Characterization of the roles of leishmanolysin and venom-allergen-like proteins during the infection of *Biomphalaria glabrata* by *Schistosoma mansoni*

by

Alèthe Lydwine Kaboré

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

in

Global Health

School of Public Health Sciences
University of Alberta

Abstract

Among the world's most significantly neglected diseases is schistosomiasis, estimated to have infected over 261 million disadvantaged people in the tropics, and placing an additional 800 million people at risk (McManus, 2012; WHO, 2015). Despite recent international efforts to eliminate schistosomiasis through extensive expansion of drug therapy programs, it remains entrenched in many developing countries, particularly in Africa, where an estimated 280,000 people die from complications related to the disease each year (Cioli, Pica-Mattoccia, Basso, & Guidi, 2014). Schistosomiasis is unusual among human diseases in that it relies upon freshwater snails for larval development and production of the human infectious developmental stage that penetrates an individual's skin when the parasite is encountered in infested water. Little is understood about the determinants of this snail-schistosome association, but perhaps the most essential observation to date is that not all snails can serve as suitable hosts for all given species of schistosomes. This leads naturally to investigations of what the underpinning factors of specificity are, and how they dictate infection establishment and maintenance. Much of our current understanding in this regard stems from studies that have focused on the snail immune response to the invading parasite. We know almost nothing however about how the schistosome facilitates infection. This gap in our understanding of the intermediate host infection process is the focus of my thesis work. I aimed to investigate two factors produced by *Schistosoma mansoni* (*S. mansoni*) during the infection of its natural snail host *Biomphalaria glabrata* (*B. glabrata*). Both molecules (*S. mansoni* leishmanolysin [SmLeish] and venom-allergen-like 3 [SmVAL3]) were chosen for in depth analysis because they possessed significant amino acid similarity to factors known to interfere with host immune responses in other host-parasite systems. Assessment of the expression of these transcripts indicated that they were expressed by

S. mansoni early during the infection of *B. glabrata*. In order to characterize the process by which SmLeish and SmVAL3 influenced infection outcome, I generated recombinant versions of each protein along with polyclonal antibodies to facilitate detection of the native proteins. SmLeish was found to be generated as a pro-peptide that could be either secreted or cleaved from the surface of a larval schistosome. Functionally, it was found to interfere with the migration of *B. glabrata* immune cells. In addition, when knocked down in the free-swimming miracidial stage of the parasite using RNA interference, it was demonstrated to have an influence on infection outcome. These results together suggest that SmLeish plays a role in *S. mansoni* infection establishment in *B. glabrata* by impeding the proper functioning of the snail immune cells at the site of early schistosome penetration. This is also the first report of a schistosome-specific factor having clearly demonstrable impact on larval infection success.

Preface

This thesis is an original work by Alèthe Lydwine Kaboré. The research project, of which this thesis is a part, received ethics approval from the University of Alberta Research Ethics Board, "Immunobiology of snail-schistosome/trematodes interactions", No. AUP00000057, June 1st, 2012.

No part of this thesis has been previously published.

Dedication

This work is dedicated to my amazing and loving family: my parents, Thomas and Andréa Marie Kaboré and my adorable sisters, Murielle, Félicie, and Clélia Kaboré, whose only existence fuels my passion for life and achievements.

Je dédie ce mémoire à mon incroyable et affectueuse famille: mes parents, Thomas et Andréa Marie Kaboré et mes adorables sœurs, Murielle, Félicie, et Clélia Kaboré, dont la simple existence motive ma passion pour la vie ainsi que mes accomplissements.

Acknowledgements

I give thanks to God, for giving me strength, an enduring spirit and faith during this journey.

I am forever grateful to my supervisor Dr. Patrick Hanington for his continued and unwavering guidance throughout the research process. I appreciate your patience in guiding me despite my initial limited laboratory experience and the fact that you were always there to support me. Patrick, you have been instrumental in building the researcher I am today; I have grown tremendously throughout this master's program and I will be forever indebted to you.

I am very thankful to my committee members, Dr. Stephanie Yanow, Dr. Michael Hawkes, and Dr. Norman Neumann for their enlightening questions and comments on my work. To my lab mates and partners in battle, Emmanuel Pila, Michelle Gordy, and Sydney Rudko, I am blessed to have learnt along your side. I am very thankful for your assistance, advice and kindness. A special thank you to Emmanuel Pila and Mahmoud Tarrabain, whose work laid the foundations for mine.

My acknowledgments to Dr. Neumann, Dr. Jeon and Dr. Stafford, and their lab members for the use of their equipment.

To my family, thank you for your support and patience during these trying years. Thank you for believing in me and always being there for me. Thank you for affording me the opportunity to pursue further studies and for showing me that I can realize my dreams.

To my dear friends Faizath, Malaïka, Nicholas and, Valery, I am very appreciative of your constant encouragement and support: past, present and future, of course!

Lastly, thank you to everyone who contributed directly and indirectly to my success in completing my degree and whose names are not mentioned here. May God bless you all, always.

Table of Contents

List of Tables

Lists of Figures

List of Abbreviations

B. glabrata	*Biomphalaria glabrata*
Bge	*Biomphalaria glabrata* embryonic ell
Breg	Regulatory B cell
DALY	Disability-adjusted life years
DNA	Deoxyribonucleic acid
cDNA	Complementary deoxyribonucleic acid
E. coli	*Escherichia coli*
E/S	Excretory / secretory
ES + LYS	Excretory / secretory and lysate
FREP	Fibrinogen-related protein
fNLP	N-formyl-methionine-leucine-phenylalanine
FPLC	Fast protein liquid chromatography
GO	Gene ontology
GP63	Glycoprotein 63
HIS	Histidine
HIV	Human immunodeficiency virus
IDT	Integrated DNA technologies
IHC	Immunohistochemistry
IHCP	Immunohistochemistry protocol
IPTG	Isopropyl β-D-1-thiogalactopyranoside
iRNA	Ribonucleic acid interference
KEGG	Kyoto encyclopedia of genes and genomes

KLH	Keyhole limpet hemocyanin
LB	Lysogeny broth
MARCKS	Myristoylated alanine-rich C kinase substrate
MIF	Migratory inhibitory factor
MRP	Myristoylated alanine-rich C kinase substrate related proteins
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PKC	Protein kinase C
PMSF	phenylmethanesulfonyl fluoride
PCR	Quantitative polymerase chain reaction
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SAM	Significance analysis of microarray
SCP	Sperm-coating protein
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEA	Soluble egg antigen
siRNA	Small ribonucleic acid interference
S. mansoni	*Schistosoma mansoni*
SmLeish	*Schistosoma mansoni* leishmanolysin
S.O.C	Super optimal broth with catabolite repression
SmVAL	*Schistosoma mansoni* venom-allergen-like

STI	Sexually transmitted disease
TAPS	Tpx/antigen 5/pathogenesis related-1/Sc7
TBS	tris-buffered saline
TBS-T	tris-buffered saline - tween
TEP	Thioester containing protein
Th1	T helper 1
Th2	T helper 2
TLRs	Toll-like receptors
Treg	Regulatory T cell
WHO	World health organization

1. Chapter 1: General Introduction

1.1 Host-parasite interactions

The multifaceted interaction between host and parasite is vital in determining the success or failure of an infection (de Morais *et al.*, 2015). Numerous parasites have demonstrated the capacity to escape, evade or suppress the immunological defense of their host in their attempt to survive and reproduce (Wyse, Oshidari, Jeffery, & Yankulov, 2013). The underpinning mechanisms of immunological interference can be passive, or it can involve active defense from the part of invading parasites to serve their existence (Schmid-Hempel, 2011, p. 191). Passive and active evasion strategies have evolved within almost every parasite group, targeting numerous immunological processes, cells and pathways. Thus, the immune defenses of a host are faced with a variety of challenges when attempting to combat a parasite. Passive mechanisms comprise parasites hiding in immuno-privileged tissues, camouflaging against immune recognition (molecular mimicry), changing surface proteins, mutating or diversification of their surface proteins, quiescence, and capsule formation (Schmid-Hempel, 2011, pp. 190–191). Whereas examples of active immune evasion strategies include: the production of specific molecules by parasites that block or modulate specific steps in the hosts' immune response, or that interfere with basic cellular functions important for host defense, such as cell motility or apoptosis (Schmid-Hempel, 2011, pp. 192–193). The strategies employed by parasites to counter a host immune response are often as important to ultimate infection success as are the host immune responses themselves, thus, understanding these processes are not only important for furthering our knowledge of specific parasitic infections, but also crucial to tailoring disease treatment as well as prevention and control approaches.

Schistosomes are flatworms of the genus Schistosoma, subfamily Schistosomatinae, and family Schistosomatidae that are dioecious and digenetic. They are extensively studied because of their role in causing human disease; schistosomiasis is estimated to infect over 260 million people worldwide (WHO, 2015). Today, three species of schistosome (*S. mansoni, S. haematobium* and *S. japonicum*) represent major health concerns in tropical and sub-tropical countries, where the principal means of control is through drug treatment, which has proven unsatisfactory in the fight to eradicate the disease thus far (Inobaya *et al.*, 2015; Ross, Olveda, Chy, *et al.*, 2014). To address the lack of sustained control to date, an integrated approach that incorporates drug administration and a focus on the snail intermediate host has arisen as an alternative; however, our understanding of schistosome larval development within the snail host lacks a mechanistic understanding of the drivers of infection success or failure.

Schistosomes elicit various immunological responses in both their definitive (human) and intermediate (snail) hosts (Bayne, Hahn, & Bender, 2001; El Ridi, Othman, & McManus, 2015; McManus & Loukas, 2008; Mitta, Adema, Gourbal, Loker, & Theron, 2012). These responses are contrasted by the fact that schistosomes have achieved a high infection prevalence and intensity in many areas where the parasites are endemic. Multiple studies have been undertaken by either investigating the interface between the schistosome and the human immune responses (El Ridi *et al.*, 2015; Fitzpatrick *et al.*, 2009) or by exploring schistosome and snail interactions (Coustau *et al.*, 2015; Gordy, Pila, & Hanington, 2015; Hanington, Lun, Adema, & Loker, 2010; Mitta *et al.*, 2012). Thus far, the vast majority of these studies have focused on the human infection due to the need for improved diagnostic tools, disease intervention strategies and the push for the development of a schistosome-specific vaccine. However, a developing field is addressing the molluscan stage of the equation. Because most biological studies of the snail

phase of the schistosome life cycle have focused on the snail response, there is a gap in our understanding of how the schistosome approaches the intermediate host stage of its life cycle (Mo, Agosti, Walson, Hall, & Gordon, 2014; Pearce, 2003). This gap requires consideration in order to provide a better understanding of all the organisms involved in the sustainment of schistosomiasis and consequently contribute to the development of better control initiatives.

1.1.1 The human immune response against schistosome infection

Schistosomiasis morbidity in humans is caused by a CD4+ T-cell-dependent immune response against trapped parasite eggs in body tissues (McManus & Loukas, 2008). Both T helper 1 (Th1) and T helper (Th2) type responses have been characterized as being dominant at different points during the infection (McManus & Loukas, 2008) (Figure 1.1). A dominant Th1 type response is mounted against the larval schistosomulae and any recently matured adult worms and therefore defines the early stages in the infection (day 1 to day 8). A dominant Th2 type response followed by a Treg and Breg mediated responses co-occur with the mature adult worms and egg production (McManus & Loukas, 2008) defining the chronic stage of the disease from day 4 onwards (Figure 1.1). This transition from Th1 toward a Th2 dominated response between day 4 and 8 of the infection is driven by antigenic changes that coincide with worm maturation and the production of soluble egg antigens (SEA) when egg production commences. Furthermore, schistosome eggs have been shown to actively modulate the type of immune response (Th2) generated during their production through specific carbohydrates on egg antigens namely polylactosamine sugar (lacto-N-Fucopentaose III) that has been revealed to perform as a Th2 adjuvant (McManus & Loukas, 2008;Pearce, Caspar, Grzych, Lewis, & Sher, 1991; Pearce & MacDonald, 2002).

The initial immune response is characterized by the presence of Th1-type cytokines, such as IL-2 and INFγ while the next stage is defined by the presence of Th2-type cytokines such as IL-4. Various studies have demonstrated that a deficiency in IL-4 leads to an extended Th1 response and prevents the occurrence of a Th2 response (Lundy & Lukacs, 2013). However, when it comes to potential protective immunity or resistance mounted against schistosomes in vaccine development studies, there are two lines of thought. The first stipulates that eliciting a Th1 type response is the best approach for creating protective immunity against schistosomes (McManus & Loukas, 2008), while the other asserts that a Th2 type (El Ridi *et al.*, 2015) response is a more effective route to induce immunity against schistosome infection. A vaccine inducing a Th1 response is expected to reduce infection burden, while a vaccine driving a Th2 response will be most effective at reducing parasite fecundity and egg production, the cause of the clinical manifestation of the disease (McManus & Loukas, 2008). Although, most attempts at vaccine production thus far have focused on creating a type 2 immune response in humans, as asserted by El Ridi *et al.* (2015), either approach has yet to yield efficient and lasting immunization against schistosomes.

1.1.2 The snail immune response against schistosome infection

In order to produce cercariae, the free-living stage of a schistosome that goes on to initiate human infections, a susceptible snail host is absolutely required. Studies focused on untangling the complex interactions that take place between snails and schistosomes have given great insight into the broader field of molluscan immunology (Pila, Gordy, *et al.*, 2016), and have provided an important context for our understanding of the evolution of immune processes. The fact that digenean trematodes almost exclusively utilize gastropods for their larval development marks an incredible opportunity to study and understand how co-evolutionary

pressures shape host-parasite associations.

Four snail cell types have been reported to play a role in the immune response of a snail (Knaap & Loker, 1990): antigen-trapping cells, reticulum cells, pore cells and haemocytes. The latter, are thought to play the most critical role. Haemocytes are the dominant immune effector cells in a snail. They engage against schistosome invasion (Bayne *et al.*, 2001) and have been shown to exist in greater numbers in snail strains that are able to successfully defend against schistosome infection when compared to their susceptible counterparts (Coustau *et al.*, 2015). There are two types of haemocytes in pulmonate molluscs: granulocytes and hyalinocytes, with the granulocytes being more essential for the anti-schistosome immune response (Bayne *et al.*, 2001; Pila, Gordy, *et al.*, 2016). While the specific mechanisms involved in snail immune defense against schistosomes are still being investigated, several molecules have been reported as playing a protective role against schistosome infection. Fibrinogen related proteins (FREPS) (Coustau *et al.*, 2015), thioester containing protein (TEP) (Coustau *et al.*, 2015), Biomphalysin (Coustau *et al.*, 2015), migratory inhibitory factor (MIF), and Toll-like receptor (BgTLR) (Pila, Tarrabain, Kabore, & Hanington, 2016) are the factors that have been demonstrated to interact with schistosomes and their excretory/secretary (E/S) products to date. Evidence suggests that both the humoral and cellular branches of the snail immune response are important for ultimate recognition, engagement, encapsulation and clearance of a schistosome sporocyst. It is postulated that a snail's ability to detect larval flatworms is associated with the release of soluble lectins (or lectin-like factors) by haemocytes that seem to recognize highly glycosylated proteins produced by these parasites. This recognition event has been shown to be followed closely by the production of peroxidases and other enzymes that are released by the haemocytes leading to parasite damage or death (Basch, 1991, pp. 182–187). The ability of the haemocytes to kill

schistosomes is nonetheless not diminished by the absence of snail plasma or incomplete coverage of the parasite surface by haemocytes, suggesting that soluble factors alone are not sufficient to protect a snail from infection (Bayne *et al.*, 2001). In some instances it has been shown that haemocytes possess the aptitude to effectively clear larval schistosomes by themselves, however since they are the primary producers of many of the known soluble factors relevant to the anti-schistosome immune response, it is difficult to isolate haemocytes from the soluble factors they produce. Nevertheless, in the case of successful snail infection, which occurs frequently under natural conditions, schistosomes obviously overcome these defense mechanisms in order to prevail and reproduce asexually within their snail hosts.

1.2 Schistosomiasis

1.2.1 Clinical manifestations

Discovered in 1851 by Theodore Bilharz (Shadab, 2015), schistosomiasis or bilharzia is currently considered the most significantly neglected tropical disease. It can manifest itself as both an acute and a chronic disease that can be further categorized based on the species of infecting schistosome and the primary route by which eggs are passed into the environment. This also determines the tissues most at risk of damage due to the infection. *S. mansoni* and *S. japonicum* cause intestinal schistosomiasis, while *S. haematobium* causes urinary schistosomiasis; these are the most significant human schistosomes (McManus, 2012). Schistosomiasis is acquired by coming in contact with schistosome-contaminated water usually because of professional or domestic activities, as well as recreational uses such as swimming (Jenkins-Holick & Kaul, 2013).

Chronic schistosomiasis occurs after recurrent exposure to schistosome cercariae in

endemic regions (Colley, Bustinduy, Secor, & King, 2014). Following pairing of the male and female worms, the female worm will initiate egg laying, a process often associated with a second onset of acute symptoms. Egg passage from the veins in which the worms reside into either the intestinal lumen or the bladder is an imperfect process during which many eggs aberrantly migrate into the tissues of the liver, spleen or bladder lining. The symptoms of chronic schistosomiasis and many of the long-term complications resulting from the infection are the result of trapped worm eggs, as opposed to the presence of mature worms themselves (Colley *et al.*, 2014). The immune response to schistosome eggs commonly results in the production of a granuloma surrounding each egg. These eggs contain proteolytic enzymes, and lead to lasting inflammation that results in the observed clinical symptoms of the chronic disease and the gradually incurred organ damage (Colley *et al.*, 2014; Jenkins-Holick & Kaul, 2013). Symptoms range from occasional abdominal pain, diarrhea, and blood loss through stool or urine, to liver and spleen enlargement (hepato-spleeno megaly), bladder and ureter fibrosis, kidney damage and bladder cancer (WHO, 2015). Urogenital schistosomiasis is a specific term coined to distinguish the specific disease that occurs when the disruption caused by the eggs of *S. haematobium* affects the reproductive organs in males and females alike, leading to genital lesions, higher susceptibility to sexually transmissible disease (STI) such as human immune-deficiency virus (HIV), and the eventual permanent outcome of infertility in the long-term (WHO, 2015). In rare occasions schistosomiasis can affect the central nervous system. Cerebral schistosomiasis can lead to headache, blurred vision, and Jacksonian epilepsy (Colley *et al.*, 2014). Sensory loss, paralysis and bladder incontinence occur when the spinal cord is affected, which is observed most frequently during the acute stages of schistosomiasis (Colley *et al.*, 2014).

Acute schistosomiasis also known as Katayama syndrome is mostly diagnosed in

individuals exposed to a schistosome infection for the first time later on in life, such as with travelers and immigrants to regions known to be endemic for schistosomiasis (Colley *et al.*, 2014). It is most commonly associated with a variety of clinical manifestations that lasts between 2 to 10 weeks including fatigue, abdominal pain and headaches, fever, myalgia and eosinophilia (Colley *et al.*, 2014). In studies comparing umbilical cord-blood lymphocytes from infants of mothers that were infected or not infected by schistosomes, there appears to be in utero sensitization against schistosomes (King *et al.*, 1998; Malhotra *et al.*, 1997). These studies established that human fetuses that are sensitized produce a Th2 type response similar to their infected mothers. This could make these individuals less likely to react with an inflammatory response when later challenged by schistosomes as children, as opposed to newly exposed individuals not sensitized in utero and thus, with no prior schistosome-induced Th2 response. This early sensitization is the primary reason that there is a lack of acute symptoms observed in those individuals living in endemic regions when exposed to schistosomes later on in life (Pearce & MacDonald, 2002).

1.2.2 Impact and control

1.2.2.1 Public health impact of schistosomiasis

In 2013, more than 261 million individuals were afflicted with schistosomiasis and required treatment. Approximately 40 million people were actually treated, which represents only about 15% coverage of the population in need of treatment (WHO, 2015). It is estimated that over 800 million people worldwide are at risk of developing schistosomiasis (McManus, 2012) with an annual death toll of about 280 000 in Sub Saharan Africa alone, the most affected region (Cioli *et al.*, 2014). Schistosomiasis is endemic to 78 tropical and sub-tropical countries in total, with 52 of these countries being classified as having a moderate to high transmission rate

(WHO, 2015) (Figure 1.2). The most recent and generous global annual burden of schistosomiasis in disability-adjusted life years (DALY) is projected at 70 million, landing slightly lower than the estimated DALY associated with HIV, while surpassing DALY estimates for both tuberculosis and malaria (King & Dangerfield-Cha, 2008; McManus, 2012; Ross, Olveda, Chy, *et al.*, 2014). This is because initial estimations of the burden of schistosomiasis as reviewed by King and Dangerfield-Cha (2008) were biased by over assessing the contribution of childhood mortality while minimizing the impact of long lasting chronic conditions especially in disadvantaged populations. Therefore, after appropriately evaluating the weight of chronic disease impact even after disease treatment and the quasi-continuous re-infection rates in endemic regions, the DALY value of schistosomiasis is now estimated as being much higher than previously thought, and is likely to more closely parallel the DALY of well-established diseases such as malaria or tuberculosis. However, because of existing co-infections, and diagnostic and surveillance paucity, the impact of schistosomiasis as expressed by DALY still requires more robust data to draw consent among scientists. For instance, for 2010, the global burden of schistosomiasis was reported in DALY values from 3.3 million to 36 million (Mo *et al.*, 2014), highlighting the inconsistent metrics used to evaluate the impact of this disease. Undoubtedly, assessments of the burden of schistosomiasis have risen over the past 10 to15 years as our understanding of the long-term impacts of the disease deepens. It is therefore not surprising that currently, schistosomiasis is considered the second most important parasitic disease after malaria (Cioli *et al.*, 2014) in terms of both morbidity and mortality impact, as malaria still occurs more frequently, and has a greater measurable impact on infected individuals and populations. Hence, the World Health Organization (2013) placed schistosomiasis as a major public health threat in endemic countries and consequently set out the audacious goal of

eliminating the disease by 2020 (Ross, Olveda, & Li, 2014).

1.2.2.1 Current control of schistosomiasis

The current schistosomiasis control paradigm focuses almost exclusively on targeted drug administration using praziquantel and has proven ineffective at reducing the overall burden of schistosomiasis thus far (King, 2009; McManus, 2012). There are a number of issues associated with such a one-dimensional approach to controlling complex parasites like schistosomes. Praziquantel only targets the mature worm stage, does not prevent reinfection, could face resistance from schistosomes, severe rebound morbidity has been reported after treatment, and very limited decrease in disease prevalence following mass drug administration has been registered (Cioli *et al.*, 2014; McManus, 2012). Mass drug administration campaigns are not sustainable because of the donor-dependent ties that exists, shortage of praziquantel, inadequate health systems to carry out morbidity control, inadequate planning of mass drug admiration campaigns, non compliance of patients and low proportion of infected individuals being treated are all challenges that the chemical control approach faces (McManus, 2012; Olveda *et al.*, 2014; Secor & Montgomery, 2005). All these direct issues faced by the mass drug administration approach are compounded upon by the fact that there are limited strategies that aim to reduce the rates of reinfection (Secor & Montgomery, 2005).

The complex life cycle of schistosomes, the multiple life stages of the parasite, and the modes of transmission all suggest that controlling schistosomiasis calls for more than one approach at a single time and even more, the inclusion of non-medical actions (Cioli *et al.*, 2014; Rollinson *et al.*, 2013). Preventing infection and reinfection either by targeting humans or the intermediate hosts is being proposed. Intervention directed towards humans can follow two routes. First, human to snail by which, schistosome eggs being passed in nature enter in contact

with water bodies and transform into miracidia ready to infect snails, and second, the route from snail to humans where the exposure of human to contaminated water causes infection. Hence, having access to clean water for drinking, for domestic, professional and recreational usage, and sanitation complemented with health promotion and education would be a great addition to the current schistosomiasis control strategy (Gray *et al.*, 2010; McManus, 2012; Rollinson *et al.*, 2013; Secor & Montgomery, 2005). Another action that has been offered for controlling schistosomiasis is targeting the intermediate molluscan hosts through molluscicide activities and environmental modifications (Gray *et al.*, 2010; Rollinson *et al.*, 2013; Secor & Montgomery, 2005). An additional point of attack of schistosomiasis would be the development and utilization of an effective vaccine.

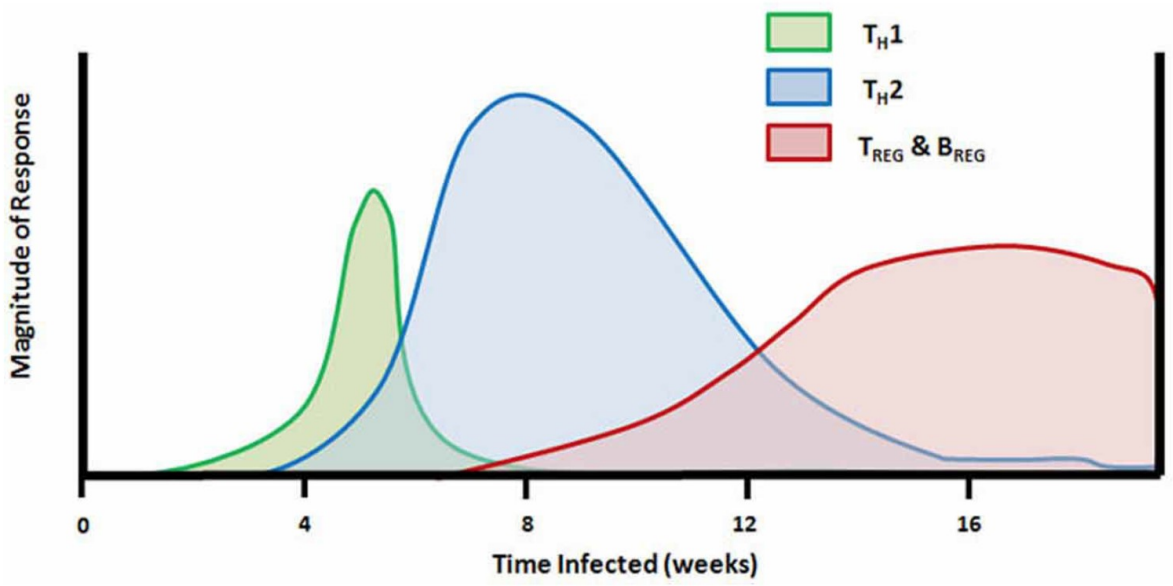
Finding new ways to control this disease is an urgent necessity. Multidimensional and integrated control measures, combining morbidity control through the use of chemical treatment and investment in transmission preclusion have proven to be most effective for elimination of schistosomes from endemic regions (Gray *et al.*, 2010; Rollinson *et al.*, 2013; Ross, Olveda, & Li, 2014; Secor & Montgomery, 2005). One aspect of transmission reduction or inhibition that is key, although under researched, focuses on improving our knowledge of the processes that underpin schistosome intramolluscan larval development, including elucidating what dictates snail and schistosome compatibility. Hence, a better understanding of how schistosomes can successfully infect and maintain infection in their snail hosts may open a path towards the development of new effective preventive approaches. Furthermore, strategies that target the transmission of the disease from snail to human have been demonstrated to be more effective compared to those that target transmission from human to snail (Chiyaka & Garcia, 2009).

1.3 Objective of the thesis

The fundamental purpose of my thesis was to increase our knowledge of the factors produced by schistosomes during their intramolluscan development in order to facilitate infection establishment and maintenance. My overarching hypothesis was that *S. mansoni* leishmanolysin (SmLeish) and *S. mansoni* venom allergen-like 3 (SmVAL3) proteins are utilized to suppress the snail defense response in order to facilitate and maintain infection. To address this hypothesis I made use of the traditional *S. mansoni – B. glabrata* model that has formed the basis for much of our understanding of schistosome larval development. Beginning with a large microarray dataset that targeted assessment of the transcripts abundantly expressed by *S. mansoni* during the early stages of intramolluscan development, I identified specific factors that possessed appeal as being involved in immune modulation. Broad assessment of the microarray data led to the identification of two factors produced by *S. mansoni*: SmLeish and SmVAL3 as promising candidates to pursue and functionally characterize for their potential role in immunomodulation of the immune response of *B. glabrata.* Two main points were initially targeted: (1) the transcript and protein abundance of leishmanolysin-like and venom-allergen-like (VAL) proteins during *S. mansoni* development in *B. glabrata*, and (2) the functional characterization of leishmanolysin-like protein in the context of its interaction with *B. glabrata* innate immune factors to suppress the snail defense response in order to facilitate and maintain infection.

The rationale for the choice and analysis of leishmanolysin-like and VAL3 proteins was a logical progression from previous studies in our laboratory showing that (a) both proteins were being expressed during intramolluscan development and, (b) previous studies undertaken in other organisms attributing an immunosuppressive function to leishmanolysin-like and VAL proteins

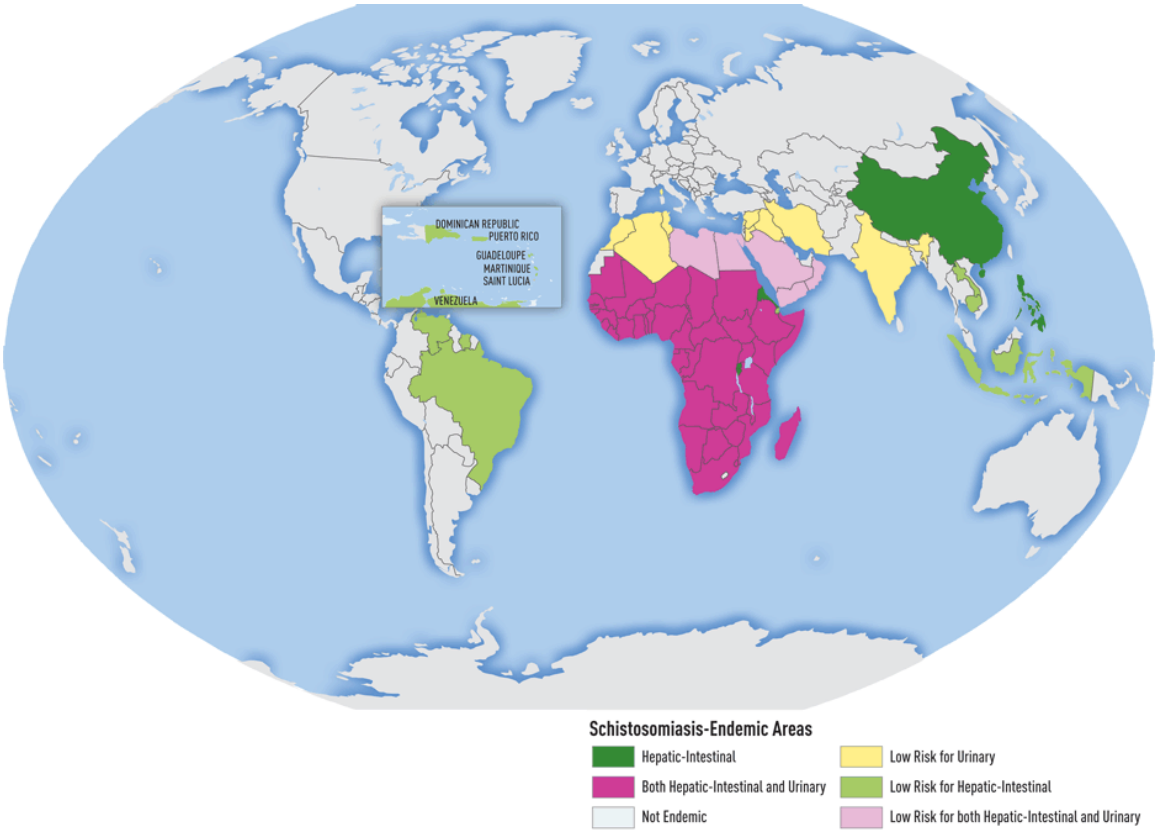
during host-parasite interactions. A functional characterization of both proteins in the context of a schistosomes' ability to interact with their hosts immunological response, offered an unprecedented opportunity to broaden the knowledge base around schistosome biology and provide insight into the mechanism underpinning the parasite contribution in the successful infection establishment and maintenance process.



From "Chronic schistosome infection leads to modulation of granuloma formation and systemic immune suppression", by L. K. Lundy and N. W. Lukacs, 2013, Frontiers in Immunology, 4, p. 3. Copyright [2013] by Lundy and Lukacs and Creative Commons (CC BY 4.0).

Figure 1.1 Timeline of TH cell mediated responses in the mouse model of schistosome infection

Subcutaneous injection of schistosome cercariae on day 0 leads to development of adult worms and egg production beginning at 4-5 weeks. The early innate and adaptive immune response to adult worm antigens is dominated by pro-inflammatory and Th1 cytokines (TNα, IL-12, and INFγ). Following egg deposition in the liver and other internal organs, the larval miracidia release soluble egg antigens (SEA) containing molecules that drive a rapid transition from Th2-dominant immunity and production of IL-4, IL-5, and IL-13. Between 7 and 8 weeks of infection, FoxP3+ T_{REG} cells and IL-10 are detectable, and the population of splenic FasL+ and CD5+ B cells begins to proliferate. Th2 response and peak granuloma formation occurs between 7 and 10 weeks of infection and is followed by granuloma down modulation and increasing fibrosis around newly deposited eggs which persists throughout the reminder of infection (Lundy & Lukacs, 2013).



From "Schistosomiasis," by Center for Disease Control and Prevention, 2015 (http://wwwnc.cdc.gov/travel/yellowbook/2016/infectious-diseases-related-to-travel/schistosomiasis). In the public domain.

Figure 1.2 Schistosomiasis global distribution

The distribution of schistosomiasis is very focal; however, surveillance for schistosomiasis is limited in most countries. Therefore, this map shades entire countries where schistosomiasis transmission has been reported. The exception to this is France where schistosomiasis has been reported on several islands, such as Guadeloupe, Martinique, and Corsica (CDC).

2. Chapter 2: Literature Review

2.1 Schistosomes

Schistosomes are flatworms of the genus Schistosoma, subfamily Schistosomatinae, and family Schistosomatidae. They are bisexual and require two hosts to complete their life cycle. Trematodes are eukaryotic, multicellular helminthes. Roughly eighty five species of schistosome have been recorded, and all are parasites of either mammals or birds; there are still likely more species as yet undiscovered as Basch (1991, p. 15) pointed out. Schistosomes are responsible for causing schistosomiasis. There are currently 6 species of schistosome recognized to infect humans: *Schistosoma haematobium, S. japonicum, S. mansoni, S. mekongi, S. intercalatum and S. guineensis* with the 3 former ones being the most common culprits. *S. japonicum, S. mansoni,* and *S. mekongi* cause intestinal schistosomiasis while *S. haematobium, S. intercalatum, and S. guineensis* cause urinary schistosomiasis.

The causative agent of the majority of schistosomiasis cases, *S. mansoni*, is also the most commonly used species in laboratory research (Protasio, Dunne, & Berriman, 2013). Its genome has been completely sequenced which allows gene expression studies, queries on gene behavior, and allows comparative genomic studies across metazoan organisms. The adult worms can be males or females and are characterized by three regions: anterior, medial and posterior. The anterior region for both sexes displays oral and ventral suckers. The double bilayer constituting the external tegument is a dynamic and interactive part of the parasite that is used in various metabolic activities such as nutrition, immune evasion, and signal transduction (Sotillo, Pearson, Becker, Mulvenna, & Loukas, 2015). The males are white, while the females are darker gray and slightly slimmer and longer in shape. *S. mansoni* has eight pairs of chromosomes including one sex pair (Bach, 1991, p.6). The diploid *S. mansoni* genome contains 11,809 genes encoding

13,197 transcripts (Berriman *et al.*, 2009) representing around 15 to 20 thousand expressed genes (Brindley, 2005). This is 363 Mbp or roughly 10% of the mammalian genome with individual chromosome sizes ranging from 18 to 73 Mbp (Brindley, 2005) and with about 40% of the genome being repetitive (Berriman *et al.*, 2009).

Schistosomes have a complex life cycle composed of numerous life stages. The adult schistosomes reside in their definitive vertebrate hosts, humans in the case of *S. mansoni*, where they sexually reproduce and yield eggs. About 50% of the eggs that are generated by a male and female worm pair are passed in the environment mainly through stool or urine. When in contact with suitable aquatic milieu, the eggs progress to hatch and release free-swimming multicellular miracidia, which in turn penetrate a suitable molluscan intermediate host (*B. glabrata* for *S. mansoni*). In molluscs, miracidia differentiate into mother sporocysts, then daughter sporocysts. After a few rounds of asexual reproduction, daughter sporocysts give rise to a second free-swimming multicellular larval stage of either male or female sex called cercariae. The cercariae proceed to locate and penetrate the definitive host through intact skin, transform into schistosomula, migrate to the hepatic portal vein and then mature adult worms (Basch, 1991, pp. 39–64) (Figure 2.1).

