The Role of Haemocytes in the Immune Response of *Biomphalaria glabrata* against *Schistosoma mansoni*: Investigations into Haematopoiesis and Pattern Recognition Receptors

by

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Abstract

Snails (Gastropoda) serve as hosts for the larval development of many medically and agriculturally important digenean trematodes (parasitic flatworms), including schistosomes, blood flukes that collectively infect more than 260 million people globally. Compatibility between snails and digeneans is often very specific such that suitable snail hosts define the geographical ranges of diseases caused by these worms. The immune cells (haemocytes) of a snail are sentinels that act as a crucial barrier to infection by larval digeneans. Haemocytes coordinate a robust and specific immunological response, participating directly in parasite killing by encapsulating and clearing the infection. However, very little is known about the production and maintenance of suitable haemocyte populations, as well as how these haemocytes engage their targets. Haemocyte proliferation and differentiation are influenced by unknown digenean-specific exogenous factors, and until recently, nothing was known about the endogenous control of haemocyte development in any gastropod model.

My doctoral research focused on characterizing endogenous drivers of haematopoiesis in *Biomphalaria glabrata* with emphasis on the evolutionarily conserved granulins. Granulins are growth factors that drive proliferation of immune cells in organisms, spanning the animal kingdom. *B. glabrata* progranulin (*Bg*GRN) was cloned, expressed and functionally characterized. Using quantitative real-time polymerase chain reactions and Western blots, I found that endogenous *Bg*GRN expression is induced at the early stages of *S. mansoni* infection. Recombinant *Bg*GRN induced proliferation of *B. glabrata* haemocytes, specifically driving the production of an adherent haemocyte subset that participates centrally in the anti-digenean defence response. Additionally, I demonstrated that susceptible *B. glabrata* snails can be made resistant to infection with *S. mansoni* by first inducing haemocyte proliferation with *Bg*GRN.

This reversal of the susceptible phenotype resulted in a 54% reduction in the number of snails successfully infected by week 7 post-challenge.

Further analysis revealed that the adherent haemocytes induced by *Bg*GRN were unique, particularly in their expression of a *B. glabrata* Toll-like receptor (*Bg*TLR). TLRs are trans-membrane proteins representing one of the groups of haemocyte surface molecules through which parasite presence is detected and the signal transduced to the haemocytes to elicit effector functions required for parasite elimination. *Bg*TLR was also found to be highly expressed at the early stages of *S. mansoni* infection like *Bg*GRN, and experimental knockdown of this TLR resulted in loss of resistance in 43% of the resistant snails challenged. Taken together, these studies (of factors that drive haemocyte proliferation and differentiation, and haemocyte surface molecules that facilitate engagement of haemocytes) significantly advance the understanding of snail immunity with respect to schistosomes, and may one day facilitate the development of tools for snail control and consequently improve the control of schistosomiasis.

Preface

This thesis is a compilation of the work done during my doctoral research. The research projects, of which this thesis is a part, received animal research ethics approval from the University of Alberta Research Ethics Office, Project Name "Immunobiology of Snail-Schistosome Interactions", No. AU00000057_REN1, AU00000057_REN2 and AU00000057_REN3.

The general introduction and discussion in chapters 1 and 6 are my original work, as well as the greater part of the literature review in chapter 2. Some sections of chapter 2, pertaining to haematopoiesis in molluscs and snail immunity, form parts of co-authored review manuscripts published as:

- Pila, E.A., Sullivan, J.T., Wu, X.Z., Fang, J., Rudko, S.P., Gordy, M.A., and Hanington, P.C. (2016). Haematopoiesis in molluscs: a review of haemocyte development and function in gastropods, cephalopods and bivalves. *Developmental and Comparative Immunology* 58,119-128; and,
- Pila, E.A., Li, H., Hambrook, J.R., Wu, X. and Hanington, P.C. (2017). Schistosomiasis from a snail's perspective: Advances in snail immunity, *Trends in Parasitology* 33(11), 845-857 respectively.

Chapter 4 of this thesis has been published as:

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I was responsible for the design of the research, performing the research and data analysis, as

well as the manuscript composition. Gordy, M.A., Phillips, V.K., Kabore, A.L., and Rudko, S.P. assisted with performing the research. Gordy, M.A. and Phillips, V.K. also assisted with data analysis while Gordy, M.A. and Rudko, S.P. contributed to manuscript composition. Hanington, P.C. was the supervisory author and was involved in research design, data analysis and composition of the manuscript.

Chapter 5 of this thesis has also been published as:

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My contribution included conception and design of the experiments, performing the experiments, data analysis and writing the manuscript. Kabore, A.L. contributed in research design, performing the experiments and analyzing the data. Tarrabain, M. assisted with performing the experiments and data analysis. Hanington, P.C. was the supervisory author and was involved in designing the research, performing the experiments, data analysis and manuscript composition.

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List of Abbreviations

α	-	Alpha
~	-	Approximately
β	-	Beta
β-PFT	-	β-pore forming toxin
%	-	Percent
A260/A280	-	Ratio of Absorbance at 260 nM to Absorbance at 280 nM
A2M	-	Alpha2 Macroglobulin
ADAMTS-7	-	A Disintegrin and Metalloproteinase with Thrombospondin motifs-7
AMP	-	Antimicrobial Peptide
ANOVA	-	Analysis of Variance
APO	-	Amoebocyte-producing Organ
APW	-	Anterior Pericardial Wall
ASB	-	Antibody Staining Buffer
ASW	-	Artificial Spring Water
Bge	-	*Biomphalaria glabrata* embryonic cell line
***Bg*GRN**	-	*Biomphalaria glabrata* progranulin
***Bg*TLR**	-	*B. glabrata* Toll-like receptor
***Bg*MIF**	-	*B. glabrata* Migration Inhibitory Factor
***Bg*Temptin**	-	*B. glabrata* temptin
***Bg*TEP**	-	*B. glabrata* Thioester-containing Protein
BLAST	-	Basic Local Alignment Search Tool
BlastP	-	Basic Local Alignment Search Tool for Protein sequences

bp	-	Base Pairs
BrdU	-	5-bromo-2′-deoxyuridine
BSA	-	Bovine Serum Albumin
°C	-	Degrees Celsius
$CaCl_2$	-	Calcium Chloride
cas9	-	CRISPR-associated protein 9
CBSS	-	Cherninès Balanced Salt Solution
CCF	-	Coelomic Cytolytic Factor
CD34	-	Cluster of Differentiation 34
cDNA	-	Complementary Deoxyribonucleic Acid
cm	-	Centimeter
CO_2	-	Carbondioxide
CREP	-	C-type Lectin-related Protein
CRISPR	-	Clustered Regularly Interspersed Palindromic Repeats
CS	-	Cut Site
C_t	-	Cycle threshold
Cu/Zn	-	Copper/Zinc
3D	-	Three-dimensional
$\Delta\Delta C_t$	-	Delta-Delta Cycle threshold
DAMP	-	Damage-associated Molecular Pattern
DAPI	-	4,6-Diamidino-2-phenylindole
DMEM	-	Dulbecco's Modified Eagle's Medium
dNTP	-	Deoxynucleotide

dpc	-	Day(s) Post-challenge
Dscam	-	Down syndrome cell adhesion molecule
dsRNA	-	Double-stranded Ribonucleic Acid
dT	-	Deoxythymine
EDTA	-	Ethylenediaminetetraacetic acid
EGF	-	Epidermal Growth Factor
EGTA	-	Ethylene-bis(oxyethylenenitrilo)tetraacetic acid
ELISA	-	Enzyme-linked Immunosorbent Assay
ERK1/2	-	Extracelluar Signal-Regulated Kinase 1 and 2
ESP	-	Excretory-secretory Product
FBG	-	Fibrinogen
FBS	-	Fetal Bovine Serum
FREP	-	Fibrinogen-related Protein
g	-	Gram or Gravity
G418	-	Geneticin antibiotic
GAPDH	-	Glyceraldehyde 3-Phosphate Dehydrogenase
GFP	-	Green Fluorescent Protein
GNBP	-	Gram-negative Binding Protein
GnRH	-	Gonadotropin-releasing Hormone
GRC	-	Guadeloupe Resistance Complex
GREP	-	Galectin-related Protein
GRN	-	Granulin
h	-	Hour(s)

HA	-	Hemagglutinin A
HCl	-	Hydrochloric acid
hpc	-	Hour(s) Post-challenge
HRP	-	Horseradish peroxidase
Hsp	-	Heat Shock Protein
ICR	-	Interceding Region
IDGF	-	Imaginal Disc Growth Factor
IgG	-	Immunoglobulin isotype G
IgSF	-	Immunoglobulin Superfamily
K, k	-	Kilo
KCl	-	Potassium Chloride
kDa	-	Kilodalton
L	-	Liter
LC-MS/MS	-	Liquid Chromatography, tandem Mass Spectrometry
LPS	-	Lipopolysaccharide
LTP	-	Larval Transformation Product
LRR	-	Leucine-rich Repeat
M	-	Molar
mA	-	Milliampere
MAPK	-	Mitogen-activated Protein Kinase
mccTLR	-	Multiple cysteine cluster TLRs
MEK	-	MAPK/ERK
Mem	-	Membrane protein

mg	-	Milligram
$MgCl_2$	-	Magnesium Chloride
MIF	-	Macrophage Migration Inhibitory Factor
m	-	Minute(s)
miRNA	-	Micro RNA
mL	-	Milliliter
mm	-	Millimeter
mM	-	Millimolar
mRNA	-	Messenger RNA
MWCO	-	Molecular Weight Cut Off
myc	-	Myelocytomatosis viral oncogene homologue
n	-	Sample size
N	-	Normal or Normality
NaCl	-	Sodium Chloride
NADPH	-	Nicotinamide Adenine Dinucleotide Phosphate
NaF	-	Sodium Fluoride
$NaHCO_3$	-	Sodium Hydrogen Carbonate or Sodium Bicarbonate
Na_2HPO_4	-	Disodium Hydrogen Phosphate
NaOH	-	Sodium Hydroxide
NF-kB	-	Nuclear factor kappa-light-chain-enhancer of activated B cells
ng	-	Nanogram
NLR	-	NOD-like Receptor
nM	-	Nanomolar

OD_{600}	-	Optical Density at 600 nM wavelength
Oligo	-	Oligonucleotide
***Ov*GRN**	-	*Opisthorchis viverrini* granulin
PAMP	-	Pathogen-associated Molecular Pattern
PBS	-	Phosphate-buffered Saline
PGRN	-	Progranulin
PGRP	-	Peptidoglycan Recognition Protein
PI3K	-	Phosphatidylinositol-3 kinase
piRNA	-	piwi-interacting RNA
PKC	-	Protein Kinase C
PMA	-	Phorbol 12-Myristate 13-Acetate
PPO	-	Prophenoloxidase
PRR	-	Pattern Recognition Receptor
qRT-PCR	-	Quantitative Real-Time Polymerase Chain Reaction
RACE	-	Rapid Amplification of cDNA Ends
r*Bg*GRN	-	Recombinant *Biomphalaria glabrata* progranulin
RHD	-	Rel Homology Domain
RISC	-	RNA-induced Silencing Complex
RNAi	-	Ribonucleic Acid Interference
RNI	-	Reactive Nitrogen Species
ROS	-	Reactive Oxygen Species
RQ	-	Relative Quantification or Fold Change
s	-	Second(s)

SD	-	Standard Deviation
SDS-PAGE	-	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEM	-	Standard Error of the Mean
siRNA	-	Short Interfering Ribonucleic Acid
SLPI	-	Secretory Leukocyte Protease Inhibitor
***Sm*PoMuc**	-	*Schistosoma mansoni* Polymorphic Mucins
sod1	-	Superoxide Dismutase 1
SP	-	Signal Peptide
SRCR	-	Scavenger-receptor Cysteine Rich
SSP	-	Snail Secretory Product
SSS	-	Sterile Snail Saline
TEP	-	Thioester-containing Protein
TGase	-	Transglutaminase
TGF–α	-	Transforming Growth Factor alpha
TIR	-	Toll/Interleukin 1 receptor domain
TLR	-	Toll-like receptor
TNF–α	-	Tumour Necrosis Factor Alpha
TBS	-	Tris-buffered Saline
TBS-T	-	Tris-buffered Saline containing 0.1% Tween-20
U	-	Unit(s)
µg	-	Microgram
µL	-	Microliter
µm	-	Micrometer

µM	-	Micromolar
V	-	Voltage
VIgLs	-	Variable Immunoglobulin and Lectin domain-containing Molecules
WHO	-	World Health Organization
WSH	-	Whole Snail Homogenate
x	-	Times

Chapter 1

General Introduction

1.1 Overview

Parasitic flatworms, also known as trematodes or flukes, cause a wide range of diseases of both medical and agricultural importance. Over 25,000 different species of trematodes exist (Esch *et al.* 2002), causing various human and animal diseases collectively known as trematodiases. These include schistosomiasis (blood fluke disease), fasciolosis, clonorchiasis, opisthorchiasis (liver fluke diseases), paragonimiasis (lung fluke disease) and echinostomiasis (intestinal fluke disease). Trematodiases present a unique challenge in terms of disease prevention and control due to the complex nature of trematode life cycles, which involve development in at least two different host organisms. However, a unifying feature in the life cycle of nearly all trematodes is the utilization of a snail as the first intermediate host in which their larval stages develop (Esch *et al.* 2002; Cribb *et al.* 2001; Bray *et al.* 2002; Abdel-Malek 1950; Von Brand and Files 1947). This is characterized by prolific clonal amplification within the snail host that greatly increases the infective potential of these parasites.

Among diseases caused by trematodes, schistosomiasis is considered the most impactful, ranking second only to malaria among parasitic diseases, in terms of morbidity and socioeconomic impact (King 2010). Schistosomiasis is caused by parasitic flatworms of the genus *Schistosoma* and affects at least 260 million people worldwide (WHO, 2016). In total, the disease occurs in 74 countries distributed in the western hemisphere, the Pacific region, the Middle East, Asia and Africa, with 85% of the cases occurring in sub-Saharan Africa (Chitsulo *et al.* 2000). Three main *Schistosoma* species cause the disease in humans which can present as

urinary or intestinal schistosomiasis. Urinary schistosomiasis is caused by *S. haematobium*, while intestinal schistosomiasis is caused by *S. mansoni* and *S. japonicum* (Gryseels *et al.* 2006). The immunopathology of the disease is mainly due to inflammatory reactions to the eggs released by adult schistosomes which live in the venous plexus of the bladder or the mesenteric venules of the bowel/rectum (Gryseels *et al.* 2006; Barsoum *et al.* 2013). Untreated cases can lead to chronic disease resulting in kidney failure, bladder cancer, secondary infertility and abortion (Rollinson 2009).

Currently, there is no vaccine available, and the major control approach for schistosomiasis is regular population-based mass treatment with the drug praziquantel, or treatment of specific risk groups such as school age children (Tchuem Tchuente *et al.* 2013; Olveda *et al.* 2014). Long term reduction in transmission remains a challenge because treatment reduces disease effects but it does not provide a lasting solution to the problem, and infection can re-establish within a short time even when human infections appear to be cleared following treatment (Rollinson *et al.* 2001). Moreover, with just a single effective drug available for treatment of schistosomiasis, the development of drug resistance is a real concern. Resistance of *S. haematobium* to standard treatment in humans have already been reported (Alonso *et al.* 2006; Silva *et al.* 2005).

Efforts to control schistosomiasis are impeded by challenges that are linked to the larval development of the parasite within snails. In the quest for global elimination of schistosomiasis, the emerging consensus is that an integrated control strategy is required instead of the current, nearly one-dimensional approach that depends on oral chemotherapy. The major components of such a strategy include: intermediate host (snail) control, reduction of human exposure to infectious water through provision of safe water, health education, monitoring of individuals

with occupational exposure, and prevention of freshwater contamination through sanitation, hygiene, and treatment of humans and zoonotic hosts (Secor 2014; WHO, 2012).

Snail control is an essential component of an integrated control approach because asexual reproduction of schistosomes within the snail results in massive amplification of the *Schistosoma* parasites. Although the requirement for snails is obligatory, there is high specificity for certain snail species (Webster and Southgate 2003; Mubila and Rollinson 2002; Stothard *et al.* 2001). Snails also exert pressure on the life cycles of some trematodes because even compatible hosts, the percentage of patent infections as a ratio of snail exposure to schistosome parasites is very low (Allan *et al.* 2013; Hamburger *et al.* 2004). Nonetheless, a recent meta-analysis of large scale schistosomiasis control attempts over the past century across the globe demonstrated that snail control is the most effective way to reduce schistosomiasis prevalence (Sokolow *et al.* 2016). Prevalence reduction of 92% was achieved in programs using extensive snail control, compared to 37% in those using little or no snail control (Sokolow *et al.* 2016). In addition, compared to vectors of other diseases such as malaria and leishmaniasis, snail dispersal over the snail lifetime in the environment is limited, unless aided by other factors such as water currents. This limited ability for dispersal is potentially advantageous from the standpoint of snail control.

This thesis is a compilation of the work done during my doctoral research, which significantly advances our understanding of the immunological determinants of a successful schistosome infection in snails, and may one day facilitate the development of tools for snail control and consequently improve the control of schistosomiasis. There are four ways that snail control has been conceived or approached by public health initiatives in the context of schistosomiasis or other trematodiases. These include: (1) application of chemicals that kill snails, known as molluscicides; (2) snail habitat modification; (3) biological control of snails

using predators, competitors, parasites and micropathogens, and (4) use of snail strains resistant to trematode infection.

Application of molluscicides is perhaps the earliest means that was introduced to control schistosomiasis through targeting of intermediate host snails. An ideal molluscicide is expected to have specific toxicity to snails, low toxicity to other organisms, no toxicity to mammals, no adverse effects if it enters the food chain, stability in storage for at least 18 months, low cost and easy measurement of its concentrations in breeding sites (Souza 1995; Sudarov and Krstic 1965). To date, no ideal molluscicides have been found. Control of snail intermediate hosts through molluscicides was explored as early as the 1920s with trials involving chemicals such as calcium cyanamide, calcium oxide, copper sulphate, sodium pentachlorophenate, n-tritylmorpholine and hydrated lime (reviewed in: Fenwick *et al.* 2006). All of the initial chemicals had various drawbacks including toxicity to non-target organisms like fish and low efficacy on the field (reviewed in: Fenwick *et al.* 2006; McCullough *et al.* 1980). Nevertheless, molluscicide was used extensively beginning from the 1950s until the advent of oral chemotherapy reduced its use. More recently, the extensive use of a molluscicide was facilitated by the discovery of niclosamide. Still in use to date, it has several optimal molluscicidal properties namely, ability to kill both snails and their eggs at concentrations less than 1 mg/L, very low toxicity to humans and livestock, relative stability to ultraviolet radiation and persistence of lethal effects for up to 24 h after application (Andrews *et al.* 1982; Foster *et al.* 1959).

Where used properly, application of molluscicides has been shown to greatly impact schistosome transmission and/or snail abundance. On the island of St Lucia for example, *S. mansoni* prevalence among children in a high-risk area was reduced from 22% to 4.3% within 5 years of niclosamide application, compared to 22% to 20% reduction in a non-molluscicide area

(Sturrock *et al.* 1974). This involved a fortnightly survey of snails and focal molluscicide of habitats in which snail colonies were detected. Snail numbers were eliminated in some static habitats but only reduced for the majority of sites being monitored. These surviving populations rebounded in as little as 12 weeks, suggesting that continued chemical pressure would be required for sustained control. Application of soapberry endod, a plant-derived molluscicide from *Phyotolacca dodecandra*, had a 6–8% reduction in *S. mansoni* prevalence in 4 years in Ethiopia (Erko *et al.* 2002). In Brazil, 3 years of molluscicide application significantly reduced snail numbers per man/minute/station from about 32 to 0.6, compared to 10.9 in the untreated area (Pieri *et al.* 1995). The impact on disease prevalence or intensity was not examined in this study.

Molluscicide application requires a high degree of training and organization in order to be successful. This includes making sure that achievable level of molluscicide will be effective against the target snails, good knowledge of transmission dynamics and how environmental variables like temperature, flow rate and vegetation can influence treatment. The cost associated with this is high, and there is always the need for reapplication since it is often not possible to eliminate all the snails in a single treatment regime (King and Bertsch 2015). In addition to these drawbacks, the current molluscicide of choice (niclosamide) also kills fish and makes treated water yellowish which raises acceptability issues in some communities (Takougang *et al.* 2007).

Despite varying successes recorded with the use of molluscicides, interest in this strategy waned substantially beginning with the discovery of anti-schistosomal drugs, particularly praziquantel in the 1970s (Fenwick *et al.* 2006), which was shown to be highly effective. Vaccine research at about the same time using radiation-attenuated cercariae and schistosomulae showed up to 80% protection in mice (reviewed in: El Ridi and Tallima 2015). The effectiveness

and ease of use of oral chemotherapy coupled with the hope that human vaccine would soon be available reduced the momentum in molluscicide use and research. However, discovery and production of an effective human vaccine have remained elusive and there is increasing recognition that chemotherapy alone will not eliminate schistosomiasis.

Habitat modification activities that have been applied to control snails include lining drainages with cement, removing vegetation on which snails feed and draining swamps and puddles where they live. Although use of this strategy alone has been shown to reduce the population of *Oncomelania* snails in some endemic areas of the Philippines and Japan (Makiya *et al.* 1982; Makiya *et al.* 1986; Ohmae *et al.* 2003), it also shows that habitat modification must be extensive for it to significantly impact schistosome transmission (Tanaka *et al.* 1983). The associated cost can be limiting for resource-limited areas and there is high risk of infection since protective tools and clothing may not be available in such areas. This risk of infection is even higher with direct removal of snails from their habitats as have been tried in China (Sleigh *et al.* 1998). Based on the low probability of great impact when used alone, habitat modification is often part of integrated schistosomiasis control strategies.

Biological control of snails has been considered at least for the last three decades, and the potential organisms can be grouped into predators, competitors, parasites and micropathogens. The importance of biological control has been reinforced by relatively recent activities such as damming of rivers and overfishing, both leading to increased prevalence of schistosomiasis due to an increase in suitable habitat and a reduction of predatory pressure on snails. The completion of the Diama dam in Senegal in 1986, which was constructed to prevent intrusion of salt water from upstream, effectively interrupted the breeding of the African river prawn (*Macrobrachium vollenhovenii*) whose larvae require estuarine waters to develop into adults. Being a voracious

consumer of snails, including those that serve as intermediate host for schistosomes (*Biomphalaria* and *Bulinus*), the epidemic of schistosomiasis that followed the construction of the dam has been associated with the prawn's decline (Secor 2014; Sokolow *et al.* 2014). Similarly, in Malawi, overfishing of the molluscivorous cichlid fish (*Trematocranus placodon*) in Lake Malawi coincided with increased transmission of schistosomiasis (Stauffer *et al.* 2006; Madsen and Stauffer 2011). These observations are drivers of research to re-stock depleted local freshwaters with molluscivorous species that once existed therein or new ones (Gashaw *et al.* 2008; Sokolow *et al.* 2014).

One of the successful deployments of snail predators to control schistosomiasis involves the North American crayfish (*Procambarus clarkii*), which was introduced for aquaculture in water bodies in Kenya. When *P. clarkii* was introduced in man-made ponds near schools, the *Bulinus* snail population was eliminated within 2 years while *S. haematobium* prevalence reduced significantly from 61% to 12 % in experimental ponds compared to 62% to 42% in control ponds in which *P. clarkii* was not introduced (Mkoji *et al.* 1999).

Snails that consume available food sources and egg masses of schistosome-transmitting snails and have higher reproductive rates have also been used successfully to out-compete the latter. Such snails (for example, *Marisa cornuarietis, Tarebia granifera, Melanoides tuberculata*) have been used at several sites in the Caribbean region and Africa to eliminate or, maintain at very low level the populations of schistosome-transmitting snails. They also prevent recolonization of sites where schistosome-transmitting snails had previously been eliminated (Pointier and Jourdane 2000; Nguma *et al.* 1982).

Parasites and micropathogens of intermediate hosts of schistosomes continue to be investigated (Duval *et al.* 2015; de Oliveira *et al.* 2004; Singer *et al.* 1994; Nassi *et al.* 1979),

with many potential candidates but of those tested in the field, only a few have been effective. The basic mode of action by these organisms is lethality and/or complete sterilization of the infected snails, which reduces their numbers over time. Nassi *et al*. (1979) achieved a near total elimination of *B. glabrata* population in Guadeloupe 15 months after introduction of the sterilizing trematode, *Ribeiroia guadeloupensis*.

Biological control can be an effective approach as the organisms will be self-sustaining once introduced. In some cases, such as the introduction of prawns, crayfish and fish, it has the added benefit of providing food and serves as a potential economic benefit for the local communities. However, there is uncertainty about the ecological impact particularly for non-native species. Also, the efficiency of this method varies according to the type of habitat, thus location-specific trials must be done as successes elsewhere cannot be automatically transposed to another location. Just as with molluscicides application and snail habitat modification, this strategy likely should be combined with others like chemotherapy to be able to eliminate schistosomiasis.

The use of snail strains resistant to schistosome infection, a genetic control technique, is a form of biological control technique using competitors. However, the type of competition here is mostly intra-specific but can also be inter-specific, and the competition platform is immunological. First proposed in the 1950s (WHO, 1954; Hubendick 1958), the premise of this strategy is that, introduction of snails resistant to schistosome infection in sufficient numbers may out-compete susceptible ones that have to contend with negative effect of infection including castration (Faro *et al.* 2013; Hechinger *et al.* 2009; Cooper *et al.* 1996). High numbers of resistant snails will also create a decoy effect, effectively 'removing' schistosome miracidia from the water since fewer compatible snail–miracidia encounters would be made, resulting in

fewer patent infections. Blocking schistosome development in the snails creates a perturbation in transmission to humans that if accompanied with measures like chemotherapy can fully interrupt transmission and eventually lead to the elimination of the disease (Woodruff 1985). Initial skepticism about its potential for success meant that this technique did not develop traction at the time it was proposed. However, there has been renewed interest over the years in this technique, perhaps due to the failure to eradicate schistosomiasis with the existing strategies or to develop a working human vaccine.

In theory, introduction of genetically resistant snails can be achieved in at least 3 different ways:

1. Selection of snail–schistosome incompatibility within the local population.
2. Importation of snails whose incompatibility to local schistosome is known.
3. Hybridization of compatible local snails with incompatible con-specifics from elsewhere (Woodruff 1985).

In addition, with the availability of advanced genomic techniques, it is now conceivable that snails can be directly modified to carry desired genes and markers for tracking them. Genomic modification technology, mediated by the CRISPR/cas9 system and transposon transformation, have already been used to modify mosquitos for resistance to *Plasmodium* infection (Gantz *et al.* 2015; Dong *et al.* 2011; Isaacs *et al.* 2011), though the success of malaria control approaches deploying these transgenic mosquitos remains to be tested. Successful use of resistant snails has the major advantage of achieving schistosomiasis control while preserving the ecological role of the snails within the ecosystem as there will only be a replacement of snail phenotype.

Transitioning this strategy from theory to practical application requires a thorough understanding of the determinants of compatibility between snails and the schistosomes that infect them. Therefore, many studies with interest in the genetic control technique have focused on various determinants of snail–schistosome compatibility, including the molecular basis and specific genes involved. Most of these studies utilize the *B. glabrata–S. mansoni* model (Coustau *et al.* 2015). From studies of this model, it has been shown that snail–schistosome compatibility is a complex, variable and heritable phenomenon (Goodall *et al.* 2006; Roger *et al.* 2008; Bonner *et al.* 2012). Furthermore, the snail immune response has emerged as an important determinant of *S. mansoni* infection success, acting in concert with host and parasite genetics and epigenetics, proteomic and transcriptomic regulation, and environmental factors (Mitta *et al.* 2017).

Progress in this area has led to the identification of many resistance-associated genes. The functional relevance of some of these genes have been determined, but many remain functionally uncharacterized. Of the former, fibrinogen-related protein 3 (FREP3) – a trematode recognition molecule secreted by the snail immune cells (Hanington *et al.* 2010; Hanington *et al.* 2012), macrophage migration inhibitory factor (MIF) – involved in trematode encapsulation and killing (Huang *et al.* 2017; Baeza Garcia *et al.* 2010), copper/zinc superoxide dismutase (Goodall *et al.* 2004), and the beta pore-forming toxin biomphalysin (Galinier *et al.* 2013) are the best characterized.

It also seems that resistance to various trematode species may be driven by a few key snail defense molecules. For instance, *S. mansoni* and *Echinostoma paraensei* are two trematode species that are separated by a wide phylogenetic distance (Olson *et al.* 2003). While *E. paraensei* can infect juveniles of both BS-90 and M-line strains of *B. glabrata* (Loker *et al.* 1987; Zhang *et al.* 2008), *S. mansoni* can only infect the M-line strain (Hanington *et al.* 2012). In

both cases, there is marked development of resistance in the snails with age, particularly to *E. paraensei*. However, when FREP3 was temporarily disrupted, resistance to infection with both parasites decreased in these snails (Hanington *et al.* 2010; Hanington *et al.* 2012). This provides an excellent model for studying the determinants of resistance to trematodes, with the implication that control of various trematode diseases could possibly be achieved by engineering snails to carry a few relevant immune genes.

Very little is known about the production and maintenance of suitable haemocyte populations within snail hosts, which are critical to a successful immune response against parasites, or how these haemocytes engage their targets. In this thesis, I have characterized two molecules that enable us to fill some of these knowledge gaps. These include *B. glabrata* progranulin, a key endogenous driver of haematopoiesis in the snail, and a Toll-like receptor – one of the haemocyte surface molecules through which parasite presence is detected and the signal transduced to the haemocytes to elicit effector functions required for parasite elimination.

The rationale for analyzing these particular molecules was based on previous studies done in our laboratory, which identified these molecules via peptide screens comparing proteins of the *S. mansoni*-resistant strain (BS-90) of *B. glabrata* to the susceptible (M-line) strain (Pila *et al.* 2016). Both molecules were found in higher abundance in BS-90 compared to the M-line snails, indicating that they might be important in the snail's immune response against schistosomes. The snail–schistosome (trematode) model is also interesting to study from an evolutionary immunological point of view because of the long-standing coevolutionary relationship that exists between this host–parasite association (Blair *et al.* 2001). The observation that nearly all of the ~ 25,000 trematode species use a snail (Bray *et al.* 2002; Esch *et al.* 2002; Cribb *et al.* 2001) leads one to hypothesize that novel immune and immune evasion strategies

would have likely evolved. Therefore, this system is well-suited for testing the idea of conserved immune features in novel immunological roles, and immune divergence that might have arisen to combat a very specific type of immunological challenge.

1.2 Outline of the thesis

This thesis focuses on understanding the role of haemocytes in the immune response of *B. glabrata* following challenge with *S. mansoni*. It is divided into six chapters and an appendix. This introductory chapter (chapter 1) gives an overview of snail control methods in the context of schistosomiasis, the aim and specific objectives of my thesis. In chapter 2, I review the literature on invertebrate immunity with emphasis on the mollusc/gastropod defence system, cellular immunity and haematopoiesis, and snail–trematode interactions. Chapter 3 presents details of materials and methods used throughout the course of the research presented in this thesis. Following this is a description of investigations into the functional roles of a *B. glabrata* endogenous growth factor, progranulin (chapter 4), and a specific *B. glabrata* Toll-like receptor (chapter 5). Chapter 6 is a general discussion of all studies, significance of findings, future research and potential application of findings. The appendix contains the description of the protein pheromone temptin in *B. glabrata*, including how it is envisioned that these studies would ultimately be used in an integrated snail control strategy for the elimination of schistosomiasis.

1.3 Aim and specific objectives of the thesis

The broader aim of my thesis was to advance our understanding of snail immunity with respect to schistosomes. Specifically, I addressed knowledge gaps related to the functional role of haemocytes as central coordinators of the snail immune response. This was achieved through

studies of factors that drive haemocyte proliferation and differentiation, and assessment of haemocyte surface molecules that facilitate engagement of haemocytes. The specific objectives of my thesis were to:

1. Molecularly and functionally characterize *B. glabrata* haemocyte growth factors, focusing on the evolutionarily conserved progranulins (*Bg*GRN).

To achieve the characterization of *Bg*GRN, the following four sub-objectives were addressed:

i. To determine *Bg*GRN domain architectural relationship to other granulins in order to investigate its possible processing and biological roles.

ii. To determine the pattern of *Bg*GRN transcript expression in *S. mansoni*-resistant (BS-90) and susceptible (M-line) *B. glabrata* snails during pathogen challenge.

iii. To develop recombinant *Bg*GRN protein and antibody and investigate the effect of recombinant *Bg*GRN on haemocyte proliferation and generation of adherent haemocytes.

iv. To assess the functional role of *Bg*GRN in the *B. glabrata* immune response against *S. mansoni* and its influence on snail phenotype.

2. Investigate the functional role of a specific *B. glabrata* Toll-like receptor (*Bg*TLR) in the immune response against *S. mansoni*.

In order to achieve this objective, three sub-objectives were addressed:

i. To determine the pattern of *Bg*TLR transcript expression in *S. mansoni*-resistant (BS-90) and susceptible (M-line) *B. glabrata* snails during pathogen challenge.

ii. To determine localization of *Bg*TLR in *B. glabrata* haemocytes and confirm protein expression.

iii. To assess the functional role of *Bg*TLR in the *B. glabrata* immune response against *S. mansoni* and its influence on snail phenotype.

Chapter 2

Literature Review[1]

2.1 Introduction

The ability to differentiate self from non-self, and to mount some form of response to inactivate or eliminate infectious agents, is one of the defining characteristics of living organisms, akin to other essential processes like nutrient acquisition and excretion of waste products. To express this universal and ancient trait (Dzik 2010; Danilova 2006), organisms utilize a variety of sensors that detect the invader, and signaling pathways to activate various effector responses that ultimately neutralize or eliminate it. Collectively referred to as the immune system, the study of these processes as a modern science, known as immunology, emerged in 1882 with the experiments of the Russian scientist, Elie Metchnikoff who identified the important effector response called phagocytosis. He initially described phagocytic cells in starfish larvae, termed macrophages, capable of engulfing rose thorns, and later documented macrophages and neutrophils in vertebrates and the migration of immune cells to sites of injury and their role in inflammation (reviewed in: Cavaillon 2011).

[1] Sections of this chapter (mollusc haematopoiesis and snail immunity) have been published in separate manuscripts:

Pila, E.A., Sullivan, J.T., Wu, X.Z., Fang, J., Rudko, S.P., Gordy, M.A., and Hanington, P.C. (2016). Haematopoiesis in molluscs: a review of haemocyte development and function in gastropods, cephalopods and bivalves. *Developmental and Comparative Immunology*, 58,119-128.

Pila, E.A., Li, H., Hambrook, J.R., Wu, X. and Hanington, P.C. (2017). Schistosomiasis from a snail's perspective: Advances in snail immunity. *Trends in Parasitology*, 33(11), 845-857.

Since then, steady progress has been made in the study of immunity in terms of the key cellular and humoral factors involved, and how the immune system functions in general. A deluge of information has been accumulated, broadening our knowledge of immunity as is evident in numerous immunological sensors of foreign agents, signal transduction molecules and effector responses that have been identified and characterized in various organisms (García-García *et al.* 2008; Danilova 2006; Hibino *et al.* 2006; De Tomaso *et al.* 2005; Krause and Pestka 2005; Palumbo 2005; Zhang *et al.* 2004; King *et al.* 2003; Nonaka and Miyazawa 2002; Dodd and Drickamer 2001; Nagai and Kawabata 2000; Aderem and Underhill 1999; Whitten *et al.* 1999; Ganz and Lehrer 1998; Lemaitre *et al.* 1996; Muta and Iwanaga 1996; Söderhäll *et al.* 1994; Ottaviani *et al.* 1993; Muta *et al.* 1991; Fuller and Doolittle 1971).

Immunity is broadly divided into two arms: innate immunity, present across invertebrate and vertebrate phyla, and adaptive immunity, characterized in vertebrates. Innate immunity, which has generally been thought of as simple and non-sophisticated, involves the use of recognition receptors, whose specificity is germline-encoded, to activate immediate effector responses. Named as pattern recognition receptors (PRRs, Janeway 1989), these receptors are non-clonally distributed on immune cells, and have evolved to recognize conserved molecular patterns associated with pathogens or cellular damage – the pathogen- or damage-associated molecular patterns (PAMPs or DAMPs, Areschoug and Gordon 2008; Taylor *et al.* 2005; Janeway and Medzhitov 2002; Medzhitov and Janeway 1997; Janeway 1989). Consequently, innate immune recognition is generic (Janeway and Medzhitov 2002; Janeway 1989).

Adaptive immunity on the other hand, has been considered as being more sophisticated because it involves fine-tuned recognition of targets based on variability and rearrangement of gene segments that encode the recognition receptors. These adaptive receptors are expressed on

specific cell types known as the T and B lymphocytes (Cooper *et al.* 1965). Lymphocytes are capable of clonal expansion and immunological memory of infection (Cooper and Alder 2006; Loker *et al.* 2004; Janeway and Medzhitov 2002).

Despite lacking the sophistication of the adaptive immune response, invertebrates are undoubtedly the most successful and abundant animals on earth, making up over 97% of known animal life, and entirely comprising all but one of the at least 30 phyla recognized in the animal kingdom (Loker *et al.* 2004; Barnes 2001). However, our current understanding of invertebrate immune mechanisms is surpassed by that of vertebrates. This is reminiscent of the scope of studies of immunity which, for the greater part of the history of the field of immunology, have disproportionately focused on few groups of organisms, particularly mammalian and avian vertebrates, and model invertebrates such as the common fruit fly *Drosophila melanogaster* and the nematode *Caenorhabditis elegans* (reviewed in: Buckley and Rast 2015; Dheilly *et al.* 2014; Hibino *et al.* 2006; Loker *et al.* 2004). Since invertebrates apparently rely on innate immunity exclusively to respond to foreign invaders, they had been presumed to be incapable of sophisticated immune responses. Early studies of immunity, based on a limited sample of organisms and taxa, shaped comparisons of invertebrate and vertebrate immune systems, and entrenched the paradigm of a simplified immune system in invertebrates despite several challenges that it posed. For instance, since invertebrates also interact with other organisms, and are equally exposed to threats posed by rapidly evolving, ubiquitous and diverse pathogens in their environments (Sweet and Bateman 2015; Loker 2010; Klaphake 2009; Johnson 1984), it seems improbable that they could survive and thrive with a limited immune system. In fact, some invertebrates such as the cnidarians are always associated with microbiota (Augustin and Bosch 2010; Dinsdale *et al.* 2008; Ainsworth *et al.* 2006). Furthermore, many invertebrates are long-

lived, and some can live for decades (Curtis 2007; Ebert and Southon 2003; Heller 1990). A simplified immune system would be disadvantageous as it could be easily countered by rapid pathogen evolution that could lead to the extinction of the species (Loker 2010).

Nevertheless, within the last three decades, there have been increases in the number of studies sampling genomic information from a greater diversity of organisms, coupled with invigorating discoveries from 'model' invertebrates (Alper *et al.* 2007; Litman *et al.* 2007; Rast *et al.* 2006; Watson *et al.* 2005; Zhang *et al.* 2004; Cannon *et al.* 2002), which have begun to unravel complex immune mechanisms in invertebrates. Increasing evidence indicate that invertebrate immunity is not only more complicated than previously thought, but also includes alternative means (to vertebrate lymphocyte-based adaptive immunity) for generating diverse and complex immune responses (Litman *et al.* 2007; Loker *et al.* 2004), and that adaptive immunity may well be considered as one part of a meta-immune system within the metazoans. Invertebrate immune strategies include: (i) expanded families of recognition receptors (Buckley and Rast 2015; Rast *et al.* 2006), (ii) extensive diversification of receptors through gene splicing (Watson *et al.* 2005), gene conversion and point mutation (Zhang *et al.* 2004), and possession of genomic architecture for diversification through post-transcriptional modifications (Buckley *et al.* 2008).

In addition, phenomena that are functionally equivalent to immunological memory have been reported in several invertebrates, including crustaceans (Sadd and Schmid-Hempel 2006; Witteveldt *et al.* 2004; Kurtz and Franz 2003; Little *et al.* 2003), insects (Rodrigues *et al.* 2010; Faulhaber and Karp 1992), and molluscs (Pinaud *et al.* 2016; Portela *et al.* 2013; Lie *et al.* 1983; Sire *et al.* 1998; Lie and Heyneman 1979; Lie *et al.* 1975a), with the underlying mechanisms beginning to be discovered (Pinaud *et al.* 2016; Wang *et al.* 2013; Netea *et al.* 2011; Pope *et al.*

2011; Rodrigues *et al.* 2010; Rowley and Powell 2007; Kurtz 2004). These discoveries about invertebrate immune capabilities, such as receptor diversification and the ability to mount a robust response to a recurrent infectious agent, once thought to be in the exclusive realm of vertebrates, obscure the distinction between innate and adaptive immunity (Flajnik and Du Pasquier 2004). However, it is worth noting that, functional immune significance is yet to be demonstrated for many of the instances of invertebrate receptor diversification and expanded families of antigen recognition molecules.

Another insight emerging from these studies is that immune processes have evolved differently in various organisms, both in terms of copy numbers and presence/absence of entire gene families, even within the same taxa (Viljakainen 2015; Hoffmann 2004). One example is the group of effector molecules classified as antimicrobial peptides, which display lineage specificity in insects. Antimicrobial peptides of the drosomycin and coleoptericin families are only present in *Drosophila* and coleopteran insects respectively (Bulet *et al.* 1991; Sackton *et al.* 2007). Melanisation is another important effector response in insects and crustaceans. Genes encoding prophenoloxidases (PPOs), which catalyze steps in the synthesis of melanin, have been found to be expanded in the mosquito *Anopheles gambiae* compared *D. melanogaster* (Christophides *et al.* 2002), both of which belong to the insect order Diptera. Nine and three genes encode for PPOs in the genomes of the former and the latter respectively. Melanotic encapsulation of *Plasmodium* parasites, and repair of injuries by swollen blood-fed adults and larvae living in swiftly running water are thought to be the potential functions of the extra *A. gambiae* PPO genes (Christophides *et al.* 2002; Muller *et al.* 1999). Therefore, the immunological 'tool kit' of an organism reflects its evolutionary trajectory, which is likely influenced by such factors as life history, lifespan, habitat and unique pathogen stressors (Dzik

2010; Danilova 2006; Loker *et al.* 2004; Christophides *et al.* 2002). Some immune effectors such as phagocytosis (Cueto *et al.* 2015; Rieger *et al.* 2012; Bosch *et al.* 2009; Dean *et al.* 2004), reactive oxygen or nitrogen species (ROI/RNI) production (Rieger *et al.* 2012; Pereira *et al.* 2001; Peskin *et al.* 1998; Adema *et al.* 1994), signaling pathways (Bosch *et al.* 2009; Salamat and Sullivan 2009; Yuan *et al.* 2009) and genes such as Toll-like receptors (TLRs, Adema *et al.* 2017; Miller *et al.* 2007; Akira *et al.* 2006; Luo and Zheng 2000) have been conserved throughout Metazoa. Beside the core set of conserved immune processes, complex features of metazoan immunity have been achieved in alternative ways (Litman *et al.* 2007) such as adaptive immunity of vertebrates (Cooper and Alder 2006; Danilova 2006), or expansion and diversification of receptors in invertebrates (Rast *et al.* 2006; Watson *et al.* 2005; Zhang *et al.* 2004).

It is also evident that gene losses (Buckley and Rast 2015; Zhang *et al.* 2010) and expansions (Zhang *et al.* 2010; Hibino *et al.* 2006) have occurred in various groups, signifying differing pressures experienced by these groups (Buckley and Rast 2015). Some immune recognition receptors such as Gram-negative binding protein (GNBP) and multiple cysteine cluster TLRs (mccTLRs) are widely distributed throughout invertebrates but do not occur within the vertebrate phylum (Buckley and Rast 2015). In contrast, nod-like receptors (NLRs), widely distributed in both vertebrates and invertebrates appear to be lost in *D. melanogaster* and *C. elegans* (Buckley and Rast 2015; Zhang *et al.* 2010). Some of these innate immune variations may be driven, or at least aided by coevolutionary interactions between pathogens and their hosts (Christophides *et al.* 2002).

In the following sections of this review chapter, I will summarize current understanding of immune responses of invertebrates in general, including the cell types, receptors, cellular

responses and other effector mechanisms involved. Then, I will discuss the process of blood cells generation (haematopoiesis) and immunity in molluscs with emphasis on Gastropoda, a class of molluscs which harbour its own specialized pathogens, the digenetic trematodes. Details of snail–trematode interactions and determinants of compatibility will be discussed. Finally, a summary of the importance of studying gastropod immunity will be presented.

2.2 Immune responses of invertebrates

2.2.1 Immune cell types

There is no common classification scheme or nomenclature for blood cell lineages across the different invertebrate phyla. Invertebrate blood cells remain largely defined by morphology and function. Owing to variability arising from different criteria used (cell size, extent of granularity, biochemical markers), cell maturation stage and differences in sample preparation techniques, confusion and discrepancies abound in the literature. Devising a universal and reliable cellular classification system remains one of the fundamental challenges in invertebrate immunology. Depending on the taxon and the classification scheme used (for example, Hartenstein 2006; Ratcliffe *et al.* 1985), a range of 1–5 major cell types are involved in innate immunity in invertebrates. While the names ascribed to these cells are variable, most are capable of phagocytosis, some perform additional functions beside immune responses, and not all can be found in all invertebrates. It is also worth noting that names given to immune cells are often similar in different invertebrates, but a similar name does not indicate a similar function.

In sponges and cnidarians, which are among the simplest metazoans (Miller *et al.* 2007), a multifunctional cell type known as amoebocyte is found in the mesoglea – a gelatinous matrix that fills the space between the two epithelia that make up the organisms. Amoebocytes (or

archaeocytes) in sponges perform multiple functions including digestion, phagocytosis and acting as a reservoir of stem cells (reviewed in: Hartenstein 2006; Müller *et al.* 1999; Van de Vyver and Buscema 1990). Some cnidarians have amoebocytes within the mesoglea (interstitial cells) capable of phagocytosis, while others use cells of the epithelium to carryout the same function. Increases in amoebocyte numbers within the mesoglea of the sea fan coral (*Gorgonia ventalina*) associate with fungal infection, and have been implicated in the antifungal melanisation reaction (Mydlarz *et al.* 2008). In another cnidarian *Hydra*, which lacks specialized and mobile phagocytes, epithelial cells can engage foreign agents via active phagocytosis (Bosch and David 1986) and secretion of potent antimicrobial peptides (Bosch *et al.* 2009). Recognition of bacterial flagellin, which is a known PAMP (Hayashi *et al.* 2001) was shown to be mediated by atypical transmembrane proteins (Bosch *et al.* 2009) similar to conventional TLRs, the difference being that the intracellular Toll/interleukin-1 receptor (TIR) and the extracelluar leucine-rich repeats (LRR) domains are present on two separate proteins. The epithelial cells in *Hydra* also show remarkable regeneration ability, following injury or tissue loss (Bosch 2007).

In Annelida, cells of the coelomic fluid (called coelomocytes or amoebocytes) can be classified into three groups: eleocytes, involved in nutrition and immunity and hyaline and granular amoebocytes, which play immune roles mainly (Bilej *et al.* 2010). Annelid coelomocytes can engage bacteria, parasites and fungi through phagocytosis and encapsulation, a coordinated response of multiple coelomocytes (Porchet-Henneré and Vernet 1992; Valembois *et al.* 1992; Valembois *et al.* 1985; Stein and Cooper 1981). PRRs, including a cytokine-like molecule known as coelomic cytolytic factor (CCF, Bilej *et al.* 1995), bind to ligands such as lipopolysaccharide (LPS), peptidoglycan and β-1,3-glucans in the cell walls of bacteria and

yeast. This results in the production of cytotoxic compounds via the PPO cascade (Procházková *et al.* 2006; Bilej *et al.* 2001; Beschin *et al.* 1999; Beschin *et al.* 1998).

In molluscs, immune cells (known as haemocytes or amoebocytes) can be classified into two types: granulocytes and agranular hyalinocytes (Castellanos-Martínez *et al.* 2014; Yoshino and Coustau 2010; Ottaviani 2006; Hine 1999; Cheng 1981). Both cell types perform immune functions such as phagocytosis (Hanington *et al.* 2010a), encapsulation (Loker *et al.* 1982) and production of reactive oxygen species (ROS, Humphries and Yoshino 2008; Lacchini *et al.* 2006), as well as other vital processes such as wound healing (Franchini and Ottaviani 2000), nerve repair (Hermann *et al.* 2005) and shell formation (Mount *et al.* 2004).

Among arthropods, three functional cell types known as haemocytes are involved in immune and stress responses. In *Dropsophila* (Insecta), these include plasmatocytes, which can engulf pathogens and apoptotic bodies, and crystal cells that are involved in degranulation and melanisation that is triggered during wound repair (Crozatier and Vincent 2011). The third haemocyte type, known as lamellocyte, which usually differentiate only in response to parasites and non-pathogen stress such as ROS, is devoted to encapsulation of parasites (Crozatier and Vincent 2011). In crustaceans, the three morphologically distinct haemocyte types are classified as: hyaline cells, semigranular cells and granular cells (reviewed in: Lin and Söderhäll 2011; Cerenius *et al.* 2010; Johansson *et al.* 2000). All cell types have been shown to be important in pathogen destruction respectively through phagocytosis, early pathogen detection and possession of granules containing immune factors such as PPO-activating system, antimicrobial peptides and the cell adhesion protein peroxinectin (Sricharoen *et al.* 2005; Johansson *et al.* 2000).

In the sea urchins (Echinodermata), which are considered to be the basal deuterostomes and sister group to the chordates (Hyman 1955), immune cells known as coelomocytes can also

be divided into three main categories based on morphology. These include phagocytes, spherule cells (also referred to as amoebocytes or morula cells) and vibratile cells (Smith *et al.* 2010). All coelomocyte types are involved in various immune functions including encapsulation, opsonization, phagocytosis, cellular clotting, cytotoxicity and antibacterial activity (Smith *et al.* 2010; Smith *et al.* 2006).

Urochordates, another group of deuterostome invertebrates, which represent close relatives of vertebrates (Delsuc *et al.* 2006), have two main types of cells called immunocytes which are involved in the immune response. These include the phagocytes and cytotoxic cells found in the haemolymph and the tunic, which envelops the larval and adult body of the organism, serving as a first barrier against pathogens (Franchi and Ballarin 2017). The phagocytes can assume both spreading and round morphology, and are characterized by fine cytoplasmic granules that are positive for lysosomal enzymes but hardly detectable with the light microscope (Cima *et al.* 2016; Ballarin *et al.* 1993). They actively move towards foreign particles and are involved in phagocytosis, encapsulation, and synthesis and release of lectins (Franchi and Ballarin 2017; Cima *et al.* 2016; Wright and Cooper 1983). The cytotoxic cells, also called morula cells have variable morphology, large granules containing PPO (Ballarin 2012) and are mainly involved in cell-mediated cytotoxicity, synthesis and release of cytokines, antimicrobial peptides and complement factors (Franchi and Ballarin 2017; Cima *et al.* 2016; Di Bella *et al.* 2011).

Variability in invertebrate cellular classification is a widely recognized problem and efforts have been targeted at better organizing this area of research. In 1985, Ratcliffe and colleagues, adopting a strictly functional approach (though with some overlap), arranged invertebrate cells into five main groups, namely, progenitor, phagocytic, haemostatic, nutritive

and pigmented cells (Ratcliffe *et al.* 1985). The progenitor cells include all small (4–10 μm), undifferentiated, often pluripotential cells that have been variously described as prohaemocytes, haemoblasts, haemocytoblasts, proleukocytes, stem cells and lymphocyte-like cells. The phagocytic cells include granular and hyaline cells involved in ingestion and intracellular killing of pathogens, classified as amoebocytes, granulocytes, macrophages, monocytes, plasmatocytes and granular cells (Ratcliffe *et al.* 1985). Haemostatic cells consist of those involved in preventing blood loss through coagulation and aggregation to seal wounds, such as cystocytes, granular cells, coagulocytes and explosive cells. Cells classified in the nutritive category include glycocytes of sponges, annelid eleocytes, and spherule cells in insects. However, immune functions are also associated with these cells. Pigment cells are of a variety of colours and contain pigments that may or may not be involved in respiration (Ratcliffe *et al.* 1985).

Hartenstein (2006) divided blood cells of invertebrates based on structure into four categories, namely, prohaemocytes, plasmatocytes, granulocytes and eleocytes. The prohaemocytes and granulocytes can essentially be placed in the progenitor and phagocytic cell categories described above. Plasmatocytes (also called hyaline haemocytes or monocytes) are described as comparable to monocyte/macrophage of vertebrates. They are phagocytic and involved in removal of apoptotic cells during development, as well as ingestion and encapsulation (reviewed in: Hartenstein 2006; Evans *et al.* 2003). Eleocytes (chloragogen cells, spherulocytes, adipohaemocytes) are a diverse group of cells containing irregularly sized and shaped lipid or crystalline inclusions, and are involved in uptake, digestion and distribution of nutrients (Hartenstein 2006) but also possess phagocytic and bactericidal properties (Cooper *et al.* 2002).

A growing number of studies have incorporated molecular markers to differentiate cell types, which is considered as the gold standard. However, the lack of specific markers in invertebrates means that antibodies against markers or antigens that are shared, or cross-react with mammalian cells are frequently used. In the gastropod mollusc *B. glabrata*, we recently determined that antibodies against a specific Toll-like receptor (*Bg*TLR) could be used to distinguish a circulating adherent, granulocytic haemocyte subpopulation that is highly phagocytic and responsible for production of ROS from other haemocytes (Pila *et al.* 2016b; Pila *et al.* 2016a). Cima *et al*. (2000) used an antibody against CD34, a haematopoietic cell marker in mammals, to confirm that the undifferentiated cell type known as haemoblast represent stem cells that circulate in the blood of the clam *Tapes philippinarum* (Cima *et al.* 2000). Engelmann and colleagues used several monoclonal antibodies developed against mammalian cell surface markers (including TNF–α, TGF–α) to assess earthworm coelomocytes. While two of the coelomocyte subpopulations could be differentially stained, a third type was negative for all the monoclonal antibodies (Engelmann *et al.* 2002). Recently, Jemaa *et al*. (2014) used antibodies against another stem cell marker (Sox2) and a haemocyte-specific enzyme (Cu/Zn superoxide dismutase) to successfully identify progenitor cells in bivalves and their differentiation into haemocytes (Jemaa *et al.* 2014).

Some early studies used markers specific to the species under study although the epitopes involved were not determined. For example, glass-adherent haemocytes of *B. glabrata* differentially stained with five monoclonal antibodies raised against surface markers on the circulating cells (Yoshino and Granath 1983). Cells expressing a surface antigen termed BGH_1 displayed distinct phagocytic and acid phosphatase activities relative to their BGH_1 expression (Yoshino and Granath 1985). Similarly, Gardiner and Strand (1999) used a panel of monoclonal

antibodies whose labelling patterns correlated with distinct classes of haemocytes in the moth *Pseudoplusia includens*.

Among invertebrates, the process of haematopoiesis is best understood in the long-standing model organisms *C. elegans* and *Drosophila*, where specific molecules involved in cell fate determination have been identified (Joshi *et al.* 2010; Lebestky *et al.* 2000). The transcription factors lozenge (Lz) and glial cells missing (Gcm) in *Drosophila* have been shown to be necessary for the development of crystal cells and plasmatocytes respectively (Lebestky *et al.* 2000). Both classes of haemocytes in turn require the *Drosophila* GATA protein Serpent (Srp) for development (Lebestky *et al.* 2000).

The availability of such specific and reliable biomarkers across other groups would improve the organization of cellular classification among invertebrates. However, it may be completely inappropriate to attempt to classify into common categories, immune cells from 30 different phyla, which incorporates at least 400 million years (Buchmann 2014) of evolution. Nevertheless, the fact that functions such as respiratory burst and phagocytosis are common to immune cells in all of these diverse organisms, is testament to how foundational they are to pathogen or parasite defense.

2.2.2 Cell-mediated immune responses

Invertebrate immune cells participate in the defence against pathogens by recognition and direct engagement through processes such as phagocytosis and encapsulation, and/or production of molecules that are cytotoxic or mediators of other elimination responses such as melanisation, clotting, inflammation and attraction of other immune cells to the site of infection.

2.2.2.1 Phagocytosis

Phagocytosis is a fundamental immune response of organisms from protozoans, where it also serves for food acquisition, to invertebrate and vertebrate animals (Cima *et al.* 2016; Cueto *et al.* 2015; Rieger *et al.* 2012; Crozatier and Vincent 2011; Smith *et al.* 2010; Aderem and Underhill 1999; Bosch and David 1986; Vogel *et al.* 1980). It is defined as a receptor-mediated process that leads to engulfment and ultimate destruction of particles, whose size is at least 0.5 μm, through actin-dependent remodelling of the cytoskeleton (Allen and Aderem 1996). Phagocytosis plays a prominent role in defence, but it is also vital in the removal of unwanted host cells to maintain homeostasis (Manaka *et al.* 2004; Zhou *et al.* 2001; Bosch and David 1986) and for feeding in some invertebrates (Vogel *et al.* 1980).

The process is initiated by specific receptors on the phagocytic cell contacting cognate ligands on the target to be engulfed (such as an invading bacterium or apoptotic cell) or engaged opsonins. This in turn activates complex signaling networks that promote actin-dependent reorganization of the plasma membrane required for local membrane extension and engulfment. The engulfment that is formed upon internalization, known as a phagosome, then fuses with the lysosome to form a phagolysosome within which the particle is destroyed or recycled in the acidic compartment (Niedergang and Chavrier 2004; Kroschinski and Renwrantz 1988). The main killing mechanisms involve ROI and RNI (Halliwell 2006), hydrolytic lysosomal enzymes and antimicrobial factors (Niedergang and Chavrier 2004). The general process of phagocytosis as described above is similar in both invertebrates and vertebrates but specific mediators such as the receptors and signaling molecules involved are very diverse (Nakanishi and Shiratsuchi 2006).

Phagocytic PRRs identified in invertebrates include members of the peptidoglycan recognition proteins (Rämet *et al.* 2002), scavenger-receptors (Rämet *et al.* 2001), and lectins (Wang *et al.* 2014; Takahashi *et al.* 2008). A homologue of the complement component C3 in the purple sea urchin (*Sp*C3) can opsonize yeast cells and increase phagocytic activity in a specific coelomocyte type (Clow *et al.* 2004) but its receptor has not been identified. In addition to these highly conserved PRRs, certain receptors with roles in phagocytosis appear to be unique to some invertebrate groups. Examples include *Drosophila* Down syndrome cell adhesion molecule (Dscam), which produces many isoforms through alternative splicing (Watson *et al.* 2005), invertebrate thioester-containing proteins (TEPs), which serve as opsonins (Stroschein-Stevenson *et al.* 2006; Levashina *et al.* 2001), and fibrinogen-related proteins (FREPs) in gastropods, which also have been shown to have opsonic properties (Hanington *et al.* 2010a). Other pathogen sensors like TLRs and NLRs participate in phagocytosis through regulation of internalization and phagosome maturation (Blander and Medzhitov 2004), and upregulation of phagocytic gene expression programmes (Doyle *et al.* 2004).

Downstream mechanisms such as signaling pathways and regulators appear to be conserved. Phagocytosis pathways such as phosphatidylinositol-3-kinase (PI3-kinase), protein kinase C (PKC) and extracellular signal-regulated kinase (ERK) are also utilized in invertebrates (García-García *et al.* 2008). The abilities to engulf targets and produce ROI/RNI were abrogated by the inhibition of these molecules in haemocytes of the mussel *Mytilus galloprovincialis* (García-García *et al.* 2008). Ras-like GTPases, mitogen-activated protein kinases (MAPKs) and NF-kB were activated following *in vitro* zymosan phagocytosis by immune cells of the ascidian *Botryllus schlosseri* (Franchi *et al.* 2013). Also, small G proteins including Rab6, Rho, Rab and

Ran GTPases have been implicated in invertebrate phagocytosis signaling (Ye *et al.* 2012; Liu *et al.* 2009; Pan *et al.* 2005).

2.2.2.2 Encapsulation

Sequestration with cellular sheaths, or encapsulation, is another cell-mediated response targeted to particles such as multicellular parasites, their eggs or larvae, which are too large for phagocytosis by a single cell. Formation of a cellular capsule around the target ensures its isolation and ultimate destruction. This immune response is prominent in arthropods (Dubovskiy *et al.* 2016; Guo *et al.* 1995) and molluscs (Nacif-Pimenta *et al.* 2012; Loker *et al.* 1982). The term nodulation is sometimes used to differentiate a very similar immune response in which the target is an aggregation of microorganisms such as agglutinated bacteria, fungi or protozoans (Satyavathi *et al.* 2014; Faraldo *et al.* 2008). An immune response analogous to encapsulation or nodulation, known as granuloma formation occurs in vertebrates, induced by the presence of persistent infectious agents like bacteria and parasites, or other foreign agents such as suture material (Kara *et al.* 2014; Davies and McKerrow 2000). Largely composed of innate immune cells (macrophages and eosinophils), granulomas and the process of their formation superficially resemble encapsulation, and provide an example of a common outcome of the immune response in invertebrates and vertebrates that is achieved through different mediators.

