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Molecular studies on susceptible and resistant *Biomphalaria glabrata* interacting with *Schistosoma mansoni*

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ABSTRACT

Identification of molecular markers involved in the interaction between *Biomphalaria glabrata* and *Schistosoma mansoni* is crucial in the control of schistosomiasis as many of these molecules are likely to play important roles in host defence and parasite infectivity. This study attempted to identify and characterise molecules associated with compatibility polymorphisms in laboratory populations of *B. glabrata* with varying susceptibilities to *S. mansoni*. First, in a series of host-parasite compatibility experiments, a longitudinal study spanning almost three decades was completed. Analyses of data comprising the infection status of snail populations that had been monitored from 1991-2017 were used to determine the pattern of *B. glabrata-S. mansoni* compatibility in multiple, laboratory-maintained, host-parasite combinations. This longitudinal study thus formed a basis for determining changes in the susceptibility and infectivity status of both the snail and parasite strains. Secondly, phylogeny of extant laboratory populations of three *B. glabrata* strains was established using the mitochondrial 16S ribosomal RNA gene. Thirdly, the three populations were characterised based on the *sod1* gene. Fourthly, using SDS-PAGE, Western Blot analysis, and mass spectrometry, haemolymph and tissue glycoproteins of these three strains of *B. glabrata* were investigated for cross-reactivity against *S. mansoni* egg antigens. Results showed that *B. glabrata* snails and *S. mansoni* parasites display varying degrees of susceptibility and infectivity and co-evolved parasites and hosts are not necessarily better adapted to each other. Analysis of the 16S rRNA indicated Brazilian and Caribbean origins of the existing snail strains. Thirty-seven alleles were found to be segregating in the *sod1* locus. There was no specific link to resistance in snails carrying the previously identified *sod1* resistance-associated B allele. Mass spectrometry analysis of two differentially expressed protein bands from *B. glabrata* were identified to be partial haemoglobin and glutathione-S-transferase (GST-mu 1) and their possible involvement in cross-reactivity with parasite glycoconjugates is suggested. Identification of compatibility linked genes and/or gene products could find useful applications in development of therapeutic control agents for schistosomiasis.

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ABBREVIATIONS

1-D SDS-PAGE	One dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis
CTAB	Cetyl trimethylammonium bromide
DNA	Deoxyribonucleic acid
GST mu1	Glutathione-S-transferase mu1
HWE	Hardy Weinberg Equilibrium
KLH	Keyhole limpet haemocyanin
LC-MS	Liquid Chromatography-Mass Spectrometry
MS	Mass spectrometry
$NaIO_4$	Sodium metaperiodate
NCBI	National Centre for Biotechnology Information
LD-PCR	Limiting dilution-Polymerase chain reaction
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
rRNA	ribosomal Ribonucleic acid
SEA	Soluble egg antigen

CHAPTER 1

GENERAL INTRODUCTION

1.1 Interactions between organisms

The relationship between species, also known as symbiosis (i.e., living together), describes some of the most intimate interactions between organisms. These interactions take three major forms in which one species exploits the capacities of another in order to surmount its own physiological limitations (Douglas, 1994). These are commensalism and mutualism (in which case either or both organisms derive some benefit from the association) or parasitism which is an antagonistic relationship (Thompson, 1988). Overlaps between these relationships occur and it is sometimes difficult to tell when there is a shift from one form to another. For example, a shift from the usually commensal relationship to parasitism has been demonstrated for the symbiotic relationship between crayfish and branchiobdellid annelids (Brown *et al*., 2002, 2012).

1.2 Host - parasite symbiosis

Parasitism has been defined as a symbiosis in which an organism (usually the smaller), referred to as the parasite, inhabits and causes harm to another organism (the host) on which the parasite depends partially or completely for metabolic needs and resource acquisition (Raffel *et al*., 2008; Ukoli, 1990). Another major hallmark of the host-parasite relationship is the parasite's ability to elicit an immune response from the host (Dineen, 1963; Ukoli, 1990). This is an expression of the antagonistic nature of the

association and indicates that the relationship is potentially harmful to the host (Zelmer, 1998).

All organisms, simple or complex, typically possess the ability to distinguish between 'self' and 'non-self' components of their bodies, a feature which enables them prevent or eliminate invading species (Wakelin, 1997). Although the term 'parasites' can be generally used for all pathogens including viruses and bacteria, it is typically restricted to pathogenic eukaryotes, specifically protozoa and helminths. Parasites are ubiquitous and hence predisposed to a wide range of hosts. In addition, they are heterogeneous in nature and affect several aspects of their hosts' behavioural, physiological, biological and ecological framework. In the same vein, host organisms of parasites are heterogeneous in terms of their diverse resistance capacity, fitness and lifespan (Barrett *et al*., 2008).

1.2.1 Host-parasite study models

In the field of ecology and wild life management, parasites have been traditionally construed as benign symbionts that normally live in equilibrium with their host. However, they become harmful and could cause high mortalities to hosts when this general host-parasite balance is disturbed by unusual environmental factors (Hudson & Dobson, 1995). Parasites are thus considered major sources of evolutionary pressure. Despite their importance in evolutionary ecology, much of the genetic factors underlying host resistance and parasite infectivity are still unknown for many host-parasite associations. Mathematical models adopted for studying host-

parasite interactions have been useful in generally elucidating these interactions.

Two models often used in explaining host-parasite interactions include the Matching alleles (MA) and the Gene-for- Gene (GFG) models (Agrawal & Lively, 2003; Grech *et al*., 2006). The MA model is based on the self-nonself recognition pattern of animal immune systems and assumes that specific host and parasite genotypes are matched more or less in a lock-and-key pattern such that specific parasite genotypes are better at infecting certain host genotypes than others (Luijckx *et al*., 2013). Thus, MA models usually result in allele frequency dynamics characterized by shorter periods and higher amplitudes (Webster & Woolhouse, 1998). The GFG models are based on studies of plant disease and postulate that infection occurs if all the resistance alleles of the host is countered by a virulence allele of the parasite, hence the number of host genotypes that the parasite may infect depends on the number of virulence allele it has and this can range from one genotype to all (Kwiatkowski *et al*., 2012).

In real life studies, the assumptions of co-evolutionary models may be observed but are not universal (Grech *et al*., 2006). In the host-parasite association between the intestinal trypanosome *Crithidia bombi*, and its bumble-bee host, *Bombus terrestris*, for instance, parasite diversity is affected by resource competition between co-infecting parasites as well as host-driven immune-mediated competition (Ulrich & Schmid-Hempel, 2012). The genetic interaction between co-evolving species is quite complex and it has been shown that the degree of specificity between genotypes of interacting species is a major determinant of the outcome of evolution. The

observation that symbionts co-habiting with parasites may play a role in the host-parasite co-evolutionary arms race further reiterates the complex nature of interacting species (Kwiatkowski *et al*., 2012).

Interactions involving medically important human, parasitic organisms have also been useful in understanding various aspect of host-parasite interaction. Under a matching-allele model, infection is based on a recognition process between host and parasites. Human parasite species, often studied as host-parasite systems range from several species of insect-borne protozoans (micro-parasites) such as *Plasmodium sp*., *Leishmania sp*. and *Trypanosoma sp*. to various helminths (trematodes and nematodes) which form a vast majority of macro-parasites including major species such as *Schistosoma sp*. *Clonorchis sp*., *Ascaris sp., Ancylostoma sp.,* etc. (Hudson & Dobson, 1995; Ukoli, 1990).

Infections caused by parasitic protozoans and helminths remain a major source of health concern globally (WHO, 2013b). Among the parasitic trematodes, the trematode, *Schistosoma sp*. is the cause of a chronic, debilitating disease, schistosomiasis in man (Utzinger *et al*., 2009) and the disease remains a global health issue associated with economic and social burdens (Colley & Secor, 2014). Although the debilitating effects of the parasitic interaction between schistosomes and man historically dates as far back as the 16th century B.C, its interaction with molluscan hosts as the intermediate host used for the completion of asexual life cycle stages was not discovered until very early in the 20th century (Adamson, 1976; Sandbach, 1976). Since this discovery, various aspects of the association between snails and trematodes, especially schistosomes has stimulated

much study necessitating their culture and maintenance in laboratories (Adema *et al*., 2012). The association between snails and digenetic trematodes has however existed for not less than 200 million years and much remains to be elucidated about the variation and complexity that mystifies the snail-schistosome interaction (Blair *et al*., 2001).

1.3 The disease, schistosomiasis

Human schistosomiasis, also known as snail fever, is a chronic, debilitating disease caused by parasitic digenetic trematodes of the genus *Schistosoma* and spread by freshwater snail intermediate hosts (hence the name snail fever). It is the most widespread of all trematode infections of man, spanning up to 75 countries and regions in the tropics and subtropics, mainly in low and middle-income countries (LMICs) of the developing world (Hotez *et al*., 2014; Lockyer *et al*., 2008). Schistosomiasis is estimated to affect more than 200 million people in these tropical climate areas and remains highly prevalent in several parts of Africa, Asia and South America, despite decades of control and elimination efforts (Sokolow *et al*., 2016).

As at 2015, the global burden of schistosomiasis was estimated to be 3.5 million DALYs (Disability Adjusted Life Years) and more than 205 million people were reported to require treatment in up to 52 countries in 2016 (WHO, 2017). In endemic areas, morbidity and mortality among children and pregnant women resulting from disease complications have been reported (Odogwu *et al*., 2006; Stothard *et al*., 2011; WHO, 2013a). The scourge of schistosomiasis is so significant that it is ranked second only to malaria as a cause of severe disease and mortality due to a parasite

infection. With 700 million people estimated to be at risk of infection, only one drug (praziquantel) certified for effective treatment and no vaccine available, research directed towards schistosomiasis control and elimination is highly prioritized (Colley *et al*., 2014; WHO, 2012).

1.3.1 Forms of disease

So far, six major snail-schistosome interactions are well known for their involvement in transmission of the two human forms of the disease, intestinal and urogenital schistosomiasis (WHO, 2017). Each species of schistosome is specifically transmitted by a suitable range of snail hosts, hence, the distribution of disease is also determined by the habitat range of the molluscan intermediate hosts (Colley *et al*., 2014). Three of the six, namely *S. mansoni*, *S. japonicum* and *S. haematobium* are of immense public health importance as they are the major cause of human infections in areas affected by the disease (Gryseels *et al*., 2006). While *S. haematobium* causes urogenital schistosomiasis, all others affect the intestine and the liver, resulting in intestinal and hepatic schistosomiasis. A summary of the pathology and important epidemiological features, including the snail hosts involved in transmission of human schistosomiasis is presented in Table 1.1

Table 1.1: Schistosomiasis disease types, causative species and snail intermediate hosts, year described, adult worm localization/associated pathology, geographical distribution and prevalence of major human schistosomes. Table adapted using information from Olveda, 2013; WHO, 2013b.

Disease type	***Schistosoma* species**	Snail host	Year	Localization of adult worms and pathology	Geographical distribution	Prevalence
Intestinal schistosomiasis	*S. mansoni*	*Biomphalaria sp.*	1907	Localize in the portal mesenteric veins; liver, spleen & gastrointestinal tract involvement leads to portal & pulmonary hypertension; may be accompanied with abdominal pain & diarrhea.	Africa, the Middle East, Caribbean Islands, Brazil, Venezuela (now Bolivarian republic of Suriname)	Most prevalent; endemic in 55 countries; >200 million infected
	S. japonicum	*Oncomelania sp.*	1904	Capillaries of the superior and inferior mesenteric veins around the walls of the bowels; pathology similar to *S. mansoni*	China, Indonesia, the Philippines	Endemic; approximately 2 million people infected
	S. mekongi	*Neotricula sp*.	1978	Similar to *S. japonicum*	Cambodia and the Lao Peoples' Democratic Republic	Less common and locally restricted;
	S. intercalatum	*Bulinus sp.*	1911	Inferior mesenteric veins and portal venous system; low location of lesions in the rectum and sigmoid colon; mild liver pathology	Democratic Republic of Congo	Locally restricted to affected regions; estimated 1.73 million infections in 1999
	S. guineensis	*Bulinus sp.*	2003	Inferior mesenteric veins; cause microscopic lesions in the intestine (especially in the rectum), the liver and genital organs	Rain forest areas of Central Africa and West Africa	Focal prevalence often overshadowed by *S. haematobium* infections
Urogenital schistosomiasis	*S. haematobium*	Bulinus *sp*.	1852	Veins of the vesical plexus around the bladder and along the ureters; results in painless haematuria; chronic cases may lead to bladder cancer	Africa, the Middle East, Corsica (France)	Highly prevalent; endemic in 53 countries; >110million people infected

1.3.2 Epidemiology of disease

Although predominant in Africa, schistosomiasis also occurs in the Americas, the Eastern Mediterranean, South East Asia and the Western Pacific. The *Schistosoma* species vary geographically in distribution and this difference is based mainly on the ecology of the snail hosts involved in transmission (Betson *et al*., 2013; Thiele *et al*., 2013). The presence of a suitable, snail intermediate host is therefore a key factor in the distribution of schistosomiasis and a major indicator of risk populations in disease surveillance and monitoring (Colley *et al*., 2014; Knight *et al*., 2014).

Natural freshwater bodies such as streams, ponds and lakes as well as man-made reservoirs and irrigation systems, when contaminated with human waste, are key sources of infection (Gryseels *et al*., 2006). Epidemiology may also vary from one region or locality to another depending on several factors some of which include socio-economic factors, human behaviour such as sanitary habits, local occupational and cultural practices, ecological and biological factors relating to man and snail hosts, acquired immunity, government policies and provision of social amenities (Bruun & Aagaard-Hansen, 2008; Odogwu *et al*., 2006).

1.4 The parasite

Schistosomes, unlike other trematodes are dioecious worms, and even more unique in that the female is permanently held close to the male in a groove referred to as the gynaecophoral canal. Adult worm of pairs of

Schistosoma mansoni live in the mammalian host's portal blood specifically in the mesenteric veins around the lower colon (Cheever & Duvall, 1974; Olveda *et al*., 2013). Adult worms are cylindrical and can measure between 7-20 mm, although female worms are longer and thinner. The male worms are white while females are greyish in appearance. The worms possess two anteriorly located terminal suckers, a complex tegument, a closed digestive tract, and reproductive organs. The males have 6-9 testes while females possess an elongated, slightly lobulated ovary which is anteriorly located. Also, schistosome eggs are distinct from that of other trematodes because they are non-operculate. Eggs of *S. mansoni* bear a lateral spine. The mean life span of adult schistosomes is about 3-5 years but can be as long as 30 years (Gryseels *et al*., 2006). The heteroxenous life cycle of *Schistosoma mansoni* involves sexual maturation of the worms in a definitive host (man or other mammal) and the development of asexual stages in a suitable intermediate host (freshwater snails) (Ukoli, 1990).

1.4.1 Sexual stages in the mammalian definitive host

The infective stages, the cercariae, are released from infected snails. These larval stages swim in water till they locate and successfully penetrate the skin of a suitable host. After successful skin penetration they lose their bifurcated tail and transform into young worms known as schistosomula (Al-Adhami *et al*., 2005). Within the first few days after infection and penetration of a capillary, the schistosomula are passively transported through the body along with blood circulation and are thereby passed repeatedly through the lungs and other systemic organs. Schistosomula are reportedly short and round shaped at day 2-5, which marks the early lung

phase of migration, however, from the 5th day, they assume a more elongated, thin shape. These are carried in the blood through the lungs to the liver (Wheater & Wilson, 1979). Young adults can be detected in the lungs by the 2nd or 3rd day post penetration and by the 15th day, they are found accumulated in the liver. In about 23 days, they migrate to the mesenteric veins where sexual maturation is attained and mating occurs. The paired worms become settled in the peri-vesical or mesenteric venous plexus and commence egg production by the 40th day (Cheng, 1986; Wheater & Wilson, 1979). Eggs are passed out to the exterior in faeces. However after extravasation, a considerable number of eggs also get lodged in tissues leading to pathology of the disease. When eggs are released into vertebrate tissues, granulomas are formed around them. These granulomas allow penetration of the eggs into the gut lumen for subsequent exit from the body in faeces (Doenhoff *et al*., 1986).

1.4.2 Asexual stages in the snail intermediate host

When eggs are released by the definitive host can remain viable for up to seven days. When the parasite eggs come in contact with water, hatching takes place and a free swimming miracidium is released from each egg. Using light and chemical stimuli, the miracidium locates and penetrates a suitable, freshwater snail intermediate host – a pulmonate snail of the genus Biomphalaria. In the region of the foot or tentacles of the snail, the miracidium transforms into a mother sporocyst and from the germ balls in the latter, daughter sporocysts are differentiated. In laboratory cultures, second generation daughter sporocysts have also been reported (Hansen *et al.*, 1974a; Hansen *et al*, 1974b; Bixler *et al.*, 2001). Also, Jourdane and

Theron (1980) have demonstrated production of new generations of sporocysts by surgical transplant of daughter sporocysts into uninfected snails (Jourdane, 1990; Jourdane & Theron, 1980).

Daughter sporocysts migrate to the host's digestive glands about 12 days after penetration of the miracidium into snail and begin to produce large numbers of furcocercous cercariae. These are shed by the snail from 4-6 weeks after infection and become free swimming in water. These free swimming cercariae must locate and penetrate the skin of a suitable definitive host (usually mammalian), within 72 hours to ensure the continuity of the cycle (Gryseels *et al*., 2006). Figure 1.1 shows the life cycle of the three major human schistosomes.

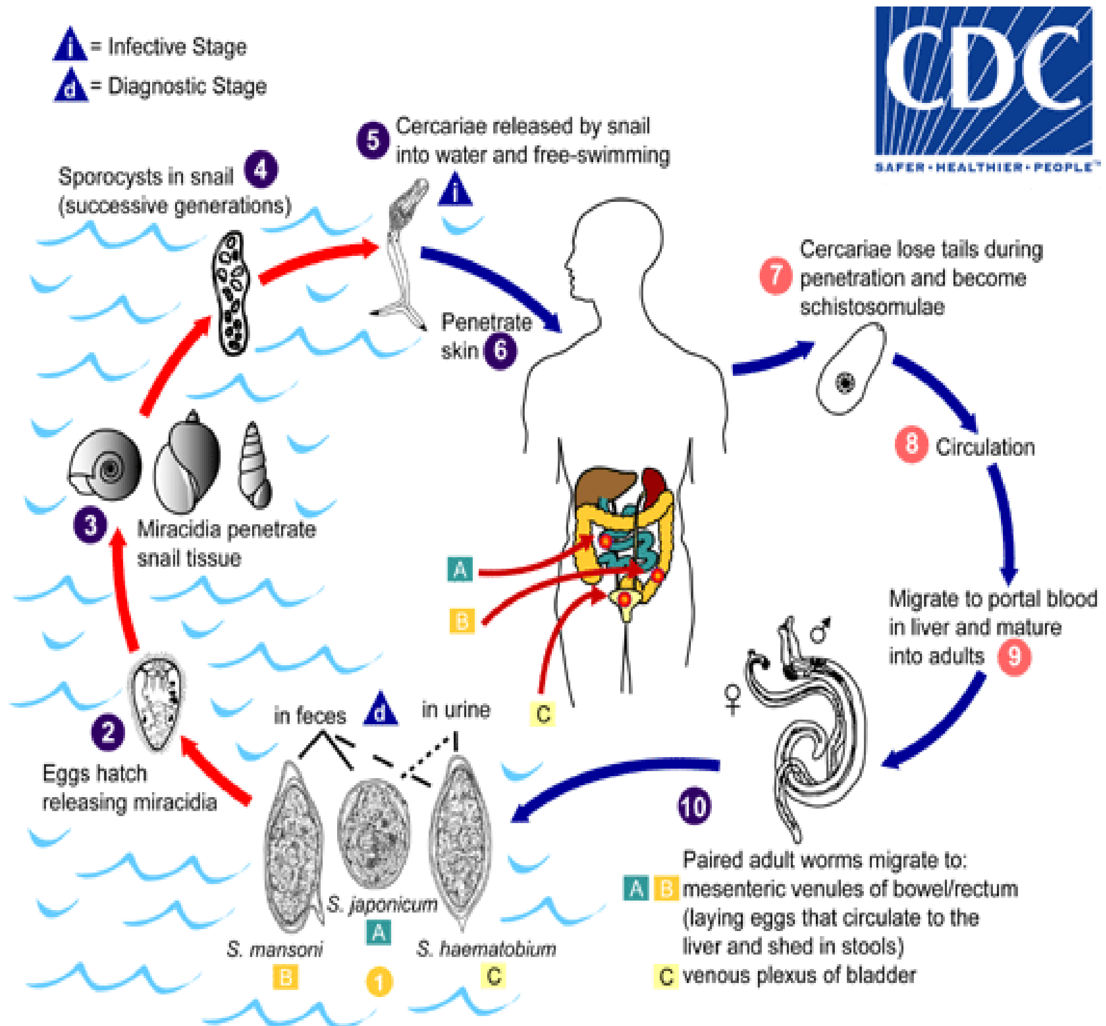

Figure 1.1: Life cycle of the three major Schistosoma species.

Source: http://www.cdc.gov/parasites/schistosomiasis/biology.html

1.5 The snail host

A vast number of gastropod snails serve as obligatory, first intermediate hosts to over five thousand species of digenetic trematodes (van der Knaap & Loker, 1990). As reported by Dejong and colleagues (DeJong *et al*., 2001), the genus *Biomphalaria,* Preston, 1910 includes thirty-four described species spanning tropical America, Africa and the Arabian Peninsula. These species vary in their roles in transmission of *S. mansoni*. Eighteen species are considered natural transmission agents of the parasite, nine are known to be refractory and the transmission status of the remaining seven have not been ascertained (Brown, 1994; Malek, 1985).

The genus *Biomphalaria* is restricted to the tropical and subtropical regions with 22 species found in the Americas and 12 species distributed across Africa, Madagascar and the Middle East. Initially, the *Biomphalaria species* were thought to have originated in Gondwanaland as far back as 100 million years ago, but considering the continental split that took place more than 70 million years back (Davis, 1980; DeJong *et al*., 2001; Pilsbry, 1911), it is thought that there should have been a distinct separation of the South American and African species. The suggested presence of *S. mansoni* before the continental split could also account for its presence in both regions (DeJong *et al*., 2001).

Allozyme-based phylogenetic studies carried out to ascertain the lineage of *Biomphalaria* in relation to the two widespread species, *B. glabrata* and *B. pfeifferi* have been contradictory. Ancestral inferences by authors have either reported *B. glabrata* as ancestral to *B. pfeifferi* (Bandoni *et al*.,

1995b; Campbell *et al*., 2000) or on the contrary, that the American species are of recent African origin (Woodruff & Mulvey, 1997). *Biomphalaria glabrata*, is currently considered to be a sole sister taxon to all African species of *Biomphalaria*. It has been suggested that *B. glabrata* or a similar ancestor of the species colonized Africa and produced all other African species including the widespread *B. pfeifferi* (Campbell *et al*., 2000; Woodruff & Mulvey, 1997). Thus, a trans-Atlantic colonization of Africa is now popularly accepted with the view that all the African species arose from a proto-*Biomphalaria glabrata* that was carried across the Atlantic presumably as juvenile snails or eggs by migratory birds more than 2 million years ago (Dejong *et al*., 2003; DeJong *et al*., 2001; Morgan *et al*., 2001).

Despite these distributions, the ability of snails to spread beyond their native domain has been reported. Documented instances include the introduction of *B. glabrata* in Egypt (Yousif *et al*., 1996) and *B. tenagophila* in Africa and Europe (Majoros *et al*., 2008; Pointier *et al*., 2005). Inadvertent introductions of snails to new territories coupled with globalization, travelling and climate change are important factors that could increase the spread of schistosomiasis, ultimately increasing the global risk of infection (McCreesh *et al*., 2015). For instance, introduction of perennial irrigations have led to the introduction of snail hosts of the schistosomiasis parasite in previously unaffected areas (Abou-El-Naga, 2013).

1.6 *Biomphalaria glabrata* Say, 1818

Although several *Biomphalaria species* can act as intermediate hosts of *Schistosoma mansoni*, *Biomphalaria glabrata* is the major host of this parasitic trematode in the Western hemisphere (Dejong *et al*., 2003).

Biomphalaria glabrata belongs to the second largest phylum of the animal kingdom, the phylum Mollusca. A representative of the Basommatophora of the Order Pulmonata, members are indigenous to freshwaters of South America and some of the Caribbean Islands. As with other species of the Class Gastropoda, these pulmonates are quite adaptive in their freshwater range of habitats. They are found in streams, ditches, wells, water reservoirs, swamps, lagoons and can survive at sewage dumping sites where they reportedly proliferate in abundance. With urbanization, their range of conducive habitats has been further increased by the development of several water resources schemes (Bayne, 2009; Jurberg *et al*., 1997; Scherrer *et al*., 1976).

1.6.1 General morphology and biology of *B. glabrata*

Biomphalaria glabrata shells are discoid and sinistral. Like other members of the subfamily Planorbinae, *B. glabrata* shells are plano-spiral and is carried upside down and therefore appears dextral, with a rounded to oval aperture. *Biomphalaria glabrata* is the largest of the genus Biomphalaria, with shell size range of 15-30 mm and sometimes reaching 40 mm and 5-6.5 whorls (Jarne *et al*., 2011). Like other Basommatophora, *B. glabrata* is hermaphroditic, thus capable of self-fertilization however, *B. glabrata* snails preferentially cross-fertilize (Dejong *et al*., 2003). Eggs are laid at high rates and a snail is capable of laying 14,000 eggs in its life span. Eggs are laid in gelatinous masses, usually on submerged vegetation and other surfaces (thus preventing desiccation). Each mass may contain 30-40 eggs (Eveland & Haseeb, 2011). Generation time is 4-6 weeks and life span ranges from 18-24 months under laboratory conditions and 15-18 months

in natural habitat. Egg production has been reported to be higher at 20-27.5°C when compared with production at 17.5°C and to be greatly reduced in constant darkness (Barbosa *et al*., 1987; Pimentel-Souza *et al*., 1990).

1.6.2 Laboratory-developed strains of *B. glabrata*

Biomphalaria glabrata is one of the most intensely studied species of freshwater snails (Mavarez *et al*., 2002). Various stocks of *B. glabrata* have been developed from field collected samples from various geographical locations and maintained in research laboratories for diverse study purposes (Pointier & Théron, 1995; Prugnolle *et al*., 2006). Field isolates and laboratory bred snails are often selected for traits such as resistance or susceptibility to parasite and pigmentation (Arijo *et al*., 2001; Lewis *et al*., 2003; Richards & Shade, 1987). Some well-known *B. glabrata* strains and isolates that have been used in various studies identified in published literature are described in Table 1.2.

Table 1.2: Names, origin and phenotypes of field isolates and laboratory strains of *Biomphalaria glabrata*.

Snail strain/isolate	Phenotype designation	Origin	Literature citation
NHM3032/BB02	Susceptible	Derived from a Belo Horizonte field isolate	http://biology.unm.edu/Biomphalaria-Genome/BB02STRAIN.html
NHM1742	Susceptible	Lab-derived from BB02	Adema *et al.*, 2006; Lockyer *et al.*, 2012
NHM3036	Susceptible	Lab-derived from BB02	Adema *et al.*, 2006; Lockyer *et al.*, 2012
M-line	Susceptible	Lab-derived	Lewis *et al.*, 2001
NMRI	Susceptible	Lab-derived	Lewis *et al*, 2001
BS-90	Resistant	Isolated from Salvador, Brazil	Paraense and Corrêa, 1963; Ittiprasert and Knight, 2012
NHM3017	Resistant	Derived from BS90	Paraense and Corrêa, 1963; Lockyer *et al.*, 2012
10-R2	Resistant (partially)	Derived from the M-line	Richards, 1973; Mulvey and Bandoni, 1994; Knight *et al.*, 1999
13-16-R1	Resistant	Derived from crosses of Brazil and Puerto Rico highly resistant isolates	Richards and Merritt Jr., 1972; Bonner *et al.*, 2012

1.6.3 *Biomphalaria glabrata* genome

Biomphalaria glabrata has a haploid number of 18 chromosomes and based on Feulgen image analysis densitometry of haemocyte samples, the snail has an estimated genome size of 931 Mb (Gregory, 2003). This size is about three times smaller than that of the human genome of 3,000 Mb (Venter *et al*., 2001). It is however about three times larger than that of the 270 Mb *S. mansoni* genome contained in 8 pairs of chromosomes (LoVerde *et al*.,

2004). Investigations have however shown extensive aneuploidy in two *B. glabrata* cell line isolates to such an extent that the total complement of chromosomes in both greatly exceeds the original cell line's diploid number of 36 chromosomes (Odoemelam *et al*., 2009). The full-length sequence of the mitochondrial genome of *B. glabrata* is 13,670 nucleotides and is the smallest mitochondrial genome characterized from molluscs so far. This includes 13 protein-coding genes, 2rRNA genes and 22 tRNA genes (DeJong *et al*., 2004).