2.2 Snail / schistosome compatibility

As is the case with most trematodes, schistosomes require suitable snail hosts in order to complete their larval development. The relationship between trematodes and gastropods is thought to have been co-evolving for at least 200 millions of years and is complex and intimate (Basch, 1991, p. 3; Blair, Davis, & Wu, 2001). Many snail-schistosome combinations yield incompatible results, meaning that there is often a low number of gastropod species infected by a human-infected schistosome in a specific geographical location (Basch, 1991, p. 179), and this

level of host specificity holds true for *S. mansoni* and *S. haematobium* when considering the definitive host, which is almost always a human. Exceptions to this rule do exist however, for example, *S. japonicum* infects only snails of the genus *Oncomelania,* but can infect over 40 mammalian hosts including mice, rabbits, pigs, cattle and humans (Basch, 1991, p. 179). Hence, the distribution of various species and strains of schistosome across the globe is closely linked to the species of snail hosts present in each location. The distribution of schistosomes causing human schistosomiasis is restricted by the availability of suitable snail intermediate hosts.

The establishment of infection between different species of schistosomes and their molluscan hosts is dependent on both the capability of the snail to mount resistance, and the parasites ability to overcome that resistance. As Richards and Shade (1987) established, in some molluscs, active recognition of the parasite followed by the parasite being cleared by the snail haemocytes is observed, while in others, the miracidia escape recognition and face minimal to zero resistance from the host. Additionally, when the miracidia do not encounter any active response from the snail, but the snail haemolymph is not favorable to the parasite development, the miracidia fail to survive. Therefore, active mollusc resistance is prevailing over a passive response; and when there is no mollusc immune response, having a hostile environment triumphs over having a suitable one for parasite development (Richards & Shade, 1987).

The factors that explain resistance and susceptibility in the snail-schistosome interface are only partially characterized, and may not be consistent in their role outside of the snail-schistosome species from which their roles were characterized. However, it is understood that there are both immunological and genetic features of resistant snails that differ from their susceptible counterparts (Bayne *et al.*, 2001). Genetic compatibility has been demonstrated by Newton (1953) through a series of reciprocal crosses between a resistant and a susceptible *B.*

glabrata which were then exposed to *S. mansoni*. Newton concluded that the resistance to *S. mansoni* infection is a genetically transmissible character. Additionally, Richards and Shade (1987) established that multiple loci, with some spread in various alleles play a role in the compatibility paradigm in each snail. Multiple studies have also supported the importance of immunological features to mount resistance: *B. glabrata* is able to recognize *S. mansoni* miracidia as foreign, in part because of the presence of specific soluble factors and immune cells dedicated to this role (Basch, 1991, p. 182; Coustau *et al.*, 2015). The immunological basis for resistance to infection is not exclusive of a role for a genetic underpinning as many of the differences found between susceptible and resistant snails has been associated with differences in the genes encoding immune-relevant proteins (Tennessen *et al.*, 2015). Adding further complexity is the fact that compatibility between snails and schistosomes can be influenced by the snail size (which is synonymous with snail age): juvenile snails are hence more susceptible to infection by schistosomes than are older ones in the case of suitable snail species/strains (Niemann & Lewis, 1990).

2.3 Host invasion strategies utilized by schistosomes

Schistosomes successfully live within their hosts (mammalian and mollusc), despite the hosts' defense mechanisms, and it is well documented that the human infection is facilitated by employing various strategies to avoid detection or immune engagement(Schmid-Hempel, 2011, pp. 187–218). In the context of intramolluscan development, some putative immunoregulatory or evasion factors have been identified through various transcriptomic and proteomic studies, however, the specific functional characterization and mechanism behind how these factors influence the snail immune response have yet to be investigated in depth. It is understood that developing schistosome larvae release an array of factors, often termed excretory/secretory (E/S)

products, that have been postulated to influence schistosome infection establishment and maintenance within the snail (Wu *et al.*, 2010) and human hosts (Fitzpatrick *et al.*, 2009). These developmentally specific factors require more comprehensive analysis to verify their involvement in infection establishment and maintenance and to ultimately identify the specific proteins contributing to these effects.

Studies on schistosome immunomodulation of the human immune response have determined that these parasites utilize both passive and active processes to maintain infection (Basch, 1991, p. 182; Schmid-Hempel, 2011, pp. 190 – 191). Investigations demonstrated that specific genes were being turned on at specific developmental stages suggesting their role in infection establishment and or maintenance (Schmid-Hempel, 2011, pp. 191–193). Analysis of the schistosome genome, transcriptome and proteome have stimulated the discovery of specific genes and proteins that have demonstrated roles in facilitating evasion or escape of the host immune response. In many other instances, these roles are predicted based on nucleotide or amino acid sequence homology with the homologous factors known to play immunosuppressive or immunomodulatory roles in other parasite systems. Mucins, elastases, serines, metalloproteases, glycans, and sperm-coating proteins (SCP) are "virulence factors" that are produced by the schistosomes and are expected to facilitate infection (Wilson, 2012). Many of these proteins are known to be produced during various stages of either the human or snail infection and are hypothesized to influence the capability of the host immune response to effectively engage with the parasite and clear the infection (Coppin *et al.*, 2003; Dvořák *et al.*, 2015; Taft & Yoshino, 2011). Further, the location in which these factors are produced is also expected to impact their function, some have been shown to be produced at the parasite surface, other excreted or secreted, and other produced within cells (Harnett, 2014). Both timing and

location/type of expression is likely critical for conveying the required functions, and would impact whether these factors are relevant at the parasite/host immune response interface as well as systemically.

Previous studies from our laboratory and others have shown that SmLeish and SmVAL are among a suite of transcripts that are up-regulated by *S. mansoni* during specific points in its intramolluscan development (Chalmers *et al.*, 2008; Hanington, Hanelt, Hines, & Loker, unpublished; Yoshino *et al.*, 2014). These data suggest that both of these proteins are relevant during the early stages of parasite establishment within the snail host, and based on known roles for homologs of both proteins that have been characterized in other host-parasite model systems, we hypothesize that their roles are likely immunomodulatory. Earlier studies from our group using a previously published *S. mansoni* oligonucleotide DNA microarray (Fitzpatrick *et al.*, 2009), established the *in vivo* transcriptional profiles of *S. mansoni* miracidia, and important intramolluscan stages of *S. mansoni* development. The transcripts identified as being either increased or decreased in expression compared to endogenous controls, using a fold change in expression of +/-1.5, were separated into 63 different functional groups based on Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis, that fall within one of the following four primary functional classifications: infection establishment and maintenance, development, cell processes, and energy and metabolism (Figure 2.2). Within the context of my thesis, the results on the infection establishment and the time period during molluscan infection are of importance.

There were 209 transcripts that were found to be increased or decreased in expression and also associated with infection establishment and maintenance (Hanington *et al.*, unpublished). Of this group, 16 were classified as having likely immunosuppressive functions based on

similarities with known immunosuppressive factors. The most prominent immunosuppressive group of transcripts acknowledged in this study shares a high degree of homology to leishmanolysin also known as glycoprotein 63 (GP63) and invadolysin. Leishmanolysin-like and invadolysin-like transcripts identified in this study are most significantly increased in expression compared to controls at day 4, being as high as 3.8 fold higher than controls. Expression is reduced on days 8 and 16 with slight elevation observed on day 32, up to 2.1 fold higher than control (Figure 2.3).

The most completely characterized of these transcripts are the venom-allergen-like proteins (VALs). VAL 3 and 7 especially were identified as displaying fluctuating expression patterns consistent with other studies performed with schistosomes (Farias *et al.*, 2012; Wu *et al.*, 2010). The expression of both VAL 3 and 7 started high, at 4.7 and 2.6 fold above control at day 2 post infection, it remained high until day 8 post infection when it reduced until day 32 when expression is again elevated to 2.7 and 2.5 fold higher than the average of a suite of commonly used endogenous controls respectively (Figure 2.3). Fifty-four endogenous controls were used for this expression analysis to create an endogenous control correction factor. The correction factor was used to normalize all the values from the array. This approach to microarray analysis has been previously used by Fitzpatrick *et al.*, (2009). Fold changes above the value of 2 were considered statistically relevant using the significance analysis of microarray (SAM) tool. The expression values reported are relative to the endogenous controls' expression values. The stage specificity of SmLeish and SmVAL proteins and the immunosuppressive role attributed to their homologues in other organisms such as various *Leishmania* species led to our decision to pursue further investigations on these molecules.

2.3.1 *S. mansoni* leishmanolysin-like protein

Analysis of the complete genome and full transcriptome of *S. mansoni* initially resulted in the identification of 17 variants of the invadolysin and leishmanolysin-like genes based on their structural similarity with the M8 metalloprotease family members (MEROPS). These variants of invadolysin and leishmanolysin proteins are similar in their composition and structure. Leishmanolysin is commonly termed invadolysin in non-*Leishmania* species, and has been identified in bacteria, plants and animals, invertebrate and vertebrates (McHugh *et al.*, 2004). These sets of homologous proteins are all classified in the M8 family of metzincin metalloproteases (McHugh *et al.*, 2004). Metalloproteases are part of a larger group of proteolytic enzyme that also encompasses aspartic, glutamic, serine, cysteine, and threonine. They can either be endopeptidases or exopeptidases (Clark *et al.*, 2010). However, invadolysin proteins specifically are usually endopeptidases without exopeptidase activity (Bouvier, Schneider, Etges, & Bordier, 1990). Metalloproteases have, in the majority of cases, a conserved zinc-binding motif of HExxH sequence, where the two histidines coordinate the zinc ion and the glutamate acts as a general base in the catalytic reaction. Metalloproteases can be categorized according to their catalytic mechanism, their substrates and products, or their structural homology (Clark *et al.*, 2010). The use of a zinc metal ion to perform hydrolysis reactions is characteristic of metalloproteases. A wide variety of physiological processes such a morphogenesis, peptide and hormones processing, cell adhesion and fusion, proliferation, migration, apoptosis, angiogenesis, inflammation, and facilitation of protozoan parasite infection are known to be carried out by metalloprotease (Clark *et al.*, 2010; Nagase, 2001).

The matrix metalloproteases (MMPs) are a group within the metalloprotease proteins, which includes leishmanolysin. They can be membrane-bound or produced as soluble factors and

have been described generally as targeting the extracellular matrix as their substrate (Massova, Kotra, Fridman, & Mobashery, 1998). When secreted, MMPs are generally secreted in an inactive state and activated when the N-terminal pro-peptide is cleaved off subsequently (Yao, Donelson, & Wilson, 2003).

One of the most studied metalloproteases is the *Leishmania* protein, leishmanolysin, also referred to as GP63. Olivier *et al.* (2012) reported GP63, a major *Leishmania* surface antigen as a zinc-dependent metalloprotease. Leishmanolysin is known to act upon the following substrates: casein, gelatin, albumin, haemoglobin and fibrinogen (Yao *et al.*, 2003). GP63 is localized at the surface of the plasma membrane of *Leishmania*, but also can be excreted and released into the host in both an extracellular as well as an intracellular (within host macrophages) context (Olivier *et al.*, 2012). Gp63 was shown to participate in a number of immunomodulatory activities that facilitate infection of host macrophages and protection of the parasite within the macrophages from degradation in the phagolysosome. It is known to assist *Leishmania* parasites in the avoidance of complement-mediated lysis of the parasite through the cleavage of C3b (Brittingham *et al.*, 1995), which interacts with macrophages using complement receptors (Mosser & Edelson, 1985) and with the fibronectin receptor to augment the internalization of the parasite (Brittingham *et al.*, 1995), and degrades the extracellular matrix, promoting parasite accelerated mobility into cells (McGwire, Chang, & Engman, 2003). More specifically, the hydrolysis of Protein Kinase C (PKC) substrates such as myristoylated alanine-rich C kinase substrate (MARCKS) and MARCKS related proteins (MRP), found in macrophages, alters the PKC signaling pathway in macrophages of the infected host leading to the inhibition of the production of anti-microbial agents like reactive oxygen species (ROS) (Olivier *et al.*, 2012). PKC is known to be responsible for the release of anti microbial agents such as ROS and

inflammatory responses is significantly impacted in macrophages infected by *Leishmania* parasites, notably because of its substrates, MARCKS/MRP, being hydrolyzed by GP63 (Olivier *et al.*, 2012). Generally, MARCKS and MRP, PKC activators, are up regulated in the presence of lipopolysaccharides, sign of invading organisms, which leads to an immune response from the host (Olivier *et al.*, 2012). The absence of activation of PKC pathway results in the absence of inflammatory immune response. Consequently, by altering its host immune response through specific PKC signaling pathway inhibition at various levels during infection, leishmanolysin creates a favorable milieu in its host to facilitate parasite survival.

Based on the crucial role played by leishmanolysin through host inflammatory response inhibition in *Leishmania* infection success (Hassani, Shio, Martel, Faubert, & Olivier, 2014), the fact that it is a protein with conserved elements among metazoan (Di Cara, Duca, Dunbar, Cagney, & Heck, 2013), and its presence at increased proportion during the intramolluscan development of *S. mansoni*, it was of interest to functionally characterize it's role during *S. mansoni* infection of *B. glabrata*. During their investigation of the array of proteins released during *S. mansoni* transformation from miracidium to sporocyst, Wu and colleagues (2010) established that *S. mansoni* released ten leishmanolysin-like peptides. *S. mansoni* genome investigations from Bos and colleagues (2009) has shown that the genome has 100 metalloprotease-containing loci divided into 19 families. *S mansoni* leishmanolysin-2 (SmLeish2) of the M08 family is the targeted protein in this project as it has the closest homology to the *Leishmania major* leishmanolysin already proven to have immunosuppressive properties, it is also one of the transcript that was observed to be up-regulated in previous microarray studies (Hanington *et al.*, unpublished). Furthermore, there is a sequence for SmLeish2 available allowing a multitude of targeted biological manipulation otherwise difficult

or impossible.

2.3.2 *S. mansoni* venom-allergen-like protein

Schistosoma mansoni venom-allergen-like (SmVAL) proteins are part of the larger family of sperm-coating protein/Tpx/antigen 5/pathogenesis related-1/Sc7 (SCP/TAPs) (Chalmers & Hoffmann, 2012). The conserved SCP/TAPs domain has been attributed the functions of cellular defense or proliferation, lipid binding anti-inflammatory scavenger of eicosanoids in blood feeding arthropod, binding the surface of liposomes containing negatively charged lipids in mammals, being required for the export of sterols *in vivo*, and binds cholesterol *in vivo* in yeast (Kelleher *et al.*, 2014). VALs are characterized by a single SCP/TAPs domain also found in bacteria, plants, animals and viruses (Kelleher *et al.*, 2014). This superfamily, which is found throughout the animal kingdom, has been associated with cell processes such as immune responses (Alexander *et al.*, 1993; Murphy, Zhang, Zhu, & Biggs, 1995), testis/sperm development (Kovalick & Griffin, 2005; Maeda, Sakashita, Ohba, & Nakanishi, 1998), envenomation (Lu, Villalba, Coscia, Hoffman, & King, 1993; Yamazaki, Hyodo, & Morita, 2003), and parasitic nematode invasion of definitive hosts (Chalmers *et al.*, 2008; Hawdon, Jones, Hoffman, & Hotez, 1996; Maizels, Gomez-escobar, Gregory, Murray, & Zang, 2001; Tawe, Pearlman, Unnasch, & Lustigman, 2000).

Cantacessi and colleagues (2009), in their review, unveiled the diversity within the superfamily of SCP/TAPs and their established and postulated roles in biological processes. Hence, SCP/TAPs proteins are found in biting insects venom and have been given a role in insect allergy though the mechanisms involved still remain to be explored; the yellow jacket protein has been given the role of trypsin inhibitor through blocking calcium-dependent ryanodine receptors (Lu *et al.*, 1993). Additionally, blood-feeding insects such as mosquitoes,

sandflies, and tsetse flies also carry SCP/TAPs proteins involved in various roles such as mediation of immune reactions in the gut in tsetse and being central to reproduction in *D. melanogaster* (Calvo, Pham, Lombardo, Arca, & Ribeiro, 2006; Charlab, Valenzuela, Rowton, & Ribeiro, 1999; Hoffman, 2006; Li, Kwon, & Aksoy, 2001; Valenzuela, Garfield, Rowton, & Pham, 2004). These proteins have also been associated with physiology of secreted process such as stabilizers of secretory products or lubricating insect mouth. SCP/TAPs can also be found in snake venom and they affect smooth muscles contraction and cyclic nucleotide-gated ion channels (Yamazaki & Morita, 2004). They can also block olfactory and retinal alpha-subunit homotetrametric channels (Yamazaki & Morita, 2004). In vertebrates such as rats, mice, monkeys, horses, SCP/TAPs are implicated in binding of sperm to oocyte and fertilization process (Jalkanen, 2005). Humans are also expressing a range of SCP/TAPs proteins that are mostly involved in sperm maturation (Kasahara, Gutknecht, Brew, Spurr, & Goodfellow, 1989). Other functions in humans includes trypsin inhibition and an involvement in tumor malignancy in astrocytes; SCP/TAPs molecules are furthermore highly expressed in leucocytes, monocytes, lung, spleen and embryonic tissues and fibrotic kidney cells and may play a role in developing fibrosis (Henriksen *et al.*, 2001; Pinkston-Gosse & Kenyon, 2007). In platyhelminth, SCP/TAPs have been closely linked to infectivity through being involved in infection initiation, establishment/and or maintenance (Cantacessi *et al.*, 2009).

S. mansoni has 29 genes that are identified as members of the VAL family, each containing the conserved α-β-α sandwich domain specific to the SCP/TAPs superfamily (Chalmers *et al.*, 2008), SmVALs are developmentally expressed and regulated (Kelleher *et al.*, 2014). *S. mansoni* VAL proteins are divided into two groups based on their predicted protein structures and gene configuration (Chalmers *et al.*, 2008). The group one SmVAL proteins

contain a signal peptide, conserved cysteine residues and lack D1 and 11 primary amino acids, while the group two members lack the signal peptide and any cysteine residues but have the D1 and 11 amino acids (Chalmers *et al.*, 2008). Group one members (SmVAL1-5, SmVAL7-10, SmVAL12, SmVAL14, SmVALl5, SmVAL18-29) are classified among secretory/excretory molecules, therefore they can be released in their immediate environment and interact with their host immune molecules and effectors (Chalmers *et al.*, 2008). Moreover Group one VALs are produced during parasitism stages and life cycle expression profiles have been done for SmVAL1, 4, 10 and linked these molecules to parasite evasion of the host immune response (Kelleher *et al.*, 2014). Group two members (SmVAL6, SmVAL11, SmVAL13, SmVAL16, SmVAL17) are linked to intracellular roles within the parasite (Chalmers *et al.*, 2008; Farias *et al.*, 2012).

SmVALs are considered vaccine candidates for human schistosomiasis. Criteria for vaccine candidacy often considers the capability of a parasite protein of modulatory roles against a host immune system through activities such as suppression or inhibition (Kelleher *et al.*, 2014). With the objective of gathering more information on SmVAL group 1 proteins, SmVAL3 that has been characterized by Chalmers *et al.* (2008) to be specific to the intramolluscan phase, was targeted for functional investigations in this research project. Chalmers and colleagues research has revealed SmVAL3 as being up regulated in schistosome eggs, miracidia and mother sporocysts. Most importantly, SmVAL3 is considerably highly present in the miracidial stage and absent in the cercarial and worm stages suggesting its predominant role being specific to the early stages of intramolluscan development of the parasite.

During the process of investigating SmVAL3 for all the specific reasons mentioned above, a publication on SmVAL9 and its function by Yoshino and colleagues (2014) was

released. Since SmVAL9 and SmVAL3 are both members of SmVAL group one and have a similar expression profiles (Chalmers *et al.,* 2008), the focus of my thesis shifted from assessing both SmLeish and SmVAL3 towards more in depth assessment of only SmLeish.

2.4 Summary

Prior to my thesis work, our understanding of the capacity for immunomodulation or immune evasion by schistosomes was confined primarily to associations that have been demonstrated to take place within the definitive host. In the following chapters I address this knowledge gap in our understanding of the biology of schistosomes with respect to their larval development in their snail intermediate host. Preliminary research that was undertaken with the aim of broadly elucidating the factors that are produced by schistosomes and have the potential to facilitate snail infection, formed the basis for my investigations into the biology and functional assessment of leishmanolysin-like and venom-allergen-like proteins. These broad assessments, coupled with observational studies that describe the stages of schistosome infection of the snail host have informed the investigations that will be described in my thesis, which supports a role for SmLeish as an immunosuppressive protein that is produced by *S. mansoni* during the early stages of infection.

2.5 Thesis objective and specific aims

2.5.1 Research objective

The objective of my thesis research is to improve our understanding of the mechanistic basis of *S. mansoni* infection of its snail host *B. glabrata.* I specifically investigated the role of leishmanolysin-like and VAL proteins during the intramolluscan infection and assessed whether any of these proteins were functioning to facilitate suppression of the snail's defense response as

a means to improve the likelihood of infection success.

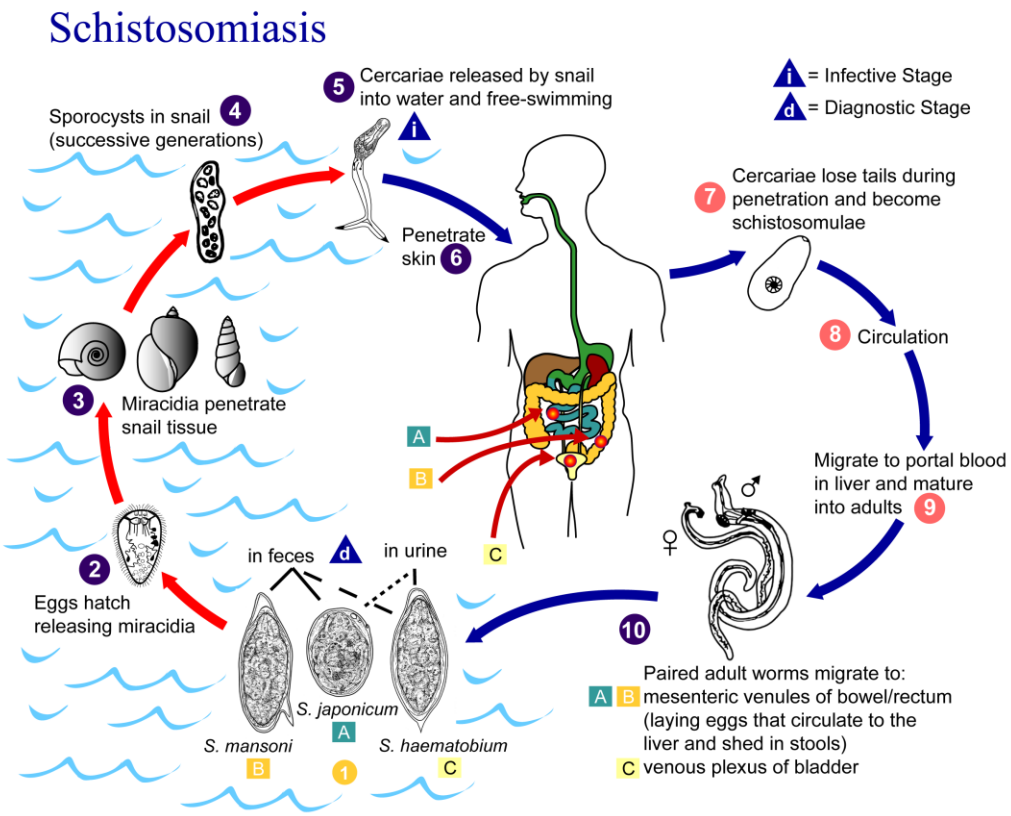
2.5.2 Hypothesis

My working hypotheses are the following:

- There will be up regulation of SmLeish protein during early infection stages in the snail host, and the presence of SmLeish will suppress the snail innate immune response, hence impacting the infection outcome.
- There will be up regulation of SmVAL3 protein during *S. mansoni* intramolluscan infection in *B. glabrata* in a way that facilitates *S. mansoni* infection establishment in *B. glabrata*.

2.5.3 Specific aims

- Establish the mRNA expression profiles of both SmLeish and SmVAL3 factors during *S. mansoni* intramolluscan development in *B. glabrata* at different time points relevant to the complete course of infection.
- Develop recombinant variants of SmLeish and SmVAL3 and antibodies that cross-react with native versions of both proteins as they are produced by *S. mansoni.*
- Use the antibodies to SmLeish to assess where and when this protein is produced using both Western blot and immunohistochemistry.
- Functionally characterize SmLeish during *S. mansoni* infection and provide insight into the mechanism underpinning that function.



From "Schistosomiasis - Biology," by Center for Disease Control and Prevention - Division of Parasitic Diseases and Malaria (DPDM), 2012 (http://www.cdc.gov/parasites/schistosomiasis/biology.html). In the public domain.

Figure 2.1 Schistosome life cycle.

Schistosome eggs hatch when in contact with fresh water and the released miracidia proceed to locate and infect fresh water snails through their head foot. After an average 4 weeks of intramolluscan development involving asexual reproduction, thousands of cercariae are released from the intermediate host and proceed to penetrate their definitive host, humans, through intact skin. The cercaria transforms into schistosomula then matures into mature worm. Both male and female parasites are required to yield eggs through sexual reproduction. About half of the produced eggs are trapped in human tissues while the other half is released into nature by urine or stool.

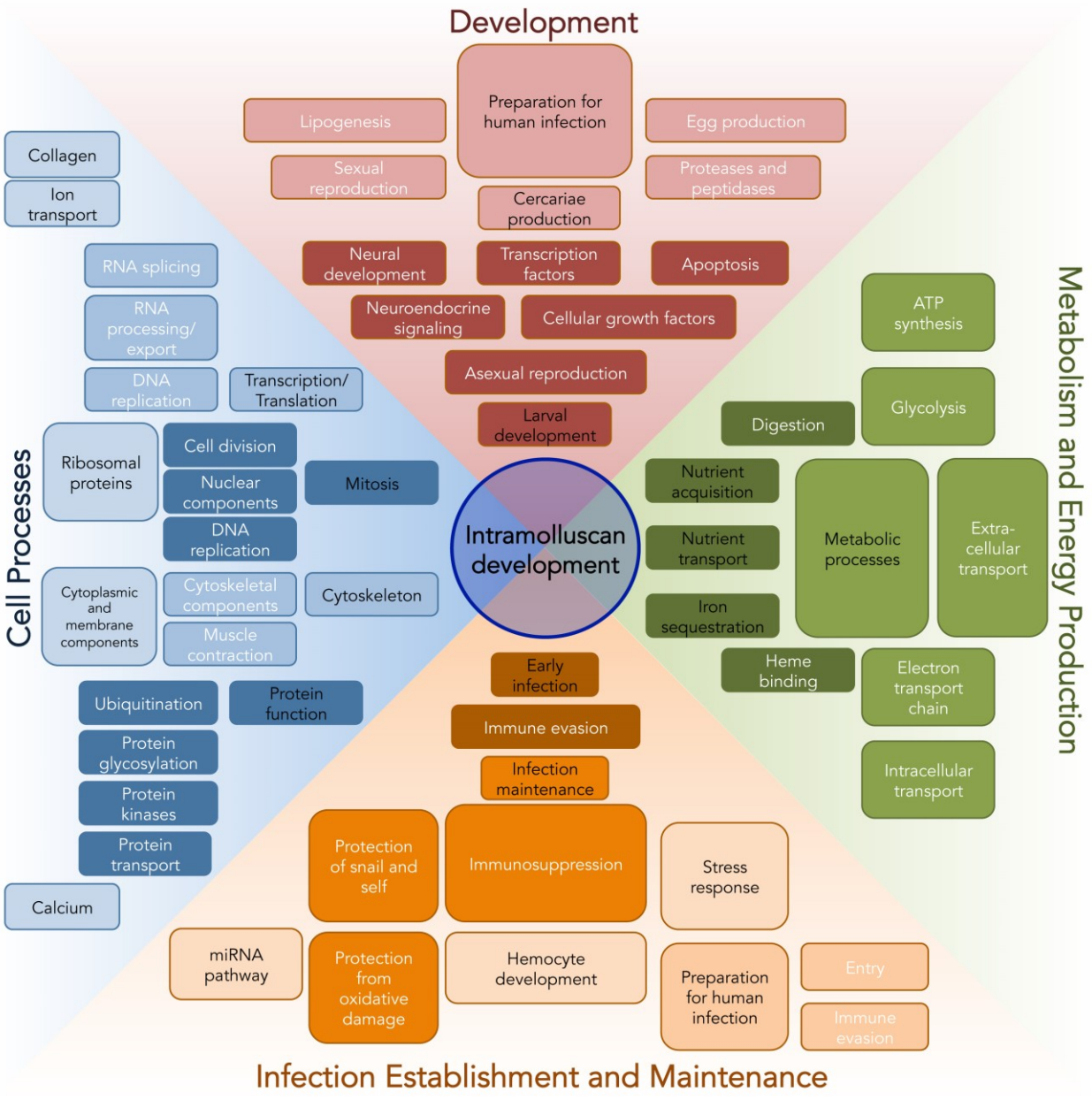


Figure 2.2 *S. mansoni* transcript array.

Transcripts were obtained from *S. mansoni* microarray analysis targeting its transcriptome during the intramolluscan life cycle stage. The transcripts were grouped into four main categories: development, metabolism and energy production, cell processes, and infection establishment and maintenance. Within each of these larger groupings, the transcripts were further broken down into classifications that most accurately represented their putative biological functions within the parasite, or the host-parasite ecosystem. Function of each transcript were estimated by combining the search results of traditional BLASTn and BLASTx, KEGG analysis and searching the GO database. (Hanington *et al.*, unpublished)

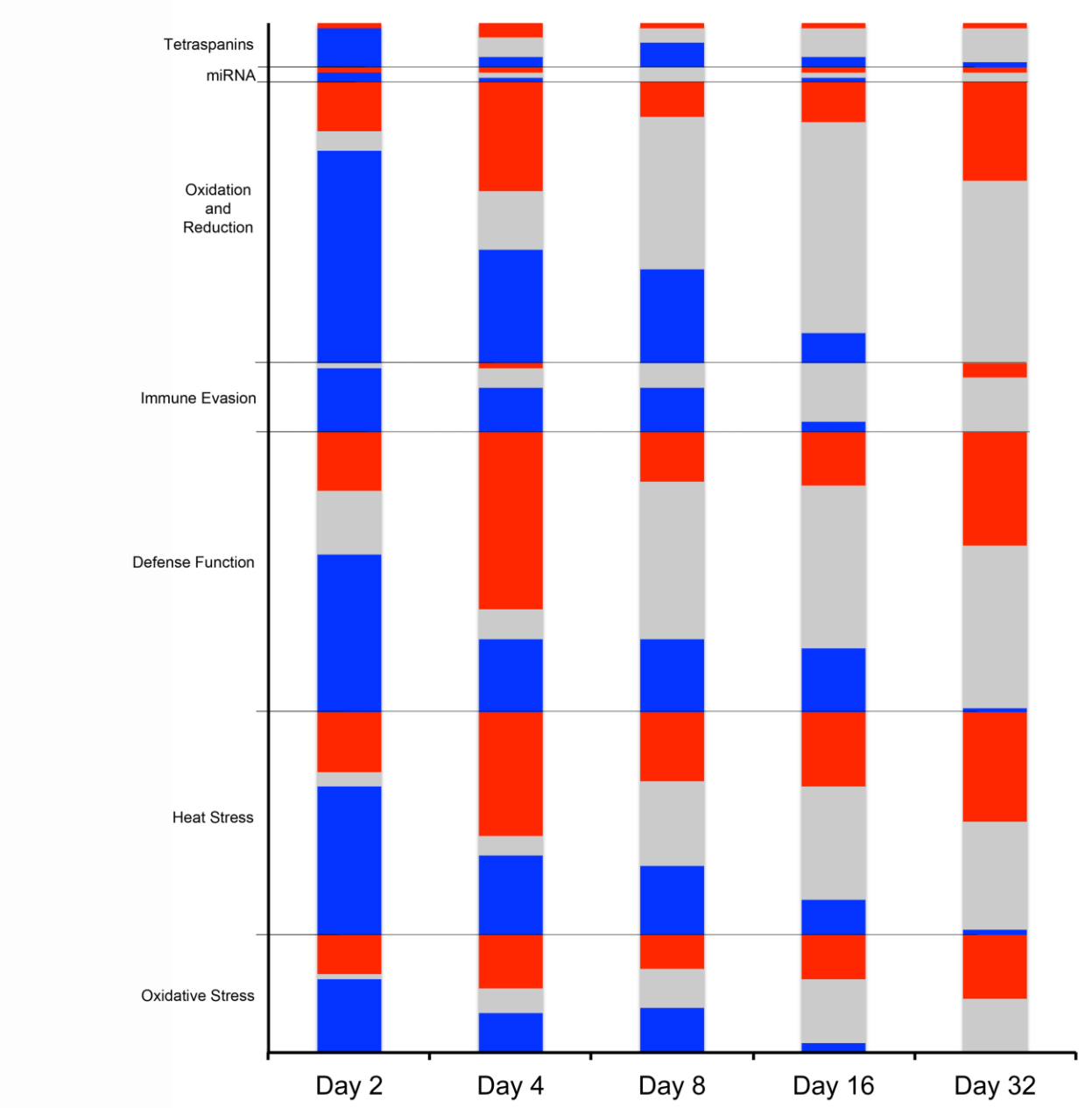


Figure 2.3 *S. mansoni* specific expression patterns of intramolluscan and larval development.

For each graph the x-axis represents the time points at which RNA was isolated from snails/parasites. The y-axis represents the specific functions of each transcript grouping presented as a percentage of the total number of transcripts identified in each primary transcript classification. Horizontal grey lines denote a change in the putative biological function of the transcript group. Each grouping is divided into three colors, red for transcripts that are expressed at higher levels than control, grey for transcripts expressed at similar levels to controls (1 to -1 fold change), and blue representing transcripts that were expressed at lower levels than controls.(Hanington *et al.*, unpublished)

3. Chapter 3: Materials and Methods

Recipes for solutions can be found in the appendix section.

3.1 Parasite, snail infection and colony maintenance

In order to obtain parasite material, the *S. mansoni* life cycle was maintained in the laboratory using *S. mansoni* NMRI strain parasites, *B. glabrata* snails, and BALB/C or Swiss Webster mice. *B. glabrata snails* were kept in artificial spring water tanks at 23-25$^{\circ C}$ and fed red leaf lettuce as needed. Mice were infected with cercariae collected from patently infected *B. glabrata.* Exposure took place in three 20 minutes partial emersions of the mice into cercariae-infested water under sterile conditions at the University of Alberta, Biological Sciences Animal Facility. All animal work observed ethical requirements and was approved by the Canadian Council of Animal Care (AUP00000057).

3.1.1 Cercariae collection

M-line strain of *B. glabrata* infected with *S. mansoni* were left overnight under an artificial light source in a tank of 500ml of artificial spring water to shed and *S. mansoni* cercariae were collected for subsequent infection of mice.

3.1.2 Collection of eggs and transformation of eggs to miracidia

Mice infected with *S. mansoni* cercariae, were euthanized 7 to 8 weeks post exposure and their liver extracted and homogenized to release *S. mansoni* eggs using an Omni Mixer Homogenizer (model number 17105 for 60 seconds). The homogenized product was added to a 2 L flask filled with artificial spring water to the top. The flask was covered in aluminum foil except the top 5 cm of the flask. Artificial light was shined at the top uncovered part of the flask to encourage the migration of newly hatched miracidia to the top of the flask thus allowing easier collection of the miracidia for subsequent use either for RNA extraction or snail infection.

3.1.3 Secretory/excretory (E/S) products collection

Schistosoma mansoni miracidia were cultivated in Chernin's balanced salt solution (CBSS; Chernin, 1963) that also contained glucose and trehalose (1 g/L each), penicillin and streptomycin (CBSS+). After 24 hours, parasite culture supernatants containing E/S (Wu *et al.*, 2009) were collected. Supernatants were sterilized with a 0.2 μm syringe filtered and concentrated. The E/S products were stored at −80°C.

3.1.4 Sporocysts collection

After 24 hours culture in Chernin's balanced salt solution as described in Chernin (1963) containing glucose and trehalose (1 g/L each), penicillin (100 μg/mL) and streptomycin (100 μg/mL) most miracidia transformed to primary sporocysts. Sporocysts were collected in two separate conditions: first from regular *S. mansoni* in order to obtain E/S products, and second from *S. mansoni* miracidia with leishmanolysin knockdown features in order to evaluate the success of the knockdown. The primary sporocysts from knockdown miracidia were kept for 5 days in conditioned complete *B. glabrata* embryonic (Bge) cell medium prepared from culture supernatants of 4-day maintained Bge cells as previously described (Yoshino and Laursen, 1995; Vermeire *et al.*, 2004). Sporocysts were collected every day until day 5 post challenge. RNA was purified and underwent reverse transcription immediately after collection of the sporocysts so that later analysis of SmLeish expression could be undertaken to confirm knockdown efficiency.

3.1.5 *B. glabrata* plasma collection

In order to detect and quantify SmLeish in infected *B. glabrata* snails, the plasma of *S. mansoni*-infected snails was collected at different time points (3 hrs, 6 hrs, 12 hrs, 1day, 2 days, 4 days, 8 day, and 16 days). Using a 200 μL pipette, snail haemolymph was extracted via the headfoot retraction method (Pila, Tarrabain, *et al.*, 2016) and stored in a 1.5 mL conical tube

held on ice. The haemolymph was subsequently centrifuged at 400 x *g* for ten minutes. The plasma was aspirated and transferred into new 1.5 mL tubes and stored at -20$^{\circ C}$ until ready for future Western blot analysis.