Encapsulation is initiated by PRRs like those involved in phagocytosis, however it is not clear how the decision is made to activate one response or the other. Upon binding of PAMPs on the target or endogenous ligands exposed by damage from the invading parasite, a network of signaling pathways induces cellular transformation from non-adhesive to adhesive states, accompanied by spreading and binding to the target and one another (reviewed in: Dubovskiy *et*

al. 2016; Lavine and Strand 2002). Cellular degranulation, which releases effector molecules and chemotactic factors then follows, and further cells attach to the growing capsules.

Encapsulation begins within minutes of invasion of the host by a foreign agent (Nacif-Pimenta *et al.* 2012; Dubovskii *et al.* 2010; Gagen and Ratcliffe 1976). Total isolation and destruction of the invader is completed within 72 h (Nacif-Pimenta *et al.* 2012; Loker *et al.* 1982; Ratcliffe and Gagen 1977). Each aspect of this highly complex process is regulated. Specific chemokines have been shown to induce cellular adhesion and spreading (Wang *et al.* 1999; Clark *et al.* 1997), and chemotaxis (Baeza Garcia *et al.* 2010). Rho GTPases (such as Rac1 and Rac2) and Jun kinase (Williams *et al.* 2006; Williams *et al.* 2005) are some of the known signaling regulators of the encapsulation process.

Killing of the parasite is carried out by effector molecules such as ROS (Hahn *et al.* 2001a; Hahn *et al.* 2001b; Nappi *et al.* 2000; Nappi *et al.* 1995) and degradative enzymes deposited by the surrounding cells (Cueto *et al.* 2015; Bayne 2009). In many arthropods, encapsulation is accompanied by the formation of a biopolymer called melanin, triggered by the PPO cascade. Certain melanisation intermediates such as o-semiquinone and o-quinone radicals are cytotoxic and probably participate in the killing of parasites (Dubovskiy *et al.* 2016; Dubovskii *et al.* 2010; Söderhäll and Ajaxon 1982). The recruitment of immune cells and termination of encapsulation occurs with the production of a basement membrane-like layer around the capsule, effectively isolating the invader from the immune system. The basement membrane is an extracellular matrix composed of components such as collagen IV, laminin and some proteoglycans which surround most tissues (reviewed in: Dubovskiy *et al.* 2016). Contents of the basement membrane show little differences within species and haemocytes normally weakly attach to self basement membrane (Nardi *et al.* 2001). However, across larger phylogenic

distances, significant differences exist. In insects, haemocytes usually fail to encapsulate undamaged tissues transplanted from conspecific individuals but would quickly encapsulate transplants from more distant species (Lackie 1988).

2.2.3 Immune receptors in invertebrates

Invertebrates express several receptors and signaling pathways that are conserved in their vertebrate counterparts. Among the major recognition receptors for which homologues have been identified across metazoans are the TLRs, NLRs, GNBP, peptidoglycan-recognition protein (PGRP), scavenger-receptor cysteine rich (SRCR) domain containing proteins, as well as various lectins, and complement-like factors (reviewed in: Buckley and Rast 2015; Dzik 2010).

TLRs are type 1 transmembrane proteins composed of an extracellular leucine-rich repeat domain, responsible for recognizing and binding to various PAMPs or DAMPs, and a conserved cytoplasmic TIR domain through which signal transduction and activation of effector functions occur. The prototypical member of this family, the Toll, plays important roles in embryonic development and innate immunity in *D. melanogaster* against fungi and Gram-positive bacteria, although it does not directly bind PAMPs (Weber *et al.* 2003). A balance of the Toll pathway and the immune deficiency pathway (Imd, which is activated in response to Gram-negative bacterial PAMPs) regulate the production of antimicrobial peptides by cells of the fat body (Lemaitre and Hoffmann 2007). The signaling of both pathways converges at the activation of NF-kB homologues (Dorsal, Dif and Relish), and together they control 71% of immune genes that are regulated in *D. melanogaster* following septic injury (De Gregorio *et al.* 2002). The genomes of some invertebrates encode large families of TLRs, suggesting that these molecules may increase the spectrum of recognition or enhance its specificity (Hibino *et al.* 2006). These

include the purple sea urchin, *S. purpuratus* (222 TLRs, Hibino *et al.* 2006; Rast *et al.* 2006) the annelid *Capitella teleta* (105 TLRs, Davidson *et al.* 2008) and the gastropod *B. glabrata* (56 TLRs, Adema *et al.* 2017). One of the *B. glabrata* TLRs was functionally characterized as part of my thesis research (Pila *et al.* 2016b). Along with additional literature review on TLRs, the results are presented in chapter 5.

Invertebrates have also evolved receptors that possess recognition mechanisms analogous to those of vertebrates. For example, histocompatibility in cnidarians (Nicotra *et al.* 2009) and protochordates (De Tomaso *et al.* 2005) is determined by unique allorecognition receptors. Two innate histocompatibility loci (*alr1* and *alr2*) have been identified in the primitive cnidarian *Hydractinia symbiotongicarpus*. These encode highly polymorphic transmembrane receptors with three immunoglobulin superfamily (IgSF)-like extracellular domains. Fusion of colonies between individuals only occurs if at least one allele from both loci is shared (Nicotra *et al.* 2009). In the colonial tunicate *B. schlosseri*, putative receptor proteins called fuhc are encoded by the fusion/histocompatibility locus (*FuHC*). One of two alleles of this highly polymorphic locus must also be shared for fusion to occur between individuals (De Tomaso *et al.* 2005). The fuhc protein interacts with other polymorphic proteins from the *fester* locus (fester and Uncle fester) in a manner analogous to the 'missing self' recognition mechanism of natural killer cells in which the fester proteins act as both activating and inhibitory receptors to fuhc (reviewed in: Ghosh *et al.* 2011).

Studies have also uncovered a number of receptors that can be considered as exclusive invertebrate innovations at immunity. Examples include SpTransformer proteins (formerly Sp185/333) expressed on the surface and perinuclear vesicles of small phagocytes and polygonal cells in *S. purpuratus* (Smith and Lun 2017; Brockton *et al.* 2008; Buckley *et al.* 2008;

Terwilliger *et al.* 2006), FREPs in gastropod haemocytes (Gorbushin *et al.* 2010; Zhang *et al.* 2004), and Dscam in insects and crustaceans (Armitage *et al.* 2017; Chou *et al.* 2009; Dong *et al.* 2006; Watson *et al.* 2005). The FREPs combine fibrinogen (FBG) and IgSF domains, which are linked to immunity as stand-alone, or domains of, innate immune factors of invertebrates and vertebrates (discussed below, Gordy *et al.* 2015).

The SpTransformer gene family which appear to be unique to echinoids, generate diverse gene products based on the absence or presence of 25 blocks of variable nucleotide sequences (12–357 bp) called 'elements', which create unique patterns of messenger RNA (mRNA). Additional diversification occurs through an unknown post-transcriptional mechanism that create single nucleotide polymorphisms and small insertions/deletions (Buckley *et al.* 2008; Terwilliger *et al.* 2006). Although their precise function is not known, their expression increases upon exposure to bacteria and distinct suites of SpTransformer proteins are induced by exposures to different pathogen types (Dheilly *et al.* 2009).

Dscams are IgSF-containing receptors which are assembled from multiple exons based on an extensive, mutually exclusive, alternative splicing mechanism that can potentially produce more than 30,000 isoforms from a single gene (Dong *et al.* 2006; Watson *et al.* 2005). Dscam1 of *D. melanogaster* contains 115 exons ordered as 20 single constant exons, and 4 exon cassettes containing variable numbers (2–48) of alternative exons (Watson *et al.* 2005). Thus, a Dscam1 mRNA comprises of all of the 20 constant exons and only one of each of the alternative exons. Dscam is expressed in secreted and membrane-associated forms (Watson *et al.* 2005), and experimentally induced Dscam deficiency (RNAi or blocking with Dscam-specific antibody) was found to impair phagocytic ability of *A. gambiae* and *D. melanogaster* haemocytes (Dong *et al.* 2006; Watson *et al.* 2005). Interestingly, *A. gambiae* exon 4 cassette (with 14 alternative

exons) produced distinct mRNA splice variants in response to challenge with bacteria, fungi and protozoan parasites, resulting in the production of receptors with increased affinity to the infectious organism (Dong *et al.* 2006).

2.2.4 Signaling pathways and transcription factors

Signaling pathways are relatively more conserved than recognition receptors between invertebrates and vertebrates. One of the evolutionarily conserved signaling cascades in innate immunity is the TLR pathway. Components of this pathway have been identified in various organisms including molluscs and insects (Adema *et al.* 2017; Zhang and Coultas 2011; Lemaitre and Hoffmann 2007; Christophides *et al.* 2002) where it has been best studied. As previously reviewed, pathways such as PI3-kinase, PKC and ERK which occur downstream of phagocytosis are well conserved (García-García *et al.* 2008), as are NF-kB, GTPases (Rab6, Rho, Rab, Ras and Ran), p38 MAPK and Jun kinases (Franchi *et al.* 2013; Ye *et al.* 2012; Liu *et al.* 2009; Williams *et al.* 2006; Pan *et al.* 2005; Williams *et al.* 2005). In addition to innate immunity, these pathways control a variety of processes including cell proliferation, differentiation, cell death and development (reviewed in: Song *et al.* 2010). The siRNA pathway identified in *C. elegans* (Lu *et al.* 2005; Schott *et al.* 2005; Wilkins *et al.* 2005) and *D. melanogaster* (Galiana-Arnoux *et al.* 2006; Wang *et al.* 2006; van Rij *et al.* 2006) is another pathway that uses molecules such as Dicer and Argonaute that are functionally and molecularly conserved.

Regarding transcription factors, cAMP response element-binding protein (CREB), a regulator of cell proliferation, survival and differentiation has been characterized in oysters in the immune response against rickettsia-like organisms (Zhu and Wu 2008). The RUNX factor

lozenge (Lz), and glial cells missing (Gcm) are important cell fate determinants in *D. melanogaster* (Lebestky *et al.* 2000). Prohaemocytes expressing Lz and Gcm develop into crystal cell and plasmatocyte lineages respectively. Both classes of haemocytes develop under the control of the *Drosophila* GATA homologue Serpent (Srp, Lebestky *et al.* 2000). Transcription factors such as ETS, GATA, C/EBP, RUNX, MYB and Zinc finger also known to be important for haematopoiesis in vertebrates have been identified in the genome of the purple sea urchin (Hibino *et al.* 2006).

2.2.5 Cytokines and growth factors

Several cytokines have been characterized in various invertebrates. These include homologues of vertebrate cytokines such as interleukin–17 (IL-17), an inflammatory cytokine cloned from oyster (Roberts *et al.* 2008), sea urchin (Hibino *et al.* 2006) and amphioxus (Holland *et al.* 2008); and macrophage migration inhibitory factor (MIF) characterized in molluscs (Huang *et al.* 2017; Baeza Garcia *et al.* 2010); transforming growth factor beta (TGF–β) and its receptor, identified in various invertebrate genomes including arthropods, molluscs, and nematodes (Herpin *et al.* 2004). Important for regulating inflammation, cellular proliferation and repair functions, TGF–β has been shown to regulate a cnidaria-dinoflagellate mutualism by suppressing immune reactions like nitric oxide production (Detournay *et al.* 2012).

Among the unique invertebrate cytokines are astakines, involved in cellular proliferation and cell fate determination in crustaceans (Lin and Söderhäll 2011), the Toll pathway activator Späetzle in insects (Dzik 2010; Lemaitre and Hoffmann 2007) and coelomic cytolytic factor–1 from the annelid *Eisenia foetida*, which has been shown to be a functional analogue of the cytokine TNF–α (Beschin *et al.* 1999). This molecule has glucan and saccharide binding

properties also implicated in the activation of the PPO cascade (Bilej *et al.* 1995). In addition, some studies based on immunoreactivity of invertebrates to recombinant vertebrate cytokines suggest that several other cytokines may be present (Ottaviani *et al.* 1996; Ottaviani *et al.* 1993).

There is limited literature on mitogenic or growth factors of invertebrates and available functional characterizations have been done mostly in insects and crustaceans. Known growth factors include imaginal disc growth factors (IDGF, Broz *et al.* 2017; Kawamura *et al.* 1999), epidermal growth factor (EGF, Hanington *et al.* 2010b; Hermann *et al.* 2005), astakines (Lin and Söderhäll 2011) and granulin (Smout *et al.* 2009; Hanington *et al.* 2008). IDGFs are chitinase-related proteins, produced in the yolk cells and fat body of *Drosophila* embryo and larva, which have been shown to stimulate proliferation of the C1.8+ cell line in cooperation with insulin (Kawamura *et al.* 1999). EGF or its receptor have been cloned in arthropods, molluscs and crustaceans where they have been implicated in growth, eye development, cell survival and axonal regeneration (Sharabi *et al.* 2013; Hermann *et al.* 2005; Freeman 1997). Astakines are homologues of vertebrate angiogenic growth factors called prokineticins (Soderhall *et al.* 2005). They were first identified in the crayfish *Pacifastacus leniusculus* but homologues have since been found in several other invertebrates (Lin and Söderhäll 2011). Two astakine proteins have been characterized from *P. leniusculus*. Astakine 1 induces cellular proliferation in the crayfish haematopoietic tissue and induce differentiation to the semigranular cell lineage, while astakine 2 induces granular cell differentiation (Lin *et al.* 2010; Soderhall *et al.* 2005). Granulins are multifunctional growth factors characterized by a highly conserved 12-cysteine motif. They have been identified and cloned in various invertebrates including slime molds, helminths, insects and molluscs (Hanington *et al.* 2008). The granulin protein of the liver fluke *Opisthorchis viverrini* has been shown to stimulate the proliferation of its host cells (Smout *et al.* 2009). My thesis

research also characterized a granulin from the gastropod *B. glabrata* (Pila *et al.* 2016a), which is presented in chapter 4.

2.2.6 Invertebrate immune effector responses

2.2.6.1 Coagulation

Coagulation or clot formation is another critical response that has been adopted by both invertebrates and vertebrates. However, there is no common evolutionary origin as the mediators of this process are different for both groups (Hanington and Zhang 2011; Bergner *et al.* 1996) and even among invertebrates (Dushay 2009; Osaki and Kawabata 2004; Muta and Iwanaga 1996), except perhaps the involvement of the cross-linking enzyme transglutaminase (TGase) at the final stage of coagulation. Basically, coagulation in invertebrates involves the formation of an insoluble matrix caused by clotting factors released from storage granules of the responding cells as they aggregate at the site of injury. It is triggered by injury or recognition of PAMPs and serves to prevent haemolymph loss (Theopold *et al.* 2004), promote wound healing (Theopold *et al.* 2004; Bursey 1977) as well as to immobilize and isolate the pathogen in a secondary barrier to prevent spreading (Matsuda *et al.* 2007; Osaki *et al.* 2002). This response has been characterized in various arthropods and other invertebrates (Dushay 2009; Scherfer *et al.* 2004; Osaki *et al.* 2002; Muta and Iwanaga 1996).

In the horseshoe crab (*Limulus polyphemus*), in which clotting has been extensively studied, PAMPs such as LPS and β-1,3-glucans, detected by clotting factors C and G respectively, activate a proteolytic cascade that results in the conversion of soluble coagulogen into coagulin gel (Muta and Iwanaga 1996). Coagulin is then stabilized by cross-linking to other proteins like proxins (Osaki *et al.* 2002) and stablins (Matsuda *et al.* 2007) in a reaction catalyzed by the

calcium-dependent enzyme TGase. The binding affinity of these stabilizing proteins to components such as LPS, lipotechoic acids and chitin enables pathogen immobilization at injury sites and wound closure (Matsuda *et al.* 2007; Osaki *et al.* 2002).

In crustaceans, insects and other arthropods, the coagulation mechanism is markedly different. Not only are the clotting factors different, the TGase-mediated cross-linking of the clotting proteins is not preceded by a proteolytic cascade (Dushay 2009; Osaki and Kawabata 2004). Several clotting proteins have been identified in insects and crustaceans. These include, lipophorin (reviewed in: Dushay 2009) and lipophorin-like proteins (Yeh *et al.* 1999; Hall *et al.* 1995), a vitellogenin-related protein (Hall *et al.* 1999), Fondue and haemolectin (Scherfer *et al.* 2006; Scherfer *et al.* 2004). RNAi-mediated knockdown of Fondue and haemolectin in *D. melanogaster* larvae caused a defect in the clotting phenotype in which bead aggregation activity and wound healing were strongly reduced or abolished (Lesch *et al.* 2007; Scherfer *et al.* 2006; Goto *et al.* 2003).

In some invertebrates, coagulation is often complemented by melanisation, and in the horseshoe crab, the link between clotting and melanisation has been shown to be due to a non-enzymatic interaction between the oxygen carrier protein haemocyanin and clotting factor B or the proclotting enzyme (Nagai *et al.* 2001; Nagai and Kawabata 2000). This interaction functionally converts haemocyanin into a phenoloxidase. Both proteins have a similar active site (Decker and Tuczek 2000) and are closely related in arthropods (Burmester and Scheller 1996). Phenoloxidase-like enzymatic activity by haemocyanin has also been reported in Kuruma prawns, *Penaeus japonicus* (Adachi *et al.* 2003) and the mollusc *Octopus vulgaris* (Salvato *et al.* 1998). Phenoloxidase activation complements coagulation in terms of wound healing and clot hardening (Bidla *et al.* 2005; Li *et al.* 2002).

2.2.6.2 Melanization

Another effective and ubiquitous immune response in invertebrates is the formation of melanin, a biopolymer formed by phenoloxidases, which is deposited on an invading pathogen or around a damaged tissue. Interestingly, melanization also occurs in vertebrates but its primary function and the key catalyzing enzyme are different. Formed by a tyrosinase-mediated reaction cascade (del Marmol and Beermann 1996), melanin in vertebrates provides protection against UV-light and other stressors such as heat and cold (reviewed in: Hill 1992). In invertebrates, phenoloxidase-mediated melanisation is activated as part of various immune processes such as encapsulation and coagulation (Ballarin 2012; Procházková *et al.* 2006; Nagai *et al.* 2001; Johansson *et al.* 2000; Nagai and Kawabata 2000; Söderhäll and Ajaxon 1982), as well as other physiological processes like hardening of exoskeleton after molting (Sugumaran 2010), camouflage and sexual displays (reviewed in: Hill 1992).

In the context of an immune response, the melanisation cascade can be triggered by injury (Altincicek *et al.* 2009) or binding of PRRs to PAMPs (Lee *et al.* 2004a; Beschin *et al.* 1998), which then activate a series of serine proteases, ultimately generating the active enzyme phenoloxidase from the zymogen PPO (Lemaitre and Hoffmann 2007; Wang *et al.* 2001; Lee *et al.* 1998). Phenoloxidase catalyze the oxidation of phenols to quinones, which then polymerize to melanin (reviewed in: Lemaitre and Hoffmann 2007; Söderhäll *et al.* 1994). As discussed above under the encapsulation response, several intermediates of the melanisation cascade are cytotoxic and these may directly kill the parasite.

The PPO system has been extensively characterized in crustaceans and insects. Genes for PPOs are among those that are differentially expanded in some insect lineages. Nine and ten genes encode for PPOs in the genomes of the mosquitoes *Anopheles gambiae* and *Aedes aegypti*

respectively, compared to only three in *D. melanogaster* (Waterhouse *et al.* 2007; Christophides *et al.* 2002). Mosquito PPO genes show distinct expression profiles in the different lifecycle stages (Muller *et al.* 1999) and in response to different pathogens (Huang *et al.* 2001; Cho *et al.* 1998). Melanotic encapsulation of *Plasmodium* and nematode parasites, constitute a major mechanism for parasite killing and elimination, enhanced by the action of PRRs such as TEP, LRIM, APLIC (Fraiture *et al.* 2009; Warr *et al.* 2006; Blandin *et al.* 2004).

2.2.6.3 Antimicrobial peptides

Antimicrobial peptides (AMPs) are potent effector molecules which act by permeabilizing microbial (bacteria, fungi and protozoa) membranes, causing efflux of solutes and lysis (Bulet *et al.* 1999; Hoffmann *et al.* 1999). The sheer number of described AMPs is remarkable – at least 900 are known to be produced by protozoans and metazoans (reviewed in: Danilova 2006). Among invertebrates, these include molecules such as mytilin, mucin, myticin in molluscs (Tincu and Taylor 2004; Charlet *et al.* 1996), defensin, drosomycin, diptericin, cecropin, gloverin in insects (Lemaitre and Hoffmann 2007; Bulet *et al.* 1999), nemapores and caenacins in nematodes (Tarr 2012), *Limulus* tachyplesin, crustacean penaedins and callinectin, as well as clavanins, styelins plicatamide, and halocyamine in urochordates (reviewed in: Tincu and Taylor 2004). Their mode of action is associated with the presence of conserved clusters of hydrophobic and cationic amino acids (Zasloff 2002), which enable their attachment and integration with membrane phospholipids, leading to pore formation or lysis (Brogden 2005). It is also thought that some AMPs might exert their activity through receptor-mediated processes and metabolic inhibition (Brogden 2005; Bulet *et al.* 1999).

In their diversity, invertebrates have evolved an array of AMPs, which exhibit many differences in terms of amino acid composition, size and structure. More than 150 AMPs have been identified in insects alone (Yi *et al.* 2014; Bulet *et al.* 1999). These can be classified into four groups: linear peptides forming α-helices and devoid of cysteine residues (cecropins), cysteine-rich peptides, proline-rich peptides, and glycine-rich peptides/polypeptides (Nappi and Ottaviani 2000; Bulet *et al.* 1999). One of the extensively characterized AMPs is defensin, a 3–5 kDa peptide belonging to the large family of cysteine-rich cationic peptides. Its spectrum of activity spans targets like bacteria (Kato and Komatsu 1996), fungi (Lee *et al.* 2004b), and protozoans (Shahabuddin *et al.* 1998). They have been shown to effect pathogen destruction through the disruption of membrane permeability barrier. Defensins have been described in phylogenetically diverse insect lineages (Bulet *et al.* 1999), other arthropods (Cociancich *et al.* 1993), molluscs (Charlet *et al.* 1996) and nematodes (Kato and Komatsu 1996).

In contrast to defensins which are widely distributed, certain AMPs are lineage-specific. For example, another cysteine-containing peptide, the antifungal agent drosomycin is only found in flies of the family Drosophilidae (Sackton *et al.* 2007), while the glycine-rich, antibacterial peptide coleoptericin is found in insects belonging to the order Coleoptera (Bulet *et al.* 1991). These lineage-specific AMPs are thought to be the result of evolutionary influences from factors such as life history, habitat and unique pathogen stressors (Dzik 2010; Danilova 2006; Loker *et al.* 2004; Christophides *et al.* 2002) given that production of AMPs is one of the innate immunity responses that has been retained throughout metazoan evolution.

In addition to AMPs, many invertebrates make use of larger proteins with potent antimicrobial properties for defence. One of such proteins is the bactericidal enzyme lysozyme which occur in all animals (Callewaert and Michiels 2010). Lysozymes primarily target Gram-

positive bacteria by hydrolyzing β-1-4-glycosidic bonds between N-acetylmuramic acid and N-acetylglucosamine of the major cell wall polymer, peptidoglycan but can also attack the cell walls of Gram-negative bacteria (Yue *et al.* 2011; Callewaert and Michiels 2010; Joskova *et al.* 2009).

2.2.6.4 Ribonucleic acid interference

Ribonucleic acid interference (RNAi) is a mechanism of gene regulation in eukaryotes initially known for its gene regulatory and immune roles in plants (Baulcombe 2004). It involves the repression of mRNA by degradation or inhibition of translation (Ding and Voinnet 2007; Hammond 2005). Studies have shown that RNAi also plays significant roles in invertebrate immunity, especially against viruses in nematodes and insects (Nayak *et al.* 2013; Galiana-Arnoux *et al.* 2006; Schott *et al.* 2005). One type of RNAi is initiated by the RNA processing enzyme Dicer (Aliyari and Ding 2009), which, acting as a PRR, senses long double-stranded RNA (dsRNA), either of endogenous or foreign origin. Dicer cleaves long dsRNA into short (21-nucleotide) dsRNA called, short interfering RNA (siRNA).

The siRNA is bound and unwound by an effector complex called RNA-induced silencing complex or RISC. One strand (sense or passenger strand) is degraded while the other strand (antisense or guide strand) is retained (Nayak *et al.* 2013; Hammond 2005). The RISC complex is guided to specific mRNA containing regions complementary to the guide siRNA strand. A catalytic component of RISC (called Argonaute) then cleaves the target mRNA. Components of siRNA pathway have been identified in *C. elegans* (Lu *et al.* 2005; Schott *et al.* 2005; Wilkins *et al.* 2005) and *D. melanogaster* (Galiana-Arnoux *et al.* 2006; Wang *et al.* 2006; van Rij *et al.*

2006) where RNAi has been studied extensively among invertebrates and shown to be involved in antiviral immunity.

RNAi-mediated gene silencing can also be initiated by small non-coding RNA molecules known as micro RNA (miRNA) and piwi-interacting RNA (piRNA, Ding and Voinnet 2007). The miRNA pathway is also initiated by a Dicer nuclease but the RNA trigger is a single-stranded RNA precursor with hairpin loop, while the piRNA pathway is independent of Dicer (Zamore 2007; Hammond 2005). Both pathways utilize an Argonaute protein as the effector component (Zamore 2007), and both have been implicated in antiviral immunity in *Drosophila* (Lau *et al.* 2009; Sabin *et al.* 2009) and mosquito (Hess *et al.* 2011; Skalsky *et al.* 2010), possibly playing redundant roles or interdependent on the siRNA pathway (Nayak *et al.* 2013).

As evident from the foregoing, the repertoire of immune responses possessed by the invertebrates as a whole is very diverse. However, the breadth of immune diversity is often not found completely in any single invertebrate group. Some responses are more elaborate in some species and in other cases, the same response may be mediated by molecules that are quite different, perhaps reflecting invertebrate heterogeneity and differing evolutionary trajectories (Loker *et al.* 2004). One group of invertebrates – the molluscs, especially gastropods, have a long-standing relationship with a specialized lineage of parasites, the digenetic trematodes (Blair *et al.* 2001). This unique relationship is interesting because it presents an opportunity to study both the importance of conserved immunological features in novel immunological roles, as well as new immunological adaptations that have arisen to combat a very specific type of immunological challenge. In the following sections, I will examine the molluscan haematopoietic and defense system in the context of interactions between gastropods and trematodes.

2.3 Haematopoiesis in molluscs

Haematopoiesis is a process that is responsible for generating sufficient numbers of blood cells in the circulation and in tissues. It is central to the maintenance of homeostasis within an animal, and is critical for defense against infection. While haematopoiesis is common to all animals possessing a circulatory system, the specific mechanisms and ultimate products of haematopoietic events vary greatly. Our understanding of this process in non-vertebrate organisms is primarily derived from those species that serve as developmental and immunological models, with sparse investigations having been carried out in other organisms spanning the metazoan lineage. As research into the regulation of immune and blood cell development advances, we have begun to gain insight into haematopoietic events in a wider array of animals, including the molluscs.

Molluscs are a large and morphologically diverse group of animals, many of which are known for their economic and biomedical importance. Some are excellent model organisms for studying neurobiology (Chen *et al.* 2014b; Hochner 2013), while several others, such as clams, oysters, squids and abalones, are economically important food sources that can be reared in aquaculture (Richards *et al.* 2015; Rojas *et al.* 2015; Lafarga de and Gallardo-Escárate 2011; Uriarte *et al.* 2011; Guerra *et al.* 1994).

Haematopoiesis in molluscs is not a well-understood process. Progress is being made in this area to define the networks of signaling pathways important for haemocyte development, as well as the endogenous factors that regulate haemocyte numbers (Salazar *et al.* 2015; Jemaa *et al.* 2014; Sullivan *et al.* 2014; Ma *et al.* 2009). Evidence so far indicates that major differences exist among the various molluscan classes in terms of haemocyte lineages and haematopoietic sites. Haemocytes in molluscs can be classified into at least two main types – the granulocytes and the

agranular hyalinocytes (Castellanos-Martínez *et al.* 2014; Yoshino and Coustau 2010; Ottaviani 2006; Hine 1999; Cheng 1981). However, within these two general types, there is variability in the number of haemocyte lineages described in various molluscs. This diversity may be in part due to some true differences, but also results from the use of different criteria (extent of granularity, cell size, ultrastructural features, cell surface/biochemical markers) and nomenclatures adopted by various researchers, as is the case for invertebrates in general, due to lack of biological markers for differentiating specific cell lineages or states of maturation. Thus, a unified classification system for haemocytes in molluscs would help to better organize this area of research.

The location where haematopoiesis takes place in molluscs varies greatly. In many gastropods, haemocyte production occurs in the pericardial region, for example in the anterior pericardial wall (Lie *et al.* 1975b) or in histologically and anatomically equivalent structures (Ottaviani 2006; Sullivan 1988; Kinoti 1971). Other reported pericardial sites of gastropod haemocyte production include the external surface of the ctenidial/pulmonary and renal veins in the pericardial cavity (Accorsi *et al.* 2014). In cephalopods, haemocytes are thought to originate from the white body, which is a multi-lobed organ that wraps around the optic bundle (Castillo *et al.* 2015; Salazar *et al.* 2015); while in the bivalves, the irregularly folded structure (IFS) of the gills has been proposed as the site of haemocyte formation (Jemaa *et al.* 2014). In many members of these molluscan groups, a haematopoietic site is yet to be described, and it is possible that more than one primary haematopoietic site exists within each of these groups.

Numerous cytokines, growth factors, receptors, intracellular signaling components, and homologues of transcription factors known to be involved in vertebrate haematopoiesis have been identified as part of genomic or transcriptional studies (Huang *et al.* 2017; Salazar *et al.*

2015; Baeza Garcia *et al.* 2010; Ma *et al.* 2009), or are assumed to be present in various molluscan groups (Ottaviani *et al.* 1993). These assumptions are based on sequence identity or immunoreactivity with antibodies raised against their vertebrate counterparts and sometimes a demonstration that they perform similar functions (Franchini and Ottaviani 2000; Granath *et al.* 1994). However, there is no described mechanism detailing the sequence of haematopoietic events for haemocyte proliferation, differentiation and maturation or cell fate determination in any molluscan group to date. Although our understanding of stages of development and types of terminally differentiated cells would be greatly aided by a haemocyte cell line, to date only short-lived primary cultures of these cells have been established, and in fact only a single immortalized cell line has been derived from molluscs, an embryonic, non-haemocyte cell line (Bge) from *B. glabrata* (Yoshino *et al.* 2013a). While a great deal of information on haemocytes now exists, there is still much to be learned about their origin and the molecules and mechanisms that guide their proliferation and differentiation.

2.4 Gastropod haematopoiesis and immune responses

Gastropoda is the most highly diversified class within the phylum Mollusca, composed of over 60,000 species of snails and slugs (Bouchet *et al.* 2005). From land to sea, gastropods live in a large variety of habitat types and face a number of challenges from both pathogenic and non-pathogenic stressors, requiring, as in all other animals, both defence mechanisms and the ability to maintain homeostasis. This diversity presents both an interesting and challenging research problem in that numerous biological processes, such as haematopoiesis, can differ from species to species, reflecting their unique life histories, habitats and pathogenic stressors.

The majority of what is known about haematopoiesis in gastropods is derived from a few species of medical or veterinary importance, especially the freshwater planorbid *B. glabrata*. This snail is the obligatory intermediate host for *S. mansoni,* one of 3 species of trematodes that cause the human disease schistosomiasis, mainly in sub-Saharan Africa, South America, and parts of Asia. This host can be infected with several other species of larval trematodes. The life cycles of nearly all of the ~25,000 species of digenean trematode (Esch *et al.* 2002) require the use of a snail intermediate host to undergo larval development (Bray *et al.* 2002; Esch *et al.* 2002; Cribb *et al.* 2001). Study of this relationship between snails and larval trematodes has been key to our current understanding of haematopoiesis and haemocyte functions in gastropods.

2.4.1 The amoebocyte-producing organ

In *B. glabrata*, the anterior pericardial wall (APW) has been proposed as a site in which haemocytes are produced (Lie *et al.* 1975b). The APW is a flat, roughly triangular sheet of tissue located ventral to the saccular kidney at the rear of the mantle cavity (Sullivan 1990), and is covered posteriorly by simple squamous pericardial epithelium and anteriorly by simple cuboidal mantle epithelium (Lie *et al.* 1976). Between the mantle and pericardium of the APW lie loose connective tissue and a haemolymph sinus. Typical cells of the connective tissue (Sminia 1972; Pan 1958) are present, including fibroblast-like cells, pore cells (rhogocytes), and haemocytes. Additionally, isolated or confluent nodules of small, mitotically active basophilic cells are found attached to the basal surface of the pericardial epithelium, and on the basis of histological, histochemical, and ultrastructural evidence appear to be haemocyte precursors (McKerrow *et al.* 1985; Jeong *et al.* 1983; Lie *et al.* 1975b). Consequently, this structure has been named the amoebocyte-producing organ or APO (Lie *et al.* 1975b).

In addition to structural evidence, transplantation studies support a haematopoietic function of the APO. Heterotopic allografts of APW, but not other organs, from schistosome-resistant to schistosome-susceptible snails confer increased non-susceptibility to infection and increased capacity to encapsulate schistosome sporocysts (Barbosa *et al.* 2006; Sullivan and Spence 1999; Sullivan *et al.* 1995), although some of this transferred resistance may be due to soluble factors produced by transplanted cells (Vasquez and Sullivan 2001). Perhaps the strongest evidence for a haematopoietic function of the APO is the observation that *B. tenagophila* recipients of APW allografts possess circulating haemocytes with a unique molecular marker from the donor (Barbosa *et al.* 2006).

The haematopoietic cells of the APO undergo increased and spontaneous mitotic division *in vivo* in certain genetic strains of snails (Richards 1975a), and as soon as 24 h after infection with larval *S. mansoni*, *Ribieroia marini*, or *Echinostoma* sp. trematodes (Joky *et al.* 1985; Sullivan *et al.* 1984; Sullivan *et al.* 1982; Lie *et al.* 1975a; Lie *et al.* 1975b). Injection of various non-self substances, including excretory-secretory products or freeze-thaw extracts of larval and adult trematodes, LPS from *Escherichia coli*, or the sulfated polysaccharide fucoidan, also stimulate cell proliferation in the APO (Sullivan *et al.* 2014; Sullivan *et al.* 2011; Sullivan *et al.* 2004; Noda 1992). Injection of water or vehicular controls of buffer alone do not stimulate significant mitotic activity in cells of the APO in *B. glabrata*. *In vitro,* when the APO is exposed to freeze-thaw extracts of *S. mansoni* or phorbol myristate acetate (PMA), proliferation can also be induced (Salamat and Sullivan 2009; Salamat and Sullivan 2008). The increased mitotic activity or mitotic burst in the APO results in its distinct enlargement, due to both hypertrophy and hyperplasia (Lie *et al.* 1975b). Jeong *et al*. (1980) reported that exposure of *B. glabrata* to miracidia of *Echinostoma lindoense*, a treatment that increases cell division in the APO (Lie *et*

al. 1976), elicited elevated concentrations of haemocytes in the haemolymph beginning at 3 days post-infection, and these authors proposed the APO as a source for this leukocytosis. As expected, immersion of snails in colchicine results in a significant increase in the number of mitotic figures observed in histological sections of the APO (Joky *et al.* 1985).

Although the mitotic burst in the APO of *B. glabrata* seems to be a response to challenge with some types of non-self, the immune-protective function of the APO is unclear. On the one hand, resistance to a challenge with normal miracidia of *E. lindoense* in snails first exposed to irradiated miracidia is always preceded by this mitotic response (Lie *et al.* 1975a), suggesting its role in so-called acquired resistance. On the other hand, schistosome-resistant snails infected with echinostome miracidia (and having an enlarged APO) actually lose their resistance to *S. mansoni* (Hanington *et al.* 2012; Lie 1982), probably as a result of interference with haemocyte function by excretory-secretory products of the larval echinostome (Loker *et al.* 1992). Moreover, the APO is mitotically unresponsive to many foreign materials that have been injected into the haemocoel, including concentrated suspensions of live Gram-positive and Gram-negative bacteria, and the increased cell division in the APO of susceptible snails following penetration by compatible schistosome or echinostome miracidia does not prevent infection (Sullivan *et al.* 1984). Finally, the 24-h delay in the onset of the mitotic response following challenge with non-self suggests that this response may not be important in the initial haemocytic encapsulation and killing of sporocysts in schistosome-resistant snails, which typically begins within several hours post-infection (Loker *et al.* 1982). Thus, rather than a protective response, the mitotic burst may represent a "second line of defense" against certain pathogens or their molecules (Sullivan *et al.* 2004), with the APO normally functioning to help maintain (along

with peripheral sites of haematopoiesis) homeostasis of haemocyte numbers. Whether the APO has any immune function beyond haematopoiesis is unknown.

A structure that is anatomically and histologically similar to the APO of *Biomphalaria* species has also been reported in a number of other pulmonated gastropods, such as, *Bulinus truncatus* (Kinoti 1971), *Helisoma trivolvis* (Sullivan 1988), *Lymnaea palustris* (Rachford 1976), and *Planorbarius corneus* (Ottaviani 2006). Although the APW appears to be a site of haemocyte production, peripheral haemocytes seem to also retain the capacity to divide, and possible haematopoietic events have also been observed in other locations in snails, including the kidney, mantle connective tissue, haemolymph, and head-foot (Sullivan 1988; Yousif *et al.* 1980; Rachford 1976; Sminia 1972; Pan 1958). The APO of *Marisa cornuarietis* is a thickened band in the roof of the lung rather than in the pericardial wall (Yousif *et al.* 1980), and *Physa virgata* does not appear to possess a defined haematopoietic organ, instead forming haemocytes in the connective tissues of the mantle (Sullivan 1988).

In *Pomacea canaliculata*, haematopoiesis occurs within the pericardial cavity, based on the observation of dividing cells along the external surface of the ctenidial/pulmonary and renal veins near their junction with the heart, and within the pericardial fluid (Accorsi *et al.* 2014). Newly formed haemocytes are thought to be stored in a saccular organ, called the ampulla, which is connected to the anterior aorta and loosely adherent to the heart. The ampulla is hypothesized to function in homeostasis of haemocyte numbers in *P. canaliculata,* by serving as a haemocyte reservoir, rather than as a site of haematopoiesis. When haemolymph was withdrawn repeatedly from this snail, haemocytes were lost from the ampulla, but dividing cells were not observed in this organ.

The specific nature and source of the stimulus eliciting mitotic division among the haemocyte precursors in the APO is not known. While it is known that certain non-self substances stimulate these cells, it is unclear whether this is in a direct or indirect manner. Recent evidence implicating granulin, an endogenous growth factor from *B. glabrata* (*Bg*GRN), in the anti-*S. mansoni* immune response (Pila *et al.* 2016a), supports an indirect response that is primarily driven by increases in the abundance of circulating growth factors, but does not exclude the possible effect of parasite mitogens *in vivo*. Receptors for mitogenic non-self substances such as LPS or for putative growth factors produced endogenously have not yet been identified in *B. glabrata*, or any other gastropod to date.

2.4.2 Haemocyte development and haematopoietic regulators

Currently, more is known about effector functions of mature haemocytes than the process of their production, stages of development, or the identity and mechanistic basis of involvement of haematopoietic regulators – growth and transcription factors, receptors, signaling pathways and cytokines. Almost nothing is understood about haemocyte precursors, particularly in terms of the growth factors driving haematopoiesis, gene expression profiles, and how commitment to specific haemocyte subsets is determined. Blast-like cells have been described in some species such as *B. glabrata, Lymnaea stagnalis, L. truncatula and Littorina littorea* (Gorbushin and Iakovleva 2006; Monteil and Matricon-Gondran 1991; Jeong *et al.* 1983; Sminia *et al.* 1983; Lie *et al.* 1975b) but these are mainly based on ultrastructure of the cells, which may be localized in the APO or in circulation, and not on any observed pluripotency or molecular markers.

2.4.2.1 Growth factors

Bona fide growth factors have begun to be identified in gastropods, largely because of gene sequence comparisons to known vertebrate growth factors. These include the endogenous growth factor granulin in *B. glabrata* previously mentioned and further discussed below (Pila *et al.* 2016a). In *Haliotis tuberculata*, porcine insulin and recombinant human EGF have been shown to influence the primary capacity of haemocytes to incorporate labelled leucine or thymidine in a concentration-dependent manner (Lebel *et al.* 1996), an observation, which suggests that homologues of these growth factors might be present in this gastropod. However, the interpretation of these initial results was complicated by the fact that the possible proliferative effect of concanavalin A used to promote the attachment of cells in the assays could not be ruled out. More recent studies have since provided more definitive evidence; an insulin receptor has been cloned from the Bge cell line, which also responds to bovine insulin by incorporating labelled methionine and thymidine (Lardans *et al.* 2001), and a *B. glabrata* EGF-related protein is one of the transcripts upregulated early on in the course of infection with *S. mansoni* (Hanington *et al.* 2010b). An EGF and its receptor have also been identified in *L. stagnalis* and they are thought to be associated with the activation and survival of resident endoneurial phagocytes, which are important for neuronal regeneration (Hermann *et al.* 2005).

2.4.2.2 Transcription factors

Transcriptional regulation of haematopoiesis is the least understood aspect of haemocyte development in gastropods. There are no studies dealing with the transcription factor profile of haemocyte precursors that I am aware of to date. A few transcription factors have been cloned, and the pattern of expression determined, but there is no direct evidence for their functional

involvement in specific proliferation, development and maturation events in gastropods. CREB is a transcription factor better known for its role in memory (Carlezon Jr *et al.* 2005), but also functions in cell proliferation and survival (Zhang *et al.* 2002). Identified in some gastropod species (Zhang and Coultas 2011; Lee *et al.* 2007; Sadamoto *et al.* 2004), it has only been studied extensively in *Aplysia* and *Lymnaea* in the context of memory. NF-kB is an evolutionarily conserved family of transcription factors that function in immune cell activation, transcription of pro-inflammatory genes, and learning and memory in the nervous system. Homologues of NF-kB have been cloned in *B. glabrata* (Zhang and Coultas 2011), and the abalone *Haliotis diversicolor* (Jiang and Wu 2007). *B. glabrata* NF-kB possesses conserved binding motifs (Humphries and Harter 2015) that suggest involvement in the immune response as would be expected from studies of other organisms, and was upregulated during *S. mansoni* challenge. While *H. diversicolor* NF-kB was downregulated during viral haemorrhagic septicaemia virus challenge, the expression of its transcript changed inconsistently with bacterial challenge over 48 h. NF-kB-like protein has been detected in the axons of *Aplysia,* but its function there is thought to be in communication, linking axons and synapses with the nuclear synthetic machinery (Povelones *et al.* 1997).

Other transcription factors playing important roles in vertebrate haematopoiesis or cell proliferation, that have been identified in gastropods, include STAT1 and 2 (Zhang and Coultas 2011) and CCAAT-enhancer binding protein (C/EBP) (Yamamoto *et al.* 2002). The functionality of the STATs has not been explored in these organisms, while C/EBP is associated with facilitation of sensory-to-motor neuron synapses in *Aplysia*. Further studies are needed in order to determine the specific roles of these transcription factors in the context of haematopoiesis in gastropods.

A recent study (Zhang *et al.* 2016) has examined the transcriptomic response in the APO of *B. glabrata* snails at 24 h post-challenge with three substances having mitogenic activity in the APO: the bacterial PAMPs LPS, peptidoglycan, and fucoidan, a sulfated polysaccharide that may mimic fucosylated glycan PAMPs on the sporocysts of *S. mansoni*. Using a 60-mer oligonucleotide *B. glabrata* microarray with 30,647 probes, this study revealed that genes involved in cellular proliferation were among the most differentially expressed, along with immune-related and detoxification genes, as well as genes with no known homologues in other organisms. Changes in gene expression were elicited by all three PAMPS with LPS having the most potent effect. Checkpoint kinase 1, a serine/threonine-specific kinase and key regulator of mitosis was found to be highly upregulated in the APO of LPS-challenged snails, indicating that it plays a role in cellular proliferation in the APO. The authors proposed that the expression of this kinase might serve as a potential genetic marker for identifying sites of haemocyte production in the snail (Zhang *et al.* 2016).

2.4.2.3 Signaling pathways

In terms of signal transduction during haematopoiesis, evidence so far indicates involvement of the MAPK pathway. A number of other immune relevant processes in haemocytes also appear to be mediated by the MAPK pathway. These include cellular adhesion, motility and spreading required for phagocytosis and encapsulation (Humphries *et al.* 2001), and regulation of the release of the cytotoxic molecules, hydrogen peroxide and nitric oxide (Humphries and Yoshino 2008; Gorbushin and Iakovleva 2007; Zelck *et al.* 2007). The MAPK/ERK pathway can be activated by protein kinase C. *In vitro* treatment of APOs from *B. glabrata* with the PKC activator PMA induced cell division in the APO in a concentration-

dependent manner and this effect was blocked when ERK1/2 inhibitor (U0126) was added (Salamat and Sullivan 2009). However, some cell division was still observed in the presence of the ERK1/2 inhibitor, indicating that other signaling pathways are likely involved in gastropod haematopoiesis.

Another MAPK (p38) has also been implicated in the induction of hydrogen peroxide production in *B. glabrata* haemocytes in response to PMA or galactose-conjugated bovine serum albumin (BSA, Humphries and Yoshino 2008), making it a potential candidate for signal transduction during haemocyte proliferation in gastropods. TLR signaling, which can also activate the MAPKs (p38 and Jun) or NF-kB, is another pathway through which mitotic activity can be achieved. In *L. littorea*, treatment of haemocytes with the TLR4 ligand, LPS, resulted in the activation of MAPKs, ERK2, and p38 (Iakovleva *et al.* 2006). As reviewed above, LPS induces cell proliferation in the APO of *B. glabrata* (Sullivan *et al.* 2011). Although the receptor-ligand relationship has not been established in this snail, analysis of its genome shows that it contains several TLRs and LRR-containing molecules, as are the signaling molecules MyD88, TRAF, IRAK and IKK (Adema *et al.* 2017). A TLR has also been identified in the disk abalone *Haliotis discus* (Elvitigala *et al.* 2013). This TLR is expressed in haemocytes more than in other tissues and its transcript is upregulated significantly upon bacterial and viral challenges. Further studies are needed in order to elucidate the signal transduction pathways involved in gastropod haematopoiesis.

2.4.2.4 Cytokines

A number of studies have assessed the presence of mammalian cytokine homologues or impact of mammalian recombinant cytokines on haemocyte function and development in

Planobarius corneus, *Viviparus ater* (IL-1α, IL-1β, IL-2, IL-6 and TNF-α) (Ottaviani *et al.* 1993), *B. glabrata* (IL-1) (Granath *et al.* 1994) and the slug, *Limax maximus* (IL-1, IL-8 and TNF-α) (Franchini and Ottaviani 2000). Detection of these homologues was largely based on immunoassays, utilizing antibodies raised against their mammalian counterparts, thus limiting our ability to interpret the true identity and function of the recognized proteins. Moreover, reports of antibody-based detection of putative cytokines in *B. glabrata* should be viewed with caution, in as much as a lectin-like protein in snail plasma non-specifically binds antibodies from several vertebrates, potentially leading to false positive results (Hahn *et al.* 1996). However, the identification and characterization of some of these factors in other invertebrates indicate that they may be ancestrally and functionally conserved (Buckley and Rast 2015; Song *et al.* 2015; Hibino *et al.* 2006). MIF (discussed below) is perhaps the only endogenous cytokine to be cloned and functionally characterized in gastropods. Multiple homologues of MIF, interleukin-17 and TNF have been identified in the *B. glabrata* genome (Adema *et al.* 2017).

All told, our current understanding of gastropod haematopoiesis is such that answers to key questions (such as stages and sequence of haematopoietic events of proliferation, differentiation and maturation) still await the results of future studies that should benefit greatly from the annotated *B. glabrata* genome (Adema *et al.* 2017).

2.4.3 Cellular immunity

The study of haemocytes, particularly their development and mechanisms of engagement and activation, only recently advanced to the point of specific functional investigations. In *B. glabrata,* most studies have focused on identifying and characterizing soluble immune factors, however, the haemocytes are critical in a snail immune response to an invading schistosome.

Without the haemocyte-driven encapsulation response, the parasite often survives and establishes infection.

2.4.3.1 Haemocyte morphotypes observed in gastropods and their role in the immune response

Morphological characterizations of gastropod haemocytes initially described two unique cell types, granulocytes and hyalinocytes. Granulocytes in *B. glabrata* are characterized by extensive production of pseudopodia in all directions and conspicuous granules, which are limited mainly to the endoplasm. These cells are adherent and measure about 24 and 16 µm in their longest and widest dimensions. They constitute about 87% of the haemocyte population (Cheng 1975). Hyalinocytes are smaller in size compared to the granulocytes and are generally spherical or slightly oval. Granules are sparse, and the only pseudopodia formed are lobose and not extensive. Hyalinocytes measure about 6.9 and 6.6 µm (in length and width) and constitute 13% of the haemocyte population (Cheng 1975). In *Viviparus ater*, only one haemocyte type has been described, the equivalent of the granulocyte. This cell is characterized by irregular form, pseudopodia formation, round or oval nucleus and abundant cytoplasm, with inclusions and numerous vacuoles (Ottaviani 1989). Some earlier studies characterized haemocyte subsets based on specific membrane antigenic differences (Yoshino and Granath 1985; Yoshino and Granath 1983).

More recent studies describe three cell types based on size, ultrastructure and internal complexity. In *B. glabrata*, three haemocyte subpopulations have been described, namely, large (> 8 µm), medium (~8 µm) and small (5–6 µm) haemocytes (Matricon-Gondran and Letocart 1999b). Large haemocytes are characterized by an asymmetrical shape, small nucleus:cytoplasm

ratio, cytoplasm with numerous mitochondria, dense particles of glycogen and prominent extensions. Medium haemocytes in comparison to the large ones are more symmetrical and have a higher nucleus:cytoplasm ratio. They have fewer organelles around the nucleus and few aggregates of glycogen particles. Small haemocytes have a high nucleus:cytoplasm ratio, are organelle-rich, with few secretory granules. In terms of haemolymph proportions, the large and medium haemocytes are almost equally numerous while small haemocytes are comparatively fewer (Matricon-Gondran and Letocart 1999b). *Biomphalaria glabrata* and *B. tenagophila* haemocytes have also been categorized into three categories (large, medium and small) based on flow cytometric analysis (Martins-Souza *et al.* 2009). Each category can also be divided into a low or high granular haemocyte based on side scatter. Small and medium haemocytes were found to be the most numerous in non-infected *B. glabrata* and *B. tenagophila,* respectively, while large haemocytes were the least numerous for both species. While the number of haemocyte subsets is in agreement with the results of Matricon-Gondran and Letorcart (1999b), there is no consensus on the precise size of what is considered small, medium or large. Based on the FSC channel values used by Martins-Souza *et al.*(2009), small and medium haemocytes would be 1–2 µm and 3–10 µm respectively, while large haemocytes would be > 10 µm.

The proportion of hyalinocytes and granulocytes differs between snail species (Ataev *et al.* 2016; Cheng 1975), and fluctuates in response to various pathogens (Ataev *et al.* 2016; Pila *et al.* 2016a; Noda and Loker 1989). The roles of these cells in an immune response include phagocytosis (Ataev *et al.* 2016; Cueto *et al.* 2015), cytotoxicity (Humphries and Yoshino 2008; Lacchini *et al.* 2006) and encapsulation (Nacif-Pimenta *et al.* 2012; Loker *et al.* 1982). These responses are enabled by intracellular synthesis of relevant proteins such as inducible immune receptors and effectors, cell adhesion molecules, and recruitment factors (Coustau *et al.* 2015;

Zahoor *et al.* 2014; Lockyer *et al.* 2012). For most of these factors, their putative functional roles in *B. glabrata* are based on inferences from transcript expression and predicted amino acid similarity to well characterized immune molecules of other organisms.

Both granulocytes and hyalinocytes can phagocytose microbes or experimental microspheres (Ataev *et al.* 2016; Cueto *et al.* 2015). Against the larger larval schistosomes, phagocytosis is limited to stripping of sporocyst microvilli and small tegumental pieces (Loker *et al.* 1982), and is perhaps more important for clearing debris from dead/dying parasites (Nacif-Pimenta *et al.* 2012; Loker *et al.* 1982; Meuleman *et al.* 1978). Larval digenean surfaces contain complex carbohydrates and glycoproteins (Guillou *et al.* 2007), some of which can be released during miracidium-to-sporocyst transformation. Monosaccharides (such as α-D-mannose and α-D-galactose), known components of these glycoproteins, initiate high rates of phagocytosis in haemocytes of *B. glabrata* when conjugated to fluorescent microspheres (Hanington *et al.* 2010a). This increased phagocytic response, mediated by *B. glabrata* FREP3 (*Bg*FREP3), provided evidence for one means through which haemocyte engagement with the parasite might occur. However, mechanistic details such as which domains of *Bg*FREP3 interact with parasite and haemocyte, and the specific haemocyte receptor involved are not known.

Regarding the anti-schistosome immune response, granulocytes seem to be most relevant because of their involvement in encapsulation, which typically results in parasite elimination. Ultrastructure and biochemical labeling of haemocyte capsules suggest that granulocytes are the primary haemocyte type in contact with the sporocyst (Loker *et al.* 1982; Bayne *et al.* 1980). Also, increased abundance of circulating haemocytes observed during schistosome infections is primarily attributed to additional circulating granulocytes (Pila *et al.* 2016a; Hanington *et al.* 2010a; Lie *et al.* 1976). High granulocytic numbers (>230 cells/μL) in adult *B. glabrata*

associate with resistance to *S. mansoni*, and the majority of circulating cells in snails with high haemocyte numbers are granulocytes (Larson *et al.* 2014). In uninfected adult snails, the average granulocytic cell numbers are in the range of 84–172 cells/μL (Pila *et al.* 2016a; Larson *et al.* 2014).

Granulocyte recruitment to the site of an invading *S. mansoni* is observable as early as 1 h in resistant snails (Nacif-Pimenta *et al.* 2012). Formation of a complete haemocyte multilayer around the sporocyst leads to its killing within 10–72 h (Nacif-Pimenta *et al.* 2012; Loker *et al.* 1982). Chemotactic factors produced by the snail and parasite are known to mediate granulocyte recruitment. The pleiotropic cytokine MIF (Huang *et al.* 2017; Baeza Garcia *et al.* 2010) has been well characterized as a haemocyte chemokine. Another potential recruitment factor is allograft inflammatory factor (*aif*), which displays increased transcript abundance during immune challenge (De Zoysa *et al.* 2010) and higher basal expression in *S. mansoni*-resistant snails (Lockyer *et al.* 2012; Larson *et al.* 2014). It is also likely that haemocytes respond to exogenous cues directly from the sporocysts (Nacif-Pimenta *et al.* 2012; Lodes and Yoshino 1990), however the identity of these factors remains unknown.

Several hypotheses have been advanced to explain the differential responses generated by individual *B. glabrata* snails to the same *S. mansoni* strain. Prominent among these is that haemocytes of compatible snails are unable to recognize the parasite and thus fail to become activated. Consequently, recruitment of more haemocytes, production of proteins required for cell-to-cell interaction, haemocyte activation and cytotoxic factors that would kill the parasite, also fail. Supporting this hypothesis is the pattern of polymorphisms found in *B. glabrata* FREPs and *S. mansoni* polymorphic mucins (*Sm*PoMucs), which fulfil several criteria as molecular determinants of snail-schistosome compatibility (Mitta *et al.* 2017). Another explanation is that

haemocytes of susceptible snails are functionally inhibited by factors in the larval transformation products (LTPs), which interfere with their motility and spreading (Lodes and Yoshino 1990), intracellular protein synthesis (Lodes *et al.* 1991) and capacity to produce ROS (Zahoor *et al.* 2009). Candidate molecules in this category include calcium-binding proteins such as calreticulin (Guillou *et al.* 2007). LTPs have also been implicated in modulation of proton channels proteins (Wright *et al.* 2017), which regulate ion balance and intracellular pH by allowing hydrogen ions to cross cellular membranes. The involvement of these channels in ROS production not only suggests a possible regulation of snail cell ROS production but also an anti-immune mechanism that could be used by sporocysts to counter snail ROS-mediated effector responses (Wright *et al.* 2017). Other hypotheses include parasite deployment of molecular mimicry to dampen snail immune recognition (Yoshino *et al.* 2013b) and differences in effector/anti-effector systems such as host ROS countered by parasite ROS scavengers (Moné *et al.* 2011).

Sporocyst killing is actively mediated by haemocytes. When plasma is replaced with culture medium, the haemocytes of *B. glabrata* 13-16-R1, which is resistant to *S. mansoni* PR-1, are still able to kill sporocysts effectively as with whole haemolymph (Hahn *et al.* 2001b). Haemocyte activation triggers production of cytotoxic molecules, the best known being the ROS. Their relevance in *B. glabrata* was conclusively demonstrated by Adema and colleagues who found that interfering with haemocyte production of ROS using an antagonist of NADPH-oxidase also compromised their ability to kill sporocysts (Adema *et al.* 1994). Currently, it is known that: (i) hydrogen peroxide and nitric oxide are the chemical species that mediate the killing of *S. mansoni* sporocysts in *B. glabrata* (Hahn *et al.* 2001b; Hahn *et al.* 2001a); (ii) of the 3 alleles for Cu/Zn superoxide dismutase (*sod1*) gene, the B allele is associated with resistance against *S. mansoni* in *B. glabrata*, and snails with the B allele express higher levels of Sod1 (Bender *et al.*

2007); (iii) snail ROS and parasite ROS scavengers likely counter one another such that compatible parasites have antioxidant capabilities matching or exceeding host oxidants (Moné *et al.* 2011). However, the precise mechanisms through which hydrogen peroxide or nitric oxides kill the sporocysts have not yet been determined.

Besides ROS, snail haemocytes can also degranulate the content of their proteolytic enzymes extracellularly and into phagosomes (Cueto *et al.* 2015; Bayne 2009). The potential cytotoxic molecules include proteases and protease inhibitors which may be relevant in disrupting the sporocyst tegument or modifying the complex glycoproteins found on their surfaces (Hanelt *et al.* 2008; Lockyer *et al.* 2007). However, as with many immune factors in *B. glabrata*, these require functional validation.

2.4.3.2 Haemocyte numbers versus functional diversity in determining compatibility with schistosomes

Maintaining sufficient haemocyte numbers is an important element of the snail immune response, but it does not appear to determine the effectiveness of the encapsulation response. Doubling or quadrupling *L. stagnalis* haemocyte concentration *in vitro* increased encapsulation size but did not kill *S. mansoni* sporocysts faster (Dikkeboom *et al.* 1988). It seems likely that haemocytes in the innermost layer of the encapsulation, those contacting the sporocyst, are actively involved in the killing process. Such haemocytes must have the relevant repertoire of receptors for recognition and capacity for cytotoxicity; implying a functionally heterogenous population. Gastropod haemocytes are thought to be functionally heterogenous in measures such as binding to parasite glycan epitopes (Yoshino *et al.* 2013b; Johnston and Yoshino 2001),

enzyme content (Larson *et al.* 2014; Bender *et al.* 2007; Goodall *et al.* 2004) and cell surface markers (Martins-Souza *et al.* 2006; Yoshino and Granath 1985).

Emerging evidence suggests that haemocyte functional differences may be the driving force behind successful immune responses. Larson *et al*. (2014) found that snails that were refractory to *S. mansoni* infection, but had low haemocyte numbers, expressed specific immune relevant genes constitutively at high abundance. Also, in one snail line that displayed high haemocyte numbers both at juvenile and adult stages, the juveniles were highly susceptible while adults were resistant; indicating that high cell numbers alone were insufficient to manifest resistance (Larson *et al.* 2014). Furthermore, it was found that newly proliferated haemocytes expressed *Bg*FREP3 at a higher proportion following an immune challenge (Hanington *et al.* 2010a), suggesting that haemocyte differentiation or maturation had occurred.

We recently began to characterize functionally distinct haemocyte subsets by assessing two haemocyte-associated factors known to play a role in *S. mansoni* immunity – *Bg*FREP3 (Hanington *et al.* 2010a) and *Bg*TLR (chapter 5, Pila *et al.* 2016b). We found that the proportion of *Bg*TLR+ haemocytes increased in snails receiving treatment with the recombinant growth factor *Bg*GRN compared to controls, whereas the proportion of *Bg*FREP3+ haemocytes did not differ significantly (chapter 4, Pila *et al.* 2016a). Furthermore, *Bg*GRN treatment before challenge reduced *S. mansoni* PR-1 infection success by 54%. Conversely, the resistant phenotype of *B. glabrata* BS-90 was significantly diminished when RNAi was used to knockdown *Bg*TLR expression (Pila *et al.* 2016b). These studies suggest that *S. mansoni* challenge of *B. glabrata* leads to haemocyte proliferation and differentiation into functionally relevant subsets. As more determinants of compatibility are functionally characterized, it will become possible to define functional haemocyte subpopulations with a better resolution in the

context of a specific immune response and to determine the conditions under which they are generated and the targets to which they are most relevant.

2.4.3.3 Haemocyte-associated receptors involved in schistosome recognition and engagement

Many receptors have been identified in snails with putative roles in the immunobiology of snail-schistosome interactions (Zahoor *et al.* 2014; Lockyer *et al.* 2012; Lockyer *et al.* 2007; Hanington *et al.* 2010b; Roger *et al.* 2008b). While some of these receptors, such as PGRPs and TLRs, are canonical PRRs, others such as variable immunoglobulin and lectin domain-containing molecules (VIgLs, Dheilly *et al.* 2015) and the Guadeloupe resistance complex (GRC, Tennessen *et al.* 2015) appear to be gastropod-specific. The broad categories of receptors include PRRs, integrin-related proteins and growth factor/cytokine-like receptors. Of these, PRRs are known to interact with *S. mansoni*.