1.7 Snail resistance or susceptibility to infection

As stated by Locker *et al*, the term 'resistant' can be applied to individual snails within a single species that are able to evade infection by a species or strain of schistosome that is normally capable of parasitizing that snail species (Lockyer *et al*., 2007). Based on this definition, *Biomphalaria glabrata* snails are termed either 'resistant' or 'susceptible' to *S. mansoni* depending on the outcome of each snail-schistosome encounter. Published studies using various field isolates and laboratory developed strains of *B. glabrata* have demonstrated that susceptibility to parasite differs between different strains of *B. glabrata* (Mulvey & Vrijenhoek, 1981; Paraense & Correa, 1963; Prugnolle *et al*., 2006; Saoud, 1965; Teofânia *et al*., 1998).

The molecular processes involved are complex and remain a source of interest to researchers for better understanding of host-parasite interactive processes (Knight *et al*., 2014). The possibility of genetic control was first proposed in 1954 as a future control strategy for schistosomiasis (Hubendick, 1958). This proposal was based on revelations from early studies (e.g., Files and Cram, 1949; Newton, 1953) which indicated that

snail hosts varied in susceptibility to schistosome parasites. Thus, the possibility of driving resistant genes into natural snail populations of *B. glabrata* remains a subject of genetic research with focus on identifying resistance genes in the snails (Allan *et al*., 2017; Mulvey & Woodruff, 1985; Tennessen *et al*., 2015). The strategic release of resistant snail populations into transmission foci, in effect, could lead to their successful integration and off-set the number of susceptible snails. This should reduce the number of susceptible snails and consequently lead to reduction in disease transmission in human hosts (Woodruff, 1985).

1.7.1 Genetic factors in snail-schistosome interaction

Intra-specific, heritable differences in *B. glabrata* have been demonstrated since the 1950s with Newton's report on geographical differences in host-parasite compatibility (Newton, 1953). His study showed that snail isolates of Puerto Rican origin were highly susceptible to parasites in contrast to isolates of Brazilian origin that were extremely refractory. Crossbreeding of these two snails revealed that snail susceptibility to the parasite is a heritable trait (Newton, 1953). These findings have since been supported over the years by other investigators (Basch, 1975; Coupel *et al*., 2015; Dias *et al*., 1988; Theron *et al*., 2008).

Also, in one of the pioneer, genetic studies on susceptibility of snails to *S. mansoni* infection (Newton 1955), the albino phenotype was linked to elevated susceptibility to infection by a Puerto Rican strain of the parasite; hence, a pattern of polygenic inheritance was proposed for this phenotype (Newton, 1955; Newton & Von Brand, 1955; Spada *et al*., 2002). Resistance

has been found to be a dominant character in many *Biomphalaria* species, however, susceptible snails may also carry certain alleles for resistance (El Naga *et al*., 2010; Mascara *et al*., 1999). Similarly it has also been suggested that the genetic regulation of susceptibility and resistance is such that some level of susceptibility actually remains in resistant snail populations (Carton *et al*., 2005; Richards & Merritt Jr., 1972).

Also, while resistance to infection in *B. glabrata* adults (e.g. the BS-90 strain which is known to be a highly resistant population) is due to a single dominant gene trait that is heritable by simple Mendelian genetics, in the juveniles it is a more complex trait controlled by five or six genes, each with multiple alleles (Lewis *et al*., 2003; Richards & Shade, 1987). Furthermore, the ability of *B. glabrata* to resist or succumb to *S. mansoni* infection is determined by the genetics of both the snail and the parasite and research have shown that the molecular basis of this complex relationship involves various genes associated with cellular and humoral functions such as receptor recognition, immune regulation, cell adhesion, stress responses, etc. (Ittiprasert *et al*., 2010; Knight *et al*., 2014).

1.7.2 Physiological and environmental factors in snail-schistosome interaction

In addition to the genetics of snail susceptibility, the physiological state of an animal could be affect its vulnerability to infection. Factors such as snail age and health and/or environmental conditions may therefore be a trigger for susceptibility. For example, susceptibility of *B. glabrata* snails to *S. mansoni* is age-dependent and adult snails have been shown to be

generally less susceptible than juveniles (Théron *et al*., 1998). It has also been demonstrated that carbohydrate depletion occurs during infection and starvation of *B. glabrata* snails due to nutrient utilisation by the parasite (Christie *et al*., 1974). In a recent study by Nelson *et al*, starved individuals were shown to have higher haemocyte counts compared to controls, however neither starvation nor increased temperature appeared to affect a resistant Salvador strain of the snails; indicated like previous authors that abnormal environmental conditions such as temperature could have both stimulatory and inhibitory effects on the internal defence system in juvenile and adult *B. glabrata* snails (Knight *et al*., 2015; Nelson *et al*., 2016).

1.7.3 Internal defence system of *B. glabrata*

According to Loker and Bayne, schistosome infection of a snail host depends solely on non-self-recognition by the snails' innate defence system (Loker & Bayne, 1982). Three major components have been associated with the internal defence responses of *B. glabrata* to larval trematodes viz., the haemocytes, soluble haemolymph factors and the sporocyst surface (Bayne *et al*., 1985; Granath Jr. *et al*., 1987). The haemocytes constitute the major cellular components of the snail's mechanism for defence and play a major role in recognition and destruction of larval stages of the parasitic trematode, *S. mansoni*.

In conjunction with humoral factors, these cellular effectors are able to recognize the invading parasite, adhere to its surface, develop a multi-layer cellular capsule and eliminate the parasite after cytotoxic activation. Humoral factors identified in *B. glabrata* include pattern recognition

receptors such as thioester containing protein, cytotoxins such as Biomphalysin (Galinier *et al*., 2013) and the variable immunoglobulin and lectin domain-containing molecules (Wu *et al*., 2017). In contrast, in susceptible snails there appears to be failure of haemocytes to detect and mount an effective cytotoxic response against invading larval stages as observed in their resistant counterparts (Mitta *et al*., 2005; van der Knaap & Loker, 1990). Differential responses of snail haemocyte to *S. mansoni* has also been demonstrated for *B. alexandrina,* a known intermediate host of *S. mansoni* in Egypt (Abou-El-Naga & Radwan, 2012; Mohamed *et al*., 2011).

Furthermore, plasma factors (Bayne *et al*., 1985; Galinier *et al*., 2013), fibrinogen-related proteins (FREPs), (Adema *et al*., 1997) and cytokine-like components (Granath Jr. *et al*., 1994) have also been shown to contribute to resistance in the snails. Studies have also acknowledged an unexpected detection of reverse transcriptase (RT) activity in *B. glabrata* but the part this enzyme plays in the haemocyte-mediated killing of parasites is as yet unknown. Indeed the factors that govern the level of snail host resistance and parasite infectivity are yet to be fully uncovered (Mitta *et al*., 2017; Raghavan *et al*., 2003).

Identification of cDNAs similar to immune genes such as peptidoglycan recognition proteins (PGRPs), macrophage migratory inhibitory factor (MIF), allograft inflammatory factors (AIF), and lipopolysaccharide binding protein/bactericidal permeability-increasing protein (LBP/BPI)-like genes in *B. glabrata* further inspired more studies on these genes which may play a functional part in snail-parasite interactions (Mitta *et al*., 2005).

Furthermore, it is now known that reactive oxygen species (ROS), nitric oxide and hydrogen peroxide in particular, mediate sporocyst killing in *B. glabrata* snails (Hahn *et al*., 2001b, 2001a). However, the precise mechanism by which this killing occurs has not been determined. Also, it has been demonstrated that the ROS of the host snails and ROS scavengers of the parasite work counteractively in a snail-schistosome encounter (Moné *et al*., 2011). Studies have further shown involvement of the Cu/Zn superoxide dismutase 1 (*sod1*) gene and alleles of this gene have been linked to susceptibility and resistance in the snails (Goodall *et al*., 2006). The presence of these alleles and the link to resistance or susceptibility in other *B. glabrata* snail populations is however not known.

Another important area of consideration for variation and immune defence and evasion in *B. glabrata* is the glycosylation of proteins involved in the immune response to the parasites. Glycosylation involves the modification of proteins post-translationally. It is an enzymatic reaction that results in the glycosidic linkage of sugars/carbohydrates to proteins (Lisowska & Jaskiewicz, 2001). The process of protein glycosylation is quite complex and highly dynamic. There are three main types of glycosylation, classified based on covalent attachment of oligosaccharide chains either to asparagine residues (N-glycosylation). This normally occurs at an asparagine residue following the consensus sequence N-X-S/T/C where X can be any amino acid except proline). The other two types of glycosylation involves the covalent attachment of oligosaccharide chains to serine or threonine residues (O-glycosylation) or to tryptophan residues (C-glycosylation).

Much of the immune recognition or immune responses to parasites in infected human or animal hosts are directed towards carbohydrate (glycan) determinants within the cell surfaces and secreted glycoconjugates; glycoconjugates are therefore important in host-parasite interactions (Nyame *et al*., 2004). Protein glycosylation in snails is therefore considered to be relevant in immune interactions between snail hosts and parasites, as is known to be the case in other host-pathogen systems (Stepan *et al*., 2012).

As demonstrated by Eberl and colleagues, glycans are major recognition targets of induced antibody production in schistosome infections (Eberl *et al*., 2001). Studies have shown that the penetration behaviour of larval miracidia in both resistant and susceptible snails for normal and irradiated parasites does not differ (Ittiprasert *et al*., 2009). There is however, growing evidence that snail susceptibility or resistance might also depend on whether or not the infective stage, the miracidium, expresses proteins and lipids that correspond to carbohydrate structures on the snail's surface (Peterson *et al*., 2009; Théron & Coustau, 2005).

The role of glycan structures of both parasite and snail in the snail-parasite encounter is also worthy of investigation as a possible basis of the differences between snail phenotypes. Knowing that glycosylation plays a key role in many types of recognition processes associated with fertilisation and development, hypersensitivity reactions, disease pathologies and cell death, these epitopes could be important molecules for future therapeutics development. Snails in particular show a broad spectrum of glycosylation abilities within their N-glycosylation patterns and this has been reported to

be a part of the host-parasite recognition process (Lehr *et al*., 2008; Staudacher *et al*., 2009). Furthermore it has been demonstrated that carbohydrate moieties on glycoproteins could play a protective role by preventing attack of free radicals on the polypeptide chains to which they are attached (Martinek *et al*., 2010). Identification of glycoproteins in the snail-schistosome host-parasite systems could further elucidate the roles of glycan epitopes in the differential defence response between *B. glabrata* snails following exposure to *S. mansoni*.

The elucidation of molecular mechanisms responsible for variability in host snails in terms of susceptibility or resistance to *S. mansoni* infection is important for understanding interactions in this host-parasite system and the possible pathological implications for the invertebrate hosts. The knowledge of host-parasite dynamics of the snail-schistosome association may find useful applications in epidemiology and control of schistosomiasis especially for the formulation of strategies and approaches for disease control. Considering the intricacies of the snail-schistosome interaction, it is of paramount importance not only to identify the factors that come into play between the snail's innate defence system and the parasite's evasion strategies, but also to understand better the pathways involved as these interactions determine success or failure of an infection (Lockyer *et al*., 2007). The interplay of these factors (both genetic and epigenetic) therefore requires continuous investigations of various parameters that can be observed under controlled experimental conditions.

1.10 Hypothesis and aims of study

The interaction between *B. glabrata* and *S. mansoni* is crucial in determining the successful transmission of the parasite to its definite host. While some strains are susceptible and are therefore able to support the parasite's development, others are resistant and hence the life cycle reaches its dead-end. This outcome has been shown to be determined by the genes of both parties (Spada *et al*., 2002) and could vary with snail age (Ittiprasert *et al*., 2010) and even environmental conditions (Ittiprasert & Knight, 2012). A key aspect of this interaction is therefore the molecular, genetic compatibility between the organisms. Also important in this snail-parasite encounter is the presence and expression of carbohydrate determinants common to both snail and parasite (Lehr *et al*., 2007, 2008). These have been shown to vary between susceptible and resistant snails and also possibly play a role in the modulation of the intermediate host's immune response and/or parasite immune evasion (Lehr *et al*., 2010). *Biomphalaria glabrata* snails used as intermediate hosts for the maintenance of the parasite life cycle have been in laboratory culture for several decades. To further elucidate molecular differences between susceptible and resistant snails, the study uses this long established host-parasite model system to investigate molecular markers associated with resistance and susceptibility in *B. glabrata*.

First, the study determined variations in *B. glabrata* susceptibility and *S. mansoni* infectivity using a multi-strain, host-parasite approach. The objective was to test if co-evolved hosts and parasites were better adapted to each other in this host-parasite system. In addition to data obtained from

experiments performed during the present study, data generated prior to commencement of the present study were collated and analysed to investigate the long-term pattern of compatibility between both organisms.

Secondly, to better understand the phylogeographical background of the existing laboratory snails stock, the study attempted to establish their phylogenetic links to other *B. glabrata* snails from published, data-based references using the 16S ribosomal RNA mitochondrial gene. Defining laboratory populations of *B. glabrata* could help clarify their genetic background, identify similar stocks in different laboratories and aid research validation.

Thirdly, the study characterised *B. glabrata* snails in terms of the alleles of the cytosolic copper-zinc superoxide dismutase 1 (*sod1*) gene which has been linked to susceptibility/resistance in the snails. The principal objective was to determine if alleles of the *sod1* gene in our laboratory populations of *B. glabrata* could also be linked to resistance and/or susceptibility in the snails. Exploring the allelic variations at this locus will help to determine reliability of the *sod1* as a marker for susceptibility/resistance in *B. glabrata* snails. The correlation between alleles identified and compatibility phenotypes of the snails were thus investigated by conducting infection experiments. Specifically, the compatibility phenotypes of the snails and the connection to the resistance-linked *sod1* allele (*sod1B*) was determined.

Fourthly, the study further aimed to characterise the total haemolymph and tissue proteins of predominantly resistant and susceptible laboratory populations of *B. glabrata* based on expression of carbohydrate epitopes cross-reacting with *S. mansoni* glycoconjugates. The first objective was to

determine if expression of cross-reacting proteins differed between resistant and susceptible snail populations. The second was to test the sensitivity of cross-reacting glycan epitopes to periodate oxidation, and the third was to identify cross-reacting glycoproteins by mass spectrometry. Identification of shared glycoproteins differentially expressed in host snails could provide useful insight to the possible roles of these molecules in the relationship between host snail and the trematode parasite.

CHAPTER 2

GENERAL MATERIALS AND METHODS

This chapter first gives a synopsis of experimental animals used in the course of this study. Since one aspect of the study involved analysis of both current and previous data, historical information on snails and parasites from which previously generated data were obtained are also provided. Parasitological methods employed in the laboratory maintenance and handling of experimental animals are also described in detail. Other materials and methods related to the molecular aspects of the study are presented in the appropriate result chapters.

2.1 Parasitology methods

2.1.1 Experimental animals

Experimental work and all data generated or analysed for this thesis required maintaining the heteroxenous life cycle of the parasitic trematode, *Schistosoma mansoni*. For laboratory maintenance purposes, the two-host transmission cycle of *S. mansoni* was kept actively in *Biomphalaria glabrata* snails (intermediate host), and CD1 strains of laboratory mice (representative definitive host). The historical records of all laboratory strains of *B. glabrata* and *S. mansoni* and the investigations in which they have been applied in this thesis are presented in Table 2.1. In this thesis, laboratory maintained snail stocks are referred to as strains and the term 'isolates' is used when referring to snail samples obtained directly from field collections with reference to published studies.

Table 2.1: Names, origin and brief history of laboratory strains of *B. glabrata* and *S. mansoni* and time period in laboratory culture. Strains used in the various chapters reported are shown. Sympatric snails and parasites (similar origin) are outlined as pairs; † indicates chapters in which previous and recent data were included in analysis. Asterisked year indicate the parasite was phased but snail was maintained till second date.

Snail strains & origin	Parasite strains & origin	Brief history of snail and parasites sources/affiliations	Period of snail maintenance (year from & to)	Applicable result chapter(s) of thesis
Bg-PR Puerto Rico	Sm-PR(or PR-1) Puerto Rico	The life-cycle of this albino snail line and its parasite was established in the UK in 1962 by S.R. Smithers of the National Institute of Medical Research (NIMR, Mill Hill, London). The snails were supplied by Bill De Witt (from the National Institute of Health, USA) along with the PR isolate of the parasite (SmPR); then to the London School of Hygiene and Tropical Medicine (LSHTM, Winches Farm Field Station, St. Albans). This snail isolate was originally established by Newton in the 1950s (Smithers & Terry, 1965).	1991-2017	Chapters 3†, 4, 5 & 6
Bg-Br Brazil	Sm-Br Brazil	Obtained from laboratory culture of the parasite (Sm-Br) and pigmented snails (Bg-BR) from Brazil Pernambico; established first in Brazil in the 1970s by Col. W. Radke, then life-cycle established from imported material at the LSHTM in 1978.	1991-2002	Chapters 3†
Bg-Eg Egypt	Sm-Eg Egypt	Pigmented snail (Bg-Eg) and parasite (Sm-Eg) obtained from Behring Research Institute, Cairo, Egypt. Life cycle established from imported material at LSHTM in 1980.	1991-2006	Chapters 3†
Bg-GP Hybrid	Sm-GP Hybrid	A pigmented snail line (hybrid) developed by pooling genetic material of the three geographical snail isolates listed above, BgPR, Bg-Br & Bg-Eg**.** This snail line was established at the University of Wales, Bangor in 1992. Hybridization was confirmed to have occurred by interbreeding these three snail isolates (Arijo, 1997). The corresponding parasite line, Sm-GP was developed from pooled genetic material of four geographically different *S. mansoni* strains from viz, Puerto Rico, Brazil Pernambico, Egypt and Kenya (Sm-PR, Sm-Br, Sm-Eg & Sm-Ken-O).	1992-2017	Chapters 3†
Bg-BH Belo-Horizonte, Brazil	Sm-BH Belo-Horizonte, Brazil	A pigmented snail isolated with the parasite, Sm-BH in Belo-Horizonte in 1967. Both supplied to the University of Wales, Bangor (by Prof. W. Hass, University of Erlangen, Germany) in 1994 where the parasite life cycle was maintained in this snail host and mice.	1994-2006	Chapters 3†
Bg-Cmp Campinas, Brazil	Sm-Cmp Campinas, Brazil	Originally, both parasite (Sm-Cmp) and albino snail (Bg-Cmp) were isolated by Paraense in 1992 from Belo Horizonte State of Minas Gerais. Both were then obtained by Prof. Maegaelhas, Campinas, Brazil who supplied them to Prof Hass, University of Erlangen, Germany for the establishment of life cycle. It was subsequently supplied to the University of Wales, Bangor 1n 1994.	1994-2004*, 2017	Chapters 3†
Bg-Abrd Brazil	Sm-Abrd Brazil	This pigmented snail, Bg-Abrd (originally from Brazil) and its parasite, Sm-Abrd (from Egypt) were supplied by Dr. L. Chappel of the University of Aberdeen.	1991-2004	Chapters 3†

Table 2.1 (continued): Names, origin and brief history of laboratory strains of *B. glabrata* and *S. mansoni* and time period in laboratory culture. Strains used in the various chapters reported are shown. Sympatric snails and parasites (similar origin) are outlined as pairs; † indicates chapters in which previous and recent data were included in analysis. Asterisked year indicate the parasite was phased but snail was maintained till second date.

Snail strains & origin	Parasite strains & origin	Brief history of snail and parasites sources/affiliations	Period of snail maintenance (year from & to)	Applicable result chapter(s) of thesis
Bg-Sen Unknown	Sm-Sen Senegal	Obtained from the LSHTM, the geographic origin of this pigmented *B. glabrata* (Bg-Sen) is unknown but the parasite Sm-Sen had been exclusively maintained in this snail by Dr. R.F. Sturrock for a short period before it was passed on to the University of Wales, Bangor for the establishment of the life-cycle. Note that Sm-Sen was isolated from field infected snail, *B. pfeifferi* snails in 1994.	1994-2004	Chapters 3†
Bg-Swan Unknown	No sympatric parasite	This pigmented snail (Bg-Swan) was supplied by Dr. Brian James, University of Wales, Swansea. The exact origin of this isolate is also unknown.	1991- 2006	Chapters 3†
No sympatric snail	Sm-Sen47 & Sm-Sen245 Senegal	These two parasite lines were isolated in 1994 from patients in Senegal. They were obtained from Dr Southgate, NHM London for research purposes at the University of Wales, Bangor.	1994-2004	Chapters 3†
No sympatric snail	Sm-Ken-O Kenya	This isolate was obtained from Prof. A Butterworth in the early 1980s without a snail host. It was however passaged successfully through the Bg-PR isolate (listed earlier).	1991- 2005	Chapter 3†
No sympatric snail	Sm-Ken-N Kenya	This parasite of Kenyan origin, was isolated from field collected *B. pfeifferi* snails in 1994. It was also provided by from Prof. A.E Butterworth, Cambridge University. The life cycle was maintained in the Bg-GP snails.	1994-2004	Chapter 3†
BgSV Salvador, Brazil	No sympatric parasite	These pigmented snails, obtained in 2002 are of Brazilian origin (Salvador, Brazil) and known to be a predominantly resistant population, possibly derived from the BS-90/13-16-R1 snail stock.	2002-2017	Chapters 3†, 4, 5 & 6
Bg-PS Hybrid	No sympatric parasite	These pigmented hybrids were derived from the inbreeding of four most susceptible isolates with overall susceptibility >90%. These included a snail isolate from Belo Horizonte (Bg-BH), one of unknown origin (Bg-Swan) and two isolates of Egyptian origin. Susceptibility of this hybrid is always ≥90%	2005-2017	Chapters 4, 5 & 6
Bg-Duss Unknown, possible hybrid	Sm-Duss Liberia	The Sm-Duss is a Liberian strain of *S. mansoni* received from Professor C. Grevelding of the University of Duesseldorf in the late 1990s. This parasite strain was originally obtained from the Bayer company laboratories where it had been maintained for half a century (Grevelding, 1995). The Bg-Duss snail isolate, also donated by Prof Grevelding, was originally from *B. glabrata* albino snail stocks also obtained from the Bayer Company laboratories. However, during the time it was cultured in Duesseldorf it was supplemented with *B. glabrata* stock from several other German and UK laboratories, hence resulting in a genetic mix. After it was acquired by Professor Doenhoff the Parasitology lab group, it has been used for the passage of the Sm-Duss parasites throughout the period of culture in our laboratory.	1998-2010	Chapters 3†

2.1.2 Laboratory culture and general maintenance of snails

The snail room and tanks were kept in optimal conditions as recommended for laboratory breeding of freshwater snails and following established snail maintenance procedures (Arijo, 1997; Chernin & Schork, 1959). *Biomphalaria glabrata* are tropical freshwater species, hence, the snail room temperature was kept at 26 ± 2°C and lighting was permanently auto-set to alternate a daily 12-hour light and 12-hour dark periods. Snails were kept in transparent, 12-litre capacity, plastic tanks each containing 10 litres of deionized water and each tank was clearly labelled. Tanks were constantly oxygenated via an aeration system with each tank provided with a sintered air filter. To avoid the development of thin shelled snails due to low pH of water, inorganic salts were added into each tank weekly. This salt solution was composed of calcium carbonate (100 g), magnesium carbonate (10 g), sodium chloride (10 g) and potassium chloride (2 g) dissolved in 3 litres of water. Growth of microorganisms in the tanks was kept at bay with a population of water fleas (*Daphnia sp*.). Cleaning of tanks was carried out as necessary, determined by regular visual inspections for accumulated debris. Dead snails (if any), were also removed from tanks.

2.1.2.1 Breeding tanks and juvenile tanks

Snail strains were bred in separate tanks each containing 20–30 snails measuring 16-20 mm shell diameter. Juveniles of 5-6 mm were harvested at intervals of two weeks. These were kept in separate tanks (juveniles tanks) for each snail strain till they reached the desired shell sizes (ranging from 9-12 mm shell diameter).

2.1.2.2 Snail feeding

Snails were fed *ad libitum* daily with commercially obtained, pelleted rabbit feed. The feed pellets were ground into powdery form and snails were fed daily according to the number of snails per tank, e.g., about 0.75–1.00g/tank/day of 100–120 snails (depending on number and sizes of snails in each tank).

2.1.3 Parasite eggs retrieval and miracidia hatching

Eggs were obtained from livers of mice 42 days post-infection (Doenhoff *et al*., 1988). The infected mouse liver was placed on a copper wire mesh (300 μM) over a beaker. The liver tissue was macerated into small pieces with a pair of scissors, then gently crushed and pressed through the wire mesh using a homogenizer while simultaneously washing with 1.8% NaCl solution. The resulting suspension containing the parasite eggs was distributed into 50 mL centrifuge tubes and centrifuged at 1250 rpm for two minutes .

The supernatant was decanted to expose the tissue pellet containing the eggs at the base of each tube. Some distilled water was added to the pellets in each tube and these were given a vigorous shake to re-suspend the pellets. Each tube was filled with distilled water, mixed and centrifuged again. This process was repeated with fresh distilled water and the final contents of the centrifuge tubes were distributed equally into four or more 10 cm petri dishes (depending on the number of tissues processed). To aid hatching into miracidia, the eggs collected and now contained in the petri dishes were left under the illumination of a light source for 30 – 60 minutes

by which time motile miracidia were observable under a dissecting microscope.

2.1.4 Experimental infection of snails

Mass infection was performed using adult snails of shell size ranging from 10-12 mm diameter selected from the juveniles tanks. For each infection experiment for instance, a single tank was set up to include equal numbers of each of a pigmented and an albino snail line (number ranged from 50-70 snails). This provided similar exposure conditions and also allowed easy identification and differentiation of strains at the point of cercariae screening. The mass infection method was adopted to ensure a larger proportion of snails phenotypes were sampled by the parasite infectivity phenotypes.

First, miracidia were hatched from eggs obtained from heavily infected mice livers as previously described (section 2.1.3). The snails to be infected were then placed in a clean tank containing 3 litres of deionized water and the suspension containing hatched miracidia was added. Each snail was thus exposed to approximately 50-100 miracidia overnight (12 hours). During the exposure period, the snails were left without artificial aeration and food in the tank. The following day, snails were rinsed with fresh water and transferred into clean tanks with 10 litres of fresh water. Snails were thereafter maintained as normal with food and aeration. Salts and a population of water fleas were added and the tanks were monitored as previously described till cercariae-screening date.

2.1.5 Determination of snail patency

Cercariae shedding from snails commences 4-6 weeks after infection. This shedding process is induced by light, hence occurs mostly at daytime (Colley *et al*., 2014; Gryseels *et al*., 2006). To determine patency, snails were individually screened 35 days after exposure to *S. mansoni* miracidia. To induce release of infective cercariae, each snail was placed in a 5 mL glass tube containing clean, deionised water under a light source for one hour. Each tube was then checked visually for actively swimming cercariae. Presence of cercariae was confirmed with a dissecting microscope and snails were either categorized as positive (shedding cercariae) or negative (not shedding cercariae). The latter were further re-examined with a dissecting microscope and separated for a re-screen after 2 weeks. Both categories were subsequently processed for haemolymph and fresh tissue extracts and/or DNA extraction depending on experimental purpose, or preserved whole at -80°C.

2.1.6 Mice maintenance and infection

The *S. mansoni* mammalian life-cycle phase was maintained in adult CD1 mice. Mice were maintained in groups of 5-6 animals per cage on a bedding of wood shavings and were fed *ad libitum* on mouse pellets. Mice infection was performed using a suspension of pooled cercariae obtained from freshly screened infected snails as described in section 2.1.5. The mice infection procedure used, simply known as the “ring method,” was originally introduced by Smithers and Terry (1965), but adapted as described by Doenhoff *et al.*, 1978. Briefly, the procedure commenced with

anaesthetizing the mouse with a single, intraperitoneal injection of sodium pentobarbitone, as determined by the bodyweight of the mouse (0.06 mg/g bodyweight per animal).

The anaesthetized animal was then placed in a customized wooden rack on its dorsal side such that its abdomen was exposed and accessible. To keep the induced animal in this position throughout the procedure, strips of cellotapes were applied across the rack. A region around the mid-section of the abdomen was clean-shaved and moistened with sterile water. The shaved portion was carefully marked out as the site of parasite infection by placing a 1 mL-capacity nickel-plated ring on it. This ring was gently but firmly secured with a strip of cello-tape such that a volume of the suspension can be deposited on the skin within the ringed-marked portion on the bare skin.