3.2 Production of Rabbit Polyclonal Antibody and Detection

3.2.1 Polyclonal antibody production

I designed three different peptide regions for targeted generation of polyclonal antibodies against SmLeish and SmVAL3. The chosen regions were selected based on presumed antigenicity (as predicted by Genscript) as well as for their position in the SmLeish and SmVAL3 amino acid sequences. The peptides were engineered by GenScript, and then used to immunize rabbits. Three different polyclonal antibodies specific for each peptide (SmLeish and SmVAL) were obtained. Each peptide was injected into a specific rabbit to create an immunological response. The animals were exsanguinated after 4 weeks and the serum IgG was purified using a Protein A/G column. Further purification was undertaken by then running the purified IgG through an immunoaffinity column bound with the specific peptides to which each polyclonal antibody was designed. These monopurified (purified against their specific peptide) antibodies were sent from Genscript along with 4 μg of plasmid containing the peptide cDNA, synthesized peptide used to develop the antibody and pre-bleed rabbit serum. Table 1 contains the sequence information for the peptide used for SmLeish antibody production. Figure 3.1 presents the location of the peptides on the SmLeish sequence.

In order to test the specificity of each generated polyclonal antibody in our possession, each antibody was used as a primary antibody during a spot blot test against their respective peptides. In the case of the antibodies generated against SmLeish, I also tested them against the

recombinant protein produced in the laboratory as well as the native protein form obtained from *S. mansoni* E/S products. Both of these tests informed the choice for the most efficient antibody, which best detected the peptide and recombinant protein.

3.2.2 Spot blot test

The spot blot protocol used was derived from Abcam (http://www.abcam.com) and was used to test the specificity of each polyclonal antibody against the specific peptide it was created against. A nitrocellulose membrane of about 7 cm by 8 cm was divided in grid by pencil to indicate the region where the spot was deposited. Then 2 μL of samples containing specific peptide were slowly pipetted in a spot onto the nitrocellulose membrane at the center of the grid and at a slow pace to minimize the area that the solution penetrated in order to restrain the spread of the sample to a diameter of 2-3 mm. The membrane was allowed to dry before proceeding to the normal Western blot steps. The results from the blotting process to test each antibody against SmLeish and SmVAL3 can be found in Figure 3.2 and 3.3.

3.3 Recombinant protein expression

To obtain the polymerase chain reaction (PCR) amplicon of the SmLeish gene sequence for use in an entry clone, 50 μL of DNA free water, 25 μL of 2 X Thermo Scientific Phusion High-Fidelity DNA Polymerase master mix, 500 nM of each of forward and reverse expression primers from Integrated DNA Technologies (IDT), and 22.4 ng/μL of the targeted synthetic DNA template in pUC57 plasmid (GenScript) were mix and distributed in 0.6 mL PCR tubes.

A Thermo Scientific Phusion High-Fidelity thermocycler was used to conduct the PCR with the following cycling parameters: initial denaturation at 98$^{\circ C}$ for 2 minutes, denaturation-annealing for 55$^{\circ C}$ for 10 seconds, and extension at 72$^{\circ C}$ for 1 minute. The denaturation-

annealing and extension steps were repeated for 25 cycles, and the final extension stage occurred at 72$^{\circ C}$ for a duration of 10 minutes. The resulting PCR product was held at 4$^{\circ C}$ at the end of the entire reaction.

The obtained PCR amplicons were then loaded on 1% agarose gel and run for 1 hour to confirm the presence of SmLeish construct band (~1500 base pairs). I was able to test the success and specificity of the amplification process of the targeted gene using this process. The target construct was excised out of the gel and the DNA extracted from the gel using GeneJET gel extraction kit from Thermo Scientific following the specifications from the manufacturer.

To produce the entry clone containing SmLeish gene sequence, the DNA extract obtained from the DNA extraction step was then cloned into the pENTR/D-TOPO vector using the pENTR™ Directional TOPO® Cloning Kit by Life Technology following the manufacturer directions. A combination of 2 μL of PCR obtained DNA of SmLeish product, 1 μL of salt solution, 2 μL of sterile water, and 1 μL of TOPO vector were left to incubate at room temperature for 5 minutes and transfer on to ice pending use for transformation reaction.

From the pENTR/D-TOPO vector, the construct was cloned into the pET-DEST42 vector using the pET-DEST42 Gateway™ Vector kit from Invitrogen following the prescribed steps. The LR recombination reaction was performed to obtain a destination vector from the entry clone. In a tube, 150 ng/μL of the entry clone, 1 μL of the destination vector, and 4.83 μL of TE Buffer (pH 8.8) were mix together, then 2 μL of clonase was added to the mix. The reaction was incubated at 25$^{\circ C}$ for 3 hours. At the end of the incubation, 1 μL of proteinase K was added and the reaction incubated at 37$^{\circ C}$ for another 10 more minutes. The end destination vector now

containing the SmLeish gene sequence was ready to be used in a transformation reaction. The sequence of the final SmLeish construct was verified using mass spectroscopy analysis.

Expression of recombinant SmLeish (rSmLeish) was accomplished using the BL21-AI™ One Shot® Chemically Competent *E. coli* Kit from Invitrogen by Life Technology, following the manufacturers specifications. The product from the precedent LR reaction (1 µL) was used to transform 25 µL of BL21 *E. coli* cells. The transformed cells were subsequently mixed with 250 µL of super optimal broth with catabolite repression (S.O.C) medium held on ice for 1 hour. Two different volumes, 200 µL and 75 µL, were plated on two different agar plates containing 100 µg/mL ampicillin at the end of the incubation. In order to identify the successful clones containing the SmLeish gene sequence, 10 colonies from each transformation plate were randomly selected and analyzed with PCR and gel electrophoresis using vector-specific primers to verify the presence of SmLeish within the transformed *E. coli*. Colonies that tested positive for the presence for SmLeish (based on size of amplicons) were then grown overnight in 5 mL LB medium containing 100 µg/mL ampicillin. A mix of equal volumes of the overnight grown cells and 100% glycerol were used to make a glycerol stock and kept at -80$^{\circ C}$ for future use.

3.4 Production and purification of recombinant protein

3.4.1 Production of recombinant protein

Following the direction provided by BL21-AI™ One Shot® Chemically Competent *E. coli* Kit, the transformed *E. coli* cells were used to express rSmLeish protein at a larger quantity. The glycerol stock was subsequently used to inoculate overnight bacterial cell culture at 37$^{\circ C}$ in 100 µg/mL ampicillin LB medium. A pilot expression experiment was carried first, by adding L-arabinose and IPTG according to the manufacturer directives to 10 mL of LB medium with 100

mg/mL ampicillin that was inoculated with 500 µL of the overnight culture. The inoculated cells were allowed to reach the mid-log phase (OD_{600} ~0.4) growth as recommended by the manufacturer before induction. The inoculated culture was allowed to grow for three more hours at 37°C in a shaking incubator. Various samples of 1ml of the culture were collected every thirty minutes to detect the time point that provided the optimal expression of protein after the incorporation of L-arabinose and IPTG. L-arabinose was added to a final concentration of 0.2% and IPTG to a final concentration of 1 mM. The various 1 mL aliquots mentioned above including one 1 mL sample at time 0 sampled before induction were centrifuged at 23232 x *g* during one minute using a microcentrifuge. The supernatants were discarded and the cell pellets stored at -20°C. SDS-PAGE and Western blot were used to analyze and contrast protein production between samples and 3 hours incubation was the best duration that yielded optimum protein amounts.

For larger scale expression and purification of the SmLeish protein, 25 mL of LB medium containing 100 mg/mL of ampicillin was grown overnight at 37°C in a shaking incubator. The overnight cell culture at an amount of 10 mL was then used to inoculate 500 mL of LB medium containing 100 µg/mL ampicillin for a total volume of 1 L. When the cultures reached their mid-log phase of growth they were induced using L-arabinose to a final concentration of 0.2% and IPTG to a final concentration of 1 mM. Cultures were then grown for 3 more hours and cell harvested through centrifugation at 23232 x *g* for 20 minutes at 4°C and stored at -20°C before purification.

3.4.2 Purification using fast protein liquid chromatography (FPLC)

The recombinant SmLeish protein was engineered to contain a N-terminal 6xHis tag and a V5 epitope to assist in purification and detection of the protein. The harvested cell pellets kept

at -20$^{\circ C}$ were allowed to thaw at room temperature then lysed to release the targeted protein that was retained in the bacterial cell inclusions. B-PER (4 mL/g of cell), the lysing reagent and PMSF (final concentration of 1 mM), the protease inhibitor, were added to the thawed cell pellet and after gentle mix left to incubate at room temperature for 15 minutes. At the end of incubation, the culture was centrifuged at 23232 x *g* for 10 minutes and the supernatant kept for the purification steps.

FPLC (AKTA Pure – GE) was used to purify the target SmLeish recombinant protein using commercial 1 or 5 mL nickel-agarose columns that bind to the 6xHIS region of the recombinant protein. The supernatant obtain after lysis and centrifuge was diluted to 400 mL using the FPLC binding buffer (recipe in the reagent section in the appendix). Following the FPCL machine manufacturer recommendations the diluted solution with the target protein, rSmLeish, was run through the FPLC machine using either 1 or 5 mL HisTrap HP affinity columns from GE Healthcare. Sample fractions (0.5 mL) were obtained from the purification process and SDS-PAGE and Western blot used to analyzed each fraction from the purification process containing protein using the anti-V5 epitope as the target. The fractions containing rSmLeish were pooled and stored at -20$^{\circ C}$ or dialyzed before storage.

The purified rSmLeish protein was dialyzed using a Slide-A-Lyzer Dialysis Cassette kit (Thermo Scientific) and by following the manufacturer protocols. Two separate overnights dialysis, using 1 X PBS were carried for each protein sample to ensure that all imidazole was removed and to concentrate the protein. The SmLeish protein concentration was measured using Qubit protein assay kit and Qubit 2.0 fluorimeter (Invitrogen) and according to the manufacturer directions.

3.5 Immunodetection using polyclonal antibodies

3.5.1 Recombinant protein

Purified and non-purified sample preparation for Western blot was achieved using induced cells or fractions obtain from the FPLC step and stored at -20$^{\circ C}$ that were then thawed at room temperature. The pellet was re-suspended in ultra pure water then vortexed for a few seconds. The N-terminal 6xHis tag and the V5 epitope on the recombinant protein were used to detect the protein using anti-His, or anti V5 antibodies (Invitrogen) or the specific antibodies designed against the protein. The proteins were separated by SDS-PAGE under reducing condition using 12% polyacrylamide gels, which was then transferred to a 0.45 μm nitrocellulose membrane from BioRad. The membrane was incubated for 1 hour in the primary antibody and secondary antibody after a 1 hour blocking time in 5% milk in TBS-T solution in between antibody incubation. To develop the membrane, an ECL Western blotting detection reagent kit (GE Healthcare) was used following the manufacturer descriptions, and an ImageQuant LAS400 detection instrument was used to visualize immunoblots following the manufacturer recommendations. The result of the detection of rSmLeish protein against its three various antibodies is shown in Figure 3.4.

3.5.2 Soluble protein

The soluble form of SmLeish as expressed by the parasite was detected by Western blot, by probing with the selected polyclonal antibody produced against the peptide fragment from GenScript. The material used for analysis of native (expressed in *B. glabrata*) SmLeish was obtained from E/S products from *S. mansoni* miracidia/sporocysts or *S. mansoni*-infected *B. glabrata* plasma. The concentrated E/S products (15 μL) or *S. mansoni*-infected *B. glabrata* plasma (15 μL) were then run on each well of a 12% SDS-PAGE gel and electrophoretically

transferred onto a nitrocellulose membrane using Bio-Rad protein electrophoresis and blotting instrument. After blocking the nitrocellulose membrane over an hour in 5% milk in TBS-T solution, the membrane was incubated with anti-SmLeish (1:5000) for another hour before washing 3 times for 5 minutes with TBS-T and once for 5 minutes with TBS and then incubation in the secondary HRP conjugated goat-anti-rabbit IgG (1:5000) for 1 hour. Before detection, the blot was washed again 3 times for 5 minutes with TBS-T and once for 5 minutes. For detection, the ECL Western blotting detection reagent kit (GE Healthcare) was used following the manufacturer guideline, and visualization was accomplished using the ABI ImageQuant LAS400 detection machine and associated software following the manufacturer specifications.

3.6 Controlled snail infection and collection

The experimental layout for extracting exposed and control snail RNA in order to obtain a time dependent expression levels of our transcript of interest is represented in Figure 3.5. In order to retrieve material for the mRNA expression profile and also infected snail tissue for microscopy targeting the intramolluscan development of *S. mansoni* in *B. glabrata* the following steps was followed.

Five juvenile *B. glabrata* snails of similar size (~8 mm shell diameter) were selected and used for each of the experimental and control groups. There were two sets of experimental groups at each of the collection time points, 5 to be used for RNA extraction and the other set of 5 for microscopy. The time points for the different collections were 0 hr (before exposure), 1hr, 3hrs, 6hrs, 12 hrs, 24 hrs, 48 hrs, 72 hrs, 4 days, 8days, 16 days, and 35 days during experimental challenges of snails.

Snails for RNA extraction were collected and stored in 1.5 mL tubes containing Trizol. Snails for histology were collected and stored in 15 mL tubes containing Railliet-Henry fixative to soften the shells. After discarding the shells, the snail tissues were transferred into 10% buffered formalin for preservation before histology and immunohistochemistry.

3.6.1 RNA extraction from snail material

Using TRIzol® Reagent with the PureLink® RNA Mini Kit (Life Technology) and following the instructions of the manufacturer, the RNA extracted from each snail tissue was stored at -80$^{\circ C}$ until it was used for cDNA synthesis for the qRT-PCR. The RNA concentration was measured using 0.2 µL of RNA and a spectrophotometer (Nanovalue Plus).

3.6.2 Complementary DNA (cDNA) synthesis from RNA

The complementary DNA (cDNA) copies from mRNA were synthesized from obtained RNA from the previous step using the Quanta Biosciences qScript cDNA SuperMix Kit. The manufacturer instructions were followed. Reagents were combined into PCR tubes or a 96-well PCR plate sitting on ice and vortexed gently before the run. The 5 X qScript cDNA SuperMix (4 µL), 1 µL of RNA template, 15 µL of RNase and DNase free water were combined in a PCR tube and the reaction run using Thermo Scientific Phusion High-Fidelity Thermocycler and following these cycles parameters: 5 minutes at 25$^{\circ C}$, 30 minutes at 42$^{\circ C}$, 5 minutes at 85$^{\circ C}$, and hold at 4$^{\circ C}$. The obtain cDNA were stored at -20$^{\circ C}$ for use in future qRT-PCR.

3.7 Primers and probes oligonucleotides design and validation

Primers were designed using the specific SmLeish gene sequence obtained from NCBI website. The gene sequence was then inputted on the qRT-PCR assay design tools from IDT to create qRT-PCR primers specific to SmLeish and SmVAL3. All primers created and used to run

qRT-PCR in the context of this research are listed in table 3. The primers were intron spanning and although DNase was use to prevent any contamination from DNA, a limitation in their utilization in transcript amplification might have been the lack of a reverse transcription control to ensure the non amplification of DNA sequences. The primers were tested to ensure the best amplification, specificity and efficiency towards their targeted DNA sequences. The position of the primers on the SmLeish sequence is represented in Figure 3.1.

In order to obtain the most efficient endogenous control for the quantification of SmLeish and SmVAL3 at various time points during *S. mansoni* intramolluscan development, *S. mansoni* GAPDH (SmGAPDH), *S. mansoni* tubulin (SmTubulin) transcripts were targeted using primers specific to each of these transcripts (Table 3). After, analyzing the specificity of each primer set via melt curve analysis (Figure 3.2), SmGAPDH was chosen for further assessment of the amplification reaction efficiency (Figure 3.6). Similar melt curve and efficiency analysis was also undertaken for SmLeish and SmVAL3 (Figure 3.7 and 3.8 respectively).

The efficiency of SmLeish and SmVAL3 qRT-PCR reactions were assessed along with SmGAPDH using serial dilutions of cDNA template generated from mRNA isolated from a snail known to be infected by *S. mansoni.* PCR efficiency was calculated using the Applied Biosystems 7500 Fast qRT-PCR analysis software and confirmed by plotting the natural log of the cDNA dilution against the average Ct values for each cDNA dilution in Microsoft Excel 2011. A 2-fold dilution series was adopted instead of the generally used 10-fold in this process in order to preserve consistency with the parameters used by the ABI software. The slope of the resulting curve was then used to calculate qRT-PCR efficiency, which is expressed as a percentage, and used by the ABI analysis software to adjust all delta delta Ct comparisons of expression and to adjust for PCR reaction efficiency differences. An efficiency of 96% was

calculated for SmGAPDH, 95% for SmLeish, and 99% for SmVAL3 qRT-PCR assays (Figure 3.9, 3.10, and 3.11). Because of the use of SmGAPDH as endogenous control, a ratio of the efficiency between SmGAPDH and SmLeish, and between SmGAPDH and SmVAL3 were most important to understand for analysis purposes and the obtained ratios were both equal to 1.

3.8 Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

The amplification reactions for SmGAPDH, SmLeish and SmVAL3 were achieved using a set of forward and reverse primers specific to each transcript and a PerfeCta Syber Green SuperMix, ROX kit (Quanta Biosciences).

Hence for each protein, 12.5 μL of the PerfeCta Syber Green SuperMix, ROX (2 X), forward and reverse primers at a final concentration of 300 nM, 5 μL of the cDNA template and 6 μL of nuclease free water were mixed on a 96 qRT-PCR wells plate on ice and then briefly vortexed and centrifuged for a minute before performing the qRT-PCR. The qRT-PCR runs were set up for 50 cycles on the 7500 Fast Real Time PCR System (Thermo Fisher Scientific). The 50 cycles used in the qPCR was not necessary for any amplification, but rather served as the endpoint of the cycling.

3.9 siRNA-mediated protein knockdown of SmLeish

S. mansoni sporocysts were emerged in a cocktail of 27-nucleotides siRNA. The siRNA was designed using the IDT siRNA design tool and they were designed to specifically target 4 different regions of the SmLeish transcript. The oligonucleotide sequences were confirmed to be unique to SmLeish by comparison to the *S. mansoni* genome. The oligonucleotide mix was suspended in Xfect transfection reagent (Clone Tech) in order to enhance delivery and sporocysts were exposed at an approximate final concentration of 6 nM. Control sporocysts

received siRNA oligo targeting green fluorescent protein (GFP), these oligonucleotides have no known homologies to expressed transcripts in *S. mansoni* and were used only to control for the engagement of the dicer complex to ensure no non-specific phenotype alterations took place. siRNA oligonucleotides information can be found in table 4.

3.9.1 Analysis of SmLeish knockdown kinetics

Confirmation of SmLeish knockdown was accomplished using the pre-existing qRT-PCR assay described above. RNA was isolated from *S. mansoni* sporocysts at 1, 2, 3, 4, and 5 days post exposure to either the SmLeish or GFP-specific siRNA oligonucleotides for 24 hours. Expression of SmLeish *in vitro* over these 5 days was assessed and compared to sporocysts exposed to GFP-specific siRNA. In each case, 10 sporocysts were used to generate pooled total RNA from which cDNA for qRT-PCR was synthesized as described above.

3.9.2 Assessment of SmLeish knockdown on *S. mansoni* infection phenotype in M-line *B. glabrata*

Fifty M-line *B. glabrata* were challenged with *S. mansoni* miracidia exposed to either SmLeish or GFP-specific siRNA oligonucleotides 24 hours prior to challenge. Snails were exposed to SmLeish-knockdown miracidia individually for 24 hours before being placed into tanks containing artificial spring water (25 snails per tank). Snails were fed biweekly a diet of red leaf lettuce, and starting at 4-weeks post challenge, were placed in 24 well plates individually and shed for 24 hours to determine whether *S. mansoni* infections had reached patency. The number of snails shedding cercariae was recorded and the snails were placed back in the tanks for another week. Cercariae shedding were assessed weekly, as was snail mortality. The

experiment culminated at week ten-post challenge. Data was assessed as a percentage of snails shedding cercariae.

3.10 Immunohistochemistry

The IHC-Paraffin protocol (IHC-P) from Abcam (http://www.abcam.com) was followed for this section. Slides with slices of *S. mansoni* exposed snail tissue in paraffin were used for the immunohistochemistry. The first phase was to deparaffinise the snail tissue sample in order to access the tissue for antibody probing. Following the IHC-P directions, the slides with infected snail tissue were incubated twice with xylene for 3 minutes, then a 1 to 1 mix of xylene and 100% ethanol for 3 minutes, followed by two 3-minutes incubations with 100% ethanol, and subsequently one 3-minutes incubation periods with 95%, 70% and 50% ethanol. To finish, the slides were rinsed with cold running water and stored in tap water in preparation for the next steps.

In a second step, the slides were submitted to an antigen retrieval process using sodium citrate buffer and a domestic vegetable steamer. About 400 mL of the buffer was warmed up using a microwave and poured into the pre-warmed vegetable steamer with a metal rack holding the deparaffinised slides. The lid was placed on and the slides and incubated for 20 minutes. The slides were soaked in cold tap water for about 10 minutes before proceeding to the immunohistochemical-staining phase. Note that an initial trial was run with various incubation durations ranging from 5 minutes to 30 minutes in order to establish the incubation time required for optimized antigen retrieval.

The immunohistochemical staining phase was done over a two-day period. On day one, the slides were washed twice for a duration of 5 minutes each in a TBS and 0.025% Tween

solution on a hand-over-hand rotor. They were then blocked using 10% normal serum with 1% BSA in TBS for two hours at room temperature on the hand-over-hand rotor. The slides were drained for a few seconds and the areas surrounding the tissue cleaned with kimwipes. The specific primary antibody (anti SmLeish, Keyhole limpet hemocyanin [KLH]) diluted in TBS with 1% BSA at a concentration of 1:250 was added to the slides and incubated overnight at 4 degree in a humidified chamber. On day two, the slides were rinsed twice for 5 minutes with a solution of TBS and 0.025% Tween with gentle agitation. The secondary fluorophore-conjugated antibody (Alexa Fluor 488) diluted in TBS with 1% BSA, following the manufacturer's recommendations, was added to the slides and incubated at room temperature for one hour. The following steps were completed in the dark. The slides were rinsed three times in TBS for 5 minutes, after which, one drop of DAPI was added on the specimen. After 5 minutes, a coverslip was added. In the process of optimization to obtain the best detection of SmLeish protein, the 3 SmLeish antibodies were used separately and as a cocktail.

Fluorescence microscopy was performed using an Axio imager A.2 microscope from Zeiss, and analyzed using Zen 2011 software, Photoshop CS5 (Adobe Systems Inc., USA).

3.11 Chemotaxis/chemokinesis

The impact of rSmLeish and *S. mansoni* E/S products on *B. glabrata* haemocyte migration was assessed using a chemokinesis assay that has been previously published (Lodes & Yoshino, 1990). This approach does not directly measure chemotaxis towards a target gradient, but instead measures the impact of a molecule of interest on cell migration behavior compared to controls. This assay was chosen because SmLeish was believed to be inhibitory to chemokinesis, and *S. mansoni*-specific targets known to be chemoattractive to *B. glabrata* haemocytes were not

available. Also, because the specific target ligand of SmLeish activity is not known, it is unknown of how it would affect chemotaxis induced by a known factor.

The assay was designed to test the impact of both SmLeish and *S. mansoni* E/S products on both M-line and BS-90 strain *B. glabrata.* BS-90 snails are resistant to *S. mansoni* infection, and thus served as a control for the infection phenotype and the hypothesized role of SmLeish in facilitating infection establishment in susceptible (M-line) snails. In all tests, haemolymph was isolated from individual snails, centrifuged at 500 x *g* for 10 minutes, and aspirating of the cell-free plasma isolated the haemocyte pellet. The haemocytes were resuspended in 200 µL of 1X PBS and then divided into two equal volumes. Each subset of haemocytes was counted using a haemocytometer (10 µl) to ensure equal cell concentrations in each subset. There was a replication for five snails per treatment, per strain.

Haemocyte chemokinesis was measured using a custom-built chemotaxis chamber. Haemocytes were always placed into the upper chamber of the apparatus. The upper chamber was separated from the lower chamber by a 5 µm pore containing membrane. All analyses consisted of staining the membrane using haemotoxilyn and eosin (H and E) and then counting the number of haemocytes that appeared on the underside of the membrane, side opposite to where the haemocytes were originally added. In each case, the experimental test was compared to a snail-matched control, which consisted of the second haemocyte subset of each snail isolation. Controls were exposed to CBSS in both the upper and lower chamber of the apparatus and thus represented the baseline haemocyte migration (chemokinesis) for each snail. These values were compared to experimental chemokinesis to generate a chemokinetic index (ratio of haemocytes that migrated in the experimental group compared to the number migrated in the control group).

The experimental groups used to test SmLeish included: 0.25 µg/mL of non-trypsinized rSmLeish, which was demonstrated to have enzymatic activity as an MMP, and 5 µg/mL *S. mansoni* E/S products. The positive control used was 1 µM fMLP which is a commonly used chemotactic agent. SmLeish, *S. mansoni* E/S and fMLP were assessed for their impact on haemocyte migration in 3 ways. Firstly, the agent was placed in the bottom well to assess attractiveness to haemocytes (in this design set-up the haemocytes will migrate to the membrane underside if attracted to the agent). Secondly, placing the agent in the top well with the haemocytes was used to assess inhibition of chemokinesis (in this case the agent would prevent migration to the underside of the membrane if it has generally anti-chemokinetic properties). The agent was considered inhibitory if the agent did not attract the haemocytes when placed in the bottom compartment and at the same time prevented haemocyte migration towards the bottom compartment when it was mixed with the haemocytes in the top compartment. Finally, the agent was placed in both wells, which is a traditional control for chemotaxis as a means to assess baseline chemokinesis. Each experimental set-up was replicated five times with independent snails supplying haemocytes for each experiment. The experimental process of the chemotaxis is summarized in Figure 3.2, and a representation of the chemotaxis set up is presented in Figure 3.13.

3.12 Matrix metalloprotease (MMP) property analysis

The Sensolyte Generic MMP Assay Kit Colorimetric (Anaspec) was used for the functional characterization of rSmLeish and the manufacturer's directions were followed. rSmLeish was incubated with the provided substrate from the kit. This chromogenic substrate is a specific substrate cleaved by MMP and has been described by the manufacturer as being a thiopeptolide molecule which cleavage releases a sulfhydryl group. This was a coupled assay

where the sulfhydryl group reaction with Ellman's reagent yields 2-nitro-5-thiobenzoic acid (TNB) as the final product. The pigment from TNB is detected using a microplate reader at 412 nm and allows with the standard curve to characterize enzyme activity. The MMP specific inhibitor was provided through the kit as well as the MMP specific activator, trypsin. The human MMP8, used as positive control, was obtained from Anaspec and used following the manufacturer's guidelines. Two main experiments were carried: (1) using two different initial concentrations (0.5 μg/mL and 1 μg/mL) of the enzyme and (2) incubating the enzyme for various lengths of times while keeping the initial concentration at 0.25 μg/mL. In both experiments the substrate concentration was kept constant in all reactions. The human MMP8 and rSmLeish were both exposed to trypsin for one hour at 37$^{\circ C}$ as an activation process and the activation process was stopped by using trypsin inhibitor (Anaspec). The inhibition of the human MMP8 and rSmLeish activity were attained by adding the MMP specific inhibitor provided by the kit to the initial enzyme before adding the substrate. After combining all relevant substances and incubating the plate at 37$^{\circ C}$ for a designated duration (30, 60, 90, 120 minutes), the 96 wells plate results were obtained using a fluorescence absorbance cuvette from SpectroMax M2 and recorded.

The standard curve was obtained using the reference enzyme provided by the manufacturer (Anaspec) and diluting it to 100, 50, 25, 12.5, 6.25, and 3.12 μM concentration, adding the MMP specific substrate and incubating the mix with the same duration as the human MMP8 and rSmLeish. The obtained reference standard curve was then used to obtain the final TNB concentration from its absorbance values. The final TNB concentration represents the enzymatic activity. A sample standard curve used in this experiment is displayed in Figure 3.14.

3.13 Statistics

The statistical significance for the knockdown efficiency, the haemocytes motility in the chemotaxis (chemokinetic index), and the metalloprotease activity of rSmLeish were tested using a t-test for independent means. The percentage of snails shedding cercariae statistical significance was tested using a z test for two independent proportions.

Table 1 Peptides used to generate SmLeish antibodies

Name	Sequence
Peptide #1	EFVANKPSIQGSRG
Peptide #2*	EEDGTPRTPRDPQT
Peptide #3	MDLENDGGSGTAF

*Peptide #2 is the one used in this study

Table 2 Recombinant protein expression primers

Name	Sequence
SmLeishmanolysin-pDest-Forward	CAC CAG GAG GAG GAG AGC CAT GGT ACC CTG TTC AAG
SmLeishmanolysin-Expression-Reverse	TTG TTT AAT TGA TCT ACG CCT G
T7 Promoter (+)	TAA TAC GAC TCA CTA TAG GG
V5 Reverse (-)	ACC GAG GAG AGG GTT AGG GAT
M13 Forward (-20)	GTA AAA CGA CGG CCA G
M13 Reverse	CAG GAA ACA GCT ATG AC

Table 3 qRT-PCR primers sequences

Primer Name	Sequence
Quantitative PCR primers	
SmGAPDH Forward	TCGTTGAGTCTACTGGAGTCTTTACG
SmGAPDH Reverse	AATATGAGCCTGAGCTTTATCAATGG
SmTubulin Forward	GCTAATAACGCTCGCGGTCATT
SmTubulin Reverse	TGCAATTTGTGGTGCTGGATAA
SmLeish Forward	CCTCATCGCTTACCAGAATGT
SmLeish Reverse	TGGTTAGTATGCGCTCGAATTA
SmLeish probe	/56-FAM/TTACAAATCCACCCACCCACTCTGG/3IABkFQ/
SmVAL3 Forward	GAGCATCGAGCATACCGAAA
SmVAL3 Reverse	CAGTTCATCGTCCCACTAAA

Table 4 siRNA oligonucleotides

Name	Sequence (positive strand)
#1	GrArArGrCrUrUrUrCrArGrArUrArUrArUrGrCrGrArGrArCrCAG
#2	CrGrArArCrUrGrUrArGrArUrGrCrUrUrUrUrGrUrGrCrUrUrAUA
#3	UrUrArCrCrUrArCrCrGrCrCrUrUrCrCrGrArArUrUrCrGrCrCAG
#4	UrCrGrArArUrCrUrUrGrUrUrArArArCrUrCrUrArCrCrUrUrCUA

Figure 3.1 *S. mansoni* leishmanolysin-2 (M08 family) gene sequence

This is the complete SmLeish2 sequence studied in this research project and obtained from the NCBI website (ID: CCD79314.1). The black highlights are the predicted trypsin cleavage sites. The pink highlight is the predicted transmembrane domain. The orange and blue lettering represent known MMP domains. The grey, green and yellow highlights are the site for which the antibodies were raised against. The red and blue highlights are the qPCR/probe primers sites. The recombinant protein sequence starts from the methionine highlight in green right above the grey highlighted section to the end of the present sequence.

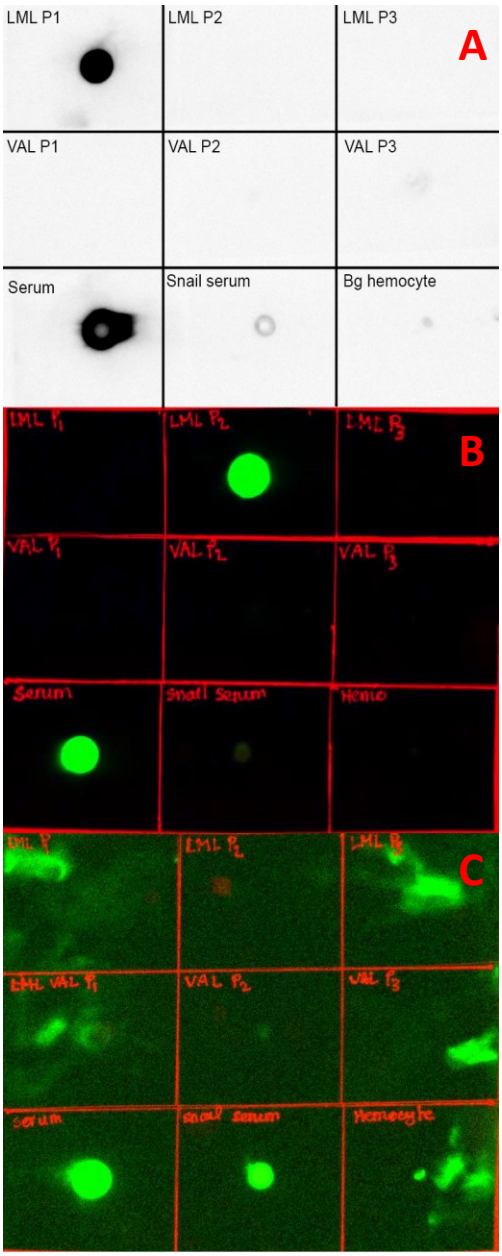


Figure 3.2 Spot test for the anti-SmLeish antibodies

Each of the three antibodies generated against SmLeish using three peptide sequences targeting three different regions of SmLeish sequence were used as primary antibodies in a Western blot. LMP P1, P2, and P3 are the peptides used to generate SmLeish antibody 1, 2, and 3 respectively. VAL P1, P2, and P3 are the peptides used to generate SmVAL3 antibody 1, 2 and 3 respectively. Serum is the serum from the rabbit used to generate the antibody and snail serum is the plasma extracted from uninfected *B. glabrata* snails. Bg hemocyte is the extracted haemocytes from *B. glabrata*. Panel A shows the result from anti-SmLeish #1 Western blot; the antibody positively recognizes peptide 1 used to create anti-SmLeish #1 and the serum from the rabbit it was raised against. Panel B displays the result from the anti-SmLeish #2 Western blot where only peptide 2 and the rabbit serum were detected by anti-SmLeish #2. Panel C shows the result from anti-SmLeish #3 Western blot: the antibody detected the rabbit serum it was raised against but also the uninfected snail serum but not P3. Anti-SmLeish #1 was the chosen antibody for subsequent tests requiring detection of SmLeish.

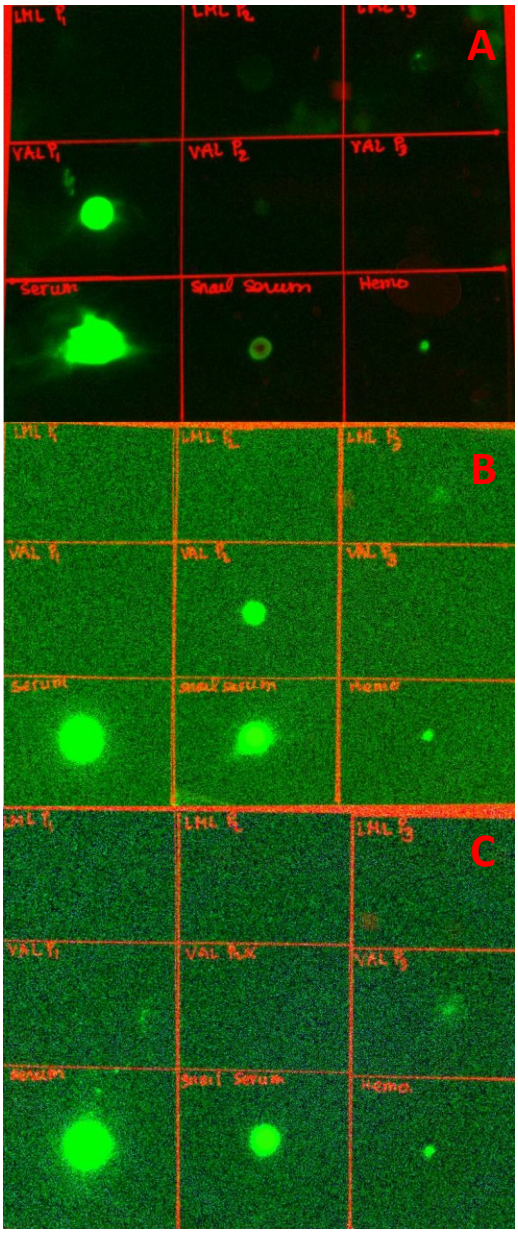


Figure 3.3 Spot test for the anti-SmVAL3 antibodies

Each of the three antibodies generated against SmVAL3 based on three peptide sequences targeting three different regions of SmVAL3 sequence were used as primary antibodies in a Western blot. LMP P1, P2, and P3 are the peptides used to generate SmLeish antibody 1, 2, and 3 respectively. VAL P1, P2, and P3 are the peptides used to generate SmVAL3 antibody 1, 2 and 3 respectively. Serum is the serum from the rabbit used to generate the antibody and snail serum is the plasma extracted from uninfected *B. glabrata* snails. Bg hemocyte is the extracted haemocytes from *B. glabrata*. Panel A shows the result from anti-SmVAL3 #1 Western blot; the antibody positively recognizes peptide 1 used to create anti-SmVAL3 #1 and the serum from the rabbit it was raised against. Panel B displays the result from the anti-SmVAL3 #2 Western blot where peptide 2, the rabbit serum and snail plasma were detected by anti-SmVAL3 #2. Panel C shows the result from anti-SmVAL3 #3 Western blot: the antibody detected the rabbit serum it was raised against but also cross reacted with the snail plasma but not the peptide it was raised against (P3). Anti-SmVAL3 #1 was the chosen antibody for subsequent tests requiring detection of SmVAL3.