B. glabrata PRRs include TLRs (Adema *et al.* 2017; Pila *et al.* 2016b), GRC (Tennessen *et al.* 2015), mannan and laminarin-binding molecules, PGRP, GNBP and lipopolysaccharide-binding protein (Yoshino and Coustau 2010; Bayne 2009) as well as VIgLs (Dheilly *et al.* 2015). The implication of *Bg*TLR in the resistance of *B. glabrata* snails against *S. mansoni* provided functional insight into how haemocytes might engage *S. mansoni* through cell-associated receptors (Pila *et al.* 2016b). The fact that signaling molecules downstream of the TLR pathway are conserved (Adema *et al.* 2017; Humphries and Harter 2015; Zhang and Coultas 2011), and that there is great diversity of TLR genes in the genome of *B. glabrata* (Adema *et al.* 2017) is indicative of their importance in the snail immune response including against schistosomes, and suggests that LRR-containing molecules are likely involved in recognition of various pathogen

types. Another putative PRR – Grctm6, a member of GRC with the hallmarks of a cell-bound receptor, has been shown to modulate the schistosome burden in *B. glabrata* (Allan *et al.* 2017).

Much remains to be discovered regarding haemocyte immune recognition, and integration with other elements of the snail immune response. Most of the identified receptors still require functional characterization to confirm an immune role. For those supported by functional data, mechanistic details such as what ligands they bind and the signaling mechanisms through which they elicit a response remain unknown. Although haemocytes are directly involved in parasite killing, humoral factors produced by these cells can act in concert or play sentinel roles.

2.4.4 Humoral factors of the *B. glabrata* immune response

During their penetration and establishment within the snail, *S. mansoni* are exposed to host haemolymph and the bevy of soluble immune effector molecules therein. Proteomic analysis of *B. glabrata* plasma revealed that an array of factors display affinity for sporocyst tegumental membrane proteins (Mem) and LTPs. These factors include PRRs such as the *B. glabrata* TEP (*Bg*TEP), cytotoxins such as biomphalysin, and VIgLs (Wu *et al.* 2017). Soluble immune effectors are involved in both the direct killing of sporocysts and preparation of haemocytes to mount a cell-mediated response.

While cell-mediated immune responses are heavily featured in primary schistosome infections, subsequent challenges seem to shift the response towards dependence on humoral effectors, highlighting their necessity and foundational role in the appearance of innate immune memory or acquired resistance (Pinaud *et al.* 2016). This is evident from the observations that primary infections lead to the encapsulation and destruction of sporocysts by host haemocytes,

whereas sporocysts in secondary immune challenge can be killed without encapsulation (Pinaud *et al.* 2016). Circulating humoral factors particularly FREPs (Pinaud *et al.* 2016; Hanington *et al.* 2012) and biomphalysin (Pinaud *et al.* 2016) have been shown to be involved in this process. Both were transcriptionally upregulated during secondary challenge (Pinaud *et al.* 2016), and transfer of primed snail plasma to naïve snails significantly decreased the prevalence of primary *S. mansoni* infections (Pinaud *et al.* 2016). Additionally, down regulation of several humoral immune factors including *Bg*FREP3 has been demonstrated to result in loss of snail resistance phenotype, supporting the role of these proteins in combatting secondary trematode infections (Hanington *et al.* 2012). The appearance of *B. glabrata* innate immune memory in response to trematode infections is a phenomenon that has been observed for the past four decades (Sire *et al.* 1998; Lie *et al.* 1983; Lie and Heyneman 1979; Lie *et al.* 1975a) though the underlying mechanisms were not well understood. Recent studies (Pinaud *et al.* 2016) have investigated the molecular mechanisms of this phenomenon and demonstrated that humoral factors are likely crucial to the appearance of innate immune memory in which invertebrates, which lack vertebrate-styled adaptive immune system, apparently demonstrate a level of 'acquired resistance' to pathogens.

2.4.4.1 Thioester-containing protein

Mounting a successful immune response requires recognizing invading pathogens. This task is accomplished in large part by PRRs like the complement C3 protein in vertebrates, and TEP homologues in invertebrates. The TEP superfamily can be separated into three families: (i) vertebrate complement proteins (C3/C4/C5), (ii) alpha2 macroglobulin (A2M) pan-protease inhibitors, and (iii) a group of TEPs unique to invertebrates. These homologous proteins share

several conserved features, including their secretion as inactive forms, activation via proteolytic cleavage, and a conserved thioester motif used to covalently bind their target. Complement proteins and invertebrate TEPs use this binding to opsonize foreign targets.

*Bg*TEP displays numerous similarities to other invertebrate TEPs. It possesses a classic thioester motif, and is cleaved prior to binding *Sm*PoMucs. It features an A2M receptor-binding domain, which in other invertebrate models has been shown to bind the A2M receptor found on circulating phagocytic cells (Stroschein-Stevenson *et al.* 2006; Blandin *et al.* 2004). These features, coupled with its association with LTPs, *Sm*PoMucs, and other Mem proteins suggest a role for *Bg*TEP in the opsonization of *S. mansoni* sporocysts (Wu *et al.* 2017; Moné *et al.* 2010). This role is also supported by functional studies of TEPs in insect models. *D. melanogaster* possesses six TEPs, which differ in their preference between opsonizing Gram-negative and Gram-positive bacteria (Stroschein-Stevenson *et al.* 2006). *Anopheles gambiae* TEP1 binds *Plasmodium berghei* ookinetes, targeting them for subsequent lysis or melanization. Also, *A. gambiae* TEP polymorphisms are linked to *P. berghei*-resistant and susceptible phenotypes (Blandin *et al.* 2004). The observation that *Bg*FREP2 can also form complexes with *Bg*TEP and *Sm*PoMucs envisions a model where *Bg*FREPs recognize invading trematodes before associating with *Bg*TEP, which then kills the parasite directly or opsonizes it for encapsulation and killing by circulating haemocytes.

2.4.4.2 Biomphalysin

Molecules that can directly lyse a target cell are important elements of the humoral immune response. Biomphalysin, a β-pore forming toxin (β-PFT) functions as one such molecule. β-PFTs constitute a category of molecules with high tertiary structure and functional

conservation, which bind to their target and heptamerize to form a β-barrel pore on the cell membrane, leading to cell lysis and death (Degiacomi *et al.* 2013; MacKenzie *et al.* 1999; Wilmsen *et al.* 1992). They are structurally characterized by a large lobe involved in binding glycophosphatidylinositol (GPI)-anchored receptors and oligomerization, and a small lobe involved in recognition of carbohydrates on the surface of target cells and transmembrane domains necessary for pore formation (Galinier *et al.* 2013; Knapp *et al.* 2010). Biomphalysin has the typical features of β-PFTs except that its small lobe does not feature a lectin-like domain, which is required for carbohydrate binding. The lack of this lectin-like domain has led to speculation that biomphalysin could function in targeting unique sporocyst antigens, though further work is required to examine this possibility (Galinier *et al.* 2013).

While prokaryotes utilize these proteins to attack host immune cells, *B. glabrata* employs biomphalysin for killing sporocysts (Galinier *et al.* 2013; Aroian and der Goot 2007; Nelson *et al.* 1999). Exposure of sporocysts to biomphalysin alone does not increase mortality unless *B. glabrata* plasma is added, suggesting the presence of a yet undetermined co-factor. *In vitro* exposure of sporocysts to combined plasma and biomphalysin results in cellular swelling, followed by sporocyst disintegration (Galinier *et al.* 2013). Biomphalysin transcript levels increase during secondary *S. mansoni* infections compared to snails undergoing a primary infection suggesting that it is a key factor in the appearance of innate immune memory in *B. glabrata* (Pinaud *et al.* 2016).

2.4.4.3 Macrophage migration inhibitory factor

Preparing haemocytes to engage invading trematodes is another requirement of an effective immune response, and MIF is a mediator of this function. MIF is a cytokine with pleiotropic

functions in mammals, including the stimulation of cell proliferation and suppression of p53-mediated apoptosis (Huang *et al.* 2017; Baeza Garcia *et al.* 2010; Calandra and Roger 2003). In gastropods, it has been identified both in *Haliotis diversicolor*, *Oncomelania hupensis*, as well as *B. glabrata* (Huang *et al.* 2017; Baeza Garcia *et al.* 2010; Wang *et al.* 2009). Just as its mammalian orthologue, *B. glabrata* MIF (*Bg*MIF), which is expressed in haemocytes, stimulates cell proliferation via activation of the ERK1/ERK2 pathway and inhibits nitric oxide-dependent, p53-mediated apoptosis in Bge cells. Moreover, knockdown of *Bg*MIF altered haemocytic behaviour in a manner that led to a significant increase in parasite burden in infected snails (Baeza Garcia *et al.* 2010), demonstrating that this cytokine influences the ability of haemocytes to effectively encapsulate *S. mansoni*.

2.4.4.4 Variable immunoglobulin and lectin domain-containing molecules

The study of *B. glabrata* RNA sequencing data from a *de novo* reference transcriptome led to identification of novel molecules that consist of one or two IgSF domains and an interceding region (ICR), suggesting a similarity to FREPs, but that instead of an FBG domain, associate with either a C-type lectin domain or a galectin domain (Dheilly *et al.* 2015). These were named C-type lectin-related protein (CREP) and galectin-related protein (GREP), respectively. Like the FREPs, the expression of CREPs and GREPs also associates with *S. mansoni* resistance in *B. glabrata* (Adema *et al.* 2017; Dheilly *et al.* 2015). Although CREPs and GREPs await functional characterization, a possible role in pathogen recognition is supported by recent proteomic studies which have identified several constitutively expressed GREPs and CREPs that are capable of binding LTPs and sporocyst tegumental proteins (Wu *et al.* 2017), and

their similarities to perhaps the most well characterized soluble immune molecules that *B. glabrata* possesses, FREPs.

2.4.4.5 Fibrinogen-related proteins

FREPs are broadly defined by the presence of a fibrinogen-related domain. These domains are conserved throughout animal evolution, and are diverse in both form and function (Hanington and Zhang 2011). Gastropods possess a unique subset of FREPs which marries a C-terminal FBG domain, connected via an ICR, to one or two IgSF domains at the N-terminus (Adema *et al.* 1997). In 1997, Adema *et al*. identified four peptides that were obtained from precipitates evoked by LTP of *Echinostoma paraensei* sporocysts in *B. glabrata* M-line snail plasma (Adema *et al.* 1997). Amino acid sequences of gel-purified proteins ~65 kDa in size were used to instruct PCR primer design that led to the discovery that the *B. glabrata* genome encoded sequences with high identity to fibrinogen-like sequences. Therefore, the ~65 kDa protein was designated a FREP (Adema *et al.* 1997).

According to the number of IgSF domains, *Bg*FREPs can be classified as: single-IgSF and tandem-IgSF *Bg*FREPs (Hanington and Zhang 2011), in which the tandemly arranged IgSF domains are joined by a small connecting region (Zhang *et al.* 2001). Whole genome analysis of *B. glabrata* indicates that *Bg*FREPs are encoded by 24 germline genes, 20 of which belong to tandem-IgSF and 4 to single-IgSF *Bg*FREPs (Adema *et al.* 2017).

A unique aspect of *Bg*FREPs is their capacity for somatic diversification that is further expanded upon by alternative splicing and multimerization in snail haemolymph (Zhang and Loker 2003). This phenomenon was first discovered because otherwise identical genomic *Bg*FREP sequence fragments occasionally differed randomly at single nucleotide positions

(Zhang *et al.* 2001; Leonard *et al.* 2001). Subsequent studies on somatic diversity focused on a region of exon 2 that encodes the IgSF1 domain of *Bg*FREP3. *Bg*FREP3 was estimated to be encoded by 3 to 5 genomic loci, but up to 45 and 37 different amplification sequences could be obtained from two snail individuals respectively (Zhang *et al.* 2004). The extent of diversity of *Bg*FREP3 IgSF1 sequences in individual snails was far greater than the estimated number of loci, suggesting a novel mechanism that increases the potential diversity of *Bg*FREPs (Zhang *et al.* 2004). Zhang *et al*. found that this diversity occurs at the somatic cell level, mainly through point mutations and gene conversion (Zhang *et al.* 2004).

Different *Bg*FREPs exhibit functional specialization in their binding preferences for particular pathogens; for example, 65–75-kDa FREPs (mainly *Bg*FREP4) associate with *E. paraensei,* whereas *Bg*FREP3 recognizes bacteria, fungi, (Zhang *et al.* 2008) and *S. mansoni* (Wu *et al.* 2017). *Bg*FREPs are capable of direct binding to *S. mansoni* Mem and LTP (Wu *et al.* 2017). As mentioned above, the highly polymorphic and variable *Sm*PoMuc proteins may be serving as targets for some *Bg*FREPs, and certainly *Bg*FREP2 (Moné *et al.* 2010). *Bg*FREPs 2, 3, and 12 are capable of binding soluble LTP (Galinier *et al.* 2017). However, the mechanism underpinning these complex formations and the targets for other *Bg*FREPs have yet to be explored, as has the implications of diversification on target recognition.

Transcript abundance of many *Bg*FREPs increases in response to infection of *B. glabrata* with *E. paraensei* or *S. mansoni* (Galinier *et al.* 2017; Adema *et al.* 2010; Hanington *et al.* 2010b), eliciting expression profiles reflective of the immune challenge (Adema 2015; Gordy *et al.* 2015). *Bg*FREPs display differential expression profiles in response to bacteria (Gram-negative or positive), but wounding does not appear to alter transcript expression (Adema *et al.* 2010). Increased expression of *Bg*FREP3 is linked to the resistant phenotype displayed by the *S.*

mansoni-resistant BS-90 strain of *B. glabrata* (Lockyer *et al.* 2012; Hanington *et al.* 2010a; Hanington *et al.* 2012; Lockyer *et al.* 2008). Specifically, *Bg*FREP3 expression was elevated compared to controls in three different types of resistance in *B. glabrata* to infection with *S. mansoni* or *E. paraensei*: BS-90 compared to M-line strains of *B. glabrata,* adult compared to juvenile *B. glabrata* M-line*,* and *B. glabrata* in which innate immune memory was induced against *E. paraensei* (Hanington *et al.* 2010a; Hanington *et al.* 2012).

RNAi-mediated knockdown of *Bg*FREP3 in adult *B. glabrata* M-line resulted in 30% of the normally resistant snails becoming infected with *E. paraensei* (Hanington *et al.* 2010a). In addition, knockdown of *Bg*FREP3 in *B. glabrata* BS-90*,* which is typically 100% refractory to *S. mansoni* challenge (PR-1 or NMRI strain), resulted in patent infections in 21% of the snails, suggesting that *Bg*FREP3 plays an important and broad role in resistance to digenean trematodes (Hanington *et al.* 2012). Increased *Bg*FREP transcript expression during secondary immune response of *B. glabrata* to *S. mansoni* suggests that *Bg*FREPs may also be involved in innate immune memory (Pinaud *et al.* 2016). Knockdown of *Bg*FREP 2, 3 and 4 was found to reduce the innate immune memory phenotype by 15%, rendering primed snails more susceptible to *S. mansoni* infection (Pinaud *et al.* 2016). Whether the diversification of *Bg*FREPs influences innate immune memory has yet to be investigated.

2.5 Comparison of molluscan immune responses with those of other invertebrates

Having discussed the diverse immune mechanisms in gastropods and invertebrates in general, here, I summarize the commonalities between molluscs and other invertebrates and highlight those immune responses that are prominently utilized in molluscs. There are several

commonalities between molluscs and other invertebrates in terms of cellular and humoral effector responses. Molluscan immune cells perform the major functions of phagocytosis (Ataev *et al.* 2016; Cueto *et al.* 2015; Hanington *et al.* 2010a), encapsulation (Nacif-Pimenta *et al.* 2012; Loker *et al.* 1982), and mediation of cytotoxic reactions through production of ROS and degranulation of hydrolytic enzymes (Cueto *et al.* 2015; Bayne 2009; Hahn *et al.* 2001a; Hahn *et al.* 2001b). Just as in other invertebrates, molluscs also deploy soluble mediators such as antimicrobial peptides (Tincu and Taylor 2004; Charlet *et al.* 1996) and factors with opsonic properties such as TEPs (Wu *et al.* 2017; Moné *et al.* 2010) and FREPs (Hanington *et al.* 2010a).

Melanization of pathogens is also used as a defence response in molluscs, both as an intracellular and extracellular effector mechanism. In the New Zealand rock oyster *Saccostrea glomerata*, melanisation occurs during encapsulation of fungal hyphae both in the extracelluar matrix and inside granulocytes that form the capsules (Aladaileh *et al.* 2007). Phenoloxidase activity has been reported in the haemolymph of several molluscs including *B. glabrata* and *B. alexandrina* (Le Clec'h *et al.* 2016; Aladaileh *et al.* 2007; Bai *et al.* 1997; Renwrantz *et al.* 1996). In *B. glabrata*, the role of phenoloxidase in immunity remains poorly understood. The enzyme is detectable in the haemolymph and its activity decreases with *S. mansoni* infection (Le Clec'h *et al.* 2016) but there is no evidence to suggest that it plays any immune role. Phenoloxidase has been shown to be involved in egg formation, and parasitic castration of the snail usually associates with a decrease of its activity in the albumen gland (Bai *et al.* 1997). Tyrosinase and laccase, which are other key enzymes of the melanotic pathway, have also been identified in molluscs, where melanisation is also involved in biomineralization and shell formation (reviewed in: Allam and Raftos 2015).

RNAi is certainly functional in mollusc since it is routinely applied in experimental gene knockdown studies (Pila *et al.* 2016b; Pinaud *et al.* 2016; Hanington *et al.* 2012). However, its importance as an antiviral defence in molluscs is not well characterized partly because we have no idea of the extent of challenge posed by viruses in many of these organisms (Loker 2010; Loker *et al.* 2004). Chen *et al*. (2014a) recently studied RNAi-mediated antiviral immunity in the scallop *Chlamys farreri* against acute viral necrosis virus (AVNV). Their findings suggest that *C. farreri* miRNA may target genes involved in a broad range of biological activities, including immunity and stress, for post-transcriptional regulation (Chen *et al.* 2014a). Nevertheless, there is evidence of many other antiviral immune components in various molluscs including those involved in recognition, signaling and effector functions (Green *et al.* 2015; Zhang *et al.* 2014; Green and Montagnani 2013).

Coagulation is not recognized as playing a prominent role in the immune response of molluscs. Haemolymph of all molluscs examined so far do not spontaneously form macroscopically visible clots (Martin *et al.* 2007; Matricon-Gondran and Letocart 1999a; Harris and Markl 1992). A process for haemolymph coagulation has yet to be described and no clotting factors have been identified in any mollusc. However, tubular, helical filaments have been observed to form during bleeding or pathogen infection in the keyhole limpet, *Megathura crenulata* (Harris and Markl 1992) and *B. glabrata* (Matricon-Gondran and Letocart 1999a) respectively. These filaments are thought to play some immune role in *B. glabrata* because, infection with *E. caproni* induces their rapid appearance. Also, haemocytes can attach and temporarily become sedentary in the bundles of parallel filaments (Matricon-Gondran and Letocart 1999a). Transglutaminase have been purified from tissue and haemocytes of some

molluscs (Nozawa *et al.* 2005; Nozawa *et al.* 2001) but they appear to function in wound healing.

2.6 Determinants of snail-schistosome compatibility

As introduced in chapter 1, not every encounter between a schistosome and a snail species deemed compatible results in the infection of the latter (Allan *et al.* 2013). Understanding the basis of this compatibility polymorphism is expected to be beneficial in the design of novel control strategies for diseases caused by snail-transmitted parasites (Lockyer *et al.* 2004b). Most studies of snail-schistosome interactions utilize the *B. glabrata–S. mansoni* model (Coustau *et al.* 2015). The snail's immune response is a major determinant of *S. mansoni* infection success based on studies of this model as already discussed. However, it also acts in concert with other factors that I will discuss next, namely, host and parasite genetics and epigenetics, proteomic and transcriptomic regulation, as well as environmental factors (Mitta *et al.* 2017).

2.6.1 Host and parasite genetics and epigenetics

Snail–schistosome or trematode compatibility is a complex, variable and heritable phenomenon (Roger *et al.* 2008b; Goodall *et al.* 2006; Richards 1975b; Richards 1975c). Variations at one or more loci in both host (Tennessen *et al.* 2015; Goodall *et al.* 2006) and parasite (Roger *et al.* 2008a; Roger *et al.* 2008b) genomes have been shown to determine whether a snail–schistosome pair would be compatible. Resistance against schistosomes is known to vary according to strain and age of the snail (Richards 1975b; Richards and Merritt 1972). Past genetic studies with inbred lines of *B. glabrata* showed that about 6 loci, each with multiple alleles are involved in resistance of juvenile snails to schistosomes (Ittiprasert *et al.* 2010; Richards and Merritt 1972); whereas, in resistant adult strains such as BS-90, 10-R2 and

13-16-R1, resistance is due to a single dominant genetic trait that is inherited in a typical Mendelian mode.

Infectivity of the parasite has also been shown to be a heritable trait. One study involving reciprocal and back crosses of two strains of *S. mansoni*, that differed in their infectivity of a strain of *B. glabrata*, found that the infectivity trait of the parasite was genetic and sex-linked, since it was passed to the progeny only in crosses that involved highly infective male but not female miracidia (Richards 1975c). Similar studies by Davies *et al*. (2001) found variations in *S. mansoni* infectivity and virulence in *B. glabrata* that were heritable and stable for over two generations (Davies *et al.* 2001).

These studies have also shown that snail strains that are deemed incompatible for one schistosome strain often remain compatible to other strains. These differences have contributed immensely to the study of immune mechanisms of the snail and parasite infective strategies. To date, several genes and genetic loci including FREPs (Adema *et al.* 1997), sod1 (Goodall *et al.* 2006; Bender *et al.* 2007), a hyperdiverse gene cluster encoding 15 genes (Tennessen *et al.* 2015) and *Sm*PoMucs (Roger *et al.* 2008a; Roger *et al.* 2008b) have been implicated in compatibility polymorphism.

The role of epigenetic mechanisms has also been demonstrated in schistosomes and snails. Despite little sequence differences, transcription levels of *Sm*PoMuc genes are different between compatible (C) and incompatible (IC) strains of *S. mansoni* to a given snail host (Perrin *et al.* 2013). This was found to be due to differential regulation of the acetylation status of histones in the promoter region of *Sm*PoMucs between C and IC strains of *S. mansoni*. Modification of this region by treatment of miracidia with trichostatin-A, a histone deacetylase inhibitor, increased *Sm*PoMucs transcription in IC but not C strains, which also correlated with

the appearance of *Sm*PoMucs combinations only observed in the IC strain (Perrin *et al.* 2013). Parasite-induced epigenetic regulation has also been described in *B. glabrata* (Arican-Goktas *et al.* 2014; Knight *et al.* 2011). The positioning of actin, ferritin and Hsp 70 genes was found to be altered concomitantly with their upregulation, following exposure to *S. mansoni* miracidia (Arican-Goktas *et al.* 2014; Knight *et al.* 2011). This non-random repositioning of genes and transcription differed between susceptible (NMRI) and resistant (BS-90) snail strains. The stress response gene Hsp 70, linked to susceptibility, was upregulated in NMRI but not BS-90 snails whereas, the injury response gene ferritin was repositioned and upregulated in both snails (Arican-Goktas *et al.* 2014).

2.6.2 Proteomic and transcriptomic regulation

Several proteins and genes that have been associated with snail–schistosome compatibility are present in both compatible and incompatible strains but for various reasons are differentially regulated (Hanington *et al.* 2012; Lockyer *et al.* 2012; Moné *et al.* 2011; Hanington *et al.* 2010a; Lockyer *et al.* 2008; Roger *et al.* 2008b; Mitta *et al.* 2005; Vergote *et al.* 2005; Lockyer *et al.* 2004a). In *B. glabrata*, some immune genes probably fail to activate or are only slightly upregulated in susceptible snails following invasion by a miracidium. In contrast, resistant snails express at a very high level these same genes and gene products (Lockyer *et al.* 2012; Lockyer *et al.* 2008; Mitta *et al.* 2005; Vergote *et al.* 2005; Lockyer *et al.* 2004a). For example, expression of sod1 was found to associate with susceptibility/resistance such that resistant (13-16-R1) snail haemocytes express significantly higher sod1 transcripts and superoxide dismutase than susceptible (M-line) snails (Goodall *et al.* 2004). It turned out that this differential expression was due to allelic differences at the polymorphic sod1 locus (Goodall *et*

al. 2006), and that one of the three sod1 alleles (B allele) when present is expressed at significantly higher level than snails lacking the allele (Bender *et al.* 2007). Interestingly, *S. mansoni* antioxidant scavengers display differential transcript expression and enzymatic activity, with higher levels correlating with high infectivity (Moné *et al.* 2011). Another compatibility determinant that is subjected to differential transcriptomic and proteomic regulation is the *Sm*PoMucs previously discussed. These proteins are differentially transcribed between *S. mansoni* strains with a high level of glycosylation (Roger *et al.* 2008a).

2.6.3 Environmental factors

Abiotic factors such as temperature, salinity, turbidity and pH have been shown to influence the outcome of snail exposure to schistosome miracidia. Based on correlations of physicochemical parameters and snail abundance (Leal Neto *et al.* 2013), optimum conditions for *B. glabrata* fall in the range of 23–27 °C (temperature), 6–7 (pH) and 400–500 mg/L (salinity). At salinity greater than 4,500 mg/L, Lwambo and colleagues found that *B. arabica* could not be infected by a Saudi Arabian isolate of *S. mansoni*; whereas, decreasing salinity increased infection rates (Lwambo *et al.* 1987). Sturrock and Upatham (1973) also observed decreased infection rates in the *B. glabrata–S. mansoni* model as salinity increased, and a similar trend for turbidity. In the same study, pH had a curvilinear effect on infection rates, with maximal infection rate at pH 7 and decreased rates at other values (Sturrock and Upatham 1973). However, these early studies did not determine whether and how the environmental parameters affected schistosome infectivity or snail susceptibility. The effect of temperature has been studied more in depth among the environmental determinants of snail–schistosome compatibility (Knight *et al.* 2015; Ittiprasert and Knight 2012; Coelho and Bezerra 2006). When *S. mansoni*-

resistant *B. glabrata* snails (BS-90 strain) were incubated at 32°C for 4 h before parasite challenge, their resistant phenotype was reversed. All of the challenged snails shed cercariae by week 7 post-challenge while none of control snails (cultured at ambient temperature, 25°C) shed cercariae (Ittiprasert and Knight 2012). Similar results were obtained in another study in which 89% of *B. glabrata* snails incubated for 12 h at 30°C became infected with *S. mansoni*. In comparison, only 1.3% of 15°C-treated snails became infected (Coelho and Bezerra 2006).

Temperature effect in the *B. glabrata–S. mansoni* model has been demonstrated to be mediated by some stress response proteins, particularly Hsp 70, Hsp 90 and reverse transcriptase domain of the retrotransposon *nimbus*, but the exact mechanism is not well understood. Genes encoding these proteins were found to be upregulated in susceptible snails upon exposure to *S. mansoni* (Ittiprasert and Knight 2012; Ittiprasert *et al.* 2009), and in snails experimentally stressed through heat pulse (Knight *et al.* 2015; Ittiprasert and Knight 2012). Resistant snails whose phenotype could be reversed with heat pulsing retained their phenotype if treated with geldanamycin, the Hsp 90-specific inhibitor, after heat pulse. Conversely, susceptible snails treated with geldanamycin before parasite exposure remained uninfected (Ittiprasert and Knight 2012).

Biotic factors can also impact snail–schistosome interactions. Both snail resistance and susceptibility to a trematode have been shown to be influenced by the presence of, or prior exposure to other trematodes. These outcomes often result from interference or suppression of the snail's immune responses by one trematode that then facilitate entry and establishment of another or antagonism between the parasites that prevents one from being able to infect the host. This has been demonstrated for both schistosomes and echinostomes. Exposure of a snail to one trematode can lead to either an increase of what is termed acquired susceptibility (Hanington *et*

al. 2012; Sousa 1992; Southgate *et al.* 1989; Lie *et al.* 1977a; Lie *et al.* 1977b) or acquired resistance to another (Sire *et al.* 1998; Lie *et al.* 1983; Lie *et al.* 1975a; Lie and Heyneman 1975). These interactions could be among determinants of the range of snail hosts used by some trematodes.

2.7 Importance of studying gastropod immunobiology

As reviewed at the beginning of this chapter, the diversity and functional capabilities of invertebrate immune systems are increasingly being realized. Several novel factors have been identified but in most cases their precise mechanism of action, or the relevance of their observed features, remain to be determined. From this standpoint of evolutionary immunology, knowledge gained from studying gastropod immunobiology will enrich our understanding of invertebrate immune mechanisms. Moreover, literature on immunity of the Metazoa is limited and dominated by two superphyla – Ecdysozoa (represented by fruitflies and nematodes) and Deuterostomia (represented by vertebrates) compared to Lophotrochozoa to which all molluscs and trematodes belong (reviewed in: Zhang and Coultas 2011). Lophotrochozoan genome sequences account for only 3.2% of the 822 genome sequences of Metazoa available at the time of writing this thesis (www.ncbi.nlm.nih.gov/genome/).

With this added knowledge, we could enhance our ability to protect molluscs and other invertebrates that are economically important sources of food from diseases, by understanding how they respond to pathogens (Hooper *et al.* 2007) or environmental conditions (Moore *et al.* 2009; Travers *et al.* 2009). Species that may be endangered or threatened (Cunningham and Daszak 1998) could be protected and better strategies designed to eliminate invasive ones (Zbikowski and Zbikowska 2009). Gastropod immunobiology has potential benefits in many

other ways, including identifying compounds with biomedical relevance, study models of pollutants/biomarkers of immune function stress, impact of climate change as well as interface between immunity and behaviour (reviewed in: Loker 2010).

Understanding the immunological determinants of snail-trematode compatibility is relevant from the perspective of curtailing diseases caused by snail-transmitted human and animal parasites because it can inform the design of strategies to block parasite transmission at the snail stage (Lockyer *et al.* 2004b). Discussions on how this can be applied in novel schistosomiasis control approaches were introduced in chapter one, and would be further discussed in chapter 6 in the context of snail immune factors characterized in this thesis.

Chapter 3

Materials and Methods

3.1 Snails

Two strains of *Biomphalaria glabrata* snails were used for the studies described in this thesis, unless otherwise stated. The BS-90 strain is often resistant to infection by many strains of *Schistosoma mansoni* (Paraense and Correa 1963; Richards and Merritt 1972), while the M-line strain is susceptible (Newton 1955; Cooper *et al.* 1994). Both snail populations were maintained in colonies in the laboratory at the University of Alberta. Snails were maintained in aerated artificial spring water (ASW, Ulmer 1970) at 23–25 °C, alternating12-h light and dark cycles, and fed red-leaf lettuce as needed.

3.2 Maintenance of *S. mansoni* lifecycle

The lifecycles of NMRI and PR-1 strains of *S. mansoni* were maintained in the laboratory by cycling between Balb/c or Swiss Webster mice and *B. glabrata* M-line snails. Both strains of mouse are competent hosts for the *S. mansoni* strains used. M-line snails were infected by exposure to 10–20 *S. mansoni* miracidia in wells of 12-well plates containing ASW. Miracidia for these infections were hatched from the livers of 6–8 weeks infected mice. After 24 h exposure, snails were returned to tanks of ASW and maintained as described above. Exposed snails were assessed weekly beginning from week 4 post-exposure for cercariae shedding. Snails were put in the individual wells of 12-well plates containing ASW and exposed to artificial (laboratory) light for 6 h, following which the wells were examined under a stereo microscope for any emerged cercariae.

Snails with confirmed patent infections were put together in 1 L of ASW and exposed to artificial light overnight. *Schistosoma mansoni* cercariae were collected and used to infect mice. Prior to exposure to cercariae, the mice were put in 'ankle'-deep warm water (37 °C) for 20 m to stimulate defaecation and urination. Faeces and urine contain substances that inhibit cercariae, resulting in poor infection. Mice were then transferred to clean 'ankle'-deep ASW containing cercariae that would remain free of faecal material for the duration of infection (20 minutes). This was repeated 2 more times, allowing mice 10-m rests between exposures. Infections took place under sterile conditions in the Animal Facility at Department of Biological Sciences, University of Alberta. At 6–8 weeks post-exposure, mice were euthanized, livers removed and immediately homogenized to release *S. mansoni* eggs. These were hatched to release miracidia for another round of snail infections.

3.3 Experimental snail infections

3.3.1 Bacterial challenge

Aeromonas salmonicida and *Escherichia coli* were grown in tryptic soy broth (1.7% bacto tryptone, 0.3% bacto soytone, 0.25% dextrose, 0.5% NaCl and 0.25% K_2HPO_4; pH 7.3) and Luria-Bertani medium (1% bacto tryptone, 0.5% yeast extract and 1% NaCl; pH 7.0) respectively. Bacteria were cultured to an optical density of 1 at 600 nM (OD_{600}), equivalent to 1.24 X 10^9 cells/mL for *A. salmonicida* and 1.20 X 10^{10} cells/mL for *E. coli*. One mL of culture was spun down at 400 X g for 5 m, washed twice in 1 mL of sterile snail saline (SSS, Table 3.3, Adema *et al.* 1993), and resuspended in 1 mL of SSS. Age-matched *B. glabrata* snails (~8 mm shell diameter) were injected with 20 µL of the bacterial suspension, while control snails received 20 µL of SSS buffer. Snails were returned to tanks and four snails each from the

experimental and control groups were collected at 1, 3, 6 and 12 h, and 1 and 2 days post-injection. Non-injected snails were collected immediately prior to injections and used as time 0 h controls. Total RNA was isolated from whole snails, converted into cDNA and used in quantitative real-time polymerase chain reaction (qRT-PCR), measuring *Bg*TLR transcript expression as described below.

3.3.2 *S. mansoni* challenge

Studies involving *S. mansoni* challenge consisted of age-matched *B. glabrata* snails (~8 mm shell diameter) that were challenged with five *S. mansoni* miracidia over a 24-h period in individual wells of 12-well plates containing ASW. Following a 24-h challenge, snails were transferred into tanks of ASW for the remainder of the study unless otherwise stated.

3.4 Generation of *Schistosoma mansoni* larval transformation products

3.4.1 Collection and hatching of *S. mansoni* eggs

Schistosoma mansoni larval transformation products (LTP), also known as excretory–secretory products (ESP), were generated from *in vitro*-transformed miracidia (Wu *et al.* 2009; Yoshino and Laursen 1995), which were hatched from eggs collected from the livers of 6–8 weeks infected laboratory mice. Dissected livers were washed for 10 m in 1.2 % NaCl containing penicillin (100 U/mL) and streptomycin sulfate (100 µg/mL), and homogenized in 200 mL of the same solution using an Omni Mixer Homogenizer (Model # 17105) in three 20 s pulses. Homogenized livers were transferred to a 250-mL beaker and filled with 1.2% NaCl. After 10 m, approximately 125 mL was removed and replaced with more NaCl solution. This washing step was repeated three times. After the third wash, the bottom sample (containing *S. mansoni* eggs) was transferred to a 2 L conical flask, wrapped with aluminium foil except for the top 5 cm of

the neck to reduce exposure to light. The flask was filled with ASW and incubated for 1 h at room temperature. A desk lamp placed on the exposed side encouraged the migration and concentration of hatched miracidia to the flask top.

3.4.2 Miracidia collection and *in vitro* transformation

Emerging miracidia were transferred with a 1 mL Pasteur pipette into 50 mL tubes and washed three times to remove tissue debris by diluting with equal volume of ASW and recollecting miracidia into new tubes. The miracidia were immobilized by a 15-m incubation on ice then centrifuged at 200 X g for 15 m. These were resuspended in Chernin's balanced salt solution (CBSS, Chernin 1963) containing penicillin (100 U/mL) and streptomycin sulfate (100 µg/mL) and 1 g/L each of trehalose and glucose (CBSS+, Wu *et al.* 2009; Yoshino and Laursen 1995). Miracidia were deposited into the wells of a 24-well plate in 1 mL aliquots. The plate was sealed with parafilm and incubated at 26 °C for 24–48 h during which transformation to mother sporocysts took place. Transformation was confirmed by the shedding of the miracidia ciliated plates and pulsating movement of the sporocysts.

3.4.3 Collection of larval transformation products

Following transformation to mother sporocysts, the CBSS containing LTP was removed, pooled and passed through 0.22 µm filters. The sample was concentrated using Pierce® concentrators 9K MWCO/7 mL (ThermoFisher Scientific) and quantified with the Qubit® protein assay kit (Life Technologies), aliquoted and stored at – 80 °C until needed.

3.5 Cell lines

3.5.1 *Biomphalaria glabrata* embryonic (Bge) cell line

The *B. glabrata* embryonic (Bge) cell line (Hansen 1976) (American Type Culture Collection NR-21959) is the only existing molluscan cell line. It shares behavioural and molecular attributes with *B. glabrata* haemocytes such as ability to recognize and phagocytose or encapsulate foreign material including trematode (Coustau *et al.* 2003; Yoshino *et al.* 1999). Cells were cultured in Corning™ 25 cm^2 flasks with plug seal caps at 26 °C in complete Bge medium (Table 3.1) as described by Odoemelam *et al*. (2009) and passaged once every 2 weeks by firm tapping or use of cell scraper to release the cells and reseeding at 1:10 dilution.

3.5.2 *Sf9* cell line

Sf9 (Life Technologies) is an insect cell line derived from the moth, *Spodoptera frugiperda*, and is widely used in the production of recombinant proteins. *Sf9* cells were cultured in Corning™ 25 cm^2 flasks with plug seal caps at 26 °C in the Sf-900 III SFM medium (Life Technologies) and passaged once every week at 1:20 dilution. Stably transfected cells, expressing *B. glabrata* progranulin (*Bg*GRN) or its cleavage fragments, were maintained in the same medium containing the selective antibiotic blasticidin (ThermoFisher Scientific) at 10 µg/mL, following selection at 50 µg/mL.

3.5.3 AD293 cell line

AD293 (Stratagene) is a human embryonic kidney cell line derived from the parental HEK293 cell line (Graham *et al.* 1977). These cells have been selected for improved adherence to tissue culture dishes, leading to flattened morphology, larger surface area and higher transfection efficiency. Cells were cultured in Corning™ 25 cm^2 flasks with 0.2 µm vent caps at

37 °C in a CO_2 incubator in Dulbecco's modified Eagle's medium (DMEM, Life Technologies), supplemented with 10% fetal bovine serum (FBS, Corning). Cells transfected with TLR plasmid constructs were cultured in the same medium containing the selective antibiotic geneticin (G418, ThermoFisher Scientific) at 1000 µg/mL for selection of positive transfectants and 500 µg/mL for routine culture.

Cells were passaged once a week at 1:10 as follows: the old medium was removed, and the cells washed once with 1x PBS (2.58 mM NaH_2PO_4, 7.68 Na_2HPO_4, 150 mM NaCl, pH7.4), then trypsinized for 1–3 m with 1 mL 0.25% trypsin-EDTA solution. Following cell dislodgement, a volume of fresh medium equivalent to the old medium was added. One part of this was transferred to 9 parts of fresh medium containing FBS at the final concentration of 10%.

3.6 Identification of *B. glabrata* progranulin (*Bg*GRN) and Toll-like receptor (*Bg*TLR)

B. glabrata pro-GRN (GenBank accession number HQ661843.1) and *Bg*TLR (JX014259.1) were identified in past studies in our laboratory as part of a *B. glabrata* protein screen. Plasma and haemocyte surface protein abundance was compared between M-line and BS-90 snails using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS analysis) as previously described (Pila *et al.* 2016a; Pila *et al.* 2016b). Briefly, differential abundances of the plasma and haemocyte surface proteins between M-line and BS-90 snails were measured as a ratio of the respective labels associated with each analyzed spectral peak. Peptides identified during analysis were initially compared to a custom Mascot database comprised of known proteins and predicted transcript translations from *B. glabrata*, *Lottia gigantica*, and *Aplysia californica*. If no matches were found using this approach, peptide fragments were used

in a BlastP search of the GenBank database in order to find the match with the highest amino acid identity.

Peptide fragments possessing the canonical cysteine-repeat pattern of GRN proteins were identified in high abundance in BS-90 snail haemolymph following *S. mansoni* challenge. The peptide fragments were used to identify the full pro-*Bg*GRN transcript sequence from the unannotated *B. glabrata* genome. Similarly, peptides relevant to *Bg*TLR, particularly the TIR domain, identified in high abundance in BS-90 snails were used to design primers for rapid amplification of cDNA ends (RACE) PCR, which successfully amplified the complete *Bg*TLR transcript.

3.7 Sequence annotations, alignments, secondary structure prediction and visualization

3.7.1 Annotation of *Bg*GRN signal peptide and cleavage sites

Using the GRN-like amino acid sequences for *B. glabrata* (*Bg*GRN, ADX33287.1), *S. mansoni* (CCD75905.1), *Schistosoma haematobium* (KGB36375.1), and *Schistosoma japonicum* (CAX73857.1), as acquired from GenBank [National Center for Biotechnology Information (NCBI)], three different programs/methods [SignalP 4.1 (Petersen *et al.* 2011), TargetP 1.1 (Emanuelsson *et al.* 2000; Nielsen *et al.* 1997), and Phobius (Kall *et al.* 2007)] were used to predict and annotate the signal peptide and cleavage sites, as recommended by UniProt (www.uniprot.org/help/signal). At least two of the methods had to agree for the prediction to be deemed correct. In this case, all three of the methods used agreed for every sequence. The Pfam (Finn *et al.* 2014) sequence search tool was used to annotate the granulin domains for each of the sequences.

3.7.2 Annotation of *Bg*TLR domains

The three programs/methods described above for *Bg*GRN were also used to analyze *Bg*TLR sequence for signal peptide and cleavage sites. The leucine-rich repeat (LRR) and the Toll/interleukin-1 receptor (TIR) domains were annotated using the LRRfinder (Offord and Werling 2013) and ScanProsite (de Castro *et al.* 2006) tools.

3.7.3 Sequence alignments

The GRN amino acid sequences for *B. glabrata*, *S. mansoni*, *S. haematobium*, and *S. japonicum,* as previously listed, were imported into the CLC Genomics Workbench 7.5.1 (www.clcbio.com). The sequences were aligned by using the CLC proprietary alignment algorithm with a gap open cost of 10, gap extension cost of 1, end gap cost "as any other," and using the very accurate (slow) setting. From there, the alignment was visually inspected for obvious inaccuracies, modified if need be, and then annotated using the same software. To determine the amino acid conservation among granulin domains within the *B. glabrata* sequence, each of the four domains were extracted as separate sequences and aligned using the same methods as described in the previous paragraph.

Determination of *Bg*TLR sequence similarity with other known TLRs from molluscs, arthropods and mammals was accomplished by using BlastP analyses (Altschul *et al.* 2005).

3.7.4 Secondary structure predictions and visualization

The *Bg*GRN amino acid sequence was submitted to the Robetta Server (robetta.bakerlab.org) (Kim *et al.* 2004) for homology modeling. The Ginzu domain prediction resulted in five domains, all using the reference parent Human granulin A [Protein Data Bank (PDB) ID code 2JYE]. Modeling of the first predicted domain (confidence = 0.45) resulted in

five predicted structures. The first structure was used for comparison purposes to human granulin A (PDB ID code 2JYE) and a partial carp granulin (PDB ID code 1QGM), with PDB files extracted from the Research Collaboratory for Structural Bioinformatics Protein Databank (www.rcsb.org) (Berman *et al.* 2000). The PDB structural information for all three proteins was loaded into PyMOL (PyMOL Molecular Graphics System, version 1.5.0.4; Schrödinger) for visualization and superimposition.

3.8 qRT-PCR assessment of transcript expression

3.8.1 Total RNA extraction from snails

Total RNA was extracted from whole snails using TRIzol® reagent in conjunction with the PureLink® RNA mini kit (Life Technologies). Snails were thoroughly cleaned with 70% ethanol-wetted Kimwipes and then immediately transferred to 1.5 mL tubes containing 1 mL TRIzol® reagent. The snails were ground with disposable pestles and incubated for 5 m at room temperature to allow complete dissociation of nucleoprotein complexes. Following the incubation, chloroform extraction, RNA binding, washing, on-column DNase treatment and elution were performed according the manufacturer's instructions (Life Technologies). Total RNA was eluted in 100 µL nuclease-free water.

3.8.2 First strand cDNA synthesis

RNA concentration was determined using the NanoVue spectrophotometer, and 1 µg was used for first-strand cDNA synthesis, combined with 4 µL of 5x qScript cDNA SuperMix (Quanta Biosciences), which contained a blend of oligo (dT) and random primers and optimized concentrations of $MgCl_2$, dNTPs, RNase inhibitor and qScript reverse transcriptase. The total volume of reaction was 20 µL. Reactions were assembled in PCR tubes and incubated at 25 °C

for 5 m, followed by 30 m incubation at 42 °C. The reaction was terminated by incubation at 85 °C for 5 m. The cDNA was diluted fivefold, and 5 µL was used as template in qRT-PCR.

3.8.3 Assessment of transcript expression

Transcript expression was assessed using qRT-PCR with gene-specific primers (Table 3.2), and the dye-based SYBR Green detection system (PerfeCTa^{TM} SYBR$^{®}$ Green FastMix^{TM}, Low ROX^{TM}; Quanta Biosciences). Primers were used at final concentrations of 0.6 µM in a reaction volume of 25 µL, including 5 µL of the fivefold diluted cDNA as template. *B. glabrata* β-actin gene was used as endogenous control, the transcript expression of which we confirmed to be stable and unaffected by parasite exposure. In experiments involving exposure to *S. mansoni* parasite, snails were confirmed to be *S. mansoni*-positive or -negative using a qRT-PCR assay targeting the parasite glyceraldehyde 3–phosphate dehydrogenase (GAPDH) gene (Boyle *et al.* 2003).

All qRT-PCRs were performed on the ABI 7500 Fast Real-Time PCR system (Applied Biosystems) using the following thermocycling conditions: initial hold at 95 °C for 10 m, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 m, with data collection every cycle. Specificity for the qRT-PCR amplicons was confirmed by continuous melt curve analysis. Relative expression of the target gene was calculated using the delta-delta cycle threshold ($\Delta\Delta C_t$) method. In order to generate values for relative expression of the target gene normalized to time 0 h, the cycle threshold (C_t) values for β-actin were subtracted from the target gene C_t for the same sample to generate ΔC_t values for all samples. Then mean ΔC_t for time 0 h was subtracted from those of the other time points of the same snail strain and treatment to generate $\Delta\Delta C_t$

values. Relative quantification (RQ) or fold change was derived from the $\Delta\Delta C_t$ values using the formula $2^{-\Delta\Delta Ct}$.

3.9 Recombinant *Bg*GRN and cleavage fragments synthesis and purification

3.9.1 Amplification of *Bg*GRN

The *Bg*GRN coding sequence was amplified with primers shown in Table 3.2 and cloned into the expression vector using the Gateway® cloning system according to the manufacturer's instructions (Life Technologies). The coding region was amplified with Phusion high-fidelity DNA polymerase in a 50-µL reaction consisting of 0.5 µM each of forward and reverse expression primers, 25 µL of 2x Phusion master mix, 0.5 µL (50 ng) of *Bg*GRN synthetic DNA template in pUC57 plasmid (GenScript), and nuclease-free water making up the remaining volume. Cycling parameters consisted of initial denaturation at 98 °C for 2 m, followed by 25 cycles of denaturation for 10 s at 98 °C, primer annealing at 55 °C for 30 s and extension at 72 °C for 1 m. This was followed by a final extension at 72 °C for 10 m.

3.9.2 Cloning of *Bg*GRN into pENTR TOPO-D entry vector

The amplified DNA was separated on 1% agarose gel to confirm size of the expected product. The band containing *Bg*GRN amplicon was excised and DNA purified from it using GeneJet gel extraction kit (ThermoFisher Scientific). *Bg*GRN DNA concentration and A260/A280 ratio were determined with NanoVue spectrophotometer. *Bg*GRN was cloned into the pENTR/D-TOPO vector by combining *Bg*GRN amplicon and pENTR vector in a 1:1 molar ratio in a total reaction volume of 6 µL, including salt solution and nuclease-free water (ThermoFisher Scientific). Following a 10-m incubation at room temperature, 2 µL of the

reaction was used to transform One Shot® TOP10 chemically competent *E. coli* cells (Life Technologies) and selected on LB plates containing kanamycin antibiotic at 50 µg/mL.

3.9.3 Sub-cloning of *Bg*GRN into pIB/V5-His-DEST expression vector

Bacterial colonies from the above transformation reaction were screened in colony PCR amplifications employing both gene- and vector-specific primers. Positive colonies were selected and grown in selective LB medium. Plasmid DNA was isolated from these clones and further verified with PCR. One entry clone was chosen from which the plasmid DNA was sub-cloned into the expression vector pIB/V5-His-DEST in a clonase recombination reaction (ThermoFisher Scientific) to produce the expression clone. The reaction was assembled with 75 ng each of pIB/V5-His-DEST vector and pENTR entry clone, Tris-EDTA buffer to a volume of 4 µL and 1 µL LR Clonase™ II enzyme. The reaction was incubated at 25 °C for 2.5 h, then 0.5 µL of proteinase K solution (2 µg/µL) was added, followed by incubation at 37 °C for 10 m to terminate the reaction. Two microliters of the clonase reaction was used to transform One Shot® TOP10 chemically competent *E. coli* cells. Transformed *E. coli* was grown on LB medium containing carbenicillin antibiotic at 100 µg/mL. Screening and selection of positive expression clones was done as described above for pENTR entry clones. The identity of *Bg*GRN was sequence-verified after cloning in the entry and expression vectors.

3.9.4 Transfection of *Bg*GRN expression vector into *Sf9* cells

Plasmid DNA was extracted from the bacterial clones containing the expression vector and transfected into the *Sf9* insect cell line using Cellfectin® II reagent (Life Technologies). Cells growing at ~ 70% confluency were gently scraped off the flask into the culture medium and seeded at 2 mL per/well in a 6-well plate. One µg of *Bg*GRN DNA was diluted in 100 µL of

unsupplemented Grace's insect medium (ThermoFisher Scientific) and incubated at room temperature for 25 m. Similarly, 8 µL of Cellfectin® II reagent was added to 100 µL of Grace's medium and incubated as above. Following 25 m incubation, diluted *Bg*GRN and Cellfectin reagent were combined and incubated for another 15 m at room temperature, then diluted with 800 µL Grace's medium. Cells, previously seeded in a 6-well plate, were washed with 2 mL of Grace's medium, taking care not to dislodge the cell monolayer. The *Bg*GRN-Cellfectin mixture was added dropwise to the cell monolayer, after which the plate was sealed with parafilm and incubated at 27 °C for 5 h. Then, Grace's medium was removed and replaced with 3 mL of *Sf9* medium. The plate was sealed and incubated for another 72 h at 27 °C.

3.9.5 Selection of *Sf9* cells stably expressing *Bg*GRN

Following 72 h of incubation at 27 °C, cells (~3 mL) were transferred from the 6-well plate into a T-25 tissue culture flask containing 3 mL of fresh *Sf9* medium. After a 3 h incubation and cell attachment, the entire medium was replaced with 6 mL of fresh *Sf9* medium containing blasticidin (antibiotic) at 50 µg/mL final concentration. Four rounds of antibiotic selection were carried out by replacing the medium every 3–4 days. Cells in the negative control sample (transfected with reagent not containing plasmid) died off completely during these rounds of selection. Following the fourth selection, the cells were allowed to grow to confluency with the antibiotic concentration reduced to 10 µg/mL.

3.9.6 Detection of recombinant *Bg*GRN

Expression of *Bg*GRN was confirmed by Western blot using antibodies against the V5 and 6x histidine tags at the C-terminal of the recombinant protein. One mL of confluent, stable transfectant culture medium was centrifuged at 400 x g for 5 m. Both the cell pellet and 40 µL of

culture supernatant were analyzed. The cell pellet was lysed with 40 µL of *Sf9* cell lysis buffer (50 mM Tris, pH 7.8, 150 mM NaCl, 1% IGEPAL CA-630). Forty µL of 2x Laemmli protein loading buffer was added to both culture medium and cell lysates. Samples were heated at 95 °C for 10 m, separated on SDS-PAGE and detected as described below in Western blots.

3.9.7 Scale up and purification of recombinant *Bg*GRN

Sf9 cells stably expressing *Bg*GRN were selected and maintained in a medium containing blasticidin at 10 µg/mL. Cultures were scaled up in 1,000 mL Corning roller bottles, and the medium containing secreted *Bg*GRN was filtered through 0.45-µm pore filters. Recombinant *Bg*GRN protein was purified from the culture medium by using a combination of HisTrap™ HP columns coupled on the AKTA Pure fast protein liquid chromatography machine (GE Healthcare) and HisPur™ Ni-NTA magnetic beads (ThermoFisher Scientific) according to the manufacturer's instructions. Purified *Bg*GRN was dialyzed against 1x PBS buffer twice for 2 h each, and then once overnight using Slide-A-Lyzer dialysis kit (Thermo Scientific).

3.9.8 Generation of recombinant *Bg*GRN cleavage products

All four granulin domains of *Bg*GRN (labeled from the amino terminal as D1, D2, D3 and D4) were amplified individually using the primers presented in Table 3.2. In addition, all possible contiguous GRN-domain combinations were amplified using combinations of the same primers. These include: D1–D2 (primers 5 and 8), D2–D3 (primers 7 and 10), D3–D4 (primers 9 and 12), D1–D2–D3 (primers 5 and 10) and D2–D3–D4 (primers 7 and 12). The identity of all amplicons was sequence-verified after cloning in the entry and expression vectors. Cloning into the shuttle and expression vectors, transfection of *Sf9* cells and Western blot detections were all done using the same procedures as described above for *Bg*GRN.

3.10 Generation of TLR reporter constructs

3.10.1 Amplification of TLRs, LRR and TIR domains

Amplifications of complete *Bg*TLR (primers 15 and 16), human TLR2 (primers 24 and 25), *Bg*TLR LRR (primers 17 and 18) and TIR (primers 19 and 20) were accomplished using ~ 300 ng of DNA template. Forward and reverse primers were modified by adding nucleotides containing restriction sites for the enzymes XmaI and SalI respectively. Reaction assembly and PCR cycling parameters were the same as described above for the amplification of *Bg*GRN.

3.10.2 TLR domain swapping

In addition to the above amplicons, two constructs were made that hybridized the LRR or TIR domains of *Bg*TLR to TIR or LRR domains of human TLR2. Fusion of the LRR domain of *Bg*TLR to human TLR2 TIR domain was accomplished using the overlap extension PCR technique, which involved two rounds of PCR. In the first round, the domains were amplified in separate reactions using primers 15 and 21 (*Bg*TLR LRR domain), and primers 23 and 25 (human TLR2 TIR domain). Primer 23 was designed such that the first 15 bp (5′ end) was the same as the last 15 bp at the 3′ of the LRR domain of *Bg*TLR. In other words, primer 23 was a chimeric, overlap extension primer in which the first 15 bp corresponded to the last 15 bp of the sense strand of *Bg*TLR LRR domain, and the rest of the nucleotides corresponded to the sense strand of transmembrane plus TIR regions of human TLR2. These products were separated on agarose gel and the DNA purified as described above. In the second round of PCR, 100 ng of each amplicon were combined and used as template with primers 15 and 25 to create a fusion construct. Fusion of human TLR2 LRR to *Bg*TLR TIR domains followed the same procedure, using primers 24 and 26 (TLR2 LRR domain), and primers 22 and 16 (*Bg*TLR TIR domain) in

the first round of PCR, and primers 24 and 16 in the second. The identity of all amplicons and integrity of cloning were verified through sequencing and restriction digests (with XmaI and SalI) of synthesized constructs after cloning into the pDisplay vector.

3.10.3 Cloning of TLR amplicons into the pDisplay vector

All amplicons described above were cloned into the XmaI/SmaI and SalI sites of pDisplay, a mammalian expression vector (ThermoFisher Scientific) that allows the expression of recombinant proteins at the cell surface fused to hemagglutinin A and *myc* epitopes (Chesnut *et al.* 1996). Individual amplicons and the pDisplay vector were first digested using XmaI and SalI-HF restriction enzymes in double digest reactions consisting of 2 µg DNA as template, 10 U of each enzyme, 6 µL of the supplied 10x CutSmart buffer and milliQ water to 60 µL total volume. Reactions were incubated at 37 °C overnight, followed by inactivation of enzymes at 65 °C for 20 m. The GeneJet gel extraction kit was used to recover the digested DNA for cloning.

Digested amplicons and the pDisplay vector were ligated using the T4 DNA ligase (ThermoFisher Scientific). Ligation reactions used 20 ng vector DNA, 3:1 (insert:vector) molar ratios and assembled in 1.5 mL tubes in total reaction volumes of 20 µL. These were incubated at room temperature in water containing some ice, allowing the temperature to slowly rise to the room temperature overnight. Reactions were terminated by adding 1 µL of 0.5 M EDTA and then diluted 5-fold with milliQ water. Two µL of the diluted reaction was used to transform One Shot® TOP10 chemically competent *E. coli* as described above for *Bg*GRN. Colony PCR was used to screen for positive clones using primers targeting both vector- and insert-specific regions. Plasmid DNA was isolated from positive clones and the integrity of the insert was verified through XmaI/SalI double digests and sequencing.

3.10.4 Transfection of TLR constructs into AD293 cells and selection of stable transfectants

AD293 cells were seeded in the wells of a 24-well plate (100,000 cells/well) the day prior to transfections. On the day of transfections, the medium was replaced with fresh medium. Transfections were done using 2 µL of TurboFect™ transfection reagent (ThermoFisher Scientific) and 2 µg of plasmid DNA. The DNA was diluted in 100 µL of DMEM, following which the transfection reagent was thoroughly mixed and added. After a 20-m incubation at room temperature, the entire mixture was added dropwise to the cells. The plate was wrapped in parafilm and incubated at 37 °C in a CO_2 incubator for 48 h.

Transfected cells were trypsinized with one-tenth of the cells was used to seed a well of 6-well plate in 3 mL complete medium. After adhering overnight, G418 antibiotic was added at the final concentration of 1,000 µg/mL. This medium was replaced every 3–4 days with fresh medium containing antibiotic such that cells were grown in selective medium continuously for one month.

3.10.5 Detection of recombinant TLR/TLR domain proteins

Confluent AD293 transfectants growing in T-25 flasks were harvested and the protein purified using Pierce™ HA-tag magnetic IP/Co-IP kit (ThermoFisher Scientific) according to the manufacturer's instruction. The cells were lysed with 500 µL of the provided lysis buffer, purified and eluted in 100 µL of elution buffer, followed by the addition of 15 µL of the neutralization buffer. Twenty µL of the provided 5x protein loading buffer was combined with 77.5 µL of the eluate and 2.5 µL of β-mercaptoethanol and used for detection on Western blots as described below.

3.11 Generation and validation of polyclonal antibodies

3.11.1 Antibody generation

A mouse anti-*Bg*GRN polyclonal antibody was generated against the recombinant *Bg*GRN (GenScript). The antibody was affinity-purified using a protein G affinity column (no. 17–0404-01, GE Healthcare) and then further purified against recombinant *Bg*GRN. It was effective for Western blot detection of native *Bg*GRN at concentrations of 1:5,000, and was found to block the proliferation-inducing effects of r*Bg*GRN at concentrations of 1:250.

Three polyclonal antibodies were generated specific for different peptides (15 amino acids long, ~ 4.4 kDa) targeting the extracellular region of *Bg*TLR. The peptide regions were selected based on presumed antigenicity as predicted by the OptimumAntigen^{TM} design tool (GenScript). Peptides were synthesized by GenScript and used to immunize rabbits. Rabbits received two boosts at 2 and 5 weeks following primary immunization. One week after the second boost, rabbits were exsanguinated and the serum IgG was affinity purified using protein A/G affinity column. These were further purified using affinity resin conjugated with the immunization peptides. The anti-*Bg*TLR antibody used in this thesis was effective for Western blot and immunocytochemical detections at concentrations of 1:1,000 and 1:200 respectively.

3.11.2 Antibody validation

The purified antibodies were supplied by GenScript along with synthesized peptides and pre-bleed serum. Specificity of the antibodies was tested against the respective peptides (or the recombinant protein for *Bg*GRN) as well as snail plasma and haemocyte lysates. Dot blots were initially used to determine whether the antibodies recognized their peptides as well as the specificity of the recognition. This was done using 2 µL of each supplied peptide, which was

slowly pipetted in one spot on a nitrocellulose membrane that was divided into a grid with a pencil. The deposited peptides were dried at room temperature for about 10 m, creating a spot of approximately 2–3 mm. The membrane was then incubated in blocking buffer and processed using the same procedure described below for Western blot following sample transfer to nitrocellulose membranes. In order to determine that the peptides were not being recognized by any pre-immune serum components, the above procedure was followed exactly with the exception that the respective pre-immune sera were used for the primary incubations in place of antibodies.

3.12 Western blot analysis

3.12.1 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Western blot detection was done using 250 ng protein suspended in Laemmli protein loading buffer (Laemmli 1970). Samples were heated at 95 °C for 10 m, then loaded on 10 or 12% (vol/vol) SDS-PAGE gels and run on the Mini Protean Tetra system (Bio-Rad) at 200 V and 180 mA. Samples were then blotted for 2.5 h onto 0.45 µm supported nitrocellulose membranes (Bio-Rad).