The cercariae suspension earlier prepared, was gently mixed to ensure that approximately equal number of cercariae (200 cercariae) are contained in each volume of suspension pipetted and applied for infection of each mouse. The same volume of suspension was then applied into the nickel-ringed portion and mice were kept in this position for 30 minutes to allow enough time for cercariae penetration. Any remaining liquid was subsequently removed using a plastic pipette and an antidote was injected to awaken the animals. The mice were kept warm to aid recovery from anaesthetic state and thereafter placed back in their cages.

2.1.7 Statistical Analysis

All statistical data were processed in GraphPad Prism version 8.0 for Windows, GraphPad Software, San Diego, California USA, (www.graphpad.com) and Microsoft Excel (2016). Details of statistical analyses are presented as performed in individual chapters.

CHAPTER 3

LONGITUDINAL STUDIES ON THE INFECTION DYNAMICS OF *BIOMPHALARIA GLABRATA-SCHISTOSOMA MANSONI* INTERACTIONS

3.1 Introduction

Biomphalaria glabrata snails exposed to miracidia of *Schistosoma mansoni* have been known to show varying levels of susceptibilities due to complex, phenotype-by-phenotype interactions between each snail and the infecting parasite (Basch, 1975; Théron & Coustau, 2005). This variation, referred to as compatibility polymorphism occurs both in field and laboratory populations (Bech *et al*., 2010). Compatibility between interacting pairs of *B. glabrata* and *S. mansoni* has been defined as a concordance between host and parasite molecules that are associated with parasite recognition and host evasion strategies (Théron & Coustau, 2005).

It has long been determined that the successful establishment of a parasite strain in its snail host is independently determined for each snail-parasite encounter and suggests compatibility of host-related and parasite-related factors (Basch, 1976). Hence, compatibility could be viewed as a reflection of both the host's susceptibility and the parasite's infectivity (Davies *et al*., 2001; van der Knaap & Loker, 1990). Population-wise, quantification of compatibility between specific host and parasite combinations can be expressed as the proportion of snails that develop patent infections (Morand *et al*., 1996; Theron *et al*., 2014).

Variations in susceptibility to *S. mansoni* as observed between and within populations of *B. glabrata* snails has also been attributed to the general concept of co-evolutionary adaptations between the host snails and the parasites (Webster, *et al*., 2007). A common hypothesis hinged on this concept and demonstrated in many studies is that snail vectors that are naturally resistant or have been selected for resistance to specific parasite strains are often highly susceptible to other schistosome strains (Kassim & Richards, 1979; Richards & Shade, 1987; Webster & Woolhouse, 1998). In other words, co-evolved host-parasite pairs (sympatric) are generally considered better adapted to each other than spatially separated (allopatric) pairs (Ebert, 1994). However, studies on the subject of co-evolution between allopatric and sympatric snail-parasite systems have been mostly limited to few numbers of snail-schistosome combinations (Richards, 1976; Theron *et al*., 2014).

While most authors agree that snail susceptibility and parasite infectivity may contribute to the outcome of each host-parasite encounter (Allan *et al*., 2017; Galinier *et al*., 2017; Rollinson *et al*., 1998), the role of co-evolution in determining compatibility between snail-parasite pairs remains relatively unclear. Variations in *B. glabrata* susceptibility to *S. mansoni* occurs both in field and laboratory snails and reduction in compatibility between snails and parasites populations during passage from field to laboratory has been reported (Theron *et al*., 2008). Infectivity of parasite populations over successive passages in laboratory snail populations have also been reported to remain relatively constant (Bech *et al*., 2010). Previous investigations by our laboratory also demonstrated that *B. glabrata* snails vary in susceptibility to *S. mansoni* strains and compatibility

between the snails and parasites appeared relatively stable within a four-year period (Arijo *et al*., 2007). The long-term effect of continuous laboratory culture on host susceptibility and parasite infectivity could further elucidate co-evolutionary processes in snail-schistosome interactions. Longitudinal data that provides information on infection status of host populations through time could provide useful insight into dynamics of host-parasite interactions. Information derived from such data could be useful in developing mathematical models that could be applied in snail-targeted surveillance and control strategies for schistosomiasis.

The main aim of this study was to further investigate compatibility between multiple sympatric and allopatric strains *B. glabrata and S. mansoni.* The objectives were to investigate the *B. glabrata-S. mansoni* relationship by testing the capability of different strains of *S. mansoni* to infect a wide range of *B. glabrata* strains and to determine the infection dynamics in the long-term. One of the questions we set out to address was if snail-parasite compatibility remain unchanged over several years in laboratory culture. Another question we sought to answer was if, on a long term, continuous passage of parasites in specific snail lines make them better adapted to infect their 'sympatric' laboratory snail hosts than other snails. To address these questions, compatibility between multiple snail and parasite strains were tested for a period ranging from 10-25 years of laboratory culture.

This chapter thus presents collation and analyses of data generated by our laboratory from 1991-2017 including those recently generated by the author (2014-2017). The investigations further elucidated compatibility between sympatric and allopatric *B. glabrata-S. mansoni* host-parasite pairs

and also determined the long term effect of continuous laboratory culture on the snail susceptibility and the parasite infectivity. Compatibility trials remain fundamentally relevant in host-parasite interaction studies and are also important in definite identification of resistance-associated molecular factors. Understanding host-parasite interactions at individual and population levels and the evolutionary consequences may be useful as epidemiological tools for predictions of disease emergence, strategic planning for disease control and biological control initiatives.

3.2 Materials and methods

3.2.1 Snails and parasites

To determine susceptibility and infectivity of hosts and parasites, 13 *Schistosoma mansoni* parasite strains were tested across a panel of 11 *Biomphalaria glabrata* snail lines. Parasite lines from similar geographical locations or that had been continuously passaged in specific snail strains for many years served as experimental equivalents of sympatric snail-schistosome combinations. Allopatric combinations were formed by testing the different snail strains with each parasite strain. Detailed, background information on all laboratory snails and parasites have strains are presented in general materials and methods (see Table 2.1). The pattern of testing the multiple strains of *S. mansoni* across a panel of *B. glabrata* strains as sympatric and allopatric host-parasite combinations are represented in Table 3.1.

Table 3.1: *Biomphalaria glabrata* and *Schistosoma mansoni* strains tested experimentally as sympatric and allopatric host-parasite combinations. Tested and untested host-parasite pairs are ticked either ✓ or x in corresponding cells. Full details of *B. glabrata* and *S. mansoni* strains are given in Table 2.1.

SCHISTOSOMA MANSONI STRAINS	*BIOMPHALARIA GLABRATA* STRAINS										
	Bg-Swan	Bg-PR	Bg-Br	Bg-BH	Bg-Sen	Bg-Eg	Bg-Abrd	Bg-Duss	Bg-GP	Bg-Cmp	Bg-SV
Sm-PR	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Sm-Br	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	x
Sm-Sen245	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	x
Sm-Ken-O	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	x
Sm-Gp	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Sm-Eg	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	x
Sm-Cmp	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	x
Sm-Sen	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	x
Sm-Sen47	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	x
Sm-BH	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	x
Sm-Abrd	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	x
Sm-Duss	✓	✓	✓	✓	x	✓	✓	✓	✓	✓	x
Sm-Ken-N	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	x

3.2.2 Snail exposure to parasite and patency screening

For each infection experiment, equal numbers each of pigmented and albino snails (50-70 snails each) measuring 10-12 mm shell diameter were randomly selected and mass infected with miracidia of the parasite strain being tested, each snail being exposed to 50-100 miracidia. Infections were performed overnight (approximately 12 hours) in clean tanks containing 3 litres of deionized water. Tanks were left without food and additional aeration during the infection period. The following day, the snails were rinsed and transferred into clean tanks containing 10 litres of fresh water. Snails were thereafter maintained as normal (as described in section 2.1.2) and tank conditions monitored daily till screening for patency was

performed. Snails were individually screened for cercariae shedding 35 days after parasite exposure (see section 2.1.5). The number of snails shedding cercariae (infected) and not shedding cercariae (uninfected) were recorded and calculated as a percentage of the total number of snails screened.

3.2.3 Determination of snail susceptibility in relation to miracidia dose

To determine the relationship between susceptibility of snails to varying numbers of miracidia, snails were exposed to known numbers of miracidia of the *S. mansoni* Sm-GP strain. These experiments were performed with snails from populations the semi-resistant strain, Bg-Cmp and a susceptible pigmented line of Bg-GP strain. Typically, four tanks, each containing 50 snails of each strain (8 tanks in all) were set up and the snails were mass-exposed to increasing numbers of miracidia; i.e., respectively 100, 200, 400 and 800 miracidia in the respective four tanks, thus exposing snails to 2, 4, 8 or 16 miracidia per snail. Snails were screened for cercarial shedding 35 days post exposure as previously described. This experiment was repeated in three trials for each of the *B. glabrata* snails (Bg-Cmp and Bg-GP) and the mean susceptibility was determined as the percentage of snails shedding cercariae (susceptible) in relation to total number of snails surviving the 35 day post-infection period.

3.2.4 Statistical analysis

The percentage of positive infections for each snail strain infected with the different parasite strains was calculated and expressed as group means ± standard error mean (SEM). The Shapiro-Wilk test was also applied to confirm the data followed a normal distribution. To test for differences between mean susceptibility and infectivity of the groups (hosts and parasites) represented in the matrix, a mixed effect analysis applying the Geisser-Greenhouse's correction was performed instead of a repeated measures (RM) one-way Analysis of Variance (ANOVA). Due to the presence of a missing value (which cannot be handled by RM ANOVA), the data was analysed by fitting a mixed model as executed in GraphPad Prism 8.0. This mixed model uses a compound symmetry covariance matrix that is fit using Restricted Maximum Likelihood (REML). This method gives the same P-values and multiple comparisons tests as RM ANOVA in the absence of missing values; in the presence of values missing completely at random, the results can be interpreted like RM ANOVA.

Statistical significance of the mean difference in susceptibility or infectivity was determined at alpha = 0.05. Levels of significance were denoted as follows: *$p \leq 0.05$; **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$ and ns to indicate non-significant values. Following ANOVA, post-hoc tests were performed when necessary using Fisher's Least Significant Difference (LSD) test with no mathematical corrections made for multiple comparison. Confidence intervals (CIs) with individual P-values were reported. For specific sympatric and allopatric combinations, paired-samples t-test and ordinary One-way ANOVA were conducted to compare mean parasite

infectivity and mean host susceptibility respectively between selected groups and a control group. In this case, Dunnett's multiple comparison test was applied (after one-way ANOVA). All analyses were performed using GraphPad Prism version 8.0.0 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com.

3.3 Results

3.3.1 Variations in snail susceptibility and parasite infectivity

Susceptibility of *B. glabrata* strains to different strains of *S. mansoni* varied between and within populations. The most susceptible snail population in terms of overall mean percentage patency was Bg-Swan (84.1%) while the least susceptible (most resistant) was Bg-Cmp (13.3%). Overall, the Bg-Cmp snails were consistently resistant to each of the 13 parasite strains tested ($p < 0.0001$) compared to the other 9 snail strains. In terms of parasite infectivity, the Puerto-Rican parasite, Sm-PR, showed the highest overall mean infectivity (79.6%) while the least infective parasite strain was a Kenyan strain, Sm-Ken-N with overall mean infectivity of 48.3%. The mean percentages of snails with patent infections measured across 11 *B. glabrata* snail lines after exposure to 13 *S. mansoni* strains are presented in Table 3.2. The table shows a ranking of snails according to an apparent difference in their susceptibility to the parasite strains as well as a ranking of the parasites according to their varying capacity to infect snail hosts.

Table 3.2: Susceptibility and infectivity of *B. glabrata-S. mansoni* sympatric and allopatric host-parasite combinations. The total number of parasite passages performed (indicated as No. of psg) and mean percentage susceptibility and infectivity ± standard error of mean (SEM) is shown for each sympatric and allopatric host-parasite pair. The snail strains are arranged in columns such that strains are ordered (left to right) from the most to the least susceptible (in terms of mean percentage patency); the parasite strains are arranged in rows in decreasing order of infectivity (top to bottom); hence, overall mean (%) snail susceptibility and parasite infectivity (Bg-SV excluded) are shown in the last row and column respectively.

	***BIOMPHALARIA GLABRATA* STRAINS**																						
	Bg-Swan		Bg-PR		Bg-Br		Bg-BH		Bg-Sen		Bg-Eg		Bg-Abrd		Bg-Duss		Bg-GP		Bg-Cmp		Bg-SV		Total mean (%)
***SCHISTOSOMA MANSONI* STRAINS**	No. of psg	% Mean ± SEM	No. of psg	% Mean ± SEM	No. of psg	% Mean ± SEM	No. of psg	% Mean ± SEM	No. of psg	% Mean ± SEM	No. of psg	% Mean ± SEM	No. of psg	% Mean ± SEM	No. of psg	% Mean ± SEM	No. of psg	% Mean ± SEM	No. of psg	% Mean ± SEM	No. of psg	% Mean ± SEM	
Sm-PR	16	89 ± 2	115	76 ± 2	35	94 ± 1	17	92 ± 3	14	91 ± 2	19	90 ± 2	8	75 ± 11	4	90 ± 6	89	74 ± 2	26	25 ± 4	40	17 ± 2	**79.6**
Sm-Br	7	97 ± 1	22	87 ± 4	25	87 ± 3	7	83 ± 3	8	92 ± 2	8	80 ± 7	12	79 ± 3	7	75 ± 6	23	71 ± 4	10	6 ± 2		n/a	**75.7**
Sm-Sen245	6	88 ± 5	31	85 ± 3	15	91 ± 2	6	77 ± 7	5	94 ± 2	13	87 ± 4	12	71± 5	2	69 ± 3	19	67 ± 5	11	26 ± 9		n/a	**75.5**
Sm-Ken-O	6	88 ± 5	18	91 ± 3	13	79 ± 6	5	88 ± 4	4	84 ± 3	5	74 ± 18	6	82 ± 4	2	73 ± 28	28	77 ± 3	5	6 ± 4		n/a	**74.2**
Sm-Eg	13	92 ± 2	35	85 ± 3	13	80 ± 5	7	82 ± 6	4	87 ± 3	14	74 ± 7	5	67 ± 13	6	63 ± 9	19	76 ± 4	8	18 ± 7		n/a	**72.8**
Sm-GP	17	78 ± 5	55	80 ± 3	36	89 ± 2	7	78 ± 6	5	81 ± 10	13	76 ± 8	9	70 ± 6	3	83 ± 9	123	77 ± 1	26	16 ± 5	21	8 ± 2	**72.4**
Sm-Cmp	6	86 ± 4	13	80 ± 5	20	81 ± 4	5	80 ± 7	8	74 ± 3	14	65 ± 6	4	66 ± 8	1	48	9	55 ± 10	37	32 ± 5		n/a	**66.7**
Sm-Sen	6	81 ± 6	53	86 ± 2	11	78 ± 7	6	70 ± 7	10	82 ± 5	11	87 ± 3	3	69 ± 18	2	34 ± 6	17	67 ± 5	6	12 ± 5		n/a	**66.6**
Sm-Sen47	13	91 ± 3	27	76 ± 4	13	74 ± 6	11	68 ± 4	5	59 ± 17	7	73 ± 8	10	65 ± 5	3	76 ± 18	17	69 ± 5	10	1 ± 0		n/a	**65.2**
Sm-BH	6	91 ± 4	40	79 ± 4	8	61 ± 9	30	79 ± 3	3	64 ± 12	9	52 ± 10	5	53 ± 12	5	62 ± 9	18	59 ± 4	8	10 ± 6		n/a	**61.0**
Sm-Abrd	7	82 ± 10	38	80 ± 3	8	65 ± 7	7	69 ± 5	5	43 ± 7	9	66 ± 7	36	76 ± 3	2	58 ± 0	8	51 ± 10	7	3 ± 2		n/a	**59.3**
Sm-Duss	12	71 ± 5	11	64 ± 5	13	68 ± 5	7	57 ± 6		n/a	10	58 ± 8	7	50 ± 6	35	62 ± 4	9	50 ± 6	4	1 ± 1		n/a	**53.4**
Sm-Ken-N	8	59 ± 9	37	61 ± 5	11	55 ± 9	6	54 ± 10	5	26 ± 15	14	62 ± 9	6	36 ± 9	4	57 ± 7	27	56 ± 5	5	17 ± 16		n/a	**48.3**
Total mean (%)		**84.1**		**79.2**		**77.1**		**75.2**		**73.1**		**72.6**		**66.1**		**65.4**		**65.3**		**13.3**		**12.5**	

Results of repeated measures ANOVA using the mixed model approach (with Greenhouse-Geisser correction) showed that mean susceptibility differed statistically significantly between snail strains (ANOVA, $F_{4.181,50.17}$ = 82.56, p < .0001). Also, mean infectivity between parasite groups were also significantly different statistically (ANOVA $F_{4.063,36.23}$ = 10.35, p < .0001). Further post-hoc tests revealed considerable statistically significant differences between mean susceptibility of Bg-Cmp snails and each of the other nine snail strains. Differences in parasite mean infectivity was also shown to be statistically different between Sm-Duss and nine other parasite strains suggesting a greater main effect of this strain on the overall infectivity parasite strains. A summary of confidence intervals and P-values of differences in mean susceptibility between snail groups and infectivity between parasite groups obtained following post-hoc tests (uncorrected Fisher's LSD test) is presented in Tables 3.3 and 3.4 respectively. Values for statistically non-significant group comparisons are not shown.

Table 3.3: Confidence intervals and P-values of differences in group mean susceptibilities compared between snail strains tested across 13 different parasite strains. Group comparisons are listed in descending order of statistical significance. Non-significant group comparisons are not shown.

Snail strains compared	95.00% CI of difference in group mean	Individual P- Value	Summary
Bg-Abrd vs. Bg-Cmp	42.92 to 62.62	<.0001	****
Bg-BH vs. Bg-Cmp	54.57 to 69.12	<.0001	****
Bg-Br vs. Bg-Cmp	56.69 to 70.85	<.0001	****
Bg-Duss vs. Bg-Cmp	41.05 to 63.11	<.0001	****
Bg-Eg vs. Bg-Cmp	51.58 to 67.03	<.0001	****
Bg-GP vs. Bg-Cmp	44.03 to 59.97	<.0001	****
Bg-PR vs. Bg-Abrd	8.756 to 17.55	<.0001	****
Bg-PR vs. Bg-Cmp	58.21 to 73.64	<.0001	****
Bg-PR vs. Bg-GP	8.846 to 19.00	<.0001	****
Bg-Sen vs. Bg-Cmp	46.95 to 72.61	<.0001	****
Bg-Swan vs. Bg-Abrd	12.51 to 23.49	<.0001	****
Bg-Swan vs. Bg-Cmp	62.22 to 79.32	<.0001	****
Bg-Swan vs. Bg-GP	12.69 to 24.85	<.0001	****
Bg-Br vs. Bg-GP	6.414 to 17.12	0.0004	***
Bg-Swan vs. Bg-BH	4.611 to 13.23	0.0007	***
Bg-Swan vs. Bg-Duss	9.503 to 27.88	0.0008	***
Bg-Br vs. Bg-Abrd	5.353 to 16.65	0.0011	**
Bg-BH vs. Bg-GP	4.678 to 15.01	0.0013	**
Bg-BH vs. Bg-Abrd	3.847 to 14.31	0.0026	**
Bg-Swan vs. Bg-Eg	3.932 to 18.99	0.0062	**
Bg-Br vs. Bg-Duss	3.242 to 20.14	0.0108	*
Bg-Eg vs. Bg-GP	1.940 to 12.68	0.0118	*
Bg-PR vs. Bg-Duss	3.612 to 24.08	0.0122	*
Bg-Swan vs. Bg-PR	0.8617 to 8.831	0.0212	*
Bg-BH vs. Bg-Duss	1.348 to 18.19	0.0265	*
Bg-Swan vs. Bg-Sen	0.9034 to 21.08	0.0354	*
Bg-Swan vs. Bg-Br	0.5197 to 13.48	0.0365	*
Bg-PR vs. Bg-Eg	0.3183 to 12.91	0.041	*
Bg-Eg vs. Bg-Abrd	0.2361 to 12.84	0.0432	*
Bg-Br vs. Bg-Eg	0.1136 to 8.809	0.0451	*

Table 3.4: Confidence intervals and P-values of differences in group mean parasite infectivity compared between parasite strains tested across different snail strains. Group comparisons are listed in descending order of statistical significance. Non-significant group comparisons are not shown.

Parasite strains compared	95.00% CI of difference in group mean	Individual P-Value	Summary
Sm-Sen245 vs. Sm-Duss	17.16 to 26.95	<.0001	****
Sm-PR vs. Sm-Duss	20.90 to 31.41	<.0001	****
Sm-Gp vs. Sm-Duss	15.15 to 23.56	<.0001	****
Sm-Eg vs. Sm-Duss	13.14 to 24.77	<.0001	****
Sm-Br vs. Sm-Duss	16.51 to 28.00	<.0001	****
Sm-PR vs. Sm-Ken-N	19.86 to 42.74	0.0002	***
Sm-Ken-O vs. Sm-Duss	13.34 to 28.18	0.0002	***
Sm-Sen47 vs. Sm-Duss	7.028 to 16.48	0.0004	***
Sm-Gp vs. Sm-Ken-N	13.98 to 35.02	0.0005	***
Sm-Sen245 vs. Sm-Ken-N	14.89 to 39.51	0.0007	***
Sm-Eg vs. Sm-Ken-N	12.11 to 36.09	0.0014	**
Sm-Ken-O vs. Sm-Ken-N	12.40 to 39.40	0.0019	**
Sm-Br vs. Sm-Ken-N	12.94 to 41.86	0.0020	**
Sm-PR vs. Sm-BH	8.761 to 28.44	0.0021	**
Sm-Eg vs. Sm-BH	5.226 to 17.57	0.0024	**
Sm-PR vs. Sm-Sen47	6.433 to 22.37	0.0027	**
Sm-Ken-O vs. Sm-Abrd	6.692 to 23.11	0.0027	**
Sm-PR vs. Sm-Abrd	9.034 to 31.57	0.0028	**
Sm-Br vs. Sm-BH	6.426 to 22.97	0.0030	**
Sm-Ken-O vs. Sm-BH	5.653 to 20.75	0.0033	**
Sm-Br vs. Sm-Abrd	6.957 to 25.84	0.0035	**
Sm-Sen47 vs. Sm-Ken-N	6.820 to 26.98	0.0043	**
Sm-Br vs. Sm-Sen47	3.703 to 17.30	0.0068	**
Sm-Cmp vs. Sm-Ken-N	6.297 to 30.50	0.0074	**
Sm-PR vs. Sm-Gp	2.303 to 11.30	0.0076	**
Sm-Sen245 vs. Sm-BH	4.772 to 24.23	0.0082	**
Sm-Sen245 vs. Sm-Abrd	5.144 to 27.26	0.0090	**
Sm-Cmp vs. Sm-Duss	3.786 to 22.73	0.0121	*
Sm-Eg vs. Sm-Abrd	3.099 to 23.10	0.0159	*
Sm-Gp vs. Sm-Abrd	3.105 to 23.90	0.0165	*
Sm-Ken-O vs. Sm-Sen47	1.999 to 16.00	0.0174	*
Sm-Gp vs. Sm-BH	2.511 to 21.09	0.0184	*
Sm-PR vs. Sm-Cmp	2.663 to 23.14	0.0191	*
Sm-Sen245 vs. Sm-Cmp	1.672 to 15.93	0.0210	*
Sm-Sen vs. Sm-Ken-N	3.087 to 33.51	0.0236	*
Sm-Sen245 vs. Sm-Sen	1.192 to 16.61	0.0282	*
Sm-Gp vs. Sm-Sen47	0.9046 to 14.30	0.0303	*
Sm-BH vs. Sm-Ken-N	1.234 to 24.17	0.0335	*
Sm-Sen245 vs. Sm-Sen47	0.9604 to 19.64	0.0342	*
Sm-Sen vs. Sm-Duss	1.027 to 25.28	0.0369	*
Sm-PR vs. Sm-Sen	0.6234 to 25.38	0.0415	*

3.3.2 Mean susceptibility of resistant strains

Results also showed that the mean percentage of infected snails for two of the snail strains, Bg-Cmp and Bg-SV was low compared to that of the most susceptible snail strain, Bg-PR, indicating high resistance in this snails. The Bg-SV snails were resistant to the parasite strains, Sm-PR (the most infective) and Sm-GP (an hybrid strain) with which they were tested. In comparison to the Bg-Cmp snails, the Bg-SV snails showed higher resistance to these two parasite strains (mean patency, 12.5% compared to 20.5% in Bg-Cmp). One-way ANOVA showed that there was a statistically significant difference in mean susceptibility between the three snail groups (ANOVA $F_{2,222} = 206.3$, $p < .0001$).

A post-hoc test (uncorrected Fisher's LSD test) further showed a statistically significant difference in susceptibility between Bg-PR snails and the Bg-Cmp and BgSV snails ($p < .0001$ in both cases). However, there was no statistically significant difference in mean susceptibility of the Bg-Cmp and Bg-SV ($p = 0.1002$). The results thus suggests that the Bg-SV snails like the Bg-Cmp, also comprise a largely resistant stock. However, successive passages of the Sm-PR parasite strain in the Bg-SV snails showed an increase in mean susceptibility of the snails at various time points during the period of investigation (Figure 3.1).

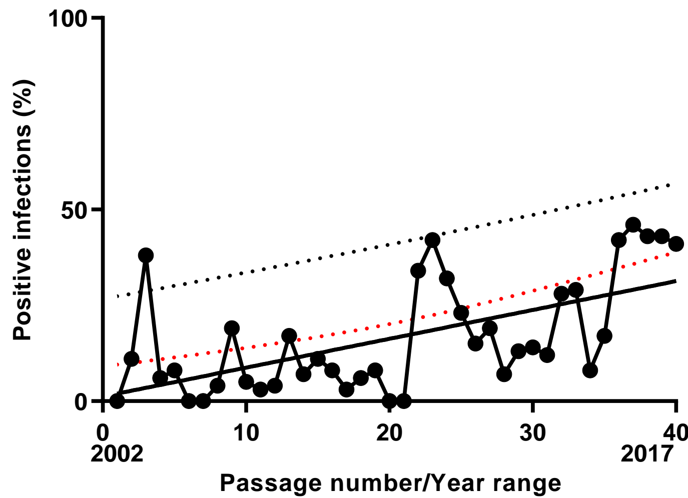

Figure 3.1: Successive passages of Sm-PR in the Bg-SV snails. 95% confidence band and prediction bands (upper limits) are shown in dotted red and black lines respectively.

3.3.3 Variability in susceptibility of sympatric and allopatric host-parasite combinations over time

Susceptibility of laboratory populations of *B. glabrata* snails to infection by different strains of *S. mansoni* varied for both allopatric and sympatric combinations. Outcome of infection percentages obtained for sympatric and non-sympatric host-parasite combinations (Figure 3.2) revealed that sympatric parasites did not necessarily demonstrate higher infectivity in their corresponding hosts in comparison to allopatric host-parasite combinations. Paired t-tests comparing Sm-Cmp in sympatric (Bg-Cmp) and allopatric (Bg-Br) hosts pairs for example, showed there was a significant difference between mean infectivity of the two snail groups ($t(11) = 13.5$, $p < .0001$). For two other sympatric-allopatric combinations compared (Sm-Abrd in Bg-Abrd compared to Bg-PR and Sm-BH in Bg-BH

compared to Bg-PR), there was no statistically significant difference in infectivity of sympatric compared with allopatric pairs ($t(22) = 1.4$, $p = 0.17$; $t(8) = 0.35$, $p = 0.7$).

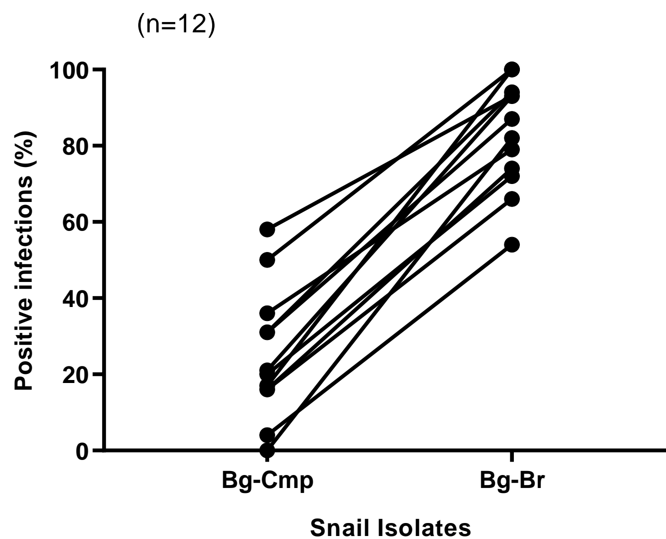

Figure 3.2: Sympatric and allopatric comparisons of mean infectivity of Sm-Cmp parasite strain in Bg-Cmp (sympatric host) and Bg-Br (allopatric host) snails. Each connected data point indicates comparison at the same time point; n represents the total number of parasite passage experiments.