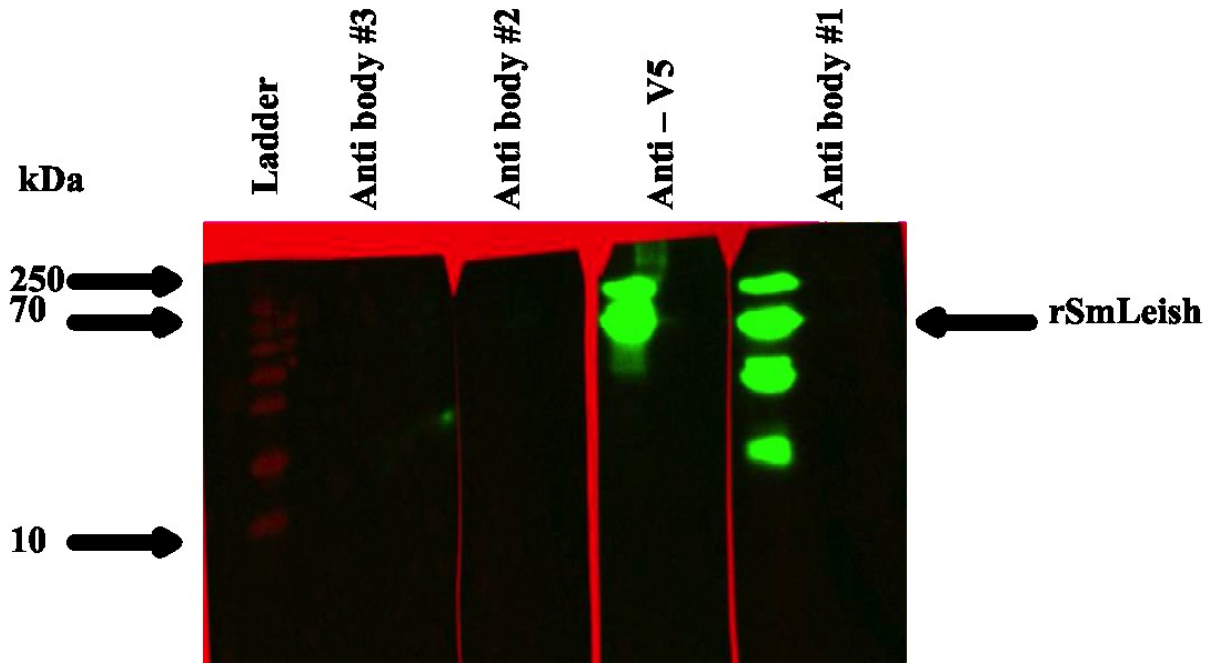


Figure 3.4 Purified SmLeish testing against anti-Smleish antibody 1, 2, and 3

Western blot testing the three different anti-SmLeish antibodies against rSmLeish results showed that anti-SmLeish #1 was the only anti-SmLeish that detected the rSmLeish. The Anti-V5 was used as a positive control to ensure the detection of the recombinant SmLeish. The various bands detected could be the products of degradation or post-transcriptional products from SmLeish. Only the constructs containing the V5 epitope after degradation are detected by the anti-V5 antibody.

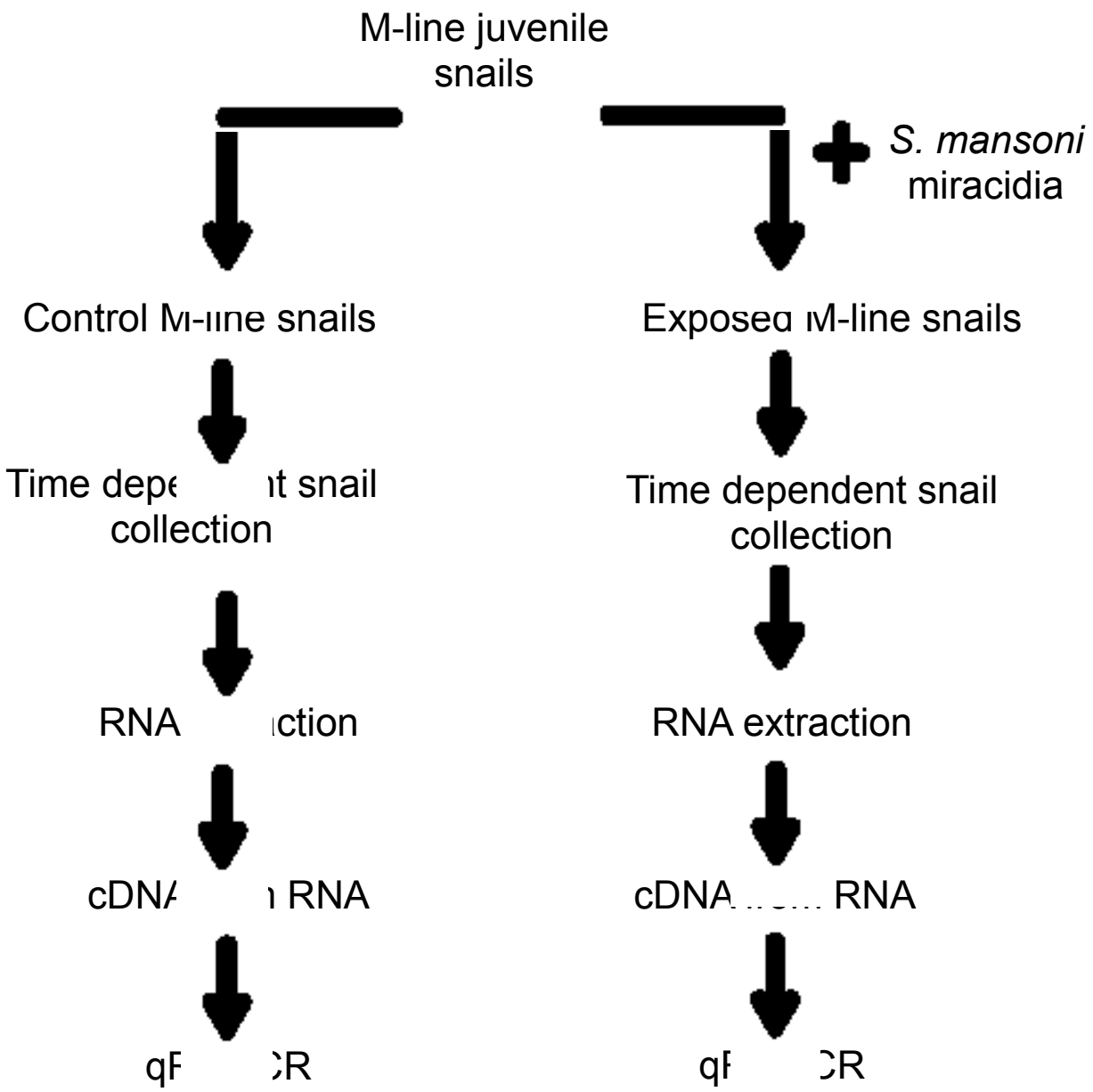


Figure 3.5 Experimental layout for RNA extraction from snail tissue

The snails for RNA extraction were collected at various time points (1, 3, and 12 hours, 1, 2, 3, 4, 8, 16, and 25 days) and stored in 1.5ml tubes containing Trizol. 5 snails were collected for exposed and control groups for the RNA extraction at each time point. RNA extracted from each snail was then used to produce cDNA that was then use as the template to run qRT-PCR reaction to quantify SmLeish and SmVAL3 transcripts.

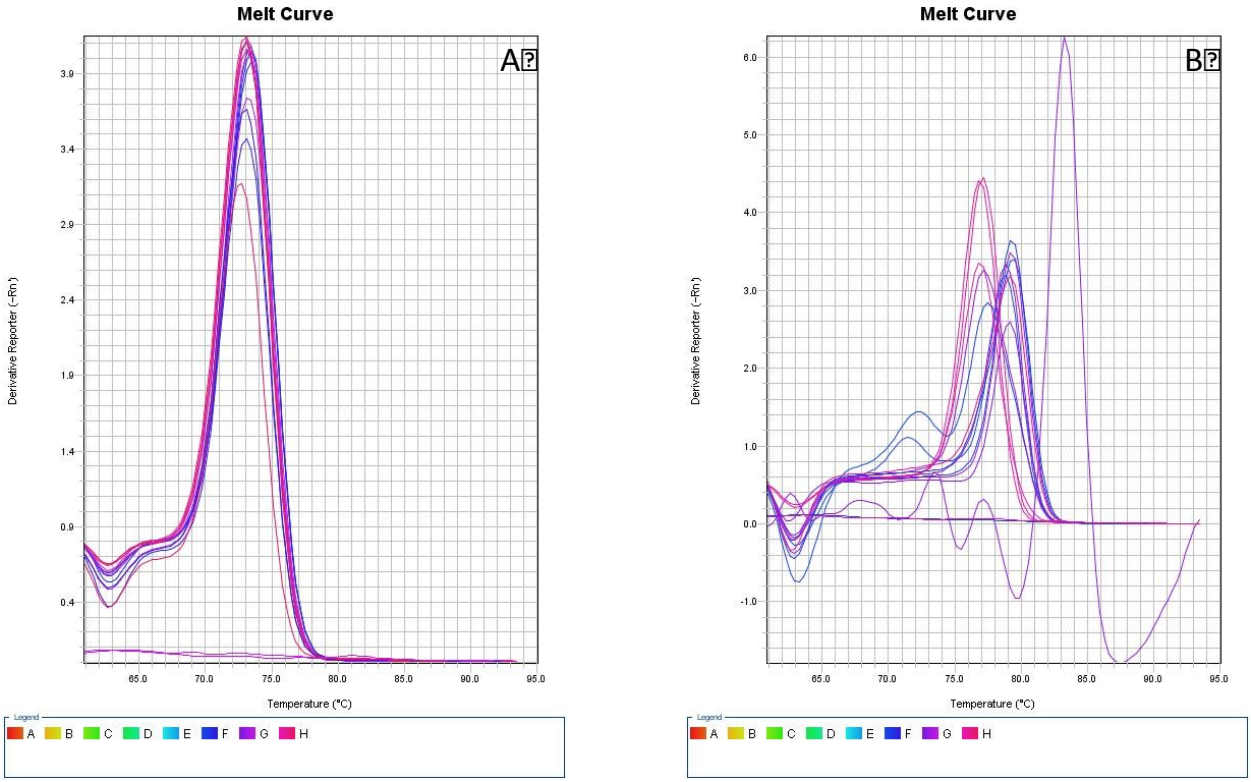


Figure 3.6 qRT-PCR melt curves for (A) SmGAPDH, and (B) SmTubulin

In order to find the best endogenous control for the study of SmLeish and SmVAL3 transcripts, two endogenous molecules were chosen and primers made to target the transcripts of each. The melt curves of the primers targeting SmGAPDH (A) and SmTubulin (B) are herein represented. The single peak in (A) demonstrates specificity for the SmGAPDH qRT-PCR assay. However the qRT-PCR assays for (B) targeting SmTubulin failed to produce a single specific product through its amplification shown by the various peaks observed. Therefore data from SmGAPDH qRT-PCR assay reactions were used in subsequent data analysis to normalized data from SmLeish and SmVAL.

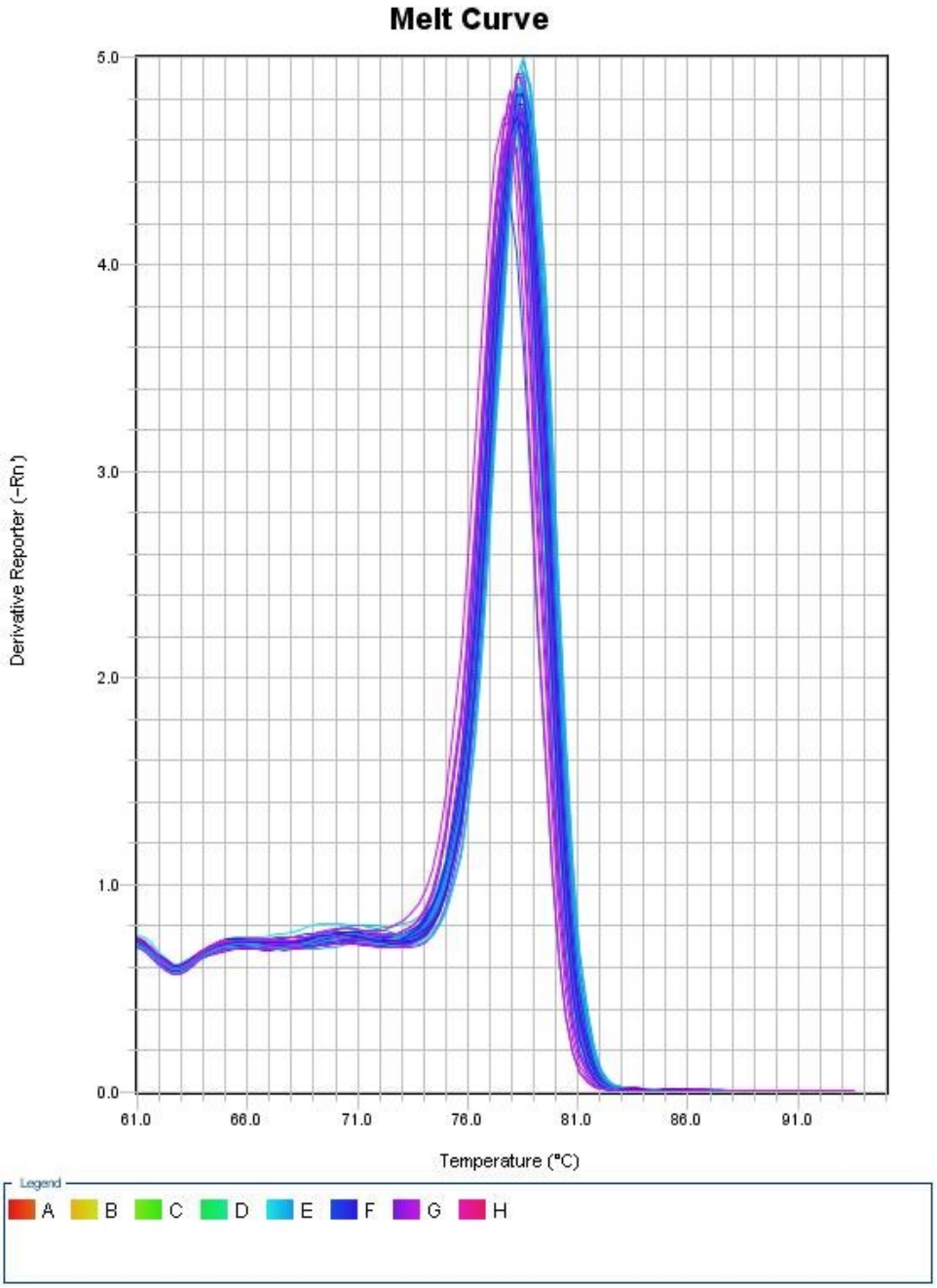


Figure 3.7 qRT-PCR melt curves for SmLeish primer set

The melt curves of the qRT-PCR assay amplification of SmLeish transcripts showed a single peak. This demonstrates the specificity of the primer set to target the SmLeish amplicon and produce a single final product.

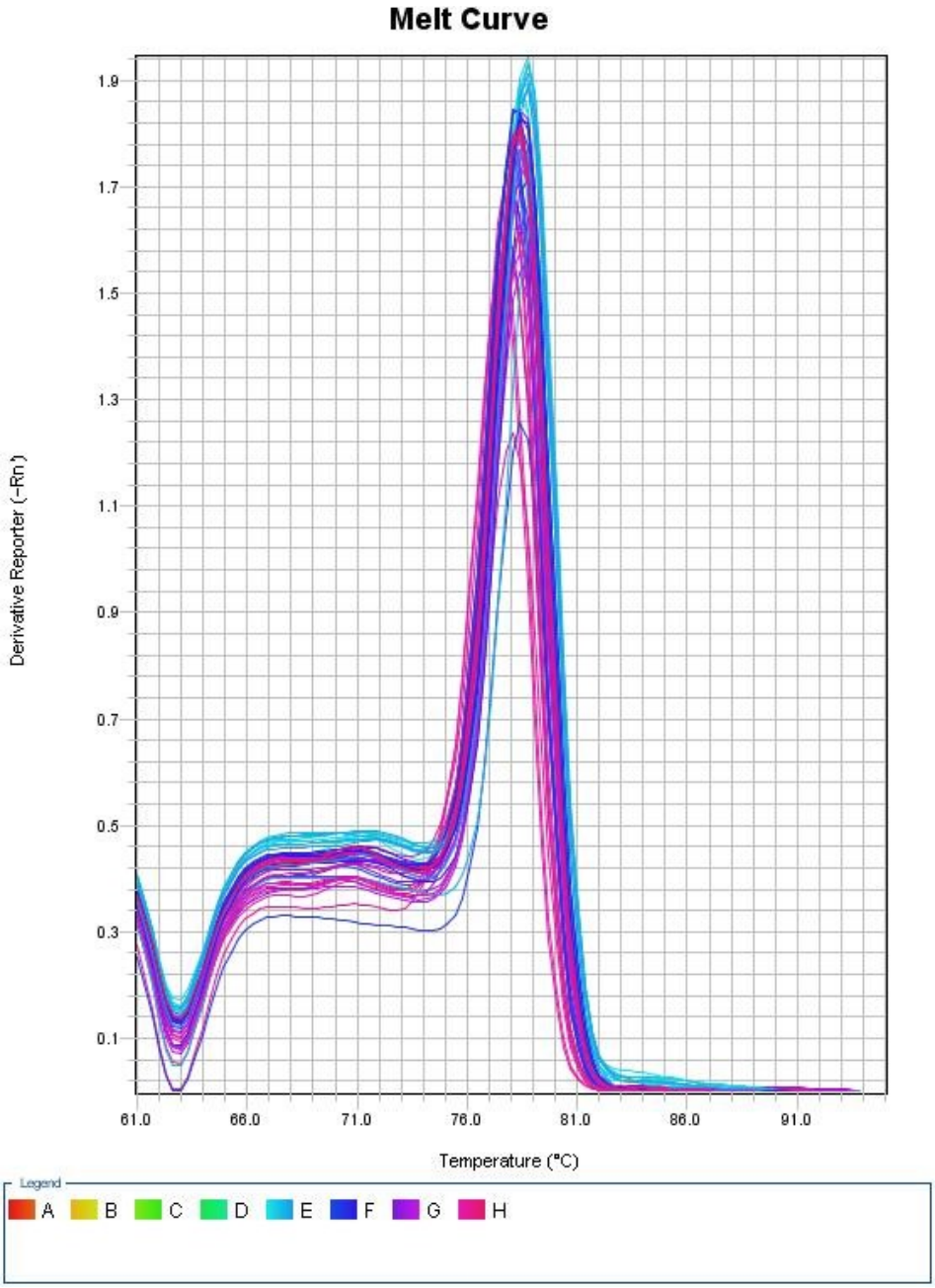


Figure 3.8 qRT-PCR melt curves for SmVAL3 primer set

The melt curves of the qRT-PCR assay amplification of SmVAL3 transcripts showed a single peak. This demonstrates the specificity of the primer set to target the SmVAL3 amplicon to produce a single final product.

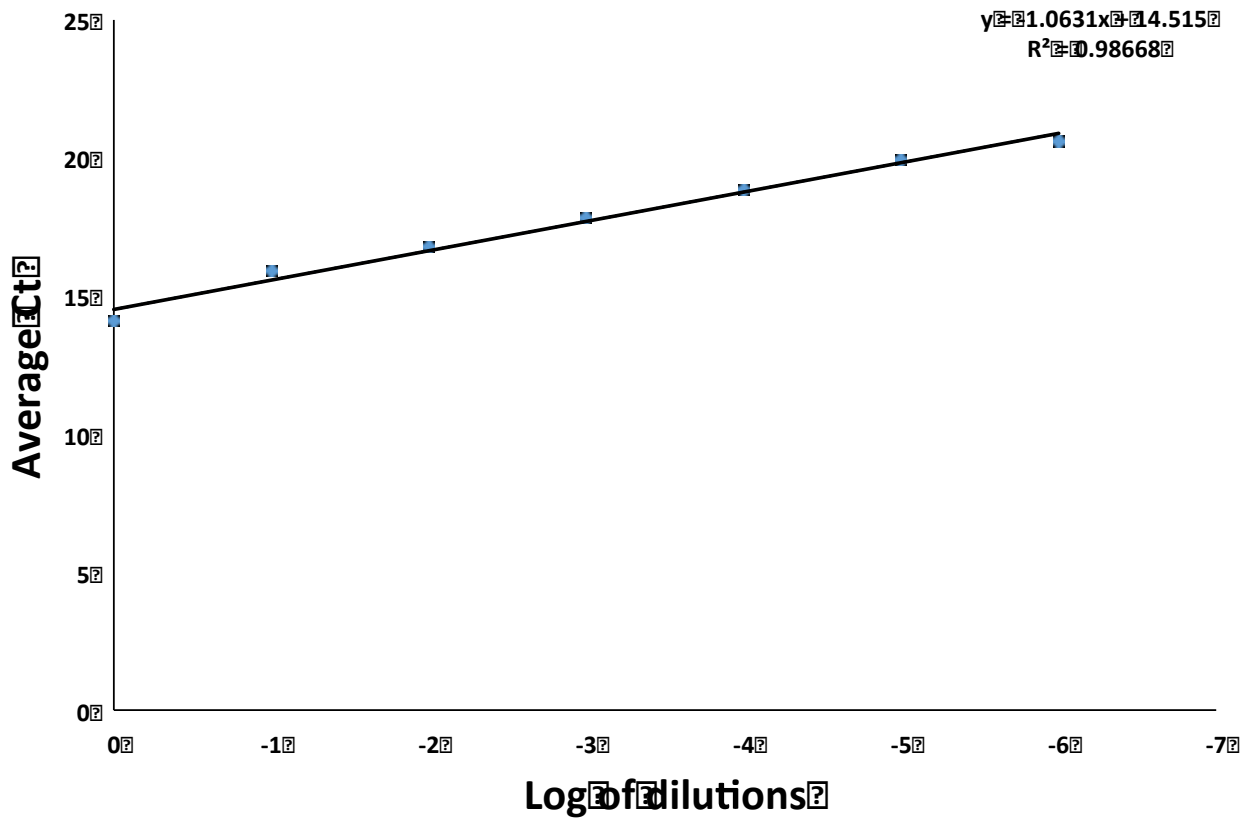


Figure 3.9 SmGAPDH primer efficiency

Comparison of the average Ct values of three replicate reactions graphed against the log(2) of the dilution of the reaction cDNA. The slope of the equation obtained from the trend line used in the efficiency equation allowed calculation of efficiency value for SmGAPDH primers. The following equation was used for the primer efficiency calculation: Efficiency = ((dilution factor)^(1/-slope))-1.

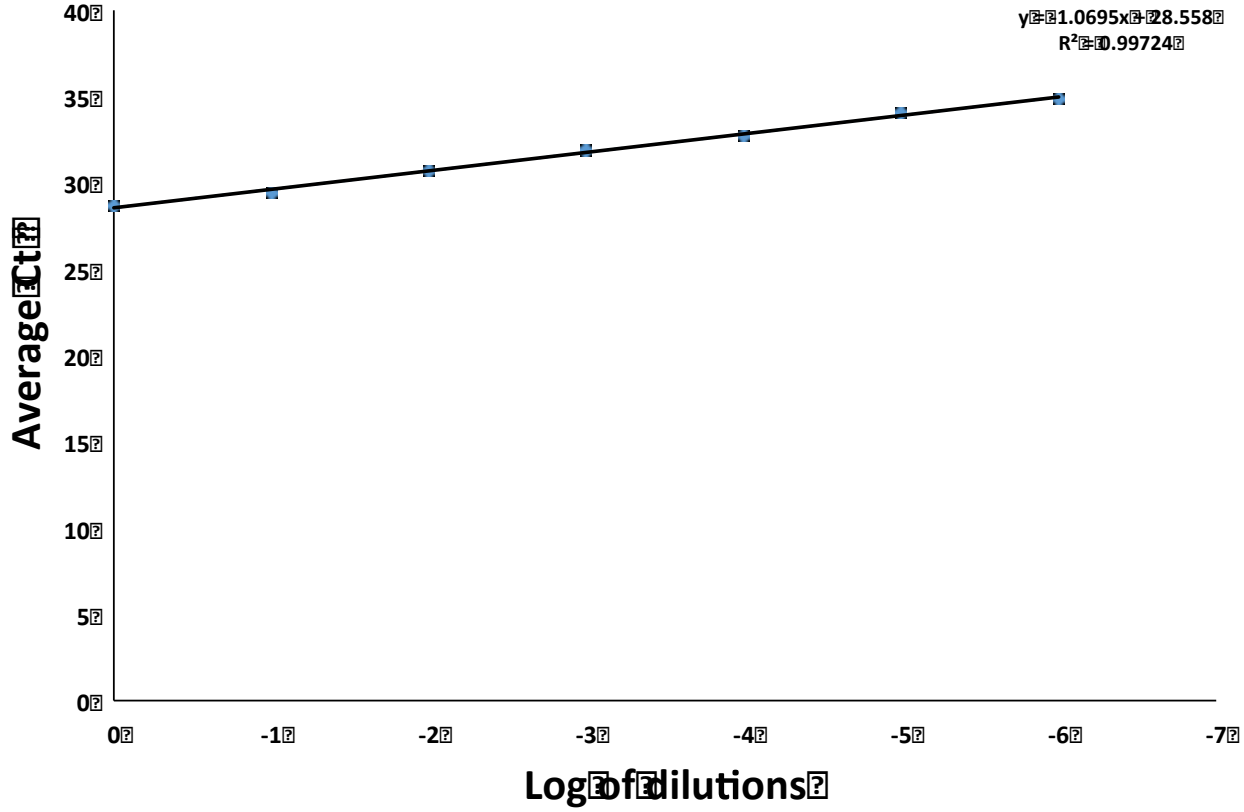


Figure 3.10 SmLeish primer efficiency

Comparison of the average Ct values of three replicate reactions graphed against the log(2) of the dilution of the reaction cDNA. The slope of the equation obtained from the trend line used in the efficiency equation allowed calculation of efficiency value for SmLeish primers. The following equation was used for the primer efficiency calculation: Efficiency = ((dilution factor)^(1/-slope))-1.

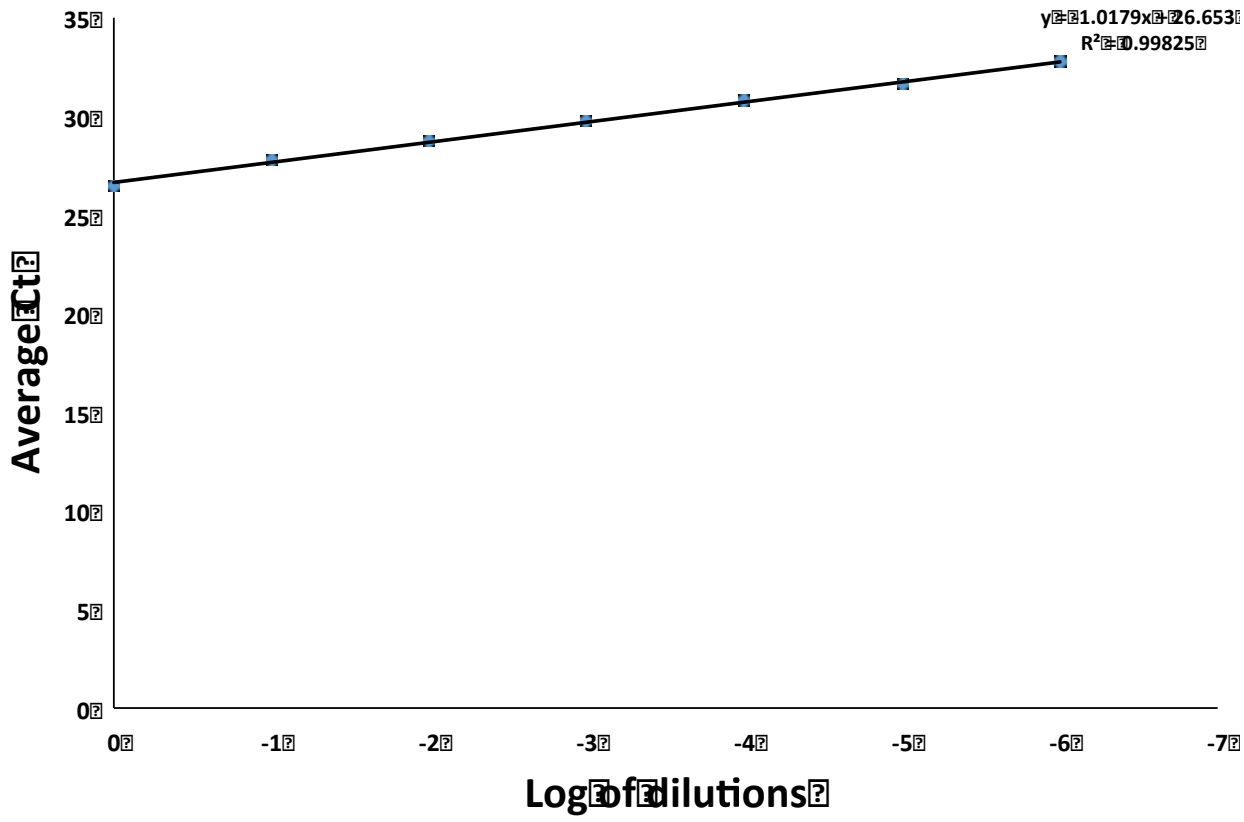


Figure 3.11 SmVAL3 primer efficiency

Comparison of the average Ct values of three replicate reactions graphed against the log(2) of the dilution of the reaction cDNA. The slope of the equation obtained from the trend line used in the efficiency equation allowed calculation of efficiency value for SmVAL3 primers. The following equation was used for the primer efficiency calculation: Efficiency = ((dilution factor)^(1/-slope))-1.

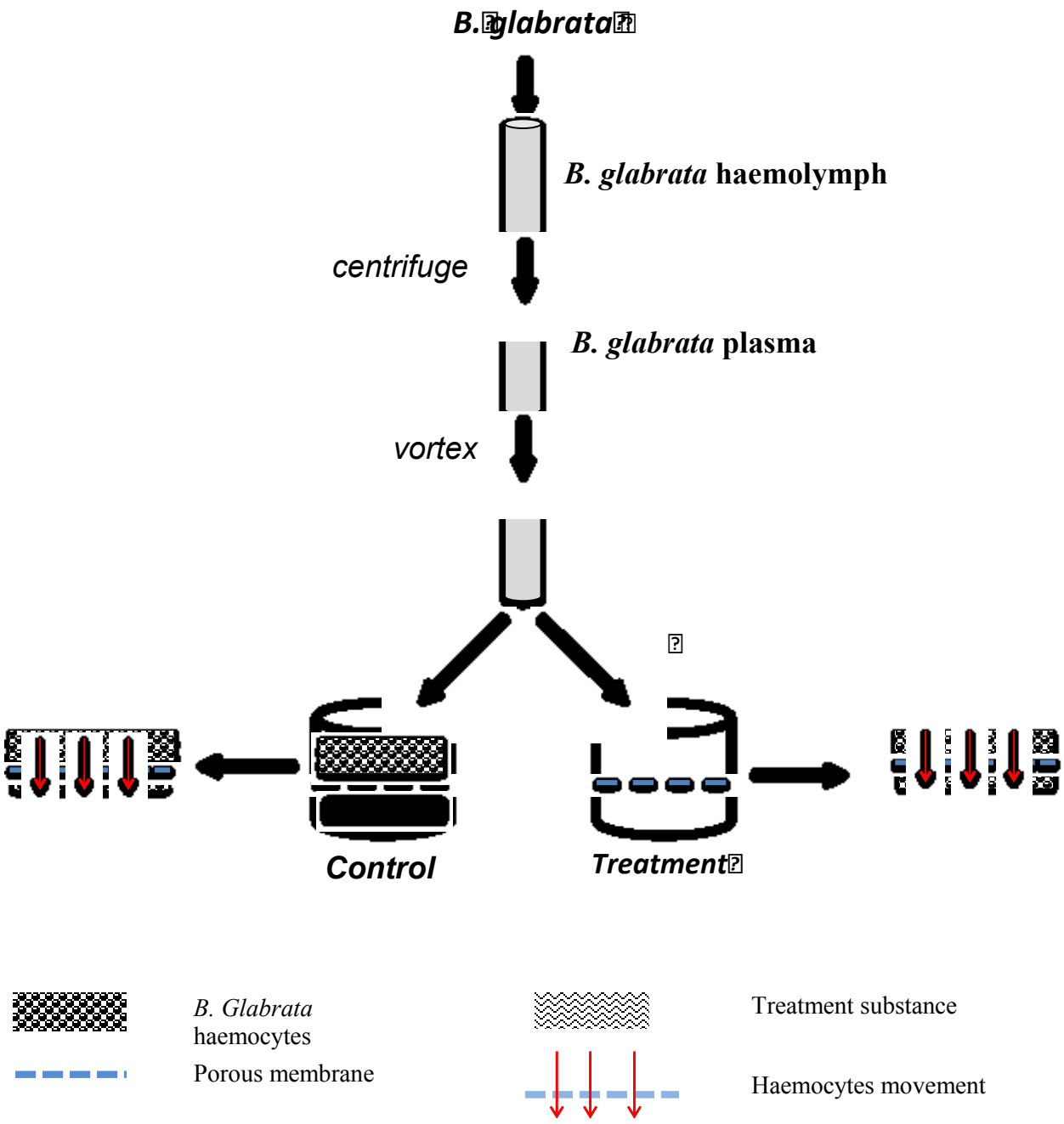


Figure 3.12 Experimental layout for chemotaxis of M-line and BS-90 snail haemocytes

For each treatment a corresponding control was obtained from the same snail haemocytes. Using either a susceptible (M-line) or resistant (BS-90) snails, the entire snail haemolymph was drained using a syringe. The haemolymph was then centrifuged down to collect the plasma. *B. glabrata* haemocytes were collected and suspended in a Bge medium, vortexed and divided into two equal quantities with one being used as control while treatment is applied to the other. After incubation duration, the membrane separating the upper and lower compartment are retrieved, haemotoxilyn and eosin (H & E) stained and the haemocytes cells on the membrane side facing the bottom chamber are counted to establish cell movement pattern. A comparison between control and treatment numbers of migrated haemocytes allows an observation of the impact of the treatment on haemocytes movement.

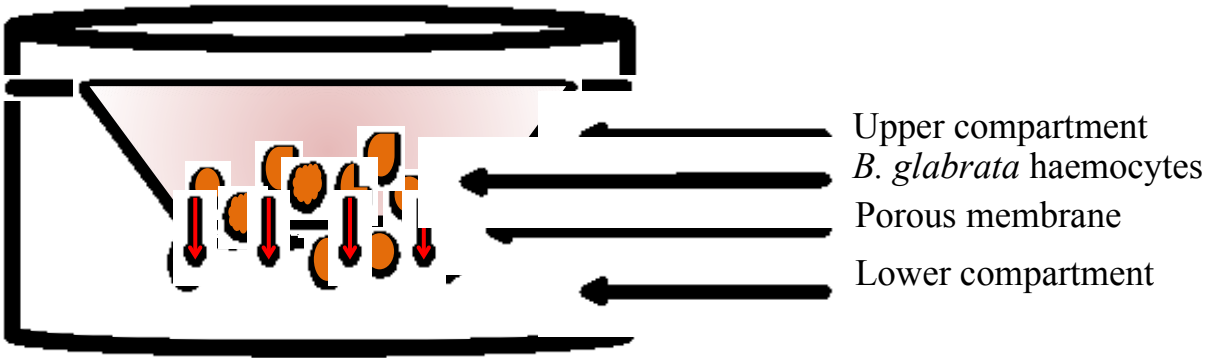


Figure 3.13 Chemotaxis apparatus set up

The chemotaxis apparatus is composed of an upper and lower compartment divided by a porous membrane (5 µM pores) that allows cell migration. *B. glabrata* haemocytes were always located in the upper compartment at the beginning of each experiment. The treatment was added to the upper, lower or both of the compartments.