3.12.2 Blocking and immunostaining

Blocking was done for 1 h at room temperature in 5% (wt/vol) powdered skimmed milk prepared in Tris-buffered saline (TBS) solution plus 0.1% Tween–20 (TBS–T buffer) before staining for 1 h in primary antibody (anti-*Bg*GRN or anti-*Bg*TLR at 1:1,000 dilutions). Membranes were washed in TBS–T buffer for 10 m, then twice for 5 m each and once in TBS solution for 5 m. Following the TBS wash, membranes were stained for 1 h in a horseradish peroxidase (HRP)-conjugated secondary antibody in blocking buffer, followed by a wash step as

described earlier. Detection was accomplished by incubating the membranes in SuperSignal West Dura Extended Duration substrate (ThermoFisher Scientific). Chemiluminescent signals were acquired on the ImageQuant LAS 4000 machine (GE Healthcare).

3.12.3 Stripping and re-probing

In order to detect *Bg*Actin which served as a protein loading control for haemocytes and Bge cell samples, antibodies were stripped from the membranes using a mild stripping buffer (15 g/L glycine, 1 g/L SDS, and 1% Tween–20, pH 2.2) after visualizing experimental samples. The membranes were incubated in the stripping buffer 2 times for 10 m each at room temperature, replacing the buffer each time. They were then washed 2 times for 10 m each in 1x PBS, and 2 times for 5 m each in 1x TBS–T. Stripped membranes were blocked and re-probed with a mouse anti-actin antibody at 1:5,000 dilution and HRP-conjugated secondary antibody using the procedure described above.

3.12.4 Haemocyte and Bge cell lysis and detection of ERK1/2 and intracellular *Bg*GRN

Primary haemocytes isolated from pools of five *B. glabrata* snails and cultured Bge cells were centrifuged once at 1,000 × *g* for 10 m. Cells were resuspended in 1× SSS buffer (Table 3.3, Adema *et al.* 1993) and centrifuged at 1,000 × *g* for 5 m. Following one wash, cells were lysed using a cell lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 30 mM NaF, and phosphatase inhibitor mixtures 2 and 3 (Sigma). Lysates were run on 12% (vol/vol) SDS gels at a concentration of 30 ng per lane. Samples were then processed and probed with mouse anti-phospho ERK1/2, and anti-total ERK1/2 antibodies (Cell Signaling Technology) at 1:2,500 dilution as described above except that blocking was done for

4 h at room temperature in 5% (wt/vol) bovine serum albumin (BSA, Sigma) prepared in TBS–T buffer before probing with the primary antibody.

3.13 siRNA-mediated gene knockdown

Snails (~8 mm shell diameter) were injected with a mixture of 27-mer short interfering RNA (siRNA) oligonucleotides (Integrated DNA Technologies) designed to specifically target four different regions of the *Bg*GRN or *Bg*TLR transcripts. The oligonucleotide sequences (Table 3.4) were confirmed to be unique by comparison with the *B. glabrata* genome (*Bg*GRN) and qPCR amplification of three sequences representing transcripts with highest shared nucleotide identity with *Bg*TLR (BGLB008702, BGLB010031, and BGLB011379). The oligonucleotide mix was suspended in Xfect transfection reagent (Clontech Laboratories) to enhance delivery, and 10–20 µL was injected directly into the snail haemocoel at an approximate final concentration of 6 nM, which was determined by estimating the volume of haemolymph within the snail. Holes in the shell resulting from the injection technique were filled with wax. Control snails received siRNA oligo targeting green fluorescent protein (GFP). The GFP oligos have no known homologies to expressed transcripts in *B. glabrata*.

In studies assaying for *Bg*GRN, siRNA-mediated knockdown was examined at the protein level in non-challenged BS-90 snails and at the transcriptional level in *S. mansoni*-challenged BS-90 snails. For *BgTLR*, knockdown effect was examined at the transcriptional level in both BS-90 and M-line snails without parasite challenge, and BS-90 snails that had been exposed to *S. mansoni*. *Bg*TLR knockdown was also examined at the protein level in Bge cells and haemocytes of BS-90 snails. For transcript knockdown, RNA was extracted from five snails for each time point, converted into cDNA, and used for qRT-PCR as described earlier. Protein

knockdown was determined by Western blot on cell-free plasma (*Bg*GRN) used directly and probed with a mouse anti-*Bg*GRN polyclonal antibody as described earlier. *Bg*TLR protein knockdown was confirmed by Western blot using both haemocytes isolated from BS-90 snails and Bge cells, which have haemocyte-like properties (Coustau *et al.* 2003; Yoshino *et al.* 1999). All proteins from haemocytes or Bge cells and cell-free plasma were quantified with Qubit protein assay kit (Thermo Fisher Scientific) according to the manufacturer's protocol.

3.14 Modulation of *Schistosoma mansoni* infection success

3.14.1 *Bg*GRN modulation of infection success

Two studies were designed to assess the importance of *Bg*GRN, and thus haemocyte number, on *S. mansoni–B. glabrata* compatibility. The first experiment used M-line *B. glabrata* snails that were injected through the shell into the haemocoel with 100 nM *Bg*GRN (n = 100 snails) or 100 nM of a recombinant protein control (*B. glabrata* Temptin produced identically to r*Bg*GRN; n = 50 snails). Injections took place 48 h before challenging each snail with five *S. mansoni* PR-1 miracidia for 24 h. Following challenge, snails from each experimental group were placed in separate tanks filled with ASW at a snail density of 25 per tank. Periodically, infection success was measured by placing snails into 12-well plates and monitoring for the shedding of cercariae over a 6-h period. Cercarial shedding was assessed once per week at 4, 5, 6, 7, and 8 weeks post-challenge. The same procedure was used to assess the impact of *Bg*GRN on the infection success of two strains of *S. mansoni* (PR-1 and NMRI) in *B. pfeifferi*, *B. sudanica* and multiple strains of *B. glabrata*. These studies used 45 snails each for the experimental group and control groups. In this study, assessment of cercariae shedding was done at a single time point of 7 weeks post-challenge.

The second experiment used BS-90 strain of *B. glabrata* that were injected with siRNA oligonucleotides specific for the knockdown of *Bg*GRN (n = 100 snails) or GFP as a control (n = 50 snails). For this study, injections took place 24 h before challenge with five *S. mansoni* PR-1 miracidia. Challenge was allowed to continue for 24 h before snails were placed in tanks, as outlined earlier, at a density of 25 snails per tank. Cercarial shedding was assessed at 4, 5, 6, 7, and 8 weeks post-challenge, for a 6 h period in 12-well plates.

3.14.2 *Bg*TLR modulation of infection success

Phenotypic influence of *Bg*TLR transcript knockdown was assessed in BS-90 snails. Snails were injected with the *Bg*TLR-specific siRNA oligonucleotide mix and after 48 h exposed individually to 5 miracidia of *S. mansoni* NMRI. Four weeks later, snails were assessed on a weekly basis, for cercariae shedding. The number of cercariae-producing infections was used as an assessment of the influence of *Bg*TLR knockdown on the resistance phenotype. *Bg*TLR knockdown snails were compared to BS-90 snails injected with the GFP-specific siRNA oligonucleotides, and to M-line snail controls challenged at the same time.

3.15 Indirect ELISA measurement of plasma *Bg*GRN

3.15.1 *B. glabrata* plasma collection

Cell-free plasma was collected from *B. glabrata* BS-90 snails through the head-foot retraction method (Sminia and Barendsen 1980). Snail shells were thoroughly cleaned with Kimwipes wetted with 70% ethanol. Then, using a 200 µL pipette fitted with a plastic tip, the snail head-foot was poked repeatedly causing the snail to retract into the shell and exude haemolymph which was pipetted out. Following exhaustive collection of plasma, snails were crushed and discarded appropriately. Haemolymph from three snails for each time point (at 0, 1,

2, 3, 4, 8, and 16 days post-challenge with *S. mansoni*) was collected and immediately placed in 1.5 mL tubes on ice. Samples were centrifuged at 1,000 × *g* for 10 m and the cell-free fraction (plasma) was collected.

3.15.2 Measurement of plasma *Bg*GRN

Plasma was diluted 1:10, 1:50, or 1:250 with 0.2 M $NaHCO_3$ (pH 9.4), and then 100 µL of sample was pipetted into a 96-well polystyrene plate. Plates were incubated at 4 °C for 24 h and then washed three times for 5 m with wash buffer (25 mM Tris, 0.15 M NaCl, 0.05 % Tween–20, pH 7.2). Then, plates were blocked with 2% (wt/vol) BSA in wash buffer for 3 h at room temperature. Following blocking, the buffer was replaced with 100 µL of blocking buffer containing anti-*Bg*GRN antibody (1:500), and incubated at room temperature overnight. Plates were washed three times for 5 m each with wash buffer and then incubated with blocking buffer containing a biotinylated secondary antibody (1:250). Plates were then covered with aluminum foil and incubated at room temperature for 1 h, washed six times for 5 m each, and then incubated in the substrate solution containing streptavidin conjugated to DyLight 649 (ThermoFisher Scientific). The reaction was allowed to proceed for 15 m and was then read using a 96-well plate reader (Molecular Devices). All plasma samples were compared with a standard curve generated using a serial dilution series of r*Bg*GRN ranging from 0.001 ng/mL to 10 µg/mL.

3.16 Elastase cleavage reaction

Recombinant *Bg*GRN (25 µg) was treated with 0.3 U/mL porcine elastase (Alfa Aeser) and used to assess whether the predicted elastase cleavage sites on *Bg*GRN were accurate. Elastase incubations took place over 5, 15, and 30 m, after which samples were run on SDS-

PAGE and then blotted for 2.5 h onto 0.45-µm supported nitrocellulose membranes (Bio-Rad). Blocking was done for 4 h at room temperature in 5% (wt/vol) BSA (Sigma) prepared in TBS–T buffer before probing with the anti-*Bg*GRN at 1:1,000 dilution. Visualization of blots was accomplished as described earlier.

3.17 Cell biological assays

3.17.1 Flow cytometric BrdU assay of haemocyte and Bge cell proliferation

Labeling of *B. glabrata* haemocytes *in vivo* with 5-bromo-2-deoxyuridine (BrdU) was accomplished by first making a BrdU stock solution. BrdU (Sigma) was prepared in 1× PBS to a working concentration of 10 mg/mL (32.6 mM). This solution was then combined with experimental and control treatments and then injected through the shell into the snail haemocoel using a 27-gauge needle in a 10–20 µL volume that was adjusted depending on the snail shell size (~8 mm shell diameter was injected with 10 µL, ~16 mm shell diameter was injected with 20 µL). The resulting hole in the snail shell was covered with wax, and the snail was returned to a clean tank of ASW for 48 h before the next manipulation. Primary *B. glabrata* haemocytes were isolated for use in proliferation bioassays using the head-foot retraction method (Sminia and Barendsen 1980).

Labeling of Bge cells with BrdU *in vitro* was accomplished by diluting a 1 mM BrdU working solution to a final working concentration of 10 µM in Bge cell culture medium. Bge cells were seeded in six-well culture plates at a density of 1×10^6 cells/mL in 2 mL volumes and allowed to adhere for 4 h before treatment and addition of BrdU. The treatments (identified below) and BrdU were administered in the same medium, which replaced normal Bge cell medium following the 4 h pre-treatment period. Labeling and treatment occurred over a 24 h

period, after which the 2 mL medium was replaced with a 0.5 mL 0.05% Trypsin-EDTA solution (Life Technologies) for 10 m to suspend the adherent Bge cells.

Treatments for Bge cells and *B. glabrata* haemocytes *in vivo* consisted of four experimental concentrations of r*Bg*GRN (25, 50, 100, and 200 nM). These treatments were compared with 100, 200, and 300 nM phorbol 12-myristate 13-acetate (PMA, Sigma) as a proliferation control treatment and with 100 nM r*Bg*GRN or 300 nM PMA combined with 1 μM or 10 μM of the MEK [MAPK (ERK 1/2)] inhibitor U0126 (Promega). These concentrations were exactly calculated for Bge cell assays and were estimated based on predicted haemolymph volume as indicated earlier for studies involving *B. glabrata* haemocytes.

For Bge cells and primary haemocytes of *B. glabrata*, the isolated cells were centrifuged at 1,000 × *g* and resuspended in 1 mL of 1× PBS + 3.7% (vol/vol) formaldehyde. Following a 15-m incubation, cells were washed three times (2 m for each wash) in 1× PBS solution. Cells were then permeabilized using a solution of 0.1% Triton X-100 (Sigma) in 1× PBS solution for 20 m, after which cells were centrifuged and resuspended in 1 mL of 1 N HCl for 10 m, followed by resuspension and incubation for 10 m in phosphate/citric acid buffer, pH 7.4 (made by combining 182 mL of 0.2 M Na_2HPO_4 with 18 mL of 0.1 M citric acid). Cells were washed three times with Triton X-100 permeabilization buffer (2 m per wash). Cells were prepared for incubation with the anti-BrdU antibody by replacing the final wash of Triton X-100 buffer with a solution of 0.1% Triton X-100, 5% (vol/vol) FBS (Corning) in 1× PBS. Cells were incubated in this solution for 1 h before replacing it with the antibody solution containing 5 μL of the provided anti-BrdU mouse monoclonal antibody conjugated to Alexa Fluor 488 (Life Technologies). Cells were incubated overnight with the antibody solution, after which they were washed three times using 1× PBS (2 m per wash), and then analyzed by flow cytometry.

In experiments involving co-labeling, haemocytes were incubated with anti-BrdU at the same time with rabbit anti-*Bg*TLR or anti-*Bg*FREP3 antibodies and further stained after washes with a goat anti-rabbit IgG secondary antibody conjugated to Alexa Fluor 680 (Life Technologies). Flow cytometric analysis of *B. glabrata* haemocytes and Bge cells was undertaken using a Beckman-Coulter Gallios flow cytometry platform. Size, granularity, and fluorescence were analyzed using the Kaluza software package (Beckman-Coulter). All washes and incubation steps described above took place at room temperature.

3.17.2 Adherent haemocyte counts

Primary *B. glabrata* haemocytes were isolated for use in proliferation bioassays and exactly 10 µL of haemolymph was mixed with 10 µL of sterile CBSS, and 10 µL of the 1:1 mixture was then placed directly onto microscope slides and incubated within a humidified chamber for 2 h at 25 °C. Following incubation, the slides were gently washed twice using sterile CBSS that was warmed to 25 °C. The adherent cells were enumerated, and the values adjusted to reflect the number of adherent (spread) haemocytes per microliter of haemolymph.

3.17.3 Phagocytosis assay

Five BS-90 snails each were injected with siRNA oligonucleotides targeting *Bg*TLR or GFP (control) and 96 h later, haemolymph was extracted from the snails and immediately mixed with ~1 x 10^6 1-µm FITC-labelled streptavidin-coated microspheres that were previously incubated with biotinylated *S. mansoni* excretory/secretory products and sporocysts (Zahoor *et al.* 2009) following the manufacturer's protocols (Bang Laboratories Inc.). Following mixing, the haemolymph was quickly deposited on a microscope slide and allowed to sit in a humidified chamber for 3 h. The slides were then washed thrice in 1× PBS and examined under a fluorescent

microscope. Haemocytes from each snail were counted from a random field of view on the slide, and 30 haemocytes for each snail were assessed for the number of beads within each cell, from which percentage phagocytosis (% of cells with one or more beads) and mean number of beads per haemocyte were calculated.

3.17.4 Immunocytochemistry

To visualize *Bg*TLR in *B. glabrata* haemocytes and Bge cells, immunocytochemistry was done using anti-*Bg*TLR polyclonal primary antibody and goat anti-rabbit IgG secondary antibody conjugated to Alexa555 fluorophore (Life Technologies). Snails were bled and 100–200 µL of haemolymph was mixed with equal volume of 4% paraformaldehyde prepared in 1× PBS. After 10 m of fixation at room temperature, haemocytes were centrifuged at 700 rpm for 5 m onto a coverslip and placed in the well of a 6-well plate. Haemocytes were washed for 2 m with 1 mL of 1× antibody staining buffer (ASB, 0.05% sodium azide, 1% BSA in 1× PBS), then blocked at room temperature for 1 h in 1% BSA prepared in 1× ASB before staining using the primary antibody (suspended in blocking buffer at 1:200 dilution) at room temperature for 1 h. Haemocytes were washed three times with 1 mL 1× ASB (5 m per wash). Staining in secondary antibody (1:500 dilution) was performed for 1 h at room temperature and washed as described for the primary antibody. Haemocytes were then mounted in a solution containing 4,6-diamidino-2-phenylindole (DAPI, GeneTex) for 5 m for the staining of nuclei. Cells from 200 µL of confluent Bge cultures were fixed and treated using the same protocol described for the haemocytes.

Control slides were treated similarly but with primary antibody staining step omitted, or both primary and secondary antibody staining steps omitted. Observation and imaging was done

under the LSM710 confocal microscope (Carl Zeiss Microimaging, Germany) at the Cross-Cancer Institute, University of Alberta. Images were processed with the accompanying ZEN 2011 software, version 7.0.0.285 and Photoshop CS5, version 12.0 x64 (Adobe Systems Incorporated, USA).

3.18 Statistical analyses.

Statistical analyses were performed using GraphPad Prism version 6.0f for Mac OS X (GraphPad; www.graphpad.com) unless otherwise indicated. Significance threshold was set at $P \leq 0.05$. For the qRT-PCR assay, all statistical comparisons were performed on mean ΔC_t values for each time point (i.e., C_t values of *Bg*GRN or *Bg*TLR normalized to actin) using one-way analysis of variance (ANOVA) with Tukey's post hoc tests. Significant differences in the proportions of snails shedding cercariae were determined using the z-test.

Table 3.1. Composition of basic Bge medium – 1,000 mL

Component	**Amount**	**Final concentration**
Schneider's *Drosophila* medium	220 mL	22%
Lactalbumin hydrolysate	4.5 g	0.45%
Galactose	1.3 g	0.13%
0.5 M phenol red	28 µL	14.1 µM
Gentamycin (50 mg/mL)	12.5 µL	20 µg/mL
milliQ water	Up to 1,000 mL	

- Adjust pH to 7.0 and filter-sterilize with 0.22 µm filters.
- To make complete Bge medium, add heat-inactivated FBS to basic Bge medium at 10% final concentration.

Table 3.2. List of primers

Primer	Description	Sequence
1	*Bg*GRN qRT-PCR	Fwd: 5′- TGA AAG TGT CGT ATG TCC TGG -3′
2		Rev: 5′- CAT CCA TAG TCC CCA GAT TGC -3′
3	r*Bg*GRN expression	*Fwd: 5′- *C ACC* **AGG AGG** ATA TTA CAG ATG TCG TGC CTC AAG ATT GTG -3′
4		Rev: 5′- GCG TAG AAC TTT GTT TGA GAA TG -3′
5	r*Bg*GRN Domain 1 expression	*Fwd: 5′- *C ACC* **AGG AGG** GAC AAC TAC ATG ACT TGC TGC AAG GCT AAT -3′
6		Rev: 5′- GT AGC AAC GGC GGT TGA TCA -3′
7	r*Bg*GRN Domain 2 expression	*Fwd: 5′- *C ACC* **AGG AGG** GAT GGA TCG ATG ACG TGC TGC CAG CTG GCT -3′
8		Rev: 5′- CT TCA CGC AGG TGC CAG CTG -3′
9	r*Bg*GRN Domain 3 expression	*Fwd: 5′- *C ACC* **AGG AGG** GGT GGA GCT ATG ACT TGC TGC AAG CTC CAG -3′
10		Rev: 5′- CT TCT TGC ACT CGC CTT GAT -3′

Primer	Description	Sequence
11	r*Bg*GRN Domain 4 expression	*Fwd: 5′- *C ACC* **AGG AGG** GAT GGT AAC ATG ACT TGC TGC AAG TTG GCC -3′
12		Rev: 5′- GG CCT TGT TGC ATG TTC CGG -3′
13	*Bg*TLR qRT-PCR	Fwd: 5′-GTC TGT CAG GTC GTT GTT CTT A-3′
14		Rev: 5′-GAT AGA CCC TCA AGC TCT GTT G-3′
15	r*Bg*TLR expression	#Fwd: 5′-CCC GGG ATG GAA CTT TTG GAA ACT CTG GA-3′
16		#Rev: 5′-GTC GAC GAC ATA GGC TGT TTT AAC ACG AC-3′
17	r*Bg*TLR LRR expression	#Fwd: 5′-CCC GGG ATG GAA CTT TTG GAA ACT CTG GA-3′
18		#Rev: 5′-GTC GAC AGG TGG TAA CAA CAG AGC TTG AG-3′
19	r*Bg*TLR TIR expression	#Fwd: 5′-CCC GGG ACC TAC GCC CTG ATC GC-3′
20		#Rev: 5′-GTC GAC GAC ATA GGC TGT TTT AAC ACG AC-3′

Primer	Description	Sequence
21	Overlap extension *Bg*TLR LRR	Rev: 5′-AGG TGG TAA CAA CAG AGC TTG AG-3′
22	Overlap extension *Bg*TLR Tm+TIR	[Δ]Fwd: 5′-TCC TTC ACT CAG GAG ACC TAC GCC CTG AT GC-3′
23	Overlap extension *Hs*TLR2 Tm+TIR	[Δ]Fwd: 5′-CTG TTG TTA CCA CCT GCA CTG GTG TCT GGC ATG-3′
24	r*Hs*TLR2 expression	[#]Fwd: 5′-CCC GGG ATG CCA CAT ACT TTG TGG-3′
25		[#]Rev: 5′-GTC GAC GGA CTT TAT CGC AGC TCT-3′
26	Overlap extension *Hs*TLR2 LRR	Rev: 5′-CTC CTG AGT GAA GGA GAG-3′
27	*Bg*Actin qRT-PCR	Fwd: 5'-GCT TCC ACC TCT TCA TCT CTT G-3'
28		Rev: 5'-GAA CGT AGC TTC TGG ACA TCT G-3’
29	*Sm*GAPDH	Fwd: 5'-TCG TTG AGT CTA CTG GAG TCT TTA CG-3'
30		Rev: 5'-AAT ATG ATC CTG AGC TTT ATC AAT GG-3’
31	BGLB008602 qRT-PCR	Fwd: 5'-GCA GTC GTA AAA GTT GTA GCA G-3'

Primer	Description	Sequence
32		Rev: 5'-CCA TGA CCA AAG GAT TTT CGA G-3’
33	BGLB010031 qRT-PCR	Fwd: 5'-CAT TTT CTA ACC TGA CCC GTT TG-3'
34		Rev: 5'-AGT AGC GGT GAT TCT GTT GG-3’
35	BGLB011379 qRT-PCR	Fwd: 5'-ACG AGA CCT TCT GTG ACA TTC-3'
36		Rev: 5'-GTT TTC TTG AAC CCA CTG CC-3’

*Forward primer modified by adding CACC nucleotides (italicized) and ribosome binding sequence (bold font) for efficient cloning and expression.

[#]Restriction enzyme cut sites (underlined) added to the forward (XmaI) and reverse (SalI) primers for cloning into the SmaI and SalI sites of the pDisplay vector.

[Δ]Boxed sequences represent 15 bp overlap with 3' end of LRR of *Bg*TLR or *Hs*TLR2 respectively for fusion via overlap extension PCR.

Table 3.3 Composition of 1x sterile snail saline – 1,000 mL

Component	**Amount**	**Final concentration**
HEPES	1.19 g	5 mM
NaOH	0.15 g	3.7 mM
NaCl	2.1 g	36 mM
KCl	0.15 g	2 mM
$MgCl_2 . 2H_2O$	0.26 g	2 mM
$CaCl_2 . 2H_2O$	0.29 g	4 mM
milliQ water	Up to 1,000 mL	

- Adjust pH to 7.8 and filter-sterilize with 0.22 µm filters.

Table 3.4. List of siRNA oligonucleotides

Description[a]	Oligonucleotide sequence
*Bg*GRN-siRNA-1	5'-GCAGUAAUCAGAACAAUGUGUUATA-3'
*Bg*GRN-siRNA-2	5'-CUACAGAUGUGAUGUGGAUCAAGGT-3'
*Bg*GRN-siRNA-3	5'-CUGAAAGUGUCGUAUGUCCUGGAGG-3'
*Bg*GRN-siRNA-4	5'-GUAUCAGCAGGAACAUGUAACAAAG-3'
siRNA-*Bg*TLR-1	5′-AGCCAAAUACUAUCGGUCAGUCUCGAC-3′
siRNA-*Bg*TLR-2	5′-GGUCAAAUUGUUAACGCUCAGGUCCAC-3′
siRNA-*Bg*TLR-3	5′-ACGCUGUUUCUGGACAUGUUAGUGGGA-3′
siRNA-*Bg*TLR-4	5′-GUGGACACACAGUUGAAACUUCUUGUC-3′
siRNA-GFP-1	5′-CCAUCAUCUUUGAAGAAGGAACAAUCUUCUUCAAAG-3′
siRNA-GFP-2	5′-AGGUAAUAAUACAGGACCCGGUGAUGGUCCUGUAUU-3′
siRNA-GFP-3	5′-AUGUUGUUACUAAUGUAGCCUUGACCUACAUUAGUA-3′

[a]Sequences are listed from the 5′ to 3′ direction of the gene targets.

Chapter 4

Characterization and Functional Analysis of *Biomphalaria glabrata* Progranulin[2]

4.1 Introduction

Granulins are well-known and evolutionarily conserved growth factors, characterized by a unique 12-cysteine (GRN) motif (Figure 4.1). Proteins containing granulin domains have been found in organisms of various taxa, from eubacteria to humans. Granulins can be found containing a single GRN motif, but are more often in a form containing tandem repeated motifs, known as the progranulin (PGRN) protein. These are secreted pro-peptides that can be subsequently cleaved into smaller functional units by elastase (Zanocco-Marani *et al.* 1999). Progranulin is functional, and has been shown to be involved in numerous biological processes including tissue repair (He and Bateman 2003), early embryogenesis, inflammation, neurobiology and cell proliferation (Ong and Bateman 2003), via activation of the MAP kinase pathway (Zanocco-Marani *et al.* 1999).

Additionally, the products of PGRN cleavage (usually ~6 kDa) can also be functional in a variety of roles that appear to be defined by the animal model in which the study is taking place, as well as the particular cleavage product under investigation. These roles include participation in, and activation of the immune response (Zhu *et al.* 2002). Ultimately, the balance between PGRN and the smaller cleavage products, is maintained by the secretory leukocyte protease

[2] A version of this chapter has been published: Pila, E.A., Gordy, M.A., Phillips, V.K., Kabore, A.L., Rudko, S.P. and Hanington, P.C. (2016). Endogenous growth factor stimulation of hemocyte proliferation induces resistance to *Schistosoma mansoni* challenge in the snail host. *Proceedings of the National Academy of Sciences of the United States of America* 113 (19), 5305-5310.

inhibitor (SLPI), which binds directly to PGRN, thereby preventing proteolysis by neutrophil elastase (Zhu *et al.* 2002). Maintaining and adjusting this balance are essential from a functional standpoint, as both the concentration of PGRN and SLPI impact upon the biological effect elicited.

Granulins drive proliferation of immune cells in organisms spanning the animal kingdom. In non-mamallian organisms, isolated studies provide limited insight into the functional role that PGRN and granulins play. Investigations of granulins in carp (Belcourt *et al.* 1993), goldfish (Hanington *et al.* 2006) and the parasitic liver fluke *Opisthorchis viverrini* (Smout *et al.* 2009) showed that they all share haematopoietic functions with their mammalian counterparts, including the induction of cellular proliferation.

The immune cells of a snail (haemocytes) are sentinels that act as a crucial barrier to infection by larval digeneans. Haemocytes coordinate a robust and specific immunological response, participating directly in parasite killing by encapsulating and clearing the infection. The central role of haemocytes in the anti-schistosome response underscores the importance of maintaining suitable numbers of circulating and tissue-resident immune cells. As previously reviewed in chapter 2, little is known about the mechanics of haematopoiesis (the process responsible for generation of blood cells) in gastropods. This limited understanding of the process suggests that haemocyte proliferation and differentiation are influenced by unknown digenean-specific exogenous factors. In *Biomphalaria glabrata*, parasite-specific factors that are excreted or secreted upon initial infection of the snail are important drivers of haematopoiesis. Following stimulation with ES products of digenean trematodes, such as *S. mansoni* (Sullivan *et al.* 2004) or *Echinostoma paraensei* (Noda 1992), the APO (Jeong *et al.* 1983) of *B. glabrata* noticeably swells, and displays an increase in mitotic events (Lie *et al.* 1976). The haemocytes

generated during these events tend to be adherent and granulocytic, and exhibit greater capacity to generate humoral factors relevant to parasite killing compared to the pre-existing population (Hanington *et al.* 2010a). Moreover, following the stimulation of haemocyte development by exogenous factors, the proportion of haemocytes with granulocytic morphology increases (Lie *et al.* 1976). These haemocytes are typically associated with parasite encapsulation (LoVerde *et al.* 1984), phagocytosis (Hanington *et al.* 2010a) and the production of ROS/RNI (Humphries and Yoshino 2008) important for parasite killing.

The second, and lesser-understood drivers of gastropod haematopoiesis, are endogenous snail growth factors that in response to infection, induce haemocyte proliferation/differentiation within the APO. Until recently, nothing was known about the endogenous control of haemocyte development in any gastropod model. Recent studies screening the transcriptome and proteome of *B. glabrata* have yielded promising candidates that possess canonical domains associated with haematopoietic factors of other organisms, both invertebrate and vertebrate (Hanington *et al.* 2010b). Past studies in our laboratory (as described in chapter 3, Pila *et al.* 2016a) comparing snail plasma proteins identified peptide fragments possessing the canonical cysteine-repeat pattern of GRN proteins in high abundance in BS-90 snail haemolymph following *S. mansoni* challenge compared to the M-line strain. These peptide fragments were used to identify the full *Bg*GRN transcript sequence from the unannotated *B. glabrata* genome. My doctoral thesis focused, in part, on advancing our understanding of the mechanics of gastropod haematopoiesis. Therefore, characterizing these evolutionarily conserved growth factors, endogenously produced by the snail, constituted a logical starting point.

In this chapter, I report on the molecular and functional characterization of a progranulin from the snail *B. glabrata* (*Bg*GRN), a natural host for the human blood fluke *Schistosoma*

mansoni. I demonstrate that *Bg*GRN transcript expression increases in the early stages of *S. mansoni* challenge, and induces the proliferation of *B. glabrata* immune cells, driving the *in vivo* development of an adherent haemocyte subset distinct from resident steady-state haemocytes. Finally, I demonstrate that susceptible *B. glabrata* snails can be made resistant to infection with *S. mansoni* by first inducing haemocyte proliferation with *Bg*GRN.

4.2 Experimental design

4.2.1 Sequence annotations, alignments, secondary structure prediction and visualization

Signal sequence and cleavage sites for *Bg*GRN and some known and predicted granulins were predicted using three different programs: SignalP 4.1 (Petersen *et al.* 2011), TargetP 1.1 (Emanuelsson *et al.* 2000; Nielsen *et al.* 1997), and Phobius (Kall *et al.* 2007). Granulin domains were annotated using the Pfam program (Finn *et al.* 2014). *Bg*GRN domain sequence alignments were done using CLC Genomics Workbench 7.5.1 (www.clcbio.com), while the secondary structure predictions and visualization were accomplished with the aid of the Robetta server (robetta.bakerlab.org) (Kim *et al.* 2004) and PyMOL (PyMOL Molecular Graphics System, version 1.5.0.4; Schrödinger) respectively as described in chapter 3.

4.2.2 Measurement of *Bg*GRN expression in *B. glabrata* snails during parasite challenge

Quantitative real-time polymerase chain reaction (qRT-PCR) was used to measure *Bg*GRN expression patterns in *S. mansoni*-challenged and control snails. Resistant (BS-90, Paraense and Correa 1963; Richards and Merritt 1972) and susceptible (M-line, Newton 1955; Cooper *et al.* 1994) snails were exposed individually to 5 miracidia of *S. mansoni* PR-1. Five snails were collected at selected time points (0, 1, 3, 12 h and 1, 2, 3, 4, 8, 16, and 35 days post challenge) that represent important milestones in the life cycle of *S. mansoni*, and encompass the

time from miracidia infection to cercarial shedding in the snail. RNA was extracted from whole snails, converted into cDNA and used in qRT-PCR as described in chapter 3.

4.2.3 Recombinant *Bg*GRN synthesis and purification

Recombinant *Bg*GRN was generated by using the Gateway® cloning system according to the manufacturer's instructions (Life Technologies). The *Bg*GRN expression plasmid was transfected into *Sf*9 cells and *Bg*GRN protein was purified from the culture medium by using a combination of HisTrap^{TM} HP columns coupled on the AKTA Pure fast protein liquid chromatography machine (GE Healthcare) and HisPur^{TM} Ni-NTA magnetic beads (ThermoFisher Scientific) as described in chapter 3.

4.2.4 Indirect ELISA measurement of plasma *Bg*GRN

Cell-free plasma was isolated from BS-90 *B. glabrata* at 0, 1, 2, 3, 4, 8, and 16 days post-challenge with *S. mansoni*, from which plasma concentration of *Bg*GRN was determined via indirect ELISA as described in chapter 3.

4.2.5 Detection of ERK1/2 and intracellular *Bg*GRN

ERK1/2 and intracellular *Bg*GRN were detected in both cultured Bge cells and primary haemocytes isolated from pools of five *B. glabrata* snails via Western blots probed with the primary antibodies: anti-phospho ERK1/2, anti-total ERK1/2 at 1:2,500 dilution (Cell Signaling Technology), or anti-*Bg*GRN at 1:1,000 dilution accordingly as described earlier in chapter 3.

4.2.6 Elastase cleavage of *Bg*GRN

The porcine elastase (Alfa Aeser) was used to assess whether the predicted elastase cleavage sites on *Bg*GRN were accurate. Twenty-five μg of r*Bg*GRN was treated with 0.3 U/mL

elastase for 5, 15, and 30 m, after which samples were run on SDS-PAGE, blotted, probed with anti-*Bg*GRN at 1:1,000 dilution and visualized as described earlier in chapter 3.

4.2.7 Flow cytometric BrdU assay of haemocyte and Bge cell proliferation

B. glabrata haemocytes and Bge cells were labelled with BrdU in combination with various treatements described below. Haemocytes were labelled *in vivo* by injection of 10 mg/mL BrdU solution through the shell into the snail haemocoel in a 10–20 µL volume, while the labelling of Bge cells was done *in vitro* with 10 µM BrdU in Bge cell culture medium. Following BrdU labelling, cells were isolated, processed and analyzed using flow cytometry as described in chapter 3.

Treatments for Bge cells and *B. glabrata* haemocytes *in vivo* consisted of four experimental concentrations of r*Bg*GRN (25, 50, 100, and 200 nM). These treatments were compared with 100, 200, and 300 nM PMA (Sigma) as a proliferation control treatment and with 100 nM r*Bg*GRN or 300 nM PMA combined with 1 µM or 10 µM of the MEK inhibitor U0126 (Promega). These concentrations were exactly calculated for Bge cell assays and were estimated based on predicted haemolymph volume as indicated earlier for studies involving *B. glabrata* haemocytes.

4.2.8 siRNA-mediated knockdown of *Bg*GRN

Snails (~8 mm shell diameter) were injected with a mixture of 27-mer siRNA oligonucleotides (Integrated DNA Technologies) designed to specifically target four different regions of the *Bg*GRN transcript. The oligonucleotide mix was suspended in Xfect transfection reagent (Clontech) to enhance delivery, and 10–20 µL was injected directly into the snail

haemocoel at an approximate final concentration of 6 nM. Control snails received siRNA oligo targeting GFP.

siRNA-mediated knockdown of *Bg*GRN was examined at the transcriptional and protein levels. For the knockdown of *Bg*GRN transcript, RNA was extracted from five snails, converted into cDNA, and used for qRT-PCR as described earlier in chapter 3. Protein knockdown was determined by Western blot on cell-free plasma used directly and probed with a mouse anti-*Bg*GRN polyclonal antibody as described earlier.

4.2.9 Adherent haemocyte counts

Snails were bled through the head-foot retraction method and 10 µL of haemolymph was mixed with 10 µL of sterile CBSS (Chernin 1963), and 10 µL of the 1:1 mixture was processed for adherent haemocyte counts as described in chapter 3. The adherent cells were enumerated, and the values adjusted to reflect the number of adherent (spread) haemocytes per microliter of haemolymph.

4.2.10 *Bg*GRN modulation of infection success

B. glabrata M-line snails were injected with 100 nM r*Bg*GRN (n = 100 snails) or 100 nM of r*Bg*Temptin as a recombinant protein control (n = 50 snails). Injections took place 48 h before challenging each snail with five *S. mansoni* miracidia for 24 h. Following challenge, snails from each experimental group were placed within independent tanks filled with ASW at a snail density of 25 snails per tank. Infection success was measured by monitoring for the shedding of cercariae over a 6-h period once per week beginning from week 4, and ending at week 8 post-challenge. The infection successes of PR-1 and NMRI strains of *S. mansoni* in multiple *B. glabrata* strains, *B. pfeifferi* and *B. sudanica* injected with *Bg*GRN were also examined. These

studies followed the same procedure as described above but involved $n = 45$ snails per group. Cercarial shedding was assessed at week 7 post-challenge.

B. glabrata BS-90 snails were injected with siRNA oligos specific for the knockdown of *Bg*GRN ($n = 100$ snails) or GFP as a control ($n = 50$ snails). For this study, injections took place 24 h before challenge with five *S. mansoni* miracidia. Challenge was allowed to continue for 24 h before snails were placed in tanks, as outlined earlier. Cercarial shedding was assessed at weeks 4 through 8 post-challenge, for a 6-h period.

4.2.11 Statistical analysis

Unless otherwise indicated, statistical significance was determined using one-way ANOVA with Tukey's post hoc tests performed using GraphPad Prism version 6.0f for Mac OS X (GraphPad; www.graphpad.com). Statistical significance threshold was set at $P \leq 0.05$. For the qRT-PCR assay, all statistical comparisons were performed on mean ΔC_t values for each time point (i.e., C_t values of *Bg*GRN normalized to actin).

4.3 Results:

4.3.1 *In silico* analysis of *Bg*GRN

4.3.1.1 *Bg*GRN-domain prediction and comparison to schistosome granulins

The *B. glabrata*, *S. mansoni*, *S. haematobium*, and *S. japonicum* progranulin proteins possess multiple granulin domains (Figure 4.1A-B; Figure 4.2). *B. glabrata* GRN has 4 predicted granulin domains characterized by amino acid residues 68–112, 158–200, 237–279, and 319–361. The four GRN domains of *Bg*GRN share a 48.9, 52.2, 60.4, and 50% amino acid identity with the three schistosome pro-GRNs. Modelling of *Bg*GRN suggests that it shares secondary and tertiary structural properties with known structures of human granulin A and carp granulin.

While the number of granulin domains differ between *Bg*GRN and the human and carp granulins, superimposition reveals predicted overlap of both the α-helices and β-sheets associated with the conserved 12-cysteine domains (Figure 4.1C).

4.3.1.2 Signal peptide and cleavage site annotations

*Bg*GRN has a predicted size of ~44 kDa, and was consistently observed as a single band in Western blot of non-manipulated snail plasma, as was recombinant *Bg*GRN (r*Bg*GRN). *Schistosoma mansoni* sporocyst ES products were not positive for GRN when probed with anti-*Bg*GRN antibody. *Bg*GRN possesses a predicted secretion signal domain in residues 1–18 (Figure 4.1B), along with five elastase cleavage sites. Four of these sites immediately follow the four granulin domains, the fifth cleavage site is predicted at the C-terminal end of pro-*Bg*GRN. Incubation of r*Bg*GRN with porcine elastase supports an elastase-mediated mechanism for processing of pro-*Bg*GRN, yielding two distinct (~18 kDa and ~8 kDa) bands that are consistent with the smaller predicted size of the *Bg*GRN cleavage products (Figure 4.3).

Longer elastase treatment appeared to produce more of the ~8 kDa cleavage product at the expense of the larger pro-*Bg*GRN (Figure 4.3). While cleavage products at these approximate sizes were not detected by Western blot analysis of plasma isolated from non-manipulated snail, cleavage products were observed in the plasma of *S. mansoni*-challenged M-line (only ~18 kDa product, Figure 4.4B), and BS-90 (both ~18 kDa and ~8 kDa products, Figure 4.4A) and haemocyte lysates (both ~18 kDa and ~8 kDa products, Figure 4.5).

4.3.2 Analysis of *Bg*GRN transcript and protein expression following *S. mansoni* challenge

The mean basal transcript abundance of *Bg*GRN in BS-90 strain of *B. glabrata* compared to M-line strain was found to be very similar following normalization to β-actin. The mean ΔC_t *Bg*GRN value in non-manipulated BS-90 snails was 7.78, SEM 0.80; $n = 5$, compared to 7.46, SEM 0.11; $n = 5$ in M-line snails. No change in *Bg*GRN expression was observed at 1 h post-challenge (hpc) with *S. mansoni* in either BS-90 or M-line snails (Figure 4.6). However, after 1 h, each snail strain displayed different expression profiles. *Bg*GRN transcript abundance increased as early as 3 hpc in BS-90 snails (RQ = 9.3, SEM 4.77), and by 12 hpc, *Bg*GRN abundance had increased significantly compared to control BS-90 snails (RQ = 26.8, SEM 5.3). Highest *Bg*GRN transcript levels appeared at 1 day post-challenge (dpc, RQ = 38.5, SEM 7.88), and then fell consistently from 2 dpc until day 35 (RQ Day 2 = 17.5, SEM 2.01; RQ Day 35 = 1.05, SEM 0.17, Figure 4.6). Compared to M-line snails challenged at the same time, the BS-90 snail *Bg*GRN transcript levels were significantly higher at 12 h and 1 dpc ($p<0.05$). None of the BS-90 snails tested positive for *S. mansoni* GAPDH after 3 dpc, a result consistent with the BS-90 snails being resistant to infection with *S. mansoni* and clearing the infection within 4 days (Sullivan *et al.* 1995).

*Bg*GRN also increased in abundance following challenge of *B. glabrata* M-line with *S. mansoni* (Figure 4.6). Increases were observed at 3 hpc (RQ = 4.3, SEM 1.26), peaking at 1 dpc (RQ 1 Day = 15.58, SEM 2.25). While *Bg*GRN transcript levels declined after 1 dpc, relative expression compared to BS-90 snails remained consistently high, with a second peak in expression at day 35 (RQ Day 2 = 8.32, SEM 1.65; RQ Day 35 = 11.11, SEM 2.70). *Bg*GRN transcript abundance was significantly higher than controls at both 1 and 35 dpc ($p<0.05$).

Ninety-six percent of the experimental M-line snails were positive for *S. mansoni* GAPDH, indicating successful infections in these snails.

Regarding protein expression, plasma *Bg*GRN lagged behind transcript expression with peak expression at 2 dpc in both BS-90 and M-line snails but with earlier appearance and higher abundance in the former (Figure 4.4).

4.3.3 *Bg*GRN protein and transcript in *B. glabrata* before and following siRNA-mediated knockdown

In both snail strains, injection of *Bg*GRN-specific siRNA oligos effectively knocked down *Bg*GRN expression, thereby reducing endogenous *Bg*GRN (Figure 4.7A). Compared to the siRNA GFP control (Figure 4.7B), the knockdown impact on plasma *Bg*GRN was noticeable by 2 days post-injection, with almost complete loss of detectable *Bg*GRN by 6 days post-injection. Knockdown of *Bg*GRN 4 days prior to challenge with *S. mansoni* resulted in an almost complete abrogation of any challenge-mediated induction of *Bg*GRN expression in BS-90 snails when compared to GFP-knockdown controls (Figure 4.7C).

4.3.4 *Bg*GRN induction of haemocyte and Bge cellular proliferation

Co-injection of BrdU with r*Bg*GRN into BS-90 snails resulted in an increased percentage of circulating haemocytes positive for BrdU (BrdU+) after 48 h compared to vehicle controls of medium alone (Figure 4.8A). The mean baseline percent BrdU+ circulating haemocytes in BS-90 snails was found to be 17.9% (SEM = 1.4%), compared to 19.8% (SEM = 1.3%), 28% (SEM = 1.6%), 34% (SEM = 2.1%) and 40.1% (SEM = 1.8%) BrdU+ haemocytes following 48-h stimulation with 25, 50, 100 and 200 nM *Bg*GRN respectively. The percentage of cells that had incorporated BrdU following *Bg*GRN injection was comparable to the positive control PMA, in

which 24% (SEM = 1.2%), 29% (SEM = 1.5%) and 38.3% (SEM = 2.1%) of circulating haemocytes were positive for BrdU after injection of 100 nM, 200 nM and 300 nM PMA respectively. Inhibition of the ERK signalling pathway by U0126 at a concentration of 10 μM abrogated both *Bg*GRN- and PMA-induced proliferation (BrdU incorporation) in circulating haemocytes (Figure 4.8A). Natural levels of circulating *Bg*GRN observed in snail plasma (12.1 nM ± 3.67) increased as a result of *S. mansoni* challenge in BS-90 snails to a peak of 110.3 nM ± 16.3 at day 2 post-challenge. Circulating *Bg*GRN returned to baseline concentrations (13.4 nM ± 3.76) by day 8 post-challenge (Figure 4.9).

*Bg*GRN elicited very similar results when applied to Bge cells (n = 6 for all treatments) for 48 h under *in vitro* culture conditions (Figure 4.8B). The mean percent BrdU+ Bge cells cultured in medium alone was 26% (SEM = 1.2%), compared to 32.8% (SEM = 1.4%), 39% (SEM = 1.1%), 43.8% (SEM = 1.3%) and 44.9% (SEM = 0.8%) after treatment with 25, 50, 100 and 200 nM *Bg*GRN. BrdU was incorporated into the DNA of 31.7% (SEM = 1.0%), 35.1% (SEM = 1.5%) and 43.4% (SEM = 1.3%) of Bge cells following treatment with PMA at concentrations of 100, 200 and 300 nM. As with primary haemocytes, the proliferation induced by *Bg*GRN and PMA was abrogated by application of 10 μM U0126.

Knockdown of *Bg*GRN in BS-90 and Bge cells 4 days prior to assessment of BrdU incorporation resulted in a non-significant, but observable reduction of BrdU+ cells in both groups [14.7% (SEM = 0.9%)] and [23.3% (SEM = 1.0%)] respectively, compared to controls (see above; Figure 4.8). Assessment of newly proliferated haemocytes using *Bg*FREP3 (Hanington *et al.* 2010a) and *Bg*TLR (Pila *et al.* 2016b) – proteins known to be associated with resistance to *S. mansoni* – indicates that *Bg*GRN-induced haemocytes have a higher percentage

of *Bg*TLR-positive cells while the proportion of *Bg*FREP3-positive cells was unaffected (Figure 4.10).

4.3.5 Confirmation of ERK phosphorylation following *Bg*GRN stimulation

Western blot analysis confirmed that induction of cellular proliferation by *Bg*GRN occurred, in part, via phosphorylation of the p44/42 MAPK in the ERK signalling pathway (Figure 4.11). Injection of 100 nM r*Bg*GRN into BS-90 snails resulted in phosphorylation of ERK1/2 at 15 m post-injection, with maximal phosphorylated ERK compared to total ERK observed at 30 and 60 m post-induction. At 90, 120, and 240 m post-treatment, the amount of phosphorylated ERK declined, but remained detectable. Total ERK levels remained constant. As with other assessments of *B. glabrata* ERK1/2 analyses by Western blot, only a single band was easily visible in these experiments (Zahoor *et al.* 2008).

4.3.6 *Bg*GRN stimulates generation of adherent haemocytes *in vivo*

The baseline number of adherent (spread) haemocytes in BS-90 snails was more than double the mean number in M-line snails [171.5; SEM = 7.3 (n = 30): 83.7; SEM = 3.8 (n = 30), respectively (Figure 4.12)]. Injection of r*Bg*GRN to a final concentration of ~100 nM in the snail haemolymph 48 h prior to haemocyte isolation, significantly increased the number of adherent haemocytes isolated from both snails [BS-90 = 216.5; SEM = 8.9 (n = 30), M-line = 158.4; SEM = 7.5 (n = 30)]. These values reflect a 126% and 189% increase in the number of circulating haemocytes in BS-90 and M-line snails respectively, increasing the number of adherent haemocytes in the M-line snails such that there was no statistically significant difference between the baseline BS-90 and M-line+r*Bg*GRN groups.

I confirmed that the increase in adherent haemocytes was the direct result of *Bg*GRN-induced proliferation by enumerating BrdU+ adherent haemocytes following *Bg*GRN injection in snails (Figure 4.13). Pre-incubation of r*Bg*GRN with a polyclonal anti-*Bg*GRN antibody blocked the increase in adherent cell numbers, confirming that the proliferative effect was the result of application of r*Bg*GRN (Figure 4.12).

Knockdown of *Bg*GRN significantly reduced the number of adherent haemocytes in BS-90 [130.8 (SEM = 6.0)], but not in M-line [76.3 (SEM = 3.1)] snails compared to GFP-specific knockdown controls [BS-90 = 176.2; SEM = 5.5, M-line = 89.1; SEM = 4.1; (Figure 4.12)]. No significant changes in adherent haemocyte numbers isolated from either BS-90 or M-line snails were measured following treatment with either 100 nM recombinant protein control (*Bg*Temptin), or a polyclonal mouse-derived isotype control (anti-HSP70).

4.3.7 Prior stimulation of haemocyte proliferation by *Bg*GRN in naturally susceptible *B. glabrata* snails significantly protects against *S. mansoni* infection

Significantly fewer M-line snails injected with r*Bg*GRN, prior to exposure to *S. mansoni*, developed patent infections compared to snails injected with a recombinant protein control (Figure 4.14A). This trend was observed throughout the course of the intramolluscan infection. The percentage of the experimental snail group (n = 100) that shed *S. mansoni* cercariae was 0%, 14%, 30%, 34% and 32% at weeks 4, 5, 6, 7, and 8 respectively. In contrast, 2%, 59%, 80%, 88% and 84% of the control snails (n = 50) shed cercariae at the same time post-challenge. The percentage of infected snails was significantly different between r*Bg*GRN injected M-line snails and those injected with the recombinant protein control from week 5 post-challenge onward (Figure 4.14A). Sporocysts were not found in any of the M-line snails that did not shed cercariae.

The knockdown of *Bg*GRN in BS-90 snails had a small impact on *S. mansoni* challenge outcome; 6, 10 and 11% shed *S. mansoni* cercariae on weeks 6, 7 and 8 respectively (n = 100), whereas none of the GFP-knockdown controls shed cercariae at any point (n = 50; Figure 4.14B).

4.3.8 *Bg*GRN-induced haemocyte proliferation also decreases susceptibility to *S. mansoni* infection in other *B. glabrata* strains and *Biomphalaria* species

One possibility associated with laboratory models such as the *B. glabrata–S. mansoni* model is that the results may not always directly translate into natural snail–schistosome associations (Mitta *et al.* 2012; Theron *et al.* 2008; Theron *et al.* 2004). To determine whether GRN may also be relevant as a determinant of compatibility in other snail–schistosome associations, haemocyte proliferation was stimulated using *Bg*GRN in four strains of *B. glabrata* (NMRI, M-line, BB-02 and BS-90), and two species of *Biomphalaria – B. pfeifferi* and *B. sudanica*, which are wild species involved in *S. mansoni* transmission in endemic regions of Africa (Brown 1994). These snails were then exposed to NMRI or PR-1 strains of *S. mansoni*. Shed cercariae counts at week 7 post-challenge indicated that significantly ($P < 0.05$) fewer snails were infected by either parasite strain for *Bg*GRN-treated *B. pfeifferi* and *B. glabrata* NMRI, M-line and BB-02 strains (n = 45). Reductions in infection success in these susceptible snails were 31%, 27%, 36% and 33% respectively for *S. mansoni* PR-1 (Figure 4.15A), and 24%, 22%, 26% and 22% for *S. mansoni* NMRI (Figure 4.15B). Only PR-1 *S. mansoni* infected *B. sudanica* snails, the proportion of which was also reduced by r*Bg*GRN injection, but this was not statistically significant. Neither parasite infected the highly resistant *B. glabrata* BS-90 snails as expected (Figure 4.15).

4.4 Discussion

Past studies of *B. glabrata* and other gastropods support a haematopoietic mechanism largely driven by exposure to exogenous factors or pathogens. However, other well-defined haematopoietic models suggest that there is an important role for endogenous growth factors in the haematopoietic process. The results of this study demonstrate that *Bg*GRN is an endogenously produced growth factor that participates in the haematopoietic events that control the production of the circulating haemocyte population. Introduction of *Bg*GRN into *B. glabrata* snails that are naturally susceptible to *S. mansoni* infection resulted in a 54% reduction in the number of snails successfully infected by week 7 post-challenge. That sporocysts were not observed upon dissection of these refractory M-line snails implies that the snails had successfully cleared any parasites.

Underpinning this impressive change in infection outcome is a significant increase in the generation of circulating adherent haemocytes, one of the important functional units of snail resistance to digenean trematode infection (LoVerde *et al.* 1984). Adherent haemocytes, the core haemocyte subset that participates in the encapsulation of *S. mansoni* sporocysts, are also highly phagocytic (Hanington *et al.* 2010a), are responsible for the production of ROS/RNI (Humphries and Yoshino 2008), and soluble immune factors that are important for successful clearance of digenetic trematodes (Galinier *et al.* 2013; Hanington *et al.* 2010a). This cell population nearly doubled following *in vivo* administration of r*Bg*GRN. Worth noting is the fact that increase in the *Bg*GRN-induced adherent haemocyte numbers in the susceptible M-line strain of *B. glabrata* paralleled the mean number of haemocytes present in the naturally refractory BS-90 strain prior to stimulation.

Whether the snails in this study defended against infection by *S. mansoni* because they had higher circulating cell numbers, or because those additional cells were immunologically distinct and conveyed new immune functionality is a central question. To address it, I used two known immunological markers of snail resistance to *S. mansoni* infection: *Bg*FREP3 (Hanington *et al.* 2012; Hanington *et al.* 2010a) and *Bg*TLR (Pila *et al.* 2016b), whose involvement in the snail's immune response is described in the next chapter (chapter 5). That *Bg*TLR was found to be proportionally higher on BrdU+ cells induced by *Bg*GRN, and *Bg*FREP3 was not, suggests that the haemocyte population induced by *Bg*GRN may be more geared towards the cellular immune response than the humoral, and that haemocyte repertoire has significant impact on the ability of a snail to clear or prevent infection (Larson *et al.* 2014).

The processing and mechanism of *Bg*GRN function is consistent with PGRNs of other organisms. *Bg*GRN possesses five predicted elastase cleavage sites that are each located immediately following one of the four 12-cysteine granulin domains. Similar to observations that have been made in studies focusing on human PGRN (Zhu *et al.* 2002), incubation of r*Bg*GRN with elastase results in the production of ~8 kDa granulin fragments that would be predicted should all cleavage sites be processed. At least one intermediate product is consistently present in *Bg*GRN elastase digests that resides at ~18 kDa, similar in size to the ~15 kDa intermediate fragment observed when human PGRN is digested with elastase (Zhu *et al.* 2002). Since porcine elastase was used to cleave r*Bg*GRN, it is possible that differences between porcine elastase and the elastase of *B. glabrata* underpin the cleavage patterns observed.

B. glabrata elastases were found to possess catalytic sites known to be active in human and porcine elastases (Figure 4.16), indicating that they do possess the canonical motifs relevant for traditional elastase function (Korkmaz *et al.* 2010). However, two of the three *B. glabrata*

elastase variants found had only incomplete sequences available, and consequently, the final catalytic residue could not be compared. Future studies could further investigate this issue as well as determine whether *Bg*GRN is processed by other enzymes that have been associated with PGRN processing in other organisms such as the neutrophil protease proteinase 3 (Kessenbrock *et al.* 2008), matrix metalloproteinase 12 (macrophage elastase, Suh *et al.* 2012) and 14 (Butler *et al.* 2008), and a disintegrin and metalloproteinase with thrombospondin motifs-7 (ADAMTS-7, Bai *et al.* 2009). Some of these enzymes have been identified in *B. glabrata* in the context of interactions with developing larval *S. mansoni* stages. ADAMTS and other zinc-dependent metalloproteinase were highly represented in snail plasma proteins that are reactive to *S. mansoni* LTP and sporocyst tegumental proteins (Wu *et al.* 2017). Proteins for these genes were differentially expressed and exclusively recovered from plasma of the NMRI strain of *B. glabrata* that is susceptible to *S. mansoni*.

Native *Bg*GRN was detectable in the plasma of unexposed *B. glabrata* only in its progranulin form; the ~18 kDa cleavage product was only observed in plasma (of both M-line and BS-90 snails) following *S. mansoni* challenge, and the ~8 kDa product was only seen in BS-90 plasma following challenge. In addition, both the intermediate ~18 kDa, and smaller ~8 kDa fragments were observed in the cytoplasmic fractions of *B. glabrata* haemocyte lysates. These observations suggest that activation or stimulation of the haemocytes (or other *Bg*GRN producing cells) leads to the cleavage of *Bg*GRN. These observations are supported by studies demonstrating that in some cases, cleavage of PGRN by enzymes such as matrix metalloproteinase (MMP)-12 occurs within the cytoplasm (Suh *et al.* 2012). Whether the cytoplasmic *Bg*GRN and observed fragments differ functionally from extracellular *Bg*GRN remains unknown. The source of the cytoplasmic *Bg*GRN, which could derive from extracellular

*Bg*GRN that enters haemocytes or post-translational processing of *Bg*GRN prior to secretion remains to be determined.

While the haemocyte receptor mediating the proliferative effects of *Bg*GRN observed in this study is unknown, there are a number of candidate receptors that warrant investigation. Even within the mammalian PGRN literature, there remains uncertainty regarding a definitive GRN receptor. Both sortilin (Zheng *et al.* 2011) and TNF receptors (Jian *et al.* 2013) have been implicated in recognition of PGRN, although recent evidence contradicts these initial reports (Chen *et al.* 2013). Toll-like receptor (TLR) 9 activation has also been found to be reliant on PGRN as a cofactor (Moresco and Beutler 2011). At present, we know very little about whether homologues of these molecules exist in *B. glabrata.* Analysis of the *B. glabrata* genome highlights the presence of genes that possess high predicted amino acid identity to the canonical domains that characterize TNF receptors and sortilin (Adema *et al.* 2017). However, whether these genes are expressed, or encode for proteins that function in a similar manner to known mammalian molecules has not yet been investigated. Of the possible candidate molecules that may interact with *Bg*GRN, TLRs are the best characterized in *B. glabrata*. A number of leucine-rich repeat domain-containing transcripts have been identified in the *B. glabrata* genome, and at least 27 of these encode *bona fide* TLRs possessing a TIR domain (Adema *et al.* 2017). While little is known about the functions of these TLRs, the fact that *B. glabrata* possesses many of the downstream components associated with TLR signalling in other organisms (Zhang and Coultas 2011), and that at least one TLR in *B. glabrata* is highly abundant on *Bg*GRN-induced adherent haemocytes is indicative that they likely retain their role as pattern-recognition receptors in *B. glabrata* and are important participants in the immune response.

While the receptor for *Bg*GRN may remain unknown, it appears that the effects of *Bg*GRN are mediated by signalling via the MAP kinase pathway and ERK1/2. The signalling pathway used by PGRN appears to be a highly conserved aspect of its biology (Ong and Bateman 2003). This is noteworthy, as it has been demonstrated that *S. mansoni* is able to interfere with the phosphorylation of ERK1/2 in susceptible snails, but not those that are phenotypically resistant (Zahoor *et al.* 2008). The kinetics of ERK1/2 phosphorylation following r*Bg*GRN stimulation of haemocytes has also been observed using human recombinant PGRN (Monami *et al.* 2006), however phosphorylated ERK1/2 is detectable for a longer time (4 h compared to ~2 h).

The 12-cysteine motif that characterizes granulins defines the protein and confers its conserved secondary and tertiary structure. It may also be responsible for its numerous biological functions (Bateman and Bennett 2009). Early investigations in carp identified three isoforms of carp GRN (Belcourt *et al.* 1993) that were later associated with mononuclear phagocytes (Belcourt *et al.* 1995). Functional studies of the carp granulin homologue in the goldfish demonstrated that this teleost granulin shares hematopoietic functions with its mammalian counterparts, inducing proliferation of myeloid cells *in vitro* (Hanington *et al.* 2006). Recent studies focusing on a PGRN produced by the flatworm parasite *Opisthorchis viverrini* demonstrate that *Ov*GRN can induce proliferation in fibroblasts of the parasite's human host and fibroblast cell lines, thereby providing a plausible mechanism for the high rates of liver cancer in individuals infected by *O. viverrini* (Smout *et al.* 2009; Papatpremsiri *et al.* 2015). The *Ov*GRN studies provide plausibility for a functional role of parasite GRNs on certain host tissues or, alternatively host GRNs on certain parasite tissues, and highlight the functional and structural conservation of granulins.