3.3.4 Infectivity patterns of sympatric host-parasite combinations

Successive passages of the Sm-Cmp parasite in its sympatric host snails, Bg-Cmp, showed variability in mean susceptibility of snails to the sympatric parasite over the passage period (Figure 3.3). With the exception of a sharp spike in mean percentage infection by the 11th passage, it appeared the

parasite was not more infective to that snail during the years of passage. The confidence bands and the upper limit prediction band show further the variability in the data.

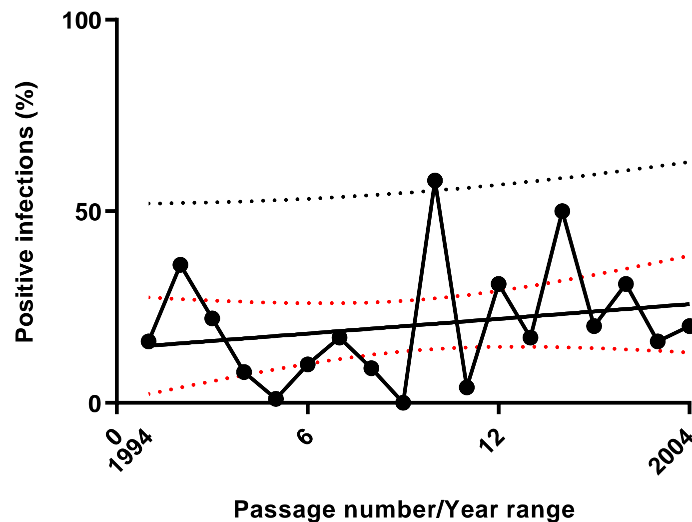

Figure 3.3: Successive passages of Sm-Cmp in Bg-Cmp from 1994-2004. Confidence bands (90%) and upper limit prediction bands (95%) are shown in dotted red and black lines respectively.

Mean susceptibility obtained from passages involving other sympatric host and parasite pairs also showed high variability in pattern observed for different pairs as shown by the prediction bands. For the Bg-Abrd & Sm-Abrd combination for instance, mean susceptibility ranged from 46-100% (excluding outlier) within a 13-year period (Figures 3.4). Similarly, mean percentages of positive infections obtained for successive passages of Sm-BH in its sympatric host, Bg-BH were also considerably variable (Figure 3.5).

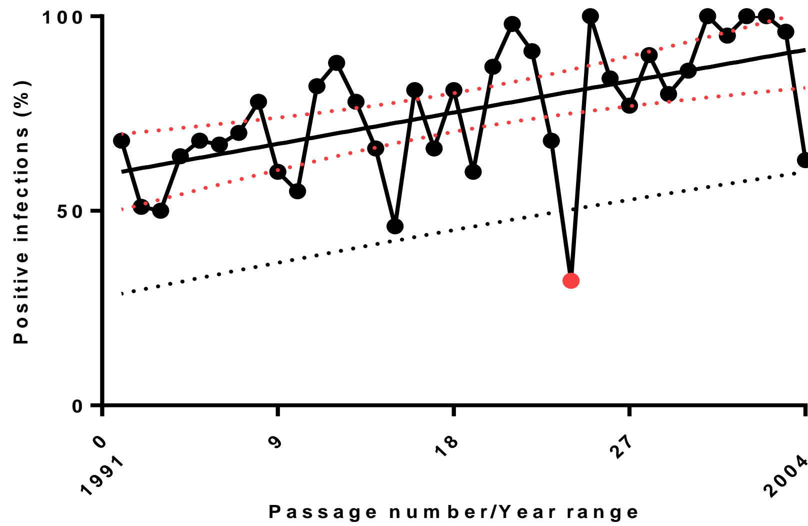

Figure 3.4: Successive passages of Sm-Abrd in Bg-Abrd from 1991-2004. Outliers are shown as red data points. Confidence and prediction (lower limit) bands (95%) are shown in dotted red and black lines respectively.

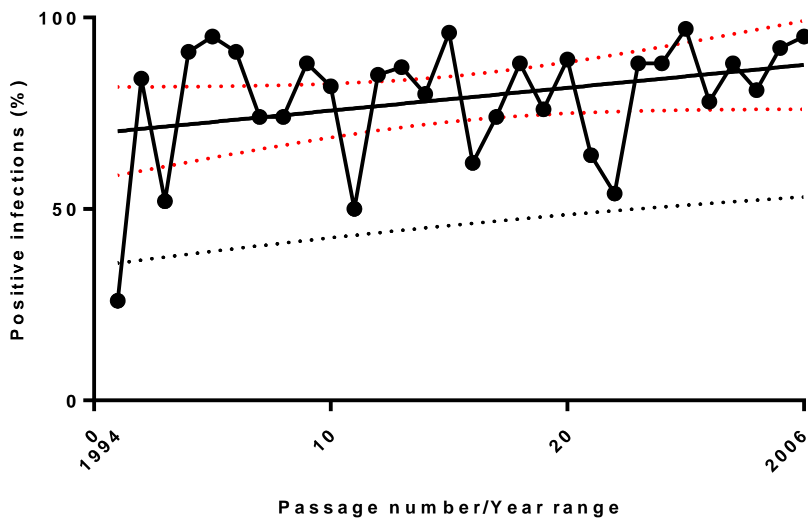

Figure 3.5: Successive passages of Sm-BH in Bg-BH from 1994-2006. Confidence and prediction (lower limit) bands (95%) are shown in dotted red and black lines respectively.

Interestingly, mean susceptibility of Bg-PR to Sm-PR appeared relatively stable for about the first decade before an irregular, somewhat oscillatory pattern of susceptibility was observed. This suggested increased variability in the data from that point on (Figure 3.6). The Bg-GP & Sm-GP hybrid, sympatric combination showed the most variable pattern of compatibility (Figure 3.7).

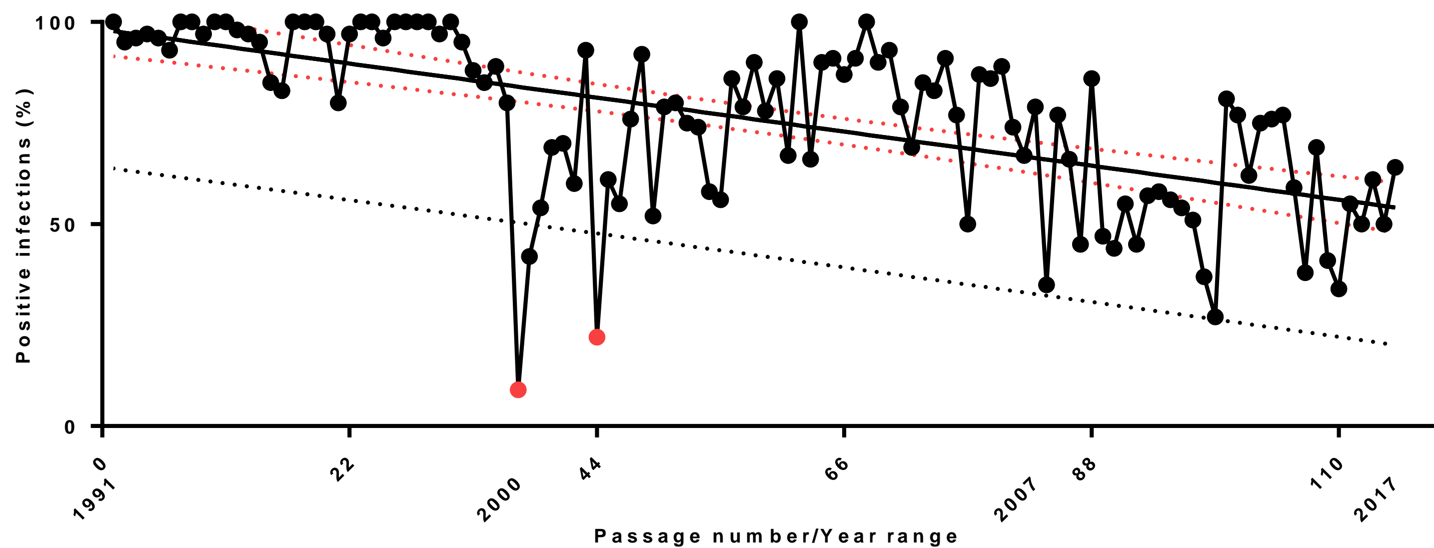

Figure 3.6: Successive passages of Sm-PR in Bg-PR from 1991-2017. Black data points show percentage of positive infections (outliers are shown as red data points). Confidence and lower limit prediction bands (95%) are shown in dotted red and black lines respectively.

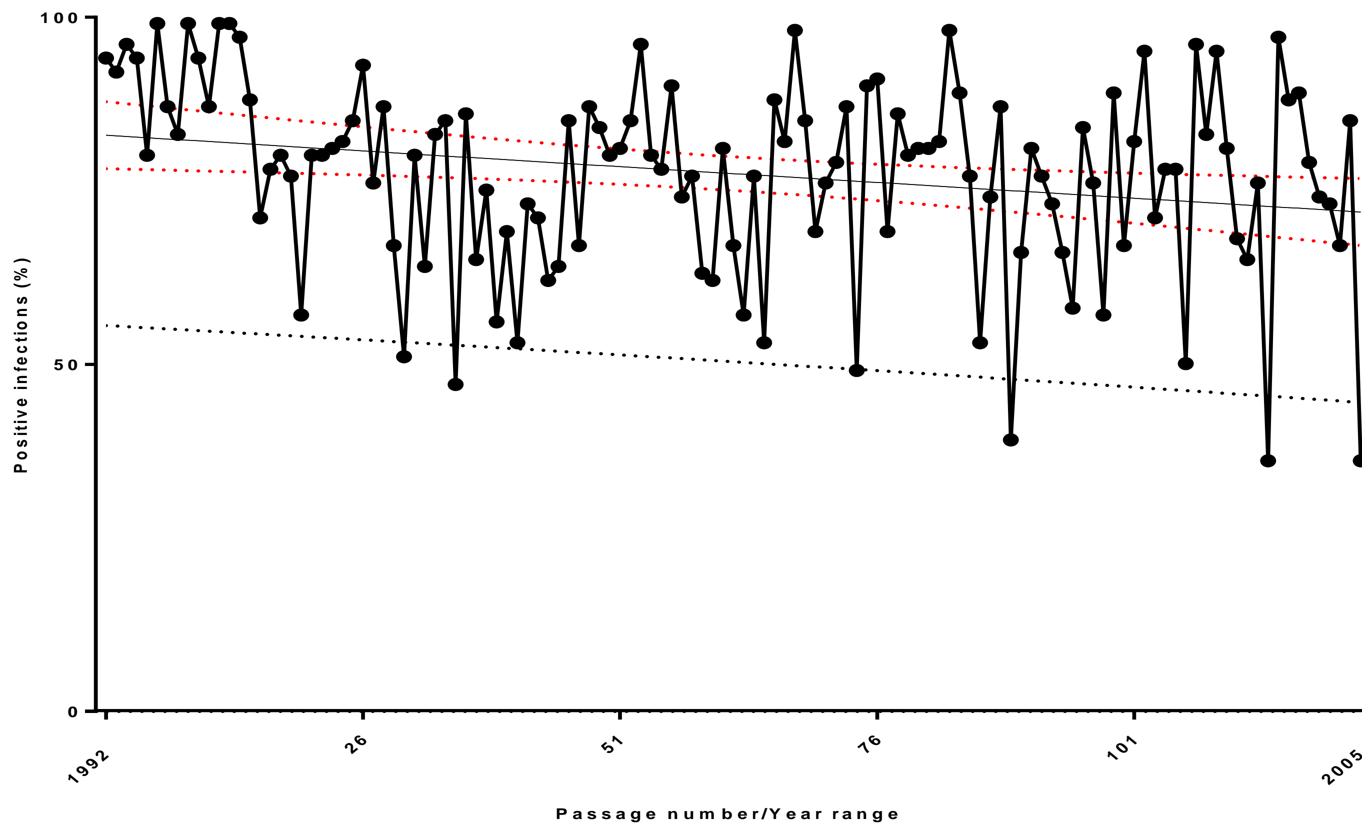

Figure 3.7: Successive passages of Sm-GP hybrid parasite in Bg-GP hybrid snails from 1992-2005. Percentages of positive infections and 95% confidence bands (dotted red lines) and lower limit prediction bands (dotted black lines) are shown .

3.3.5 Comparison of mean susceptibility between hybrid snail and parent stocks

The overall mean susceptibility of the hybrid snail, Bg-GP was found to be less than those of each of the parental stock from which it was developed (Bg-Br, Bg-PR, and Bg-Eg) indicating slightly higher resistance (see Table 3.2). One-way ANOVA revealed a statistically significant difference between the groups means (ANOVA $F_{3,16} = 4.028$, $p < .05$). Post-hoc test (Dunnett's test for multiple comparison) revealed a statistically significant difference in mean susceptibility between the hybrid Bg-GP and Bg-Br ($p = 0.0138$). There was however no statistically significant difference in mean susceptibility between this hybrid (Bg-GP) and the other two parent strains (Bg-PR and Bg-Eg) from which it was derived ($p > .05$).

A similar analysis comparing the average parasite infectivity between the hybrid parasite, Sm-GP and the four parental parasite isolates that made up its gene pool was also performed. In this case however, difference in mean infectivity between the four parasite groups were not statistically significant (ANOVA $F_{4,15} = 0.2566$, $p = 0.901$, ns).

3.3.6 Effect of number of miracidia on variation in host susceptibility

Using samples of predominantly resistant (Bg-Cmp) and highly susceptible (Bg-PR) snail strains, variation in mean susceptibility (mean ± SEM) of individual snails to 2, 4, 8 and 16 miracidia (averaged from three independent experiments) was determined (Figure 3.8). The results showed that number of susceptible snails increased with the number of miracidia applied up to the a dose of 8 miracidia per snail. Mean susceptibility then almost plateaued for the Bg-GP and decreased for the Bg-Cmp respectively. This indicated that host susceptibility was partly dependent on the number of miracidia encountered by the snail only to a maximum point.

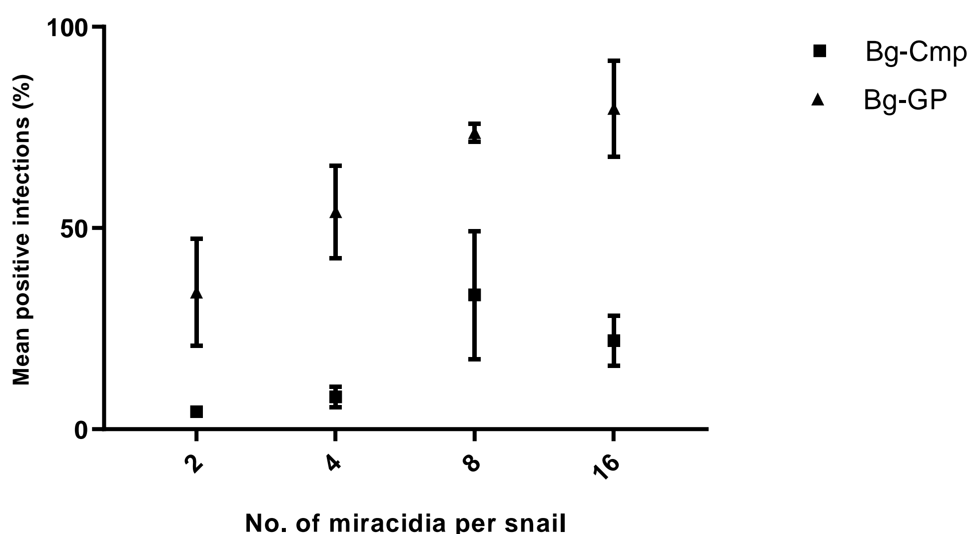

Figure 3.8: Mean patency per snail (± SEM) obtained for *B. glabrata* strains (Bg-Cmp and Bg-GP) exposed to increasing number of Sm-GP miracidia

3.4 Discussion

The compatibility between multiple *B. glabrata* laboratory snails and multiple strains of *S. mansoni* was investigated to elucidate the pattern of interaction and co-adaptation between sympatric and allopatric host-parasite pairs after several years of laboratory maintenance. The results showed high variability in infection dynamics of host-parasite pairs and indicated that sympatric host-parasite pairs were not necessarily co-adapted to each other.

Variable patterns of snail susceptibility and parasite infectivity

The results obtained in these investigations suggests that laboratory populations of *B. glabrata* and *S. mansoni* maintain considerable variation in susceptibility to different parasite strains. All snail-schistosome combination tested resulted in variable numbers of positive infections throughout the years of study. Given that resistance and susceptibility is a heritable trait, the results support suggestions that snails and parasites may maintain a degree of genetic heterogeneity even after several generations and many decades of laboratory culture. Genetic heterogeneity has been similarly reported in laboratory and field populations of *B. glabrata* by other authors (Margaret Mulvey & Bandoni, 1994).

Furthermore, while both the group means of the snail hosts and parasites were shown to differ statistically in terms of variations in snail susceptibility or parasite infectivity, follow up multiple comparison tests revealed these differences were more evident in snail groups rather than the parasite groups. In other words, host susceptibility to parasites generally produced a statistically more significant main effect than parasite infectivity to host.

Involvement of both host and parasite genotypes in variability in susceptibility has been demonstrated in other *B. glabrata-S. mansoni* co-evolution studies. For example, Webster and colleagues demonstrated that *B. glabrata* resistance to *S. mansoni* is a temporary, natural phenomenon that is due to rapid counter adaptations by parasites (Webster *et al*., 2007). Considering the longer generation times required in the mice in comparison to the snail, it is possible that the co-adaptational changes at this point is an indication of corresponding changes in parasite virulence after several passages (spanning several years) in the definitive hosts, mice. This supports suggestions that that genetic heterogeneity in *S. mansoni* though relevant, is limited and so the genetics of snail hosts are likely to have greater effect on schistosomiasis epidemiology than the genetics of the parasite (Vidigal *et al*., 1994). Other authors using a malaria host-parasite study system have similarly demonstrated that most of the variance in virulence, resistance and transmission potential was more attributable to host main effects than parasite main effects (Grech *et al*., 2006).

Also, there was generally, apparent fluctuations in mean percentages of infected snails, indicating varying susceptibility of the snail hosts to the different parasite strains during the years of laboratory maintenance. Our findings showed most snail combinations maintained mid to high susceptibility, however susceptibility varied widely from 0% in resistant snails to 100% in susceptible ones. This was in line with findings of other authors who have similarly shown that *B. glabrata* snail susceptibility to *S. mansoni* could vary widely from 0% to 100% (Coupel *et al*., 2015; Theron *et al*., 2014). Also, while mean susceptibility in the Bg-SV (which was considered a predominantly resistant strain) appeared to increase with time

during the period of study, the susceptible Bg-PR and Bg-GP snail strains seemed to increase in resistance with increasing number of infection passages. These changes in susceptibility pattern observed especially for the Bg-SV and Bg-PR snails was further indication that increased levels of variability in patency could occur after many years of laboratory maintenance. Such increased variability in susceptibility of snails to infection could suggest a degree of co-adaptation occurring between host and parasite. This is however unlikely, given the relatively short periods between some passages. Instead, the trend observed may more likely be an indication of changes in allele frequencies as often observed in simple frequency-dependent selection models. Vidigal and co-workers have also reported a lack of correlation between the length of time of laboratory maintenance of snails and the extent of heterogeneity (Vidigal *et al*., 1994).

Furthermore, passage experiments conducted to test the hypothesis that co-evolved species of parasite and hosts are better adapted to each other showed that this may not always be the case. The pattern of susceptibility observed for successive passages of sympatric parasites in corresponding snails (e.g. Bg-Cmp and Sm-Cmp host-parasite combination), showed that this snail did not rapidly become better adapted to its sympatric parasite isolate, Sm-Cmp. In all combinations tested, compatibility appeared to follow an oscillatory pattern with peaks of high susceptibility followed by low resistance. This could suggest some form of evolutionary arms race or the result of bottlenecking events resulting from inadvertent selection. This pattern is also similar to those obtained by Webster *et al.*, 2007. It is also important to note that in snail-schistosome interactions, there are possible

costs to fitness which could also affect host susceptibility (Davies *et al*., 2001).

Another explanation for the oscillatory pattern observed similarly in both sympatric and allopatric pairs as seen in the Sm-Abrd/Bg-Abrd and Sm-BH/Bg-BH pairs or the SmPR/Bg-SV pairs is possibly due to correct match between host and parasite genotypes. This is based on the matching allele model which proposes that the success or failure of each snail-parasite encounter depends on matching between the host and the parasite genotypes (Agrawal & Lively, 2003; Basch, 1975; Theron *et al*., 2008; Théron & Coustau, 2005). Interestingly, susceptibility in the Bg-PR snails remained fairly constant till about the 40th passage when a sharply oscillating pattern was observed for the infection outcomes. This change could be the result of a bottleneck event at the time causing a shift in the allelic composition of the laboratory populations. At this point, it is important to consider how the genetics of the parasite could also affect the outcome of exposure in each host-parasite encounter. In the arms race between host and parasite, the role of the latter in driving infection patterns is also important.

An interesting observation from the results was made from comparison of the mean patency of susceptible snails, Bg-PR with those of resistant Bg-Cmp and BgSV snails. The latter two were comparable in overall resistance to parasite infection, however, they showed a trend toward susceptibility with time while the susceptible Bg-PR snails showed a converse trend toward resistance during the study period. The extent of variability in the data and the observed trend towards resistance or

susceptibility was also noted from the upper and lower limit prediction bands. A possible explanation for this is that frequencies of resistant alleles in the susceptible population of snails were increasing while in the resistant population, the reverse was the case. The oscillatory pattern observed in susceptibility could supports this explanation. Also, genetic drift resulting from bottleneck in the laboratory could have caused changes in the frequency of alleles, leading to the cyclic pattern observed.

Also, comparison of the hybrid snail isolate (Bg-GP) with the parental stocks from which it was developed, showed that the hybrid was less susceptible to the schistosome parasite lines compared to its three parents. This indicates that resistant genes are carried in susceptible snail populations as has been demonstrated by other authors (Lewis *et al*., 2003).

Miracidia dose effect on susceptibility

Infection rates in snails were shown to increase with increase in the number of miracidia used for infection. However, after the number of miracidia was increased beyond 8 miracidia per snail, susceptibility appeared to almost reach a plateau in the susceptible Bg-GP snails while it slightly decreased in the Bg-Cmp resistant strain. These results indicate a limit or threshold at which an increase in number of miracidia would have relatively little or no effect on the percentage of snails infected. These observations are also in line with the findings of Theron and colleagues using a dose response curve by which it was demonstrated that snail infection rates were directly proportional to number of parasites to a maximum of 20 parasites after which infection rates levelled out on a plateau (Theron *et al*., 2014).

In this study, a mass infection technique was performed with multiple snails exposed to miracidia of sympatric or allopatric parasite strains. The mass infection of snails has the advantage that multiple snails with wider range of phenotypes are exposed to wider range of parasite infectivity phenotypes, hence there is increased chance of matching between the wider range of phenotypes provided by sympatric and allopatric snails and parasites. In other words, if the snail phenotype (sympatric or allopatric) is matched by any of the parasite phenotypes, then infection occurs. This method increases the likelihood of infection for each snail in the batch of snails infected (choice of snail is increased and likelihood of each miracidium finding a possible host is also increased).

On the other hand, single snail exposures eliminate competition effects but may lead to higher mortality due to multiple larval penetration and overburdening of the host defences; it may however be beneficial in experiments that require measurements of specific physiological parameters of infected snails. Potentially, all *B. glabrata* snails can be infected if they come in contact with the right miracidia as shown in this study. This has also been demonstrated by other host-parasite infection studies (Basch, 1975; Mitta *et al*., 2012). The mass infection method also provides similar exposure conditions for all the snails and may more accurately reflect snail susceptibility and/or parasite infectivity as would probably occur in natural populations.

This study however has other limitations, foremost of which are those associated with longitudinal data collection. The infection experiments were performed in a time period spanning almost three decades. During this time, changes in research location, (meaning snails had to be transferred

to a new laboratory) could have created some bottleneck. Also, due to the long term nature of data collection, subtle changes in physiological status of host populations as well as inadvertent selection and other bottlenecks in laboratory could affect the pattern of compatibility observed. To minimise any form of experimental bias, uniform conditions were maintained for experimental animals. This study thus further shows the complex nature of the *B. glabrata-S. mansoni* host-parasite relationship as a result of heterogeneity of *B. glabrata* snails. While it cannot be categorically concluded that the diversity observed in the various strains are fully representative of the heterogeneity of natural populations, the extent of variability in the present data suggests that laboratory snail populations could also be highly heterogeneous. The genetic heterogeneity of the extant snail populations in terms of phylogeography is the focus of the next chapter.

In this chapter, the data clearly showed that all strains of *B. glabrata* are potential hosts of *S. mansoni*. The general implications of this especially with regards to risk of disease spread needs to be highlighted in public health forums. The patterns observed in this novel longitudinal study demonstrates that the susceptibility of snails and the infectivity of the parasites are highly variable, irrespective of sympatric or allopatric nature of interacting host-parasite pairs and may have dire implications for the transmission of schistosomiasis.

CHAPTER 4

PHYLOGEOGRAPHY AND GENETIC DIVERSITY OF LABORATORY-CULTURED *BIOMPHALARIA GLABRATA* POPULATIONS

4.1 Introduction

Biomphalaria glabrata has a wide geographical range in the New World, where it serves as an important snail intermediate host of *Schistosoma mansoni* and based on phylogeographical studies, is divided into a northern and southern group, separated by the Amazon river (Mavarez, *et al*., 2002). Intra-species differences have also been reported both in natural and laboratory populations of *B. glabrata* and this variability has been shown in a number of studies (Richards, 1976; Mulvey and Bandoni, 1994; DeJong *et al.*, 2001; Dejong *et al.*, 2003). One of the factors that influence the distribution of genetic diversity within species is the history of the species (Mavarez, *et al*., 2002) and previous studies have indicated that high genetic differentiation among natural populations of *B. glabrata* may be the consequence of habitat discontinuity and demographic instability (DeJong *et al*., 2001; Vidigal *et al*., 1994).

Previous research for example involving allozyme studies (Bandoni *et al*., 1995a; Mulvey & Vrijenhoek, 1981), Random Amplified Polymorphic DNA analysis, RAPDs (Vidigal *et al*., 1994) and DNA sequence data (DeJong *et al*., 2001; Mavarez, *et al*., 2002) have shown that *B. glabrata* snails are a genetically diverse species. Distribution of genetic diversity within populations of snails have also been shown to be temporally stable with limited gene flow observed for snails sampled across multiple microhabitats in schistosomiasis-endemic communities (Thiele *et al*., 2013). Also,

regional variations have been reported within the species and genetically diverse groups/haplotypes have been observed for *B. glabrata* populations in South America and the Caribbean (Dejong *et al*., 2003). Also, laboratory strains of *B. glabrata* have been reported to show high levels of polymorphism despite inbreeding (Mulvey & Vrijenhoek, 1981) and similar stocks of snails maintained in different laboratories have shown high genetic variability among populations (Mulvey & Bandoni, 1994).

Considering the plethora of schistosomiasis-related researches world-wide, laboratory-culture of *B. glabrata* with its naturally-associated parasite, *S. mansoni* is common practice as it provides the required controlled conditions for experiments. Hence, as a model for host-parasite interaction studies, routine maintenance of the *S. mansoni* lifecycle is often required and may go on for many years in research laboratories as exemplified in our laboratory. During such extended periods of *B. glabrata* maintenance, the snails undergo several generations and experimental passages of parasites that have passed through both the snail intermediate and vertebrate definitive hosts (usually mice or hamsters) and are frequently exchanged between researchers. Phylogeographical information on such snail populations are sometimes lost through the years or become relatively unclear as snails are transferred between laboratories for establishment of breeding stocks. Hence, investigations to determine or clarify genetic backgrounds of laboratory populations become necessary.

A useful approach to the study of parasite-transmitting organisms in recent times involves genetic methods that inform on the phylogeographical associations of organisms and aim to establish possible evolutionary

connections among and within species (Avise, 2009). Phylogenetic studies provide insight into molluscan lineages based on genomic data and could thereby offer explanations regarding the heterogeneity observed in schistosomiasis transmission patterns (Thiele *et al*., 2013; Tuan *et al*., 2012). While initial phylogenetic analysis of field and newly acquired laboratory strains could help to determine origin and relatedness of different isolates, periodic analyses of laboratory populations could provide useful information on genetic changes occurring over long periods of laboratory culture due to genetic drift or selection.