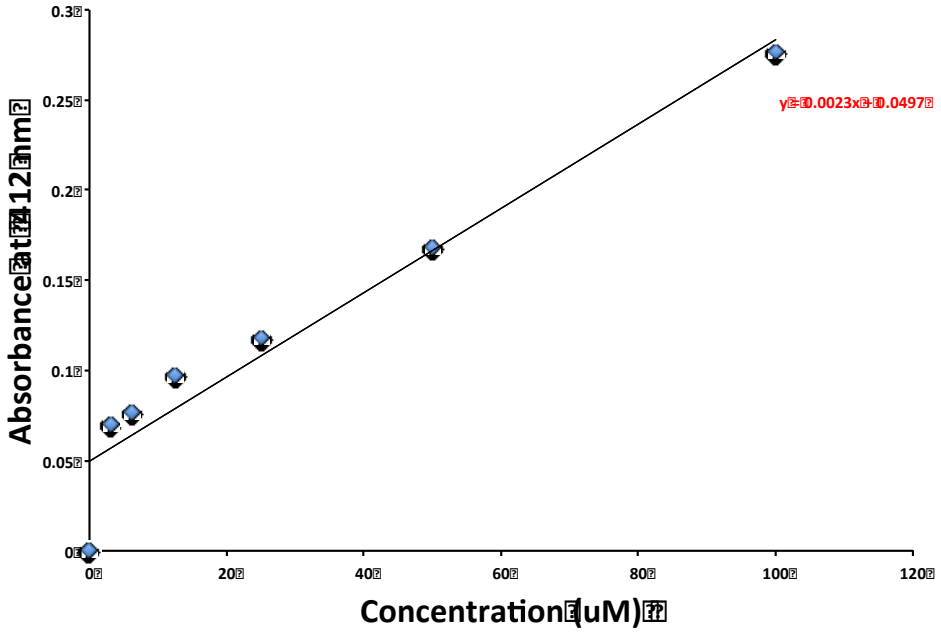


Figure 3.14 Standard curve of concentration dilutions vs. their respective absorbance

The absorbance of the dilution series of a standard enzyme from Anaspec was measured at 412 nm. The obtained values were used to create a standard curve. The standard curve was used to extrapolate the enzymatic activity of metalloprotease enzymes (rSmLeish and human MMP8).

4. Chapter 4: *Schistosoma mansoni* Leishmanolysin

4.1 *Schistosoma mansoni* leishmanolysin - Introduction

Leishmanolysin was an appealing target to pursue in functional analyses related to *S. mansoni* infection of the snail host for two primary reasons: (1) as a MMP SmLeish shares many similarities with enzymes known to influence immune responses in other organisms, and (2) SmLeish is very similar in primary animo acid structure to the leishmanolysin of *Leishmania* which has clear functions in the suppression of macrophage function (Olivier *et al.*, 2012). Using past studies in other host-parasite model systems as a guide, I addressed the primary objective of this part of my thesis to investigate whether SmLeish was playing an immunosuppressive or immunomodulatory role within *B. glabrata,* thereby facilitating *S. mansoni* infection success. Consequently, various reagents were developed and experiments were conducted in order to answer this question. The first set of experiments was executed to determine if the rSmLeish protein that I generated had MMP activity. This was accomplished by modifying an existing MMP activity assay designed for assessing mammalian MMPs, such that it served to demonstrate that SmLeish was able to cleave a known MMP specific substrate. The hypothesis was that rSmLeish will degrade the MMP specific substrate and that the MMP specific inhibitor will impede the rSmLeish enzymatic activity of substrate degradation. The second group of experiments was designed to measure the abundance of SmLeish transcript and protein during *S. mansoni* infection of *B. glabrata* using qRT-PCR and Western blots. The hypothesis was that SmLeish transcript and protein levels would be detected at an increased level during the establishment stage of the parasite life cycle. I also aimed to detect SmLeish within the tissues of *S. mansoni*-infected *B. glabrata* with the goal of visualizing the location of SmLeish during the intramolluscan infection. The last collection of experiments were conducted based on the

understanding that the snail haemocytes were agents that interacted with the parasite and that within susceptible snails, their activity was negatively affected by the presence of parasites or their E/S products (Connors & Yoshino, 1990; Lodes & Yoshino, 1990). Therefore, I performed a chemotaxis experiment by exposing the generated rSmLeish to *B. glabrata* haemocytes (from resistant and susceptible snails) to determine whether SmLeish influenced the ability of haemocytes to migrate, and to further understand whether haemocytes from susceptible and resistance snail strains were affected differently by SmLeish. Finally, the impact of siRNA-mediated knockdown of SmLeish was assessed in an experiment where SmLeish was knocked down in *S. mansoni* miracidia. They were then used to infect susceptible *B. glabrata* snails. If SmLeish was important for infection establishment and maintenance in this infection model, I expected that abrogating its expression would result in less snails being successfully infected by SmLeish knockdown parasites compared to controls.

4.2 *Schistosoma mansoni* leishmanolysin - Results

4.2.1 SmLeish metalloprotease activity

In order to test if SmLeish has metalloprotease activity, purified rSmLeish was incubated with synthesized, mammalian metalloprotease substrate. Various incubation times and various initial rSmLeish concentrations were used in experiments in order to assess how incubation time and/or concentration impacted rSmLeish enzymatic activity. Because previous studies have reported most metalloproteases to be expressed as a pro-peptide that are subsequently activated by cleavage, often by trypsin, we included a condition under which rSmLeish was incubated with trypsin to determine if rSmLeish required activation. We were unsure whether the rSmLeish reflected the natural pro-SmLeish protein, thus I included both a trypsin treated and not treated rSmLeish as part of the experimental design. Human MMP8 was used as a positive control; this

was the closest matching human MMP in the Genbank database when SmLeish was compared using BLASTp. It was also essential to recognize that because of the absence of known activators and inhibitors specific to SmLeish to date, trypsin and the MMP specific inhibitor from our testing kit, that are specific to mammals were used to test rSmLeish. Thus, species-specific differences in enzyme activity could limit the functional response observed by rSmLeish in this test.

The MMP specific chromogenic substrate is a thiopeptolide which when cleaved by an MMP releases a sulfhydryl group that then reacts with Ellman's reagent to give 2-nitro-5-thiobenzoic acid (TNB) as a final product. The pigment of the TNB is detected using a microplate reader at 412 nm. Using the standard curve and the values obtained from the microplate read, the MMP enzyme activity was obtained.

Activated human MMP8 successfully cleaved the substrate in a time and concentration dependent fashion (enzymatic activity of 63 after 120 minutes incubation compared to only 9 after 30 minutes incubation), and cleavage was dependent on activation by trypsin (Figure 4.1). Inhibition of human MMP8 activity by the MMP specific inhibitor provided with the kit from Anaspec, significantly reduced substrate cleavage (6 was the highest enzymatic activity value registered after 120 minutes incubation) (Figure 4.1). Hence, the trypsin was efficient at activating the human MMP8 specific and the MMP inhibitor efficient at drastically decreasing the human MMP08 proteolytic activity.

After incubating both forms of the rSmLeish protein with the MMP specific substrate for 30, 60, 90, and 120 minutes, the trypsinized rSmLeish enzymatic activity was 18, 13, 21, and 28 at 30, 60, 90, and 120 minutes respectively (Figure 4.2), while the non-trypsinized form

registered 0, 11, 14, and 18 at 30, 60, 90, and 120 minutes respectively (Figure 4.3). The inhibited version of the trypsinized form of rSmLeish protein activity was 12, 5, 10 and 12 (Figure 4.2) while the inhibited non-trypsinized activity was 0, 1, 2, and 0 at 30, 60, 90 and 120 minutes correspondingly (Figure 4.3). The difference in enzymatic activity between inhibited and non-inhibited for the non-trypsinized form of rSmLeish was statistically significant from 1 to 2 hours of incubation time ($p = 0.018$ for 1 hr, $p=0.031$ for 1.5 hrs, and $p=0.001$ for 2 hrs). The difference in enzymatic activity between inhibited and non-inhibited for the trypsinized form of rSmLeish was also statistically significant from 1 to 2 hours of incubation time ($p = 0.020$ for 1 hr, $p = 0.005$ for 1.5 hrs, and $p = 0.007$ for 2 hrs).

Observing the concentration dependent incubation data, the trypsinized rSmLeish activity was 57 with an initial concentration of 0.5 μg/mL and 59 with an initial concentration 1 μg/mL (Figure 4.4). However, the inhibition of the trypsinized form of SmLeish led to the following values for its enzymatic activity: 0 with an initial concentration 0.5 μg/mL and 34 with an initial concentration 1 μg/mL (Figure 4.4). The non-trypsinized rSmLeish recorded 61 at the initial concentration of 0.5μg/mL and 97 at the initial concentration of 1μg/mL, whereas it's inhibited form enzymatic activity value was 27 and 17 for the initial concentration of 0.5 μg/mL and 1 μg/mL respectively (Figure 4.5). Due to lack of repeated measurement of this experiment, statistical significance of the enzymatic activities of either trypsinized or non-trypsinized rSmLeish with the initial concentration 0.5 μg/mL and 1 μg/mL could not be verified.

4.2.2 Detection and quantification of SmLeish

The next line of inquiry was to assess the transcript and protein expression levels of SmLeish at various periods during the intramolluscan development of *S. mansoni*. Assessing transcript expression allowed the measurement of SmLeish in many snails over the entire course

of larval *S. mansoni* development, providing a comprehensive picture of SmLeish expression dynamics. Protein abundance was measured by Western blot, and due to the confines of this technique and the fact that SmLeish was produced as both a parasite-associated and soluble protein, *S. mansoni*-infected snail plasma and homogenate were used to confirm that the transcript expression pattern were paralleled by the SmLeish protein.

In order, to be able to detect the presence of SmLeish and measure its quantity at the transcript level, primers and probes were designed and qRT-PCR carried out using infected whole-snail tissue sampled at various intervals during the infection process to obtain the RNA template from which cDNA was synthesized. For all transcript expression studies, five different snails at each time point were compared to five time-matched control snails, and SmLeish expression was normalized to the endogenous control SmGAPDH, a housekeeping gene, that was used to both demonstrate the presence of *S. mansoni* in the sampled snails and an internal template loading control. Comprehensive validation of SmGAPDH was performed previously (Taft & Yoshino, 2011) but was confirmed in our studies as well (Figure 3.6).

In the quest to obtain the optimal primers and probes for SmLeish detection during qRT-PCR, the oligonucleotide primer pair was tested on cDNA generated from infected snail RNA. Based on the ability of the pair to specifically amplify the targeted SmLeish sequence, and by analyzing the melt curves and reaction efficiency, I was able to assess the primers/probe set to be used in subsequent qRT-PCR analysis of SmLeish expression as optimal.

The SmGAPDH qRT-PCR results (Figure 4.6) demonstrates the detection of SmGAPDH in miracidium (34.8 average Ct) and in successfully infected susceptible (M-line) snails (34.6 average Ct at 1 hour post exposure to 15.4 average Ct at day 35 post exposure). Snails not

challenged by *S. mansoni* present no Ct values for SmGAPDH from 1 hour to 35 days. The same experiment was done using resistant snails (BS-90 strain). By day four post exposure of the snails to *S. mansoni* miracidia, the resistant snails had no detectable SmGAPDH expression, suggesting that these snails had cleared the infection (Figure 4.7).

After establishing the ability to detect the endogenous control, SmGAPDH in infected *B. glabrata* snails only, the transcript levels of SmLeish during *S. mansoni* intramolluscan development at different time points were assessed (Figure 4.8). Frist level analysis was achieved using the delta delta Ct method, in which the average Ct values for SmLeish were normalized to the average Ct values of SmGAPDH in order to eliminate any variation in the amount of cDNA used but even more to obtain an accurate value in gene expression change that is not associated with the increase of parasite material within the snail as the intramolluscan stage progresses. Then, SmLeish expression was further normalized to the expression values of free-swimming miracidia to provide a fold change in expression value (Rq).

At 1 hour post challenge, an 8.24 fold change in transcript expression was observed, followed by a 16.33 fold change detected at 3 hours. The maximum fold change relative to *S. mansoni* miracidia was registered at half a day (33.23 fold change). The fold change in SmLeish transcript abundance was then observed to decrease to 10.23 fold change at day 3 and 0.35 fold change at day 35. By day 35 post challenge, SmLeish expression profile drops to its lowest values but stays above miracidial levels of SmLeish.

Since transcript expression is not always synonymous with protein production, establishing an expression profile for the SmLeish protein was required. I was also uncertain as to whether SmLeish was functional as a membrane-bound pro-protein, or whether it could also

be functional in a secreted fashion as has been demonstrated for some MMPs. To assess soluble, secreted SmLeish, plasma from infected snails sampled at various times during *S. mansoni* development in *B. glabrata* was run on denaturing and reducing SDS-PAGE and analyzed by Western blot to characterize the presence of secreted SmLeish protein (Figure 4.9). A 48 kDa secreted SmLeish was detectable from 12 hours to 8 days post challenge, with the greatest abundance of SmLeish protein observed at 12 hours and 2 days post challenge, suggesting a higher quantity of SmLeish protein being secreted/excreted at these specific periods of the infection. SmLeish has a number of possible trypsin cleavage sites (Figure 3.1), suggesting that there could be various possible sizes of a cleaved or secreted version. The 48 kDa SmLeish detected from the infected snail plasma is the expected size of the secreted native protein that would be produced by cleavage of SmLeish pro-peptide at the trypsin cleavage site that is computationally assessed as being the most likely trypsin cleavage site (Expasy Prosite).

Whole *S. mansoni*-infected snail homogenates from various time points of the parasite intramolluscan development were also run on denaturing and reducing SDS-PAGE and analyzed by Western blot to characterize the presence of pro-SmLeish protein (Figure 4.10). The expected pro-peptide form of SmLeish expected around 125 kDa was detected at a comparable abundance throughout the various targeted time points (3,6,12 hours and 1,2,4,8, and 16 days). The soluble form of SmLeish was also detected around 48 kDa as expected at all time points analyzed except 3 and 6 hours; these latter observations are in line with the *S. mansoni*-infected snail plasma SDS-PAGE and Western blot analysis in Figure 4.9.

To determine whether SmLeish was directly associated with the invading larval *S. mansoni,* I used specific antibody against SmLeish to localize the protein in *B. glabrata* housing a sixteen day-old infection of *S. mansoni.* The anti-SmLeish primary antibody was designed to

recognize both the predicted soluble SmLeish as well as the putative pro-SmLeish protein. Infected snail tissue taken at sixteen days post challenge was fixed and then rehydrated and observed using fluorescence microscopy (Figure 4.11). We were able to observe the presence of SmLeish protein where schistosome sporocysts were located in the snail tissue and nowhere else. At sixteen days post challenge, *S. mansoni* mother sporocysts are expected to be fully developed, generating daughter sporocysts and to be located in the snail head foot. SmLeish was detected around the mother sporocysts; which is concordant with its hypothesized membrane-bound location for the protein. DAPI staining allowed the detection of cell nuclei, while the anti-KLH, which detects keyhole limpet hemocyanin, present in snail plasma, served as a positive control for our fluorescence microscopy assay and demonstrated that the developed assay and process was working properly.

4.2.3 Functional characterization of SmLeish

After establishing the metalloprotease activity of SmLeish and its upregulated expression, both in terms of transcript and protein, the next logical step led to investigating the actual impact that SmLeish would have on the snail defense system. Since, haemocytes are the major effectors in snail defense, studying the interaction between SmLeish and *B. glabrata* haemocytes was the understandable next step.

4.2.3.1 Chemotaxis/chemokinesis

Previous studies have shown that *S. mansoni* E/S products have a visible effect on the morphology and functional capabilities of *B. glabrata* haemocytes (Coustau & Yoshino, 1994; Lodes & Yoshino, 1990). The most obvious of these effects is a noticeable change in the ability of haemocytes to adhere to a substrate and move towards a sporocyst *in vitro.* These effects, importantly, are not noticed when the same *S. mansoni* or trematode E/S products are incubated

with the haemocytes of a snail species or strains that naturally resist infection (Nacif-Pimenta *et al.*, 2012). These observations led to the conclusion that SmLeish may be one of the factors in E/S products that inhibits haemocyte function, as it is produced in a soluble form. To test this, I followed protocols that had been used to test the impact of *S. mansoni* E/S products on haemocyte migration (chemokinesis) (Lodes & Yoshino, 1990). In order to establish whether SmLeish is capable of influencing the biological function of *B. glabrata* haemocytes, purified non-trypsinized rSmLeish was used in a chemokinesis experiment in presence of *B. glabrata* haemocytes isolated from *S. mansoni*-resistant (BS-90) or susceptible snails (M-line). *S. mansoni* secretory/excretory products (*S. mansoni* ES+LYS), and N-formyl-methionine-leucine-phenylalanine (fLMP), a broadly used chemotactic peptide were used, the former to test the influence of the entire excreted/secreted proteome of *S. mansoni* and the later as a positive control. Three different experimental groups were set up to test the impact of rSmLeish, *S. mansoni* ES+LYS and fLMP on haemocyte movement across a porous (5µM pore size) polycarbonate membrane. In all cases haemocytes are added initially into the top compartment, separated from the bottom compartment by the membrane. The experimental substance was either in the bottom compartment, mix in with *B. glabrata* haemocytes cells in the top compartment, or placed in both the top and bottom compartments at the same time.

Haemocyte migration through the membrane onto the bottom surface of the membrane was assessed microscopically and compared to snail-matched controls to generate a chemokinetic index in which any value above one means that there was more migration of *B. glabrata* haemocytes in the treatment group compared to the control group.

When the fLMP was in the bottom compartment, there was a larger amount of *B. glabrata* haemocytes travelling to the bottom compartment from the upper compartment for both

resistant and susceptible snails alike with chemotactic index of 2.54 for BS-90 snails, and 2.26 for M-line snails (Figure 4.12). The comparison of the observed chemokinetic index between fLMP being in the bottom compartment and when it is located in both upper and bottom compartments (control set up) for resistant and susceptible snail haemocytes showed statistical significance ($p = 0.0004$, $p = 0.0020$, respectively). The haemocytes from both resistant and susceptible snails are significantly attracted to fLMP. In the second case where fLMP was in the top chamber mix with *B. glabrata* haemocytes, migration within the treatment group was slightly less than the control group for both resistant and susceptible snails with a chemokinetic index of 0.86 for M-line snails and 0.68 for the BS-90 snails (Figure 4.12). The comparison of the chemokinetic index between fLMP being in the top compartment to when it is located in both upper and bottom compartments for resistant snail haemocytes showed statistical significance ($p = 0.004$) while there was no statistical significance for the susceptible snail chemokinetic index ($p = 0.077$). The haemocytes from resistant and susceptible snail are significantly attracted to fLMP. When fLMP was in both chambers there was a minor increase in migration of haemocytes in the treatment compared to the control group for resistant and susceptible snails similarly with the chemokinetic index of 1.18 for M-line snails, and 1.32 for BS-90 snails (Figure 4.12). The attraction of resistant and susceptible snails' haemocytes towards fLMP had no significant difference ($p = 0.366$, $p = 0.369$, and $p = 0.303$ for the respective set up of fLMP in the lower, upper or both compartments when comparing resistant versus susceptible snail motility). Hence the impact of the positive control was similar on both snail strains.

Placing *S. mansoni* ES+LYS in the bottom compartment led to the migration of more haemocytes towards the bottom compartment in treatment compared to control for the resistant snails (chemokinetic index of 1.52), while there was a lower migration of the treated susceptible

haemocytes compared to their controls (chemokinetic index of 0.61) (Figure 4.12). The statistical comparison of chemokinetic index between the BS-90 haemocytes movement when the *S. mansoni* ES+LYS is in the bottom compartment to the case where *S. mansoni* ES+LYS were in both the bottom and upper compartments showed significance ($p = 0.0001$) while the susceptible snail haemocytes movement were not statistically significant ($p = 1.167$). Additionally, comparing resistant and susceptible snail haemocytes motility showed significant difference ($p < 0.00001$) in the impact that *S. mansoni* ES+LYS in the bottom compartment has on both snail strains. Indeed, BS-90 haemocytes were attracted towards the *S. mansoni* ES+LYS while M-line haemocytes were not attracted. In the experiment where the *S. mansoni* ES+LYS were in the top chamber mix with *B. glabrata* haemocytes, both treated resistant and susceptible snails' haemocytes registered less migration than their respective controls (chemokinetic index of 0.46 for M-line, and 0.31 for BS-90) (Figure 4.12). The results were statistically significant when compared to the case of *S. mansoni* ES+LYS in both compartments ($p = 0.0092$ for M-lines and $p = 0.0002$ for BS-90). When assessing the impact of *S. mansoni* ES+LYS impact on resistant and susceptible snails' haemocytes chemokinetic indexes, there was statistical significance on the impact between how the strains are affected when in presence of *S. mansoni* ES+LYS in any of the set ups ($p < 0.00001$, and $p = 0.030$ for *S. mansoni* ES+LYS in the bottom and upper compartment respectively). In the case of *S. mansoni* ES+LYS in both compartments, even though a higher migration rate of treated haemocytes was registered from both resistant and susceptible snails compared to the previous set ups, the treatments still had less motility than the controls (chemokinetic index of 0.73 for M-line snails, and 0.83 for BS-90 snails) (Figure 4.12).

For the experimental set up with non-trypsinized rSmLeish all three treatments registered less mobility than the controls for both the resistant and susceptible snail's haemocytes although,

the treated resistant snail haemocytes had a slightly higher mobility (chemokinetic index of 0.83, 0.83, and 0.90 respectively) than the susceptible snail haemocytes (chemokinetic index of 0.66, 0.31, and 0.51 respectively) (Figure 4.12). The statistical analysis comparing the chemokinetic index of both resistant and susceptible snails' haemocytes from the non-trypsinized rSmLeish being in the bottom compartment or in the upper compartment to the case where non-trypsinized rSmLeish was in both compartments, showed significance in the observation of the susceptible snails' haemocytes response ($p = 0.0455$, $p= 0.0007$ for rSmLeish in the bottom and upper compartment respectively) but not in the resistant snails' response ($p = 0.3582$, $p = 0.3687$ in the bottom and upper compartment respectively). Furthermore, comparing the impact of non-trypsinized rSmLeish between the resistant and susceptible snails showed that there is significant difference ($p = 0.0247$, $p < 0.00001$, and $p = 0.0009$ for the cases where rSmLeish is at the bottom, upper and both compartments)

4.2.3.2 rSmLeish Knockdown

Knockdown of SmLeish expression in *S. mansoni* miracidia before exposing them to susceptible snails was carried out as another means to test the role that this protein plays during *S. mansoni* infection establishment and development within *B. glabrata.* Knockdown of SmLeish was confirmed by qRT-PCR of day 1 to day 5 *in vitro* cultivated sporocysts, following a 24 hour *in vitro* incubation of *S. mansoni* miracidia in a solution of the knockdown oligonucleotides. Knockdown specificity was compared to control parasites that were incubated with GFP-specific siRNA oligonucleotides. The siRNA oligonucleotides used for both SmLeish and control GFP knockdown studies were 27 nucleotides long, which can be engaged by the dicer complex. Thus, the GPF control oligonucleotides served to ensure that the induction of the RNAi machinery within the parasite did not lead to non-specific knockdown effects on infection outcome.

The injection of GFP siRNA oligonucleotides did not decrease the overall expression of SmLeish over the course of sporocyst *in vitro* development (Figure 4.13). Hence, the value in fold change of the SmLeish transcript increased by 11.64 fold by day 5 post GFP-siRNA injection. As demonstrated in previously mentioned results, SmLeish transcript expression is expected to increase during the first hours and days of the intramolluscan development of *S. mansoni* (Figure 4.8), the discrepancy between the levels of transcripts at specific time points in these studies and the GFP knockdown control experiments is expected to be the result of the *in vitro* nature of the GFP studies, which do not mimic the intramolluscan environment evaluated in Figure 4.8.

Knockdown of SmLeish was successful and led to a measurable reduction in SmLeish expression throughout the *in vitro* development of the *S. mansoni* sprorcysts (Figure 4.13). The fold change in the SmLeish transcript expression went from 1.84 fold change at day 1 post knockdown to 0.31 fold change at day 5 following knockdown. Thus, the leishmanolysin transcript expression went from an almost 200% increase compared to miracidia levels on day 1 (1.84) to being reduced at about 30% compared to miracicial levels by day 5. Comparing between the GFP-knockdown and the SmLeish knockdown levels of SmLeish expression, the maximum difference in SmLeish expression between the two treatment groups is at day 4 and is 10.75-fold expression difference. Day 3, 4 and 5 SmLeish transcript expressions were statistically different between the controls and the SmLeish knockdown groups ($p = 0.0039$, $p = 0.0002$, and $p = 0.0003$ for day 3, 4 and 4 respectively).

A representation of the percentage of shedding snails between the control and treatment groups from the expected beginning of snail's shedding period (four weeks post exposure) up to ten weeks after is represented in Figure 4.14. To assess the biological relevance of SmLeish

during *S. mansoni* infection of *B. glabrata* we exposed M-line snails to SmLeish or GFP-knockdown miracidia and then allowed the infection to progress to patency. Shedding of cercariae is the ultimate metric of downstream infection potential of a schistosome, as cercariae go on to initiate new human infection, thus, we believe that this measurement is the most essential and informative. Two assessments were made in this study, the first was the time to shedding of cercariae for snails in each treatment group, the second was the number of snails, out of the 50 in each group that reached patency and shed cercariae. The snails exposed to *S. mansoni* miracidia with knockdown SmLeish had a delayed shedding (week 5) compared to the control group, which started shedding at week 4. Furthermore, the percentage of shedding snail was always less in the SmLeish treatment group compared to the GFP control group. Week six, 7, 8 and 10 data of the SmLeish knockdown are significantly different to the comparable data from the controls ($p = 0.0317$, $p < 0.0001$, $p = 0.0015$, and $p = 0.0337$ for week 6, 7, 8, and 10 respectively).

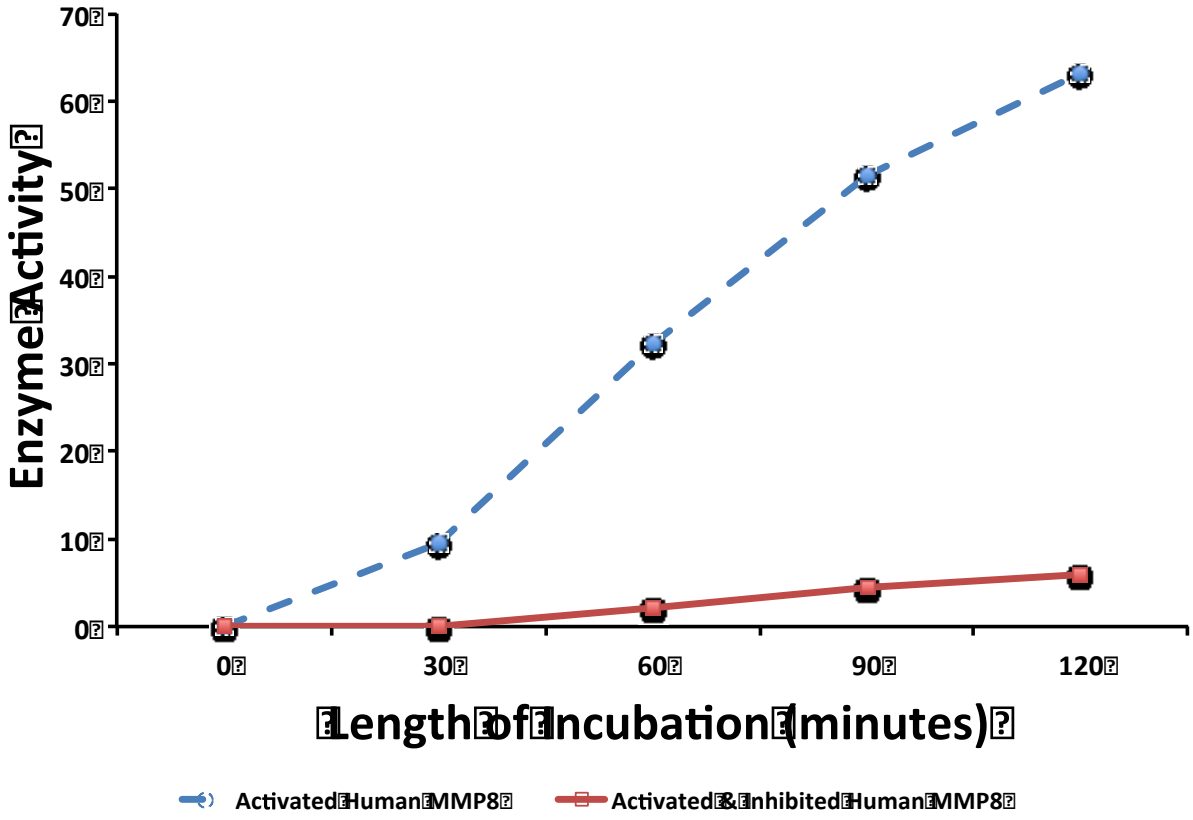


Figure 4.1 Human MMP8 vs. inhibited human MMP8 enzymatic activity - time dependent

A representation of the enzymatic activity of human MMP8 in the presence of MMP specific substrate in relation to incubation duration. The enzymatic activity was measured using the absorbance values of TNB, the final product of the substrate break down by human MMP8 using a standard curve. Both initial concentrations were 0.25µg/mL. The human MMP8 was activated using trypsin and the substrate degradation process measured every 30 minutes for 2 hours. The activated human MMP8 enzyme was inhibited using MMP specific inhibitor and the substrate degradation process measured every 30 minutes for 2 hours. The activation using trypsin was efficient on activating the human MMP8 while the inhibition using an MMP specific inhibitor reduced the human MMP8 enzymatic activity.

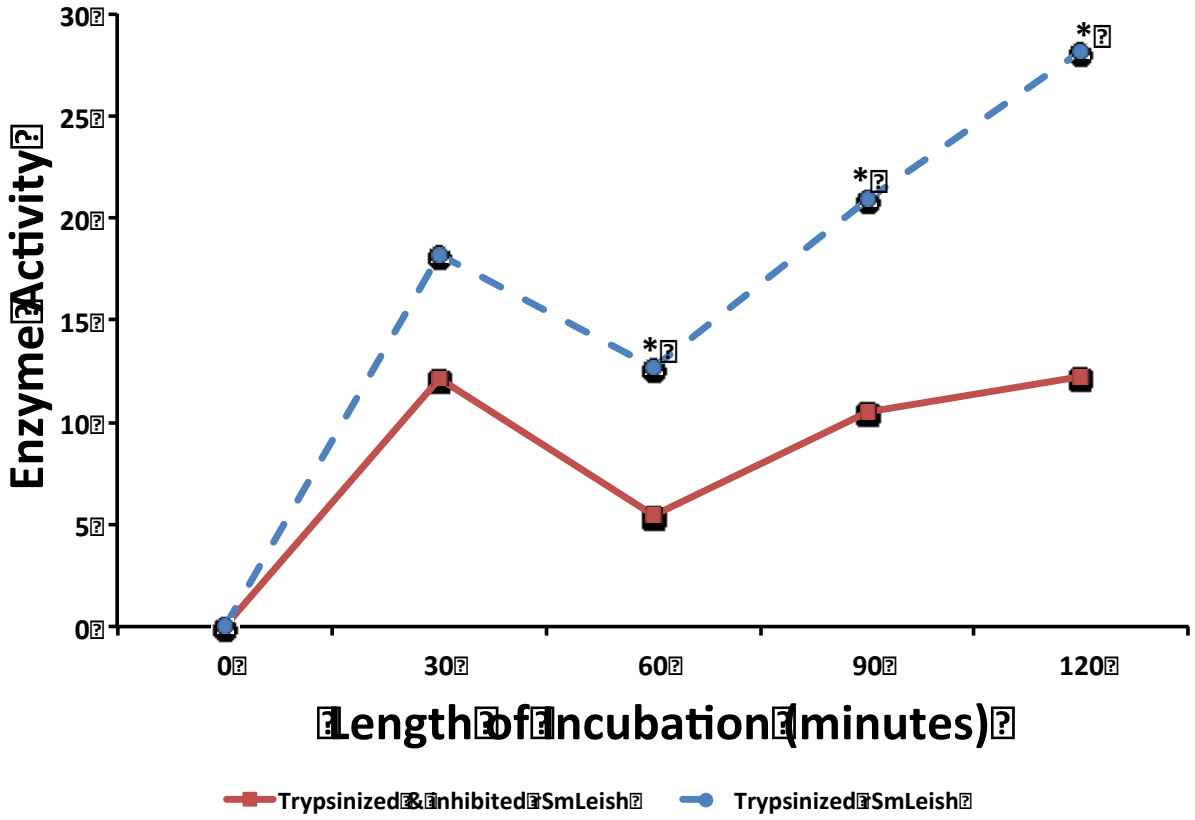


Figure 4.2 Trypsinized vs. trypsinized & inhibited rSmLeish enzymatic activity - time dependent

A representation of the enzymatic activity of trypsinized rSmLeish in the presence of MMP specific substrate in relation to incubation duration. The enzymatic activity was measured using the absorbance values of TNB, final product of the substrate break down by rSmLeish using a standard curve. An initial concentration of 0.25 µL of rSmLeish was used for each reaction. rSmLeish was activated using trypsin and the substrate degradation process measured every 30 minutes for 2 hours. The trypsinized rSmLeish was inhibited using an MMP specific inhibitor and the substrate degradation process measured every 30 minutes for 2 hours. The activation using trypsin and inhibition were partially effective on rSmLeish protein. The annotated points with asterisk represent a statistically significant difference between enzymatic activity of inhibited and non-inhibited trypsinized rSmLeish ($p < 0.05$).

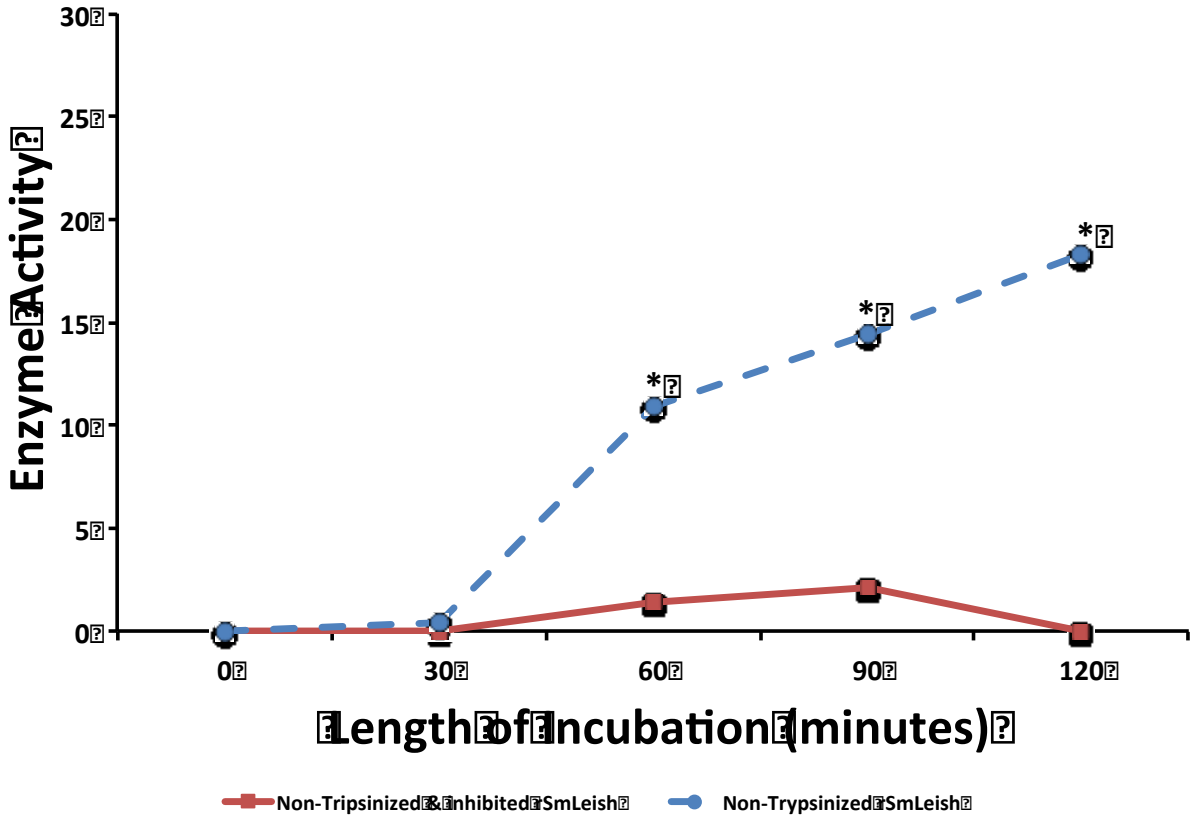


Figure 4.3 Non-trypsinized vs. non-trypsinized & inhibited rSmLeish enzyme activity – time dependent

A representation of the enzymatic activity of non-trypsinized rSmLeish in the presence of MMP specific substrate in relation to incubation duration. The enzymatic activity was measured using the absorbance values of TNB from the substrate break down by rSmLeish using a standard curve. An initial concentration of 0.25ul of rSmLeish was used for each reaction. Non-trypsinized rSmLeish substrate degradation process was measured every 30 minutes for 2 hours. The non-trypsinized rSmLeish was inhibited using an MMP specific inhibitor and the substrate degradation process measured every 30 minutes for 2 hours. The non-trypsinized rSmLeish was able to break down the MMP specific substrate and its inhibition was significantly effective at reducing proteolytic activity. The annotated points with asterisk represent a statistically significant difference between enzymatic activity of inhibited and non-inhibited non-trypsinized rSmLeish.