The variety of biological functions that PGRN is capable of is impressive, and it is likely that *Bg*GRN may also participate in wound healing, immunological defence and neuronal development in *B. glabrata*. Despite its pleiotropic function, I have demonstrated that *Bg*GRN is an important growth factor in *B. glabrata,* driving haemocyte proliferation and development of an adherent haemocyte subset that is central to the defense of the snail against *S. mansoni*. This represents a number of firsts for the *B. glabrata–S. mansoni* infection model and for the field of gastropod immunobiology. *Bg*GRN is the first functionally characterized endogenous growth factor of any gastropod mollusc, and this represents the first gain-of-resistance study in a snail–digenean infection model that uses a defined factor to induce snail resistance to infection.

In conclusion, while the list of factors and genes that influence compatibility between *B. glabrata* and *S. mansoni* continues to grow, this study demonstrates that haematopoietic events regulated by endogenous factors such as granulin can significantly impact the outcome of *S. mansoni* infection, and that not all haemocytes are equal when considering their role in the immune response.

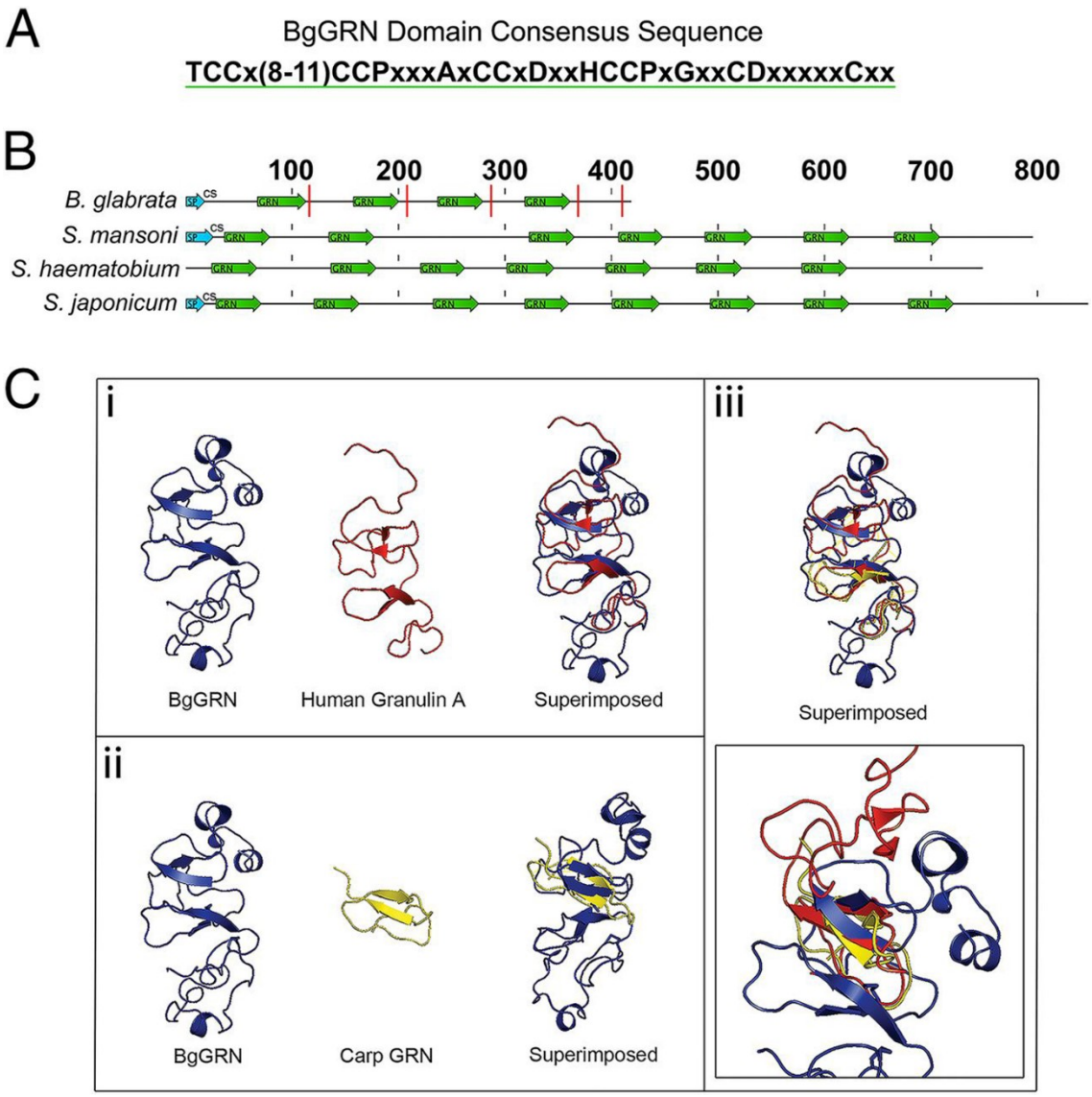

Figure 4.1. *In silico* prediction of *Bg*GRN granulin domain architecture compared to some known and predicted granulins. A. The consensus sequence of the *Bg*GRN granulin domain highlighting the canonical 12-cysteine motif that defines granulin proteins. B. Reference diagram highlighting the predicted locations of the granulin domain (green arrows) in the predicted progranulin protein sequences of *B. glabrata, Schistosoma. mansoni, S. haematobium,* and *S. japonicum.* Predicted signal peptides (SP) are highlighted in blue arrows and are followed by the predicted cut site (CS) for the signal peptide. Predicted elastase cleavage sites for *Bg*GRN are highlighted with a red line intersecting the protein at the approximate cut site. C. The predicted 3D structure of *Bg*GRN compared to the two known crystal structures for human (i) and carp (ii) granulins, with a focus on the similar organization of beta-sheets and alpha-helices (iii).

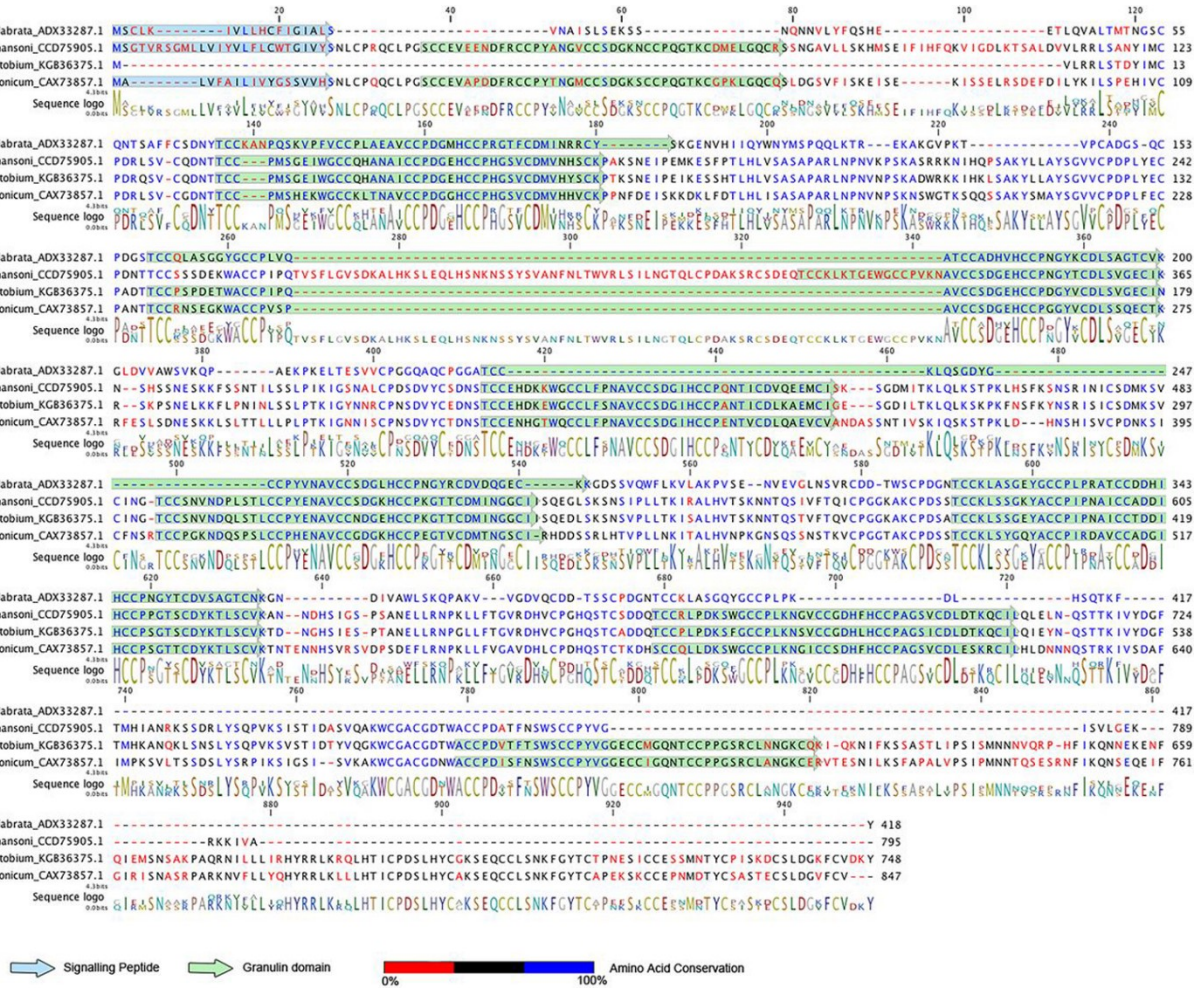

Figure 4.2. Protein alignment of *Bg*GRN with pro-GRNs of *S. mansoni, S. haematobium* and *S. japonicum*. Comparison of the amino acid conservation between the pro-*Bg*GRN and pro-GRN proteins of three schistosomes highlights the conservation of the 12-cysteine motif that defines members of the GRN family. Granulin domains are highlighted in green and highlight the high amino acid conservation of the GRN domains between the three schistosomes. A large stretch of amino acids present in the *S. mansoni* pro-GRN breaks the second GRN domain, and so does an additional amino acid stretch present in all three schistosome pro-GRNs in domain three. *Bg*GRN and *S. japonicum* granulin possess predicted secretion signal peptide domain in residues 1-18 and cleavage sites between residues 18 and 19. *S. mansoni* granulin has a predicted signal peptide from residue 1-26 and cleavage site between 26 and 27. The *S. haematobium* granulin sequence does not possess a traditional signal peptide that can be identified using predictive software.

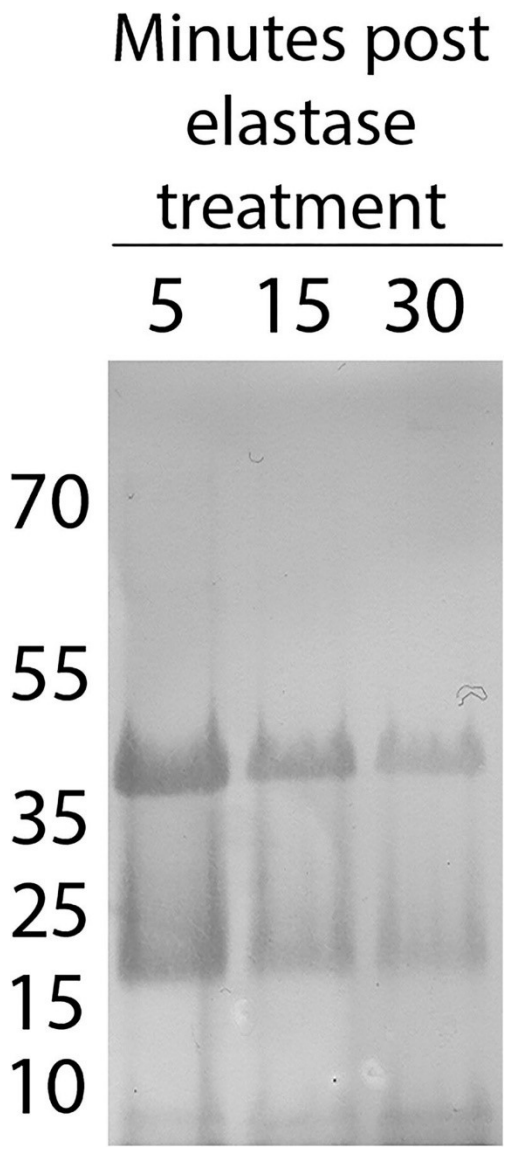

Figure 4.3. Elastase cleavage of r*Bg*GRN yields two differently sized cleavage products. Twenty-five µg of r*Bg*GRN was treated with 0.3 U/mL elastase for 5, 15, and 30 m, after which samples were run on SDS-PAGE, and analyzed via Western blots. Cleavage resulted in the appearance of two distinct products at ~18 kDa and 8 kDa.

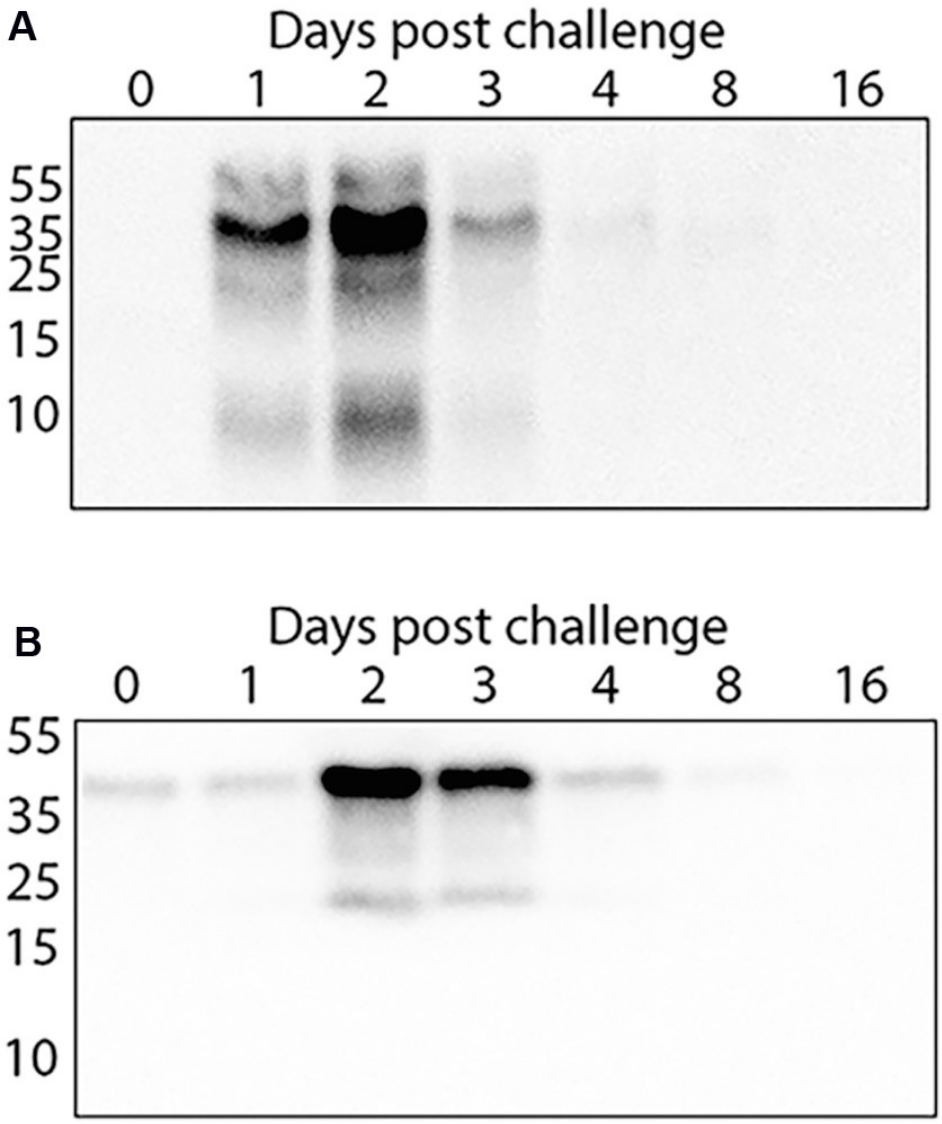

Figure 4.4. Western blot analyses of *S. mansoni*-challenged snail plasma. BS-90 (A) and M-line (B) snails were challenged with 5 *S. mansoni* miracidia. Following the indicated durations in days post-challenge, plasma was isolated and analyzed through Western blots using a mouse anti-*Bg*GRN polyclonal antibody. Sample loads in each lane were normalized to protein concentration. Numbers on the left represent molecular weight markers in kDa.

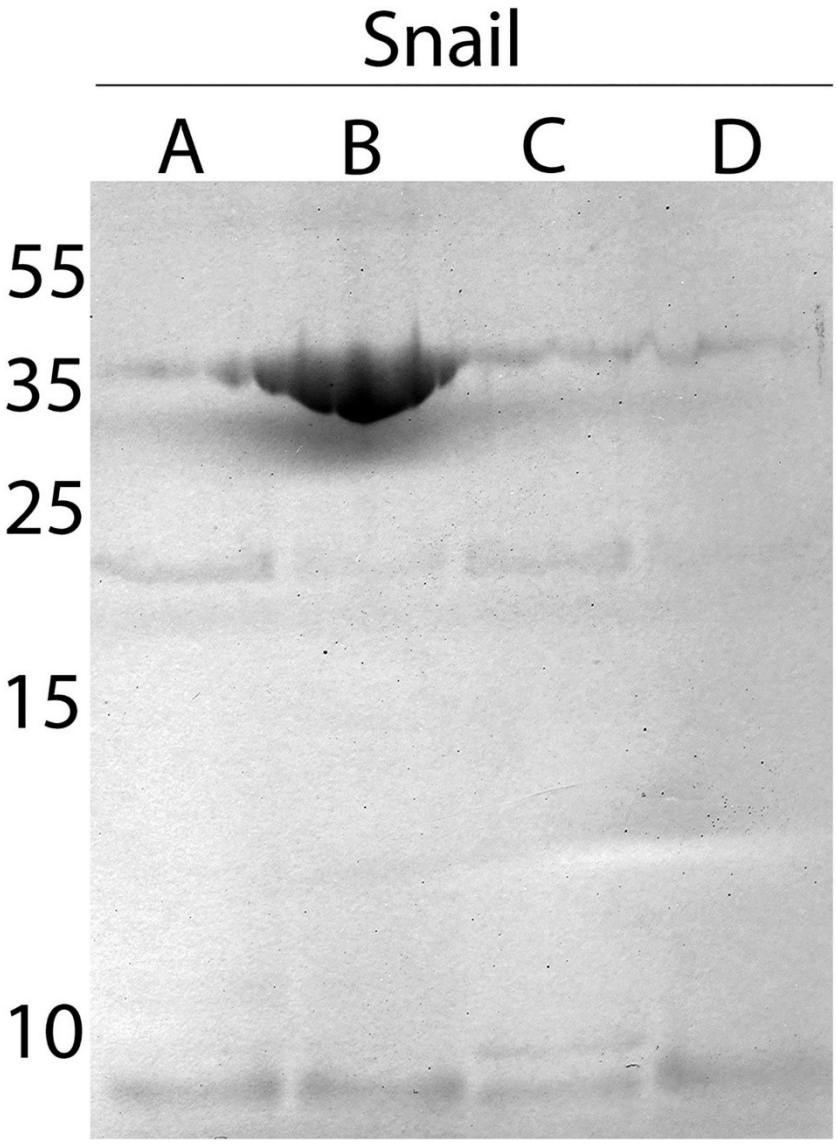

Figure 4.5. Cleavage of *Bg*GRN appears to take place intracellularly within haemocytes. Western blot analysis using anti-*Bg*GRN antibody was carried out on haemocytes isolated from four different pools (A-D) of 5 snails. The results suggest that the ~18 kDa and 8 kDa cleavage products of *Bg*GRN are present within haemocytes.

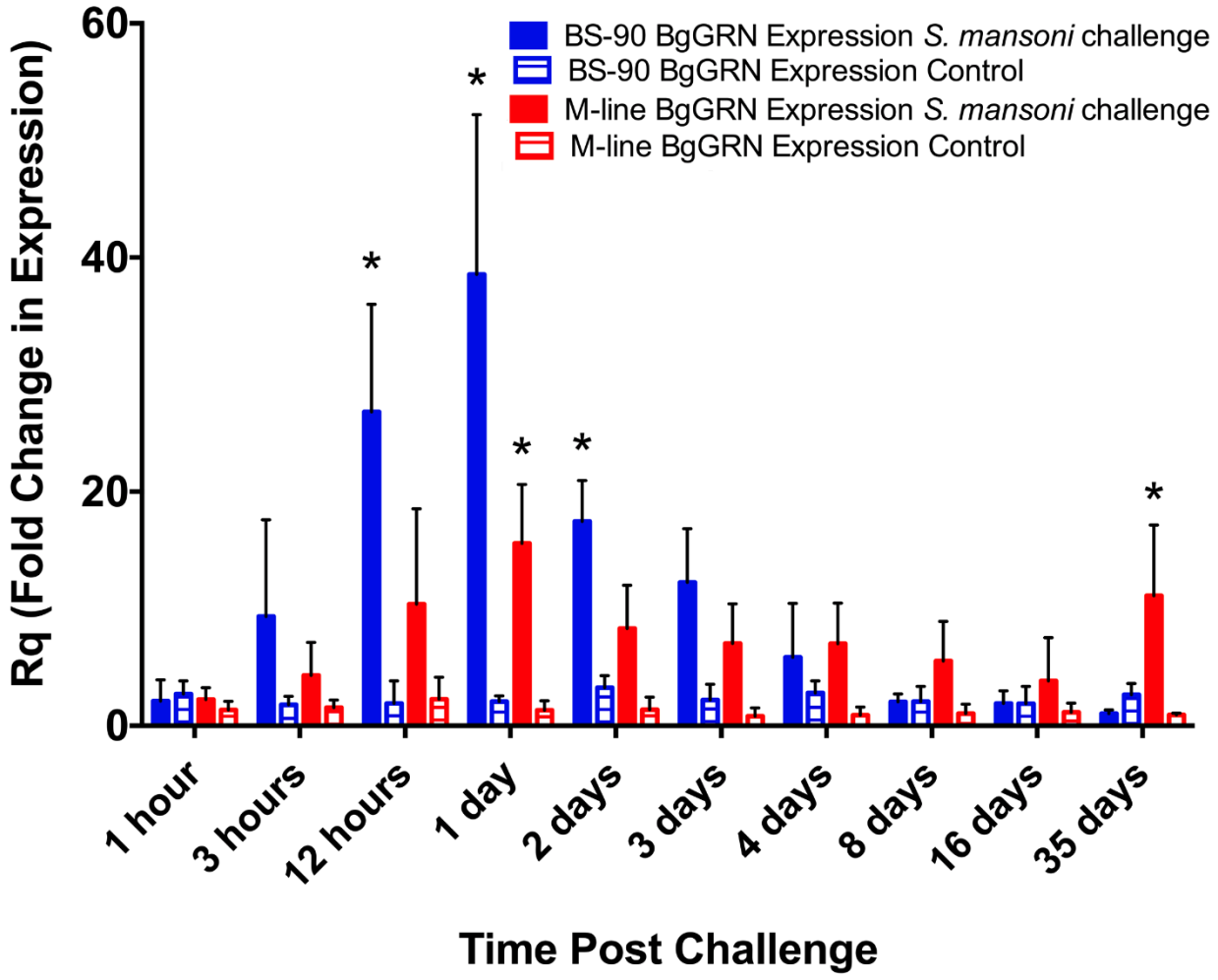

Figure 4.6. Quantitative RT-PCR analysis of *Bg*GRN expression in *B. glabrata* at critical time points during the entire intramolluscan development of *S. mansoni*. Fold change of *Bg*GRN expression in BS-90 (blue bars, *n*=3 for each time point) and M-line (red bars, *n*=5 for each time point) strains challenged with *S. mansoni* (solid bars) or, not-challenged, control (hatched bars) following normalization to a time zero control group (5 snails) and then to the endogenous control β-actin. Bars represent standard error. * = within strain significant difference between *S. mansoni*-challenged and not-challenged snails at the specific time point ($p<0.05$). The *Bg*GRN transcript expression data was derived from one experiment.

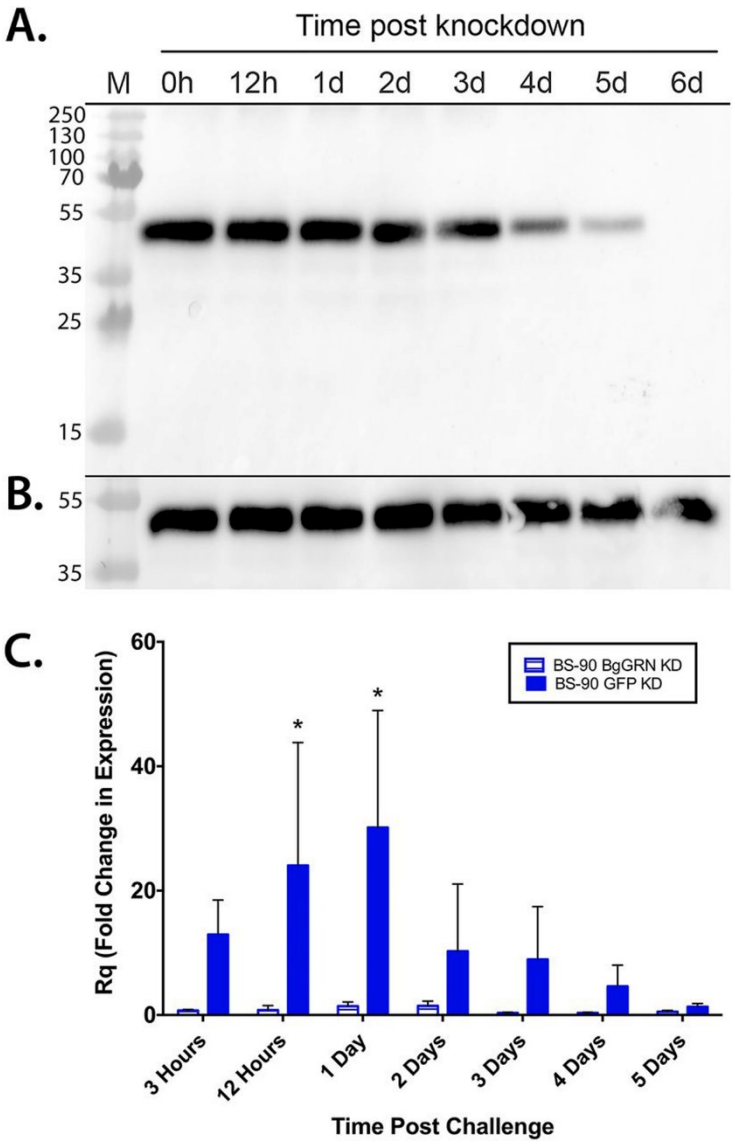

Figure 4.7. Kinetics of siRNA-mediated knockdown of *Bg*GRN *in vivo.* A. Western blot assessment of *B. glabrata* progranulin abundance in *B. glabrata* BS-90 plasma following injection of *Bg*GRN-specific siRNA. B. Knockdown efficacy was compared to control BS-90 snails injected with siRNA oligos specific for green-fluorescent protein (GFP). C. qRT-PCR assessment of *Bg*GRN transcript abundance in *B. glabrata* BS-90 injected with siRNA oligos specific for *Bg*GRN (hatched bars; *n*=5 for each time point) or GFP (solid bars; *n*=5 for each time point) 4 days prior to challenge with *S. mansoni*. Both *Bg*GRN and GFP knockdown samples were normalized to both a time zero control group (*n*=5) and to the endogenous control β-actin. Bars represent standard error. * = a significant difference between *Bg*GRN knockdown and GFP knockdown group means at indicated time point.

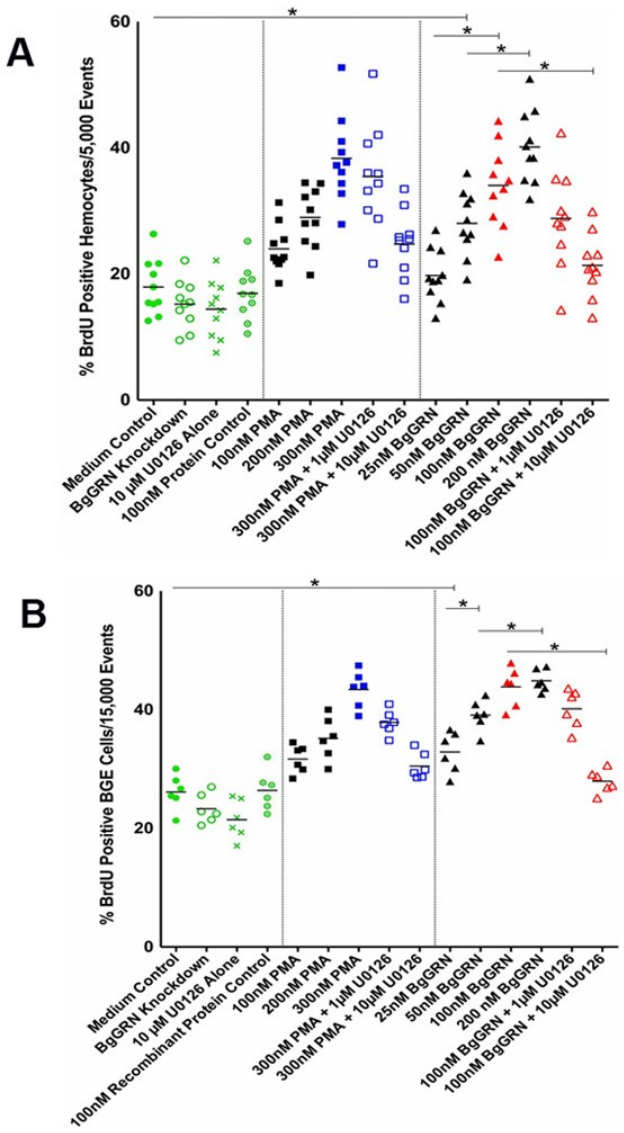

Figure 4.8. *Bg*GRN-induced proliferation of circulating *B. glabrata* BS-90 haemocytes and Bge cells. A. Measurement of the percent BrdU positive BS-90 primary haemocytes (out of 5,000 total events; $n = 10$ independent trials for each treatment) following 48-h stimulation. B. Measurement of the percent BrdU positive Bge cells (out of 15,000 total events; $n = 6$ independent trials for each treatment) following 24-h stimulation. Exposures to 25, 50, 100 and 200 nM r*Bg*GRN were compared to 100, 200 and 300 nM PMA (positive controls) and negative controls (medium, knockdown, recombinant protein and inhibitor only controls). Increases in the % positive BrdU cells were r*Bg*GRN dose-dependent, with an apparent plateau at 100nM. The proliferation-inducing effect of r*Bg*GRN was abrogated by addition of the ERK1/2 blocker U0126 at 10µM, but not at 1µM. Important comparisons of significance are highlighted and indicated by *.

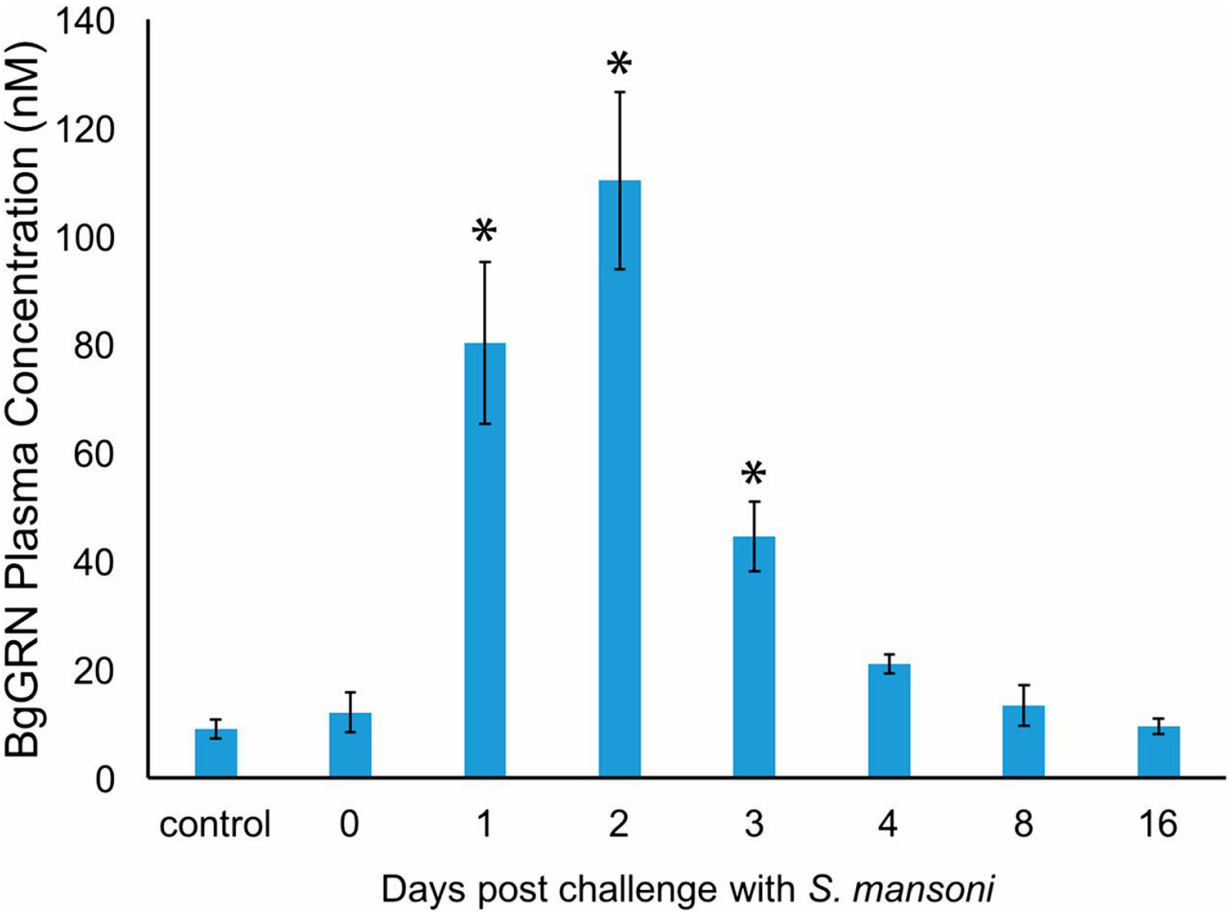

Figure 4.9. Circulating *Bg*GRN in *B. glabrata* BS-90 snail plasma. Indirect ELISA was used to assess the concentration of *Bg*GRN in BS-90 snail plasma at 0, 1, 2, 3, 4, 8, and 16 days post-challenge with *S. mansoni*. Plasma from three different snails was used for independent assessments at each time point. All plasma samples were compared to a standard curve generated using known concentrations of r*Bg*GRN. Bars represent standard deviation. * represents significance compared to control (not challenged) snails. *Bg*GRN plasma concentration was assessed in one experiment.

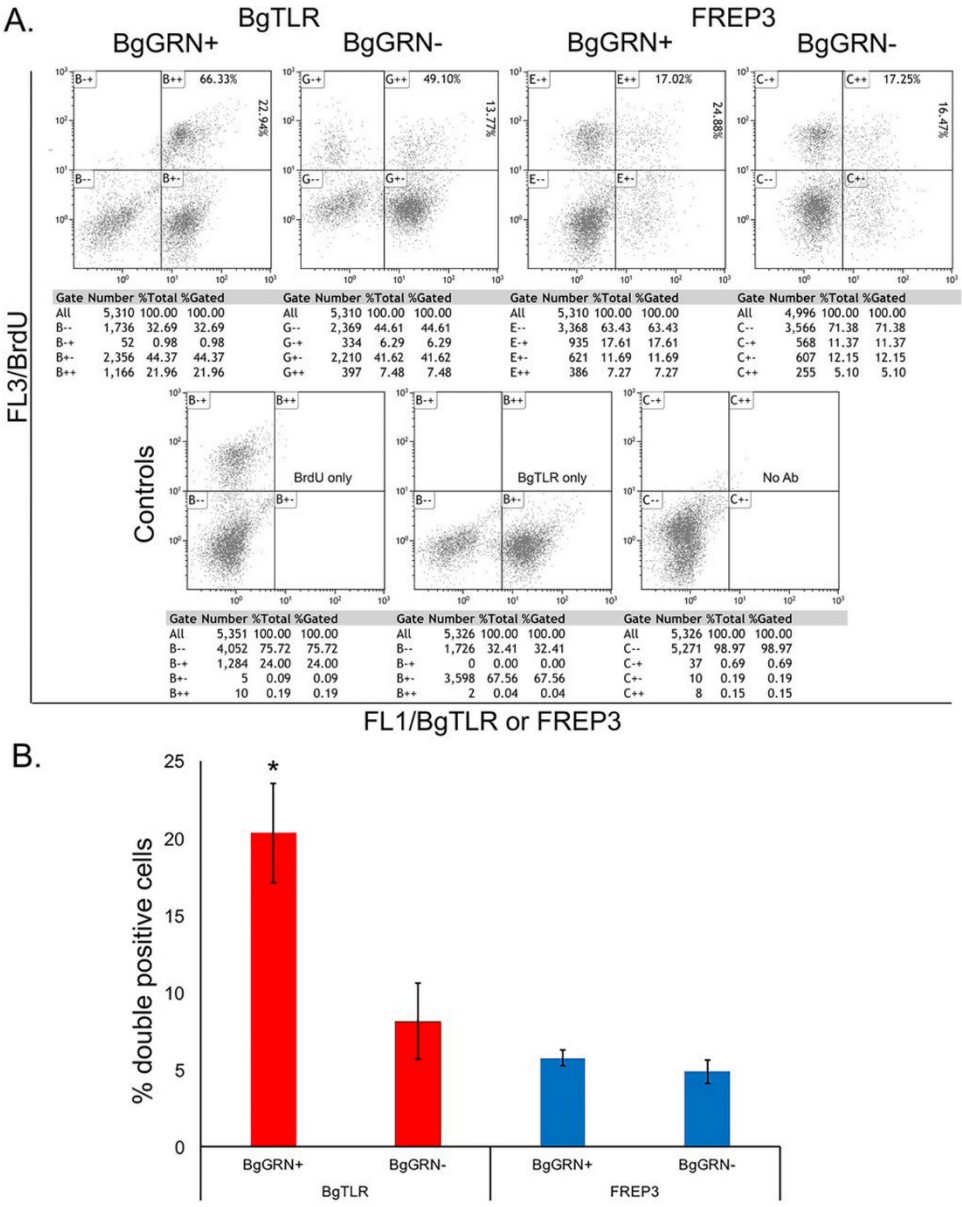

Figure 4.10. *Bg*GRN-induced haemocyte proliferation increases the proportion of *Bg*TLR-positive cells. BS-90 snails were co-injected with BrdU and r*Bg*GRN or BrdU and medium-only as control. After 48 h, haemocytes were isolated, processed and co-stained for BrdU and immune protein of interest. (A) Co-labelling of BrdU and *Bg*TLR or FREP3. Controls include single staining for BrdU, *Bg*TLR or no antibody. For the co-labelled profiles, the top right box shows the proportion of double positive adherent haemocytes that are *Bg*TLR- or FREP3-positive respectively (top) and BrdU-positive (right). (B) Plot of percent double positive cells for the different treatments derived from panel A in a single experiment.

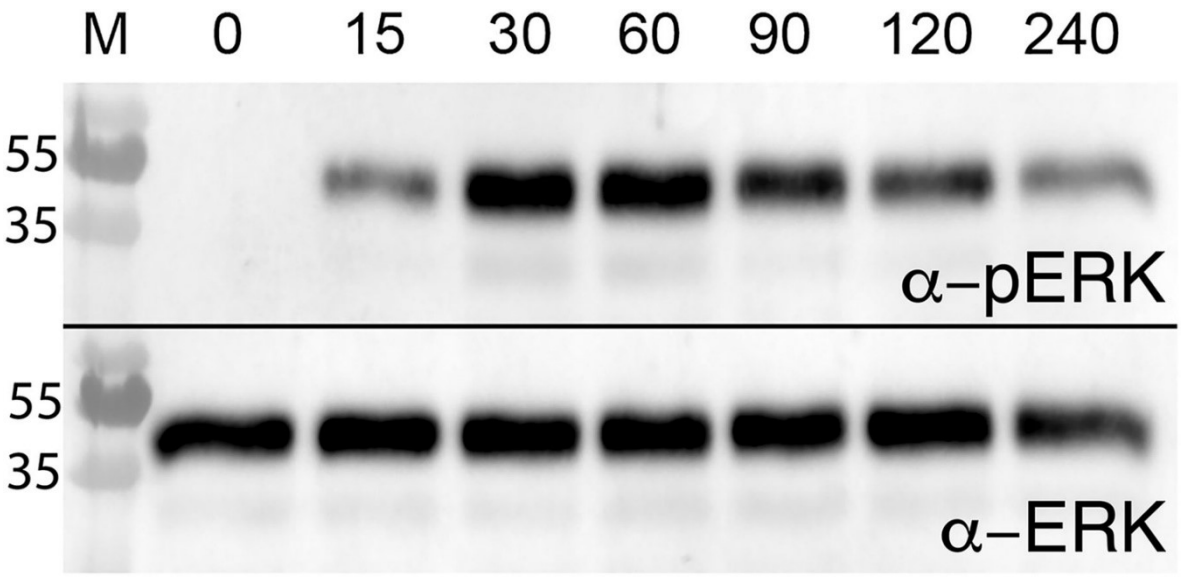

Figure 4.11. Proliferative effect of r*Bg*GRN relies on signaling via ERK1/2 and the MAPK pathway. Western blot analysis of ERK1/2 was carried out following r*Bg*GRN injection into BS-90 snail haemocoels. Using an anti-phosphorylated ERK1/2 antibody, ERK phosphorylation was assessed over 240 m at the indicated time points in minutes. Numbers to the left represent molecular weight marker (M) sizes in kDa.

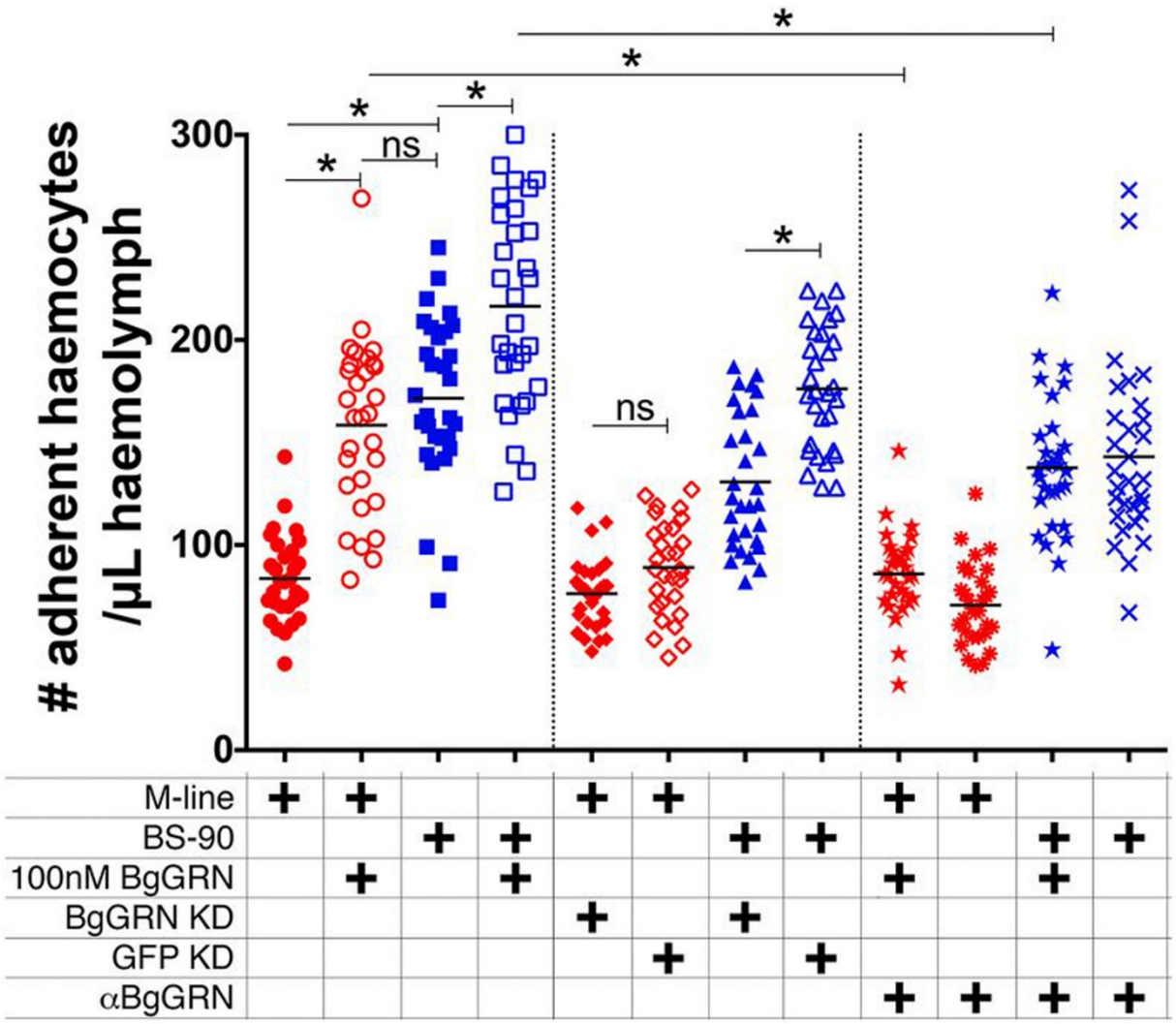

Figure 4.12. Effects of r*Bg*GRN, knockdown of *Bg*GRN and anti-*Bg*GRN polyclonal antibody on the number of adherent haemocytes. Snails were injected either with 100 nM r*Bg*GRN (induction) or r*Bg*GRN pre-incubated with anti-*Bg*GRN antibody (α*Bg*GRN, abrogation treatment) and 48 h later, haemocytes were isolated and used for adherent haemocyte counts. Knockdown snails received siRNA oligos against *Bg*GRN or GFP (control) 48 h prior to the above treatments. Important comparisons of significance are highlighted and indicated by *. Adherent haemocyte numbers represent data derived from one experiment.

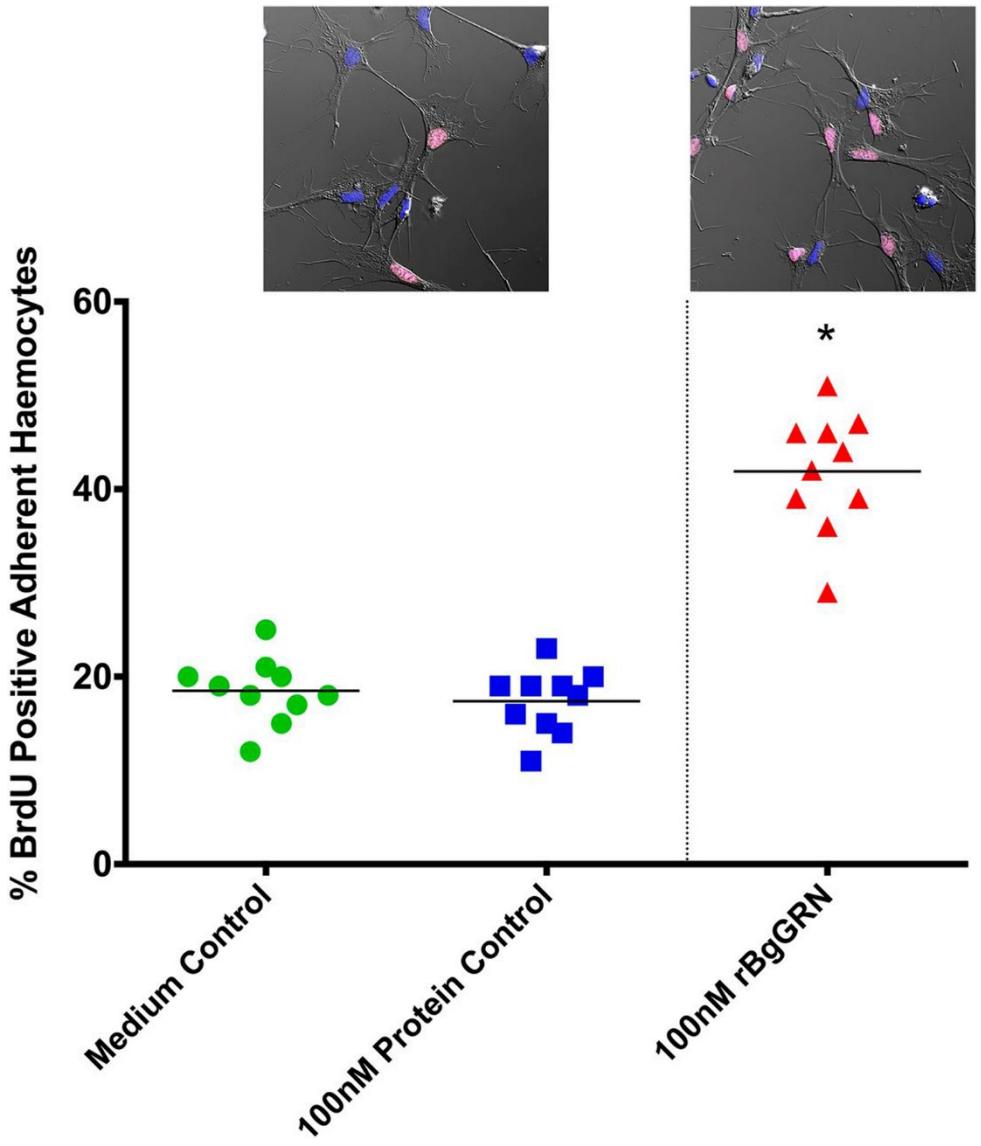

Figure 4.13. Increase in adherent haemocytes are the direct result of *Bg*GRN-stimulated haemocyte proliferation. *B. glabrata* BS-90 snails were co-injected with BrdU and r*Bg*GRN or BrdU and medium-only as control. After 48 h, haemocytes were isolated and placed directly onto microscope slides to adhere for 2 h in a humidified chamber. Following incubation, haemocytes were fixed, permeabilized and stained with anti-BrdU mouse monoclonal antibody. BrdU-positive haemocytes were counted out of 100 adherent haemocytes from random fields of view (insets) and percentages expressed in a graphical form. BrdU-positive cells (new) are shown in pink while already existing haemocytes are in blue.

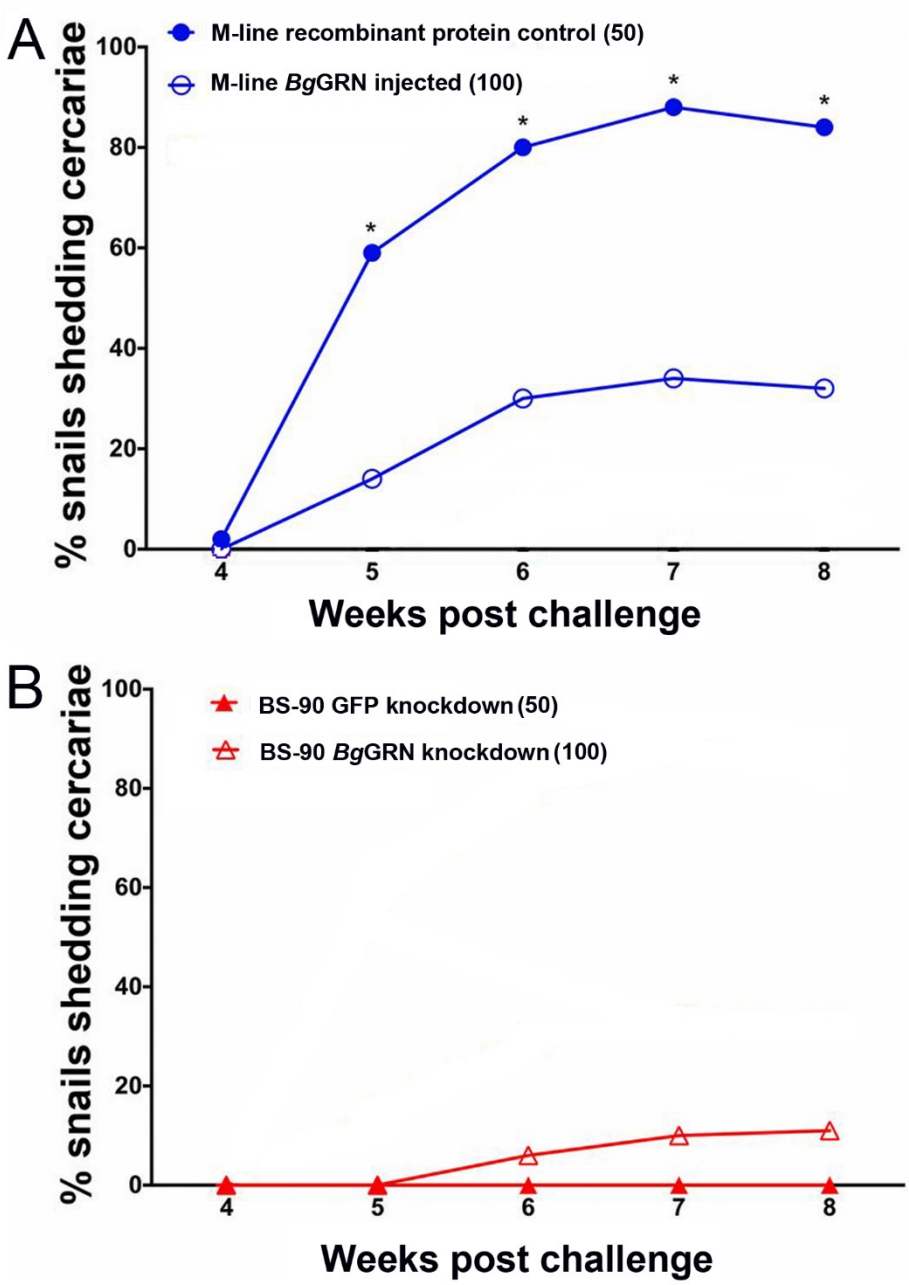

Figure 4.14. *Bg*GRN induces the proliferation and development of adherent haemocytes to the point that susceptible M-line *B. glabrata* are able to defend against *S. mansoni* infection. A. Injection of r*Bg*GRN into M-line *B. glabrata* 4 days prior to challenge with *S. mansoni* significantly (*) impacted *S. mansoni* infection success. B. Complementary knockdown of *Bg*GRN in BS-90 *B. glabrata* also altered *S. mansoni* infection success rate, albeit not significantly. *Bg*GRN injection and knockdown data represent one experiment each.

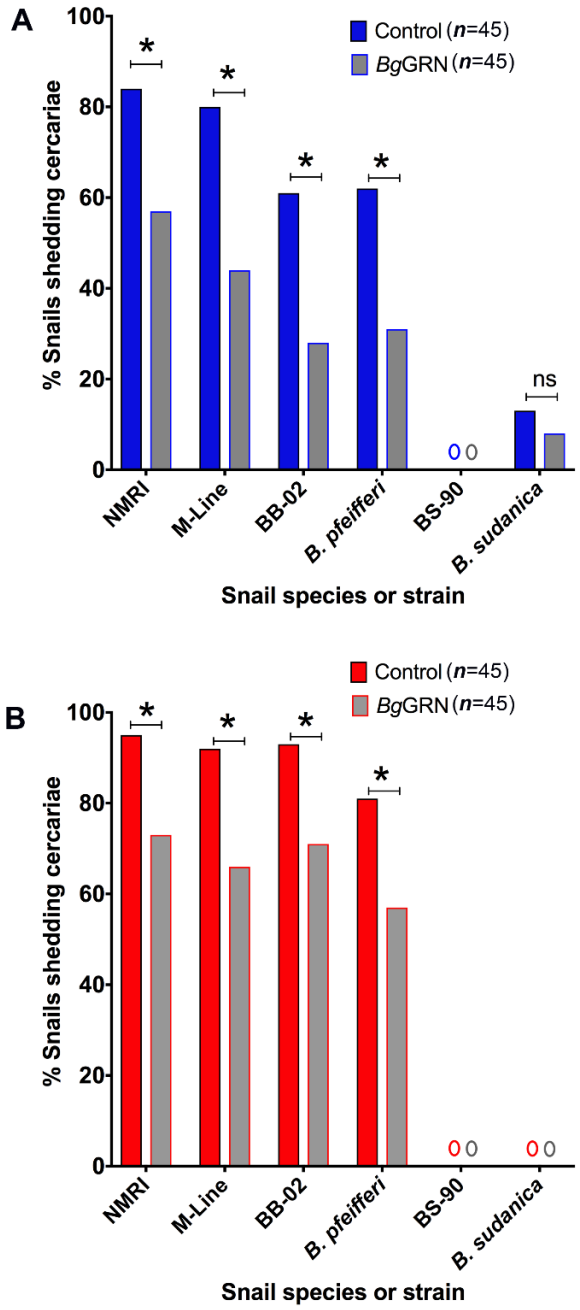

Figure 4.15. Recombinant *Bg*GRN treatment reduces schistosome infectivity in other snail-schistosome interactions. Injection of r*Bg*GRN into *Biomphalaria* snails [*B. glabrata* (NMRI, M-line, BB02 strains) and *B. pfeifferi*] 4 days prior to challenge with *S. mansoni* PR-1 (A) or NMRI (B) significantly (*) impacted *S. mansoni* infection success. *Bg*GRN injection in *B. sudanica* also altered *S. mansoni* PR-1 infection success rate, albeit not significantly. BS-90 snails served as resistant phenotype control for the *S. mansoni* parasite strains used ($n = 45$ for all groups in one experiment).

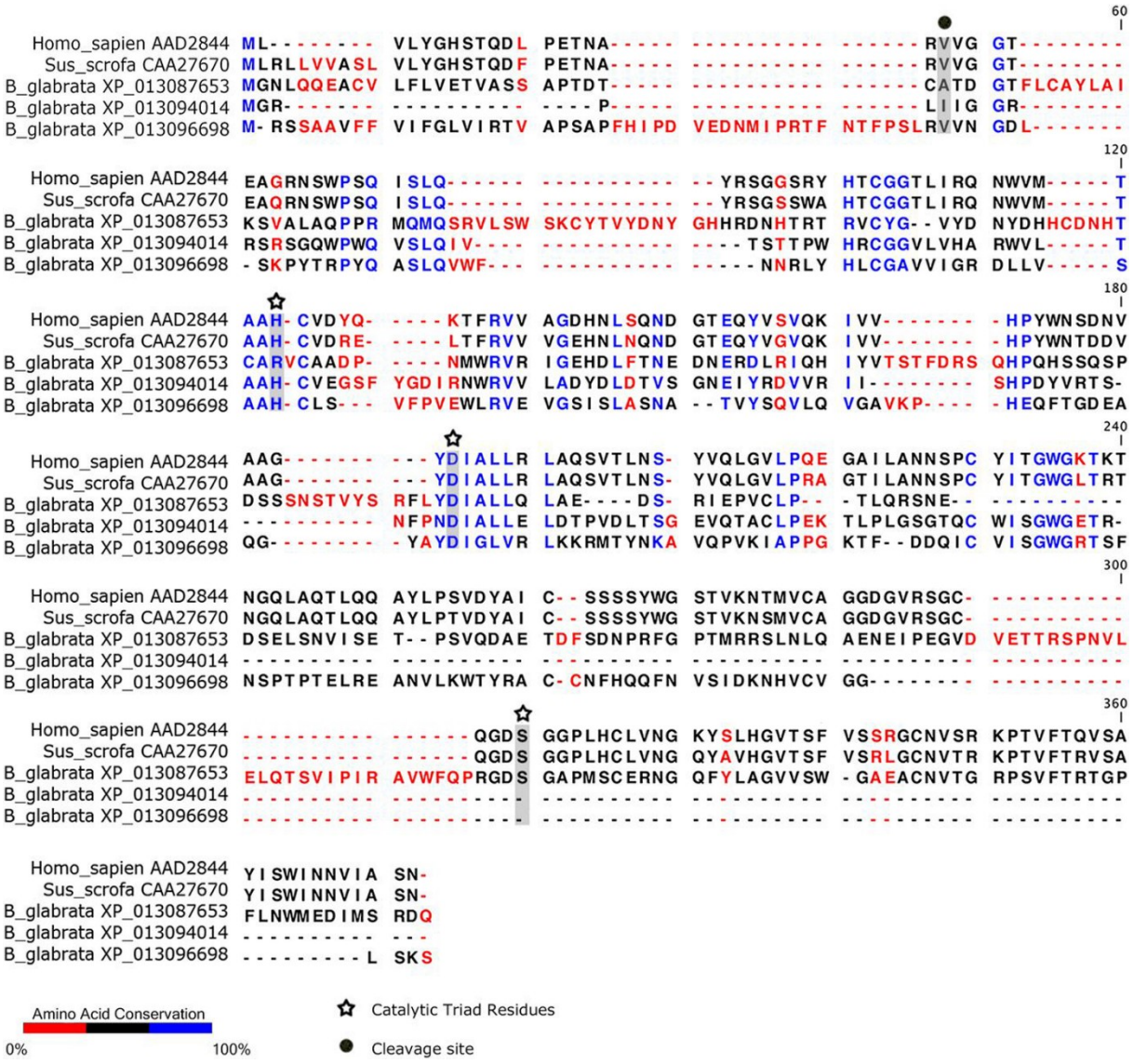

Figure 4.16. Amino acid alignment of human, porcine and *B. glabrata* elastases. Analysis of the predicted amino acid sequence of *B. glabrata* elastase suggests that it possesses all relevant catalytic sites known to be active in human and porcine elastases. Only incomplete sequences were available for two of the three *B. glabrata* elastase variants, therefore, the final catalytic residue could not be compared. *Homo sapiens* elastase (AAD2844.1), *Sus scrofa* elastase(CAA27670), and the three *B. glabrata* predicted elastase proteins (XP_013096698, XP_13094014, XP_013087653) were aligned in CLC Genomics workbench using the alignment tool with a gap open cost of 10, a gap extension cost of 1.0, and an end gap cost of 'as any other' using the very accurate (slow) option. The active sites were identified using the NCBI conserved domains database.