In our laboratory, *B. glabrata* snails have been in laboratory culture and maintenance for over two decades. Prior to this time, and as previously presented (Table 2.1), the parental stocks of the existing laboratory populations have historically been exchanged between researchers and laboratories. During these years of laboratory maintenance, not less than ten strains of *B. glabrata* have been bred with thousands of generations of snails produced through consistent breeding schemes. Furthermore, these laboratory strains of *B. glabrata* historically differed in time and sources of acquisition, the number of individuals that constituted the founding populations, and for hybrids, the parental snails used in development of hybrid strains. Also, during this period, some of the strains have either been phased out and/or replaced by other strains during laboratory transition periods.

At the onset of the present study, populations of three strains of *B. glabrata* (BgPR, BgPS and BgSV) were being used for maintaining the life cycle of a Puerto Rican strain of *S. mansoni* (PR1) and constituted the source of

sample materials used for investigations reported in this and subsequent chapters of this thesis. It was however not known if these three populations each formed a homogeneous stock and if they were linked to popular, published laboratory stocks used by other researchers. This study therefore sought mainly to establish phylogenetic links of the three strains with other *B. glabrata* strains from other published studies. The objectives were to determine variations in nucleotide sequences between the currently investigated strains and other strains/isolates from and to clarify information on historical and genealogical roots especially since this information had become debatable over time and in the case of the hybrid BgPS strain, partially unknown.

Diversity of the present laboratory stock was thus determined using the 16S rDNA nucleotide sequences of *B. glabrata* in comparison to previously published haplotypes of laboratory and field strains from various locations in South America and the Caribbean that were available on the National Centre for Biotechnology Information (NCBI) data repository. Notably included for comparison were well published *B. glabrata* strains like the M-line, 13-16-R1 and 10-R2 laboratory developed hybrids and the BB02 field obtained isolate (Davids & Yoshino, 1995; Hahn *et al*., 2001b; Hanelt *et al*., 1997; Tennessen *et al*., 2015). Investigating the diversity and establishing the phylogeny of the three *B. glabrata* strains, BgPR, BgPS and BgSV will help in defining the present laboratory stock as well as clarify their history. Information so derived could help to ascertain historical and genealogical roots especially when such information become debatable over time and in the case of the hybrid BgPS strain for example, partially unknown. Understanding genetic diversity within and among laboratory

populations of *B. glabrata* could also help determine truly similar snail stocks, aid research replicability and interpretation of results from various laboratory research groups.

4.2 Materials and Methods

4.2.1 Laboratory snails and NCBI reference sequences

For phylogeny reconstruction, recently generated (this study) and previously published 16S rRNA nucleotide sequences were used. Snails were obtained from laboratory maintained populations of three strains of *Biomphalaria glabrata*, viz., BgPR, BgPS and BgSV. For this investigation, twenty-one snails were used to represent each of the *B. glabrata* strains BgPR (n=6), BgPS (n=7) and BgSV (n=8) from the laboratory populations. Background information on these strains are provided in Chapter 2 (Table 2.1).

To provide a basis phylogeographical analyses, 48 nucleotide sequences (obtained from GenBank) of the partial 16S rRNA mitochondrial gene from 33 populations of *B. glabrata* were included in the analyses. These comprised field and laboratory snails sampled from different geographical domains of *B. glabrata* in South America and the Caribbean (Table 4.1). In addition, one sequence each for *B. pfeifferi, B. alexandrina B, camerunensis* and *B. tenagophila* were included as outgroups, hence the entire dataset comprised 73 sequences. Names of all snail specimen, NCBI accession numbers, source country and author references of the entire dataset are presented in Table 4.1.

Table 4.1: Names, accession numbers and geographical origin of *Biomphalaria glabrata* strains and *Biomphalaria sp.*. included in 16S rRNA phylogenetic analysis (Figure 4.1). Accession numbers and names of laboratory strains are shown in normal font while field isolates are shown in italics.

Species & strain names	Accession number	Source population and country	Author reference
B. glabrata			
BgPS	-	Hybrid (mix of four strains, two Egypt, one Brazil & one of unknown origin)	This study (n=7)
BgPR	-	Puerto Rico	This study (n=6)
BgSV	-	Salvador, Brazil	This study (n=8)
	AF449597	Colibri, Brazil	Mavarez *et al*, 2002
	AF449598	Salvador, Brazil	" "
	AF449599	Belo Horizonte, Brazil	" "
	AF449600	Caripe, Venezuela	" "
	AF449601	Los Narajos, Venezuela	" "
	AF449603	Tres Lances, Venezuela	" "
	AF449605	Chuao, Venezuela	" "
	AF449606	Calicanto, Venezuela	" "
	AF449607	Zuata, Venezuela	" "
	AF449608	Punta Cabito, Venezuela	" "
	AF449609	Vallet, Guadeloupe	" "
	AF449610	Maupeou, Martinique	" "
	AF449611	Vieux Fort, Guadeloupe	" "
	AF449612	Grand Camp, Guadeloupe	" "
	AF449613	Pierrot, Saint Lucia	" "
	AY030206	Jarabacoa, Dominican Republic	Dejong *et al*, 2001
	AY030207	Rio Grande, Puerto Rico	" "

Table 4.1 (*continued*): Names, accession numbers and geographical origin of *Biomphalaria glabrata* strains and *Biomphalaria sp*. included in 16S rRNA phylogenetic analysis (Figure 4.1). Accession numbers and names of laboratory strains are shown in normal font while field isolates are shown in italics.

Species & strain names	Accession number	Source population and country	Author reference
B. glabrata			
	AY030208	Salvador, Brazil	" "
	AY030209	Aragua, Venezuela	" "
	AY198080	Guadeloupe	Dejong *et al*, 2003
	AY198081	Guadeloupe	" "
	AY198082	Venezuela	" "
	AY198083	Venezuela	" "
	AY198084-198089	Brazil	" "
10-R2	AY198090	Hybrid	" "
	AY198091	Brazil	" "
	AY198092	Brazil	" "
13-16-R1	AY198093	Hybrid	" "
M-LINE	AY198094	Hybrid	" "
	AY198095-AY198104	Brazil	" "
	AY204640	Belo Horizonte, Brazil	" "
1742	AY380531	Hybrid	Dejong *et al*, 2004
BB02	AY737280	Brazil	
	DQ084845	Imbaba, Egypt	Jørgensen *et al*, 2007
***Biomphalaria sp*.**			
B. tenagophila	*AF449615*	Contagem, Brazil	Mavarez *et al*, 2002
B. alexandrina	*AY030204*	Giza, Egypt	Dejong *et al*, 2001
B. pfeifferi	*AY198056*	Senegal	Dejong *et al*, 2003
B. camerunensis	*AY198079*	Cameroon	Dejong *et al*, 2001; 2003

4.2.2. DNA Extraction

Genomic DNA was extracted from tissue slices obtained from the head-foot region of individual snails using a hexadecyltrimethylammonium bromide (CTAB)-based protocol (Goodacre & Wade, 2001) with lysis buffer containing 2% CTAB, incubated with proteinase K (20mg/mL) and ß-mercaptoethanol (0.2%) at 55°C for a minimum of 2 hours (ensuring complete digestion of the tissues). This was followed by extraction with Chloroform-Isoamyl Alcohol (24:1). At this point, 500 µL of Chloroform-Isoamyl Alcohol was added to each tube containing the digested tissue slice sample of each snail, mixed by inverting the tubes for 5 minutes and then centrifuged for 5 minutes at 13,000 rpm.

The aqueous layer formed (which contained the DNA), was carefully removed with a pipette and transferred into a freshly labelled tube. The DNA was subsequently precipitated with 2.5 volumes 95% ethanol (icecold) and 1/10 volume 3 M NaAc and left overnight at -80°C. After 10-15 minutes centrifugation at 13,000 rpm, the ethanol was removed and the DNA pellet was washed with 70% ethanol (cold) before air drying. The pellet was thereafter re-suspended in 200 µL Tris (pH8), boiled for 10 minutes at 95°C, cooled at room temperature, placed in the fridge for about 30 minutes and finally stored at -20°C. (see outlined protocol in Appendix 1). DNA extract from individual samples were quantified with the Nanodrop 1000 spectrophotometer (Thermo Scientific, U.K).

4.2.3 PCR amplification conditions

Individual PCR amplification of the partial 16S rRNA gene (approximately 450bp) was performed for each snail. Amplification reaction of the 16S rRNA fragment included primer pairs 16sar (5′- CGC CTG TTT ATC AAA AAC AT-3′) and 16sbr (5′- CCG GTC TGA ACT CTG ATC AT-3') (Okusu *et al*., 2003; Xiong & Kocher, 1991). Primers were prepared by Sigma-Aldrich Company Ltd, U.K. Each amplification was performed using BIOTAQ™ DNA Polymerase (Bioline Company, UK) in a 25 µL reaction using 0.5 unit of Taq DNA polymerase (Bioline Company, U.K) with 200 µM dNTPs, 1.5 mM - 2.5 mM $MgCl_2$, 0.2 µM of each primer and 2.5 µL of 10x reaction buffer. Reaction cycles were carried out using the PTC100™ programmable thermal cycler (Bio-Rad, U.K). Cycling conditions included one cycle of 96°C for 2 minutes, 94°C for 30 seconds, 50°C (annealing) for 1 minute, 72°C for 2 minutes followed by 35 cycles of 94°C for 30 seconds, 50°C for 1 minute and ending at the final step of 72°C for 2 minutes. Holding temperature for PCR products was set to 20°C. Negative controls (reaction mixes with no DNA) were included for each PCR.

4.2.4 Gel Electrophoresis and DNA visualization

Successful amplification was verified on ethidium bromide-stained gel electrophoresis. Approximately 5 µL of PCR product was analysed in 1.5% agarose gel prepared with TBE (Tris/Borate/EDTA) buffer. To aid visualization of the amplified DNA target bands, 5 µL of ethidium bromide (EtBr) was added to the gel mixture. Gels were electrophoresed at 80-100V

and bands visualized and photographed with a transilluminator (Bio Rad Gel Doc System, Bio Rad, U.K).

4.2.5 PCR product purification and sequencing

The remaining end products of PCR amplification (20 µL) were purified using the QIAquick PCR purification kit (Qiagen, U.K) according to manufacturer's instructions and then stored at -20°C till use. Samples were prepared for sequencing following company specifications. Sequencing was performed by Macrogen Europe Laboratory on an ABI 3730XL sequencer.

4.2.6 Sequence assembly

Sequence chromatographs obtained for the 21 laboratory snail specimen were visually cross-checked for base calling errors and corrected using the DNA editing and viewing software, Trev (Bonfield *et al*., 2002) and the Groups, Algorithms and Programming version 4.8.8 (GAP 4) (https://www.gap-system.org) applications of the Staden package (Staden *et al*., 2000). Primer sites were removed and all sequencing data obtained were imported and aligned in the Genetic Data Environment (GDE) (Smith *et al*., 1994).

As a basis for comparison of nucleotide variations, generated sequences were searched using the Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov) and compared with the 16S rRNA sequences of *B. glabrata* available in the National Centre for Biotechnology Information (NCBI) GenBank nucleotide database. The reference sequences were imported and simultaneously aligned with the sequences obtained

from our laboratory samples. All nucleotide sequences (intra-specific and outgroups) included in the final data set were aligned and trimmed to equal lengths and imported into MEGA version 7 software (Kumar *et al*., 2016) for phylogeny reconstruction.

4.2.5 Determination of sequence variation and phylogenetic analysis

Analyses of DNA polymorphism and generation of haplotype data were performed using DnaSP version 6.0 (Rozas *et al*., 2017). Estimated statistics included the following parameters: number of polymorphic sites (S), total number of mutations (Eta), number of haplotypes (h), haplotype diversity (Hd), mean number of nucleotide differences (k), nucleotide diversity (Pi) and nucleotide polymorphism (θ) per site from Eta. To test for non-neutral evolution patterns within our laboratory populations, Tajima's (1989) D test (Tajima, 1989) based on the number of mutations was also performed using DnaSP version 6.

Phylogeny reconstruction was conducted and estimates of evolutionary divergence between the sequences were determined using MEGA 7 (Kumar *et al*., 2016). Evolutionary history and divergence were inferred using the Maximum Likelihood (ML) method based on the Hasegawa-Kishino-Yano (HKY) model (Hasegawa *et al*., 1985). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join (Saitou & Nei, 1987) and BioNJ (Gascuel, 1997) algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete

Gamma distribution was applied to model evolutionary rate variations among sites (4 categories (+G, parameter = 0.1581) while allowing for invariable evolutionarily sites ([+I 43.09% sites). For tree construction, support for internal nodes was determined by bootstrapping (1000 replicates) (Felsenstein, 1985). The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. Also evolutionary divergence was estimated as number of base substitutions per site from averaging over all sequence pairs between groups (also using the MCL model 1000 replicates bootstrap). Overall, the analysis involved 69 *B. glabrata* and four outgroup 16S rRNA nucleotide sequences (total 73 sequences). All positions containing gaps were excluded from the analysis, hence the final dataset comprised a total of 376 nucleotide positions.

Furthermore, using DnaSP version 6, similarity of samples' origins/localities was determined, by calculating the nearest neighbour statistic (S_{nn}) between geographic populations. Probability was obtained by applying a permutation test with 1000 replicates and levels of significance obtained were denoted as ns, not significant; *, $0.01<P<0.05$; **, $0.001<P<0.01$ and ***, $P<0.001$. This statistic, (i.e., S_{nn}) is a measure of how often the closest neighbours of sample sequences are found to be from the same location in geographic space. This is usually relevant when samples for genetic analysis are obtained from two or more localities. The value of S_{nn} is expected to be close to 1 when the populations at two localities are highly differentiated and near one-half when the populations at the two localities are part of the same panmictic population (Hudson, 2000).

4.3 Results

4.3.1. Intra-species variation of *B. glabrata* populations

This study presents analyses of polymorphisms using 73 nucleotide sequences of the partial 16S rRNA gene; 69 from *Biomphalaria glabrata* obtained from three populations of our laboratory stock (BgPR, BgPS and BgSV), 33 populations from published nucleotide sequences (NCBI database). Out of a total of 407 sites analysed (376 sites excluding gaps), 34 were variable and 27 were parsimony informative. Pair-wise number of substitutions ranged from 0 to 4.18% with an overall average of 1.84%. With the exception of BgSV where a statistically significant value was obtained (-1.701*, $p < 0.05$), Tajima's test indicated neutral evolution in all other intraspecific comparisons made ($p > 0.10$) (Tajima, 1989).

4.3.1.1 Overall haplotype diversity among populations

Overall, haplotype diversity was high among the snail populations suggesting high genetic diversity. Polymorphism data analysis revealed 20 haplotypes in the entire dataset. Overall haplotype (Hd) was 0.919 (excluding outgroups) and nucleotide diversity (Pi) was 0.018. Out of the 20 haplotypes, 10 (50%) were unique to samples obtained from laboratory-maintained populations, 6 (30%) to field-obtained samples while 4 (20%) were common to both the laboratory and field-sampled snails. Haplotype diversity of the combination of all laboratory strains (this study and published references), only slightly lower (0.878) than those of field isolates (0.890).

4.3.1.2 Diversity within current laboratory populations

The three laboratory populations sampled in this study (i.e., comprising of the BgPR, BgPS and BgSV strains) were found to belong to four of the twenty haplotypes identified. Haplotype diversity within the populations of the BgPR, BgPS and BgSV strains were 0.733, 0.714 and 0.250 respectively. Nucleotide diversity was however low within each sampled population. A summary of parameters estimated from analyses of DNA polymorphism, including nucleotide divergences for each group is given in Table 4.2.

Table 4.2: Summary of DNA polymorphisms based on comparison of partial 16S rRNA nucleotide sequences of laboratory and field strains of *B. glabrata*. Summary of parameters for the currently examined laboratory strains are shown in italics; combined analyses of laboratory and field reference sequences appear in normal font below the double line. Parameter totals for current and reference populations are differentiated as parameter totals 1, 2 & 3.

Strains/group	No. of sequences	Polymorphic sites (S)	No. of mutations (Eta)	No. of haplotypes (h)	Haplotype diversity (Hd)	Average no. of nucleotide differences (k)	Nucleotide diversity (Pi) ($x10^{-2}$)	θ/site from Eta ($x10^{-2}$)
BgPR	*6*	*9*	*9*	*3*	*0.733*	*4.267*	*1.102*	*1.019*
BgPS	*7*	*9*	*10*	*3*	*0.714*	*5.000*	*1.299*	*1.060*
BgSV	*8*	*8*	*8*	*2*	*0.250*	*2.000*	*0.517*	*0.797*
Parameters total 1	*21*	*12*	*13*	*4*	*0.738*	*4.633*	*1.210*	*0.943*
Laboratory strains (references only)	27	24	27	14	0.923	6.575	1.744	1.858
Laboratory strains (all)	48	24	27	14	0.878	5.848	1.551	1.614
Field isolates (references only)	21	21	23	10	0.890	5.024	1.308	1.665
Parameters total 2 (without outgroups)	69	27	30	20	0.919	6.737	1.836	1.695
Parameters total 3 (with outgroups)	73	52	60	24	0.928	6.535	1.757	3.318

4.3.2 Phylogenetic relationships among haplotypes

Phylogeny reconstruction using all 73 sequences showed a geographical division of all 20 haplotypes of the *B. glabrata* sequences into four main clades; two Brazilian clades (Brazil I and Brazil II), one clade for the Greater Antilles region and one representing Venezuela and Lesser Antilles region (Figure 4.1). These four clades appeared to form two main lineages. The Brazil I haplotypes formed one lineage. This lineage however appeared to have given rise to the two clades, the Greater Antilles and the Brazil II clades. The second lineage clearly included all haplotypes of the Venezuelan and Lesser Antilles clade. The Brazil I also appeared ancestral to the Venezuelan and Lesser Antilles clade.

4.3.2.1 Haplotype distribution

The first major clade, the Brazil I clade comprised nine haplotypes split into two subclades, one comprising haplotypes H1-H7. This appeared as sister clade to a second subclade which comprised haplotypes H8 & H9. All but one (Accession number DQ084845) of the individuals that formed the first subclade were samples from laboratory populations while the second subclade included two field obtained samples. The second major clade, the Brazil II clade comprised four haplotypes, H11-H14 with H11 forming sister taxa to the other three haplotypes. All samples from the present study clustered variously within the two Brazilian clades.

The third major clade, the Greater Antilles clade included a sole haplotype (H10) with sample representation from Dominican Republic and Puerto Rico

while the fourth major group (the Venezuelan & Lesser Antilles clade) comprised six haplotypes (H15-H20). With the exception of one sample (Accession number AY198098) from Brazil, all samples within this clade were either of Venezuelan or Lesser Antilles decent.

The phylogenetic tree clearly shows the clustering of samples from this study in relation to published 16S rRNA reference sequences of *B. glabrata*. The three *B. glabrata* strains (BgPR, BgPS and BgSV) sampled from our laboratory populations were represented within four haplotypes (i.e., 20% of the overall number of haplotypes in entire dataset), indicating a general lack of diversity. Three of these, haplotypes H1, H7 and H9 fell within Brazil I clade and the fourth, H11 within Brazil II. The BgPR occurred within three haplotypes (H1, H7 and H9), BgPS within three (H7, H9 and H11) and the BgSV within two (H1 and H9). Only samples of the BgPS population and two published samples of Brazilian origin were found clustered within the fourth haplotype, H11 of the Brazil II clade. A maximum likelihood tree showing the distribution of haplotypes within the four major geographical clades is shown in Figure 4.1.

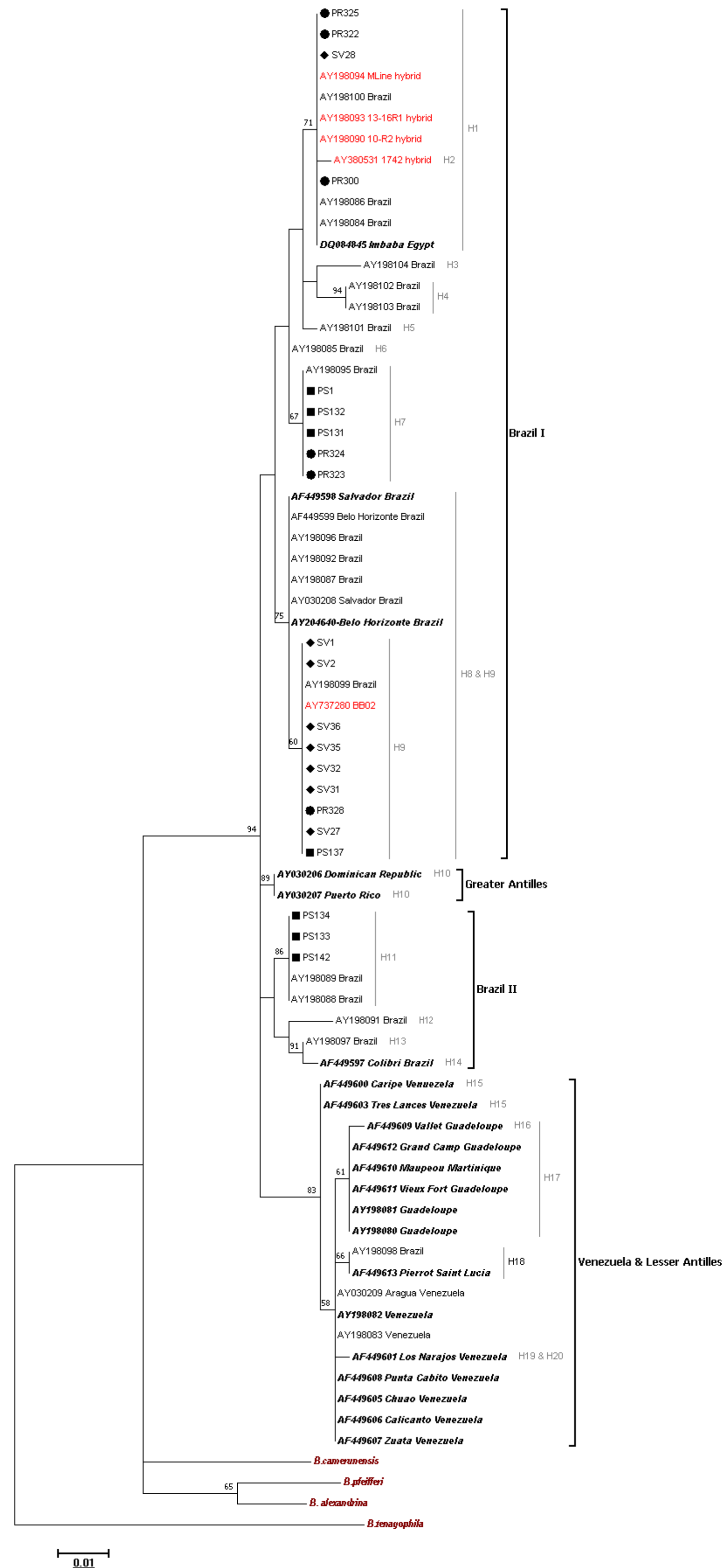

Figure 4.1: Maximum likelihood (ML) tree of 16S rRNA sequence data of *Biomphalaria glabrata*. Distribution of haplotypes H1-H20 within the four main geographical clades (right brackets) are shown. Current laboratory strains BgPR, BgPS & BgSV are indicated with circle, square & diamond shapes respectively. Accession numbers of reference sequences are shown for laboratory strains (normal black font), field isolates (bold, italic black font) and well published strains/isolates (red font). *Biomphalaria sp.* (outgroups) are indicated in maroon font. Bootstrap values >50% are indicated next to branches.

The mean evolutionary distances between the three strains used in the present study (21 sequences) was 0.0126 while for the entire *B. glabrata* sequence dataset analysed (69 sequences), the mean evolutionary distance estimate was 0.0184. For brevity, comparisons were made specifically between haplotypes H1,H7,H9 and H11 (to which the strains BgPR, BgPS and BgSV of the three populations sampled belonged) and other haplotypes. The other haplotypes in this comparison refer to all other Brazilian haplotypes (grouped as Brazil 1 (others) and Brazil II others) and Greater Antilles and Venezuelan-Lesser Antilles clades.

Results indicated that highest mean divergence (0.0245) was between haplotype H1 (of Brazil I) and the Venezuelan-Lesser Antilles clade. Interestingly, the lowest divergence (0.0082) was recorded between haplotype H11 (of Brazil II clade) and the single haplotype H10 of the Greater Antilles clade. Estimates of evolutionary divergence between the four major clades and haplotypes H1,H7,H9 and H11 and the outgroup sequences are presented in Figure 4.2.

SERIAL NUMBER	GROUP/OUTGROUP	SERIAL NUMBER											
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1	*B. tenagophila*		0.0222	0.0200	0.0202	0.0196	0.0197	0.0193	0.0196	0.0215	0.0199	0.0198	0.0189
2	*B. pfeifferi*	0.0874		0.0140	0.0123	0.0136	0.0137	0.0094	0.0137	0.0129	0.0139	0.0134	0.0143
3	**Brazil I (H9)**	0.0774	0.0483		0.0138	0.0035	0.0064	0.0130	0.0052	0.0072	0.0052	0.0058	0.0056
4	*B. camerunensis*	0.0805	0.0424	0.0479		0.0139	0.0139	0.0120	0.0139	0.0144	0.0143	0.0138	0.0147
5	Brazil I (others)	0.0762	0.0471	0.0084	0.0497		0.0042	0.0128	0.0042	0.0068	0.0048	0.0052	0.0040
6	**Brazil I (H1)**	0.0746	0.0457	0.0138	0.0482	0.0099		0.0132	0.0063	0.0083	0.0070	0.0066	0.0048
7	*B. alexandrina*	0.0746	0.0277	0.0426	0.0396	0.0427	0.0429		0.0132	0.0114	0.0124	0.0124	0.0125
8	Greater Antilles	0.0746	0.0456	0.0109	0.0481	0.0106	0.0138	0.0428		0.0066	0.0044	0.0046	0.0054
9	Venezuela & Lesser Antilles	0.0875	0.0458	0.0205	0.0539	0.0208	0.0245	0.0369	0.0178		0.0058	0.0062	0.0080
10	**Brazil II (H11)**	0.0780	0.0487	0.0109	0.0512	0.0118	0.0166	0.0397	0.0082	0.0150		0.0020	0.0061
11	Brazil II (others)	0.0790	0.0477	0.0145	0.0502	0.0150	0.0175	0.0428	0.0109	0.0181	0.0045		0.0063
12	**Brazil I (H7)**	0.0710	0.0485	0.0109	0.0510	0.0093	0.0084	0.0396	0.0109	0.0234	0.0137	0.0164	

Figure 4.2: Estimates of evolutionary divergence between *B. glabrata* haplotype groups and outgroups. Mean distance between groups (lower diagonal) and standard error estimates (upper diagonal) are shown. Groups are listed in serial numbers 1-12. Bold font shows clades & haplotypes (in parenthesis) of individuals from the present study; outgroup species names are in italics. Number of base substitutions per site from averaging over all sequence pairs between groups were calculated using the maximum composite likelihood (MCL) model, applying a bootstrap procedure of 1000 replicates. Analysis involved 73 nucleotide sequences.

Given the distribution of the BgPR, BgPS and BgSV snails within the clades, the geographical links of the snails were further determined by estimating the nearest neighbour statistic (S_{nn}). For the three laboratory populations sampled (BgPR, BgPS and BgSV), S_{nn} was found to be 0.583 ($p = 0.002$**), indicating that the populations were all from the same geographical location. Overall estimation of this statistic however suggested that all four clades (Brazil I, Brazil II, Greater Antilles and Venezuela & Lesser Antilles) were highly geographically differentiated (S_{nn}=0.996, $p < 0.001$***). Estimated S_{nn} values further indicated various levels of geographical differentiation between the Brazil II and the Greater Antilles clades (S_{nn}=1.000, $p = 0.016$ *), the Greater Antilles and Venezuela & Lesser Antilles clades (S_{nn}=1.000, $p = 0.013$*) and the Brazil I and Brazil II clades (S_{nn}=1.000, $p < 0.001$***).

4.4 Discussion

Intra-species genetic diversity of the partial 16S rRNA gene was investigated in three laboratory populations of *B. glabrata* in order to clarify/establish their phylogeographical links to published field and laboratory stocks. and determine the haplotype diversity of the populations. Results showed high haplotype diversity within the currently sampled laboratory strains and published reference sequences of *B. glabrata*. Furthermore, phylogenetic analyses of the three investigated laboratory strains supported the Brazilian and/or Puerto Rican ancestry of the snails.