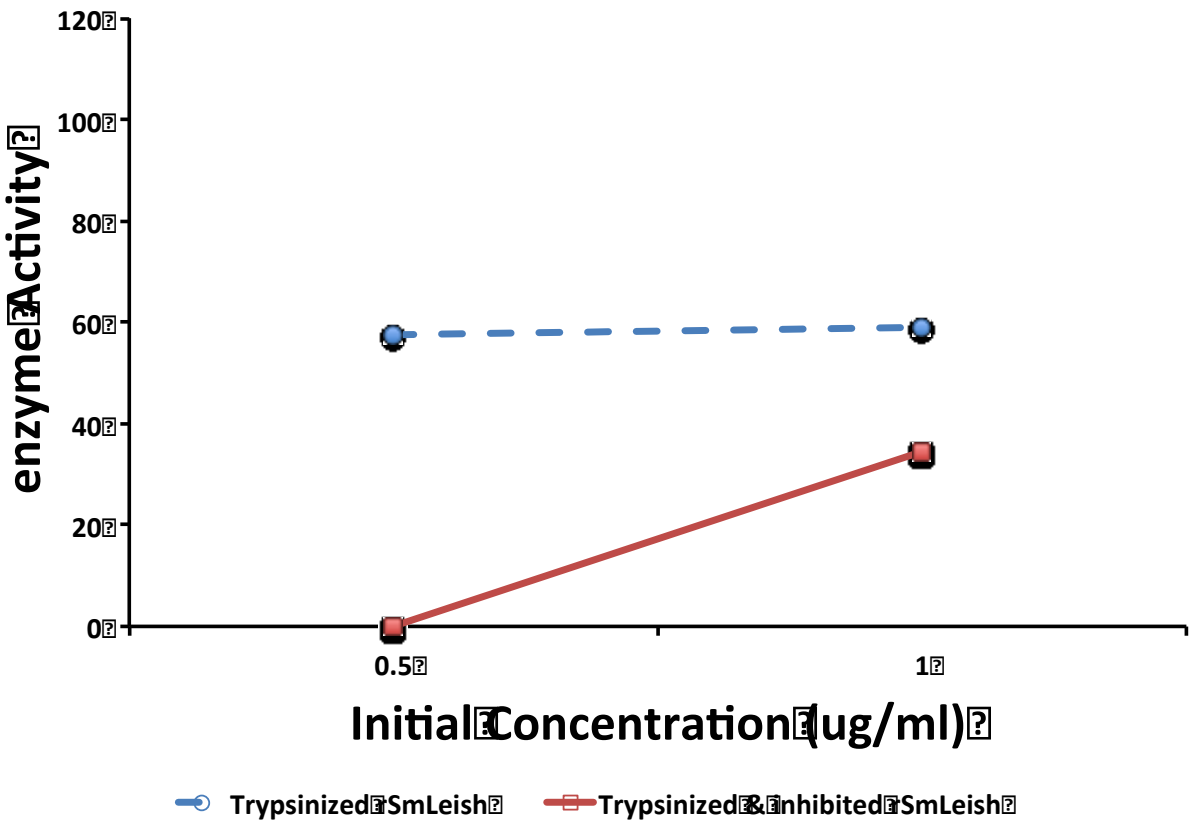


Figure 4.4 Trypsinized vs. trypsinized & inhibited rSmLeish enzymatic activity - concentration dependent

The x-axis represents the initial trypsinized rSmLeish concentrations and the y-axis represents the trypsinized rSmLeish proteolytic activity. The trypsinized rSmLeish activity was measured using the absorbance values from TNB, final product of the substrate break down by rSmLeish. The trypsinized rSmLeish substrate degradation process was measured after 2 hours incubation from two different initial concentrations of 0.5 and 1 µg/mL of rSmLeish. Increased concentration had a positive impact on trypsinized rSmLeish although minimal.

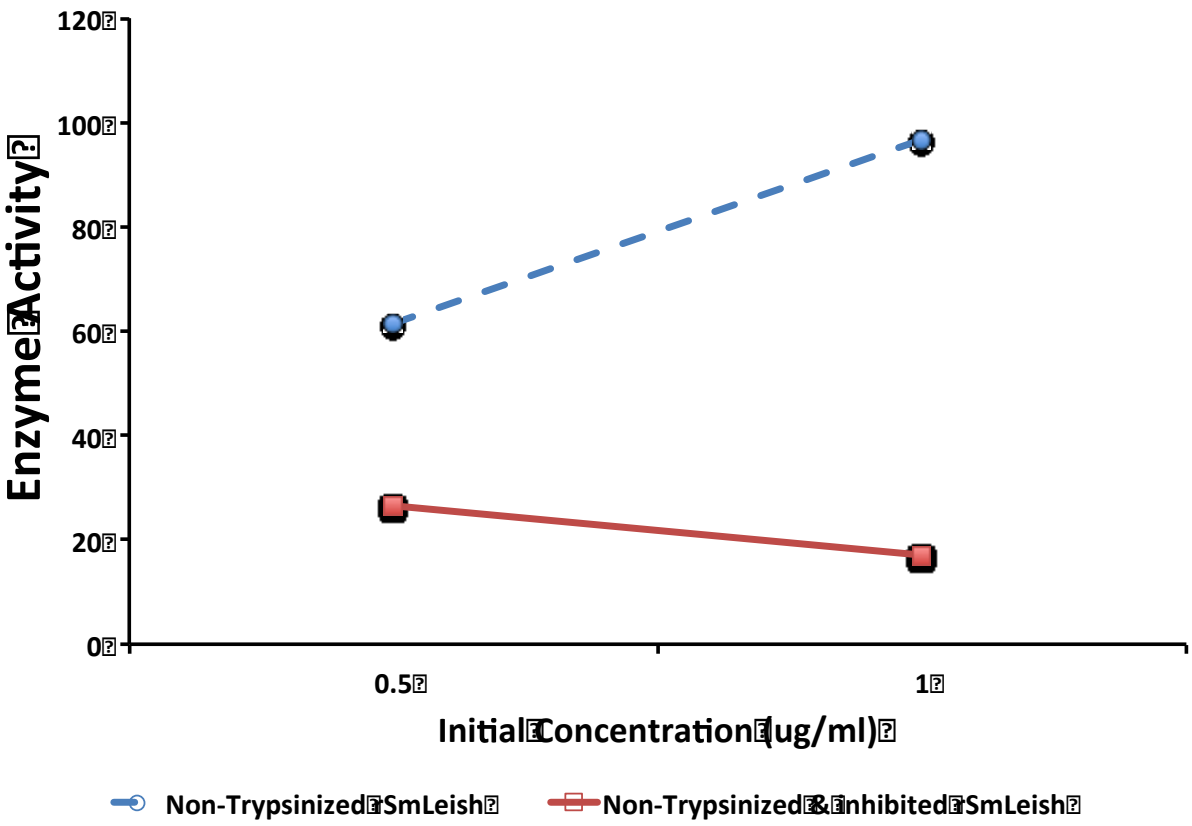


Figure 4.5 Non-trypsinized vs. non-trypsinized rSmLeish enzymatic activity - concentration dependent

The x-axis represents the initial non-trypsinized rSmLeish concentrations and the y-axis represents the non-trypsinized rSmLeish proteolytic activity. The activity of non-trypsinized rSmLeish was measured using the absorbance values from TNB, final product of the substrate break down by non-trypsinized rSmLeish. The non-trypsinized rSmLeish substrate degradation process was measured after 2 hours incubation with two different initial concentrations of 0.5 and 1 µg/mL of non-trypsinized rSmLeish. Increased concentration had a pronounced impact on non-trypsinized rSmLeish. The non-trypsinized and inhibited rSmLeish activity stayed limited in both of the initial concentrations.

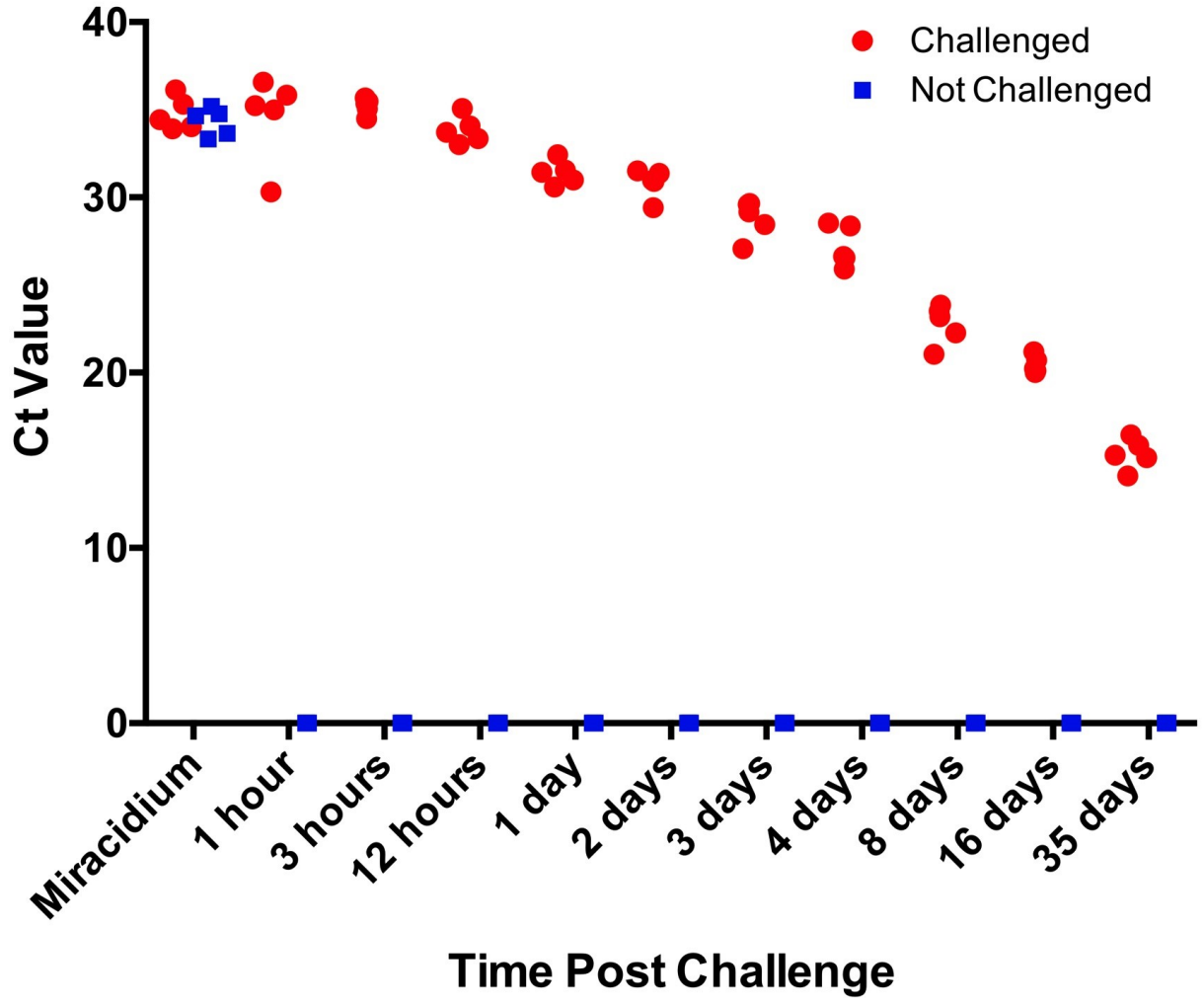


Figure 4.6 Ct values for SmGAPDH transcript expression during *S. mansoni* development in *B. glabrata* (M-line) from miracidium to 35 days post infection

Graphical representation of qPCR Ct values for SmGAPDH during the intramolluscan stage of *S. mansoni* for challenged and unchallenged susceptible (M-line) *B. glabrata*. The blue squares represent SmGAPDH transcript Ct values in the control (unchallenged) snails and the round red dots represent the SmGAPDH transcript Ct values for the challenged snails. SmGAPDH is only present in *S. mansoni* miracidium and *B. glabrata* infected with *S. mansoni* tissue. There is a continuous increase of SmGAPDH during the course of *S. mansoni* infection in *B. glabrata* as the parasite biomass increases. *S. mansoni* miracidium contain less SmGAPDH than the various stages of *S mansoni* in the Snail. The amount of dots and squares at each time point represents the amount of snails used for the qRT-PCR. Hence although five snails were collected per time point, only those that were successfully infected were kept for the data analysis of challenged snails in this graph for the experimental groups. High Ct values are synonymous with low transcript values.

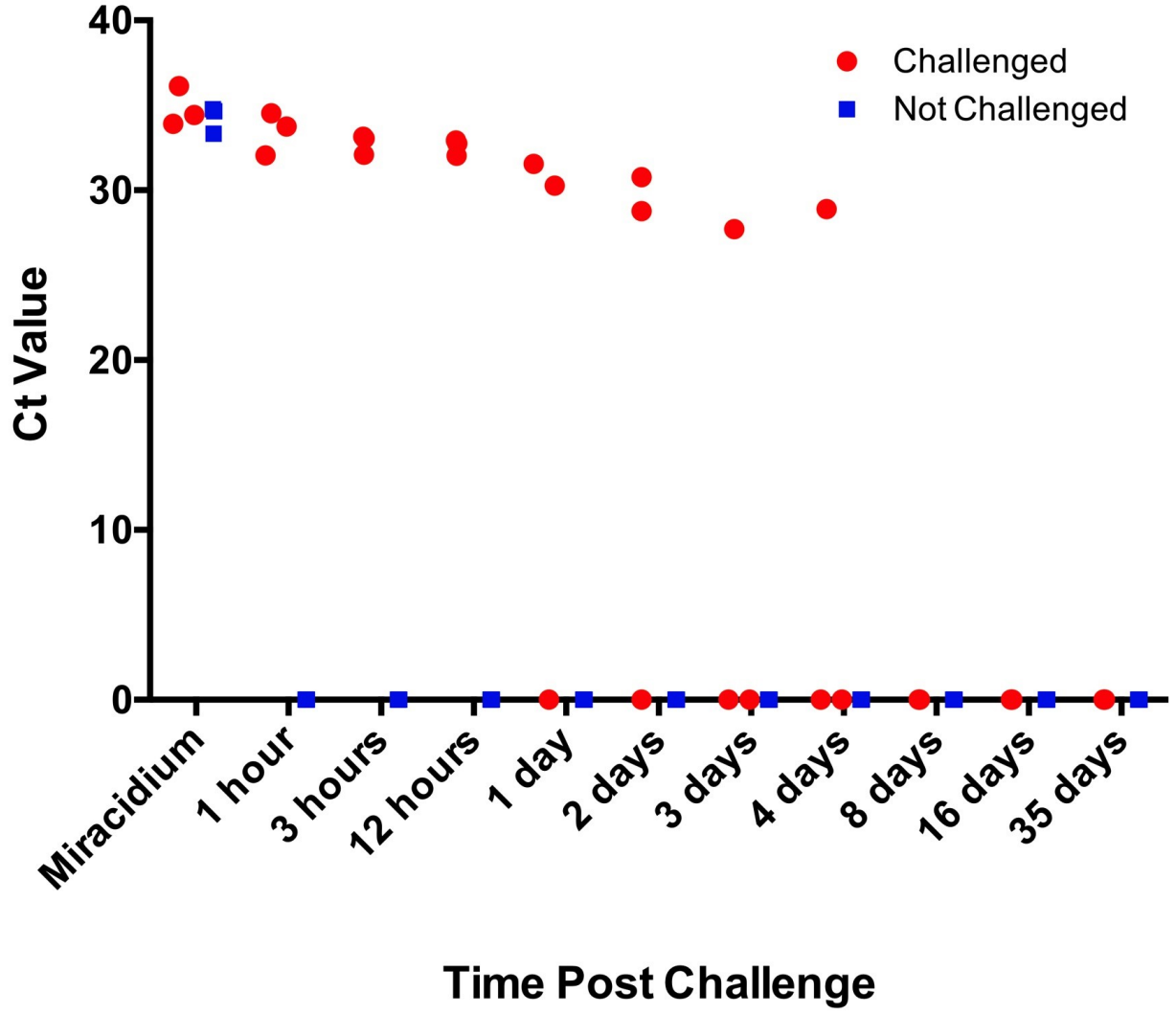


Figure 4.7 Ct values for SmGAPDH transcript expression during *S. mansoni* development in *B. glabrata* (BS-90) from miracidium to 35 days post infection

Graphical representation of qPCR Ct values for SmGAPDH during the intramolluscan stage of *S. mansoni* for challenged and unchallenged resistant (BS-90) *B. glabrata*. The blue squares represent SmGAPDH transcript Ct values in the control (unchallenged) snails and the round red dots represent the SmGAPDH transcript Ct values for the challenged snails. SmGAPDH is only present in *S. mansoni* miracidium and *B. glabrata* infected with *S. mansoni* until day 4. There is a continuous increase of SmGAPDH transcripts until *S. mansoni* is cleared from the BS-90 *B. glabrata* snails. The amount of dots and squares at each time point represents the amount of snails used for the qRT-PCR. Hence although five snails were collected per time point, only those that were successfully infected were kept for the data analysis of challenged snails in this graph for the experimental groups. *S. mansoni* miracidium contain less SmGAPDH than the various stages of *S mansoni* in the Snail. High Ct values are synonymous with low transcript values.

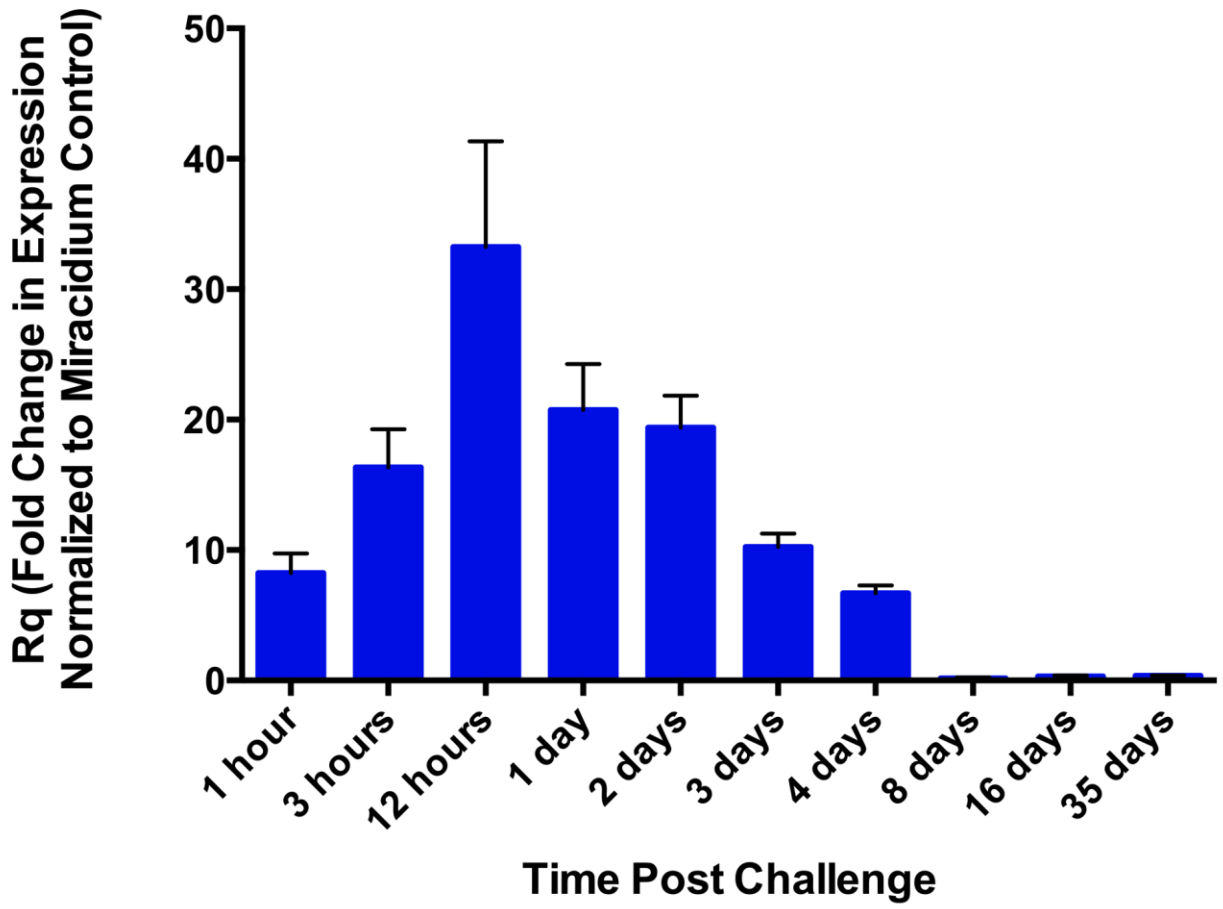


Figure 4.8 SmLeish transcript expression in fold change during *S. mansoni* development in *B. glabrata* from pre-exposure to 35 days post-successful infection

Transcript expression evolution of SmLeish during *S. mansoni* development in *B. glabrata*. The SmLeish expression in Rq was normalized to corresponding SmGAPDH expression Rq values then to the miracidia level of SmLeish. SmLeish transcript expression increases steadily post exposure to pick at half a day of *B. glabrata* infection by *S. mansoni*. The quantity of SmLeish transcript then lowers until it reaches values close to zero towards the end of the intramolluscan development.

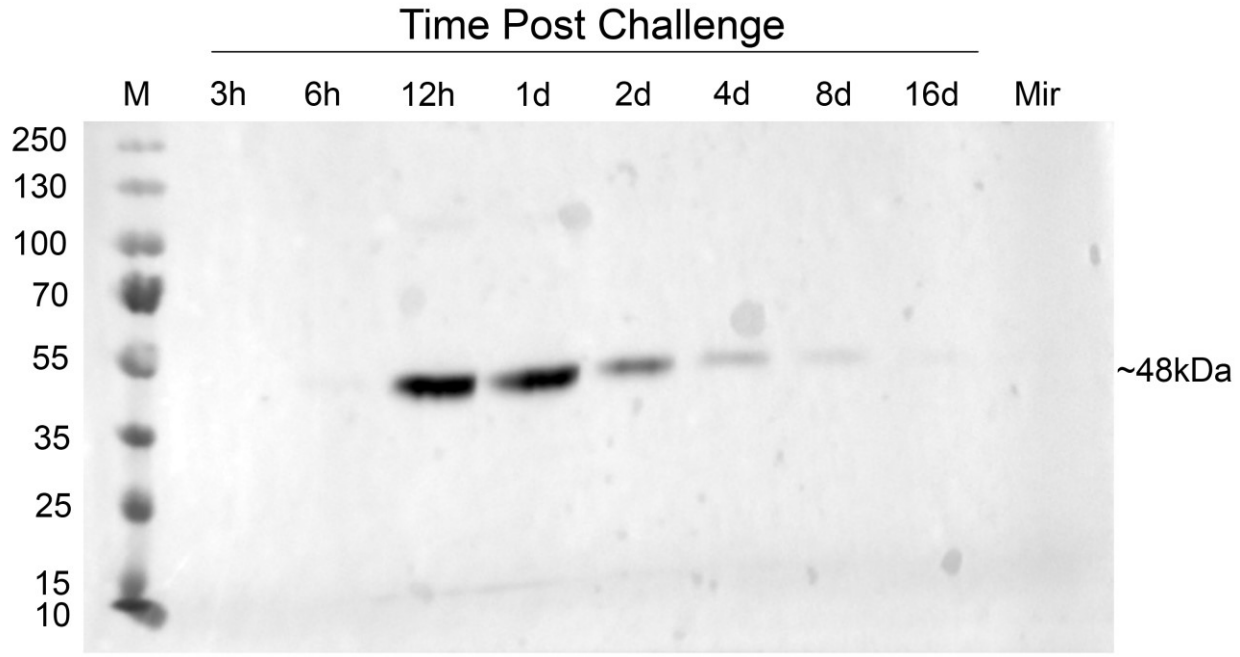


Figure 4.9 SmLeish protein expression during *S. mansoni* development in *B. glabrata* from pre-exposure to 35 days post-successful infection in *B. glabrata* plasma

M represents the ladder (kDa) and Mir stands for miracidium. The protein expression detected at about 48kDa, the expected soluble SmLeish size, is at its highest at 12 and 24 hours post exposition of the snail to *S. mansoni* parasites. The detection was done using specific designed antibodies against SmLeish. These findings indicate that SmLeish protein is excreted/secreted in the snail environment.

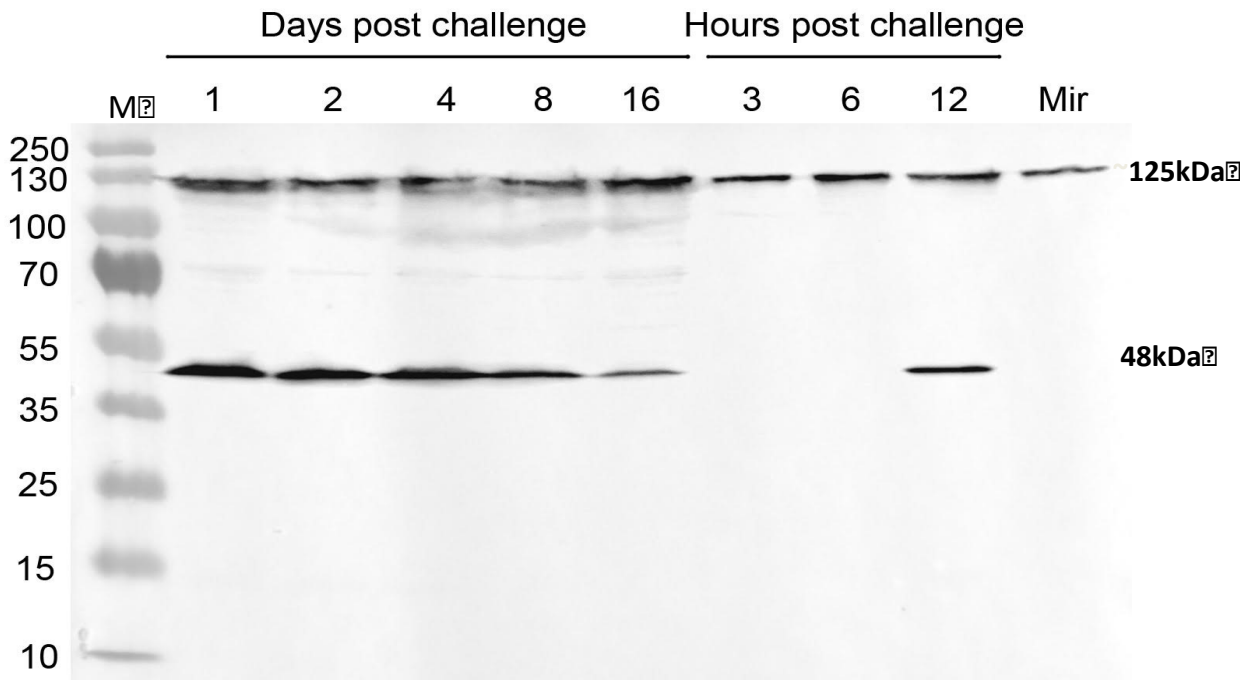


Figure 4.10 SmLeish protein expression during *S. mansoni* development in *B. glabrata* from pre-exposure to 35 days post successful infection in *B. glabrata* homogenate

The first column (M) represents the ladder (kDa) and Mir stands for miracidium. The soluble SmLeish protein expression was detectable around 48 kDa at all time points except 3 and 6 hours post exposition of the snail to *S. mansoni*. The pro-peptide form of SmLeish expected at around 125 kDa was detected at all time points tested. These findings support that SmLeish protein is excreted/secreted in the snail but also exist in a pro-peptide form throughout the intramolluscan stage of the parasite life cycle.

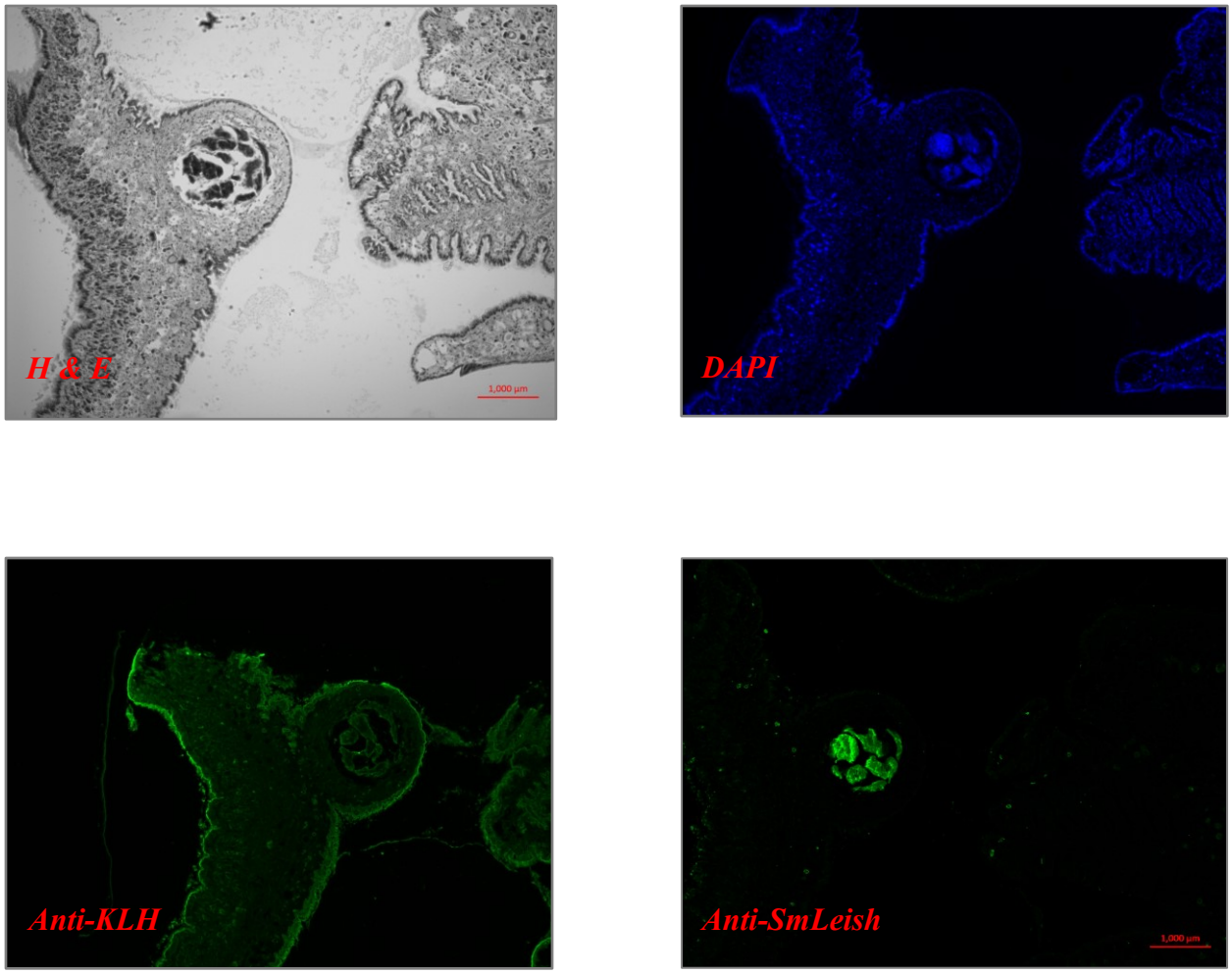


Figure 4.11 SmLeish detection in snail tissue hosting 16 days old *S. mansoni* sporocysts using immunohistochemistry

SmLeish detected in infected snail tissues around *S. mansoni* mother sporocysts in 16 days infected *B. glabrata* tissue. Haemotoxilyn and eosin (H&E) staining was the initial staining that allowed the observation of the tissue in order to locate which ones were infected. Anti KLH antibody as a positive control, allowed visualization of the presence of Keyhole limpet haemocyanin (KHL), a protein found in snail haemolymph. The DAPI staining allowed visualization of the cell nuclei. SmLeish was localized around *S. mansoni* mother sporocysts at the head foot and nowhere else within the snail tissue using and assay containing SmLeish specific antibody.

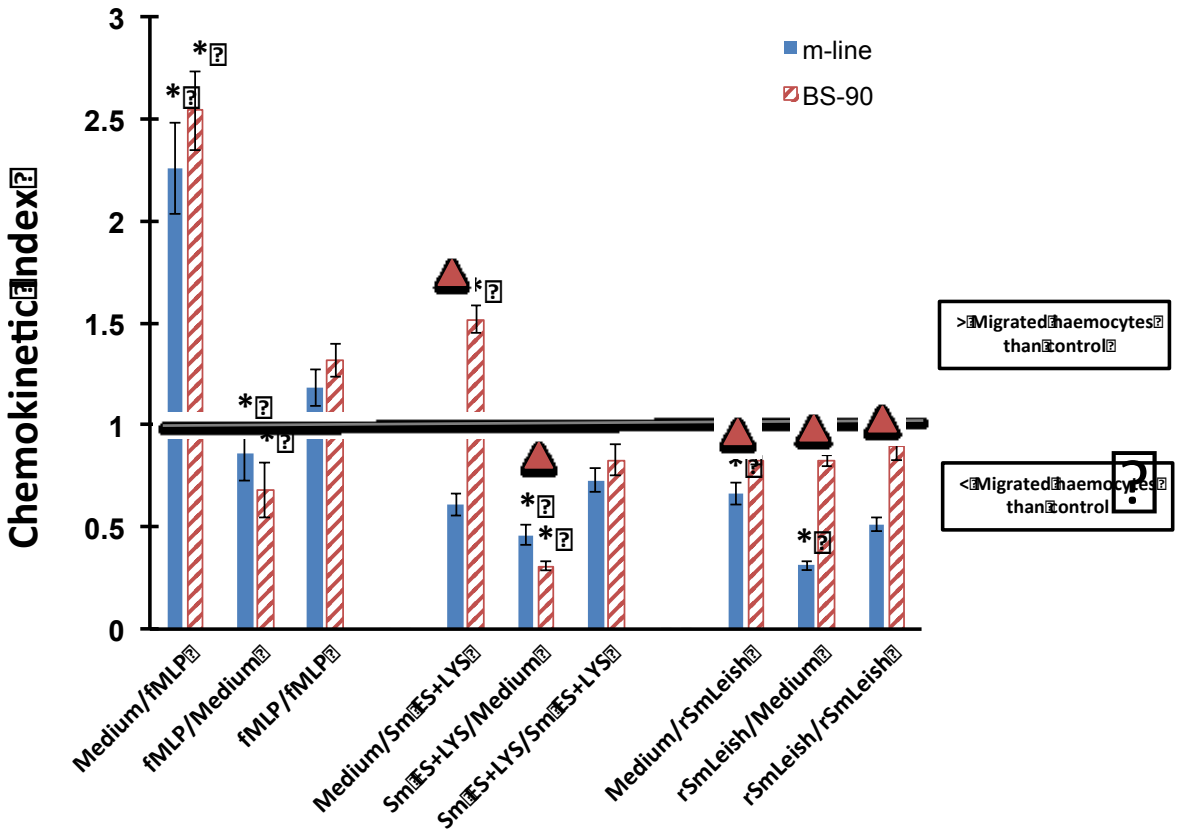


Figure 4.12 *B glabrata* haemocytes movement based on various treatments with rSmLeish, *S. mansoni* E/S and lysate, and fLMP

The chemotaxis index (the ratio of haemocyte migration in the experimental group to the control group) is represented for various treatment and their controls. Resistant snail (BS-90) haemocytes are presented in orange-hatched histograms and susceptible snail (M-line) haemocytes in solid blue histograms. The medium (snail haemocytes) was always located in the upper compartment, while the treatments, fMLP, *S. mansoni* excretory/secretory & lysate (Sm ES+LYS), and rSmLeish, were alternatively put in the bottom, upper and both compartments. Both snail type haemocytes were attracted to fMLP and traveled towards the bottom compartment containing fMLP or stayed in the upper compartment containing fLMP. BS-90 haemocytes were significantly attracted to the Sm ES + LYS, while treated M-line haemocytes registered less activity that their controls conterparts. Mixing Sm ES + LYS with the haemocytes from both the BS-90 and M-line snails resulted in less motility towards the bottom compartment in the treatment group compared to the controls. rSmLeish reduce the haemocytes migration in treatment groups for the susceptible and resistant snails compared to their control groups. The susceptible snails are more negatively impacted by the rSmLeish. The asterisk represents statistically significant chemokinetic index differences between the cases where treatment was either applied in the bottom or upper compartment and cases of treatment located in both compartments (control set up). The red triangles represent statistical significant chemokinetic index differences between resistant and susceptible snail data within the same case, based on the impact of the agents.

