Kirk, R.S., Rollinson D. and Walker A.J. (2009). Nitric oxide production by *Biomphalaria glabrata* haemocytes: effects of *Schistosoma mansoni* ESPs and regulation through the extracellular signal-regulated kinase pathway. Parasites and Vectors 2:18:1-10.

[7] Xth European Multicolloquium of Parasitology, Paris 2008
[8] Kingston University Life Science, Postgraduate Research Seminar (July 2009)

10.3 Appendix: Co-supervision of an undergraduate laboratory-based project

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Nitric oxide production in the snail *Biomphalaria glabrata*, a host for the parasite *Schistosoma mansoni*

Independent investigation showing a general increase in nitric oxide (NO) production in susceptible *B. glabrata* haemocytes when challenged with PMA (1 µM), laminarin (5 mg/ml), LPS (5 mg/ml) or zymosan A (5 mg/ml) compared to controls (DAF-FM only) over 2 h. Experiments were carried out in triplicate on two separate days using pooled haemolymph from susceptible snails (as outlined in the Material and Methods section 2.6). The project independently confirmed that extracellular signal-regulated kinase (ERK) inhibition by U0126 (10 µM over 2 h) reduced NO levels in susceptible snail haemocytes.

10.4 Appendix: List of Publications arising from PhD study

Zahoor Z, Davies AJ, Kirk RS, Rollinson D, Walker AJ. 2008. *Schistosoma mansoni* disrupts cell defence signalling in schistosome-susceptible *Biomphalaria glabrata*. *The Malacologist,* 13

Zahoor Z, Davies AJ, Kirk RS, Rollinson D, Walker AJ. 2008. Disruption of ERK signalling in *Biomphalaria glabrata* defence cells by *Schistosoma mansoni*: Implications for parasite survival in the snail host. *Developmental & Comparative Immunology*, 32(12):1561-1571.

Zahoor Z, Davies AJ, Kirk RS, Rollinson D, Walker AJ. 2009. Nitric oxide production by *Biomphalaria glabrata* haemocytes: effects of *Schistosoma mansoni* ESPs and regulation through the extracellular signal-regulated kinase pathway. *Parasites and Vectors*, 2(18):1-10

Zahoor Z, Davies AJ, Kirk RS, Rollinson D, Walker AJ. 2010. Larval excretory-secretory products from the parasite *Schistosoma mansoni* modulate HSP70 protein expression in defence cells of its snail host, *Biomphalaria glabrata*. *Cell Stress & Chaperones,* DOI 10.1007/s12192-010-0176-z

Glossary

Antibody	Immunoglobulin produced that binds to specific antigen
Antigen	Molecule that is recognised by a T cell receptor or antibody
Antigen presenting cell (APC)	A cell that presents processed antigens
B cell	A lymphocyte that is produced in the bone marrow and is involved in the humoral immune response
cDNA library	A pool of complementary DNA clones produced by cloning total messenger RNA from single source
Cell signalling pathway	A system of communication that governs basic cellular activities
Cercaria	A larval stage of a parasite
Chemotaxis	Migration of cells along a concentration gradient of an attractant
Cilia	An organelle found in eukaryotic cells
Classical pathway	The mechanism of complement activation
Cytokine	Substances that are secreted by immune cells which carry signals locally
Definitive host	A host in which the parasite reaches maturity
Dendritic cells	An antigen presenting cell
Differentially expressed gene (DEG)	A difference in gene expression between two samples
Digenea	The subclass of Platyhelminthes
Encapsulation	The coordinated response of cells to engulf a large particle or pathogen
Excretory-secretory products (ESPs)	A range of molecules released or excreted from a parasite into the hosts' environment
Excretory pore	Pore on the surface of some invertebrates through which the excretory system opens
Exocytosis	Discarding particles that are too big to diffuse through the plasma membrane
Fluke	A common name for a trematode
Gene expression	The transcription of the information contained within a DNA sequence
Haemocyte	An invertebrate defence cell
Helminth	Parasitic worm
Humoral response	A reaction mediated by B lymphocytes, may involve the production of antibodies
Immunomodulation/ Immunoregulation	Adjustment of the immune response or defence mechanisms
Immunopatholgy	The branch of immunology that deals with pathologies of the immune system
Inflammation	The body's reaction to infection or injury, may involve pain, swelling and redness
Innate immune response	An evolutionarily conserved defence response
Interferon	A cytokine with anti-viral properties that can regulate immune responses
Interleukin	Cytokine usually secreted by leukocytes

Intermediate host	A host that harbours the parasite only for a short transition period, during which some developmental stage is completed, but not to sexual maturity
Larva	Immature stage
Larval transformation proteins (LTPs)	Isolated protein fractions from the schistosome larval stage
Lymphocyte	A white blood cell
Macrophage	A phagocytic blood cell that is involved in cellular immune defence responses
Microarray	A tool for analysing gene expression; a small membrane or glass slide containing samples of many genes arranged in a regular pattern
Miracidium	A free-living motile ciliated stage of a digenean parasite
Mollusca	A phylum composed of a large group of animals having no backbone, soft unsegmente bodies, sometimes covered with a hard shell
Molluscan defence reaction	The defence response of molluscs against pathogens
Nematode	Unsegmented worm, usually pseudocoelomate
Oligonucleotides	A short sequence of nucleotides (RNA or DNA) typically with twenty or fewer base pair
Parasite	An organism that lives on or within and at the expense of another organism
Pathogen	A microorganism that injures its host
Pathogenic	Giving origin to disease or symptoms of disease
Phagocytosis	The process by which phagocytes engulf and digest cells or particles
Platyhelminthes	Unsegmented flat worm
Proteasome	A cellular organelle with protease activities that degrades mature proteins in an ATP-dependent manner
Resistant snails	Snails which are resistant to pathogenic infection
Respiratory burst	Oxygen dependent increase in metabolic activity within a phagocytic cell
Schistosomes	Small flukes that live in blood vessels of its infected definitive host
Susceptible snails	Snails which are susceptible to pathogenic infection
Sporocyst	A sac-like larval stage where asexual reproduction may take place
T cell	Lymphocyte that has been processed through the thymus gland
Tolerance	Diminished or absent capacity to make a specific response to an antigen
Tumour necrosis factor	Cytokine that has cytotoxic and immunoregulatory effects
Vector	The type of intermediate host for parasites, bacteria, protozoa and viruses
Virulence	Capacity of an organism to overcome host defences

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