4.4.1 Haplotype composition and diversity

The presence of two or more haplotypes within each of the currently sampled laboratory population (BgPR, BgPS and BgSV) was contrary to previous studies which showed single haplotypes found in most populations sampled (Dejong *et al*., 2003). For the BgPS snails, the presence of multiple haplotypes was not unexpected due to their hybrid nature. This strain was developed from inbreeding of four susceptible strains of *B. glabrata* thought to be from three different geographical origins, one of which was unknown.

The BgPR are albino snails which could have mixed background of Brazilian and Puerto Rican snails. Half of the BgPR samples shared the same haplotypes with the published hybrid strains, M-Line (AY198094), the 13-16-R1 (AY198093), the 10-R2 (AY198090) hybrid strains (haplotype H1) suggesting strong links with these hybrids. The *B. glabrata* M-line strain is reported to have resulted from a cross between *B. glabrata* from Puerto

Rico (a susceptible population) and offspring of resistant snails from Salvador, Brazil (DeJong *et al*., 2004; Newton, 1955). Furthermore, it has been reported that the M-line stock are not genetically homogeneous within populations (Mulvey & Bandoni, 1994). Similar to the M-line strain is the 1742 strain (Campbell *et al*., 2000). It is also worthy of note that the M-line strains were also reported to have later been used as females in a cross with resistant Puerto Rican strains to create the resistance-selected hybrids, the partially resistant 10-R2 and the resistant 13–16-R1 (Dejong *et al*., 2003; Richards, 1973; Richards & Merritt Jr., 1972). The ML tree clearly corroborates the reported history of these hybrids.

In contrast to the BgPS and BgPR strains, all but one of the BgSV sequences clustered with haplotype H9, indicating the BgSV probably formed a more homogeneous population than the other two sampled populations. The overall haplotype diversity within each strain differed. While it was high in the BgPS and BgPR strains, diversity between BgSV haplotypes was low further supporting the homogeneous nature of the latter. In addition, multiple haplotypes could result from outcrossing due to migrations between populations. A plausible explanation that could be considered is if accidental transfers of strains between aquaria have occurred during routine maintenance procedures. In the case of the BgPR and BgSV snails, however, such accidental transfer would have been easily detected due to the different pigmentation phenotypes of the two strains. The presence of one BgPS sample with haplotype H9 could however be a result of accidental transfer. While utmost care is taken to avoid this (e.g., different strains separated in shelves, thorough washing and drying to remove any eggs)

such transfer of snail between aquaria is not impossible since the strains are maintained in the same room.

A comparison of haplotype distribution between sequences of laboratory (this study and references) and field obtained samples showed that number of haplotypes was higher in the laboratory samples than those of field samples. This was however a reflection of the presence of nucleotide sequences of hybrids within the populations analysed. *Biomphalaria glabrata* populations are sometimes selected for resistance or susceptibility for experimental purposes and development of hybrid populations in the laboratory are not uncommon. In this investigation, five hybrid populations, including one from our laboratory were included in the analyses. Also, polymorphism data showed high haplotype diversity but low nucleotide diversity within the three populations currently sampled. Typically, this could suggest some form of bottlenecking event which may have been followed by population expansion (e.g., as a result of multiple founder events and selections).

Considering that the snails have been under laboratory maintenance for several years, there is likelihood that such bottlenecking conditions could occur especially as a result of intensive breeding schemes for the continuous maintenance parasite life cycle and inadvertent selection. However, given that the overall diversity in comparison to sequences from other laboratory and field obtained samples were similar, it is also possible that the snails have maintained some level of genetic variation in spite of several bottlenecking and founding events. Moreover, aquarium stocks and field

populations have been reported not to differ markedly in levels of genetic variation (Bandoni *et al*., 1995b; Mulvey & Vrijenhoek, 1981).

4.4.2 Phylogeny and relationships between haplotypes

While the present phylogenetic analysis suggests that the BgPR and BgSV laboratory strains are closely related to *B. glabrata* of Brazilian descent, it is also indicated that the hybrid BgPS belonged to a second Brazilian clade which showed close links with haplotypes of Caribbean origin (Puerto Rico and Dominican Republic). The overlapping appearance of Brazilian and Caribbean haplotypes has been previously reported (Dejong *et al*., 2003). The origin of one of the snails from which the BgPS laboratory hybrid was derived was unknown however, this result suggests sister status with the highly divergent group, Brazil II haplotypes H12, H13 and H14 that appear closer to snails of Caribbean origin than those of major Brazilian descent (Brazil I).

The overlapping distribution pattern of Brazilian haplotypes across geographical regions has been suggested to be due to introduction of freshwater snails into these areas through migration. Such introduction of snail species into freshwaters is not uncommon and has been reported in the Caribbean (Pointier, 1999) and in African locations (Pflüger, 1982). In the work of Dejong, *et al*. (2003), presence of natural populations from Brazil clustering with populations from Lesser Antilles was similarly attributed to long-distance colonisation events. Substantial diversity of *B. glabrata* snails has been shown by other authors with distinct clades identified within the species (Dejong *et al*., 2003; Mavarez, *et al*., 2002). In most populations sampled by these authors, unique haplotypes were

identified and there were more Brazilian haplotypes than other geographical regions, suggesting that the Brazilian populations formed a more diverse group. Studies of Brazilian *B. glabrata* snails using RAPDs have also indicated that *B. glabrata* are considerably heterogeneous (Vidigal *et al*., 1994). The clustering of most of the BgSV sequences (except one) within a specific haplotype suggests their ancestral links to field isolates from Salvador, Brazil which is consistent with their historical record. The tree also shows the ancestral link of the BB02 strain to other field strains from Belo-Horizonte, corroborating its origin (Adema *et al*., 2017).

Based on the objectives of the study, sampling was limited to minimum of six individuals per population. While phylogeny showed a distinct structuring of haplotypes into geographical lineages similar to previously identified clades, the divisions are meant to define the ancestral links of the haplotypes considering the poor resolution of the Brazil II haplotypes. Given that laboratory populations are usually bottlenecked, the low number of haplotypes found may be indicative of existing low diversity among snail populations. The current diversity is however not inviolable as differences in gene flow has also been suggested to play an important role in population differentiation and in restoring genetic diversity in populations that are demographically unstable (Mavarez *et al*., 2002).

This chapter further served to define the current laboratory strains based on the 16S rRNA haplotypes in an attempt to clarify the history the laboratory populations. These results answer the questions on the number and diversity of haplotypes present in the current laboratory stock of snails, thereby giving an insight into how the snails compare to previously

published *B. glabrata* snails from other laboratories and different geographical locations. The findings thus provide information on neutral diversity and could be useful for routinely characterizing laboratory snail populations in order to determine phylogenetic relationships between the various strains of *B. glabrata* used in research laboratories.

Establishing phylogenetic links of *B. glabrata* populations could aid better comparison and interpretation of results obtained in various laboratories. The variations in this gene however does not provide a link to the fitness of snails since they are selectively neutral. The next chapter investigates an adaptive locus that may provide insight into the evolutionary potentials of the population. Understanding the genetic diversity of laboratory populations could provide further insight into the dynamics of snail-schistosome interactions.

CHAPTER 5

INVESTIGATIONS OF THE *SOD1* GENE AS A MARKER FOR RESISTANCE/SUSCEPTIBILITY IN *BIOMPHALARIA GLABRATA*

5.1 Introduction

In *Biomphalaria glabrata*, resistance or susceptibility to *Schistosoma mansoni* is an heritable trait that may be controlled by many loci (Bayne, 2009; Lockyer *et al*., 2007). There is also evidence to suggest that just a few genes are mainly involved in determining resistance in the snail (Moné *et al*., 2011; Richards & Merritt Jr., 1972). The presence of both resistant and susceptible phenotypes within populations of *B. glabrata* has been crucial in the quest to find genes associated with resistance since the possibility of introducing such genes into field populations to control parasite transmission was suggested almost six decades ago (Hubendick, 1958). Identifying various compatibility genes involved in host-parasite interaction therefore remains a key component of schistosome research.

To date, however, only one loci, the copper/zinc superoxide dismutase 1 (*sod1*) has been demonstrated to show strong polymorphic associations with the compatibility phenotypes (resistance or susceptibility) in this snail-parasite interaction (Goodall *et al*., 2006). As an important component of the cellular defence, the mobile cells, known as haemocytes, act by encapsulation of invading schistosome larvae. These cells are also responsible for subsequent killing of parasites through production of reactive oxygen and nitrogen species. Specifically, studies investigating the underlying molecular mechanisms of compatibility phenotypes in *B. glabrata* snails (Hahn *et al*, 2001a), found that the cytotoxic response of

haemocytes of resistant snails differed from that of susceptible ones in the event of *S. mansoni* parasite invasion. Furthermore, findings revealed the involvement of reactive oxygen species, hydrogen peroxide (H_2O_2), the enzymatic product of the cytosolic Cu/Zn superoxide dismutase (*sod1*) in this differential parasite clearance (Hahn *et al*, 2001b). Polymorphism in the *sod1* gene has thus led to suggestions of possible links to differential expressions of the gene and susceptibility and resistance in snails.

Subsequently, studies conducted by Goodall and other workers (Bender *et al*., 2007; Goodall *et al*., 2006) showed a direct link between polymorphism in the *Cu/Zn superoxide dismutase* (*sod1*) gene and *B. glabrata* resistance-susceptibility phenotypes in relation to *S. mansoni* infection. The initial findings by Goodall *et al* using resistant 13-16-R1 and susceptible M-line (Oregon) hybrid strains of *B. glabrata* identified three alleles, A, B and C and indicated that the B allele was unique to the resistant 13-16-R1 strain (Goodall *et al*., 2006). After further investigations using only the 13-16-R1 resistant snails, the B allele was demonstrated to have a strong link with the resistant phenotype and the C allele with the susceptible phenotype of *B. glabrata* snails (Goodall *et al*, 2006).

Subsequently, a fourth allele, previously mentioned by Goodall and colleagues, was identified and reported as allele D by other researchers (Tennessen *et al*., 2015), working with the same resistant hybrid population (13-16-R1). In the latter study, significance of allele A in relation to allele C in determining susceptibility was also clarified and both alleles were suggested to be functionally equivalent (i.e., both associated with susceptibility). Furthermore, the possibility that the D allele may also

determine resistance was suggested (Tennessen *et al*., 2015). The link of *sod1* allele B (and possibly, allele D) to resistance and *sod1* alleles A and C to susceptibility remains un-tested in other *B. glabrata* populations. Identification and characterisation of genes linked to compatibility in the snail vectors could further elucidate their roles in adaptive or evolutionary potentials of snail host populations and provide more insight into the dynamics of the host-parasite interactive processes.

This study aimed to investigate the *sod1* alleles segregating in populations of *B. glabrata* currently being maintained in our laboratory. Our principal objective was to determine if the B allele link to resistance could also be corroborated in other laboratory populations of *B. glabrata.* These snail populations have been in laboratory culture for many years and have also been demonstrated to vary in resistance and susceptibility to *S. mansoni.* Investigating this link in other *B. glabrata* populations could provide further insight for future applications in marker-assisted selections and for development of genetic-based vector control strategies.

5.2 Materials and methods

5.2.1 Snail hosts and parasite

Experiments for this study were carried out using snails sampled from laboratory populations of the three *B. glabrata*, strains, namely, BgPR, BgPS and BgSV. These snails have been in laboratory culture for the maintenance of *S. mansoni* life cycle for more than a decade. Patency data obtained from previous and present laboratory studies was a vital source of information on the general susceptibility status of the snails. At the time this study

commenced, the BgPR strains constituted a generally susceptible population (but varied periodically in susceptibility, sometimes tending toward resistance as reported in chapter three) while the BgSV predominantly exhibited the resistant phenotype (patency <25%). The BgPS strains on the other hand had consistently maintained a high degree of susceptibility to parasite infection. All three populations were sampled for characterisation of *sod1* alleles. All snail infections were performed with the PR-1 strain of *S. mansoni*.

5.2.2 Infection experiments and sampling criteria

For the this investigation, five infection experiments were performed to determine susceptibility of individual snails to the PR-1 strain of *S. mansoni*. Samples of susceptible (infected, shedding cercariae) and resistant (not infected, not shedding cercariae) individuals were then obtained from the three populations for genotyping. Thus, snails sampled for genotyping were obtained from these five infection experiments.

Four of the infection experiments involved individuals sampled from populations of the BgPR and BgSV strains (identified as experiments 1-4). In addition, a fifth infection experiment (experiment 5) involving individuals from the highly susceptible BgPS strain was also performed. For each of the four infection experiments (i.e., experiments 1-4), equal numbers (except for experiment 2) of the BgPR and BgSV snails were typically used such that total number of snails exposed per tank ranged from 50-70 snails.

Thus, for infection experiment 1, number of BgPR and BgSV snails were 35 snails each (total 70), 25 BgPR and 45 BgSV for experiment 2 (total 70),

30 snails each for experiments 3 (total 60) and 30 snails each for experiment 4 (total 60). Experiment 5 was performed using 50 BgPS snails.

For all infection experiments, snails ranging from 10-12 mm shell diameter were mass exposed to miracidia overnight (approximately 12 hours) in tanks containing 3 litres of deionized water, each snail being infected with 50-100 miracidia. At the end of each infection experiment (i.e., 35 days post-exposure), all surviving snails were individually screened for parasite infection. The susceptible (infected, shedding cercariae) or resistant (not infected, not shedding cercariae) phenotype was then assigned to each snail. Details of snail infection and screening procedures can be found in general materials and methods (Section 2.1.4).

Secondly, in order to identify the *sod1* alleles present in laboratory populations of the three *B. glabrata* strains, snails for which susceptibility status (infected, susceptible or not infected, resistant) had been previously determined were used for genotyping. For each infection experiment the sampling plan was to genotype equal numbers obtainable for infected (susceptible) and uninfected (resistant) snails per strain. This was set to a minimum of 10 snails each for either infected or uninfected groups. If this number was not obtainable for any group in an infection experiment, number obtainable for each group was selected for genotyping. This was set to a maximum of 25. In all, 135 individuals (81 BgSV, 42 BgPR and 12 BgPS) were genotyped. Thirdly, frequencies of the *sod1* alleles in the snails were determined in order to identify possible links between different alleles and resistance in snails.

5.2.3 PCR amplification and genotyping of *B. glabrata sod1* gene

To detect polymorphism at the *sod1* locus, the fourth intron was amplified by PCR. Using previously designed *sod1* primers the 3' terminal intron of the Cu/Zn *SOD1* gene (*sod1*) was amplified in a two-step PCR process (nested PCR). The first amplification (primary PCR) targeted a 460bp sequence using the first set of primers, 1F and 1R. The second PCR amplification with a second set of primers, F-(VIC) and R-(VIC) resulted in amplification of approximately 240bp, a shorter fragment of the region of interest.

A key target for identification and differentiation between the previously published resistant allele B and the other alleles A, C and D was a two base pair insertion/deletion event within the polymorphic region of the fourth intron (Bonner *et al*., 2012; Tennessen *et al*., 2015). Details of primer pairs 1F and 1R (Goodall *et al*., 2006) and F- & R- (VIC) (Bonner *et al*., 2012) used for primary and secondary PCR respectively are shown in Table 5.1.

Table 5.1: Details of primers used in DNA amplification, sequencing and genotyping

Primer name	Direction	Primer sequence (5' – 3')	References
1F	Forward	GGTGATGATGGTGTTGCTGA	Goodall *et al*, 2006; Lockyer *et al*, 2012
1R	Reverse	GATACCAATGACACCACAAGCTAA	Goodall *et al*, 2006; Lockyer *et al*, 2012
F(VIC)	Forward	TCATTGGTCGCAGCTTAGTG	Bonner *et al*, 2012
R(VIC)	Reverse	GTCCTGTCATGTAGCCACCA	Bonner *et al*, 2012

Each amplification was performed using BIOTAQ™ DNA Polymerase (Bioline Company, UK) in a 25 μL reaction which included either 1 μL of DNA or primary PCR product as template, 200 μM dNTPs, 1.5mM $MgCl_2$, 0.2 μM of each primer, 0.5 units of Taq DNA polymerase (Bioline Company, U.K) and 2.5 μL of 10x reaction buffer. Cycling conditions for the PTC100™ programmable thermal cycler for both primary and secondary PCR amplifications were: 1 cycle of 94°C for 2 minutes, 94°C for 30 seconds, 58°C (annealing) for 30 seconds, 72°C for 90 seconds followed by 35 cycles of 94°C for 30 seconds, 58°C for 30 seconds, 72°C for 90 seconds and ending with a final step of 72°C for 10 minutes. At the end of the cycle, PCR products were held at 20°C until they were removed from the machine.

5.2.4 Identifying heterozygotes and Limiting Dilution PCR (LD-PCR) genotyping

To separate and amplify single template DNA from heterozygotes, the limiting dilution PCR was performed on each sample as a nested PCR amplification. This method is widely applicable due to its technical simplicity and because it circumvents the problem of excessive, competing targets. This method has been used successfully to separate and detect multiple pro-viral DNA molecules (Wade *et al*., 1998). Here, the method was adapted for amplification and separation of alleles of the *sod1* gene intron. First, a direct amplification of each snail sample was conducted as described in section 5.2.3. After sequencing, chromatograms were examined. Samples with a mixed template show double peaks resulting in base miscalling and were assigned a heterozygote status. All samples were then re-amplified

using a limiting dilution approach which involved a series of 10 fold serial dilutions (to achieve single molecule amplification of DNA) with the first set of primers (1F and 1R). This served as the end-point and working dilution for each sample. For amplifications performed using limiting dilution, amplification of a single molecule of template DNA was considered successful if ≤20% of each sample’s amplicons were successfully amplified. The second reaction resulted in the amplification of approximately 240bp, a shorter fragment of the region of interest. A key target for identification and differentiation between the resistant allele B and the susceptible alleles A and C was a two base pair insertion/deletion event within the polymorphic region of the fourth intron (Bonner *et al*., 2012; Goodall *et al*., 2006).

5.2.5 PCR product visualisation, purification and sequencing

Amplified targets were detected on agarose gel (1.5% agarose prepared with TBE (Tris/Borate/EDTA) buffer. Approximately 5 µL of PCR product was analysed by gel electrophoresis with 5 µL of ethidium bromide (EtBr) added to the gel mixture to aid visualization of the amplified DNA target bands. Gels were electrophoresed at 80-100V and bands visualized and photographed with a trans-illuminator (Bio Rad Gel Doc System, Bio Rad, U.K). End products of PCR amplification were purified using the Qiagen PCR purification kit (Qiagen, U.K) according to manufacturer’s instructions. Following company specifications for sequencing PCR products, six replicates each of the products of each LD-PCR amplification were sent to Macrogen Europe Laboratory for sequencing in both directions using primers F-(VIC) and R-(VIC).

5.2.6. Sequence analysis

Nucleotide sequences of the 3' terminal intron of *SOD1* (forward and reverse strands) were assembled using Trev version 1.9-r and GAP 4 Version 4.8.8 (https://www.gap-system.org) applications of the Staden package (Staden *et al*., 2000). The sequences were then imported into the Genetic Data Environment (GDE) (Smith *et al*., 1994) for alignment. To determine polymorphic sites for the identification of alleles, published sequences for *sod1* alleles were also downloaded from the NCBI data base and included as references in the alignment. All aligned sequences were visually cross-checked and corrected as necessary.

5.2.7. Allele nomenclature

Nucleotide sequences were imported into DnaSP version 6 and haplotype data was generated to identify the various haplotypes present. After analysis of nucleotide data, each sequence variant identified was considered an allele and assigned a haplotype number. The previously identified *sod1* alleles A, B, C and D (Goodall *et al*., 2006; Tennessen *et al*., 2015) are here referred to as haplotypes H1-H4. Newly identified variants were given haplotype numbers H5 on. This change to nomenclature was necessary as the number of haplotypes (alleles) identified exceeded the number of letters in the alphabet.

5.2.8 Statistical analysis

Statistical significance of the differences in percentages of infected individuals in the five experiments were determined using the Fisher's exact

test as implemented in GraphPad Prism version 8.0 for Windows, GraphPad Software, San Diego, California USA, (www.graphpad.com). The genotype-phenotype link to resistance was also tested using the Fisher's exact test. This test was performed to test the hypothesis of no association between infection outcomes (resistant, shedding cercariae or susceptible, not shedding cercariae) and presence of *sod1* genotypes. Associations were determined to be significant at $p < 0.05$.

5.2.9 Genotypic, phylogenetic and network analysis

Deviation from Hardy-Weinberg equilibrium was determined in relation to the *sod1* gene haplotypes by Fisher's exact test using the Markov Chain algorithm as implemented in the web version of GenePop version 4.2 (Raymond & Rousset, 1995; Rousset, 2008). Genotype matrices were also generated for each sampled population.

Phylogenetic analyses were conducted in MEGA7 (Kumar *et al*., 2016). Evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei, 1987) and evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2004). The nucleotide sequence of each allele (here referred to as haplotype) was used for phylogeny reconstruction. Network analysis was performed on NETWORK 5.0.0.3 software (http://www.fluxus-engineering.com) for building phylogenetic networks using the median joining approach (Bandelt *et al*., 1999) and incorporating all nucleotide sequences the three populations. Both phylogeny reconstruction and haplotype network building

were based on 214 nucleotide sequences representing a total of 38 haplotypes.

5.3 RESULTS

5.3.1 Multi-strain characterisation of *Biomphalaria glabrata sod1* gene

This investigation set out to identify the *sod1* alleles (haplotypes) in laboratory populations of three laboratory strains of *B. glabrata* and to determine their link to resistance or susceptibility phenotypes in snails exposed to *S. mansoni*. Results revealed considerable polymorphism and variability in *B. glabrata sod1* phenotype-genotype association with identification of 34 additional alleles within the investigated locus.

5.3.1.1 Variations in *B. glabrata* susceptibility

The first stage of the study involved infection experiments to determine the phenotype of individual snail samples in terms of susceptibility or resistance to *S. mansoni*. Results revealed considerable variation in percentage of snails infected in each infection experiment for the BgPR and BgSV snails and high susceptibility in the BgPS snails. Out of the 260 snails used in the four infection experiments involving BgPR and BgSV strains, 195 survived and were individually assessed for parasite infection. Percentage of positive infections in the four experiments ranged from 50-77% (experiment 1, 77%; experiment 2, 55%; experiment 3, 50% and experiment 4, 61%) in BgPR snails and 14-46% (experiment 1, 14%; experiment 2, 42%;

experiment 3, 46% and experiment 4, 43%) in BgSV snails. For the fifth experiment involving 50 BgPS snails, 39 surviving snails were screened for infection and 95% were infected. Percentages of positive infections in relation to dates of experiment are shown in Figure 5.1. Overall, estimated mortality for the three strains ranged from 20-25%.

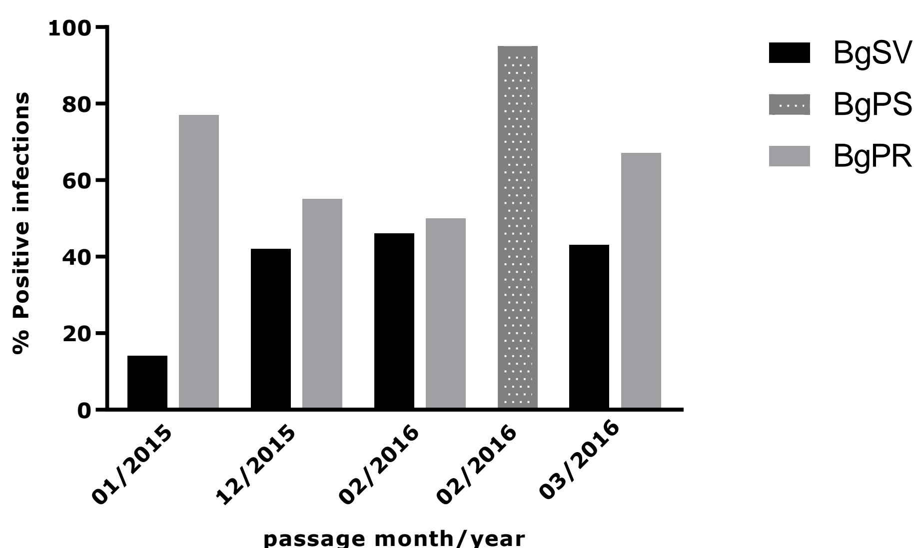

Figure 5.1: Percentage susceptibility of *B. glabrata* strains BgSV (black bars), BgPR (grey bars) and BgPS (patterned bar) to PR-1 *S. mansoni* strain. Each paired black and grey bars represent individual infection experiments. Single patterned bar represents the infection experiment of BgPS. Month and year of infection experiments are indicated on the X-axis.

Although the BgSV snails showed a lower number of infected (susceptible) individuals in the first infection experiment (<20%), this appeared to change in subsequent experiments indicating a decrease in resistance. Fisher's exact tests confirmed there was a statistically significant difference in percentage susceptibility of BgSV snails between the first infection

experiment and the next two infection experiments ($p < 0.05$). Statistical differences in percentages of infected individuals observed between experiments 1-4 are presented in Table 5.2.

Table 5.2: Statistical differences in susceptibility of BgSV & BgPR strains between infection experiments. Levels of significance are shown in parenthesis.

Comparison	BgSV strain	BgPR strain
Experiment 1 – 2	0.0282 (*)	0.2038 (ns)
Experiment 1 – 3	0.0284 (*)	0.0980 (ns)
Experiment 1 – 4	0.0492 (*)	0.3228 (ns)
Experiment 2 – 3	0.8068 (ns)	>0.9999 (ns)
Experiment 2 – 4	>0.9999 (ns)	0.7521 (ns)
Experiment 3 – 4	>0.9999 (ns)	0.7303 (ns)

5.3.1.2 Extensive polymorphism in the 4th intron of *sod1*

Analysis of nucleotide sequences of the three laboratory populations sampled revealed that the *sod1* locus displayed high polymorphism. Thirty-seven haplotypes were identified in 135 snails genotyped and these are denoted as haplotypes H5-H38. These included 34 new haplotypes of which haplotype H5 was most common. Nucleotide sequences of the haplotypes consisted of 15 polymorphic sites (indel positions included). This brings the total number of haplotypes found in the 3' terminal *sod1* intron to 38, with a total of 22 variable sites.

Three of the four previously identified *sod1* haplotypes (H1, H2 and H4 corresponding to alleles A, B and D respectively), were found in the three currently sampled snail population. Haplotype H3 (allele C) was not found.

A distinguishing feature of the previously reported, resistance-linked haplotype H2 (i.e., allele B) (Goodall *et al*, 2006) is a two-base pair (bp) insertion (Bonner *et al*., 2012). These insertions were also observed in haplotypes H18 and H33. All detected *sod1* haplotypes and nucleotide bases at each polymorphic site are listed in Table 5.3. The names and accession numbers of previously reported haplotypes are included where available.

Table 5.3: Polymorphism of the *sod1* intron and haplotype designations. Previously published alleles are represented as haplotypes H1-H4 (with accession numbers and published designated allele names A-D indicated alongside); H5-H38 represent newly identified alleles; additionally detected polymorphic positions (this study) are underlined.

ACCESSION NO./HAPLOTYPE DESIGNATION	VARIABLE SITE NUMBERS																					
	4	**13**	**18**	**21**	**22**	**48**	**81**	**93**	**<u>98</u>**	**113**	**<u>119</u>**	**129**	**<u>135</u>**	**137**	**141**	**145**	**<u>157</u>**	**<u>170</u>**	**185**	**194**	**195**	**252**
DQ239577 (A) – H1	A	A	T	G	A	T	G	C	A	T	A	T	G	A	A	C	G	T	G	-	-	A
DQ239578 (B) – H2	A	A	C	G	A	T	G	T	A	-	A	C	G	T	T	T	G	T	G	T	T	G
DQ239579 (C) – H3	T	C	T	A	T	T	G	T	A	-	A	C	G	T	T	C	G	T	C	-	-	G
Reported (D) – H4	T	C	T	A	T	A	T	T	A	-	A	C	G	T	T	C	G	T	G	-	-	G
H5	A	A	T	G	A	T	G	T	C	T	A	T	G	A	T	C	G	A	G	-	-	G
H6	A	A	T	G	A	T	G	T	A	T	G	T	G	A	T	C	A	T	G	-	-	G
H7	A	A	T	G	A	T	G	T	C	T	A	T	A	A	T	C	G	A	G	-	-	G
H8	A	A	T	G	A	T	G	C	A	T	G	T	G	A	A	C	A	T	G	-	-	G
H9	G	A	T	G	A	T	G	T	A	-	A	C	G	T	T	T	G	T	G	-	-	G
H10	A	A	T	G	A	T	G	T	C	T	A	T	G	A	T	C	A	T	G	-	-	G
H11	A	A	T	G	A	T	G	T	C	T	A	T	G	A	T	C	G	T	G	-	-	G
H12	A	A	T	G	A	T	G	T	A	T	G	T	G	A	T	C	A	A	G	-	-	G
H13	A	A	T	G	A	T	G	T	C	T	G	T	G	A	T	C	G	T	G	-	-	G
H14	A	A	T	G	A	T	G	T	C	T	G	T	G	A	T	C	A	A	G	-	-	G
H15	A	A	T	G	A	T	G	T	A	T	G	T	G	A	T	C	G	A	G	-	-	G
H16	A	A	T	G	A	T	G	T	A	-	A	T	G	A	T	T	G	T	G	-	-	G
H17	A	A	T	G	A	T	G	T	A	T	A	T	G	A	T	C	G	T	G	-	-	G
H18	A	A	C	G	A	T	G	T	A	-	A	C	G	A	T	T	G	T	G	T	T	G
H19	A	A	C	G	A	T	G	T	C	T	A	T	G	A	T	C	G	A	G	-	-	G
H20	A	A	T	G	A	T	G	T	C	-	A	T	G	A	T	T	G	A	G	-	-	G

Table 5.3 (continued): Polymorphism of the sod1 intron and haplotype designations. Previously published alleles are represented as haplotypes H1-H4 (with accession numbers and published designated allele names A-D indicated alongside); H5-H38 represent newly identified alleles; additionally detected polymorphic positions (this study) are underlined.