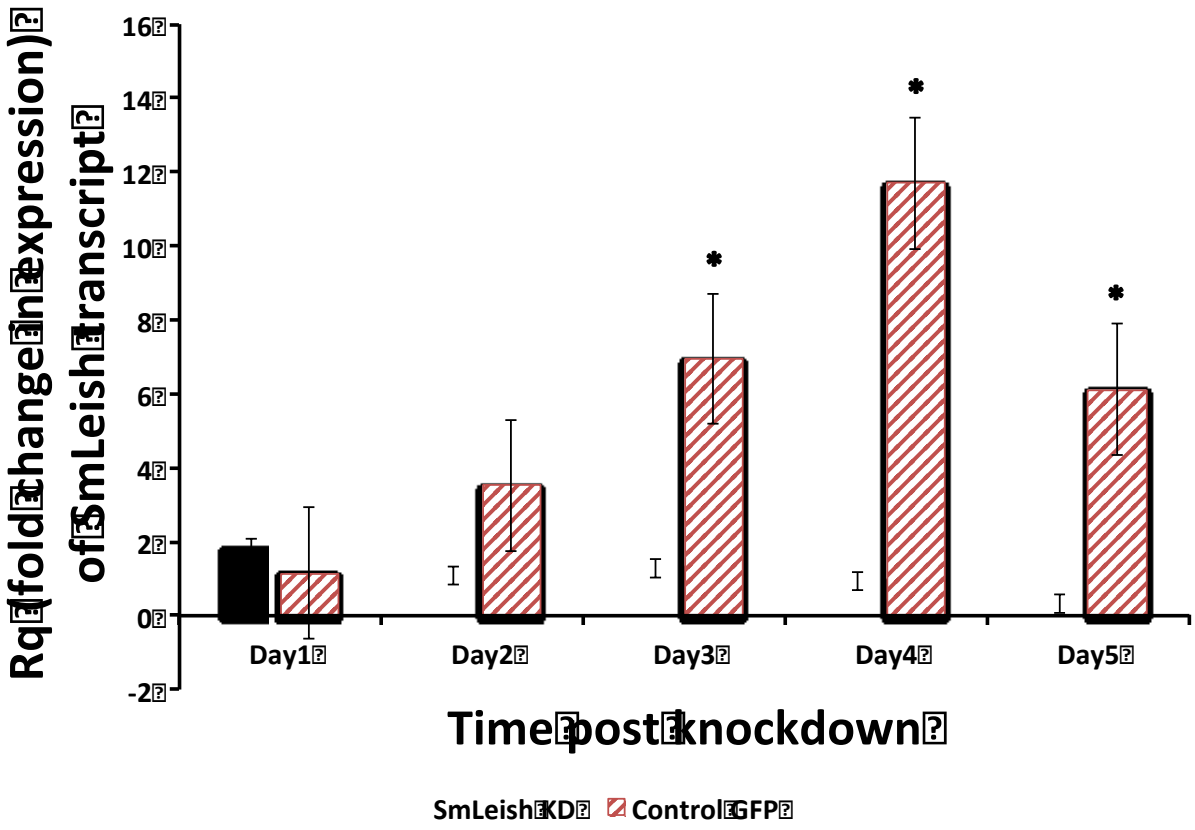


Figure 4.13 Daily fold change in SmLeish transcript levels in GFP & SmLeish knockdown miracidia treatment

SmLeish expression (Rq) is represented as a function of days post-knockdown. The control SmLeish transcript level (red histograms) increased consistently until day 4 before decreasing at day 5 post-exposure to the green fluorescent protein (GFP) siRNA. The SmLeish expression was efficiently reduced in rSmLeish knockdown parasites (blue histograms) compared to the GFP control. The effect of the knockdown was noticeable as early as day 2 after exposure to the rSmLeish knockdown siRNA. A t-test of the data concluded that the SmLeish transcript expression between control and experimental group was significant ($p<0.05$) for days 3, 4, and 5 between. This is highlighted on the graph by the asterisk.

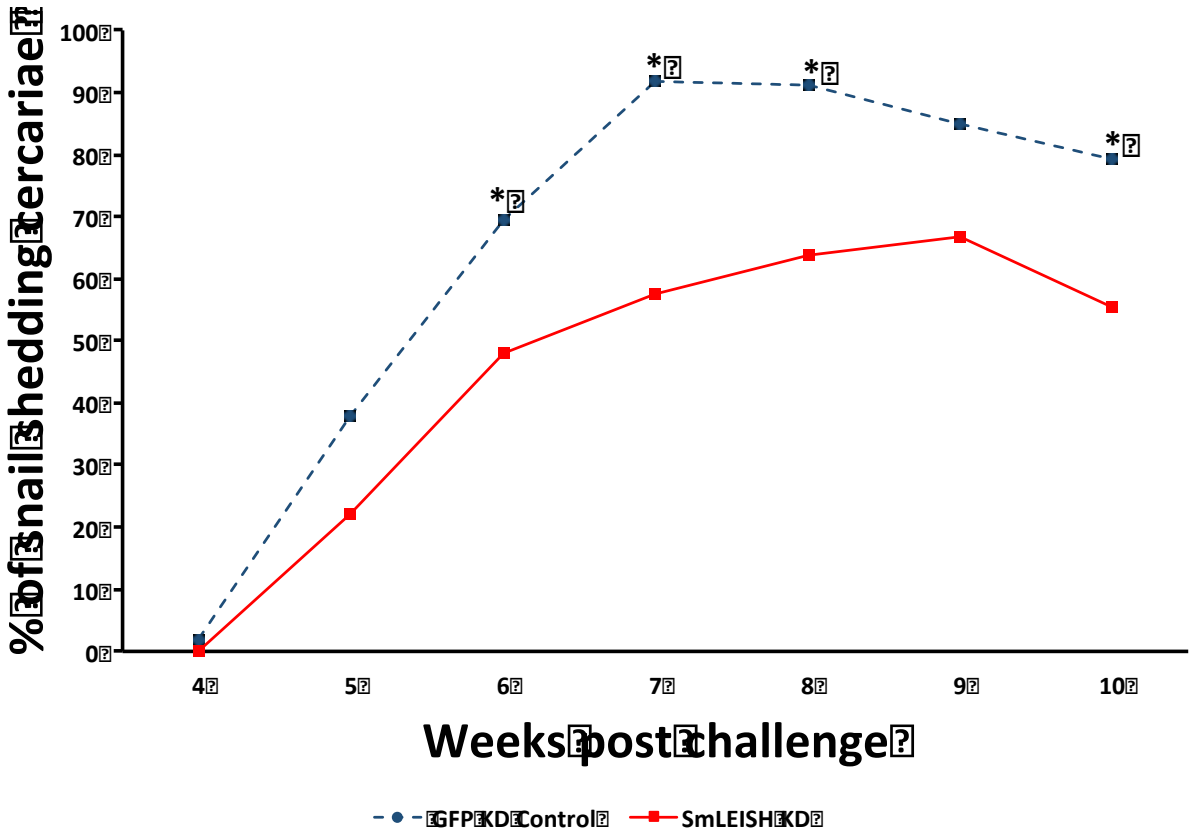


Figure 4.14 Percentage of snails shedding cercariae after being challenged with leishmanolysin knockdown S. mansoni miracidia taken at different weeks post challenge

Percentage of snail shedding cercariae as a function of time post challenge (weeks). At the end of the intramolluscan stage of the parasite where cercariae are expected to shed form the snail, the proportion of snails exposed to rSmLeish knockdown parasite significantly ($p < 0.05$) shed less cercariae than the control snails at week 6,7,8 and 10 (represented with the asterisk on the graph). A z-test for two independent proportions was performed to obtained the significance values.

4.3 *Schistosoma mansoni* leishmanolysin – Discussion

In this section, I have presented my data on the functional characterization of SmLeish. Broad conclusions that can be drawn from this data imply that SmLeish is produced as a pro-protein that is expressed at the surface of the *S. mansoni* sporocyst, but is also either cleaved or secreted by the parasite during the early stages of the intramolluscan infection. These data support a role for both a local and systemic action of SmLeish within *B. glabrata.* The putative function is likely congruent with known functions of other MMPs, most probably leishmanolysin itself, as SmLeish shares its highest amino acid identity to leishmanolysin and as I have demonstrated, also possesses MMP activity. While the assessment of MMP activity using the human MMP8 assay did not provide a complete picture regarding how SmLeish is functionally activated or as to what its substrate may be, the fact that it was able to cleave a MMP specific substrate implies parallel functions. Ultimately, SmLeish was functionally able to prevent the migration of haemocytes from a susceptible *B. glabrata* snail, which was an expected result based on past observations of the impact of parasite E/S products on haemocyte morphology and capacity to migrate towards and encapsulate a sporocyst. Given this functional impact on haemocytes, I predicted that prevention of SmLeish expression would likely influence infection outcome, which was confirmed by knockdown of SmLeish, thereby providing direct functional association between SmLeish and *S. mansoni* infection success.

4.3.1 rSmLeish metalloprotease activity

Both the trypsinized and non-trypsinized forms of rSmLeish tested demonstrated MMP enzymatic activity, though at slightly different rates. After 120 minutes incubation time, the trypsinized form enzymatic activity was 28, while the non-trypsinized form enzymatic activity registered 18. The human trypsin may not be well suited to cleave SmLeish, although few

anticipated trypsin cleavage sites exist within the protein sequence. Trypsin might still have the capability to target rSmLeish through cleavage at specific sites that may or may not necessarily be the usual activation cleavage sites that discards the N-terminal signal portion of the peptide. Hence, some fragments from the trypsinized rSmLeish might be active while others are not active. The fact that MMP activity was registered in the non-trypsinized rSmLeish suggests the generated rSmLeish was produced in an already active form. This latter explanation is possible, as the recombinant protein that was generated did not include part of the N-terminal region of the complete SmLeish protein. In summary, trypsin seems able to activate SmLeish although not to a great extent.

The data from the MMP inhibition for both forms of rSmLeish displayed significantly less activity than their respective non-inhibited counter-parts overall. Although, the inhibition impact increased as incubation length increased for both forms of rSmLeish, from 60 minutes to 120 minutes incubation duration, the inhibited non-trypsinized rSmLeish was more impacted compared to the typsinized rSmLeish. Indeed, the gap between inhibited and non-inhibited activity after 120 minutes was 18 for the non-trypsinized rSmLeish compared to 16 for trypsinized rSmLeish and statistically significant ($p<0.05$). The MMP inhibitor (Anaspec) effectively reduced the enzymatic activity of both types of rSmLeish. These results suggest that rSmLeish carry MMP activity because of its ability to interact with known substrate and inhibitors of MMP and yield results as would be expected of any mammalian MMP.

The time and concentration kinetics of rSmLeish demonstrate increased activity with longer exposure of the enzyme to a substrate, and with increased enzyme concentration relative to the substrate. This might have lasting impact in the context of host interaction for if the targeted substrate of SmLeish is produced by its hosts, snails in this circumstance, at a slow rate

or exists at a stagnant amount and given that SmLeish does not become saturated, *S. mansoni* will degrade the majority if not all of the substrates pool within the snail with time. Alternatively, increases in SmLeish production at key points during *S. mansoni* development could functionally limit specific aspects of the snail defense response. Because the intramolluscan stage lasts for four to eight weeks, it allows a substantial amount of time for SmLeish to degrade potential snail targets. However, an activation mechanism for SmLeish in the context of an *S. mansoni* infection remains unclear, and persistent activation of SmLeish, or an increase in activity that corresponds to increases in expression of the protein would be dependent on co-production of the activation agent (perhaps trypsin).

Most importantly, even with potential unsuitability of the substrate and inhibition substance for *S. mansoni*, rSmLeish clearly has MMP activity that is time and concentration dependent. This finding is relevant in the context of host immune response modulation by *S. mansoni*. As a metalloprotease, SmLeish could have the ability to breakdown key molecules from the snail immune defense factors, which would hinder the snail aptitude to effectively, interact, encapsulate and clear the parasite. Focusing future research in finding the specific targets of SmLeish within the snail immune factors will noticeably increase our understanding of the snail-schistosome dynamics and how a schistosome specific factor interacts with its molluscan host.

4.3.2 SmLeish expression and detection

Before undertaking any assessment of SmLeish transcript expression during intramolluscan development, I had to design an assay that both allowed us to detect infected snails that were not shedding cercariae (prepatent) and also account for the rapid and extensive multiplication of parasite tissue within the snail. *S. mansoni* tubulin, and SmGAPDH were

assessed and ultimately SmGAPDH was selected to serve as the endogenous and infection control for these studies. SmGAPDH detection was found to be specific to *S. mansoni* miracidium and successfully infected snails only, which was important in that it allowed us to only analyze SmLeish transcript abundance in those snails that were harboring an *S. mansoni* parasite within. The unchallenged snails display no presence of SmGAPDH transcripts, clearly showing that there is no cross-reactivity between these *S. mansoni*-specific primers and the GAPDH of *B. glabrata*. There is also an increase in SmGAPDH transcript abundance as the infection progresses in *B. glabrata* demonstrating the proportional increase of SmGAPDH with the parasite biomass increase throughout asexual reproduction within the snail. This increase is also mirrored by an increase in the SmGAPDH DNA, which indicates that the increase in transcript abundance is not due to an increase in relative expression within the *S. mansoni* cells, but an increase in the cell number (Taft & Yoshino, 2011). The level of SmGAPDH was at its lowest in the miracidia before snail entry.

After establishing the SmGAPDH assay, the next step was to quantify the transcript levels of SmLeish during *S. mansoni* intramolluscan development at different time points. The data displays an increase in SmLeish expression very early on in the infection establishment process, which culminates at twelve hours post challenge. The expression of SmLeish stays fairly high until day four of the infection progress. By the end of the infection cycle in the snail SmLeish expression profile drops to its lowest values but stays above miracidial levels. The increase in SmLeish transcript production early on during the intramolluscan development of *S. mansoni* in *B. glabrata*, suggests that this specific protein plays a role in the early stages of the infection process. It is important to consider that SmLeish expression was assessed relative to SmGAPDH, and because SmGAPDH expression continues to increase throughout the infection

it is likely that SmLeish is also present in increased abundance at the protein level due to the continual increase in parasite biomass.

In some cases, transcript abundance cannot be directly related to functional protein levels. This is particularly true in the case of proteins initially produced as pro-peptides prior to activation by enzymatic processing. Further, SmLeish, as a MMP, was thought to be produced as both a membrane-bound and surface cleaved protein, making it difficult for transcript expression assessment alone to inform on putative function in terms of location relative to the parasite and chronology of the infection. The data for the protein expression profile (Figure 4.9), where an increase in SmLeish was observed in the snail plasma at twelve hours, followed by one and two days post challenge demonstrate the presence of SmLeish as a secreted or cleaved protein that has the potential for functional impact throughout the circulation of the snail. It also implies that as a soluble protein, SmLeish could be functional from as early as half a day post infection of *B. glabrata* by *S. mansoni*, thus very strategic in early infection establishment, as we know that most resistant snails clear the infection within the first 2-4 days post challenge. Indeed, the expression profile of SmGAPDH in resistant *B. glabrata* (BS-90) shows no detection of the parasite after day 4 (Figure 4.7). These observations are concordant with the hypothesized role of rSmLeish helping in evading or suppressing snail immune response. It should be noted that the Western blot data showing SmLeish within the plasma of *B. glabrata* should not be considered as being directly attributed to the parallel increases in transcript expression outlined in Figure 4.8. The transcriptional analyses cannot distinguish between a soluble or membrane bound SmLeish protein, and thus, the increase in transcript expression cannot be directly linked to the increase in soluble SmLeish protein.

SmLeish has a predicted inside-outside transmembrane domain from amino acid positions 6-24. Thus, we predict that it has the capacity for functions in close proximity to the *S. mansoni* sporocyst additional to the role it might play systemically. This hypothesis is further supported by the fact that we have shown that SmLeish retains functionality without trypsin activation. To assess the pro-protein under natural conditions two experimental approaches were undertaken. The first was to attempt to visualize SmLeish in whole infected snail protein extracts. The second was to develop an immunohistochemistry assay that would specifically visualize SmLeish within the intramolluscan environment. The data from the immunohistochemistry has been successful and demonstrates SmLeish signal in close association with an *S. mansoni* mother sporocysts at day 16-post challenge (this is the only time point for which we have found a sporocyst within the headfoot tissue to date). The detection of SmLeish in the vicinities of the parasite indicates that SmLeish is likely also membrane bound. It seems likely, when considering the data together, that SmLeish is functional in two forms within *B. glabrata.*

4.3.3 Chemotaxis/chemokinesis

The observations from the chemokinesis experiments demonstrate that haemocytes from both resistant and susceptible snails are motile cells and are attracted to fMLP. It is also clear that the haemocytes from resistant and susceptible snails react differently when in the presence of *S. mansoni* ES+LYS. While the BS-90 haemocytes are still mobile and not deterred by *S. mansoni* ES+LYS, the M-line haemocytes have a reduced mobility. In fact, BS-90 haemocytes appear to be attracted by the *S. mansoni* E/S products, whereas M-line haemocytes motility is inhibited. Exposure of haemocytes to rSmLeish also suggests that rSmLeish negatively impacts the movement of the haemocytes from both resistant and susceptible snails with a higher impact on

susceptible snail haemocytes. In the case of the rSmLeish being in the same compartment as the *B. glabrata* haemocytes there is a greater immobility from the susceptible snail haemocytes compared to their control counterparts, which supports the suggested negative impact that leishmanolysin has on the movement of susceptible snail haemocytes. From this experiment we can draw two important conclusions about SmLeish biology; the first is that SmLeish negatively impacts haemocyte movement, particularly haemocytes from susceptible M-line snails. The second conclusion is that there is a fundamental difference in how resistant snail haemocytes engage with *S. mansoni* ES+LYS, which we know contain SmLeish. BS-90 haemocytes are attracted to these E/S products, whereas M-line haemocytes are not attracted. Because SmLeish does not appear to attract BS-90 haemocytes, we can conclude that SmLeish is likely not the attractive component of E/S products to BS-90 haemocytes. Confirmation that SmLeish is the only inhibitor of chemokinesis in E/S products that is impacting M-line haemocytes cannot be assessed from this experiment. Unfortunately, because the target ligand for SmLeish associated with *B. glabrata* is not known, we cannot make any conclusions regarding the mechanism(s) of haemocyte movement inhibition. However, given that cell movement towards a target is based on sensing a chemical gradient, and also requires adhesion of the cell membrane to a substrate, it is possible that SmLeish interferes with one of both of these two processes. The working hypothesis, subject of future studies moving forward, is that SmLeish could be enzymatically cleaving cell-surface proteins that are involved in cell movement. Moreover, we know that MMPs have been shown to act upon fibrinogen, which is a significant component of fibrinogen-related proteins (FREPs) produced by *B. glabrata* and known to be chemotactic. Thus, SmLeish may also be acting to prevent FREPs from recruiting haemocytes to the invading parasite by degrading chemotactic factors produced by the snail. SmLeish may also be preventing cell

movement, which requires extracellular matrix components, traditionally known as MMP substrates.

4.3.4 rSmLeish knockdown

The knockdown of SmLeish was confirmed using qRT-PCR and an *in vitro* assay to harvest sporocysts that had been exposed to siRNA specific for SmLeish or GFP as a control. The injection of GFP siRNA did not decrease the overall expression of SmLeish over the course of the infection, and these sporocysts displayed an increase in SmLeish expression as they progressed through five days *in vitro*. As demonstrated in previously mentioned results, SmLeish transcript expression is expected to increase during the first crucial hours and days of the intramolluscan development of *S. mansoni* miracidia into sporocysts. However, because this is an *in vitro* assay, and does not reflect the intramolluscan conditions in which SmLeish expression was measured in the above studies, directly comparable results cannot be expected. In the SmLeish knockdown group there is an evident reduction in the abundance of SmLeish transcripts compared to the GFP control group, supporting the effectiveness of the developed knockdown assay specifically targeting SmLeish within *S. mansoni* when administered to miracidia *in vitro* for 24 hours. This substantial decrease in SmLeish transcript expression demonstrates that effectively knockdown of SmLeish by day 2-3 post *in vitro* culture (day 3-4 post initial incubation with the siRNA oligonucleotides). SmLeish protein abundance in this experiment was not assessed due to limited access to live sporocysts for this study, and as such cannot confirm protein level knockdown of SmLeish. Moreover, transcript or protein knockdown kinetics during *S. mansoni* intramolluscan development was not assessed. Obtaining such information would have required using much more snail material in the experimental design in order to have enough snails to extract snail plasma from at various time points and still keep

enough snail going through the complete cycle in order to observe the outcome of infection through shedding of cercariae. Future studies will assess knockdown kinetics in parasites that are within snails to confirm that the knockdown effects are parallel to what was observed *in vitro.*

If SmLeish is a central regulator of the *B. glabrata* immune response during infection, preventing its expression during the infection process should have a measurable impact on infection success. Reaching patent infection in the snail culminates with the release of cercariae, which are the unit of human infectivity for *S. mansoni.* A reduction in the number of M-line snails reaching patent infections when challenged with *S. mansoni* in which SmLeish was knocked down would indicate an important role in the infection process. M-line snails were challenged with *S. mansoni* that were exposed to siRNA for SmLeish or GFP and then held for 4 weeks before weekly assessment of cercarial shedding. SmLeish knockdown parasites were significantly diminished in their capacity to produce patent infections in snails, highlighting the importance of SmLeish in facilitating infection. While the infection dynamics mirrored the GFP controls in that patent snail infection increased over time until week 8, the maximum percent patent infections was significantly lower in the SmLeish knockdown group from week 6 onward. This result is noteworthy as being the first demonstration of a parasite-specific factor that clearly impacts infection success in the snail host. The fact that there was not complete abrogation of patent infections suggests that either SmLeish is not the only parasite-specific factor that dictates infection success, or that our knockdown at the protein level was not complete and that some parasites retained the capacity to produce enough SmLeish. This will be tested in the future by assessing SmLeish protein knockdown success. However, given that a number of other putative factors appear to be expressed by *S. mansoni* during the intramolluscan development (for

example SmVAL), it seems likely that SmLeish is one of a number of immunomodulatory factors that influence infection outcome.

5. Chapter 5: *SmVAL3 transcript* and Protein Expression During the Intramolluscan Stage

5.1 *Schistosoma mansoni* venom-allergen-like 3 - Introduction

Previous studies of the *S. mansoni* transcriptome and proteome have demonstrated numerous members of the VAL family in schistosomes to be up regulated and stage specific (Wu *et al.*, 2010). Furthermore, VAL proteins have a variety of established roles in different organisms including their involvement in infection establishment and or maintenance for helminthes (Chalmers *et al.*, 2008). During the intramolluscan development of *S. mansoni*, VAL3 has been shown to be present. In order to confirm such previous claims and establish whether SmVAL3 is a key player during *S. mansoni* infection of *B. glabrata*, transcript and protein produced at various intramolluscan development time points were measured.

5.2 *Schistosoma mansoni* venom-allergen-like 3 - Results

Variation in SmVAL3 transcript expression was quantified along with the parasite intramolluscan development (Figure 5.1). It was noticeable that at hour one post challenge, the transcript expression of SmVAL3 was at its highest point relative to miracidial expression (1.70 fold increase). In relative terms, this was the highest point of SmVAL expression throughout the entire intramolluscan development of *S. mansoni* in *B. glabrata*. By twelve hours the transcript expression of SmVAL3 decreased to less than one fold (0.63 fold increase) and continued to decrease, and by thirty-five days SmVAL3 transcript expression approached the miracidial levels (0.0013 fold).

In the instance of SmVAL3 protein expression and detection in infected snail plasma, Western blot analysis allowed the visualization of SmVAL3 presence in *B. glabrata* plasma at

various time points during *S. mansoni* intramolluscan development (Figure 5.2). Most of the SmVAL3 protein expression happened between day 2 and day 4 and is represented by a protein of around 24 kDa in size, which is expected based on the amino acid sequence. The protein expression profile mirrors the transcript expression profile closely.

5.3 *Schistosoma mansoni* venom-allergen-like 3 - Discussion

Both the transcript and protein expression profiles of SmVAL3 demonstrate an increase in abundance within the early stages of *S. mansoni* intramolluscan development, suggesting SmVAL3 is excreted/secreted and also has a potential role in parasite intramolluscan development in *B. glabrata*. These same regulation patterns have been shown in previous studies investigating the entire or individual SmVAL family members expression across the parasite life stages or at a specific stage. Indeed, while investigating SmVAL3, a study focused on the functional role and expression of SmVAL9 was published by Yoshino and colleagues (2014). SmVAL9 is classified within the same subgroup one as SmVAL3 and hence expected to play a similar role. Yoshino *et al.*, (2014) concluded that SmVAL9 was up regulated in *S. mansoni* mother sporocyst but is present at even higher abundance in miracidia. They also attributed a property of creating a favorable environment within the host to SmVAL9 through regulating the host cell MMP and tissue inhibitors of metalloproteinase production. This influences the tissue remodeling of the snail host through the increase in tissue invasion and disrupting lethal anti-parasite redox activity from the snail immune response. For this reason, I did not pursue further investigation on the actual role that SmVAL3 played in the infection of *B. glabrata* by *S. mansoni* and rather focused on SmLeish, which still requires investigation. This being the case, future investigations into SmVAL3 may still be warranted in light of the now described role of

SmLeish in *S. mansoni* infection as SmVAL may be modulating the role of SmLeish given the discovery that it influences snail MMPs.

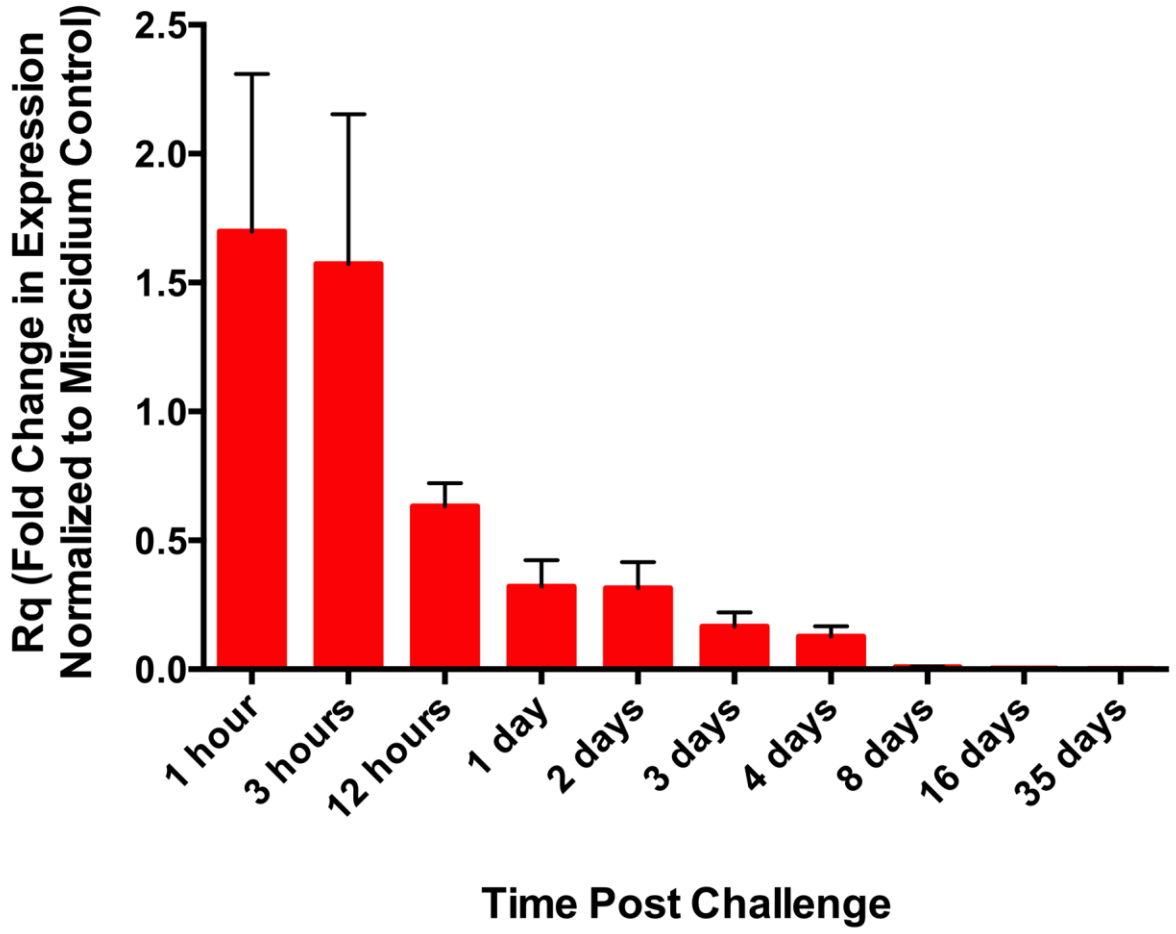


Figure 5.1 SmVAL3 transcript expression during *S. mansoni* development in *B. glabrata* from pre-exposure to 35 days post-successful infection

The x-axis is the time line from post exposure to 35 days post challenge. The y-axis shows the Rq values of SmVAL3 transcript expression. The SmVAL3 transcript expression is at its highest at 1-hour post challenge of the snail with *S. mansoni*. These findings indicate that SmVAL3 may be playing a role during the early stages of infection and that SmVAL3 transcripts are being produced very early on during *S. mansoni* intramolluscan development.

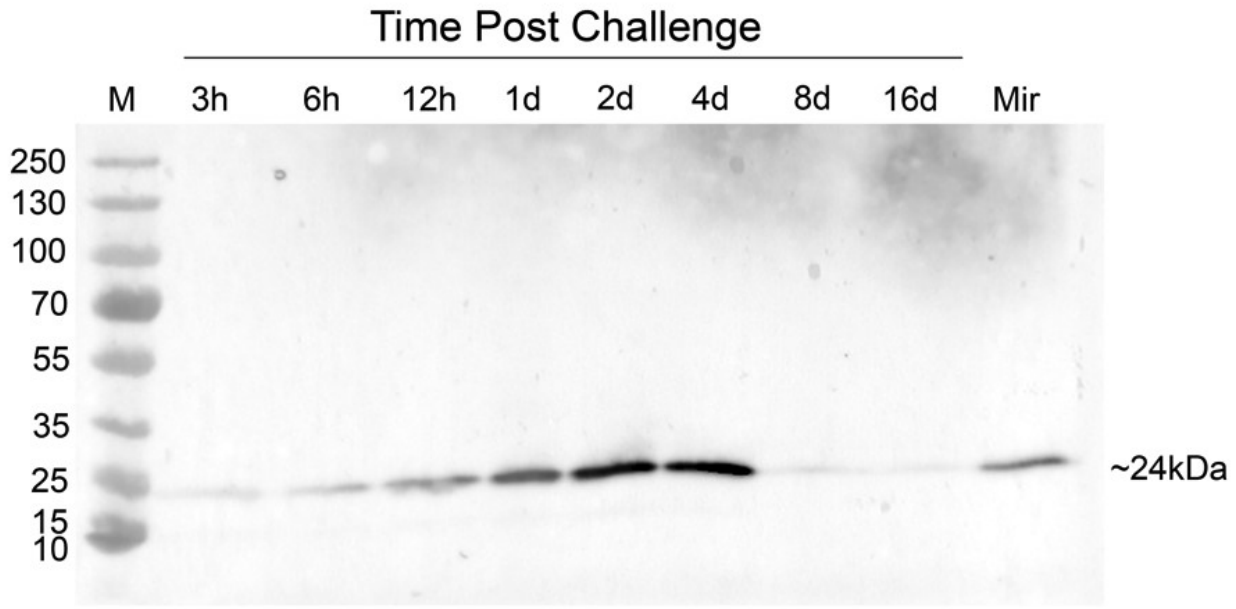


Figure 5.2 *S. mansoni* venom-allergen-like 3 protein expression during *S. mansoni* development in *B. glabrata* from pre-exposure to 35 days post-successful infection

M represents the ladder (kDa) and Mir represents the miracidia. The protein expression is at its highest at day 2 and 4 post exposition of the snail to *S. mansoni*. These findings indicate that SmVAL3 protein is excreted/secreted in the snail plasma and that it may play a role during the early stages of infection.

6. Chapter 6: General Discussion & Conclusion

6.1 General discussion

It is well established that the immune response of *B. glabrata* is capable of successfully defending the snail against infection by *S. mansoni* (Coustau *et al.*, 2015; Hanington *et al.*, 2010; Pila, *et al.*, 2016). However, it is also well known that resistance to infection is not the norm, and that typically, particularly under natural conditions; suitable snails exposed to *S. mansoni* miracidia become infected and shed cercariae. Whereas snail resistance to infection has been attributed to the production and engagement of specific immune features of the snail (some of which are not present in the same abundance or form in susceptible snails), much about how the parasite fits into the infection outcome equation are yet to be understood. Past observational studies have revealed that a phenotypic level the haemocytes of a susceptible snail are impacted differently than those of a resistant snail when in the presence of a *S. mansoni* sporocyst or its products. The difference is observable at many levels, but is most obvious when monitoring the ability of the haemocytes to adhere to a substrate, and move towards and ultimately encapsulate the sporocyst (Lodes & Yoshino, 1990). In addition, various studies on the snail-schistosome interaction have led to the understanding that schistosome E/S products can diminish the snail immune response (Lodes & Yoshino, 1990). Yoshino and Lodes in 1988 established that *B. glabrata* haemocyte responds to schistosome larval E/S products by increasing the expression of specific molecules, although the resistant snail response turns out to be toxic to the parasite, compared to susceptible snails which has no measurable negative effect. Subsequently, Lodes and Yoshino (1990) followed up with the discovery that schistosome E/S products inhibits M-line snail haemocytes motility. This present study expands these discoveries by demonstrating that a specific E/S product, SmLeish, inhibits M-line *B. glabrata* haemocyte motility. There is a

specific factor (SmLeish) produced by *S. mansoni* that explains at least partially these observations. Indeed, this work established that SmLeish inhibits susceptible *B. glabrata* haemocytes movement and with its detection within infected *B. glabrata* plasma, SmLeish is in direct contact with haemocytes and therefore capable of influencing their movement systemically. Understanding the specific mechanism behind this process requires more investigation, however, this represents an important step in the characterization of the parasite engagement of the snail immune response, and suggests that SmLeish produced during this interaction could be negatively impacting the capacity of the snail to defend against infection.

The initial finding that spurred investigation into SmLeish was the identification of a transcript that was significantly increased in expression during the early stages of *S. mansoni* intramolluscan infection and shared a high nucleotide identity with GP63, also known as leishmanolysin. Leishmanolysin has been demonstrated to be a critical factor in the infection establishment and maintenance of *Leishmania sp.* within host macrophages. Although, leishmanolysin, also referred to as invadolysin, has been studied to some extent in many organisms, *S. mansoni* is not among those species for which we have any information. The decryption of the *S. mansoni* genome revealed that the parasite possesses multiple variant of leishmanolysin-like genes. Furthermore, various studies including previous ones from our laboratory demonstrated that the expression of leishmanolysin is stage specific as showed in Figure 2.3 and reported by Wu and colleagues (2010). Since the revealed functions of leishmanolysin proteins in organisms such as *Leishmania major* demonstrate its key role in infection establishment and or maintenance, it became an interesting target for further evaluation in the context of *S. mansoni* infection. The choice to focus on the intramolluscan stage of the parasite life cycle was motivated by the lack of data related to that stage of schistosome

development, especially with respect to the mechanisms employed by the parasite to infect a snail. Furthermore, the intent to open another school of thought around schistosomiasis control strategies that targets the intramolluscan stage of the parasite life cycle and at the same time offers new information that could apply to the mammalian stage of the disease were additional reasons behind the focus of the intramolluscan life stage of the parasite. It is worth noting that leishmanolysin is particularly effective at preventing activation of human macrophages and effectively directs these immune cells towards an alternative activation state that makes them unable to drive a proinflammatory immune response to which they are central (Olivier *et al.*, 2012). Since this inflammatory response is considered important during the early stages of *S. mansoni* invasion of the human host (during cercariae penetration), and macrophages are also central immune cells during the fight against the schistosomulae stage, it is possible that some variants of SmLeish may also play a role during *S. mansoni* infection of the human host.

SmLeish transcript and protein expression increased during *S. mansoni* larval development in *B. glabrata*. Its detection in infected snail plasma and localization around *S. mansoni* sporocysts in infected snail tissue convey that SmLeish is most important during the early stages of larval development. Moreover, the capacity of SmLeish to inhibit susceptible *B. glabrata* haemocytes from properly migrating, communicates that this protein may be actively preventing snail haemocytes from entering in contact with the larval stages of the parasite by preventing them from moving towards the parasite and interact with it. In the case of pronounced knockdown of the expression of SmLeish, *S. mansoni* infection success decreases leading to a delayed larval development and less overall cercariae production ultimately meaning that fewer larval parasites were successfully infecting normally susceptible *B. glabrata* snails. Hence,

SmLeish is implicated in *S. mansoni* infection establishment and success in *B. glabrata* by potentially interfering with haemocytes ability to locate and encapsulate the parasite.