Chapter 5

Molecular and Functional Characterization of a *Biomphalaria glabrata* Toll-like Receptor[3]

5.1 Introduction

One of the major evolutionarily conserved pathways in innate immunity is the Toll/Toll-like receptor (TLR) pathway. TLRs are trans-membrane proteins composed of an extracellular leucine-rich repeat domain, responsible for pathogen recognition and a conserved cytoplasmic Toll/IL-1 (TIR) domain, which is responsible for signal transduction and activation of effector functions. In 2007, Zhang and colleagues (Zhang *et al.* 2007) published homologues of Gram-negative binding protein (GNBP) and peptidoglycan recognition protein (PGRP) in *Biomphalaria glabrata*. Both are extracellular components that can activate TLR and the related immune-deficiency (IMD) pathways, respectively. Later, they also identified the nuclear factor kappa B (NF-kB), a major downstream transcription factor in the TLR pathway. NF-kB expression patterns in resistant snails infected with schistosome parasites was found to be consistent with the early immune response patterns critical for parasite killing (Zhang and Coultas 2011). Humphries and Harter (2015) made the first identification of binding motifs of *B. glabrata* NF-kB and showed through electrophoretic mobility shift assays that the Rel homology domain (RHD) of *B. glabrata* NF-kB bound the identified kB motifs, as well as a consensus vertebrate kB motif (Humphries and Harter 2015). However, it was not known whether, and how, TLR was involved in regulating parasite infection in this snail.

[3] A version of this chapter has been published: Pila, E.A., Tarrabain, M., Kabore, A.L. and Hanington, P.C. (2016). A novel Toll-like receptor (TLR) influences compatibility between the gastropod *Biomphalaria glabrata* and the digenean trematode *Schistosoma mansoni*. *PLoS Pathogens* 12 (3), e1005513.

While comparing haemocyte surface proteins between *Schistosoma mansoni*-susceptible and resistant *B. glabrata* snails, past studies in our laboratory discovered peptides of a TLR-like protein, some of which displayed a high amino acid sequence identity with the TIR domain of known vertebrate TLRs. Peptides associated with this protein were at a significantly higher abundance in the resistant snail strain. These findings along with the fact that this TLR-like protein appeared to be the only one found in the snail genome at the time informed its initial choice as one of the immune genes to investigate as part of my doctoral research. Furthermore, as discussed in chapter 4 (characterization of progranulin in *B. glabrata*), the proportion of haemocytes positive for this TLR increased in snails injected with recombinant progranulin (r*Bg*GRN) compared to controls; prompting me to further investigate this putative TLR.

In this chapter, I present the results of the first functional characterization of a TLR in the snail *B. glabrata*. TLRs play a key role in the innate immune response by directly recognizing a variety of pathogens (typically bacteria, viruses and fungi) or factors that are released during cell stress, damage or death and transducing signals to the immune cells (Bowie and O'Neill 2000). The leucine-rich repeat (LRR) region of TLRs recognizes conserved motifs on pathogens known as the pathogen-associated molecular patterns (or PAMPs) such as bacterial lipopolysaccharide, flagellin protein and genomic DNA containing unmethylated CpG motifs (Hoshino *et al.* 1999; Hayashi *et al.* 2001; Hemmi *et al.* 2000), viral DNA (Hochrein *et al.* 2004), fungal zymosan (Takahara *et al.* 2004) and the *Plasmodium* pigment haemozoin (Coban *et al.* 2005). The Toll/TLR pathway can also be activated by host endogenous ligands or molecules that signal tissue damage – the so-called damage-associated molecular patterns (DAMPs). These include molecules such as uric acid, fibrinogen, mitochondrial DNA, heat-shock proteins 60 and 70 and fibronectin (Bryant *et al.* 2015). *Drosophila* Toll, the founding member of the TLR family is not

a direct recognition receptor. Instead, pathogen components trigger the activation of protease cascades that then lead to the cleavage of the Toll ligand – Späetzle (Michel *et al.* 2001). The cytoplasmic region of TLRs which shares homology with the interleukin-1 receptor [known as a Toll/interleukin-1 receptor (TIR) domain, (Hoshino *et al.* 1999)] is responsible for transducing signals from pathogen recognition to the immune cells leading to the activation of the effector functions. This pathway is evolutionarily conserved from nematodes to mammals (Akira *et al.* 2006).

The *B. glabrata* TLR that I have characterized in this study (GenBank accession number: JX014259.1, herein after referred to as *Bg*TLR) possesses complete LRR and TIR domains, and I demonstrate here the involvement of *Bg*TLR in the immune response of *B. glabrata* against *S. mansoni.* This implies that *Bg*TLR has a novel functionality with respect to helminth parasites, making it unique among known TLRs, one of the most evolutionarily conserved pattern-recognition receptors and cognate signalling pathways in immunity.

5.2 Experimental design

5.2.1 Measurement of *Bg*TLR expression in *B. glabrata* snails during parasite challenge

Quantitative real-time polymerase chain reaction (qRT-PCR) was used to measure *Bg*TLR expression patterns in *S. mansoni*-challenged and control snails. Resistant (BS-90, Paraense and Correa 1963; Richards and Merritt 1972) and susceptible (M-line, Newton 1955; Cooper *et al.* 1994) snails were exposed individually to 5 miracidia of *S. mansoni* NMRI. Five snails were collected at selected time points (0, 1, 3, 12 h and 1, 2, 3, 4, 8, 16, and 35 days post challenge) that represent important milestones in the life cycle of *S. mansoni*, and encompass the

time from miracidia infection to cercarial shedding in the snail. RNA was extracted from whole snails, converted into cDNA and used in qRT-PCR as described in Chapter 3.

5.2.2 *Bg*TLR knockdown and phenotypic influence on resistance

Snails (~ 8 mm shell diameter) were injected with a cocktail of 27-mer siRNA oligonucleotides designed to specifically target 4 different regions of the *Bg*TLR transcript or, oligonucleotides targeting the green fluorescent protein (GFP) as knockdown control. The effect of siRNA-mediated knockdown of *Bg*TLR was assessed in both BS-90 and M-line snails without parasite challenge, and BS-90 snails that have been exposed to *S. mansoni*. Five snails were collected for each time point [at 0, 3, 12 h and 1, 2, 3, 4 and 5 days (challenged BS-90); 0, 3, 12 h and 1, 2, 3, and 4 days (non-challenged BS-90 and M-line) post-injection]. RNA extraction, cDNA synthesis and qRT-PCR were performed as described Chapter 3. *Bg*TLR protein knockdown was confirmed at 1, 2, 3, 4, 5 and 6 days post-injection by Western blot using both haemocytes isolated from BS-90 snails and the Bge cell line, which has haemocyte-like properties (Coustau *et al.* 2003; Yoshino *et al.* 1999).

Phenotypic influence of *Bg*TLR transcript knockdown was assessed in BS-90 snails. Snails were injected with *Bg*TLR-specific siRNA oligonucleotide mix as described above and after 48 h exposed individually to 5 miracidia. Four weeks later (and weekly thereafter), snails were assessed for cercariae shedding, which was used as an indicator of the influence of *Bg*TLR knockdown on the resistance phenotype. *Bg*TLR knockdown snails were compared to BS-90 snails injected with the GFP-specific siRNA oligonucleotides, and to M-line snail controls challenged at the same time.

5.2.3 Phagocytosis assay

Five BS-90 snails each were injected with siRNA targeting *Bg*TLR or GFP (control) and 96 h later, haemolymph was extracted from the snails by the head-foot retraction method and processed in a phagocytosis assay as described in Chapter 3.

5.2.4 Immunocytochemistry

To visualize *Bg*TLR in *B. glabrata* haemocytes and Bge cells, immunocytochemistry was done using an anti-*Bg*TLR polyclonal antibody raised in rabbits against the extracellular domain of *Bg*TLR (GenScript), and a goat anti-rabbit IgG secondary antibody conjugated to Alexa555 fluorophore (Life Technologies) as described in Chapter 3.

5.2.5 Statistical analysis

To determine significant differences in *Bg*TLR transcript levels, one-way analysis of variance (ANOVA) with Tukey's post-hoc tests were performed using GraphPad Prism version 6.0f for Mac OS X (GraphPad Software, California USA, www.graphpad.com). Significant differences in the proportions of snails shedding cercariae were determined using the z-test. Statistical significance threshold was set at $P \leq 0.05$.

5.3 Results

5.3.1 *Bg*TLR displayed increased transcript expression in BS-90 snails following challenge with *S. mansoni*

To determine whether *Bg*TLR transcript expression was modulated by *S. mansoni* challenge, I used qRT-PCR to measure its expression patterns in schistosome-challenged snails,

which were compared to control non-challenged snails at each experimental time point, and normalized against the endogenous control of β-actin expression. *Bg*TLR expression was rapidly induced in the *S. mansoni*-resistant (BS-90) snails responding to *S. mansoni* challenge, with transcript abundance significantly increasing 14-fold as early as 12 h post challenge compared to the 0 h time point. The highest value recorded was a 27-fold increase at 1 day post-challenge, which was significantly different from all other time points ($P < 0.05$). This was followed by reduced *Bg*TLR transcript abundance that returned to pre-exposure levels by day 8 post-challenge (dpc, Figure 5.1). In contrast, *S. mansoni* challenge did not induce significant changes in *Bg*TLR transcript expression in the susceptible (M-line) snails (Figure 5.1). Non-exposed BS-90 and M-line snails expressed very similar levels of *Bg*TLR transcript as determined by comparison of absolute expression (C_t values), which ranged approximately from 7.2 to 7.5.

5.3.2 Predicted structure of *Bg*TLR

The full-length coding sequence of *Bg*TLR is composed of 3,546 nucleotides. The open reading frame predicts a protein of 1,181 amino acid residues. Its ectodomain contains 23 LRR motifs spanning residues 3–725 as predicted by LRRfinder (Offord and Werling 2013) and ScanProsite tools (de Castro *et al.* 2006). The TIR domain is encoded by residues 899–1,037, preceded by a transmembrane region on residues 849–870 (Figure 5.2). There is no identifiable signal peptide in *Bg*TLR based on current predictive software. BlastP analyses (Altschul *et al.* 2005) indicate that the TIR region of *Bg*TLR shares the highest identity to other molluscan TLR TIR regions (65–88%), followed by those of arthropods (51–58%). Among the mammals, TLRs having closest TIR region identities to *Bg*TLR are TLR 4 (40%), TLR 3 (38%) and TLR 13

(37%). The LRR region of *Bg*TLR is much more variable, with identity range of 27–43% only to other molluscan and arthropod TLRs.

Amplification specificity for *Bg*TLR was confirmed using qRT-PCR melt curve and customized BLAST sequence analyses (Figure 5.3 and Figure 5.4) while siRNA knockdown specificity was assessed by measuring the transcript abundance of the three sequences in the *B. glabrata* genome that shared the highest nucleotide identity with *Bg*TLR using *Bg*TLR knockdown cDNA as a template (Figure 5.5). The peptide fragment used for the synthesis of the antibody used in this study for Western blot and immunocytochemistry analyses was also confirmed to be specific by testing the binding of peptide pre-incubated antibody to *Bg*TLR (Figure 5.6).

5.3.3 siRNA-mediated knockdown of *Bg*TLR

Injection of an siRNA oligonucleotide cocktail targeting the extracellular LRR region of *Bg*TLR (Figure 5.7) into M-line and BS-90 snails induced a measurable knockdown of *Bg*TLR expression in both snail strains. Maximum knockdown was observed at 3 days post-injection with transcript levels of 0.09 and 0.04-fold compared to the time 0 h control for M-line and BS-90 strains respectively. Both were significantly different from time 0 h control ($P < 0.05$, Figure 5.8B). Knockdown effect of *Bg*TLR siRNA was sustained in BS-90 snails following *S. mansoni* challenge with *Bg*TLR transcript levels reducing to 0.4-fold at 3 days post-injection. In comparison, snails injected with siRNA oligonucleotides targeted to GFP as a control displayed a 3-fold *Bg*TLR expression increase at 3 days post-injection (Figure 5.8A). *Bg*TLR knockdown kinetics at the protein level in BS-90 snails lagged behind transcript knockdown with an

observable reduction in *Bg*TLR protein abundance at 4 days post-injection as determined by Western blots using both haemocytes isolated from BS-90 snails and the Bge cells (Figure 5.9).

5.3.4 *Bg*TLR immunolocalization in haemocytes and Bge cells

Immunocytochemical studies using an antibody developed against the extracellular region of *Bg*TLR (Figure 5.7) indicate that it is expressed in the majority of haemocytes found in the circulation of *B. glabrata*, although some haemocytes appeared not to express *Bg*TLR (Figure 5.10). In the Bge cell line, all cells appear to be *Bg*TLR-positive with differences in staining intensity among cells (Figure 5.11). *Bg*TLR positive signals were detected primarily on the extracellular surface of the plasma membrane, and faint immunolocalization was observed in the cytoplasm of both haemocytes and Bge cells (Figure 5.11). Intracellular localization may be due to *Bg*TLR trafficking between the cellular membrane and intracellular compartments similar to TLR 4 expression in human monocytes (Latz *et al.* 2002) or, it may indicate that *Bg*TLR has a role in the intracellular environment. Future studies focused on identifying the *Bg*TLR ligand(s) might clarify its subcellular localization and possible intracellular role.

5.3.5 Knockdown of *Bg*TLR in BS-90 snails decreases haemocyte phagocytic response

Knockdown of *Bg*TLR significantly ($P<0.05$) decreased the mean number of *S. mansoni* sporocyst ES products/lysate-coated beads phagocytosed by individual haemocytes of BS-90 snails. Snails injected with siRNA oligonucleotides targeting *Bg*TLR and 96 h later assessed for phagocytic activity averaged 13.1 beads per haemocyte, whereas, GFP knockdown control snails averaged 17 beads per haemocyte (Figure 5.12A). A breakdown of the number of beads observed in each haemocyte demonstrated that haemocytes from *Bg*TLR KD snails were much less likely to take in more than 25 beads per haemocyte compared to the GFP controls (Figure 5.12B). It is

worth noting that based on the assay used, the phagocytic events reported here do not differentiate beads that were associated with cell membranes or actively captured and partially engulfed by haemocytes from completely internalized beads.

5.3.6 Knockdown of *Bg*TLR in resistant BS-90 snails yields cercariae-producing *S. mansoni* infections

Knockdown of *Bg*TLR abrogated the resistant phenotype of BS-90 snails to *S. mansoni* infection. Snails injected with siRNA oligonucleotides and 2 days later challenged with *S. mansoni*, developed patent infections that led to cercariae shedding in ~43% of BS-90 snails over two trials, whereas none of the control BS-90 snails (injected with GFP siRNA) shed cercariae (Figure 5.13). BS-90 snails that were injected with siRNA targeting *Bg*TLR started shedding cercariae one week prior to the susceptible M-line snails, which served as a benchmark for infection success. At week 4 post-exposure, 2% of the BS-90 *Bg*TLR knockdown snails shed cercariae. This increased to 43% at week 5 and then decreased to 22% at week 7 when the experiment was terminated. The proportions of BS-90 *Bg*TLR knockdown snails shedding cercariae at weeks 5, 6 and 7 were statistically significant ($P<0.05$) compared to BS-90 GFP knockdown snails. In comparison, M-line snails started shedding cercariae only at week 5 (46%), increasing to 56% at week 7 (Figure 5.13). There was no statistically significant difference at week 5 in the proportions of snails shedding cercariae between BS-90 *Bg*TLR knockdown and the M-line susceptible control.

Injection of siRNA oligonucleotides resulted in an overall mortality of 27%, 17% and 19% for BS-90 *Bg*TLR knockdown, BS-90 GFP knockdown and the M-line susceptible controls respectively. It is worth noting that these mortalities might have impacted the percentages of

shedding snails which were calculated out of the total starting number. Since snails were not tracked individually over the course of the experiment, these percentages likely underestimate shedding snails that were lost in the mortalities.

5.3.7 Challenge with Gram-negative bacteria does not induce strong expression of *Bg*TLR transcripts

To gain insight into the range of PAMPs detected by *Bg*TLR, thus guiding future experiments that might be designed to screen for *Bg*TLR ligands, I measured *Bg*TLR transcript expression in *B. glabrata* snails responding to injection with two Gram-negative bacteria – *Aeromonas salmonicida*, a known aquatic pathogen (Byers *et al.* 2002) and *Escherichia coli*, a common bacterial species with both pathogenic and commensal strains (Picard *et al.* 1999). As shown in Figure 5.14, neither bacteria elicited strong *Bg*TLR transcript levels in BS-90 or M-line snail strains compared to the response observed in *S. mansoni* challenge (Figure 5.1). There were no distinct expression patterns, levels did not exceed 1.5-fold relative to the time 0 h control, and all were not statistically significant ($P > 0.05$).

5.4 Discussion

In this study, I have characterized a TLR in *B. glabrata* that I demonstrate is immunologically relevant during the anti-schistosome immune response of the snail. This study provides significant evidence that *Bg*TLR is involved in regulating parasite infection in this snail. *Bg*TLR expression was increased as early as 3 h following *S. mansoni* challenge in the resistant (BS-90) snails, lasting up to 3 days before returning to pre-exposure levels (Figure 5.1). This pattern of expression is consistent with the involvement of *Bg*TLR in the immune response because the duration coincides with the critical period for the clearance of parasite infection in

incompatible strains (Sullivan *et al.* 1995; Hahn *et al.* 2001; Lie *et al.* 1980). Moreover, the means by which *Bg*TLR was initially identified suggested that it was present in higher abundance in BS-90 snails when compared to M-line. This is further supported by siRNA-mediated knockdown of *Bg*TLR, which also had maximal effect within this duration (Figure 5.8A-B). Forty-three percent of resistant snails that received *Bg*TLR-specific siRNA oligonucleotides before *S. mansoni* challenge went on to shed cercariae after a 4-week incubation period. On the other hand, resistant snails that did not receive *Bg*TLR-specific siRNA but were challenged with *S. mansoni* cleared the infection within 4 days. This was determined by a qRT-PCR assay targeting the parasite glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene, which failed to detect parasite product beyond day 4 (Kabore 2016). Conversely, in susceptible (M-line) snails, GAPDH expression was still detectable after 4 days post-infection and increased throughout the incubation period.

The siRNA oligonucleotides used to knockdown *Bg*TLR transcript are specific as shown with qRT-PCR amplification (Figure 5.5) of the three predicted transcripts sharing the highest nucleotide identity with *Bg*TLR available on the Vectorbase database for *B. glabrata* (www.vectorbase.org). However, their cross-reaction with BGLB008602 leaves the possibility that the phenotypic knockdown effects observed may be due to either or both of these proteins. BGLB008602 is 100% identical to *Bg*TLR but lacks a 1195 bp region of *Bg*TLR and cannot be functionally distinguished from *Bg*TLR using our siRNA knockdown approach. I have reason to believe that both may be splice variants of the same gene based on my finding that the 'missing' 1195 bp region is currently annotated as introns 8 and 9 in the *B. glabrata* genome (www.vectorbase.org). As shown by Western blot (Figure 5.7 and Figure 5.6), no band representative of the size predicted for BGLB008602 (~127 kDa) is observed, suggesting that its

effect, if any, is minimal or, is functioning in a different context than the anti-*S. mansoni* immune response. Should these transcripts be true splice variants, the difference in size would most likely impact their interaction with PAMPs or endogenous ligands since the differential region is in the extracellular domain and contains LRR motifs commonly required for ligand recognition.

Currently, we do not know the precise recognition mechanism by which *Bg*TLR is involved in the snail immune response. That is, what PAMPs or perhaps endogenous ligands it recognizes and how this signal is transduced to the haemocyte. TLRs have been characterized in a number of other molluscs such as the disk abalone, Hawaiian bobtail squid, Zhikong scallop, soft-shell clam, mussel and oyster (Elvitigala *et al.* 2013; Qiu *et al.* 2007; Mateo *et al.* 2010; Toubiana *et al.* 2013; Zhang *et al.* 2013), however this is the first functional identification of a TLR in a gastropod. To date, all molluscan TLRs that have been functionally assessed are thought to be involved in antimicrobial immune responses. This is typical of most TLRs as they recognize PAMPs ranging from lipids, proteins, lipoproteins and nucleic acids (Akira *et al.* 2006). An interesting feature of *Bg*TLR is that it is induced in response to *S. mansoni* challenge. This suggests that it might be involved in recognizing PAMPs that are shared between schistosomes and microbes. However, an intriguing possibility is that *Bg*TLR may be exclusive for schistosome (or digenean trematode)-specific epitopes. This possibility is supported by my preliminary bacterial challenge experiments, which found no significant induction of *Bg*TLR expression in response to *A. salmonicida* or *E. coli*. Should this be the case, it will mark a significant finding in the field of invertebrate immunology, and add a new PAMP to the TLR ligand canon. To the best of my knowledge, no anti-parasitic recognition/response involving TLRs have been characterized in an invertebrate.

There are two possible sources of exogenous ligand(s) that might form the basis of TLR-based recognition in *B. glabrata* with respect to schistosomes. The first includes the complex carbohydrates, which are major components of larval teguments (Lehr *et al.* 2007; Lehr *et al.* 2008; Peterson *et al.* 2009). Secondly, during larval transformation, complex glycoproteins are also released with the other larval transformation products (Wu *et al.* 2009), which might be recognized by *Bg*TLR. Larval carbohydrates have been shown to elicit functional immune responses such as the induction of ROS production in haemocytes (Hahn *et al.* 2000; Lacchini *et al.* 2006; Zelck *et al.* 2007; Humphries and Yoshino 2008) and phagocytosis (Fryer *et al.* 1989). Both parasite larval carbohydrates and glycoproteins can bind to snail plasma and haemocyte proteins *in vitro* (Johnston and Yoshino 1996; Johnston and Yoshino 2001; Castillo and Yoshino 2002; Castillo *et al.* 2007).

It also remains possible that the *Bg*TLR ligand(s) might be endogenous. This is also the case for the founding member of the TLR family (*Drosophila* Toll) and for some mammalian TLRs. In *Drosophila,* the Toll does not function as a direct recognition receptor of a microbial pattern. Instead, its ligand (Späetzle) is activated first through proteolytic cleavage by pathogen-triggered protease cascades (Michel *et al.* 2001). Examples of molecules that serve as endogenous ligands abound in mammals. These include uric acid, mitochondrial DNA, heat-shock proteins 60 and 70, fibronectin and fibrinogen (Bryant *et al.* 2015). In mice, evidence suggests that TLR4 recognizes fibrinogen to induce chemokine secretion in macrophages (Smiley *et al.* 2001). Based on this evidence, one candidate group of molecules that may serve as an endogenous ligand for *Bg*TLR is the fibrinogen-related proteins, which are highly diversified family of lectins functionally linked to the anti-parasite response in *B. glabrata* (Hanington *et al.*

2010; Hanington *et al.* 2012). I have started making TLR reporter constructs (Figure 5.16) that can be used in future studies to screen for and identify *Bg*TLR ligand(s).

In terms of functional immune responses driven by *Bg*TLR, experimental results support the involvement of phagocytosis as a downstream functional response elicited following engagement, and ultimately resulting in the prevention of parasite infection. Knockdown of *Bg*TLR in BS-90 snails significantly reduced the mean number of beads phagocytosed per haemocyte from 17 to 13 (Figure 5.12A), although the overall percentage of phagocytic haemocytes did not differ from GFP knockdown control snails. This, and the fact that parasites are too large to be phagocytosed by a single haemocyte indicate that other effector responses might also be activated by *Bg*TLR. Phagocytosis together with cytotoxicity and encapsulation are the major haemocyte responses that are induced as a result of receptor recognition of PAMPS in the snail (Yoshino and Coustau 2010). It is likely that the role of *Bg*TLR in the snail immune response against *S. mansoni* is through the combined effects of these processes. However, the involvement of encapsulation and cytotoxicity remains to be investigated in the context of *Bg*TLR.

The major pathways that are known to be activated downstream of TLR are present in *B. glabrata,* and for some, their active involvement in the snail immune response have been demonstrated. These major pathways include the NF-kB, MAPK/ERK and phosphatidylinositol-3 kinase (PI3K). MAPK, ERK and PI3K pathways have been shown to be involved in cellular adhesion, motility and spreading required for phagocytosis and encapsulation (Humphries *et al.* 2001), as well as in regulating the release of hydrogen peroxide and nitric oxide molecules used in cytotoxicity (Zelck *et al.* 2007; Humphries and Yoshino 2008). No functional role has been demonstrated for NF-kB although its expression is consistent with involvement in an immune

response (Zhang and Coultas 2011). *Bg*TLR may be associated with any of these pathways or their combination in order to activate the effector responses in haemocytes, and this is an area actively under investigation.

*Bg*TLR appears to be selectively expressed in certain haemocytes only (Figure 5.10), and differentially on cells of the Bge cell line. The relationship between *Bg*TLR-negative haemocytes with the other haemocyte subsets has not yet been explored. However, expression of pattern recognition receptors on haemocyte subsets is not unique to *Bg*TLR. A member of the fibrinogen-related proteins (FREP3) has been shown to be expressed only by subsets of haemocytes which increase in number during an immune response in the snail (Hanington *et al.* 2010). The fact that these immunologically relevant factors are not equally present on all haemocytes of known morphological subset alludes to the likelihood that there is far greater functional diversity within a haemocyte morphotype than is currently understood.

In conclusion, the studies presented here have demonstrated a connection between *Bg*TLR and the resistance phenotype of *B. glabrata* with respect to *S. mansoni* challenge. By characterizing the receptor which is a major component of the TLR pathway, my findings complement others that have characterized both upstream and downstream components of the pathway (Zhang and Coultas 2011; Zelck *et al.* 2007; Fryer *et al.* 1989; Humphries *et al.* 2001), and provides conclusive evidence that this pathway is fully conserved and functionally relevant in the immune response of *B. glabrata*. It also paves the way for further studies not only to identify the ligands, specific immune responses induced, and the haemocyte subsets involved, but also to determine the role of the other *B. glabrata* TLRs and leucine rich repeat containing molecules.

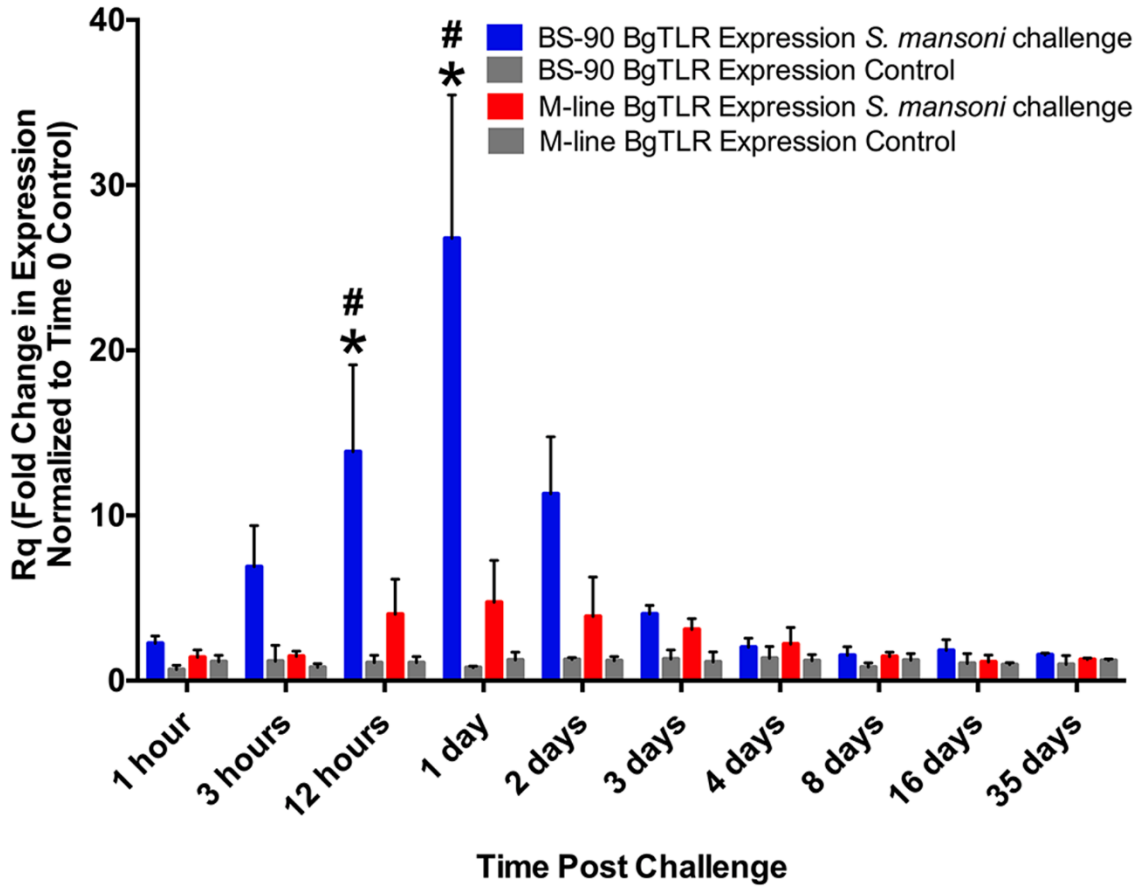

Figure 5.1. *Bg*TLR transcript expression with and without *S. mansoni* challenge. Snails (BS-90 and M-line strains) were individually exposed to 5 miracidia or left unexposed (control). Five snails were collected at indicated time points (in one experiment) over the incubation period of the parasite. RNA was extracted from whole snails, converted to cDNA and *Bg*TLR expression was measured by qRT-PCR. Expression was quantified in fold changes normalized to time 0 h controls. Bars represent standard error ($n = 5$). Asterisk (*) indicates significant difference ($P < 0.05$) between experimental and control samples, while hash (#) indicates significant difference between BS-90 and M-line snails at the respective time points.

1	ATG	CAA	CTT	TTG	GAA	ACT	CTG	GAG	ATC	TCT	CAC	AGC	TCA	TTA	AGA	AAT	TTG	TTC	54
1	**M**	**Q**	**L**	**L**	**E**	**T**	**L**	**E**	**I**	**S**	**H**	**S**	**S**	**L**	**R**	**N**	**L**	**F**	**18**
55	TCA	CTC	TGT	TCC	TAC	ACG	TCT	TTA	AAG	AAT	CTG	AAC	TTG	TCC	TTC	AAC	CAT	TTG	108
19	**S**	**L**	**C**	**S**	**Y**	**T**	**S**	**L**	**K**	**N**	**L**	**N**	**L**	**S**	**F**	**N**	**H**	**L**	**36**
109	GCC	AAT	CTT	GAA	GAT	CTC	GGC	ATC	AAC	TGT	GGA	GGG	AAA	TCT	TTA	CAC	AAT	CTT	162
37	**A**	**N**	**L**	**E**	**D**	**L**	**G**	**I**	**N**	**C**	**G**	**G**	**K**	**S**	**L**	**H**	**N**	**L**	**54**
163	GAG	AGT	CTT	GAC	ATG	CGC	AAT	AAC	CTC	CTG	ACA	GAG	ATT	CCA	AAT	TGG	TTG	TCT	216
55	**E**	**S**	**L**	**D**	**M**	**R**	**N**	**N**	**L**	**L**	**T**	**E**	**I**	**P**	**N**	**W**	**L**	**S**	**72**
217	GAG	AAT	TTG	CTG	AAC	CTT	CAC	TAT	TTG	TAT	TTA	TCT	GGA	AAT	CTC	ATT	GAA	AAC	270
73	**E**	**N**	**L**	**L**	**N**	**L**	**H**	**Y**	**L**	**Y**	**L**	**S**	**G**	**N**	**L**	**I**	**E**	**N**	**90**
271	TAT	GAC	CAC	CTC	CCG	TTG	AAG	AAC	TTT	TCG	AGT	TTA	TAC	TTG	ATG	GAT	CTT	TCC	324
91	**Y**	**D**	**H**	**L**	**P**	**L**	**K**	**N**	**F**	**S**	**S**	**L**	**Y**	**L**	**M**	**D**	**L**	**S**	**108**
325	AAC	AAT	TCT	CTG	ACT	GAG	ATA	AAG	AAA	GAT	TTT	TTG	TTA	GGG	TGT	GAC	AAT	CTG	378
109	**N**	**N**	**S**	**L**	**T**	**E**	**I**	**K**	**K**	**D**	**F**	**L**	**L**	**G**	**C**	**D**	**N**	**L**	**126**
379	CAA	TAC	TTA	TAC	ATG	TCC	AGA	AAT	CCA	ATT	ATC	TAC	ATC	CAA	AGA	CAA	TTT	CTG	432
127	**Q**	**Y**	**L**	**Y**	**M**	**S**	**R**	**N**	**P**	**I**	**I**	**Y**	**I**	**Q**	**R**	**Q**	**F**	**L**	**144**
433	AAA	GCA	GTT	TCA	AAT	TTG	GTC	GAA	CTG	GAA	ATG	GTG	GAG	TCG	AGA	CTG	ACC	GAT	486
145	**K**	**A**	**V**	**S**	**N**	**L**	**V**	**E**	**L**	**E**	**M**	**V**	**E**	**S**	**R**	**L**	**T**	**D**	**162**
487	AGT	ATT	TGG	CTA	GAG	ATA	TCG	GAT	ATT	TCT	AAA	CGA	CTT	CGA	ATT	TTA	AAT	TTG	540
163	**S**	**I**	**W**	**L**	**E**	**I**	**S**	**D**	**I**	**S**	**K**	**R**	**L**	**R**	**I**	**L**	**N**	**L**	**180**
541	TCT	AGA	AAC	AGA	CTT	ACT	AAA	ATC	AAT	GAG	AAC	ACA	ATG	TCA	GAC	CTG	CGA	TTA	594
181	**S**	**R**	**N**	**R**	**L**	**T**	**K**	**I**	**N**	**E**	**N**	**T**	**M**	**S**	**D**	**L**	**R**	**L**	**198**
595	GAA	GTC	TTG	AAT	GTC	AGC	TAT	AAT	AGA	ATA	GTT	GGT	CTC	AAT	AGC	AAT	GCC	TTT	648
199	**E**	**V**	**L**	**N**	**V**	**S**	**Y**	**N**	**R**	**I**	**V**	**G**	**L**	**N**	**S**	**N**	**A**	**F**	**216**
649	GGG	TCT	CAG	ACA	AAT	CTC	ATC	ACA	TTG	GAC	TTA	TCT	TAT	AAC	TTA	ATT	ACA	GAC	702
217	**G**	**S**	**Q**	**T**	**N**	**L**	**I**	**T**	**L**	**D**	**L**	**S**	**Y**	**N**	**L**	**I**	**T**	**D**	**234**
703	GTG	CCT	GTC	AGA	TTT	TCT	CAA	AAT	ATG	ACC	AAT	TTG	GTT	CAC	CTT	CTA	TTA	AAT	756
235	**V**	**P**	**V**	**R**	**F**	**S**	**Q**	**N**	**M**	**T**	**N**	**L**	**V**	**H**	**L**	**L**	**L**	**N**	**252**
757	AAC	AAC	AAC	ATC	AAA	GTT	GTC	CAA	AGC	GAA	GCA	TTC	ATG	GGT	TTG	GGA	AAA	TTG	810
253	**N**	**N**	**N**	**I**	**K**	**V**	**V**	**Q**	**S**	**E**	**A**	**F**	**M**	**G**	**L**	**G**	**K**	**L**	**270**
811	GAG	AGC	TTG	GAT	CTT	AGC	TTC	AAC	AGT	TTA	CAA	GAA	TTG	ATG	CCG	CAA	GTA	GTT	864
271	**E**	**S**	**L**	**D**	**L**	**S**	**F**	**N**	**S**	**L**	**Q**	**E**	**L**	**M**	**P**	**Q**	**V**	**V**	**288**
865	GGG	ACC	TTG	GAG	CAC	ATT	GTC	AAT	GTG	AAT	CTA	AGC	TAT	AAC	CAT	CTC	AGA	GTA	918
289	**G**	**T**	**L**	**E**	**H**	**I**	**V**	**N**	**V**	**N**	**L**	**S**	**Y**	**N**	**H**	**L**	**R**	**V**	**306**
919	TTG	AAC	AGC	GAT	CTT	TTC	TTC	AAG	TTC	AAA	CAG	ATG	AAG	CAC	CTC	AAT	GTA	TCT	972
307	**L**	**N**	**S**	**D**	**L**	**F**	**F**	**K**	**F**	**K**	**Q**	**M**	**K**	**H**	**L**	**N**	**V**	**S**	**324**
973	CAC	AAT	GCT	CTT	CAA	GAG	TTG	CCT	TTT	CTA	TAC	GGT	AAT	GTG	GCT	CTC	CAG	GAT	1026
325	**H**	**N**	**A**	**L**	**Q**	**E**	**L**	**P**	**F**	**L**	**Y**	**G**	**N**	**V**	**A**	**L**	**Q**	**D**	**342**
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343	**L**	**D**	**A**	**S**	**F**	**N**	**N**	**I**	**T**	**K**	**V**	**I**	**A**	**Q**	**T**	**F**	**Q**	**D**	**360**
1081	CTT	AAA	GAA	CTG	CAA	ACC	ATT	TCG	CTG	AGC	CAT	AAT	TTG	CTG	AGT	TCT	CTC	CCT	1134
361	**L**	**K**	**E**	**L**	**Q**	**T**	**I**	**S**	**L**	**S**	**H**	**N**	**L**	**L**	**S**	**S**	**L**	**P**	**378**
1135	TTC	AGG	ATG	TTT	AAA	GGG	TGT	GAC	AAT	GTG	AAA	ACT	ATT	TAC	TTG	TCC	TTC	AAT	1188
379	**F**	**R**	**M**	**F**	**K**	**G**	**C**	**D**	**N**	**V**	**K**	**T**	**I**	**Y**	**L**	**S**	**F**	**N**	**396**
1189	TTG	CTG	AGC	CAT	TTA	GAT	GAC	GAT	TTT	TTT	ACG	AGT	TCT	CCA	CGA	CTG	ACG	TTC	1242
397	**L**	**L**	**S**	**H**	**L**	**D**	**D**	**D**	**F**	**F**	**T**	**S**	**S**	**P**	**R**	**L**	**T**	**F**	**414**
1243	ATA	GAT	CTG	TCC	CAC	AAT	AAG	ATC	ACT	GCC	ATG	AAC	AAT	ATA	TTC	AGA	TAT	TTA	1296
415	**I**	**D**	**L**	**S**	**H**	**N**	**K**	**I**	**T**	**A**	**M**	**N**	**N**	**I**	**F**	**R**	**Y**	**L**	**432**
1297	AAC	CAT	TTA	AAA	TTC	CTT	CAA	TTA	AGT	TAC	AAC	AAA	ATT	ACC	ACC	CTG	CTT	AGA	1350
433	**N**	**H**	**L**	**K**	**F**	**L**	**Q**	**L**	**S**	**Y**	**N**	**K**	**I**	**T**	**T**	**L**	**L**	**R**	**450**
1351	AAC	CAG	CTG	CCG	AGA	TCT	CTT	GAG	ACA	TTG	GAT	ATA	AGC	AAT	AAC	AAT	ATC	CAC	1404
451	**N**	**Q**	**L**	**P**	**R**	**S**	**L**	**E**	**T**	**L**	**D**	**I**	**S**	**N**	**N**	**N**	**I**	**H**	**468**
1405	CAA	ATA	TCT	TCA	CAT	ACC	TTT	AAG	ACT	TTG	TCT	AAC	CTG	AGA	TAC	GTG	GAC	CTG	1458
469	**Q**	**I**	**S**	**S**	**H**	**T**	**F**	**K**	**T**	**L**	**S**	**N**	**L**	**R**	**Y**	**V**	**D**	**L**	**486**
1459	AGC	GTT	AAC	AAT	TTG	ACC	ACA	CTG	TCT	CAG	GAT	GAG	GTT	GAG	ATA	GCT	TAC	AAC	1512
487	**S**	**V**	**N**	**N**	**L**	**T**	**T**	**L**	**S**	**Q**	**D**	**E**	**V**	**E**	**I**	**A**	**Y**	**N**	**504**

1513	CTG	TTG	TCT	AAA	CCC	ACC	TTC	AAT	CTT	GTG	TAC	AAT	CCC	CTG	GTC	TGT	GAT	TGT	1566
505	**L**	**L**	**S**	**K**	**P**	**T**	**F**	**N**	**L**	**V**	**Y**	**N**	**P**	**L**	**V**	**C**	**D**	**C**	**522**
1567	AAA	CTG	GAA	TGG	CTG	AAA	GAC	TGG	TAT	GAC	GGG	AAA	TTC	AAA	GAC	ACG	GGA	ACT	1620
523	**K**	**L**	**E**	**W**	**L**	**K**	**D**	**W**	**Y**	**D**	**G**	**K**	**F**	**K**	**D**	**T**	**G**	**T**	**540**
1621	CTG	CCC	ACT	TTT	CAG	ACC	ACT	TTA	ACT	TAC	GGA	TGC	ATT	AGC	CCA	TTG	TAT	TCT	1674
541	**L**	**P**	**T**	**F**	**Q**	**T**	**T**	**L**	**T**	**Y**	**G**	**C**	**I**	**S**	**P**	**L**	**Y**	**S**	**558**
1675	ACC	AAA	ATG	CCC	ATC	ACC	TCT	CTG	AGG	AGT	GAC	GAG	TTT	CTT	TGC	CAC	TAC	GAG	1728
559	**T**	**K**	**M**	**P**	**I**	**T**	**S**	**L**	**R**	**S**	**D**	**E**	**F**	**L**	**C**	**H**	**Y**	**E**	**576**
1729	AAA	CAC	TGC	GAC	AAA	ACG	TGC	GTG	TGC	TGT	GAC	TAC	GAC	GTG	TGC	CAT	TGC	AAG	1782
577	**K**	**H**	**C**	**D**	**K**	**T**	**C**	**V**	**C**	**C**	**D**	**Y**	**D**	**V**	**C**	**H**	**C**	**K**	**594**
1783	TAC	ACC	TGC	CCT	TCC	TCC	TGC	CAG	TGC	TAC	ATC	GGC	GAC	AAG	TTC	CTC	AAC	ATT	1836
595	**Y**	**T**	**C**	**P**	**S**	**S**	**C**	**Q**	**C**	**Y**	**I**	**G**	**D**	**K**	**F**	**L**	**N**	**I**	**612**
1837	CAC	CAG	GTT	CAC	TGC	TTC	AAC	GCC	AAC	CTC	ACT	GAC	GTG	CCC	GGA	AAA	ATA	CCA	1890
613	**H**	**Q**	**V**	**H**	**C**	**F**	**N**	**A**	**N**	**L**	**T**	**D**	**V**	**P**	**G**	**K**	**I**	**P**	**630**
1891	GAA	GGT	GCT	ACT	CTA	CTC	CGC	CTT	GAT	GGC	AAC	AAC	CTC	CCT	AGC	CTC	CGT	GAA	1944
631	**E**	**G**	**A**	**T**	**L**	**L**	**R**	**L**	**D**	**G**	**N**	**N**	**L**	**P**	**S**	**L**	**R**	**E**	**648**
1945	CAT	TCC	TTT	CTT	GGT	CTG	ACC	CAC	GTC	GTG	GAT	CTT	TAT	CTA	AAC	AAC	AGC	CAC	1998
649	**H**	**S**	**F**	**L**	**G**	**L**	**T**	**H**	**V**	**V**	**D**	**L**	**Y**	**L**	**N**	**N**	**S**	**H**	**666**
1999	ATC	CAC	ACT	GTG	GAA	AAC	AAT	ACT	TTT	AAA	GGA	ATG	AAG	TCT	GTC	AGG	TCG	TTG	2052
667	**I**	**H**	**T**	**V**	**E**	**N**	**N**	**T**	**F**	**K**	**G**	**M**	**K**	**S**	**V**	**R**	**S**	**L**	**684**
2053	TTC	TTA	AAC	AAT	AAT	CTG	TTG	ACG	ATC	ATA	TCT	CCC	GGT	GTC	TTT	AGC	GGG	TTG	2106
685	**F**	**L**	**N**	**N**	**N**	**L**	**L**	**T**	**I**	**I**	**S**	**P**	**G**	**V**	**F**	**S**	**G**	**L**	**702**
2107	GAG	AAT	CTG	GAG	AGA	ATT	TTT	CTT	CAG	AAC	AAC	TTT	ATA	AGT	TTG	ATA	GAC	CCT	2160
703	**E**	**N**	**L**	**E**	**R**	**I**	**F**	**L**	**Q**	**N**	**N**	**F**	**I**	**S**	**L**	**I**	**D**	**P**	**720**
2161	CAA	GCT	CTG	TTG	TTA	CCA	CCT	TAT	CTT	TAC	CTA	ATT	AAC	CTT	AGG	GAA	AAT	GAT	2214
721	**Q**	**A**	**L**	**L**	**L**	**P**	**P**	**Y**	**L**	**Y**	**L**	**I**	**N**	**L**	**R**	**E**	**N**	**D**	**738**
2215	CTC	AAC	ACG	CTC	CCT	ATA	GAT	GGT	CTT	TGG	GGA	TTT	GTC	AAT	CGT	TCT	AGG	GAA	2268
739	**L**	**N**	**T**	**L**	**P**	**I**	**D**	**G**	**L**	**W**	**G**	**F**	**V**	**N**	**R**	**S**	**R**	**E**	**756**
2269	AGT	GGT	TTG	AAA	GTT	CGA	TTT	TCT	CTG	AGT	CAA	AAT	CCT	TAT	AGC	TGT	CAG	CTT	2322
757	**S**	**G**	**L**	**K**	**V**	**R**	**F**	**S**	**L**	**S**	**Q**	**N**	**P**	**Y**	**S**	**C**	**Q**	**L**	**774**
2323	GAC	TTT	GTT	TGT	AAG	TTT	GTT	CTG	TTT	ATC	AGA	GAC	AGC	GCG	GAC	TGC	ATT	GAA	2376
775	**D**	**F**	**V**	**C**	**K**	**F**	**V**	**L**	**F**	**I**	**R**	**D**	**S**	**A**	**D**	**C**	**I**	**E**	**792**
2377	GAC	ATC	TCA	GAC	ATT	AAG	TGT	AGT	TCT	AAT	AGC	CTC	GGT	CAG	CAA	AGT	TAC	TAT	2430
793	**D**	**I**	**S**	**D**	**I**	**K**	**C**	**S**	**S**	**N**	**S**	**L**	**G**	**Q**	**Q**	**S**	**Y**	**Y**	**810**
2431	CAA	GAT	GGA	TTT	ACT	TTG	CTG	GAC	TTT	CAG	ATT	GAA	CTC	TGT	AGC	GAG	AAC	CAA	2484
811	**Q**	**D**	**G**	**F**	**T**	**L**	**L**	**D**	**F**	**Q**	**I**	**E**	**L**	**C**	**S**	**E**	**N**	**Q**	**828**
2485	TCG	TTT	CCC	ACT	AAC	ATG	TCC	AGA	AAC	AGC	GTG	CAC	TCT	TCT	TCA	GCC	AAG	GGA	2538
829	**S**	**F**	**P**	**T**	**N**	**M**	**S**	**R**	**N**	**S**	**V**	**H**	**S**	**S**	**S**	**A**	**K**	**G**	**846**
2539	GAA	ACC	TAC	GCC	CTG	ATC	GCC	GCA	TGT	GTA	GTC	ATA	GCT	TTC	GGT	CTG	GCC	CTG	2592
847	**E**	**T**	**Y**	**A**	**L**	**I**	**A**	**A**	**C**	**V**	**V**	**I**	**A**	**F**	**G**	**L**	**A**	**L**	**864**
2593	CTG	ATA	GTG	GCC	TAC	ATG	AAC	AGA	GAC	TTC	CTG	CAG	GTT	CTC	TGT	TTC	ACG	CGT	2646
865	**L**	**I**	**V**	**A**	**Y**	**M**	**N**	**R**	**D**	**F**	**L**	**Q**	**V**	**L**	**C**	**F**	**T**	**R**	**882**
2647	TTC	GGT	CTT	CGT	GTT	TTC	AAA	ATG	GCA	AAG	GCT	ACT	GAG	GAC	AAT	GAC	AGG	CCT	2700
883	**F**	**G**	**L**	**R**	**V**	**F**	**K**	**M**	**A**	**K**	**A**	**T**	**E**	**D**	**N**	**D**	**R**	**P**	**900**
2701	TAT	GAT	GCC	TTC	ATC	TCC	TAC	AGC	AGC	AAG	GAC	GAG	GAC	TTT	GTG	ATC	CAC	CAG	2754
901	**Y**	**D**	**A**	**F**	**I**	**S**	**Y**	**S**	**S**	**K**	**D**	**E**	**D**	**F**	**V**	**I**	**H**	**Q**	**918**
2755	CTG	GCG	CCC	AGG	CTG	GAG	AAC	GGC	GAC	AAG	AAG	TTT	CAA	CTG	TGT	GTC	CAC	TAC	2808
919	**L**	**A**	**P**	**R**	**L**	**E**	**N**	**G**	**D**	**K**	**K**	**F**	**Q**	**L**	**C**	**V**	**H**	**Y**	**936**
2809	AGG	GAC	TTT	CCT	GTG	GGT	GCC	TGC	ATC	GCT	GAA	ACC	ATC	GTT	CGA	AGT	GTG	GAG	2862
937	**R**	**D**	**F**	**P**	**V**	**G**	**A**	**C**	**I**	**A**	**E**	**T**	**I**	**V**	**R**	**S**	**V**	**E**	**954**
2863	GCC	AGT	AAG	CGG	ACT	ATA	CTG	GTT	GTA	TCT	GAT	AAC	TTT	CTA	GAC	TCT	GAG	TGG	2916
955	**A**	**S**	**K**	**R**	**T**	**I**	**L**	**V**	**V**	**S**	**D**	**N**	**F**	**L**	**D**	**S**	**E**	**W**	**972**
2917	TGT	CGC	TTT	GAG	TTT	CAG	ACT	GCT	CAC	CAA	CAG	GTT	TTG	AAT	GAG	AGG	AGA	AAT	2970
973	**C**	**R**	**F**	**E**	**F**	**Q**	**T**	**A**	**H**	**Q**	**Q**	**V**	**L**	**N**	**E**	**R**	**R**	**N**	**990**
2971	CGG	GTC	ATA	TTG	ATC	CTC	ATG	CAC	GAC	CTT	GAC	ACC	GAG	AAA	CTG	GAC	AGT	ACC	3024
991	**R**	**V**	**I**	**L**	**I**	**L**	**M**	**H**	**D**	**L**	**D**	**T**	**E**	**K**	**L**	**D**	**S**	**T**	**1008**

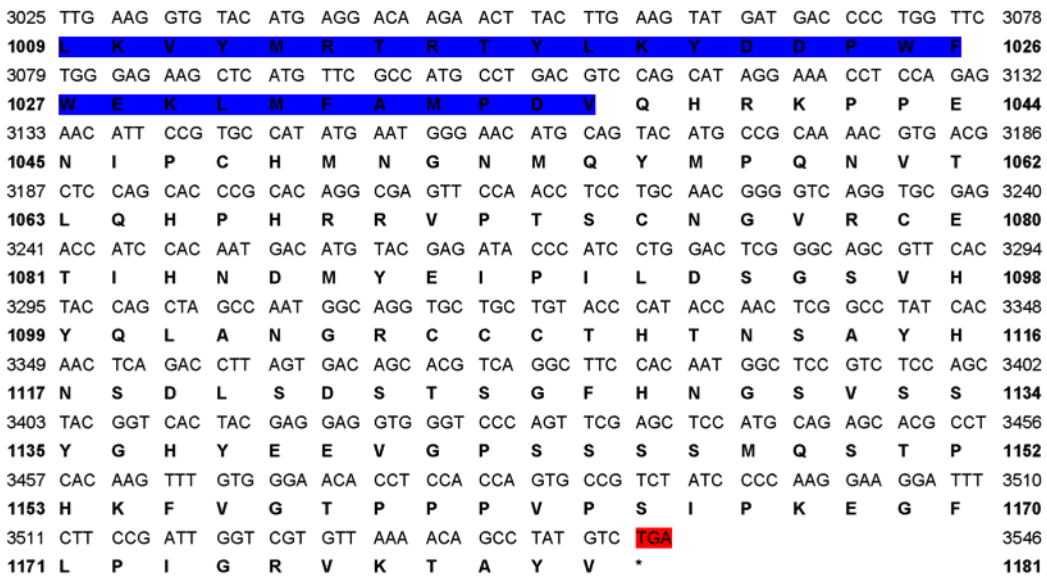


Figure 5.2. Annotated *Bg*TLR coding sequence. The entire open reading frame is shown with alternating lines of nucleotide codons and corresponding amino acid residues. Colour codes represent the start codon (bright green), leucine-rich repeat motifs (pink), transmembrane region (grey), TIR domain (blue) and the stop codon (red).

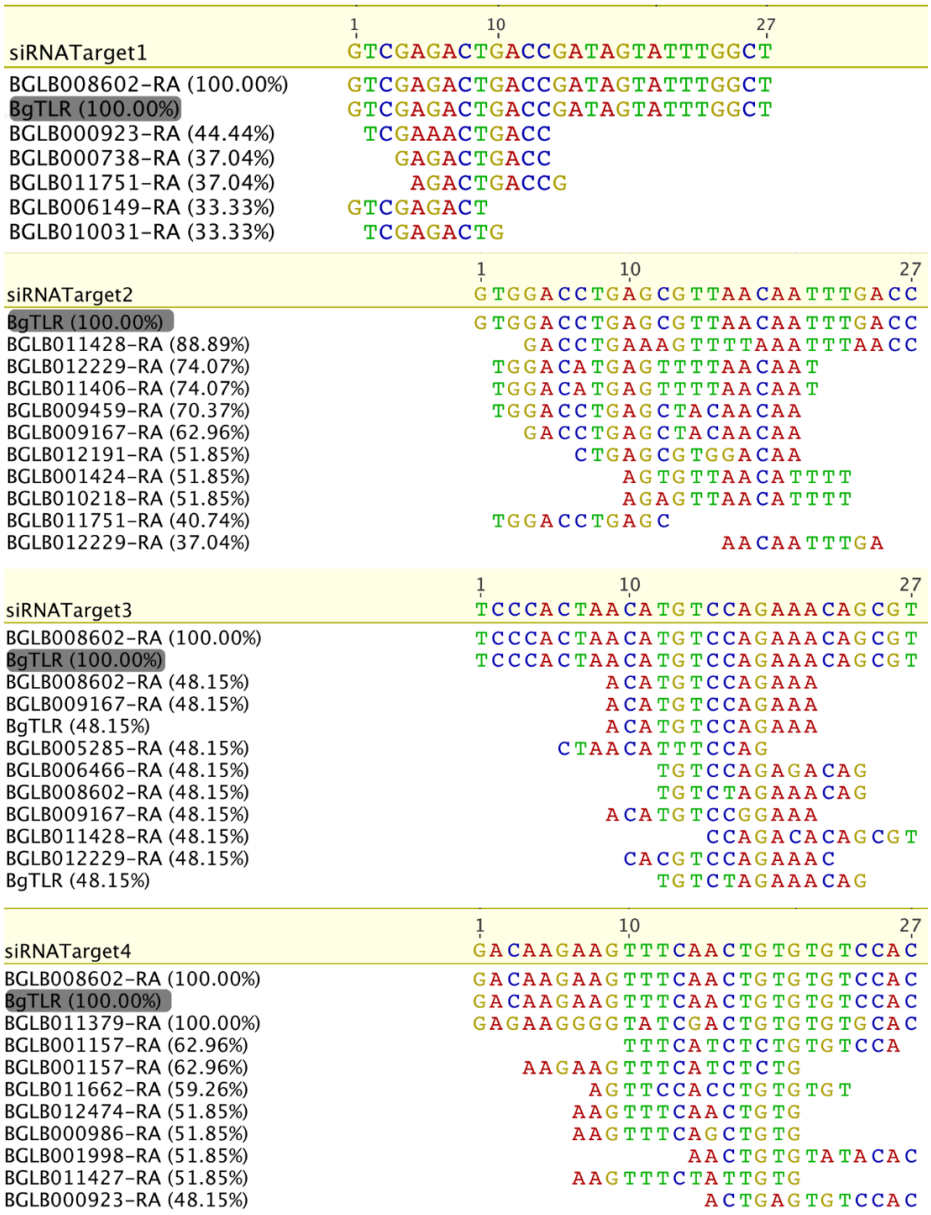


Figure 5.3. Nucleotide BLAST of siRNA oligonucleotides targeting *Bg*TLR. Sequences representing transcripts with high shared nucleotide identity with *Bg*TLR were retrieved from VectorBase (www.vectorbase.org) and used to create a custom database against which the siRNA sequences were searched. *Bg*TLR is highlighted in grey for each siRNA target results. Percentages in brackets represent the query coverage. Note that *Bg*TLR and BGLB008602-RA are 100% identical in all aspects except that the latter lacks the region between nucleotides 608-1,489 (possibly splice variants). Most of the retrieved transcripts have nucleotide conservations of less than 50% to any of the siRNA targets. Shown in the figure is the alignment of 10 top sequences.

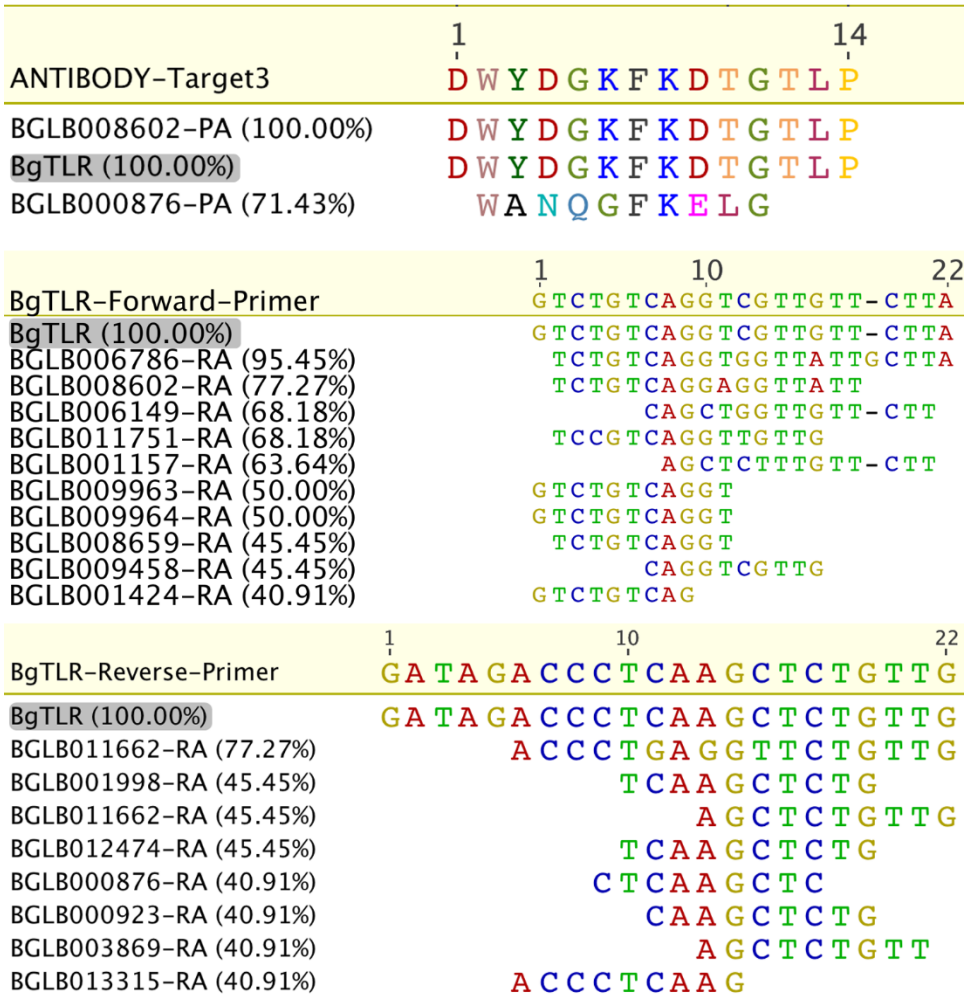


Figure 5.4. BLAST analysis of anti-*Bg*TLR cognate peptide sequence and qRT-PCR primers respectively. Sequences were analyzed similarly as described for Figure 5.3. *Bg*TLR is highlighted in grey for each alignment, displaying up to 10 top sequences. Custom database and BLAST analyses were done using Geneious version 6.1.6 (www.geneious.com) (Kearse *et al.* 2012).