ACCESSION NO./HAPLOTYPE DESIGNATION	VARIABLE SITE NUMBERS																					
	4	13	18	21	22	48	81	93	98	113	119	129	135	137	141	145	157	170	185	194	195	252
H21	A	A	T	G	A	T	G	T	A	-	A	C	G	A	T	T	A	T	G	-	-	G
H22	A	A	T	G	A	T	G	T	A	T	A	T	G	A	T	C	A	T	G	-	-	G
H23	G	A	T	G	A	T	G	T	A	-	A	T	G	A	T	C	A	T	G	-	-	G
H24	G	A	T	G	A	T	G	T	A	T	A	T	G	A	T	C	A	T	G	-	-	G
H25	A	A	T	G	A	T	G	T	A	-	A	T	G	A	T	C	G	T	G	-	-	G
H26	G	A	T	G	A	T	G	T	C	-	A	T	G	A	T	T	G	T	G	-	-	G
H27	G	A	T	G	A	T	G	T	A	T	A	T	G	A	T	T	G	T	G	-	-	G
H28	G	A	T	G	A	T	G	T	C	-	A	T	G	A	T	C	G	T	G	-	-	G
H29	A	A	C	G	A	T	G	T	C	-	A	T	G	A	T	C	G	T	G	-	-	G
H30	A	A	C	G	A	T	G	T	C	T	A	T	G	A	T	C	G	T	G	-	-	G
H31	A	A	C	G	A	T	G	T	C	-	A	T	G	A	T	C	G	A	G	-	-	G
H32	A	A	T	G	A	T	G	T	C	-	A	T	G	A	T	C	G	A	G	-	-	G
H33	A	A	C	G	A	T	G	T	A	-	A	C	G	T	T	C	G	A	G	T	T	G
H34	A	A	C	G	A	T	G	T	A	T	A	T	G	T	T	C	G	A	G	-	-	G
H35	A	A	C	G	A	T	G	T	C	-	A	C	G	T	T	C	G	A	G	-	-	G
H36	A	A	C	G	A	T	G	T	C	T	A	T	G	T	T	C	G	A	G	-	-	G
H37	A	A	T	G	A	T	G	T	C	-	A	T	G	A	T	C	G	T	G	-	-	G
H38	A	A	T	G	A	T	G	T	A	T	A	T	G	A	T	C	G	A	G	-	-	G

5.3.1.3 Conformation to Hardy-Weinberg expectations

The HWE was determined in relation to *sod1* gene polymorphism. Overall, the three *B. glabrata* laboratory populations sampled showed deviations from Hardy-Weinberg equilibrium as determined by Fisher's exact test using the Markov Chain algorithm (GenePop version 4.2). This deviation was highly significant ($p < 0.0001$) and was attributable to an overall deficit of heterozygotes (Table 5.4).

Table 5.4: Summary of Hardy-Weinberg probability test. Inbreeding coefficient (F_{IS}) estimates are shown by strain of *B. glabrata*. Estimates obtained by the Markov Chain (MC) method as determined by the methods of Weir and Cockerham (W&C) and Robertson and Hill (R&H) are shown.

Snail strain	*p*-value (HWE)	*p*-value (Heterozygote deficit)	F_{IS} (W&C)	F_{IS} (R&H)
BgSV	0.00001	0.0001	0.1588	0.1168
BgPR	0.00001	0.0405	0.1950	0.0422
BgPS	0.00001	0.0863	0.1784	0.1326

5.3.1.4 Distribution of the *sod1* haplotypes

The most frequent haplotype (allele) found was one of the newly identified haplotypes, haplotype H5 (allele-wise frequency, 48.5%) with highest numbers of homozygote and heterozygote individuals (genotype-wise frequency, 61.5%). This haplotype was mostly found in the BgSV snails (known to be predominantly resistant) compared to the more susceptible BgPR and BgPS snails. The frequencies of three of the four previously reported haplotypes (i.e., alleles A, B and D, designated as haplotypes H1, H2 and H4 respectively) in the currently sampled laboratory snails were H1 (1.1%), H2 (14.8%) and H4 (0.4%). The fourth, haplotype H3 (allele C) was completely absent.

The frequency of the resistance-associated haplotype H2 (allele B) was high compared to the other three reported haplotypes (ranked second in overall frequency, after haplotype H5). Frequencies of each of the other haplotypes were generally low (<10%). Also, approximately 71% of all haplotypes were low frequency alleles (frequencies <1%). Allele-wise (haplotypes) and genotype-wise (homozygotes and heterozygotes) distribution of all haplotypes in the three populations sampled in relation to the compatibility status of snails (resistant i.e., not infected, not shedding cercariae or susceptible i.e., infected, shedding cercariae) is presented in Tables 5.5 and 5.6 respectively. The genotype matrices of the sampled populations can be found in appendix 9, Figures S5A, S5B and S5C).

Table 5.5: Distribution of 37 sod1 haplotypes (alleles) identified in BgSV, BgPR and BgPS laboratory populations of *B. glabrata*. Total numbers and percentages of haplotypes of resistant (uninfected, not shedding cercariae) and susceptible (infected, shedding cercariae) snails are shown in the last row and column. Numbers and percentages (in parenthesis) are also indicated for haplotypes with >1% frequency across populations. Names of previously reported alleles are indicated in parenthesis alongside their haplotype designation.

Haplotype/ (Allele) name designation	**BgSV**		**BgPR**		**BgPS**		**Total number (%)**
	Number resistant	Number susceptible	Number resistant	Number susceptible	Number resistant	Number susceptible	
H1 (A)	1	0	0	0	0	2	3 (1.11)
H2 (B)	20 (7.41)	5 (1.85)	4 (1.48)	8 (2.96)	0	3 (1.11)	40 (14.81)
H3 (C)	0	0	0	0	0	0	0 (0.00)
H4 (D)	0	0	0	0	0	1	1 (0.37)
H5	61 (22.59)	51 (18.88)	6 (2.22)	10 (3.70)	0	3 (1.11)	131 (48.52)
H6	5 (1.85)	0	1	4 (1.48)	0	4 (1.48)	14 (5.19)
H7	1	0	3 (1.11)	6 (2.22)	0	2	12 (4.44)
H8	0	0	0	0	0	1	1 (0.37)
H9	0	0	7 (2.59)	13 (4.81)	0	0	20 (7.41)
H10	0	0	0	1	0	0	1 (0.37)
H11	0	0	0	1	2	2	5 (1.85)
H12	0	0	1	1	0	0	2 (0.74)
H13	0	0	1	0	0	0	1 (0.37)
H14	0	0	2	0	0	0	2 (0.74)
H15	0	0	0	0	2	2	4 (1.48)
H16	0	0	1	0	0	0	1 (0.37)
H17	0	0	1	1	0	0	2 (0.74)
H18	2	0	0	0	0	0	2 (0.74)
H19	2	3 (1.11)	0	0	0	0	5 (1.85)
H20	0	0	0	1	0	0	1 (0.37)
H21	0	0	0	1	0	0	1 (0.37)
H22	0	0	1	1	0	0	2 (0.74)
H23	0	0	0	1	0	0	1 (0.37)
H24	0	0	0	1	0	0	1 (0.37)
H25	0	0	0	1	0	0	1 (0.37)
H26	0	0	0	1	0	0	1 (0.37)
H27	0	1	0	1	0	0	2 (0.74)
H28	0	1	0	0	0	0	1 (0.37)
H29	0	1	0	0	0	0	1 (0.37)
H30	0	1	0	0	0	0	1 (0.37)
H31	0	3 (1.11)	0	0	0	0	3 (1.11)
H32	0	0	0	1	0	0	1 (0.37)
H33	1	0	0	0	0	0	1 (0.37)
H34	1	0	0	0	0	0	1 (0.37)
H35	1	0	0	0	0	0	1 (0.37)
H36	1	0	0	0	0	0	1 (0.37)
H37	0	0	1	0	0	0	1 (0.37)
H38	0	0	1	0	0	0	1 (0.37)
Total number (%)	96(35.56)	66(24.44)	30(11.11)	54(20.00)	4(1.48)	20(7.41)	270(100)

Table 5.6: Genotype-wise (homozygotes and heterozygotes) distribution of 37 *sod1* haplotypes identified in BgSV, BgPR and BgPS laboratory populations of *B. glabrata*. Genotypes of individuals with the most frequent haplotype H5 (dotted underline) and the resistance-linked haplotype H2 (thin underline) are indicated. Thick underline indicates individuals having both haplotypes. Total numbers and percentages of resistant (uninfected, not shedding cercariae) and susceptible (infected, shedding cercariae) snails are shown in the last row and column.

Genotypes	BgSV		BgPR		BgPS		Total number (%)
	Number resistant	Number susceptible	Number resistant	Number susceptible	Number resistant	Number susceptible	
H1H4	0	0	0	0	0	1	1 (0.74)
H1H5	1	0	0	0	0	0	1 (0.74)
H1H8	0	0	0	0	0	1	1 (0.74)
H2H2	1	0	0	0	0	0	1 (0.74)
H2H5	17	5	1	2	0	1	26 (19.26)
H2H6	1	0	0	0	0	0	1 (0.74)
H2H7	0	0	2	6	0	2	10 (7.41)
H2H9	0	0	1	0	0	0	1 (0.74)
H5H5	20	23	2	2	0	1	48 (35.56)
H5H6	2	0	0	0	0	0	2 (1.48)
H5H7	1	0	1	0	0	0	2 (1.48)
H5H9	0	0	0	2	0	0	2 (1.48)
H5H20	0	0	0	1	0	0	1 (0.74)
H5H32	0	0	0	1	0	0	1 (0.74)
H6H6	0	1	0	1	0	2	4 (2.96)
H6H9	0	0	0	1	0	0	1 (0.74)
H6H10	0	0	0	1	0	0	1 (0.74)
H6H38	0	0	1	0	0	0	1 (0.74)
H9H9	0	0	3	5	0	0	8 (5.93)
H11H12	0	0	0	1	0	0	1 (0.74)
H11H15	0	0	0	0	2	2	4 (2.96)
H12H37	0	0	1	0	0	0	1 (0.74)
H13H14	0	0	1	0	0	0	1 (0.74)
H14H22	0	0	1	0	0	0	1 (0.74)
H16H17	0	0	1	0	0	0	1 (0.74)
H17H25	0	0	0	1	0	0	1 (0.74)
H18H18	1	0	0	0	0	0	1 (0.74)
H19H19	1	0	0	0	0	0	1 (0.74)
H19H31	0	3	0	0	0	0	3 (2.22)
H21H22	0	0	0	1	0	0	1 (0.74)
H23H24	0	0	0	1	0	0	1 (0.74)
H26H27	0	0	0	1	0	0	1 (0.74)
H27H28	0	1	0	0	0	0	1 (0.74)
H29H30	0	1	0	0	0	0	1 (0.74)
H33H34	0	1	0	0	0	0	1 (0.74)
H35H36	0	1	0	0	0	0	1 (0.74)
Total number (%)	45(33.3%)	36(26.7%)	15(11.1%)	27(20.0%)	2(1.48%)	10(7.41%)	135(100%)

5.3.1.5 Phylogeny reconstruction

Phylogenetic analysis of genotyped samples incorporating all haplotypes showed high variation within the *sod1* locus for the three *B. glabrata* strains studied (Figure 5.2).

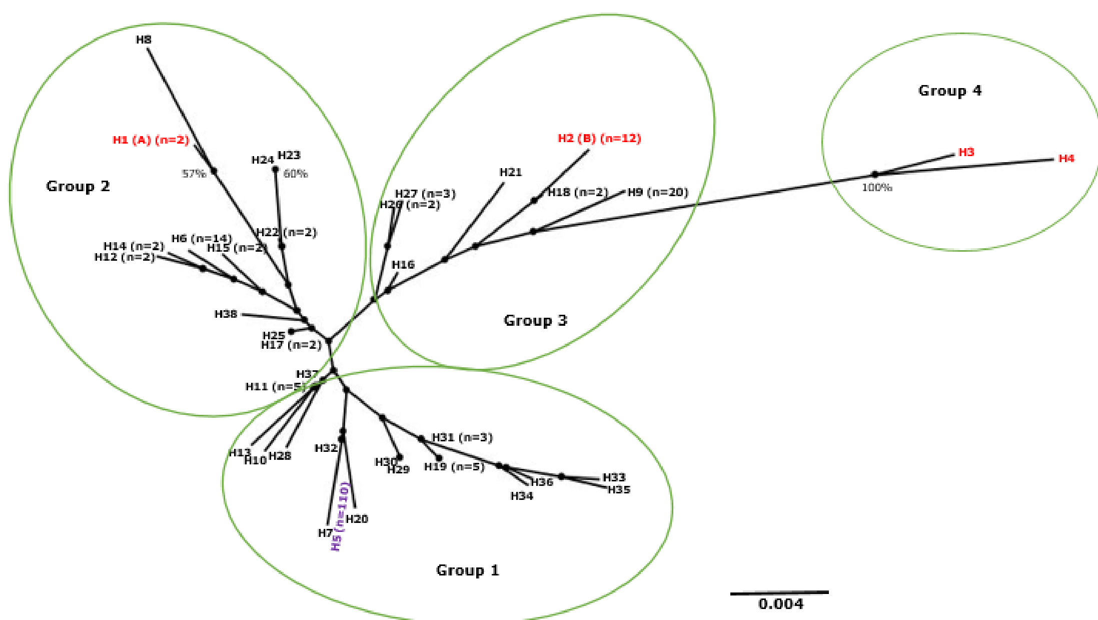

Figure 5.2: Neighbour-joining radial phylogenetic tree of the *sod1* fourth intron of *B. glabrata* incorporating sequences of all 38 haplotypes identified to date. The tree is drawn to scale and is unrooted. The percentage of replicate trees (bootstrap support values) in which the taxa clustered together are shown next to branches (values below 50% are not shown). Published, resistance-linked allele B and susceptible alleles A, C & D (i.e., haplotypes H1-H4) are shown in red font. Newly identified alleles, haplotype H5 (most abundant) and others H6-H38 are shown in purple and black fonts respectively. Green ovals indicate hypothetical division of haplotypes into groups.

For the purpose of description, the haplotypes appear divisible into four major groups. Group one contained 17 haplotypes (≈46%) and included the most common haplotype H5. Group 2 contained 12 haplotypes (≈32%) which included one of the previously published alleles (allele A, i.e., haplotype H1). Group 3 had 7 haplotypes (≈19%) and included the resistance-associated allele B (haplotype H2). Group 4 consisted of 1 haplotype (≈3%) which had also been previously reported, haplotype H1 (allele D) and also contained the published allele C (not found in the currently investigated populations). Generally, the bootstrap support for the tree was weak and only Group 4 appeared to be strongly supported with bootstrap value of 100%.

Haplotype network

Although overall haplotype diversity was high (0.719), there was however, a low-level of divergence between individual nucleotide sequences. Haplotype diversity within each *B. glabrata* strain was 0.455, 0.860 and 0.842 for the BgSV, BgPR and BgPS snails respectively. The most common haplotype H5 appeared closely linked within a larger cluster of 32 haplotypes, including the H1 (allele A). The BgPR snails had a higher number of haplotypes (23), approximately 61% compared to the 16 haplotypes found in the BgSV population (≈42%) even though more of the BgSV snails were sampled. The BgPS had nine haplotypes (≈24%) of total number of haplotypes identified. Figure 5.3 shows the network of all haplotypes found in relation to the previously reported haplotypes as well as the distribution of haplotypes among the three strains.

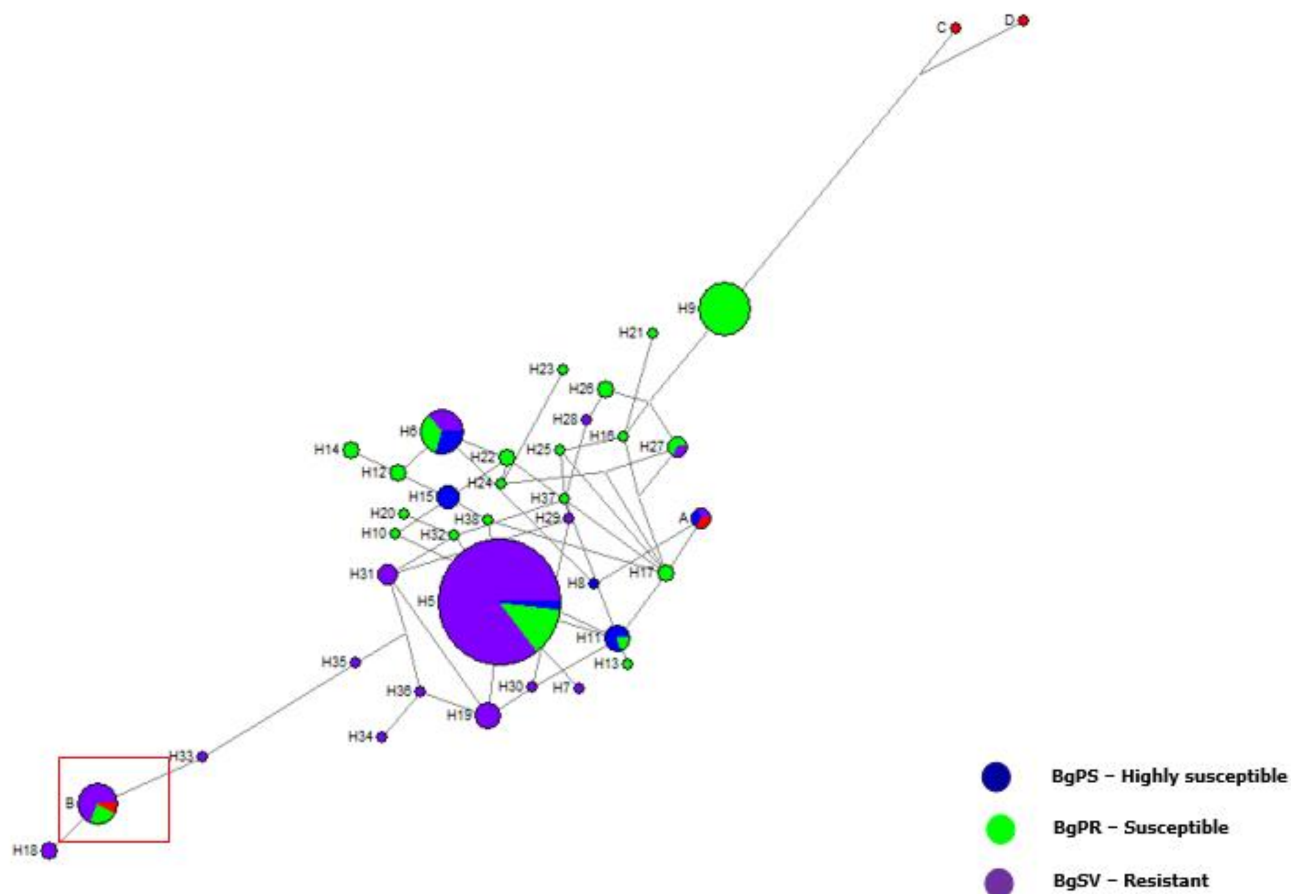

Figure 5.3: Median-joining network of 38 *sod1* haplotypes from three laboratory populations of *B. glabrata*, BgPS, BgPR & BgSV. Red pie slices indicate positions of reference sequences of alleles A, B, C & D (representing haplotypes H1- H4) in relation to additionally identified alleles within the three populations (denoted as haplotypes H5-H38). The size of each circle is proportional to the representative number of sequences. The resistance-linked allele B is indicated in the red box.

5.3.2 Genotype link to resistance/susceptibility phenotype

With the large numbers of low frequency alleles found in the present study and the low or complete absence of individuals having the previously reported haplotypes H1, H2, H3 and H4 (Table 5.5), there was little or no statistical power to determine definite association between these haplotypes and the resistance or susceptibility phenotypes. Instead, we tested to see if there was a link to resistance in snails having the haplotype H2 (representing the published, resistance-associated allele) using Fisher's exact test (two-tailed). The test result (Table 5.7) showed no statistically significant link to resistance in snails that had the H2 haplotype and those that did not have this haplotype (p = 0.0591).

Since we found haplotype H5 to be the most abundant in this study, its possible association with resistance or susceptibility was similarly tested (Table 5.8). The results showed that the association between the snails with the haplotype H5 and resistance was statistically significant (p = 0.0207*), indicating this haplotype may have an influence in determining outcome of infection in the snails. Relative risk was calculated to determine the strength of the association also indicated there was a reduced risk of infection in individuals. Relative risk of infection in haplotype H5 individuals was estimated to be 0.680 (95% CI: 0.502, 0.924) indicating that individuals possessing the haplotype H5 were 32% less likely to be infected with the parasite than those lacked this haplotype. Furthermore, we tested to determine if there was an association between the genotypes (i.e.,

heterozygotes or homozygotes states) and resistance in the snails. Results showed that resistant homozygotes (not infected, not shedding cercariae), were generally not statistically different from resistant heterozygotes (Table 5.9) in terms of their susceptibility to parasite (p = 0.862).

Table 5.7: Contingency tables comparing susceptibility of allele B (haplotype H2) carriers with non-carriers.
(Fishers exact test: p = 0.591 ns)

	Susceptible (Number positive)	Resistant (Number negative)	Total
Haplotype H2 present	16	23	39
Haplotype H2 absent	57	39	96
Total	73	62	135

Table 5.8: Contingency tables comparing susceptibility of haplotype H5 carriers with non-carriers.
(Fisher's exact test: p = 0.0207*)

	Susceptible (Number positive)	Resistant (Number negative)	Total
Haplotype H5 present	38	45	83
Haplotype H5 absent	35	17	52
Total	73	62	135

Table 5.9: Contingency table comparing susceptibility of homozygotes and heterozygotes
(Fisher's exact test: p = 0.8627 ns)

	Susceptible (Number positive)	Resistant (Number negative)	Total
Homozygote	35	28	65
Heterozygote	38	34	70
Total	73	62	135

5.4 Discussion

This chapter reports the investigations of *sod1* alleles (haplotypes) in three laboratory strains of *B. glabrata* that varied in their degree of resistance and/or susceptibility to *S. mansoni*. The link between haplotypes and the susceptibility/resistance of individual snails i.e., whether snails were resistant (uninfected) or susceptible (infected) was also investigated. Results revealed 37 haplotypes including 34 previously unidentified haplotypes in the populations studied of which haplotype H5 was the most common. The study found no statistical significant link of haplotype H2 (allele B) to resistance in the snails; H2 (allele B) was previously reported to be linked to resistance by Goodall *et al* (2006).

5.4.1 Allelic (haplotype) variation and host susceptibility

The results obtained in this study did not show a significant association between the previously published haplotype H2 (*sod1* B allele) with resistance or susceptibility in the snails. Thirty-seven haplotypes, H5-H38 were identified in the present study (including 34 newly identified haplotypes) of which haplotype H5 was the most common. This novel finding is contrary to the pioneer study by Goodall *et al* who reported only four alleles (haplotypes) in a predominantly resistant snail population (Goodall *et al*., 2006; Tennessen *et al*., 2015). The differences between the snail stocks used offers a logical explanation for most of the variation in alleles identified in the present study compared to the Goodall *et al* study. One of the snail strains used in our study, BgPS is a highly susceptible hybrid of mixed origin (Brazil and an unknown source, probably Caribbean

as inferred in chapter four results of this thesis). The BgPR snails have historical links to Puerto Rican and Brazilian hybrids and the BgSV snails are to our knowledge, and as confirmed in the previous chapter, of Brazilian origin. The 13-16-R1 resistant, hybrid *B. glabrata* snails used in the Goodall *et al* study are a mix of snails of Brazilian and Puerto Rican origin and genetic background (Bonner *et al*., 2012; Richards & Merritt Jr., 1972). This is corroborated by results of chapter four of this thesis which showed the BgSV for example was of a different haplotype to the 13-16-R1 snails used by the Goodall *et al* study (Goodall *et al*., 2006).

Certain aspects of the results obtained in the current study are in line with previous studies. For instance, genotype-wise, the Goodall *et al* study reported a complete lack of susceptible snails with the homozygote B allele and a low percentage of resistant homozygotes having this allele. This was consistent with our findings as none of the individuals having the resistant B allele (haplotype H2) was susceptible. However contrary to their finding with respect to strong association of allele B with resistance in snails, our data revealed no statistical significance association between resistance and the allele B (haplotype H2). Also, there were generally more heterozygote than homozygote resistant individuals (Goodall *et al*., 2006) as was also found in this study. In addition, our study found no statistical significant difference between heterozygote and homozygote individuals in terms of susceptibility (numbers infected/shedding cercariae) or resistance (numbers not infected/not shedding cercariae).

Furthermore, majority of the haplotypes found in the present study were rare and the low numbers of snails with the previously reported haplotypes

(as well as the complete absence of haplotype H3) in this study was also notable. Small populations are particularly likely to be prone genetic drift. This could lead either to an infrequently occurring allele being lost or becoming the only allele present at a particular gene locus. The low frequency of haplotype H4 (allele D) in this study (<1% frequency) was however in agreement with previous studies that have also reported it as rare (Goodall *et al*., 2006; Tennessen *et al*., 2015). In our study, haplotype H5 was the most abundant and in the populations, similarity in numbers of infected (susceptible) and uninfected (resistant) snails having this haplotype may simply indicate changes in allele frequencies at the time the infection experiments were performed. The high variability in snail susceptibility and/or parasite infectivity as shown by the results of chapter three corroborates this view. Since the present study found a statistical significant association between presence of haplotype H5 (the most common haplotype found) and resistant phenotype in the snails, we are inclined to suggest that some form of balancing selection could have given rise to the abundant haplotype H5 (considering that a higher than expected diversity could indicate the presence of balancing selection). However, such abundance of an allele and the lack of another could also signify loss due to genetic drift.

An important factor that could be considered is the reduced effect of genetic background on the correlation between *sod1* alleles and resistance as demonstrated in one study (Bonner *et al*., 2012; Tennessen *et al*., 2015). While it was somewhat surprising that the snails showed departure from HWE as estimated statistically (since *B. glabrata* snails are preferential out-crossers), it was not totally unexpected. Laboratory populations are not free

from bottlenecks and potential sources of deviation from the principles of HW could be genetic drift, inadvertent selection and mutation.

For each experimental sampling for infection, snails were randomly sampled from the breeding populations and grown to desired sizes in preparation for exposure to parasites. Hence, the gene pool of the selected lot will influence both allele frequencies and consequently the outcome of the experiment. This is clearly shown by the deficit of heterozygotes observed in this study as indicated by F_{IS} values. When populations are small, allele frequencies could drift away from equilibrium and consequently affecting genotype frequencies. A larger study sample could probably confirm the frequencies of alleles in the population but this was beyond the scope of the present study which sought to identify the haplotypes and determine the link to resistance in the system.

The differences in snail stock based on genetic background and other experimental variations (for instance, the 13-16-R1 studies used hamsters as definitive host in comparison to mice used in our study) could explain some of the differences in results obtained by the present study. Severe genetic bottlenecks in the parasite could also have led to reduction in compatibility after numerous laboratory passages.