6.2 Conclusion

The investigation of the role played by SmLeish and SmVAL3 proteins in *S. mansoni* intramolluscan development through this project brought forward important points that will be useful for further mechanistic investigations in this field. During my thesis work, I effectively demonstrated that both proteins are up regulated early on during the infection establishment process in *B. glabrata* at the transcript and protein levels, and that SmLeish exists as both an excreted/secreted and parasite-associated protein. This correlates with the conclusions from previous studies that showed that VAL3 transcripts are up-regulated in the molluscan stage of *S. mansoni* (Chalmers *et al.*, 2008). Additional analysis of SmLeish provides us with compelling evidence that SmLeish possesses metalloprotease activity, although there is still the need to identify the specific substrates, activation and inhibitory agents and the ideal conditions required for an optimum function of SmLeish. My research also uncovered the impact that SmLeish has on *B. glabrata* haemocyte motility. Indeed, exposing *B. glabrata* haemocytes to rSmLeish revealed a reduction in haemocyte movement, thereby providing a context for the mechanism underpinning its role during infection. This suggests that when expressed within *B. glabrata* environment, SmLeish might serve as an immobilizing agent that keeps the snail immune effector cells away from *S. mansoni*, limiting their ability to encapsulate and dispose of the invading parasite. Future studies will benefit from using various concentration of SmLeish in order to discover if this impact on *B. glabrata* haemocytes is concentration dependent. The knockdown experiment demonstrated that a significant reduction in SmLeish expression in *S. mansoni* before *B. glabrata* infection, impacts the infection outcome. A longer intramolluscan

stage and a reduced shedding were definitively observed as the result of knocking down SmLeish in *S. mansoni*, implying the necessity of SmLeish in a successful and complete infection of *B. glabrata* by *S. mansoni*. This marks the first alteration of infection outcome in the *S. mansoni-B. glabrata* model by modulating the expression of a parasite-specific factor.

Taken together, my thesis findings strongly point towards SmLeish playing a role in the process of *S. mansoni* infection of *B. glabrata*, through interfering with haemocyte activity. Because SmLeish has metalloprotease characteristics it is predictable that it might interact with other snail defense molecules through degradation, thereby rendering them inoffensive toward the parasite. It will be essential in future work to evaluate the extent to which SmLeish contributes to infection success and the specific physiological mechanism behind its activity. This molecule can prove to be key in understanding the immune modulation by *S. mansoni* and the control of infection in molluscs and possibly in humans as well, and may form the basis for new, future preventive or curative actions towards the control of schistosomiasis, a significant public health disease that has eluded control measure thus far.

Reference

Alexander, D., Goodman, R. M., Gutrella, M., Glascock, C., Weymann, K., Friedrich, L., Ryals, J. (1993). Increased tolerance to 2 oomycete pathogens in transgenic tobacco expressing pathogenesis-related protein-1a. *Proceedings of the National Academy of Sciences of the United States of America*, *90*(15), 7327–7331. http://doi.org/10.1073/pnas.90.15.7327

Basch, P. F. (1991). *Schistosomes: development, reproduction, and host relations*. New York (USA): New York (USA) : Oxford University Press, 1991.

Bayne, C. J., Hahn, U. K., & Bender, R. C. (2001). Mechanisms of molluscan host resistance and of parasite strategies for survival. *Parasitology*, *123 Suppl*, S159–S167. http://doi.org/10.1017/S0031182001008137

Berriman, M., Haas, B. J., LoVerde, P. T., Wilson, R. A., Dillon, G. P., Cerqueira, G. C., El-Sayed, N. M. (2009). The genome of the blood fluke *Schistosoma mansoni*. *Nature*, *460*(7253), 352–8. http://doi.org/10.1038/nature08160

Blair, D., Davis, G. M., & Wu, B. (2001). Evolutionary relationships between trematodes and snails emphasizing schistosomes and paragonimids. *Parasitology*, *123 Suppl*(1999), S229–43. http://doi.org/10.1017/S003118200100837X

Bos, D. H., Mayfield, C., & Minchella, D. J. (2009). Analysis of regulatory protease sequences identified through bioinformatic data mining of the *Schistosoma mansoni* genome. *BMC Genomics*, *10*, 488. http://doi.org/10.1186/1471-2164-10-488

Bouvier, J., Schneider, P., Etges, R., & Bordier, C. (1990). Peptide substrate specificity of the membrane-bound metalloprotease of *Leishmania*. *Biochemistry*, *29*(43), 10113–10119.

http://doi.org/10.1021/bi00495a015

Brindley, P. J. (2005). The molecular biology of schistosomes. *Trends in Parasitology*, *21*(11), 533–536. http://doi.org/10.1016/j.pt.2005.08.015

Brittingham, A., Morrison, C. J., McMaster, W. R., McGwire, B. S., Chang, K. P., & Mosser, D. M. (1995). Role of the *Leishmania* surface protease gp63 in complement fixation, cell adhesion, and resistance to complement-mediated lysis. *Journal of Immunology (Baltimore, Md. : 1950)*, *155*(6), 3102–3111. http://doi.org/10.1016/0169-4758(95)80054-9

Calvo, E., Pham, V. M., Lombardo, F., Arca, B., & Ribeiro, J. M. C. (2006). The sialotranscriptome of adult male *Anopheles gambiae* mosquitoes. *Insect Biochemistry and Molecular Biology*, *36*(7), 570–575. http://doi.org/10.1016/j.ibmb.2006.04.005

Cantacessi, C., Campbell, B. E., Visser, a., Geldhof, P., Nolan, M. J., Nisbet, a. J., Gasser, R. B. (2009). A portrait of the "SCP/TAPS" proteins of eukaryotes - Developing a framework for fundamental research and biotechnological outcomes. *Biotechnology Advances*, *27*(4), 376–388. http://doi.org/10.1016/j.biotechadv.2009.02.005

Chalmers, I. W., & Hoffmann, K. F. (2012). Platyhelminth Venom Allergen-Like (VAL) proteins: revealing structural diversity, class-specific features and biological associations across the phylum. *Parasitology*, *139*(10), 1231–1245. http://doi.org/10.1017/S0031182012000704

Chalmers, I. W., McArdle, A. J., Coulson, R. M., Wagner, M. a, Schmid, R., Hirai, H., & Hoffmann, K. F. (2008). Developmentally regulated expression, alternative splicing and distinct sub-groupings in members of the *Schistosoma mansoni* venom allergen-like

(SmVAL) gene family. *BMC Genomics*, *9*, 89. http://doi.org/10.1186/1471-2164-9-89

Charlab, R., Valenzuela, J. G., Rowton, E. D., & Ribeiro, J. M. (1999). Toward an understanding of the biochemical and pharmacological complexity of the saliva of a hematophagous sand fly *Lutzomyia longipalpis*. *Proceedings of the National Academy of Sciences of the United States of America*, *96*(26), 15155–15160. http://doi.org/10.1073/pnas.96.26.15155

Chernin, E. (1963). Observations on hearts explanted in vitro from the snail *Australorbis glabratus*. *The Journal of Parasitology*, *49*(3), 353–364. Retrieved from http://www.jstor.org/stable/3275797

Chiyaka, E. T., & Garcia, W. (2009). Mathematical analysis of the transmission dynamics of schistosomiasis in the human-snail hosts. *Journal of Biological Systems*, *17*(03), 397–423. http://doi.org/10.1142/S0218339009002910

Cioli, D., Pica-Mattoccia, L., Basso, A., & Guidi, A. (2014). Schistosomiasis control: praziquantel forever? *Molecular and Biochemical Parasitology*, *195*(1), 23–29. http://doi.org/10.1016/j.molbiopara.2014.06.002

Clark, I. M., Rowan, A. D., Ugalde, A. P., Ord, G. R., Quir, P. M., Puente, X. S., & Carlos, L. (2010). Matrix Metalloproteinase Protocols, *622*. http://doi.org/10.1007/978-1-60327-299-5

Colley, D. G., Bustinduy, A. L., Secor, W. E., & King, C. H. (2014). Human schistosomiasis. *The Lancet*, *383*(9936), 2253–2264. http://doi.org/10.1016/S0140-6736(13)61949-2

Connors, V. a, & Yoshino, T. P. (1990). In vitro effect of larval *Schistosoma mansoni* excretory-secretory products on phagocytosis-stimulated superoxide production in hemocytes from *Biomphalaria glabrata*. *The Journal of Parasitology*, *76*(6), 895–902.

http://doi.org/10.2307/3282811

Coppin, J.-F., Lefebvre, C., Caby, S., Cocquerelle, C., Vicogne, J., Coustau, C., & Dissous, C. (2003). Gene expression changes in *Schistosoma mansoni* sporocysts induced by *Biomphalaria glabrata* embryonic cells. *Parasitology Research*, *89*(2), 113–9. http://doi.org/10.1007/s00436-002-0643-2

Coustau, C., Gourbal, B., Duval, D., Yoshino, T. P., Adema, C. M., & Mitta, G. (2015). Advances in gastropod immunity from the study of the interaction between the snail *Biomphalaria glabrata* and its parasites: A review of research progress over the last decade. *Fish & Shellfish Immunology*, *46*(1), 5–16. http://doi.org/10.1016/j.fsi.2015.01.036

Coustau, C., & Yoshino, T. P. (1994). *Schistosoma mansoni*: modulation of hemocyte surface polypeptides detected in individual snails, *Biomphalaria glabrata*, following larval exposure. *Experimental Parasitology*. http://doi.org/10.1006/expr.1994.1053

de Morais, C. G. V., Castro Lima, A. K., Terra, R., Dos Santos, R. F., Da-Silva, S. A. G., & Dutra, P. M. L. (2015). The dialogue of the host-parasite relationship: *Leishmania spp.* and *Trypanosoma cruzi* Infection. *BioMed Research International*, *2015*, 324915. http://doi.org/10.1155/2015/324915

Di Cara, F., Duca, E., Dunbar, D. R., Cagney, G., & Heck, M. M. S. (2013). Invadolysin, a conserved lipid-droplet-associated metalloproteinase, is required for mitochondrial function in *Drosophila*. *Journal of Cell Science*, *126*(Pt 20), 4769–81. http://doi.org/10.1242/jcs.133306

Dvořák, J., Fajtová, P., Ulrychová, L., Leontovyč, A., Rojo-Arreola, L., Suzuki, B. M.,

O'Donoghue, A. J. (2015). Excretion/secretion products from *Schistosoma mansoni* adults, eggs and schistosomula have unique peptidase specificity profiles. *Biochimie*, 1–11. http://doi.org/10.1016/j.biochi.2015.09.025

El Ridi, R., Othman, A. a., & McManus, D. P. (2015). Editorial: The schistosomiasis vaccine – It is time to stand up. *Frontiers in Immunology*, *6*(July), 4–6. http://doi.org/10.3389/fimmu.2015.00390

Farias, L. P., Rodrigues, D., Cunna, V., Rofatto, H. K., Faquim-Mauro, E. L., & Leite, L. C. C. (2012). *Schistosoma mansoni* venom allergen like proteins present differential allergic responses in a murine model of airway inflammation. *PLoS Neglected Tropical Diseases*, *6*(2), e1510. http://doi.org/10.1371/journal.pntd.0001510

Fitzpatrick, J. M., Peak, E., Perally, S., Chalmers, I. W., Barrett, J., Yoshino, T. P., Hoffmann, K. F. (2009). Anti-schistosomal intervention targets identified by lifecycle transcriptomic analyses. *PLoS Neglected Tropical Diseases*, *3*(11), e543. http://doi.org/10.1371/journal.pntd.0000543

Gordy, M. A., Pila, E. A., & Hanington, P. C. (2015). The role of fibrinogen-related proteins in the gastropod immune response. *Fish and Shellfish Immunology*, *46*(1), 39–49. http://doi.org/10.1016/j.fsi.2015.03.005

Gray, D. J., McManus, D. P., Li, Y., Williams, G. M., Bergquist, R., & Ross, A. G. (2010). Schistosomiasis elimination: lessons from the past guide the future. *The Lancet. Infectious Diseases*, *10*(10), 733–6. http://doi.org/10.1016/S1473-3099(10)70099-2

Hanington, P. C., Hanelt, B., Hines, J., & Loker, E. S. (n.d.). Transcriptional profiling of

Schistosoma mansoni sporocysts during their intramolluscan development in *Biomphalaria glabrata*. Unpublished.

Hanington, P. C., Lun, C. M., Adema, C. M., & Loker, E. S. (2010). Time series analysis of the transcriptional responses of *Biomphalaria glabrata* throughout the course of intramolluscan development of *Schistosoma mansoni* and *Echinostoma paraensei*. *International Journal for Parasitology*, *40*(7), 819–831. http://doi.org/10.1016/j.ijpara.2009.12.005

Harnett, W. (2014). Secretory products of helminth parasites as immunomodulators. *Molecular and Biochemical Parasitology*, *195*(2), 1–7. http://doi.org/10.1016/j.molbiopara.2014.03.007

Hassani, K., Shio, M. T., Martel, C., Faubert, D., & Olivier, M. (2014). Absence of metalloprotease GP63 alters the protein content of *Leishmania* exosomes. *PLoS ONE*, *9*(4). http://doi.org/10.1371/journal.pone.0095007

Hawdon, J. M., Jones, B. F., Hoffman, D. R., & Hotez, P. J. (1996). Cloning and characterization of ancylostoma-secreted Protein a novel protein associated with the treansition to parasitism by infective hook larvae. *Journal of Biological Chemistry*, *271*(12), 6672–6678.

Henriksen, A., King, T. P., Mirza, O., Monsalve, R. I., Meno, K., Ipsen, H., Spangfort, M. D. (2001). Major VenomAllergen of YellowJackets, Ves v 5: Structural characterization of a pathogenesis-related protein superfamily. *Nature Genetics*, *39*(11), 438–448. http://doi.org/10.1002/prot.XXXX

Hoffman, D. R. (2006). Hymenoptera venom allergens. *Clinical Reviews in Allergy & Immunology*, *30*(2), 109–128. http://doi.org/10.1385/CRIAI:30:2:109

Inobaya, M. T., Olveda, R. M., Tallo, V., McManus, D. P., Williams, G. M., Harn, D. a, Ross, A. G. (2015). Schistosomiasis mass drug administration in the Philippines: lessons learnt and the global implications. *Microbes and Infection / Institut Pasteur*, *17*(1), 6–15. http://doi.org/10.1016/j.micinf.2014.10.006

Jalkanen, J. (2005). Mouse cysteine-rich secretory protein 4 (CRISP4): a member of the crisp family exclusively expressed in the epididymis in an androgen-dependent manner. *Biology of Reproduction*, *72*(5), 1268–1274. http://doi.org/10.1095/biolreprod.104.035758

Jenkins-Holick, D. S., & Kaul, T. L. (2013). Schistosomiasis. *Urologic Nursing*, *33*(4), 163–170. http://doi.org/10.7257/l053-816X.2013.33.4.163

Kasahara, M., Gutknecht, J., Brew, K., Spurr, N., & Goodfellow, P. N. (1989). Cloning and mapping of a testis-specific gene with sequence similarity to a sperm-coating glycoprotein gene. *Genomics*, *5*(3), 527–534. http://doi.org/10.1016/0888-7543(89)90019-0

Kelleher, A., Darwiche, R., Rezende, W. C., Farias, L. P., Leite, L. C. C., Schneiter, R., & Asojo, O. a. (2014). *Schistosoma mansoni* venom allergen-like protein 4 (SmVAL4) is a novel lipid-binding SCP/TAPS protein that lacks the prototypical CAP motifs. *Acta Crystallographica Section D: Biological Crystallography*, *70*(8), 2186–2196. http://doi.org/10.1107/S1399004714013315

King, C. H. (2009). Toward the elimination of schistosomiasis. *The New England Journal of Medicine*, *360*(2), 106–109. http://doi.org/10.1056/NEJMp0808041

King, C. H., & Dangerfield-Cha, M. (2008). The unacknowledged impact of chronic schistosomiasis. *Chronic Illness*, *4*(1), 65–79. http://doi.org/10.1177/1742395307084407

King, C. L., Malhotra, I., Mungai, P., Wamachi, A., Kioko, J., Ouma, J. H., & Kazura, J. W. (1998). B cell sensitization to helminthic infection develops in utero in humans. *The Journal of Immunology*, *160*(7), 3578–3584. Retrieved from http://www.jimmunol.org/content/160/7/3578.abstract\nhttp://www.jimmunol.org/content/160/7/3578.full.pdf

Knaap, W. P. W. Van Der, & Loker, E. S. (1990). Immune mechanisms in trematodes-snail interactions, *6*(6), 175–182.

Kovalick, G. E., & Griffin, D. L. (2005). Characterization of the SCP/TAPS gene family in *Drosophila melanogaster*. *Insect Biochemistry and Molecular Biology*, *35*(8), 825–35. http://doi.org/10.1016/j.ibmb.2005.03.003

Li, S., Kwon, J., & Aksoy, S. (2001). Characterization of genes expressed in the salivary glands of the tsetse fly, *Glossina morsitans*. *Insect Mol Biol*, *10*(April 2000), 69–76. Retrieved from http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=11240638

Lodes, M. J., & Yoshino, T. P. (1990). The effect of schistosome excretory-secretory products on *Biomphalaria glabrata* hemocyte motility. *Journal of Invertebrate Pathology*, *56*(1), 75–85. http://doi.org/0022-2011(90)90147-X [pii]

Lu, G., Villalba, M., Coscia, M. R., Hoffman, D. R., & King, T. P. (1993). Sequence analysis and antigenic cross-reactivity of a venom allergen, antigen 5, from hornets, wasps, and yellow jackets. *Journal of Immunology (Baltimore, Md. : 1950)*, *150*(7), 2823–2830. Retrieved from

http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=8454859&retmode=ref&cmd=prlinks

Lundy, S. K., & Lukacs, N. W. (2013). Chronic schistosome infection leads to modulation of granuloma formation and systemic immune suppression, *4*(February), 1–19. http://doi.org/10.3389/fimmu.2013.00039

Maeda, T., Sakashita, M., Ohba, Y., & Nakanishi, Y. (1998). Molecular cloning of the rat Tpx-1 responsible for the interaction between spermatogenic and Sertoli cells. *Biochemical and Biophysical Research Communications*, *248*(248), 140–146. http://doi.org/10.1006/bbrc.1998.8918

Maizels, R. M., Gomez-escobar, N., Gregory, W. F., Murray, J., & Zang, X. (2001). Immune evasion genes from larval nematodes. *International Journal for Parasitology*, *31*.

Malhotra, I., Ouma, J., Wamachi, A., Kioko, J., Mungai, P., Omollo, A., … King, C. L. (1997). In utero exposure to helminth and mycobacterial antigens generates cytokine responses similar to that observed in adults. *Journal of Clinical Investigation*, *99*(7), 1759–1766. http://doi.org/10.1172/JCI119340

Massova, I., Kotra, L. P., Fridman, R., & Mobashery, S. (1998). Matrix metalloproteinases: structures, evolution, and diversification. *FASEB J.*, 1075–1095.

McGwire, B. S., Chang, K. P., & Engman, D. M. (2003). Migration through the extracellular matrix by the parasitic protozoan *Leishmania* is enhanced by surface metalloprotease gp63. *Infection and Immunity*, *71*(2), 1008–1010. http://doi.org/10.1128/IAI.71.2.1008-1010.2003

McHugh, B., Krause, S. a, Yu, B., Deans, A.-M., Heasman, S., McLaughlin, P., & Heck, M. M.

S. (2004). Invadolysin: a novel, conserved metalloprotease links mitotic structural rearrangements with cell migration. *The Journal of Cell Biology*, *167*(4), 673–86. http://doi.org/10.1083/jcb.200405155

McManus, D. P. (2012). Schistosomiasis in 2012: current status and key research priorities required for control leading to elimination. *Expert Review of Anti-Infective Therapy*, *10*(11), 1233–6. http://doi.org/10.1586/eri.12.121

McManus, D. P., & Loukas, A. (2008). Current status of vaccines for schistosomiasis. *Clinical Microbiology Reviews*, *21*(1), 225–242. http://doi.org/21/1/225 [pii]\r10.1128/CMR.00046-07

Mitta, G., Adema, C. M., Gourbal, B., Loker, E. S., & Theron, A. (2012). Compatibility polymorphism in snail/schistosome interactions: from field to theory to molecular mechanisms. *Developmental and Comparative Immunology*, *37*(1), 1–8. http://doi.org/10.1016/j.dci.2011.09.002

Mo, A. X., Agosti, J. M., Walson, J. L., Hall, B. F., & Gordon, L. (2014). Schistosomiasis elimination strategies and potential role of a vaccine in achieving global health goals. *American Journal of Tropical Medicine and Hygiene*, *90*(1), 54–60. http://doi.org/10.4269/ajtmh.13-0467

Mosser, D. M., & Edelson, P. J. (1985). The mouse macrophage receptor for C3bi (CR3) is a major mechanism in the phagocytosis of *Leishmania* promastigotes. *Journal of Immunology (Baltimore, Md. : 1950)*, *135*(4), 2785–2789.

Murphy, E. V., Zhang, Y., Zhu, W., & Biggs, J. (1995). The human glioma pathogenesis-related

protein is structurally related to plant pathogenesis-related proteins and its gene is expressed specifically in brain tumors. *Gene*, *159*(1), 131–135. http://doi.org/10.1016/0378-1119(95)00061-A

Nacif-Pimenta, R., de Mattos, A. C. A., da Orfanó, A. S., Barbosa, L., Pimenta, P. F. P., & Coelho, P. M. Z. (2012). *Schistosoma mansoni* in susceptible and resistant snail strains *Biomphalaria tenagophila*: in vivo tissue response and in vitro hemocyte interactions. *PLoS ONE*, *7*(9), 1–12. http://doi.org/10.1371/journal.pone.0045637

Nagase, H. (2001). Metalloproteases. In *current protocols inpProtein science* (pp. 1–13). Hoboken, NJ, USA: John Wiley & Sons, Inc. http://doi.org/10.1002/0471140864.ps2104s24

Newton, W. L. (1953). The inheritance of susceptibility to infection with *Schistosoma mansoni* in *Australorbis glabratus*. *Experimental Parasitology*, *2*, 242–257.

Niemann, G. M., & Lewis, F. a. (1990). *Schistosoma mansoni*: influence of *Biomphalaria glabrata* size on susceptibility to infection and resultant cercarial production. *Experimental Parasitology*, *70*(3), 286–292. http://doi.org/10.1016/0014-4894(90)90110-X

Olivier, M., Atayde, V. D., Isnard, A., Hassani, K., & Shio, M. T. (2012). *Leishmania* virulence factors: focus on the metalloprotease GP63. *Microbes and Infection*, *14*(15), 1377–1389. http://doi.org/10.1016/j.micinf.2012.05.014

Olveda, D. U., Olveda, R. M., McManus, D. P., Cai, P., Chau, T. N. P., Lam, A. K., Ross, A. G. P. (2014). The chronic enteropathogenic disease schistosomiasis. *International Society for Infectious Diseases*, *28*(2014), 193–203. http://doi.org/10.1016/j.ijid.2014.07.009

Pearce, E. J. (2003). Progress towards a vaccine for schistosomiasis. *Acta Tropica*, *86*(2-3), 309–

313. http://doi.org/Doi 10.1016/S0001-706x(03)00062-7

Pearce, E. J., Caspar, P., Grzych, J. M., Lewis, F. A., & Sher, A. (1991). Down regulation of Th1 cytokine production accompanies induction of Th2 responses by a parasitic helminth, *Schistosoma mansoni*. *J Exp Med*, *173*(1), 159–66. http://doi.org/10.1084/jem.173.1.159

Pearce, E. J., & MacDonald, A. S. (2002). The immunobiology of schistosomiasis. *Nature Reviews Immunology*, *2*(7), 499–511. http://doi.org/10.1038/nri843

Pila, E. A., Gordy, M. A., Phillips, V. K., Kabore, A. L., Rudko, S. P., & 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*, 201521239. http://doi.org/10.1073/pnas.1521239113

Pila, E. A., Tarrabain, M., Kabore, A. L., & Hanington, P. C. (2016). A Novel Toll-Like Receptor (TLR) influences compatibility between the gastropod *Biomphalaria glabrata* , and the digenean trematode *Schistosoma mansoni*, 1–23. http://doi.org/10.1371/journal.ppat.1005513

Pinkston-Gosse, J., & Kenyon, C. (2007). DAF-16/FOXO targets genes that regulate tumor growth in Caenorhabditis elegans. *Nature Genetics*, *39*(11), 1403–1409. http://doi.org/10.1038/ng.2007.1

Protasio, A. V., Dunne, D. W., & Berriman, M. (2013). Comparative study of transcriptome profiles of mechanical- and skin-transformed *Schistosoma mansoni* Schistosomula. *PLoS Neglected Tropical Diseases*, *7*(3), 1–10. http://doi.org/10.1371/journal.pntd.0002091

Richards, C. S., & Shade, P. C. (1987). The genetic variation of compatibility in *Biomphalaria*

glabrata and *Schistosoma mansoni*. *Journal of Parasitology*, *73*(6), 1146–1151.

Rollinson, D., Knopp, S., Levitz, S., Stothard, J. R., Tchuem Tchuenté, L. A., Garba, A., Utzinger, J. (2013). Time to set the agenda for schistosomiasis elimination. *Acta Tropica*, *128*(2), 423–440. http://doi.org/10.1016/j.actatropica.2012.04.013

Ross, A. G. P., Olveda, R. M., Chy, D., Olveda, D. U., Li, Y., Harn, D. a, Williams, G. M. (2014). Can Mass Drug Administration Lead to the Sustainable Control of Schistosomiasis? *The Journal of Infectious Diseases*, *211*, 1–7. http://doi.org/10.1093/infdis/jiu416

Ross, A. G. P., Olveda, R. M., & Li, Y. (2014). An audacious goal : the elimination of schistosomiasis in our lifetime through mass drug administration. *Lancet*, *6736*(14), 1–2. http://doi.org/10.1016/S0140-6736(14)61417-3

Schmid-Hempel, P. (2011). Parasite immune evasion and manipulation of host phenotype. In *Evolutionary Parasitology Evolutionary Parasitology: The Integrated Study of Infections, Immunology, Ecology, and Genetics*. http://doi.org/10.1093/acprof:oso/9780199229482.001.0001

Secor, E. w., & Montgomery, S. P. (2005). Something old, something new: is praziquantel enough for schistosomiasis control? *Medicinal Chemistry*, *7*, 450–461.

Shadab, A., Ahmed, H., Editor, C., & Stuart, M. (2015). Schistosomiasis.

Sotillo, J., Pearson, M., Becker, L., Mulvenna, J., & Loukas, A. (2015). A quantitative proteomic analysis of the tegumental proteins from *Schistosoma mansoni* schistosomula reveals novel potential therapeutic targets. *International Journal for Parasitology*, *45*(8), 505–16. http://doi.org/10.1016/j.ijpara.2015.03.004

Taft, A. S., & Yoshino, T. P. (2011). Cloning and functional characterization of two calmodulin genes during larval development in the parasitic flatworm *Schistosoma mansoni*. *The Journal of Parasitology*, *97*(1), 72–81. http://doi.org/10.1645/GE-2586.1

Tawe, W., Pearlman, E., Unnasch, T. R., & Lustigman, S. (2000). Angiogenic activity of *Onchocerca volvilus* recombinant proteins similar to vespid venom antigen 5, *109*, 91–99.

Tennessen, J. A., Th??ron, A., Marine, M., Yeh, J. Y., Rognon, A., & Blouin, M. S. (2015). Hyperdiverse gene cluster in cnail host conveys resistance to human schistosome parasites. *PLoS Genetics*, *11*(3), 1–21. http://doi.org/10.1371/journal.pgen.1005067

Valenzuela, J. G., Garfield, M., Rowton, E. D., & Pham, V. M. (2004). Identification of the most abundant secreted proteins from the salivary glands of the sand fly *Lutzomyia longipalpis*, vector of Leishmania chagasi. *The Journal of Experimental Biology*, *207*(Pt 21), 3717–3729. http://doi.org/10.1242/jeb.01185

WHO. (2013). Schistosomiasis progress report 2001–2011 and strategic plan 2012–2020, 80.

WHO. (2015). *Schistosomiasis*. Retrieved from http://www.who.int/mediacentre/factsheets/fs115/en/#

Wilson, R. A. (2012). The cell biology of schistosomes: a window on the evolution of the early metazoa. *Protoplasma*, *249*(3), 503–518. http://doi.org/10.1007/s00709-011-0326-x

Wu, X., Sabat, G., Brown, J. F., Zhang, M., Taft, A., Harms, A., & Yoshino, T. P. (2010). Proteomic analysis of *Schistosoma mansoni* proteins released during in vitro miracidium-to-sporocyst transformation. *Molecular and Biochemical Parasitology*, *164*(1), 32–44. http://doi.org/10.1016/j.molbiopara.2008.11.005.Proteomic

Wyse, B. a, Oshidari, R., Jeffery, D. C., & Yankulov, K. Y. (2013). Parasite epigenetics and immune evasion: lessons from budding yeast. *Epigenetics & Chromatin*, *6*(1), 40. http://doi.org/10.1186/1756-8935-6-40

Yamazaki, Y., Hyodo, F., & Morita, T. (2003). Wide distribution of cysteine-rich secretory proteins in snake venoms: isolation and cloning of novel snake venom cysteine-rich secretory proteins. *Archives of Biochemistry and Biophysics*, *412*(1), 133–141. http://doi.org/10.1016/S0003-9861(03)00028-6

Yamazaki, Y., & Morita, T. (2004). Structure and function of snake venom cysteine-rich secretory proteins. *Toxicon*, *44*(3), 227–231. http://doi.org/10.1016/j.toxicon.2004.05.023

Yao, C., Donelson, J. E., & Wilson, M. E. (2003). The major surface protease (MSP or GP63) of *Leishmania sp.* Biosynthesis, regulation of expression, and function. *Molecular and Biochemical Parasitology*, *132*, 1–16. http://doi.org/10.1016/S0166-6851(03)00211-1

Yoshino, T. P., Brown, M., Wu, X. J., Jackson, C. J., Ocadiz-Ruiz, R., Chalmers, I. W., Hoffmann, K. F. (2014). Excreted/secreted *Schistosoma mansoni* venom allergen-like 9 (SmVAL9) modulates host extracellular matrix remodelling gene expression. *International Journal for Parasitology*, *44*(8), 551–563. http://doi.org/10.1016/j.ijpara.2014.04.002

Appendix

Reagents

Artificial spring water

Four stock solutions were mixed to create the artificial spring water: Solution A, B, C, and D. For a final volume of 40 L: 20 mL of solution A, 100 mL of solution B, 100 mL solution C, and 50 mL of solution D were mix together and brought to the final volume of 40 L with deionized water. For 1 L of stock solution A, 0.25g of ferric chloride was dissolved with deionized water to the final volume of 1 L. For 1 L of stock solution B, 11g of anhydrous calcium chloride were dissolved in deionized water to the final volume of 1 L. For 1 L of stock solution C, 10g magnesium sulfate was dissolved in deionized water to the final volume of 1 L. For 1 L of stock solution D, 34 g of potassium phosphate monobasic was dissolved in distilled water to 500 mL; then the pH equilibrated to 7.2 using sodium hydroxide. 1.5 g of ammonium sulfate was added, and the volume brought to 1 L using deionized water.

Growth media

For 250 mL of agarose plate: 2.5 g of tryptone, 1.25 g of yeast extract, 2.5 g of sodium chloride, 3.75 g of agar were mix to a final volume of 250 mL using water, and ampicillin was added so the final concentration was 100 μg/mL.

For 1.8 L of LB Medium: 18 g of tryptone, 9 g of yeast extract, 18 g of sodium chloride, and deionized water were mix to final volume. Ampicillin was added to a final concentration of 100 μg/mL.

Western blot buffers

To obtain 12% SDS-Page Gel for 2 gels, the running Gel composition was the following: milli-Q water (5.8 mL), 1 M tris at pH 8.8 (8 mL), 40% acrylamide (6 mL), 10% APS (0.108 mL), 20% SDS (0.108 mL), and TEME-D (0.02 mL). For the Stacking Gel, 5.7 mL of milli-Q water, 1.14 mL of 1 M tris at pH of 6.8, 0.7 mL of 40% Acrylamide, 0.076 mL of 10% APS, 0.04 mL of 20% SDS, and 0.01 mL of TEME-D were mixed.

For the SDS-Page loading buffer (4X) at an approximated final volume of 10 mL: 5 mL of 0.5 M tris-HCL (pH 6.8), 4 mL of glycerol (100%), 0.8 mL of β-mercaptoethanol, 0.04 g of bromophenol blue, and 0.8 g of SDS were combined together.

To obtain 1 L of 1 X Running Buffer: 1 M tris (25 mL), 20% SDS (5 mL), glycine (14.2633 g) were combined and bought to 800ml with deionized water and the pH adjust to 8.3. The final volume of 1 L was obtained adding deionized water.

For 1 L 1 X Transfer Buffer: 25 mL of 1 M tris, 200ml of methanol, 5 mL of 20% SDS, and 14.2633 g of glycine were combined. Then deionized water was added to a volume of 800 mL and the pH adjusted to 8.3. The entire mix was then brought to a final volume of 1 L with deionized water

To obtain the Washing Buffers, 1 X tris-buffered-saline (TBS) buffer obtained by diluting 10 X TBS 10:1 with deionized water. The 1 X TBS-Tween buffer was prepared by diluting 10 X TBS 10:1 with deionized water and subsequently adding 1ml of tween 20%.

To obtain 1 L of 10X TBS: 60.6 g tris, 87.6 g NaCl were mixed using deionized water. The pH was adjusted to 7.6 with 1 M HCl, before adding deionized water to a final volume of 1

L. The tris Buffers saline-tween (TBS-T) solution was obtained by diluting 0.05% Tween20 in 1 X TBS.

Northern blot buffer

For 1% TAE Agarose Gel: 100ml of 1X TAE to 1g of agarose. The solution was microwaved to melt the Agarose, allowed to cool slightly, and then poured in a gel cast before leaving it to solidify.

For 1 L of Running Buffer: 100ml of 1 M tris HCL (pH 7.5), 20 mL of 0.5 M EDTA (pH 8.0) were incorporated and brought to a final volume of 1 L using milli-Q water.

Fast protein liquid chromatography (FPLC) buffers

All buffers solutions have a pH of 7.4 and filtered using 0.45 mm pore membrane.

For the Binding Buffer for 2 L: 20mM of sodium phosphate (5.5196g), 500 mM of sodium chloride (NaCl) (58.44 g), 20 mM Imidazole (2.7232g) were diluted to about 1.8 L with milli-Q water. After ensure that all solid particles were completely dissolved the pH was adjust to 7.4 by adding1 M HCL or 1 M NaOH solution as required. The solution was brought up to the final volume of 2 L using milli-Q water.

For 1 L Wash Buffer for: 20 mM of sodium phosphate (5.5196 g), 500 mM of sodium chloride (58.44 g), 40 mM Imidazole (2.7232 g) were diluted to about 0.8 L with milli-Q water. After ensuring that all solid particles were completely dissolved, the pH of the solution was adjust to 7.4 by adding 1 M HCL or 1 M NaOH as required and the solution brought up to the final volume using milli-Q water.

To obtained 1 L Elution Buffer: 20m M of sodium phosphate (5.5196 g), 500 mM of sodium chloride (58.44 g), 500 mM Imidazole (34.04 g) were diluted to about 0.8 L with milli-Q water. After ensuring that all solid particles were completely dissolved, the pH of the solution was adjusted to 7.4 by adding either 1 M HCL or 1 M NaOH as required and the entire mix was brought to a final volume of 1 L using milli-Q water.

For 20% ethanol (1 L): 200 mL of 100% Ethanol, and 800 mL of milli-Q water were combined.

Bovine serum albumin/tris buffers saline-tween (BSA/TBS-T):

0.1% BSA and TBS-T was mixed together.

Fixative: Railliet-Henry's fluid

Distilled water (930 mL), sodium chloride (6 mg), formaldehyde (37%) (50 mL), and glacial acetic acid (20 mL) were combined together.

Neutral-buffered formalin (10%)

Formalin (37-40% Formaldehyde) and buffer phosphate solution were combined to obtain the final composition of 10% formalin.

Buffer (phosphate) solution 1 L

Sodium phosphate monbasic (3.31 g), sodium phosphate dibasic heptahydrate (33.77 g) were dissolved in deionized water to a final concentration of 1 L

Chernin's balance solution 1 L

Sodium chloride (2.80 g), potassium Chloride (0.15 g), disodium phosphate anhydrous (0.07 g), magnesium sulfate heptahydrate (0.54 g), calcium chloride dehydrate (0.53 g), sodium

Bicarbonate (0.05 g), and phenol Red (5 mL) were mix together using milli-Q water to a final volume of 1 L.

Sodium citrate buffer

For a 10M sodium citrate, 0.05% tween 20 at a final of pH 6.0: tri-sodium citrate (dihydrate) (2.94 g) was dissolved in 1000 mL of distilled water. The pH was adjusted to 6.0 with 1 N HCl. 0.5 mL of Tween 20 was subsequently added to the mixture and mixed well. The solution was stored at room temperature for a maximum of 3 months or at 4°C if longer storage was required.