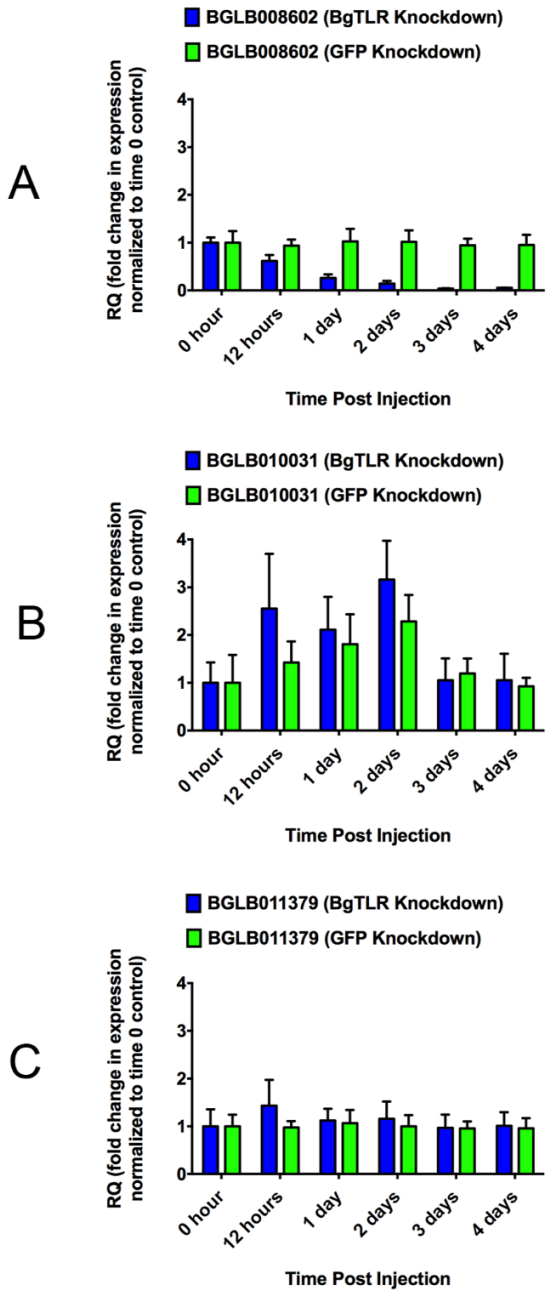


Figure 5.5. Expression of transcripts with the highest nucleotide identity with *Bg*TLR following *Bg*TLR siRNA-mediated knockdown. Quantitative RT-PCR was performed targeting 3 transcripts that appear to be the most similar to *Bg*TLR: BGLB008602 (A), BGLB010031 (B) and BGLB011379 (C) using cDNA generated from BS-90 *Bg*TLR and GFP siRNA knockdown samples as templates. *Bg*TLR siRNA did not have knockdown effect on these TLRs except on the putative splice variant (BGLB008602), which displayed a pattern similar to *Bg*TLR knockdown.

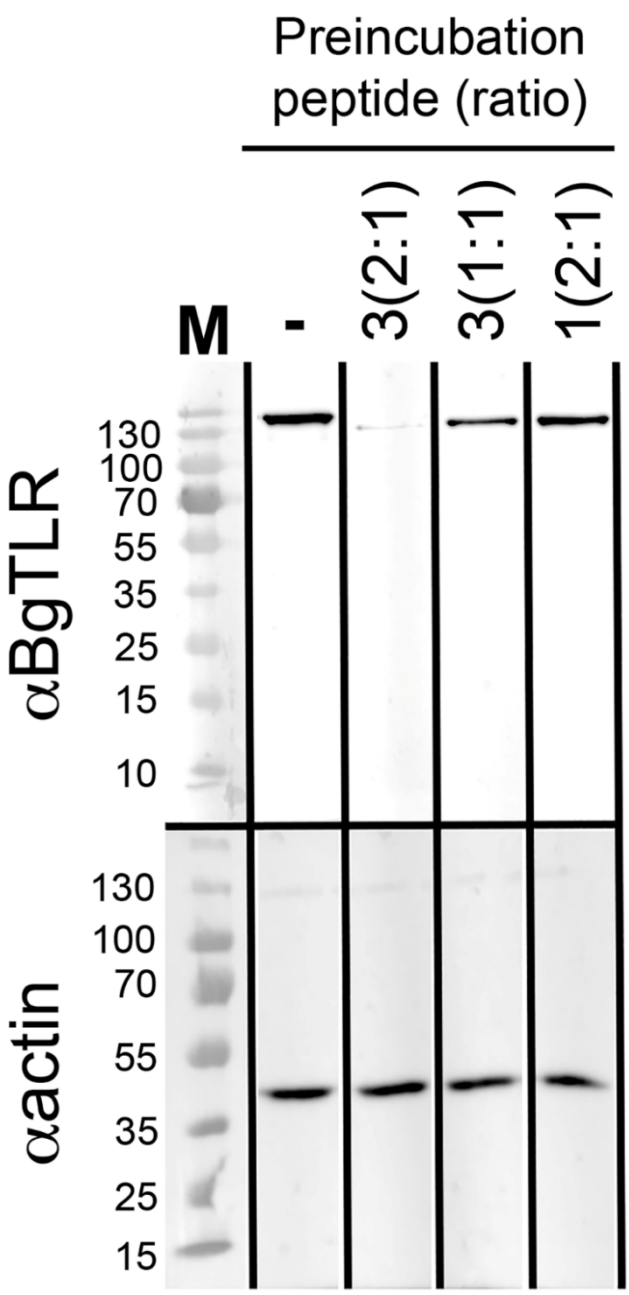


Figure 5.6. *Bg*TLR antibody detection of its cognate peptide. Protein extracts from BS-90 haemocytes were run on duplicate SDS-PAGE gels, then transferred to nitrocellulose membranes and probed with *Bg*TLR antibody used in this study without pre-incubation with its cognate peptide [-], pre-incubation with the peptide at 2:1 (peptide:antibody) molar ratio [3(2:1)], equal molar ratio [3(1:1)] or with an alternative *Bg*TLR antibody targeting a different peptide [1(2:1)]. *B. glabrata* actin served as protein loading control. M = molecular marker, with sizes in kDa shown to the left.

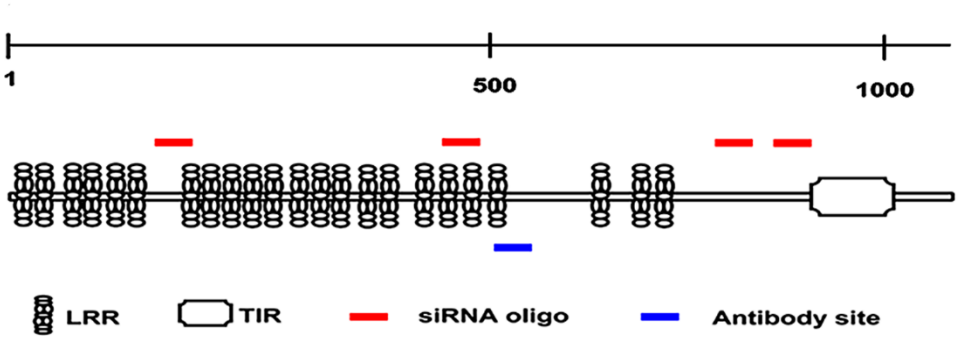


Figure 5.7. Graphic view of *Bg*TLR. Depicted domains are not drawn to scale. Shown are the leucine-repeat motifs (LRR) and Toll/IL-1 (TIR) domain. Red bars indicate the approximate positions targeted by siRNA oligonucleotides used to inject the snails, while the blue bar indicates the targeted position of the antibody used.

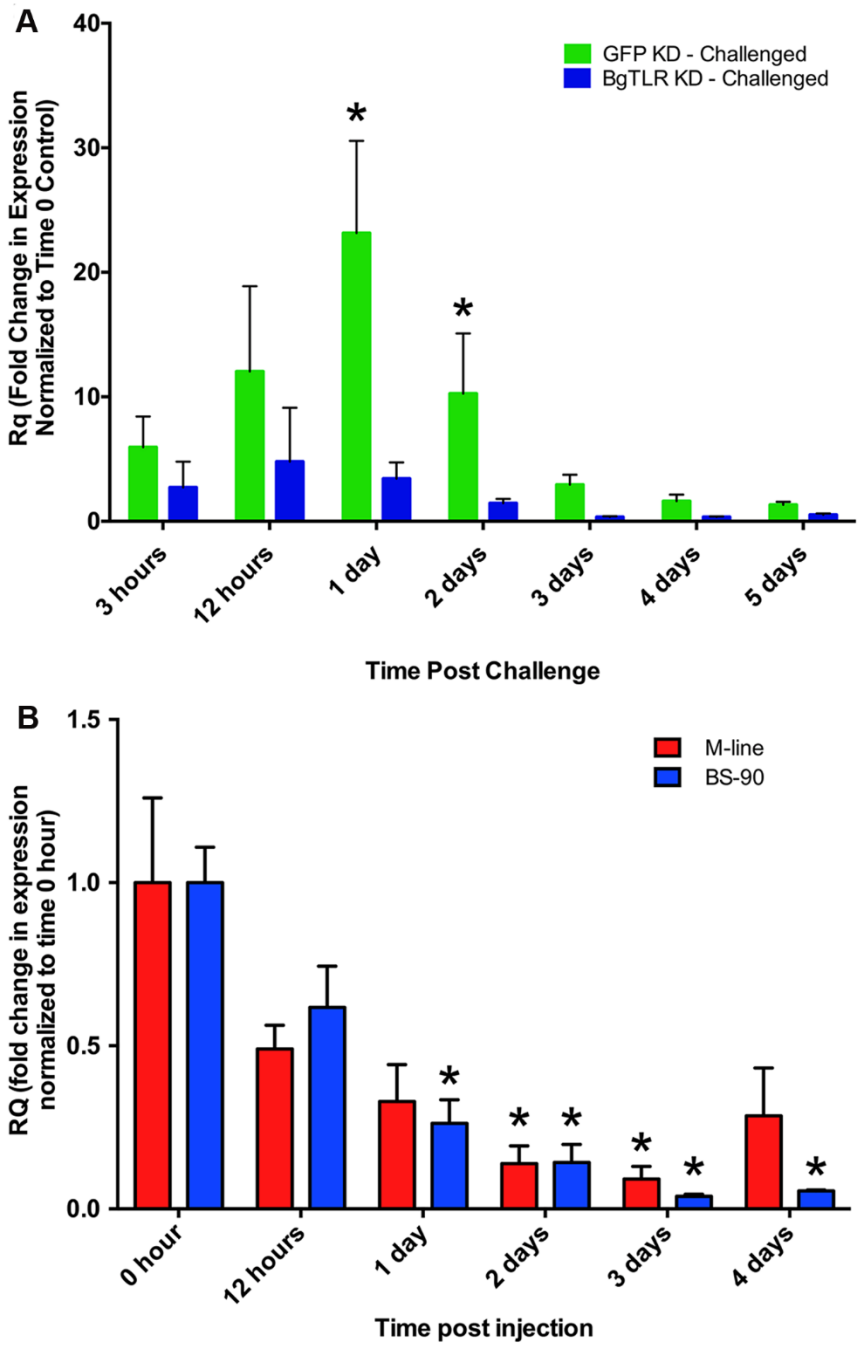


Figure 5.8. siRNA-mediated knockdown of *Bg*TLR transcripts. (A) Knockdown of *Bg*TLR or GFP (control) in BS-90 snails responding to *S. mansoni* parasite challenge. (B) Knockdown of *Bg*TLR in unexposed M-line and BS-90 snails. Five snails were collected for each time point in B and C for RNA extraction and cDNA synthesis. Bars represent standard error (n = 5). Asterisks indicate significant difference ($P < 0.05$) from 0-h time points.

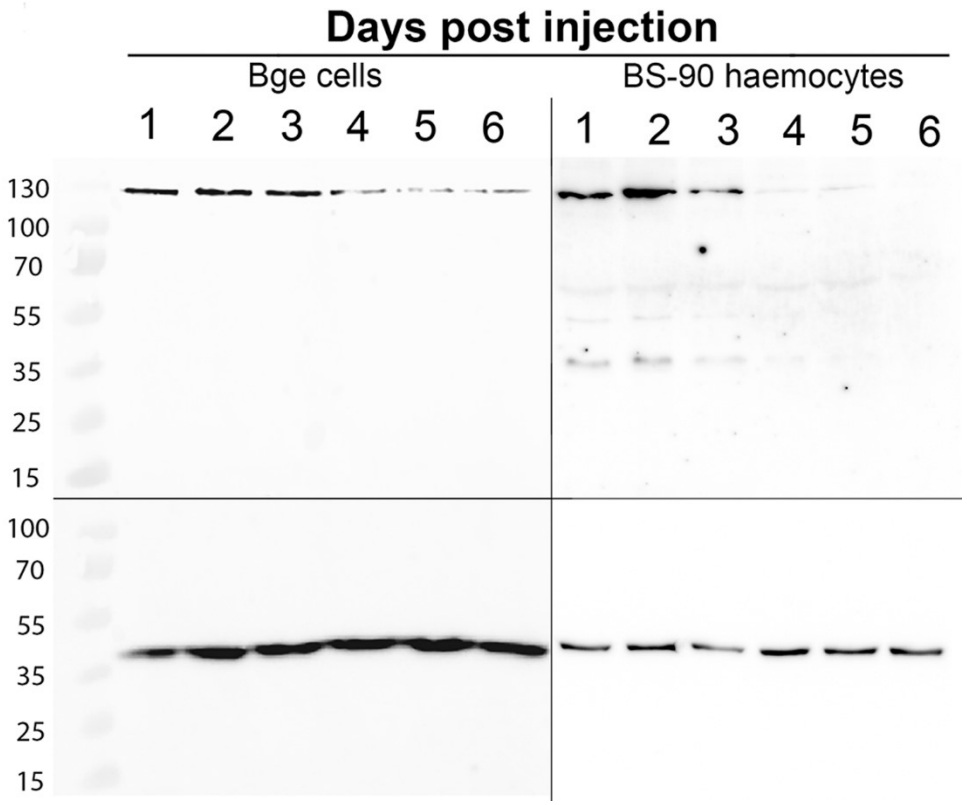


Figure 5.9. Confirmation of *Bg*TLR protein knockdown. *Bg*TLR (~135 kDa) knockdown in Bge cells and BS-90 haemocytes respectively. Protein was detected via Western blot with primary antibody developed against the extracellular region of *Bg*TLR. *Bg*Actin (~42 kDa) served as the loading control. Numbers on the left represent molecular weights in kDa corresponding to the molecular marker in the proceeding lane.

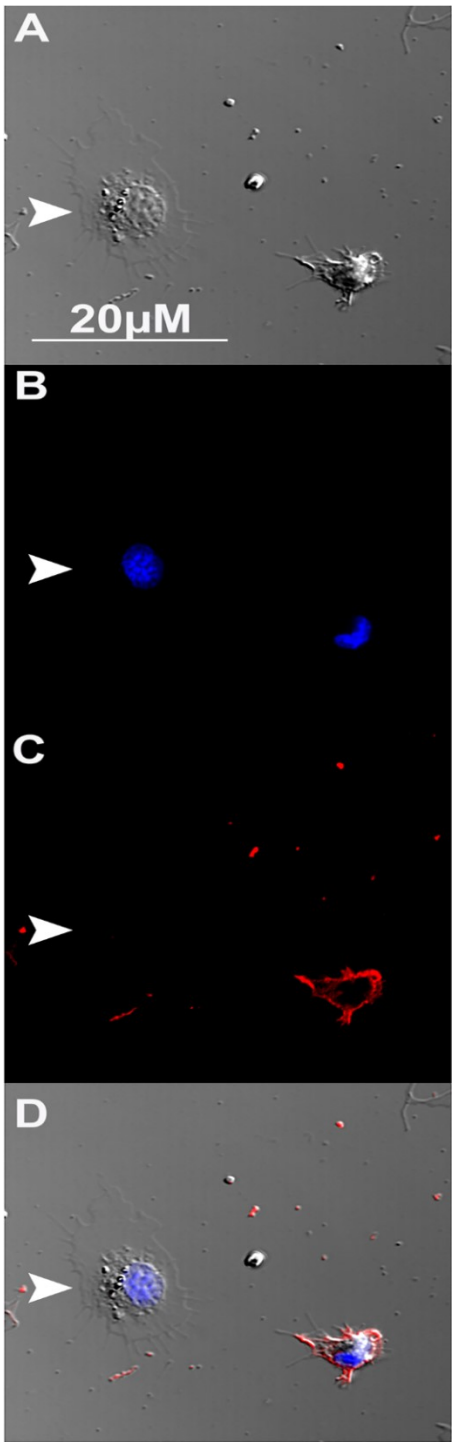


Figure 5.10. *Bg*TLR is not expressed on all haemocytes. Bright field view (A) of two adjacent haemocytes labelled with the nuclear stain DAPI (B) and anti-*Bg*TLR primary antibody (C). The merged view (D) shows that *Bg*TLR protein was only expressed on the haemocyte on the right of the panel. Scale bars represent 20 µM.

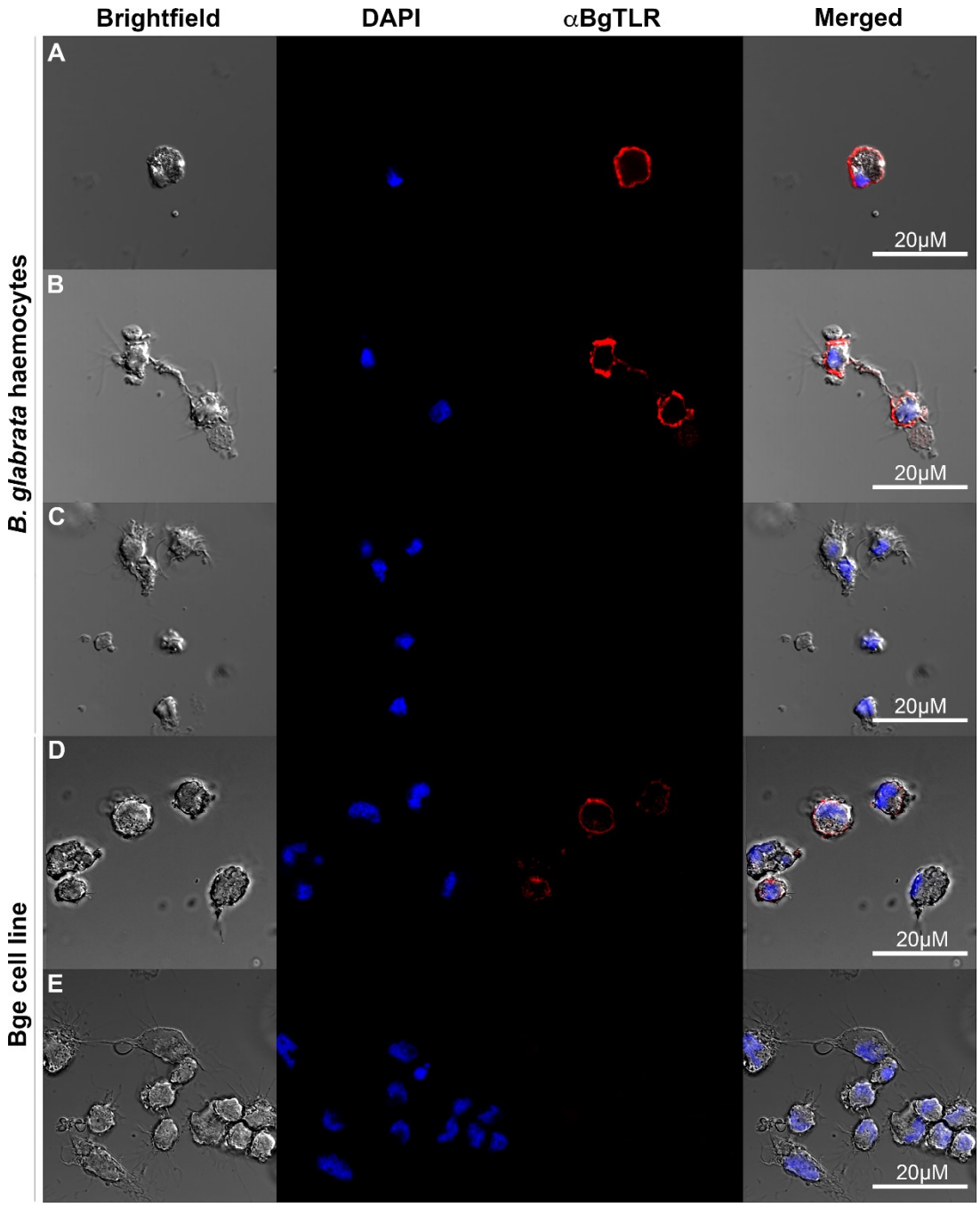


Figure 5.11. *Bg*TLR immunolocalization in haemocytes and Bge cells. Haemocytes (A-C) and Bge cells (D-E) were labelled with DAPI and anti-*Bg*TLR primary antibody. Control haemocyte (C) and Bge (E) samples were stained with DAPI but the primary antibody step omitted. Scale bars represent 20 μM.

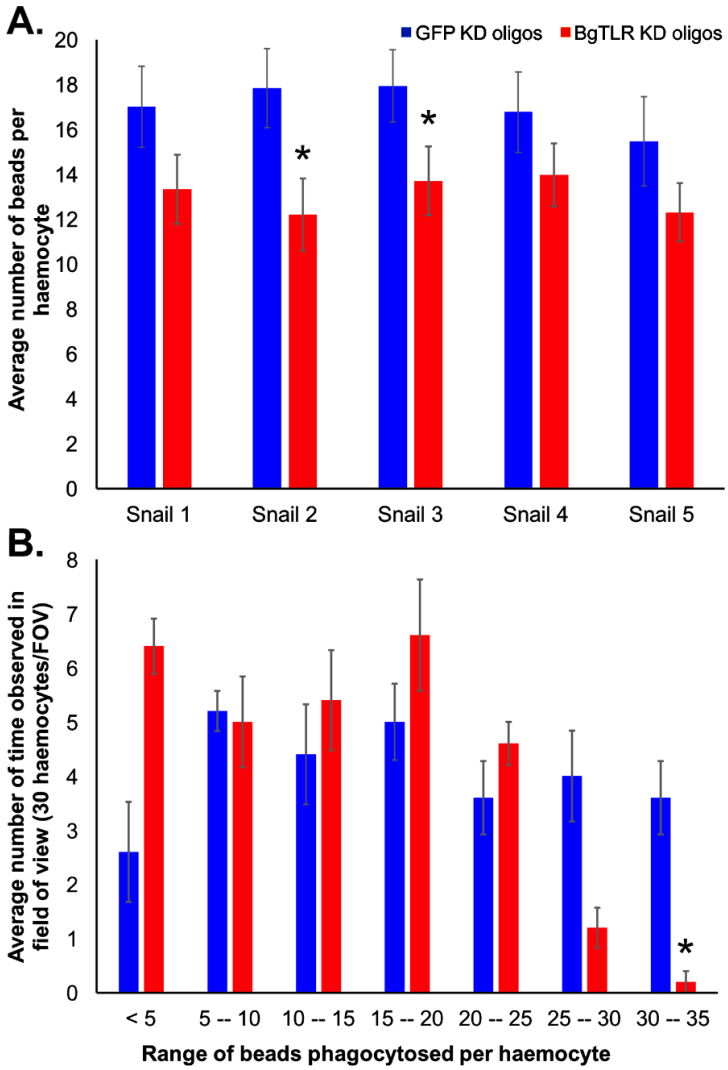


Figure 5.12. Effect of *Bg*TLR knockdown on haemocyte phagocytic response. (A) Mean number of phagocytosed beads per haemocyte. Five BS-90 snails each were injected with siRNA targeting *Bg*TLR or GFP (control) and 96 h later, haemolymph was extracted from the snails and immediately mixed with ~1 x 10^6 1-µm FITC-labelled streptavidin-coated beads pre-incubated with biotinylated *S. mansoni* excretory/secretory products and sporocysts. After 3 h, haemocytes from each snail were counted from a random field of view on the slide, and 30 haemocytes for each snail were assessed for the number of beads within each cell from which mean number of beads per haemocyte was calculated. Asterisk (*) indicates significant reduction ($P < 0.05$) in the mean number of beads per haemocyte in *Bg*TLR knockdown snails. (B) Frequency of number of beads observed in 30 haemocytes from one field of view (FOV). Asterisk (*) indicates significant difference ($P < 0.05$) in the average number of beads phagocytosed between *Bg*TLR and GFP knockdown. Phagocytic events represent a mixture of haemocyte-associated beads and those completely internalized.

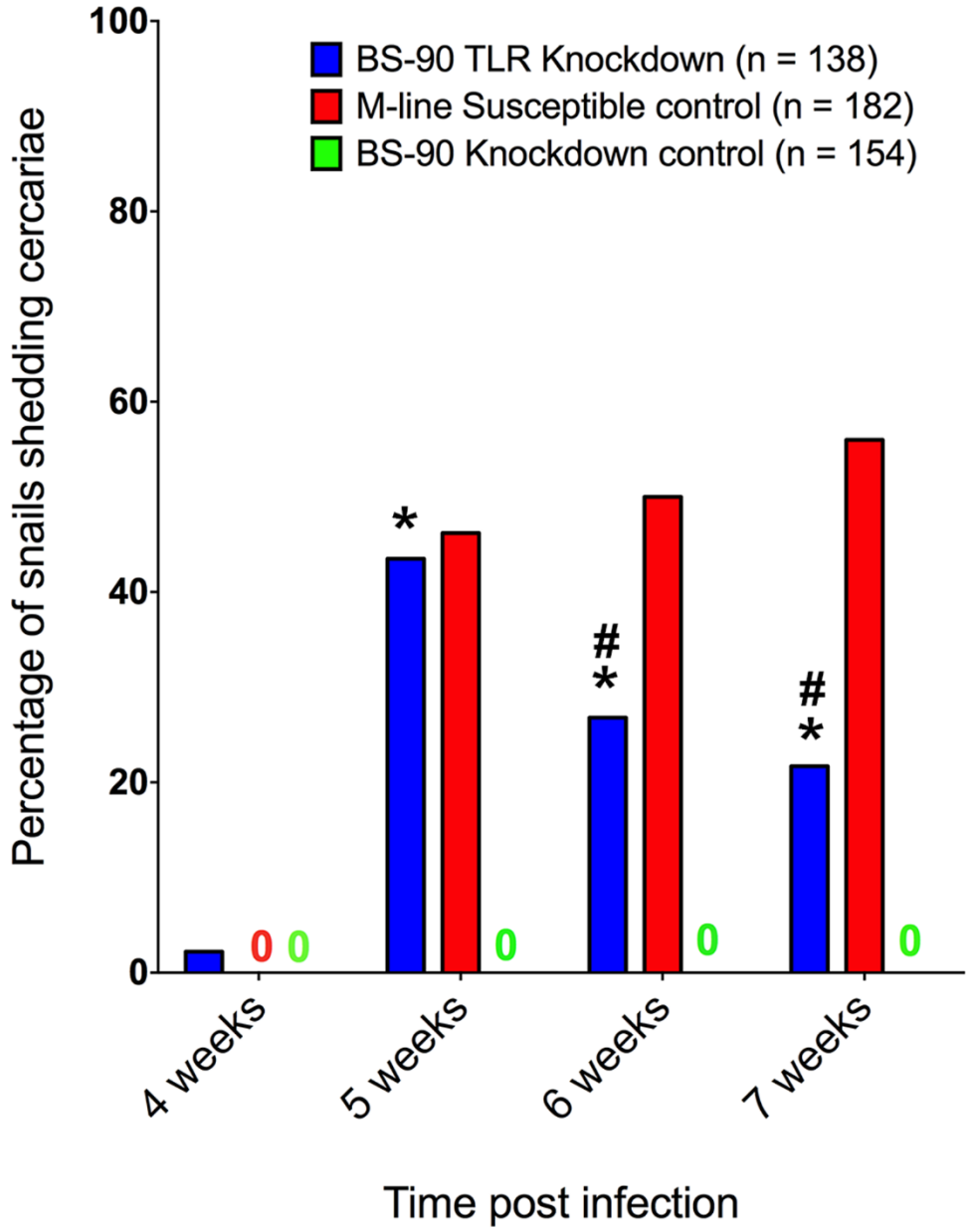


Figure 5.13. Influence of *Bg*TLR knockdown on *S. mansoni*-resistant phenotype of BS-90 snails. BS-90 snails were injected with *Bg*TLR or GFP (knockdown control) siRNA oligonucleotide mix and after 48 h exposed individually to ~5 miracidia. Non-injected M-line snails served as the susceptible controls for parasite viability. The number of cercariae-producing snails was expressed as a percentage of the starting number (*n*) of snails. Figure represents pooled data from two independent experiments. Asterisk (*) indicates significant difference from BS-90 GFP knockdown control for each week while hash (#) indicates significant difference between BS-90 *Bg*TLR knockdown and M-line susceptible control for each week.

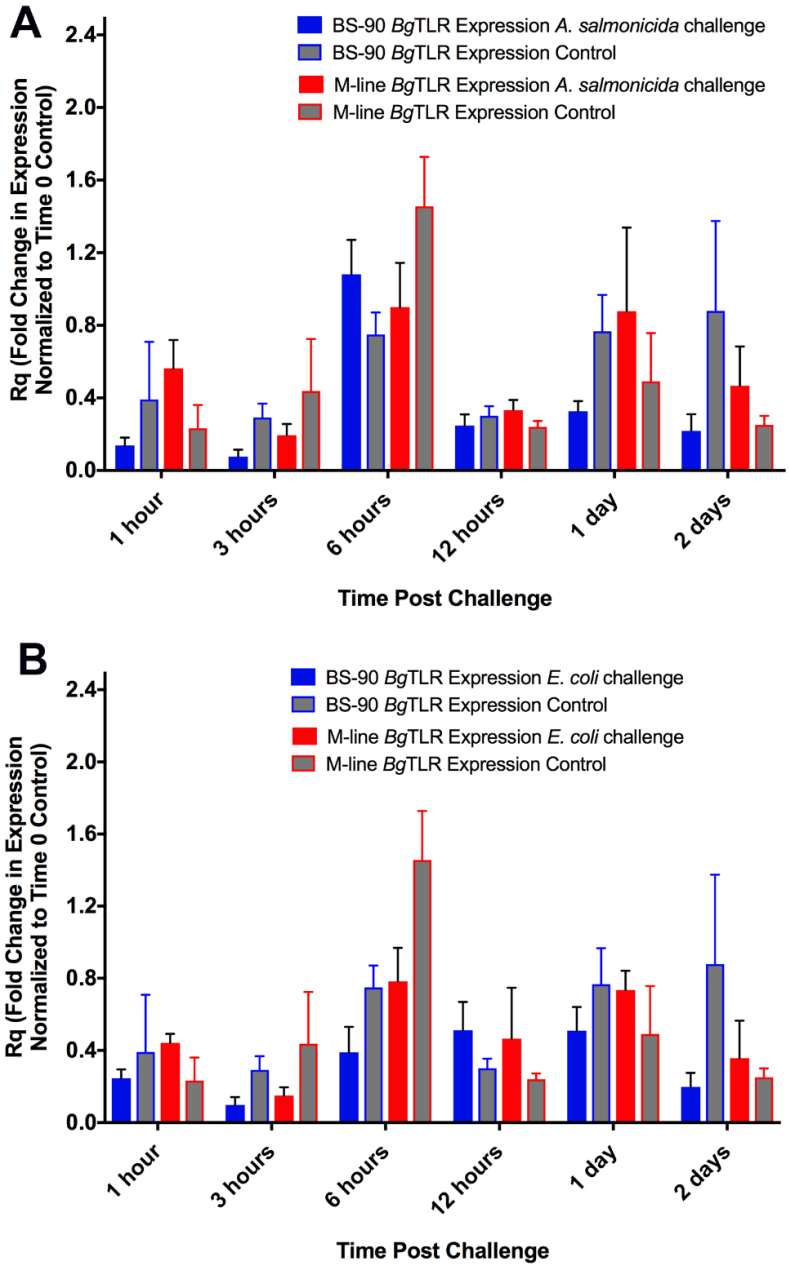


Figure 5.14. *Bg*TLR transcript expression with and without bacteria challenge. Snails (BS-90 and M-line strains) were individually injected with 2.48 X 10^7 *A. salmonicida* cells (A), or 2.40 X 10^8 *E. coli* cells (B), or injected with sterile snail saline (control). Four snails were collected at indicated time points post-injection of bacteria. RNA was extracted from whole snails, converted to cDNA and *Bg*TLR expression was measured by qRT-PCR. Expression was quantified in fold changes normalized to time 0 h controls. Bars represent standard error ($n = 4$ in one experiment).

Chapter 6

General Discussion

6.1 Overview of findings

Circulating immune cells are essential for the survival of all animals possessing a circulatory system. In molluscs, including snails, these circulating cells (or haemocytes) play critical roles in defending against infections, as well as performing other vital functions such as wound healing, tissue remodelling, shell formation and repair (Hermann *et al.* 2008; Martin *et al.* 2007; Gorbushin and Iakovleva 2006). The snail immune response is centrally coordinated by haemocytes, which directly engage trematode parasites through encapsulation, phagocytosis, ROS production, and degranulation of hydrolytic enzymes (Ataev *et al.* 2016; Cueto *et al.* 2015; Nacif-Pimenta *et al.* 2012; Humphries and Yoshino 2008). Haemocytes also secrete soluble mediators such as biomphalysin that have been shown to induce *S. mansoni* sporocyst disintegration and elimination (Pinaud *et al.* 2016; Galinier *et al.* 2013). These diverse immune and physiological functions, in addition to senescence and migration across epithelia, often deplete haemocyte numbers, necessitating a process for their basal production, and regulation of haemocyte proliferation.

Research into haematopoiesis in molluscs has advanced to the point where mechanistic investigations of growth factors, signaling pathways, cytokines and receptors are being undertaken, although largely focused on developmental and immunological model molluscs, or those that are economically important food sources (Castillo *et al.* 2015; Jemaa *et al.* 2014; Baeza Garcia *et al.* 2010; Salamat and Sullivan 2009; Tirapé *et al.* 2007). But molluscan haematopoiesis remains poorly understood, compared to our understanding of the process in

mammalian vertebrates (Baron and Fraser 2005) and other invertebrates such as *D. melanogaster* (Lebestky *et al.* 2000), which does not necessarily translate across such vast evolutionary distance. We do not have a complete description ofthe entire process in any mollusc, and currently know more about the functions of mature haemocytes than how they are produced. For gastropod molluscs, which are obligate intermediate hosts for most digenean trematodes (Olson *et al.* 2003; Cribb *et al.* 2001), the immune response is a major determinant of infection success by these parasites. Interest in characterizing the key players of the immune response in gastropods is borne by the potential benefit this may confer, by enhancing our ability to design better control strategies for diseases such as schistosomiasis. Moreover, the long-standing host–parasite association that exists between gastropods and trematodes presents an interesting immunological situation in which we can test the idea of immune conservation and immune divergence.

To advance our understanding of snail immunity with respect to schistosomes, the studies comprising my thesis focused on factors that drive the proliferation and differentiation of haemocytes, which are vital in determining the outcome of the interaction between *B. glabrata* and *S. mansoni*. In addition, I assessed haemocyte surface molecules that facilitate the engagement of haemocytes at the interface of this interaction. I functionally characterized the evolutionarily conserved growth factor progranulin to address the knowledge gap relating to the functional role of haemocytes in this snail.

In silico predicted *Bg*GRN 3D structure showed a similar organization of β-sheets and α-helices compared to known crystal structures for human and carp granulin (chapter 4, Pila *et al.* 2016a), indicative of a possible similar processing and biological roles. Consistent with this prediction, the analysis of *Bg*GRN transcript and protein expression showed that it was

upregulated during *S. mansoni* challenge in both the *S. mansoni*-resistant (BS-90) and susceptible (M-line) strains of *B. glabrata*. Higher *Bg*GRN expression levels were elicited in BS-90 than M-line snails, as was earlier appearance of *Bg*GRN cleavage products in the former, within the timeframe critical for elimination of the parasite (chapter 4, Hahn *et al.* 2001b; Sullivan *et al.* 1995; Lie *et al.* 1980).

Administration of recombinant *Bg*GRN into *Biomphalaria* snails induced haemocyte proliferation and drove the production of a granulocytic haemocyte subset involved in anti-trematode immune response. When *S. mansoni* challenge was preceded by induction of cellular proliferation via recombinant *Bg*GRN administration, the susceptible phenotype of *B. glabrata* strains and *B. pfeifferi* was significantly reversed such that *S. mansoni* miracidia were up to 54% less successful at infecting these snails (chapter 4). Initial characterization of the newly generated haemocytes indicated that these haemocytes are functionally different, at least based on the expression of *Bg*FREP3 and a *Bg*TLR, both of which are known to be associated with resistance to *S. mansoni* (Pila *et al.* 2016b; Hanington *et al.* 2010). *Bg*GRN-induced haemocyte proliferation increased the proportion of *Bg*TLR-positive cells compared to those positive for *Bg*FREP3 (chapter 4).

Based on this, I focused on functionally characterizing *Bg*TLR, which like *Bg*GRN, was found to be upregulated at time points critical for parasite elimination or establishment; and more so in the BS-90 snails (chapter 5, Pila *et al.* 2016b). When I knocked down *Bg*TLR using RNAi before exposing BS-90 snails to *S. mansoni* miracidia, about 43% of the snails developed patent infections. This demonstrates that haemocytes bearing this *Bg*TLR are functionally relevant in the anti-*S. mansoni* immune response, and that *Bg*GRN treatment drives the production of these relevant cells.

6.2 Significance of findings

The results of studies undertaken during my doctoral research represent significant strides in the field of gastropod immunobiology. Not only do they constitute a string of firsts for this field, they provide evidence for the involvement of endogenous growth factor stimulation of haemocyte proliferation, functional diversity among known haemocyte morphotypes and mechanistic insight into recognition and engagement of the parasite by haemocytes. Until the publication of my studies on *Bg*GRN, nothing was known about endogenous control of haemocyte development in any gastropod model. We knew that larval digenean trematodes (Joky *et al.* 1985; Sullivan *et al.* 1984; Sullivan *et al.* 1982; Lie and Heyneman 1975; Lie *et al.* 1975) or LTPs (Sullivan *et al.* 2004; Noda 1992) stimulated haemocyte proliferation in the APO, but whether this was a direct mitogenic effect of parasite factors or, through snail endogenous factors that, in response to infection induced haemocyte proliferation was unknown. Evidence of *Bg*GRN-induced haemocyte proliferation, as well as increases in the abundance of circulating *Bg*GRN and appearance of the cleavage products during immune response against *S. mansoni*, supports an indirect stimulation of the APO, although it does not exclude the possible effect of parasite mitogens *in vivo*.

The role of haemocyte numbers versus functional diversity is a fundamental topic in gastropod immunobiology (Larson *et al.* 2014). Evidence from the *Bg*GRN studies support functional differences as the driving force behind successful immune responses. *Bg*GRN treatment induced the proliferation of haemocytes relevant in the anti-*S. mansoni* immune response, and explains why haemocyte numbers alone do not seem to determine the effectiveness of the encapsulation response or *S. mansoni* infection success (Larson *et al.* 2014; Dikkeboom *et al.* 1988). That *Bg*TLR was also found to be selectively expressed in certain haemocytes,

provides further evidence that greater functional diversity exists within a known haemocyte morphotype than previously understood (Hanington *et al.* 2010).

The *Bg*GRN studies were also the first gain-of-resistance studies of a snail-trematode model using a defined snail-specific factor. Prior studies of the determinants of compatibility between snails and schistosomes, using defined factors, succeeded in reversing the resistance phenotype against *S. mansoni* (Hanington *et al.* 2012; Ittiprasert and Knight 2012; Hanington *et al.* 2010). Production of schistosome-resistant snails is the ultimate goal for studies with the aim of blocking schistosome transmission at the molluscan stage. Therefore, *Bg*GRN provides an excellent pathway for achieving this goal, which may also incorporate the suite of other resistance-associated factors through the immunologically relevant haemocyte subsets it induces to proliferate.

Findings from the *Bg*TLR studies provided the missing link to past studies that characterized upstream and downstream components of the pathway in *B. glabrata* (Humphries and Harter 2015; Zhang and Coultas 2011; Zelck *et al.* 2007; Humphries and Yoshino 2008; Humphries *et al.* 2001). The conclusive evidence that this pathway is functionally relevant in *B. glabrata* provided insight into how haemocytes might engage *S. mansoni* through cell-associated receptors. Studies of *B. glabrata* resistance-associated factors with respect to schistosome infections until then had functionally characterized humoral factors only. These include soluble recognition molecules such as *Bg*FREPs (Hanington *et al.* 2012; Hanington *et al.* 2010), and effector molecules such as *Bg*MIF (Baeza Garcia *et al.* 2010) and biomphalysin (Galinier *et al.* 2013). While soluble receptor and effector functions are important, recognition at the haemocyte surface and subsequent engagement are not well understood. Implication of *Bg*TLR in the snail's immune response is a step towards filling this knowledge gap about how parasite presence is

detected and the signal transduced to the haemocytes to elicit effector functions required for parasite elimination.

6.3 Model for how snails defend against schistosomes

Based on available data in the literature and the work presented in this thesis, I propose the following model for how snails defend against schistosomes (Figure 6.1). This is a modification of the model proposed by Gordy *et al*. (2015) in order to integrate the role of *Bg*GRN and *Bg*TLR in the anti-schistosome immune response. Haemocytes, the central coordinators of the snail's immune response, develop from progenitors within the APO or the anterior pericardial wall (Figure 6.1A, Jeong *et al.* 1983). They can be found in the circulation as well as in tissues (Ataev *et al.* 2016; Hermann *et al.* 2008). At least two haemocytes subsets exist (Ataev *et al.* 2016; Adema *et al.* 1992), and the most common is an adherent granulocytic cell (Figure 6.1A, Lie *et al.* 1976; Cheng 1975). Their development can be driven endogenously by growth factors like *Bg*GRN (Pila *et al.* 2016a). *Bg*GRN drives adherent haemocytes development from the APO and the haemocyte subset that develops in response to *Bg*GRN is positive for a specific Toll-like receptor (*Bg*TLR) that is important for engagement and killing of the schistosome sporocyst (Figure 6.1C). These *Bg*TLR+ haemocytes could engage the sporocyst (via unknown ligands) and activate specific effector responses relevant for parasite killing (Figure 6.1B–C).

Haemocytes can also be driven to develop following challenge by a schistosome (Joky *et al.* 1985; Sullivan *et al.* 1984; Sullivan *et al.* 1982; Lie and Heyneman 1975; Lie *et al.* 1975). Haemocytes generated following schistosome challenge abundantly produce *Bg*FREPs (Figure 6.1D), particularly, *Bg*FREP3, *Bg*FREP7 and *Bg*FREP13 (reviewed in: Gordy *et al.* 2015).

*Bg*FREP3 is a soluble, lectin-like molecule that recognizes schistosome surface proteins and is important for the ultimate killing of the parasite (Hanington *et al.* 2010). *Bg*FREPs appear to engage with a set of polymorphic mucins expressed by schistosomes (Moné *et al.* 2010). The *Bg*FREP–*Sm*PoMuc complex also engages a thioester-containing protein (*Bg*TEP) that closely resembles complement C3 (Figure 6.1C–D).

Recognition, mediated by *Bg*TLR or *Bg*FREP-opsonized sporocysts, begins the encapsulation process which leads to the recruitment of additional haemocytes and phagocytosis of the sporocyst tegument/production of ROS (Figure 6.1B-C, reviewed in: Pila *et al.* 2017a; Gordy *et al.* 2015).

6.4 Future research

The research undertaken for my doctoral thesis was focused on factors that drive haemocyte proliferation and differentiation, and assessment of haemocyte surface molecules that facilitate engagement of haemocytes with the parasite. The results of this research certainly demonstrate the involvement and importance of the evolutionarily conserved factors *Bg*GRN and *Bg*TLR in the immune response of *B. glabrata* against *S. mansoni*. However, further studies are required, especially to fill in the mechanistic details of the involvement of these resistance-associated factors.

Future studies I envisage for *Bg*GRN include: (i) determination of the effect of *Bg*GRN elastase cleavage products on ROS production, chemotaxis, phagocytosis and encapsulation, and (ii) identifying the receptor for *Bg*GRN. In addition, all the studies on *Bg*GRN and its cleavage products can be replicated using other trematodes and snail–schistosome combinations. This will give us insight as to whether *Bg*GRN can drive resistance against trematode species that are

separated by a wide phylogenetic distance such as between schistosomes and echinostomes (Olson *et al.* 2003), in which case granulin might be used as a catchall strategy for controlling digenetic trematode-transmitted diseases based on resistance genes.

For *Bg*TLR, its role in other immune functions (encapsulation and ROS production) would be the next set of experiments I would propose, followed by studies focused on screening for its ligand(s). As proposed for *Bg*GRN, figuring out the relevance of *Bg*TLR in the immune response against other trematode species would be worthwhile. Furthermore, it would be interesting to determine the role of the other numerous TLRs and LRRs found in *B. glabrata*.

6.4.1 Determine the immunological effects of *Bg*GRN elastase cleavage products

In this thesis, all *Bg*GRN studies involving recombinant protein were done using progranulin – the form in which the protein is made, but this can be subsequently cleaved into smaller functional units by elastase as reviewed in chapter 4 (Zanocco-Marani *et al.* 1999). Determining the immunological effects of *Bg*GRN cleavage products is the next logical step because both progranulin and the cleavage products are functional, with the effects of the cleavage products sometimes opposite to progranulin. The cleavage products can participate in, and activate immune responses but their effects can be antagonistic to each other (reviewed in: Cenik *et al.* 2012).

Recombinant constructs for various combinations of *Bg*GRN domains are in place in *Sf*9 cell clones (Figure 6.2). These recombinant *Bg*GRN granulin domains can be purified and used for the respective immune assays (ROS production, chemotaxis, phagocytosis and encapsulation). To test for the effect on ROS production, snail haemocytes can be isolated and incubated with each cleavage product. Then ROS production can be measured using a

commercial kit, such as Amplex^{TM} Red hydrogen peroxide/peroxidase kit, which contains a fluorogenic probe that gives a fluorescent product when oxidized by ROS produced from the cells. Chemotaxis can also be tested using a commercially available apparatus. This consists of two chambers separated by a 5-µm pore membrane filter. Each *Bg*GRN domain construct as a test chemotactic sample can be added to the bottom chamber while the cells are added in the top chamber. After a period of incubation, membrane filter would be carefully removed after aspirating media from the top chamber. The filter can be placed on a microscope slide (bottom side up), fixed and stained for enumeration. For phagocytosis, I envision that snails will be co-injected with fluorescent beads and the cleavage products or beads alone prior to haemocyte isolation and assay for presence of beads within them. Encapsulation assays may consist of exposure of haemocytes pre-incubated with the cleavage products to *in vitro*-transformed *S. mansoni* sporocysts. All of the proposed experiments can include the full-length *Bg*GRN for comparison.

6.4.2 Identify the receptor for *Bg*GRN

Identifying the receptor for *Bg*GRN can be approached in two ways. The first approach would be to screen candidate receptors based on the mammalian granulin literature, such as TNF–α receptor and sortilin (Jian *et al.* 2013; Zheng *et al.* 2011). Genes that possess high predicted amino acid identities to both proteins are found in *B. glabrata* genome (Adema *et al.* 2017). Possible interactions between these proteins and *Bg*GRN can be tested by using a genetic interaction system such as the yeast two-hybrid system (Fields and Song 1989). This would require the generation of chimeric constructs because the candidate interacting partners need to be expressed as a fusion to separate modules of a transcription factor. When reconstituted

through the binding of the proteins, this transcription factor would activate a reporter gene under its control (Fields and Song 1989).

The second approach I propose is to take advantage of the V5 tag on recombinant *Bg*GRN to anchor it on an anti-V5 purification column. Membrane proteins can be isolated from snail haemocytes at a time of heightened *Bg*GRN expression, and presumably its receptor, as occurs during the immune challenge with *S. mansoni* parasite. The isolated membrane proteins can be passed through the column with immobilized *Bg*GRN. The resulting *Bg*GRN-receptor complex can then be eluted by the addition of V5 peptides and analyzed by mass spectroscopy.

6.4.3 Determine the effect of *Bg*TLR on immune functions

The involvement of phagocytosis as a downstream functional response was supported by the *Bg*TLR studies but the overall percentage of phagocytic haemocytes did not differ from the GFP knockdown control snails. Also, it is known that for parasites, which are too large to be phagocytosed by a single haemocyte, other cellular responses such as encapsulation and ROS production are the more prominent participants in the effector response (Nacif-Pimenta *et al.* 2012; Hahn *et al.* 2001a; Hahn *et al.* 2001b; Loker *et al.* 1982). Therefore, it is important to determine whether *Bg*TLR activates these responses. Testing for these responses should be straightforward. Snails could be injected with siRNA oligos against *Bg*TLR or GFP as control prior to isolation of haemocytes and exposure to parasite LTPs which should elicit ROS production. ROS can be measured as described above for *Bg*GRN cleavage products. For the encapsulation reaction, the isolated haemocytes can be exposed to *in vitro*-transformed *S. mansoni* sporocysts, after which haemocyte behaviour such as spreading and percentage of sporocysts encapsulated can be determined.

6.4.4 Screen for *Bg*TLR ligands

Identifying the ligand(s) recognized by *Bg*TLR is essential because, with the possibility that it might be recognizing PAMPs that are digenean trematode-specific, a new PAMP could be added to the TLR ligand canon. Anti-parasitic recognition/response involving TLRs have not been characterized in an invertebrate to the best of my knowledge. Identifying *Bg*TLR ligands would also clarify its subcellular localization and possible intracellular role since faint immunolocalization was observed in the cytoplasm of both haemocytes and Bge cells (chapter 5).

Human TLR2/*Bg*TLR chimeric fusion constructs, full length *Bg*TLR and truncated LRR and TIR domains have been cloned in the pDisplay vector and are expressed in AD293 cells (Figure 6.3), which do not express endogenous Toll-like receptors (Schmidt *et al.* 2010). Initial screening could involve exposure of these cells to LTPs derived from *in vitro*-transformed *S. mansoni* miracidia and potential endogenous ligands such as *Bg*FREPs (Smiley *et al.* 2001). TLR response/signaling can be determined by direct measurement of phosphorylated NF-kB, a downstream transcription factor onto which TLR signaling converge. This can be measured using commercially available kits such as phospho-NF-kB p65 (ser536) InstantOne^{TM} ELISA (eBioscience). Following the initial tests with crude LTPs, the screening could be refined by progressively testing size-separated LTPs while retaining positive fractions. The proteins present in these positive fractions could then be identified using mass spectroscopy. Information garnered from the studies proposed here would advance our understanding of the mechanisms of involvement of these two proteins in gastropod immunobiology.

6.5 Potential application of findings

As reviewed in chapter 2, beyond enriching the literature on invertebrate immune capabilities, particularly of the lophotrochozoans, the findings from studies presented herein can be integrated into current control approaches to reduce human and animal disease burden caused by schistosomes in endemic regions. A necessary step towards elimination of any infectious disease is to break the transmission cycle. For schistosomiasis and other trematodiases, we know that the snails present a significant opportunity that can be exploited because of the parasite's requirement for specific snail hosts, which also act as a bottleneck point (Allan *et al.* 2013; Hamburger *et al.* 2004). We could diminish snail susceptibility to trematodes by altering the balance of snail populations in the field towards the resistant ones.

The ability of *Bg*GRN to mediate gain-of-resistance in *Biomphalaria* snails against *S. mansoni*, and potentially against other trematodes, would prove useful in this regard. Snails could be engineered to overexpress *Bg*GRN using genomic modification technologies such as CRISPR/cas9 system, which has already been used to modify mosquitoes for resistance against *Plasmodium* infection (Gantz *et al.* 2015; Dong *et al.* 2011; Isaacs *et al.* 2011). This modification could drive increased resistance to schistosome infection in snails. The modified snails can then be massively bred and released in a locality after eliminating the remaining individuals through snail-specific pesticides or removal using attractants.

Snail replacement is proposed so that the ecological impact on the intervention areas might be as minimal as possible. In one of the projects carried out during my doctoral research, I determined that an endogenously produced pheromone, *Bg*Temptin, is an attractant of *B. glabrata* (see appendix, Pila *et al.* 2017b). This provides a tool that can be further developed and used in the snail replacement effort, in the form of a pheromone trap. The trap can be deployed

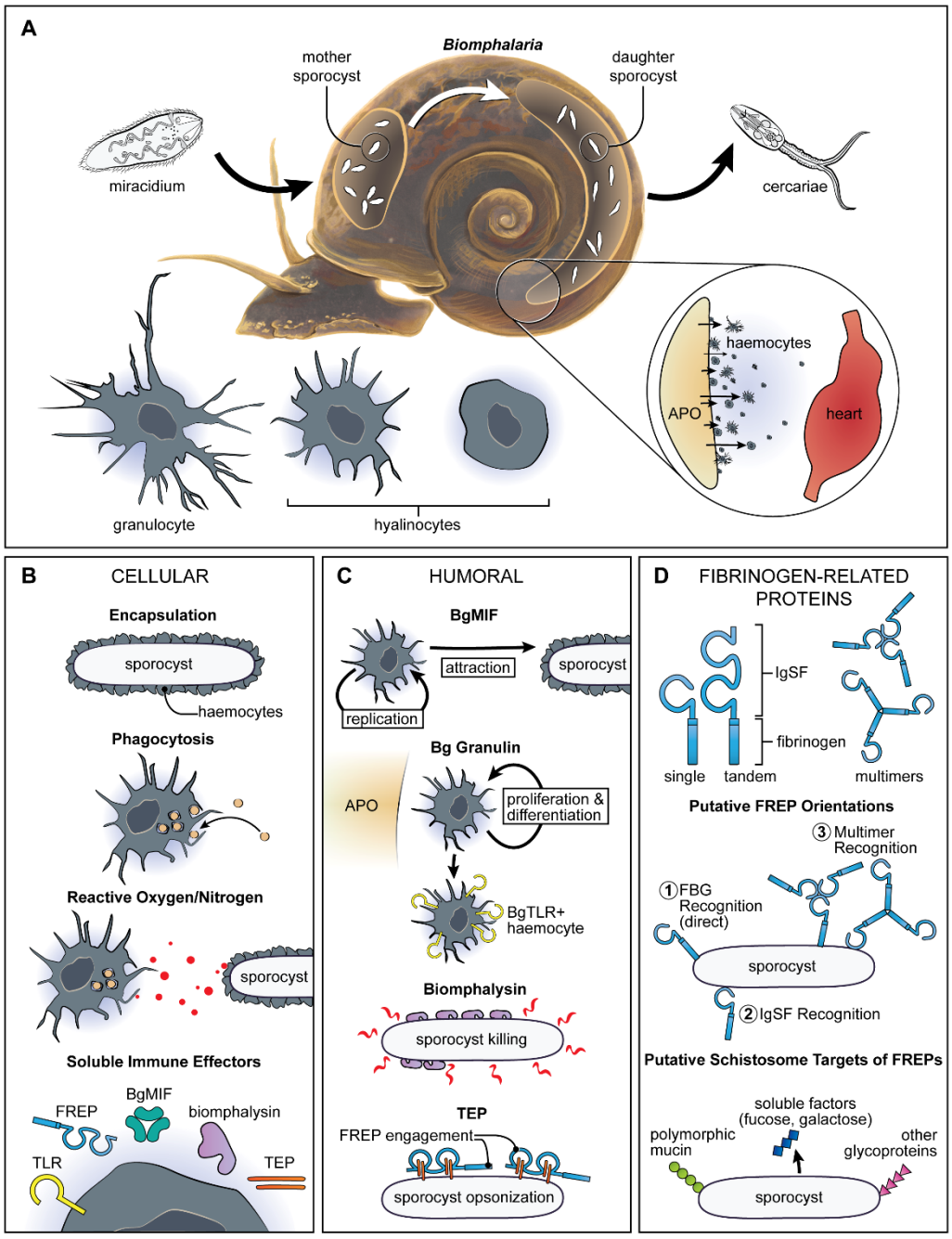
either alone and the attracted snails physically removed, or in combination with molluscicides bound to the trap (Thomas *et al.* 1980), potentially minimizing the off-target effects of molluscicides alone. Combining snails of enhanced resistance against schistosomes with snail elimination via pheromone traps and persistent mass drug administration, we may break the transmission cycle for *Schistosoma* parasites, and pave the way for achieving sustained control of human schistosomiasis.

There are studies including a field trial which indicate in principle that the approach of using resistant snails in an integrated control strategy is feasible. Cross-breeding of a highly resistant strain of *B. tenagophila* (Taim lineage) with a highly susceptible strain (Joinville) resulted in a generation of snails that had infection rates up to five times lower than the susceptible parental strain (Rosa *et al.* 2004; Rosa 2002). When these same strains were raised together in the presence of the parasite – *S. mansoni*, the survival rate of the resistant strain increased, while the susceptible strain suffered a negative selection pressure (Rosa *et al.* 2006). A recent field study found that susceptibility of *B. tenagophila* offspring to *S. mansoni* infection reduced from 38.6% to 2.1% within 14 months of introduction of resistant conspecifics (Marques *et al.* 2014). It is worth noting that this study neither incorporated elimination of resident snails nor treatment of the human population, both components of which are likely to have greater impact on disease prevalence and snail infection rates.

6.6 Summary

The studies presented in my thesis show that the endogenous growth factor *Bg*GRN is relevant in the immune response of *Biomphalaria* snails against *S. mansoni*. *Bg*GRN stimulated the proliferation of an adherent, granulocytic haemocyte subset that is typically associated with

parasite encapsulation, phagocytosis, and the production of ROS/RNI important for parasite killing. Initial assessment of these haemocytes using two resistance-associated factors (*Bg*FREP3 and *Bg*TLR) revealed differences in the proportion of haemocytes bearing these factors, thus, signifying functional differences within the newly generated haemocyte subset. By functionally characterizing *Bg*TLR, I was able to demonstrate its involvement as a haemocyte surface molecule that facilitate engagement of haemocytes with the parasite. Collectively, these studies have advanced our understanding of snail immunity with respect to schistosomes.



Adapted from Pila *et al*. (2017a).

Figure 6.1. Proposed model for how snails defend against schistosome infections based on defined elements of *B. glabrata* immune response. (A) Locations of intramolluscan schistosome stages and the amoebocyte-producing organ (APO). In a successful infection, miracidia penetrate the snail, often at the head-foot region, where they shed their ciliated

epidermal plates and transform into mother sporocysts. Mother sporocysts migrate to the digestive gland and replicate into many daughter sporocysts, which further replicate and eventually produce the cercariae that emerge from the snail. The APO is a flat sheet of tissue in the anterior pericardial wall, which produces haemocyte precursors that mature into granulocytes and hyalinocytes. Haemocyte development can be driven by endogenous factors like *Bg*GRN (*Bg*TLR+ haemocytes) or induced by infection (*Bg*FREP+ haemocytes). (B) In incompatible snails, the sporocysts are eliminated through haemocyte-mediated immune responses, including encapsulation, phagocytosis, and production of cytotoxic molecules (reactive oxygen/nitrogen species). Haemocytes also produce soluble immune effectors. (C) Humoral factors produced by haemocytes include chemotactic (*Biomphalaria glabrata* migration inhibitory factor, *Bg*MIF), proliferation and differentiation (granulin) signals, and molecules capable of direct killing of sporocysts (biomphalysin) or opsonization (thioester-containing protein, TEP, and fibrinogen-related protein, FREP). (D) Fibrinogen-related proteins. Single or tandem-immunoglobulin superfamily (IgSF) FREPs may directly recognize targets through the fibrinogen (FBG) or IgSF domains. FREP recognition may also be dependent or enhanced by multimer formations, perhaps through the FBG or IgSF domains. The various FREP configurations may then directly recognize sporocyst targets (soluble factors like galactose, polymorphic mucins, or other glycoproteins) or via mediators like the snail TEP.

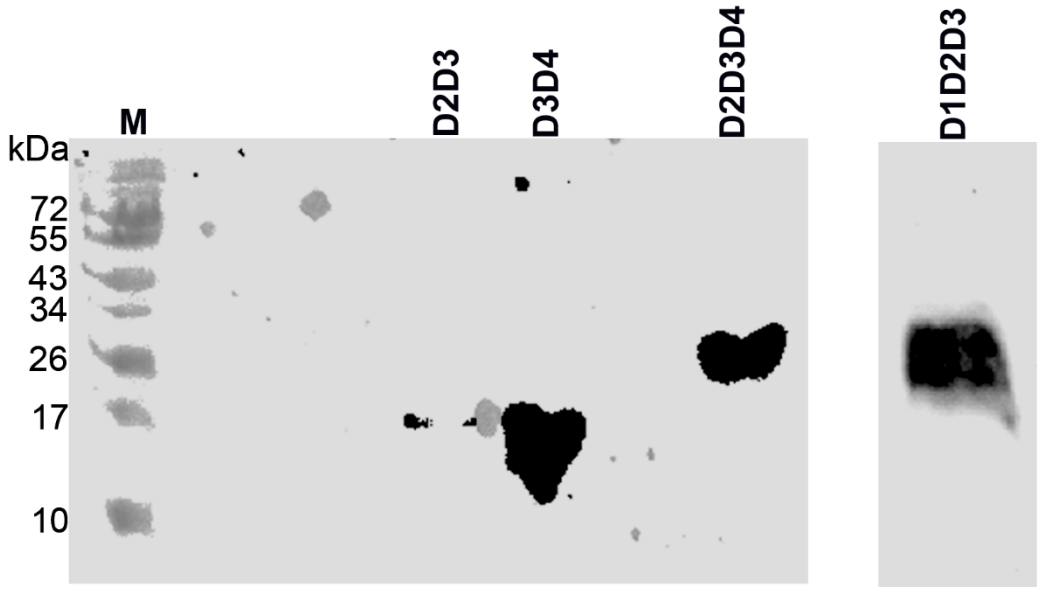


Figure 6.2. Generation of recombinant *Bg*GRN cleavage fragments. Nucleotide sequences representing various *Bg*GRN granulin-domain combinantions were cloned into the pIB/V5-His-DEST Gateway® expression vector, which was transfected into the *Sf*9 cells. Protein expression was detected in cell lysates via Western blots using a primary antibody against the V5 tag. M = molecular weight marker, with sizes shown to the left in kDa.

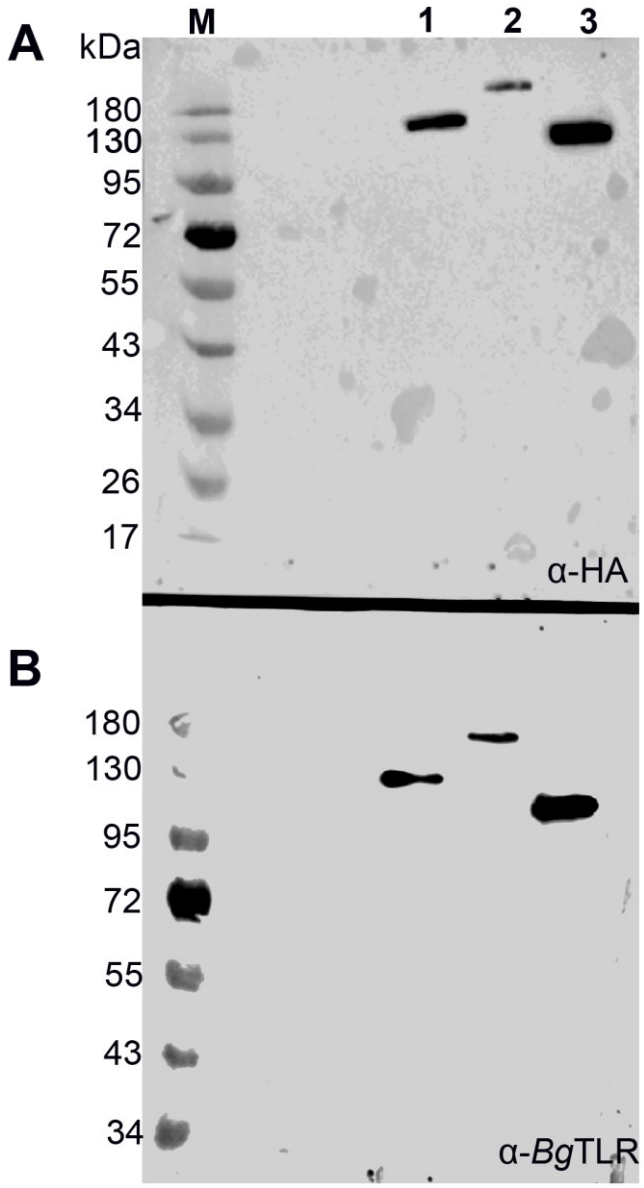


Figure 6.3. Confirmation of stable TLR fusion protein expression in AD293 cells. Complete or truncated *Bg*TLR coding sequences and chimeric *Bg*TLR constructs were cloned into the pDisplay vector and transformed into TOP10 *E. coli* cells. Plasmid was purified and used to transfect AD293 cells. After a month of selection on geneticin antibiotic, protein was purified from cell lysates and detected on Western blot. (1) *Bg*TLR LRR fused to human TLR2 TIR (~107 kDa). (2) Complete *Bg*TLR (~135 kDa). (3). *Bg*TLR LRR (~83 kDa). Protein was detected with primary antibody developed against the fused hemagglutinin tag (A) or the extracellular region of *Bg*TLR (B). M = molecular marker, with sizes in kDa shown on the left.

6.7 References

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Appendix

The Protein Pheromone Temptin is an Attractant of *Biomphalaria glabrata*[4]

A.1 Introduction

Chemosensory communication is an evolutionarily conserved process utilized by most of the major groups of living organisms including plants (Alborn *et al.* 1997), protozoans (Alimenti *et al.* 2011), cnidarians (Howe and Sheikh 1975), molluscs (Cummins *et al.* 2004), annelids (Gaudron *et al.* 2007), arthropods (Yew and Chung 2015), fish (Sorensen *et al.* 2005), amphibians (Kikuyama *et al.* 1995), reptiles (reviewed in: Mason and Parker 2010) and mammals (reviewed in: Stowers and Kuo 2015). Volatile chemical substances called pheromones, released and detected by organisms of the same species, are commonly used by terrestrial organisms for various communication purposes ranging from aggregation for reproduction, alarm signals, food foraging, and migration (Brant *et al.* 2015; Traynor *et al.* 2015; Painter *et al.* 2004; Ichinose *et al.* 2003). Pheromones of peptide or protein nature are advantageous for communication in aquatic environments and have been described in a wide range of animals (reviewed in: Cummins and Bowie 2012) including molluscs (Kuanpradit *et al.* 2012; Cummins *et al.* 2011; Boal *et al.* 2010; Painter *et al.* 2004; Browne *et al.* 1998).

Within the phylum Mollusca, chitons (Polyplacophora), which are generally considered to be an early derived molluscan taxon (Sigwart and Sutton 2007), are thought to have a gonadotropin-releasing hormone (GnRH)-like peptide pheromone based on the findings that they can be stimulated to release ripe gametes following exposure to lamprey GnRH-1 or tunicate

[4] A version of this appendix has been published: Pila, E.A., Peck, S.J. and Hanington, P.C. (2017). The protein pheromone temptin is an attractant of the gastropod *Biomphalaria glabrata*. *Journal of Comparative Physiology A*, 203(10), 855-866.

GnRH-2 at concentrations of 1.0 mg/L (Gorbman *et al.* 2003). Protein pheromones are also messengers of chemical communication in the most modern molluscan group (Cephalopoda), represented by squids, cuttlefishes and octopuses. For instance, extreme male-male aggression during the mating season of the squid, *Loligo pealeii,* has been shown to be triggered by a protein contact pheromone (β-microseminoprotein) embedded in the eggs (Cummins *et al.* 2011). Chemosensory communication is also critical in the behaviour of bivalves and gastropods. Protein pheromones have been implicated among these two molluscan groups in processes like conspecific spawning in oysters (Rice *et al.* 2002) and clams (Munro *et al.* 1983), aggregation in species of *Aplysia* (Painter *et al.* 2004) and the abalone *Haliotis asinina* (Kuanpradit *et al.* 2012), alarm behaviour and sex recognition in the apple snail, *Pomacea canaliculata* (Takeichi *et al.* 2007; Ichinose *et al.* 2003), as well as stimulation of larval settlement and metamorphosis in oysters (Browne *et al.* 1998; Zimmer-Faust and Tamburri 1994).

The gastropod *Biomphalaria glabrata* is an intermediate host for *Schistosoma mansoni*, a parasitic flatworm that causes intestinal schistosomiasis in humans. Schistosomiasis is a chronic, devastating disease that affects over 260 million people worldwide (WHO, 2016) and has so far eluded control efforts in endemic regions that are often dominated by oral chemotherapy. The schistosome life cycle requires the parasite undertake larval development within specific species of *Biomphalaria* snails (Bray *et al.* 2002; Esch *et al.* 2002; Cribb *et al.* 2001), a process during which extensive asexual amplification takes place. The importance of the snail host during schistosome development has made the intra-molluscan stage of *Schistosoma* species a target for studies seeking to develop novel methods that can be used in an integrated schistosomiasis control strategy (Adema *et al.* 2012). Application of molluscicides, as an alternative control strategy that targets the snail intermediate hosts, have been shown to have great impact on snail

numbers and schistosome prevalence (Erko *et al.* 2002; Pieri *et al.* 1995; Sturrock *et al.* 1974; Foster *et al.* 1959). However, the cost associated with this strategy, due to the high degree of training and organization required for success (King and Bertsch 2015), is a major drawback along with environmental effects such as the killing of non-target organisms (Takougang *et al.* 2007; Takougang *et al.* 2006). An alternative strategy, to use species-specific attractive factors as a means for luring the snail for removal from the water, or away from areas of human activity, holds great potential for success, but the identity and functional mechanisms of relevant factors produced by *B. glabrata* are not known.

Most studies of *B. glabrata* chemoreception have examined the attractive potential of exogenous factors released from components of the snail's environment, such as macrophytes and epiphytic algae. These include certain amino acids, sugars, short-chain carboxylic acids, and extracts of the snail's plant food sources, such as lettuce (Thomas *et al.* 1989; Thomas 1986; Thomas *et al.* 1983; Bousfield *et al.* 1980; Uhazy *et al.* 1978). Little is known about chemoattractive factors produced by the snail, but the available evidence indicates that specific chemical communication takes place in this snail, presumably, through protein pheromones or some other chemical mediators. For example, *B. glabrata* fecundity can increase with the introduction of conspecifics into their environment, even without physical contact, or transfer of sperm (Vianey-Liaud and Dussart 2002). However, there appears to be uncertainty on the nature and involvement of specific mediators. One study found that individuals of *B. glabrata* can follow short-lived (10 to 30 m) mucus trails produced by themselves or other conspecific individuals, but not those from another species (*Lymnaea stagnalis*, Townsend 1974). Whereas, another study found evidence of both intraspecific chemoattraction of *B. glabrata* and

interspecific attraction of *B. glabrata* to *Helisoma trivolvis,* but no evidence of *B. glabrata* mucus trail following or attraction to egg masses (Marcopoulos and Fried 1994).

To further elucidate chemosensory communication in *B. glabrata*, I have examined the behavioural responses to a temptin-like protein produced by the snail *B. glabrata* (*Bg*Temptin). Temptin was first identified and characterized for its role in attraction of the marine gastropod *Aplysia californica* (Cummins *et al.* 2004). Mating aggregations are typical of this hermaphroditic slug and have been shown to be initiated by the laying of egg cordons, coinciding with the release of a mixture of protein pheromones: attractin, enticin, temptin and seductin, which associate to form larger complexes (Cummins and Bowie 2012; Cummins *et al.* 2005). Although protein pheromones have been described in gastropods and other molluscs, none have been characterized in *B. glabrata*. In 2006, Adema and colleagues identified and deposited one expressed sequence tag of temptin in GenBank (accession number: FC857191.1). A search of the recently sequenced *B. glabrata* genome (Adema *et al.* 2017) at the onset of this study (Preliminary genome assembly, version 4.0.3 – contigs) produced a significant alignment (E value ≤ 0.01) only to temptin, among the four attraction pheromones produced by *Aplysia*. Furthermore, temptin peptides were found to be among the components of egg masses produced by *B. glabrata* (Hathaway *et al.* 2010), suggesting that *Bg*Temptin is expressed and potentially functions in a role similar to that found in *Aplysia*. This prompted me to investigate its influence on the behavioural responses of *B. glabrata,* with the objective to determine whether temptin – a known component of a pheromone complex in *Aplysia* that is capable of attraction - would be able to attract *B. glabrata* on its own.

I report on the functional characterization of a temptin-like protein from the snail *B. glabrata,* and demonstrate that recombinant *Bg*Temptin (r*Bg*Temptin) is attractive to this snail, and that the effect is specific and dependent on the concentration of the protein.

A.2 Experimental design

A.2.1 Recombinant *Bg*Temptin synthesis and purification

The *Bg*Temptin coding sequence was cloned into the expression vector using the Gateway® cloning system per the manufacturer's instructions (Life Technologies). Briefly, total RNA was isolated from whole snail tissue of the M-line strain of *B. glabrata* using Trizol according to the manufacturer's instructions (Life Technologies). The RNA was converted into cDNA in an oligo-dT-primed synthesis according to the manufacturer's protocol (ThermoFisher Scientific). The coding region of *Bg*Temptin was amplified from this cDNA template using Phusion high-fidelity DNA polymerase (ThermoFisher Scientific) with the following primers: *Bg*Temptin-forward: *CAC CAG GAG G*AT CTT C**AT AAT GG**A ATT TGT TTG CCT TGT TGT G (modified by adding *CACC* nucleotides, *ribosome binding* and **kozak** consensus sequences accordingly for efficient cloning and expression) and *Bg*Temptin-reverse: CGG CTC CAA CTT GTC GCA ATC. Cloning into the entry and expression vectors, *Sf*9 cells transfection and protein purification were done as described in the main text (chapter 3). Purified *Bg*Temptin was concentrated using Pierce® concentrators 9K MWCO/7 mL (ThermoFisher Scientific) and quantified with the Qubit® protein assay kit (Life Technologies), aliquoted and saved at – 80°C until needed.

A.2.2 Western blot detection of r*Bg*Temptin

Western blot detection of recombinant r*Bg*Temptin was accomplished using culture medium containing secreted protein. Five mL of confluent culture medium was passed through 0.22 µm filter and then concentrated 10-fold using 9K MWCO concentrators. Protein samples were suspended in 4x Laemmli protein loading buffer, heated at 95 °C for 10 m, and 30 µL was loaded per lane on 10% (vol/vol) SDS-PAGE. Sample separation and immunoblotting were done as described in chapter 3.

A.2.3 Detection of *Bg*Temptin complex formation

To test whether *Bg*Temptin complexes with other proteins, purified r*Bg*Temptin was combined with samples obtained from two sources: snail secretory products (SSP) and whole snail homogenates (WSH). SSP was prepared by putting 20 adult snails in 60 mL artificial spring water (ASW, Ulmer 1970) overnight (~17 hours). The snail-conditioned water and any secreted product was then collected and filtered through 0.22-µm pore size filters and then concentrated 30-fold using 9 MWCO/7 mL concentrators (ThermoFisher Scientific). WSH was prepared by homogenizing 10 adult snails (whose shells had been removed) in 60 mL of cell lysis buffer (50 mM Tris, pH 7.8, 150 mM NaCl, 1% IGEPAL CA-630). Homogenized sample was incubated for 30 m in the lysis buffer at room temperature, after which it was filtered then concentrated 2-fold. The protein concentration in SSP and WSH was 386 µg/mL and 5,200 µg/mL respectively based on the Qubit® protein assay kit (Life Technologies).

Recombinant *Bg*Temptin was incubated with SSP or WSH in the following ratios (wt/wt in µg): 1:1, 1:2, 1:5, 1:10, 2:1 and 5:1 in 0.6 mL tubes for 2.5 h at room temperature on a Labquake shaker (ThermoFisher Scientific) to allow potential complexes to form. After the incubation, a volume of sample containing 1 µg total protein was withdrawn from each tube. A

protein loading buffer (without β-mercaptoethanol) was added to the withdrawn sample to a final volume of 40 µL. Twenty-five µL (625 ng) of this sample was then separated on 10% (vol/vol) SDS-PAGE and processed as described above for Western blot analysis, detecting the V5 epitope associated with r*Bg*Temptin.

A.2.4 Attraction tests

A lane maze (Figure A.1a) was used to test the attractive potential of r*Bg*Temptin to *B. glabrata* snails and to determine the optimal concentration to be used in the T-maze tests. The apparatus was constructed from white polyvinyl chloride sheets 60 cm long, 25 cm wide and 10 cm deep, with a volume of 15 L. It was divided into 5 lanes, each measuring 60 cm x 5 cm x 10 cm (length x width x depth respectively). Each lane was rinsed with 500 mL of ASW before and in between tests to minimize the potential effect of any residual test substance. Individual lanes were filled to a depth of 1 cm with ASW (500 mL) and sat for 2 m, allowing the water to calm. A test substance was added at one end, and after 5 m of diffusion, a snail was introduced. The diffusion rate of r*Bg*Temptin was inferred from testing the addition of food colouring at the same amount and time as temptin. Test substances used included: 2 g banana (positive control), no stimulus (negative control), 1 snail, 1 snail + 5 egg masses, *Bg*Temptin at 0.25 nM, 0.5 nM, 1 nM, 2 nM, 4 nM and 8 nM final concentrations. This range of concentrations was chosen to encompass the amount that has been used in similar studies in *Aplysia* (Cummins *et al.* 2005).

Each snail was placed directly in the middle of its trough (lane), with its head towards the 60 cm side. A Nikon D90 camera with 18–55 mm lens was set on a tripod at one 60 cm side to cover the entire surface of the apparatus, and to take one picture per m for 1 h (60 in total). The lanes were divided into four equal quadrants of 15 cm each. Snail location in the lanes was

tracked using the 1-m snapshots which were used to determine the amount of time spent in each quadrant. These numbers were converted and expressed as percentage of time spent in the treatment or non-treatment halves of the lane. This way, the addition of test substances could be randomized, while maintaining consistency in data acquisition. The snails used for trials on one day were put in a separate tank and not used for other trials for at least 4 days. Each test consisted of one snail per lane. The tests were then repeated 8–22 times for the various assay treatments using new snails or ones that had not been used in an assay for at least 4 days.

A T-maze (Figure A.1b) was used to test the specificity of r*Bg*Temptin as an attractant of *B. glabrata* in the presence of alternative substances. It was constructed with the same material as the lane maze with a total volume of 8.5 L. Each arm of the apparatus was 25 cm long, 10 cm wide and 10 cm deep. The apparatus was rinsed with ASW before and in between tests. It was filled to 1 cm depth with ASW (1000 mL) and allowed to settle for 2 m. Test substances were added at the ends of the two top arms and, after 5 m of diffusion, a snail was introduced at the end of the bottom arm. Just as with the lane maze, a camera was set on a tripod, capturing the entire surface of the apparatus, to track snail movement via 1-m snapshots. The amount of time the snail spent in each arm of the T-maze, in a 30-m period (based on inferred time required for complete diffusion of test substances), was calculated from the acquired pictures and expressed as percentages. Test substances were randomly assigned to the arms of the T-maze, and snails from one trial were not used for at least 4 days. Each test consisted of one snail per lane and the tests were repeated 10 times for the various assay treatments using snails as described for the lane maze above. Size-matched (8–10 mm shell diameter), sexually mature *B. glabrata* M-line snails were used for all experiments.

A.2.5 Sequence alignments and phylogenetic methods

Amino acid sequences for *Bg*Temptin, 12 *B. glabrata* predicted temptin-like genes (XP_013062304.1, XP_013078118.1, XP_013085977.1, XP_013085978.1, XP_013085980.1, XP_013085982.1, XP_013085986.1, XP_013086708.1, XP_013086707.1, XP_013086709.1, XP_013094712.1 and XP_013095488.1), and the 4 known proteins of the *Aplysia* pheromonal complex [*A. californica* temptin (AAP73787.1), attractin (AAD00569.1), enticin (AAN83923.1) and seductin (AAN83922.1)], as acquired from GenBank [National Center for Biotechnology Information (NCBI)], were imported into CLC Genomics Workbench 8.0.3 (www.clcbio.com). A multiple sequence alignment was produced using the CLC proprietary algorithm, at the very accurate (slow) setting, with a gap open cost of 10, gap extension cost of 1 and end gap cost "as any other." A maximum likelihood tree was generated from this alignment also with CLC Genomics. The initial tree for the heuristic search was obtained by the neighbour joining method, and the WAG protein substitution model, with gamma distribution parameter set to 1.0, number of substitution rate categories to 4, and using 1,000 bootstrap replicates. The WAG model, which uses amino acid frequencies and substitution rates that have been empirically derived from alignments of 3,905 amino acid sequences from 182 protein families, provides a better estimate of the evolutionary process, is applicable to a broader range of protein sequences and not prone to systematic errors compared to models estimated using counting methods (Whelan and Goldman 2001).

A.2.6 Statistical analysis

Results of the attraction tests were analyzed using GraphPad Prism version 6.0f for Mac OS X (GraphPad Software, California USA, www.graphpad.com). To compare the amount of

time spent by the snail in the two halves of the lane maze, paired sample *t*-tests were performed. The time spent by snails in the three arms of the T-maze apparatus was compared using the Kruskal-Wallis test followed by Dunn's post hoc test with correction for multiple comparisons. The statistical significance threshold was set at $P \leq 0.05$.

A.3 Results

A.3.1 Western blot detection of *Bg*Temptin

Full-length *Bg*Temptin consists of 126 amino acid residues and is 98% identical to the FC857191.1 clone. The lysine, tyrosine and serine residues at positions 2, 90 and 103 of FC857191.1 are substituted by glutamic acid, aspartic acid and arginine respectively in *Bg*Temptin. *Bg*Temptin is predicted to have a signal peptide with a cleavage site between position 16 and 17 (Petersen *et al.* 2011) and a molecular weight of ~12 kDa for the cleaved protein (Gasteiger *et al.* 2003). Western blot detection of native *Bg*Temptin (Figure A.2) shows a single band at ~18 kDa. Reduced samples also appeared as single bands but of a slightly larger molecular weight (~20 kDa, Figure A.2).

A.3.2 *Bg*Temptin attraction of *B. glabrata* snails is concentration-dependent

The attraction of *B. glabrata* snails to r*Bg*Temptin was initially evaluated in lane maze tests (Figure A.3), with the end containing the test substance designated as the treatment half. Six concentrations of *Bg*Temptin (0.25 nM, 0.5 nM, 1 nM, 2 nM, 4 nM and 8 nM) were tested alongside banana, a food bait (Long 1996) as positive control. As expected, when the snails were introduced into lanes with no test substance added, the time spent between the first and second halves of the lanes (mean = 47.45%, SD = 19.71% and mean = 52.55%, SD = 19.48%

respectively) were not significantly different (P = 0.6837), indicating that snail movement was random in the absence of attractant and not in preference to either side of the apparatus. Similar results were obtained with the addition of 2 nM bovine serum albumin (BSA) as a protein control (not shown). At 0.25 nM *Bg*Temptin concentration, snails spent about 12.48% more time in the treatment than the non-treatment halves of the lanes (mean = 56.24%, SD = 14.43% and mean = 43.76%, SD = 12.43% respectively). Time spent in the treatment half of the lanes increased to 23.42% and 20.85% at r*Bg*Temptin concentrations of 0.5 nM and 1 nM respectively (0.5 nM treatment half: mean = 61.16%, SD = 18.19%, non-treatment half: mean = 38.84%, SD = 18.87%; 1 nM treatment half: mean = 60.43%, SD = 29.67%, non-treatment half: mean = 39.57%, SD = 24.06%).

At 2 nM *Bg*Temptin concentration, snails spent about 62% more time in the treatment half, which was statistically significant (P < 0.01; treatment half: mean = 81.05%, SD = 13.06%, non-treatment half: mean = 18.95%, SD = 13.06%; Figure A.3). Time spent in the treatment half of the lanes compared to the non-treatment half increased to 74.16% and 74.72% at *Bg*Temptin concentrations of 4 nM and 8 nM respectively (4 nM treatment half: mean = 87.08%, SD = 9.56%, non-treatment half: mean = 12.92%, SD = 9.56%; 8 nM treatment half: mean = 87.36%, SD = 6.13%, non-treatment half: mean = 12.64%, SD = 6.13%). These differences were also significant (P < 0.01). The three highest concentrations of r*Bg*Temptin resulted in snail attraction that was greater than that of the 2 g banana piece (positive control), which also significantly (P < 0.01) attracted the snails to the treatment half (treatment half: mean = 70.77%, SD = 25.35%, non-treatment half: mean = 29.23%, SD = 15.05%). The attractive effect of r*Bg*Temptin reached a plateau at around 4 nM, since the 8 nM concentration did not produce appreciable difference in the percent-time spent in the treatment half of the lane maze. In other assay trials, snails spent

significantly more time (P = 0.0302) in the treatment half when another snail was introduced in place of a test substance (treatment half: mean = 67.50%, SD = 29.04%, non-treatment half: mean = 32.50%, SD = 9.27%), but a snail plus 5 egg masses were not attractive (treatment half: mean = 40.00%, SD = 23.58%, non-treatment half: mean = 60.00%, SD = 25.87%; P = 0.5720).

A.3.3 *Bg*Temptin attraction of *B. glabrata* snails is specific

To further confirm the attractive effect and specificity of r*Bg*Temptin, snails were exposed in a T-maze to 2 nM recombinant protein as well as various other assay conditions. When exposed to both banana (positive control) and 2 nM r*Bg*Temptin, the snail spent significantly more time (44%) in the banana arm than *Bg*Temptin (32%), compared to the blank end (23%; P = 0.0135; Figure A.4b). Time spent in the *Bg*Temptin arm significantly increased to 53% in exposures with 2 nM BSA protein control (24%; P = 0.0015) comparable to similar trials involving banana (Figure A.4c–d). Similarly, snails spent more time in the high *Bg*Temptin (2 nM) arm compared to low *Bg*Temptin (0.25 nM) when introduced from blank (52%; adjusted P = 0.0202) or BSA (55%) arms (P = 0.0001; Figure A.4e–f). Snails introduced into the T-maze arm already containing an attractant could not be lured away by another attractant. Recombinant *Bg*Temptin at a concentration of 2 nM did not attract snails away from banana (P < 0.01) just as banana did not attract the snails introduced in the r*Bg*Temptin-containing arm (P < 0.01; Figure A.4g–h). In these trials, snails spent on average 85% of the time in the attractant in which they were introduced. Denatured r*Bg*Temptin (heated at 95 °C for 10 m before application) was not significantly attractive to the snails (Figure A.4i–k). As was expected considering the results of the lane maze tests, time spent by the snails in the three arms of the T-maze was not significantly different when no test substance was added (P = 0.7667; Figure A.4l).

A.3.4 *Bg*Temptin complex formation and potential partners

Temptin functions as part of a pheromone complex in *Aplysia*, thus, I was interested in determining whether r*Bg*Temptin binds to other proteins produced by *B. glabrata*. I initially searched the *B. glabrata* genome (Adema *et al.* 2017) available on the Vectorbase website (www.vectorbase.org) and analysed GenBank for sequences that shared nucleotide similarity to other known molluscan pheromones. While these analyses identified 12 predicted temptin-like genes (Figure A.5) with the conserved residues relevant for fold and function (Cummins *et al.* 2007, Figure A.6), they did not identify genes with homologies to the other *Aplysia* pheromones known to complex with temptin (attractin, enticin and seductin). Among the *Aplysia* pheromones, only attractin has a solved crystal structure to which I could compare the identified *B. glabrata* temptin-like sequences. Also, the highly conserved attractin motif IEECKTS which is critical for its function (Painter *et al.* 2004) is lacking in the temptin-like sequences of *B. glabrata* (Figure A.7), meaning that structural homology with attractin could be positively ruled out. I initially carried out protein threading using the Phyre^2 programme (Kelley *et al.* 2015) (http://www.sbg.bio.ic.ac.uk/phyre2). The output of this approach did not yield conclusive results. Either the percent identities or confidence of the match were very low or both (data not shown). Moreover, the matches were to unknown proteins or those belonging to superfamilies not clearly connected to chemosensation. Therefore, I resorted to phylogenetic analysis comparing the predicted protein sequences of the temptin-like genes with *Bg*Temptin, *Aplysia* temptin, enticin, attractin and seductin. As shown in Figure A.5, the phylogenetic tree resulting from these sequences can be grouped into 2 main clades. Clade I consists of most of the temptin-like sequences including *Bg*Temptin and *Aplysia* temptin (*Ac*Temptin). The remaining proteins of *Aplysia* pheromone complex are segregated in clade II with three temptin-like sequences

(XP_013085977.1, XP_013085982.1 and XP_013085980.1; Figure A.5). However, the bootstrap support for both clades was 30% only.

Since the *in silico* strategies yielded results with low confidence only, I proceeded to conduct protein association and pull-down assays using V5/His-tagged r*Bg*Temptin as a bait protein with snail secretory products (SSP) and whole snail homogenate (WSH) as sources of prey proteins. Western blot detection of r*Bg*Temptin after incubation with prey samples shows the appearance of a higher molecular weight band, indicating that r*Bg*Temptin binds to at least one other partner (Figure A.8). In both SSP and WSH, a single higher molecular weight band appeared between the 34 and 43 kDa, putting the size of the (yet to be identified) partner between 16 and 25 kDa. The ratio of 5 µg bait to 1 µg prey sample produced the most prominent band by Western blot analysis among the ratios tested (Figure A.8).

A.4 Discussion

Chemosensory communication enables numerous processes, some of which are essential for the survival or reproductive fitness of an organism. Expectedly, the phenomenon is highly conserved through evolution even though the specific mediators can be remarkably divergent. It is especially important in organisms in which there is little or no development of vision (reviewed in: Cummins and Bowie 2012). Beyond their biological importance, these compounds can potentially be applied in the control of pest species or vectors/intermediate hosts of disease-causing agents. For schistosomiasis, there are many areas of research focusing on prevention and treatment of human infection by schistosomes. Some of this research is focused on the snail host, either on the ability of the snail to resist infection (Pila *et al.* 2016a; Pila *et al.* 2016b; Galinier *et al.* 2013; Hanington *et al.* 2012; Baeza Garcia *et al.* 2010; Goodall *et al.* 2006), or finding a way

to draw snails away from areas of human activity in the water (Thomas *et al.* 1989; Thomas 1986; Thomas *et al.* 1983; Bousfield *et al.* 1980; Uhazy *et al.* 1978). The latter strategy requires identification and understanding of factors that are specifically attractive to the snail host.

In this study, I generated recombinant temptin, a protein produced by *B. glabrata* (*Bg*Temptin) with homology to a known molluscan pheromone, and have examined its snail attraction potential. Western blot analyses indicate that the observed size of native recombinant *Bg*Temptin is slightly higher than the predicted size of the mature protein plus detection tag (Figure A.2, Figure A.8). This difference could be due to the signal peptide being retained on the mature protein or the impact of post-translational modifications (such as glycosylation) on the protein size. *Bg*Temptin is predicted to have 3 N-linked glycosites (at positions 47, 65 and 92) as predicted by GlycoEP (www.imtech.res.in/raghava/glycoep/submit.html) (Chauhan *et al.* 2013). This potential glycosylation in the protein could impact on the observed size. Nevertheless, the signal peptide, if retained on the mature protein, does not on its own entirely interfere with r*Bg*Temptin function, or the ability of the protein to form complexes with other proteins secreted by *B. glabrata*.

Reduced and denatured r*Bg*Temtpin migrated more slowly on SDS-PAGE than native protein (Figure A.2). In its native conformation, r*Bg*Temptin molecules may have bound less SDS and thus retained its structural compactness as it migrated through the gel. This effect has been observed in *Escherichia coli* β-barrel membrane protein (Kleinschmidt *et al.* 1999), human cystic fibrosis transmembrane conductance regulator (Rath *et al.* 2009) as well as lysozyme (Dunker and Kenyon 1976), and is associated with factors like disulphide bond formations which can reduce SDS binding by up to 2-fold (Therien *et al.* 2001). *Bg*Temptin has six cysteine residues, all of which are predicted to be disulphide bonded (Ceroni *et al.* 2006)

(http://disulfind.dsi.unifi.it/), with confidence of disulfide bonding state prediction ranging from 6 to 8 (0 = low, 9 = high). Three intramolecular disulphide bridges are also predicted between the cysteine pairs: 5 and 73, 13 and 93, and 34 and 121 (Ceroni *et al.* 2006), with confidence of connectivity value of 0.5753 (real values range from 0 to 1). Disruption of r*Bg*Temptin conformational structure through heating and reducing agents may be responsible for the anomalous migration on the SDS-PAGE (Figure A.2).

The results of this study demonstrate that r*Bg*Temptin attracts *B. glabrata* snails and that this attraction is dependent on the concentration of r*Bg*Temptin with an apparent plateau at 4 nM. Exposure of the M-line strain of *B. glabrata*, which is susceptible to numerous strains of *S. mansoni*, to r*Bg*Temptin resulted in 81% (lane maze) and at least 70% (T-maze) time spent at the r*Bg*Temptin source. All snails used in the present study were not infected with *S. mansoni*. It is important to determine whether *B. glabrata* would respond consistently to *Bg*Temptin during infection, under various physiological states or varying external factors like pH and temperature. Whether infected snails will respond similarly to *Bg*Temptin is an interesting question because it will provide insight to the function of *Bg*Temptin and the possible impact on its use in schistosomiasis transmission areas. It is well known that the parasite eventually castrates infected *B. glabrata* (Faro *et al.* 2013; Cooper *et al.* 1996), so it is possible that temptin may lose its effectiveness as an attractant if it functions as a sex pheromone, or still be effective if it is purely an aggregation pheromone. It is also possible that through the long co-evolution that has taken place between *Biomphalaria* snails and the schistosomes that infect them, that *S. mansoni* may have developed chemosensory mechanism that exploit *Bg*Temptin as a way to locate suitable snail hosts (Liang *et al.* 2016). Any of these outcomes would be informative and important to

characterize in order to predict how *Bg*Temptin might perform as a snail attractant in a natural water body.

As in other studies (Marcopoulos and Fried 1994), *B. glabrata* snails were significantly attractive to conspecific individuals. An unexpected finding of this study is the non-attraction of egg masses to *B. glabrata*. Contrary to findings in *Aplysia* (Painter *et al.* 1991) and other gastropods(reviewed in: Runham 1992), egg masses did not attract the snails used in this study, despite the previous finding that temptin is a component of egg masses (Hathaway *et al.* 2010). I found an exact match of only 3 of the 4 peptide sequences for temptin described by Hathaway *et al*. to *Bg*Temptin, indicating that the two proteins are not exactly the same. This may account for why the egg masses were not attractive. Since only 5 egg masses were used in each test involving 500 mL of water, it is also possible that the concentration of *Bg*Temptin present was below the limit of detection by the snails. This is probable since *Bg*Temptin at a concentration of 0.25 nM was consistently not attractive to the snails (Figure A.3, Figure A.4e–h). Egg masses were also found to be unattractive to snails in a *B. glabrata* chemoattraction study even at 2 egg masses per 55 mL of water (Marcopoulos and Fried 1994). Another possibility is that other temptin-like proteins are released that increase the effective concentration that the snails respond to. The results of the complex formation assays (Figure A.8) support this possibility. Currently I do not know precisely where *Bg*Temptin is produced in the snail, but based on the fact that a temptin protein has been detected in *B. glabrata* egg masses (Hathaway *et al.* 2010), it may be also associated with the reproductive organs and likely produced in the albumen gland like *Aplysia* temptin (Cummins *et al.* 2004).

Recombinant *Bg*Temptin itself was attractive to *B. glabrata*, unlike in *Aplysia,* where temptin functions as part of a pheromone complex that also contains attractin, enticin and

seductin proteins (Cummins *et al.* 2005; Cummins *et al.* 2004). The results of this study suggest that non-reduced and non-denatured r*Bg*Temptin did not form homomeric complexes (Figure A.2, Figure A.8), indicating that the protein either functions on its own as monomer or forms a heterocomplex. Indeed, pull down assays confirmed that *Bg*Temptin forms a heterocomplex with as yet unknown partner(s) present within the snail and also secreted into the environment (Figure A.8). It will be worthwhile to determine the identity of the partner(s) in the heterocomplex, which could also be one of the other temptin-like proteins produced by *B. glabrata*. Moreover, it would be interesting to determine whether pre-incubation with snail-conditioned water or the actual partner(s) forming the complex might increase the attractiveness of r*Bg*Temptin to *B. glabrata*. It also remains possible that *Bg*Temptin forms homomeric complexes in the snail with the participation of cofactors that may not be present in our studies.

While *Bg*Temptin alone was attractive to the snails, it is important to also determine the role of the other temptin-like genes – whether they are redundant in function, if they would increase the attraction by forming a complex or, other molecular interactions, and if *Bg*Temptin is attractive to *S. mansoni* miracidia. Substances that are specifically attractive to *B. glabrata* have the potential for use in pheromone traps where they can be deployed either alone and the attracted snails physically removed, or in combination with molluscicides bound to the trap (Thomas *et al.* 1980), potentially minimizing the off-target effects of molluscicides alone. Characterization of *Bg*Temptin, an endogenously produced attractant, as done in this study, provides another tool that can be further developed and used in the effort to eliminate schistosomiasis.

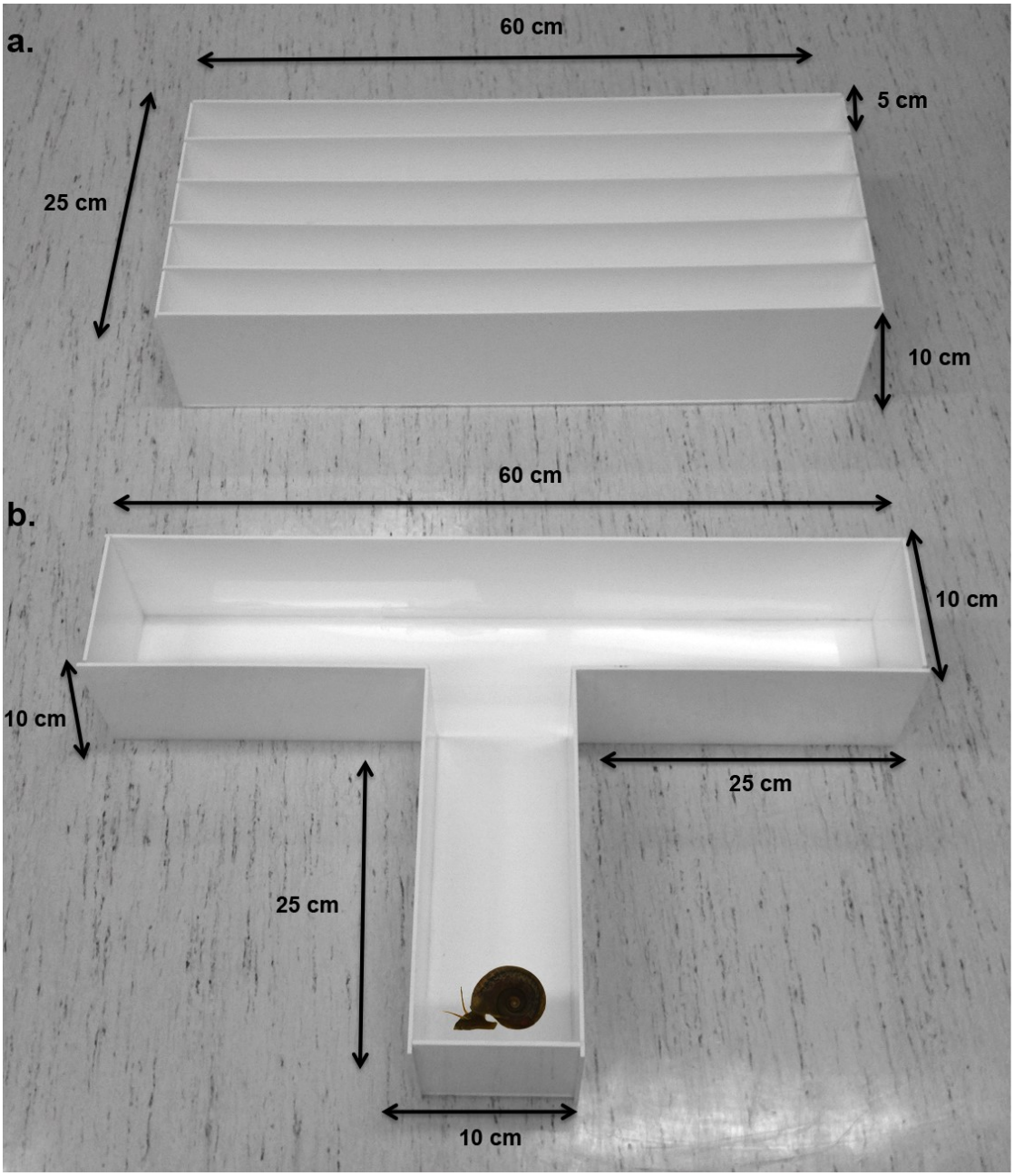

Figure A.1. Setup for the snail attraction tests. The lane maze (a) and T-maze (b) were constructed from white polyvinyl chloride sheets. They were filled with artificial spring water (ASW) to a depth of 1 cm and allowed to settle for 2 m. A test substance was introduced at one end (lane maze) or the two top arms (T-maze) and after 5 m of diffusion, a snail was introduced at the middle of the lane with its head towards the 60 cm side (lane maze), or the end of the bottom arm (T-maze). Both lane and T-mazes were rinsed before and in between tests, and were set under a Nikon D90 camera that took pictures at 1 m intervals.

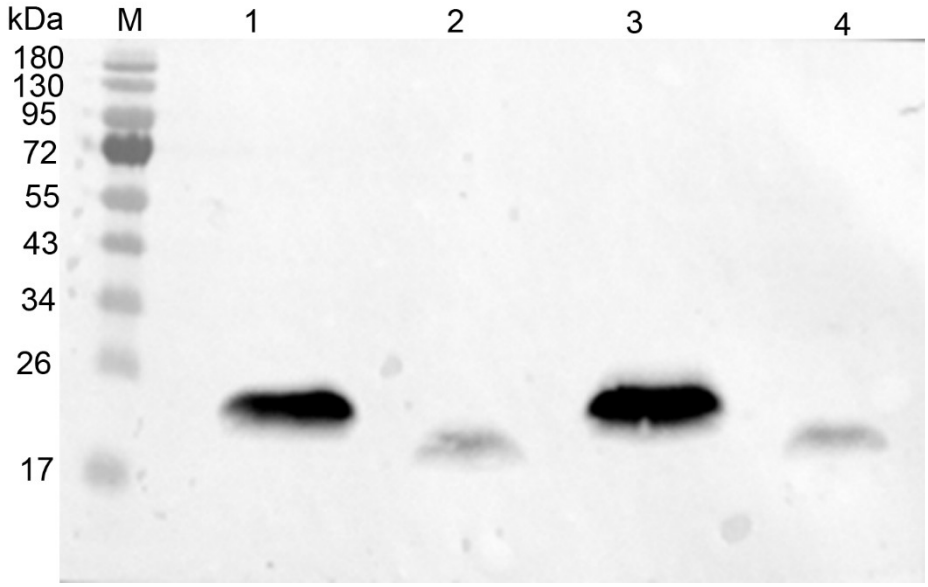

Figure A.2. Western blot detection of *Bg*Temptin. Equal volumes of concentrated protein samples were separated on 10% SDS-PAGE, blotted onto a 0.45 µm nitrocellulose membrane and detected with a primary antibody against the V5-epitope tag fused with recombinant *Bg*Temptin. (M) Molecular weight marker with sizes shown in kDa to the left. (1) Samples suspended in Laemmli protein loading buffer with β-mercaptoethanol and heated at 95 °C for 10 m. (2) Samples suspended in loading buffer without β-mercaptoethanol (no reducing agent) and not heated. (3). Samples suspended in loading buffer with β-mercaptoethanol but not heated. (4) Samples suspended in loading buffer without β-mercaptoethanol but heated at 95 °C for 10 m.

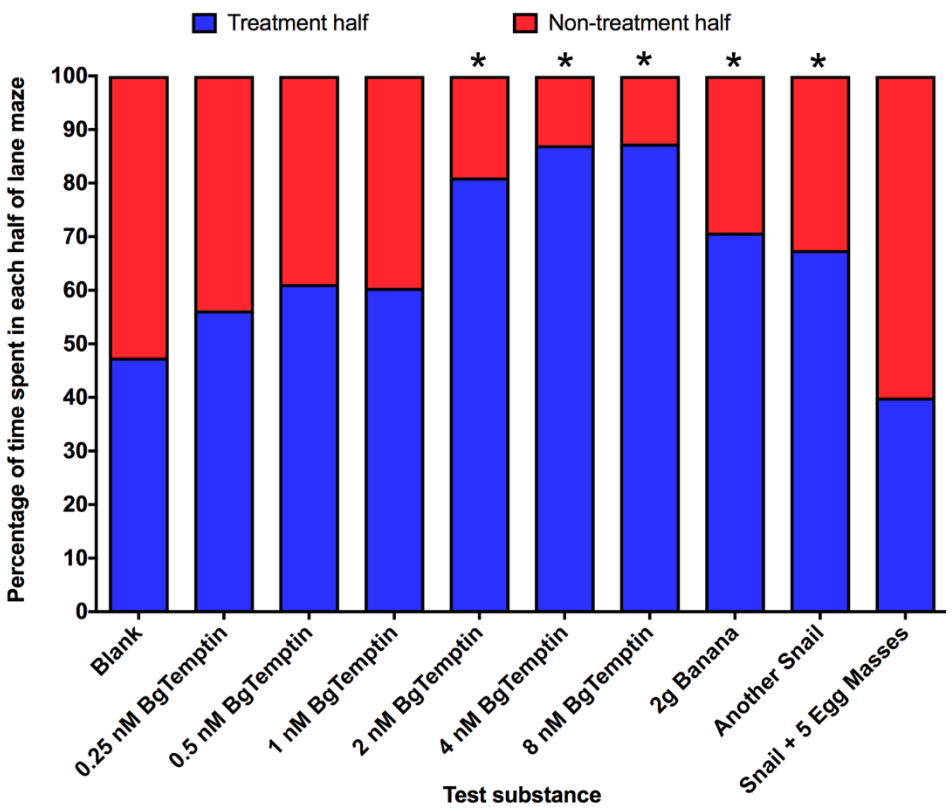

Figure A.3. Percentage of time spent in each half of the lane maze by *B. glabrata* snails under various test substances. Six concentrations of *Bg*Temptin (0.25 nM, 0.5 nM, 1 nM, 2 nM, 4 nM and 8 nM) were tested alongside a food bait (banana) as positive control, another snail, and a snail + 5 egg masses. No test substance was added in a blank test (negative control). The end of the lane containing a test substance was designated as the treatment half and the opposite end as the non-treatment half. Asterisks (*) indicate statistically significant differences between time spent in the first versus the second halves ($P < 0.05$).

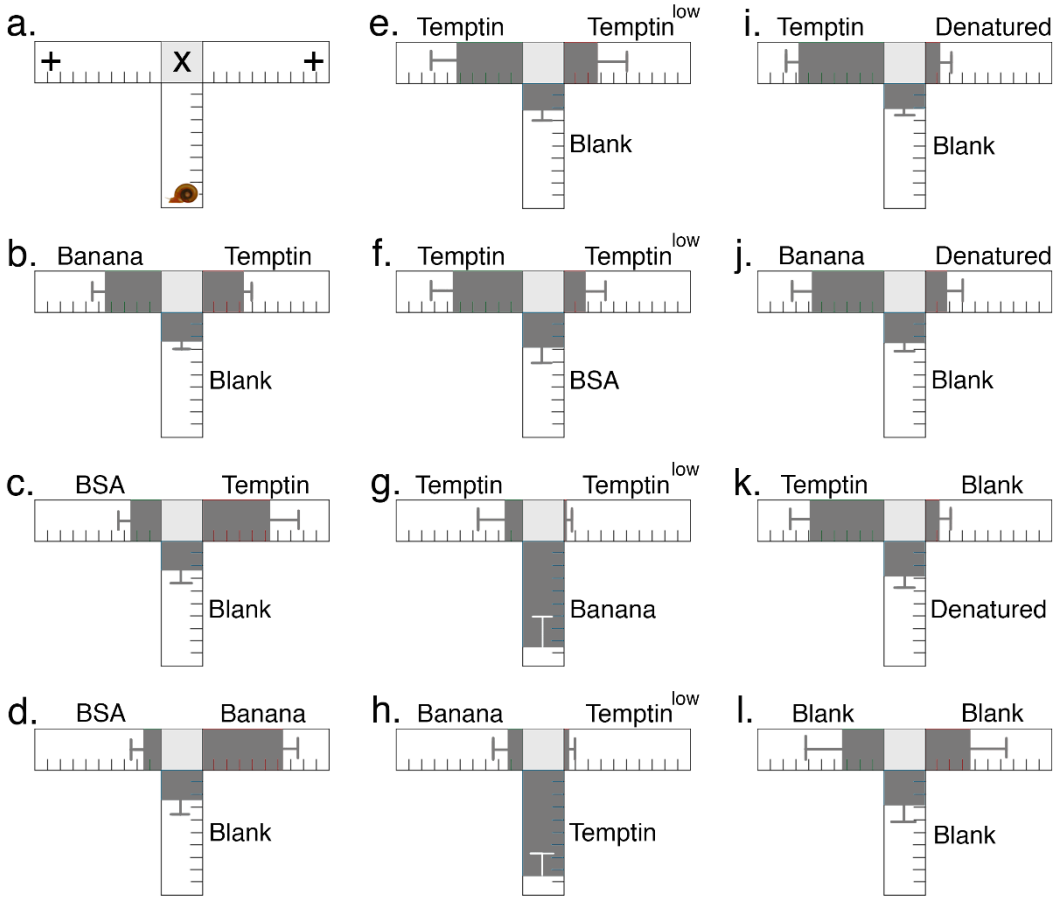

Figure A.4. Specificity of *B. glabrata* attraction to *Bg*Temptin in a T-maze. (a) Setup of the assay showing where test substances and snails were introduced. (b) Comparison of *Bg*Temptin and banana (a food bait). (c–d) Comparisons of *Bg*Temptin and banana respectively to BSA (protein control). (e–f) Comparisons of high *Bg*Temptin (2 nM) with low *Bg*Temptin (0.25 nM) in the presence of a blank and 2 nM BSA respectively. (g–h) Tests showing that snails introduced in a T-maze arm already containing an attractant could not be lured away by another attractant. (i–k) Denatured *Bg*Temptin (heated at 95 °C for 10 m before application) loses attractive potency. (l) In the absence of a test substance, time spent by the snails in each arm of the T-maze is similar. Snails were always introduced at the end of the bottom arm while the introduction of test substances (+) varied among the arms of the T-maze. The scale of each arm represents 100% of time spent in that arm, and is divided into ten sections, representing 10 % each. Percentage of time spent by the snail in each arm is denoted by grey filling of the area bound by the corresponding tick mark. Bars represent standard deviation (SD).

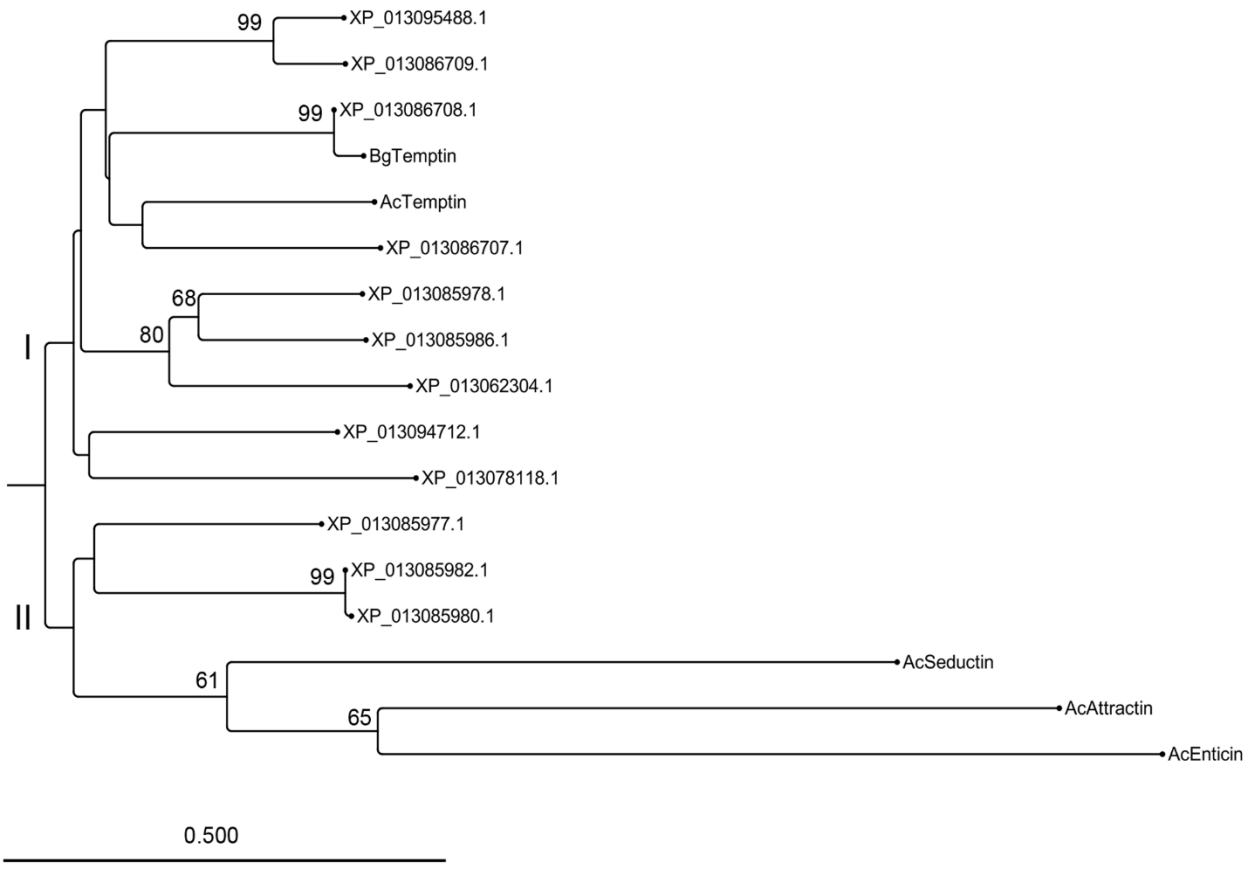

Figure A.5. Maximum Likelihood Phylogram of *B. glabrata* temptin-like sequences and *Aplysia californica* pheromonal protein sequences. Amino acid sequences for *Bg*Temptin, 12 *B. glabrata* predicted temptin-like genes (XP_013062304.1, XP_013078118.1, XP_013085977.1, XP_013085978.1, XP_013085980.1, XP_013085982.1, XP_013085986.1, XP_013086708.1, XP_013086707.1, XP_013086709.1, XP_013094712.1 and XP_013095488.1) and the 4 known proteins of the *Aplysia californica* pheromonal complex [temptin (AAP73787.1), attractin (AAD00569.1), enticin (AAN83923.1) and seductin (AAN83922.1)]. Bootstrap values greater than 50% are shown at the tree branches. The scale bar represents 0.5 amino acid substitutions per site. I to II represent the phylogenetic clades.

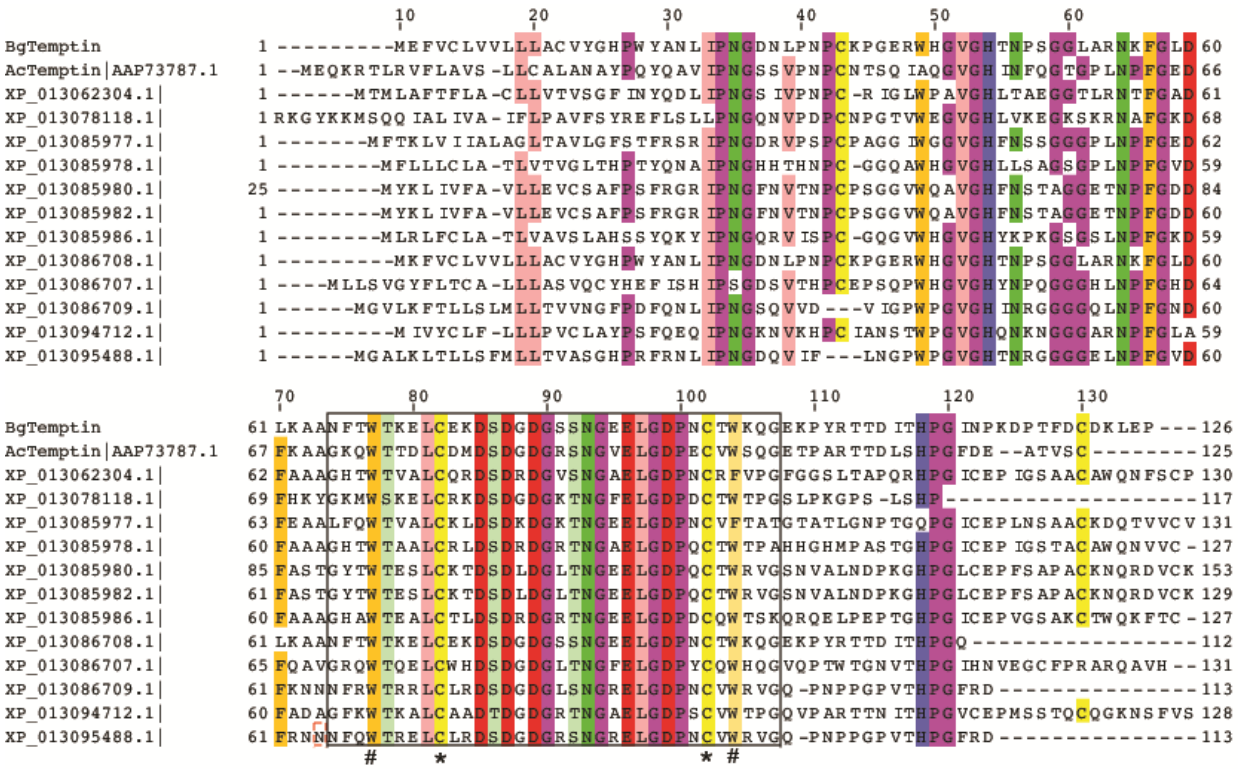

Figure A.6. Alignment of *Bg*Temtin, *Aplysia californica* temptin (*Ac*Temptin) and 12 temptin-like sequences predicted in *B. glabrata*. Columns with identity of greater than 60% are coloured. The region proposed to be relevant for temptin fold and function is shown in the box. The cysteine residues involved in disulfide bonds and the flanking tryptophan residues (Cummins et al., 2007) are denoted by * and # respectively.

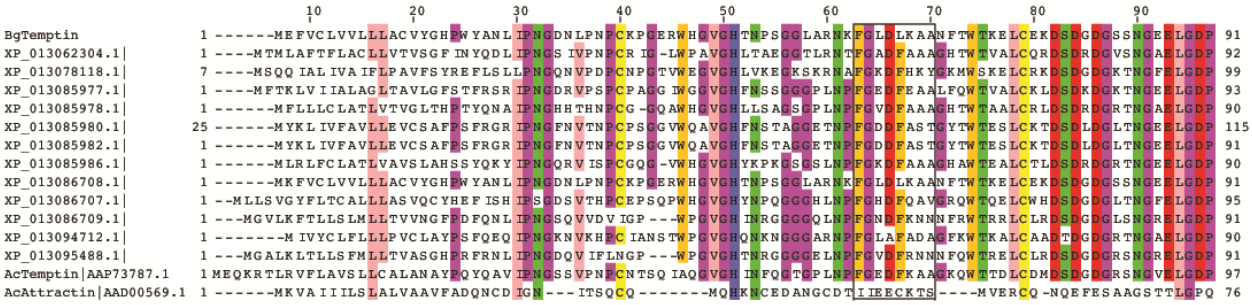

Figure A.7. Alignment of *Aplysia californica* attractin (*Ac*Attractin), temptin (*Ac*Temptin) and temptin-like sequences from *B. glabrata*. Columns with identity of greater than 60% are coloured. The highly conserved attractin motif (IIEECKTS) is shown in the box.

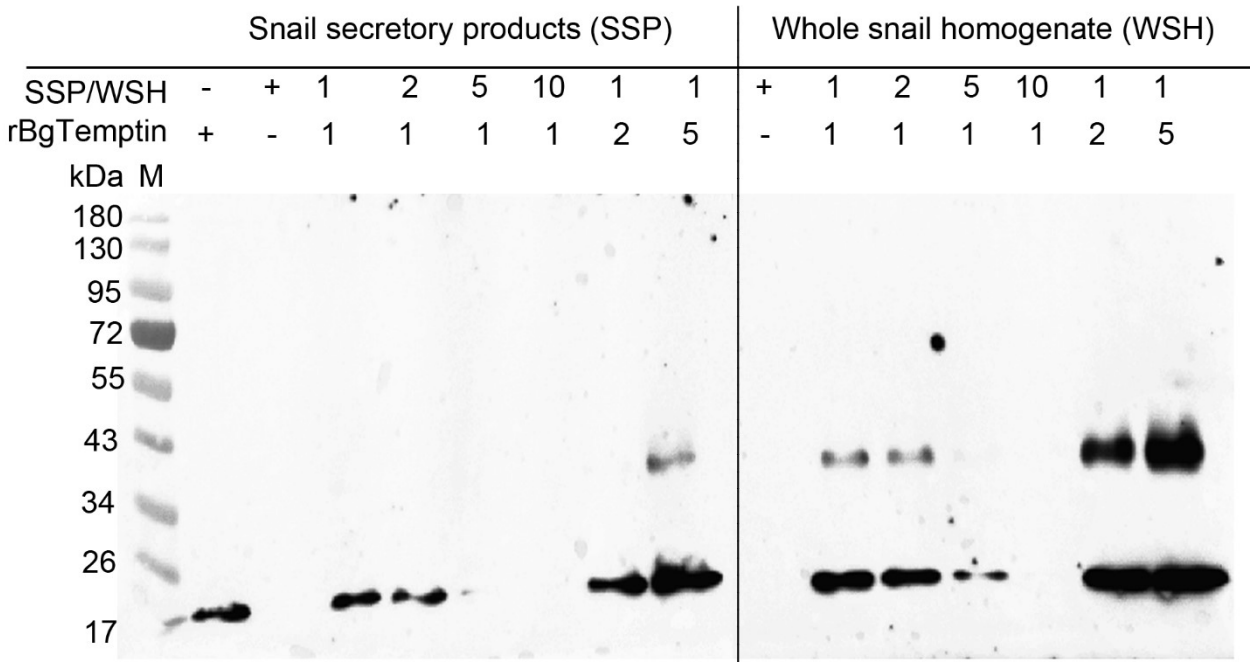

Figure A.8. *Bg*Temptin complex formation. Recombinant *Bg*Temptin was combined with SSP or WSH for 2.5 h. Then, 625 ng of the combined protein was suspended in protein loading buffer without β-mercaptoethanol, separated on 10% SDS-PAGE and detected with a primary antibody against the V5-epitope tag fused with recombinant *Bg*Temptin. (M) Molecular weight marker with sizes shown in kDa to the left. Numbers at the top of image represent ratios of µg protein combined and incubated. + and – indicate that a particular sample was added or not respectively.