5.4.2 Marked changes in host-parasite compatibility

Laboratory experiments in which snails were exposed to *S. mansoni* infection revealed significant variation in the percentage of BgSV snails infected between experiments 1 and experiments 2, 3 and 4. This variation may suggest changes in allele-frequency between the sampling period. Laboratory populations of snails in particular are generally prone to

bottlenecking conditions and founder effects which could trigger allelic loss or changes in allelic frequencies over periods of laboratory cycles. The time interval between infection experiments 1 and experiments 2, 3 and 4 were 11, 13 and 14 months respectively. The non-significant p-values obtained for percentage of snails infected between infection experiments for which sampling took place within two to three month further supports this view. Both genetic and environmental factors have been implicated in variations in compatibility outcomes of *B. glabrata-S. mansoni* encounters (Theron *et al*., 2008). The difference in percentage outcome of infected BgSV snails between the first experiment and subsequent experiments could suggest the occurrence of a laboratory bottleneck that could have resulted in possible changes in allele frequency during this period.

The results obtained here also appear to support the matching allele hypothesis based on the outcome of infections per experiment. This hypothesis submits that the outcome of each individual snail-parasite encounter depends on the matching of host and parasite genetic factors. (Richards & Shade, 1987; Théron & Coustau, 2005) as well as other complex molecular components (Richard Galinier *et al*., 2017). The difference between percentage of positive infections obtained in the first experiment and the subsequent ones for the BgSV and BgPR snails could therefore be an indication of changes in matched host and parasite genetic components. Interestingly, more than 80% of the snails in the first infection experiment with BgSV snails were found to be resistant (not infected). However more research comparing the genotypes of both hosts and

parasites during snail-schistosome interactions may be needed to make such a connection between matching of host and parasite genotypes.

5.4.3 Relationships among *sod1* haplotypes

Although this study found no strong links of the *sod1* B allele or other specific alleles to susceptibility or resistance to *S. mansoni* in the *B. glabrata* snails studied, it provides a *sod1* based phylogeny that could be used in establishing *sod1* allele groups for *B. glabrata* snails. The pattern shown by the haplotype network suggests the possibility of ongoing directional evolution in these laboratory populations. Although the snails were under no form of intentional selection, it is likely that there was some form of inadvertent selection taking place in breeding snail tanks. If indeed there was ongoing selection pressure of such nature, allele frequencies could have been shifted in favour of a specific phenotype at a point in time. Such homoplastic shift towards a *sod1* phenotype that can neither be categorized as resistant or susceptible may constitute a form of overall advantage for heterozygotes who could be carriers of both a resistant and a susceptible allele. The large number of alleles identified in the *sod1* locus as obtained from the present study may also lend credence to this notion. Since involvement of hydrogen peroxide has been implicated in cytotoxic parasite clearance in resistant *B. glabrata*, genetic and cellular factors that could influence its production should be considered important in variability of host susceptibility (Adema *et al*., 1994; Lockyer *et al*., 2007).

Also with the low variations between nucleotide sequences coupled with small fragment size and too few synapomorphies in the data, the tree produced is not robust enough to make definite inferences on the ancestral

links of the various haplotypes. More data from field and laboratory snails may therefore be required to understand the evolutionary relationships of the haplotypes.

This study demonstrates for the first time extensive polymorphism of the *B. glabrata sod1* and provides fresh insight to the relevance of the *sod1* gene locus as a marker for susceptibility or resistance in *B. glabrata* snails. Further investigations using field isolates are needed to determine the effects of *sod1* polymorphism in natural populations. This could determine the eventual use of the *sod1* or other related genes in marker assisted control interventions snail populations. In addition, investigations of protein products of genes and their expression in host-schistosome encounters could give further insight other molecules of immune relevance. The next chapter thus investigates proteins of immune relevance. Identifying these proteins could lead to identification of other genetic markers that could play key roles in determining compatibility polymorphisms in host-parasite interaction of *B. glabrata-S. mansoni* combinations.

CHAPTER 6

INVESTIGATIONS OF IMMUNE-RELEVANT PROTEINS AND GLYCOPROTEINS IN *BIOMPHALARIA GLABRATA*.

6.1 Introduction

In the host-parasite interaction of *Biomphalaria glabrata* and *Schistosoma mansoni*, the snail haemolymph represents a major line of molecular defence (internal defence system) against invading parasites and is therefore a major determinant of snail susceptibility or resistance to the parasite (Abou-El-Naga & Radwan, 2012; Alves de Mattos *et al*., 2011). Within the haemolymph are circulating snail haemocytes (the primary immune effector cells) which are crucial for immune surveillance (Oliveira *et al*., 2010). These cells migrate into tissues after parasitic infection (Barçante *et al*., 2012; Martins-Souza *et al*., 2011) and their destructive action has been shown to differ in resistant and susceptible snails, with active killing of sporocyst occurring only by haemocytes from resistant snails (Hahn *et al*., 2001b).

Studies in the last decade have also shown that snail haemocytes express carbohydrate-binding proteins (lectins) which may be involved in mediating cytotoxic killing of larval schistosomes (Castillo *et al*., 2007; Yoshino *et al*., 2008). These molecules, variable immunoglobulin and lectin domain the containing molecules include fibrinogen-related proteins (FREPs), C-type lectin-related proteins (CREPS) and Galactin-related proteins (GREPs) (Dheilly *et al*., 2015). It has thus been hypothesized that the internal defence mechanism of snail hosts is triggered by a lectin-based system for

recognition of non-self carbohydrates (glycans) determinants. Such a system will favour successful parasite establishment if host and parasite share glycan epitopes present in the host defence molecules (Martins-Souza *et al*., 2011; Mitta *et al*., 2012; Yoshino *et al*., 2012).

Biomphalaria glabrata haemolymph is a rich source of glycoproteins. Together with the haemocytes, soluble haemolymph factors (plasma/cell free haemolymph) are one of the major components associated with the internal defence responses of *B. glabrata* to larval trematodes (Bayne *et al*., 1985; Granath Jr. & Spray, 1987). The plasma of *B. glabrata* snails contain proteins and glycoproteins with putative immune functions that may also be involved in mediating defence responses during a snail-parasite encounter (Yoshino *et al*., 2012). Both the haemolymph and haemocytes of different strains of *B. glabrata* have been shown to exhibit differences in antigenicity to *S. mansoni* however, expressed haemolymph-like antigens of parasite origin have been found commonly in both susceptible and resistant snails (Yoshino & Bayne, 1983). As a whole, *B. glabrata* is considered a viable source of glycan-based antigens that could potentially serve as biomarkers in detection of human and animal parasitic infections as well as development of prophylactics (Chacón *et al*., 2002).

Despite the array of immune molecules for host defence, successful establishment of parasite occurs in some hosts. The mechanisms through which larval schistosomes evade immune recognition are however not fully understood and remain a subject of interest in the field of molecular parasitology. One possibility is that successful evasion occurs through manipulation of the host snail’s internal defence system. An hypothesis for

this manipulative mechanism is that the parasites evade host recognition by expressing haemolymph-like antigens within their glycoproteins and glycolipids (Yoshino & Bayne, 1983). In the human definitive host, response to schistosome larval and egg stages (parasite antigens) is mainly directed towards glycans (Eberl *et al*., 2001) but in the invertebrate snail intermediate hosts, the nature of this glycan-based antigenicity remains largely unclear. A previous study revealed that N-glycans of *B. glabrata* haemolymph glycoproteins share carbohydrate (glycan) epitopes that could play a role in cross-reactivity of these glycoproteins with *S. mansoni* glycoconjugates. These epitopes were found to consist of three core monosaccharide constituents, namely, fucose (Fuc), N-acetylgalactosamine (GalNAc) and N-acetylglucosamine (GlcNAc) and included (β1-2)-linked core xylose (Xyl) and/or terminally located Fuc(α1-3)GalNAc(β1-4)[±Fuc(α1-3)]GlcNAc(β1-)] units (Lehr *et al*., 2007).

Furthermore, using *B. glabrata* strains of Puerto-Rican and Salvador origins, shared glycan epitopes between the host snail and *S. mansoni* were demonstrated to be more expressed in susceptible than resistant snails (Lehr *et al*., 2010). Given the variability in among snail strains as observed in the previous chapters (both compatibility and in relation to *sod1* polymorphism) we asked if such variation also occurred between glycoproteins of the three snail strains (BgPS, BgPR and BgSV) snail strains occurred in the interaction between glycoproteins of different snail strains, that varied in susceptibility to *S. mansoni* and parasite glycoconjugates. In the previous chapters, investigations showed considerable variations in susceptibility to *S. mansoni* within and among populations of *B. glabrata* in our laboratory as well as polymorphism in a compatibility-related gene (as

seen in chapter three and chapter five results). Two of the strains used are considered to be of similar stock to those used in the Lehr *et al* study (Lehr *et al*, 2010) while the third, BgPS is a hybrid, highly susceptible strain. We consider that this strain BgPS would show a highly expressed pattern of cross-reactivity with *S. mansoni* glycoconjugates since expression of shared glycans have been demonstrated to more expressed in susceptible than resistant snails (Lehr *et al*., 2010).

The main aim of this study was to investigate glycoproteins of *B. glabrata* cross-reacting with glycoconjugates of *S. mansoni*. The first objective was to determine differences in glycoprotein patterns and expression between susceptible and resistant snails based on their cross-reactivity to parasite antigens using three strains of *B. glabrata*. Two of these three strains, BgPR and BgSV are of similar stock populations as those used in the study by Lehr and colleagues (Lehr *et al*, 2010) and had shown considerable variation in susceptibility to *S. mansoni* infection, while the third, BgPS is a highly susceptible strain (as seen in previous chapters).

The second objective was to determine the sensitivity of shared glycan epitopes between *B. glabtara* and *S. mansoni* to periodate oxidation. Hence, periodate oxidation of carbohydrates was performed (Eberl *et al*., 2001). Treatment of nitrocellulose membranes with $NaIO_4$ prior to application of antibody probes has been used to demonstrate removal of carbohydrate structures on proteins (Doenhoff *et al*., 2016). When performed under acidic pH, $NaIO_4$ oxidizes the hydroxyl group of sugars to aldehydes. These aldehydes can be subsequently converted to alcohols in the presence of a reducing agent such as sodium tetrahydroborate ($NaBH_4$) thereby preventing recognition of the glycan epitopes by antibodies (Alarcón de

Noya *et al*., 2000). Understanding the nature and composition of *B. glabrata* plasma glycans could provide further insight into molecular factors mediating self-nonself recognition during snail-schistosome interactions.

The third objective was to purify differentially expressed proteins and/or glycoproteins of *B. glabrata* based on their cross-reactivity with *S. mansoni* glycoconjugates. Using 1-D SDS-PAGE, Western immunoblotting and MS methods, the purification and identification of glycoproteins of *B. glabrata* cross-reacting with *S. mansoni* glycoconjugates was attempted. Investigations of glycoproteins involved in snail susceptibility and resistance mechanisms could lead to better understanding of molecular factors involved in host-parasite interactions and add to existing knowledge of the role of glycoproteins in the evolution of susceptibility or resistance in host-parasite interactions.

6.2. Material and methods

6.2.1 Snails

Snails used for this study were sampled from three laboratory-bred populations of *B. glabrata* comprising three different strains (as described in previous chapters), viz., the predominantly resistant Salvador strain (BgSV), the susceptible Puerto-Rican strain (BgPR) and the highly susceptible, hybrid (BgPS) strains. Full descriptions of these strains are provided in Table 2.1 of section 2.1.1. Investigations were performed using plasma (cell-free haemolymph) and tissue extracts of the snails. Except where otherwise stated, unexposed snails were used for investigations.

6.2.2 Haemolymph extraction and plasma preparation

Haemolymph was extracted from seven similar-sized snails (10-12 mm) for each of the three strains of *B. glabrata* (i.e., BgPS, BgSV and BgPR), by gentle shell crushing between two slides (Peña & Adema, 2016). Exuded haemolymph was pipetted carefully into 1.5 mL Eppendorf tubes. Approximately 200 µL of haemolymph was pooled from seven snails per strain. Each tube was shortly centrifuged at 4000 rpm for 15 minutes thereby removing haemocytes and cell debris (Lehr *et al*., 2007). The resulting supernatant (plasma) was stored in 50 µL aliquots and stored at –20°C for SDS-PAGE and Western Immunoblot analyses.

6.2.3 Preparation of snail tissue extract

Proteins were extracted from freeze dried snail tissues of four exposed snails that had been determined to be either infected (positive, that is shedding cercariae) or uninfected (negative, not shedding cercariae). Briefly, each lyophilized tissue was re-suspended in 200 µL of 0.05M Tris HCl (pH 7.5) in a 1.5 mL Eppendorf and homogenized with a sterile pestle. The tubes were centrifuged at 14,000 rpm for 3 minutes. The supernatant was transferred into fresh tubes and stored as aliquots of 40 µL at -20°C.

6.2.4 Estimation of protein concentration

Protein concentrations of snail haemolymph, tissue extracts and parasite antigens were estimated using the Bio-Rad DC protein assay (Bio-Rad, U.K)

which is adapted from Lowry *et al* (1951). This was either performed using a microplate reader or a cuvette based spectrophotometer. In both cases, 5 serial dilutions each of bovine serum albumen (BSA) and of each unknown sample were prepared. BSA was used as comparative standard for the unknown samples and a blank reading was included using distilled water. BioRad D_C protein assay reagents A and B were mixed with the samples (25 µL of A and 200 µL of B for microplate readings or 100 µL of A and of 800 µL of B for cuvettes). The samples were allowed to stand for 15 minutes at room temperature and then absorbance was read at wavelength of 750 nm or nearest. Recorded readings were input into Microsoft excel and plotted to obtain standard curves from which the concentration of each sample was estimated.

6.2.5 Antigens and antibodies

Schistosoma mansoni soluble egg antigens (SEA) used were obtained from our laboratory stock of antigen (stored at -80°C), prepared for various research applications and use by our laboratory. These antigens were prepared using the method described by Doenhoff *et al.* (1988) and involved the extraction of SEA from the livers and intestines of infected mice harbouring adult worms. Also, antisera raised against *S. mansoni* lifecycle stages (eggs, cercariae and adult worms) that had been produced in rabbits through an immunization procedure (Dunne *et al.*, 1986; Modha *et al.*, 1988) were used. Protocols are provided in appendices 3 and 4.

6.2.6 Production of rabbit anti-HRP and rabbit anti-KLH

Horse radish peroxidase (HRP) and keyhole limpet haemocyanin (KLH) applied as immunogens for rabbits, were commercially obtained from Sigma-Aldrich, U.K. Each immunogen (10 mg/mL solution) was emulsified in a 1:1 ratio of immunogen to Freund's adjuvant and injected into the rabbits as described in appendix 4. The sera samples obtained were also processed as described and stored at –80°C.

6.2.7 Dot Blot screening of antisera

Preliminarily, nine polyspecific antisera were tested for cross-reactivity with snail plasma proteins using the dot blot technique (Beyer, 1984). Briefly, the nitrocellulose membrane to be used was divided into grids to indicate spots to be blotted. Two microliters of snail plasma was then applied in the centre of each grid. The membrane was allowed to dry and then blocked in non-fat dry milk dissolved in Tris Buffered Saline (TBS) for 1 hour at room temperature. This was followed with primary and secondary antibody incubation as described later for western immunoblotting. Cross-reactivity with snail haemolymph or tissue extract would indicate the presence of some shared epitopes between the *B. glabrata* snails and larval stages of the parasite. Three of the most serologically cross-reactive rabbit antisera, two raised against the parasite eggs (see section 6.2.4 for anti-sera production) and one against keyhole limpet haemocyanin (KLH) were selected for the current investigation. A list of antisera tested is presented in Table 6.1.

Table 6.1: List of rabbit anti-sera tested for cross-reactivity using Dot blot method

Anti-serum code	Description	Protein concentration (mg/mL)
BR14	Anti-Keyhole Limpet Haemocyanin (Anti-KLH)	50.52
BR84	Anti-*S. mansoni* egg antigen (Anti-SEA)	48.01
BR23	Anti-Trichloroacetic extract (Anti-TCA) of Cercaria Transformation Fluid (CTF)	23.21
BR58	Anti-adult worm	35.06
1025Z	Anti-SEA	35.54
1155A	Anti-TCA extract of adult worm	38.51
1018D	Anti-SEA	50.52
1025O	Anti-*S. mansoni* cercariae	35.28
993P	Anti-*S. mansoni* cercariae	55.21

6.2.8 One-Dimensional Sodium Dodecyl Polyacrylamide Gel Electrophoresis (SDS-PAGE) of protein samples

This electrophoretic technique uses the principle of protein separation through the gel on the basis of differences in their relative molecular sizes. Gels of 9 x 6 x 0.75 mm containing either 8% or 12% acrylamide were prepared and one-dimensional sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (Laemmli, 1970) using the Bio-Rad mini gel system (full details of provided in Appendix 5). The protein profiles of the three snail strains were thus compared on SDS-PAGE gels. Three repeats each were performed for all samples analysed using SDS-PAGE.

6.2.8.1 Preparation and casting of resolving and stacking gels

Protein mixtures are separated in an electrophoretic field by their size. To do this, the first step was to prepare and cast a resolving gel that allows the movement and separation of the protein sample though this gel matrix. 8% or 12% gel mixtures were prepared in falcon tubes for resolution of larger or smaller proteins respectively (see appendix 6, Table S1a). The resolving gel mixture was then thoroughly mixed by inverting the tube and applied between the glass plates. This was then left to polymerize at room temperature for 30-60 minutes. For optimum protein resolution, the second step was the preparation and casting of the stacking gel on top of the resolving gel. The stacking gel mixture was prepared in a separate tube and inverted for thorough mixture before casting (see appendix 6, Table S1b). This gel served as the template for creating the wells in which protein samples were loaded for appropriate run through lanes subsequently formed. The lower concentration of acrylamide, reduced pH and different ionic content of the stacking gel causes the proteins in sample mixtures to be initially concentrated into a band within the first few minutes of commencing electrophoretic run and thereafter, move into the resolving part of the gel. See appendix 6 for detailed protocol and components used for the preparation of both gels.

6.2.8.2 Sample treatment and loading for SDS-PAGE

To determine differences in protein profiles of the snail strains, the pooled plasma samples were compared on SDS-PAGE. Also, proteins from tissue extracts of infected (positive for cercariae shedding) and uninfected

(negative for cercariae shedding) snails were also compared on SDS-PAGE for identification of differentially expressed proteins. Three repeats of these protein profile experiments were performed. For each snail strain, 10 μg of protein from each pooled sample of snail haemolymph or 5 μg of tissue extract was treated with 1/4 volume of reducing buffer (containing β-mercaptoethanol) and allowed to boil for 5 minutes. After cooling, the samples were kept on ice until ready for gel loading. Molecular size markers were loaded in at least one well and sample loading buffers were applied to empty wells. Electrophoresis was performed at 80-120V (usually lasted for 1.5-2 hours) in a gel electrophoresis tank containing 1x running buffer.

6.2.8.3 Protein visualization on gel

Gels containing the electrophoresed proteins were stained with Coomassie blue or Simply-Blue safe stain (for better MS compatibility) following manufacturer's instructions or for further investigations, transferred onto a nitrocellulose membrane for electro-transfer of proteins.

6.2.8.4 Excision and elution of protein targets

Proteins of interest were carefully excised from a de-stained gel using a sterile lancet. Each sliced protein bands were then chopped into small bits, transferred into an Eppendorf tube. The gel pieces were incubated overnight at 37°C in a volume of elution buffer containing 0.06M Tris-HCl and 10%SDS, pH 7.0 (Beyer *et al*., 2008). The tube was then centrifuged at the same temperature for 30 minutes at 14,000 x g. The supernatant was carefully pipetted into a fresh tube and used immediately or stored at -20°C for use within three days.

6.2.9 Western immunoblotting of proteins

Proteins were electro-blotted from the SDS-PAGE gel onto nitrocellulose membranes (Amersham) based on the method of Towbin *et al*., 1979. (Towbin *et al*., 1979) as adapted and described by (Maizels *et al*., 1991). Briefly, the gel containing electrophoresed proteins, gel-sized matched nitrocellulose membrane, 2 fibre pads and two sheets of filter paper were incubated for 15-30 minutes in cold (4°C), 1X transfer buffer. These were arranged to form a gel sandwich between gel cassettes. With the dark side of the cassette unit flat on the work surface, the sandwich was arranged in this order: fibre pad, filter paper, gel, nitrocellulose membrane, filter paper, fibre pad. Ensuring no bubbles were formed between the gel and membrane, the sandwich is complete by tightly closing the cassette and placing it into the cassette module (See appendix 7). The unit was placed into an electrophoresis tank filled with cold (4°C), 1X transfer buffer. The tank was placed on stirrer and a magnetic bar was inserted. Electro-transfer was performed at 70 V, 350-400 mA for 2 hours at 4°C.

6.2.10 Blocking and antibody incubation

After electro-blotting, membranes were blocked overnight by incubating in non-fat dry milk dissolved in Tris Buffered Saline (TBS) at 4°C on a rocker. Primary incubation of blotted membranes to detect cross-reacting glycoproteins was carried out with polyspecific rabbit sera, BR84, 1025Z (both anti-SEA) and BR14 (anti-KLH) in a 1:20,000 dilution of antibody in TBS. Membranes were incubated overnight at 4°C with gentle agitation then washed 3 times for 5 minutes in TBST (TBS and 0.5% Tween 20). This was

followed immediately with secondary antibody incubation of membranes with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG. Incubation was performed in a 1:1000 dilution of antibody in TBS solution for 2 hours at room temperature with gentle agitation (see appendix I). Blots were again washed 3 times for 5 minutes each in TBST and developed for visualization of immunoblotted proteins.

6.2.11 Visualization of proteins on immunoblotted membranes

Chromogenic detection of proteins was based on the HRP enzyme system coupled with the substrate, 4 –chloro-I-naphthol (Beyer, 1984; Hawkes *et al*., 1982). To visualize proteins electro-transferred on the membranes, the chromogenic substrate was dissolved in 4 mL ethanol. The solution was then diluted with warm 20 mL TBS solution (warmed in a microwave oven for 20 seconds at 800W) and 10 µL of hydrogen peroxide was added. This final solution was then spread evenly on the electro-blotted nitrocellulose membrane and incubated for 5-20 minutes on a rocker until clear bands of protein become visible. The membranes were then rinsed twice in water to stop the staining reaction. Visible bands were visually classified as either weak or strong as depicted by the intensity of the reaction.

6.2.12 Sodium metaperiodate treatment of electroblotted membranes

In order to determine if cross-reactivity was due to glycan epitopes shared between the host and parasite, blots were pre-treated with sodium metaperiodate which is an oxidising agent known to destroy carbohydrate

epitopes present on glycoproteins. This technique is an adaptation of Eberl *et al.* (2001) and was performed as described by Hamilton *et al.* (1999). Electro-blotted nitrocellulose membranes were equilibrated for 30 minutes in 50 mM sodium acetate (NaAc) buffer (pH 4.5). The equilibrated membrane, containing transferred protein was cut in two halves. One half was treated with 10 mM sodium metaperiodate ($NaIO_4$) in 0.05 M NaAc buffer pH 4.5 while the second half was treated with NaAc buffer only.

Both incubation dishes were placed on a rocker in the dark for one hour at room temperature. The membranes were thereafter washed twice with NaAc buffer pH 4.5, and incubated at room temperature in sodium borohydride solution for 30 minutes. The membranes were each washed in phosphate buffered saline (PBS, 1xPBS) twice followed by five washes in 1xTBST. Blocking and antibody detection were performed as described for western immunoblotting above. All gel electrophoresis and Western immunoblots of snail and tissue proteins were repeated minimum of three times.

6.2.13 Mass spectrometry analyses of proteins

For mass spectrometry, proteins were extracted from unexposed snails. Tissues were processed as described earlier in section 6.2.3. After gel electrophoresis (SDS-PAGE) protein bands that were visually determined to show enhanced staining on SDS-PAGE gels and were determined to be cross-reactive with parasite antigens as observed on Western immunoblotted membranes were chosen for analysis. The gel bands were excised and sent for analysis according to specified guidelines. All mass

spectrometry procedures were performed by BSRC Mass Spectrometry and Proteomics Facility, University of St. Andrews St Andrews, Fife (see Appendix 8 for procedure).

6.2.14 Bioinformatics and analyses of identified proteins

The MS/MS raw data file generated via the 'Create mgf file' script in PeakView (Sciex) was analysed using the Mascot search algorithm (Matrix Science), against the NCBInr database (Aug 2016), both including all species (93482448 sequences) and restricting the search to *Biomphalaria glabrata* (31470 sequences). Parameters included trypsin as the cleavage enzyme, carbamidomethyl as a fixed modification of cysteines and methionine oxidation as a variable modification. The peptide mass tolerance was set to 20 ppm and the MSMS mass tolerance to ± 0.05 Da. A protein was accepted as identified if it had 2 or more peptides with Mascot Ion Scores above the Identity Threshold ($p < 0.05$) and, for those proteins identified by only 2 peptides, the MSMS spectral assignments match most of the peaks in the MSMS spectra.

Proteins identified by MASCOT search were also subject to further analyses using the Basic Local Alignment Search Tool (BLAST) http://blast.ncbi.nlm.nih.gov/Blast.cgi on the NCBI database. Glycosylation sites predictions were determined using GlycoEP software for prediction of N- and O- linked glycosylation sites (Chauhan *et al*., 2013).

6.3 RESULTS

6.3.1 Dot blot selection of anti-sera

Out of nine rabbit anti-sera tested using the dot blot technique, five were cross-reactive with snail proteins. The results showed that rabbit anti-sera raised against *S. mansoni* eggs and KLH were the most cross-reactive with *B. glabrata* plasma proteins tested. The most cross-reactive anti-sera, 1025Z, BR84 and BR14 were therefore selected for subsequent investigations with SDS-PAGE and Western Blot (Table 6.2).

Table 6.2: Cross-reactivity of rabbit anti-sera with *B. glabrata* proteins. Symbols, + and – are used to indicate cross-reactive and none cross-reactive sera respectively. Multiple + indicates intensity

Anti-serum code	Description	Cross-reactivity
BR14	Anti-Keyhole Limpet Haemocyanin (Anti-KLH)	++
BR84	Anti-*S. mansoni* egg antigen (Anti-SEA)	++
BR23	Anti-Trichloroacetic extract (Anti-TCA) of Cercaria Transformation Fluid (CTF)	+
BR58	Anti-adult worm	-
1025Z	Anti-SEA	+++
1155A	Anti-TCA extract of adult worm	-
1018D	Anti-SEA	-
1025O	Anti-*S. mansoni* cercariae	-
993P	Anti-*S. mansoni* cercariae	+

6.3.2 Plasma protein profiles of *B. glabrata* snails are fundamentally identical.

To determine if there were differences in snail plasma profiles, the protein profiles of plasma samples of unexposed BgPS, BgPR and BgSV snails were compared using SDS-PAGE. Results showed that the serological protein profiles of *B. glabrata* were similar for the three strains as determined on Coomassie blue-stained SDS-PAGE (Figure 6.1). A high molecular weight molecule greater than 100 kDa, was clearly evident in total plasma of the snails.

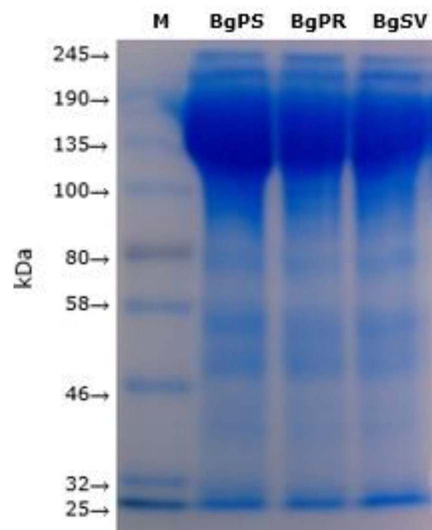

Figure 6.1: Coomassie-blue stained SDS-PAGE of *B. glabrata* plasma proteins. Each sample load contained 10 µg of plasma proteins from highly susceptible (BgPS), susceptible (BgPR) and resistant (BgSV) strains. Lane M represents molecular weight standards

6.3.3 Intra-species variation in serological recognition

Although the Coomassie-stained gel showed similar banding patterns across the three snail isolates, variation in intensity of cross-reactivity of plasma proteins with glycoconjugates from *S. mansoni* eggs was observed in the electro-blotted membranes, indicating differences in expression of carbohydrate (glycan) epitopes. There was a higher degree of cross-reactivity in the plasma of BgPR snails compared to BgPS and BgSV counterparts. Cross-reactivity of snail glycoproteins with *S. mansoni*-derived molecules as observed on Western immunoblot of plasma of resistant (BgSV) and susceptible (BgPS & BgPR) strains of *B. glabrata* probed with anti-SEAs 1025Z and BR84 are shown in Figures 6.2 and 6.3 respectively.

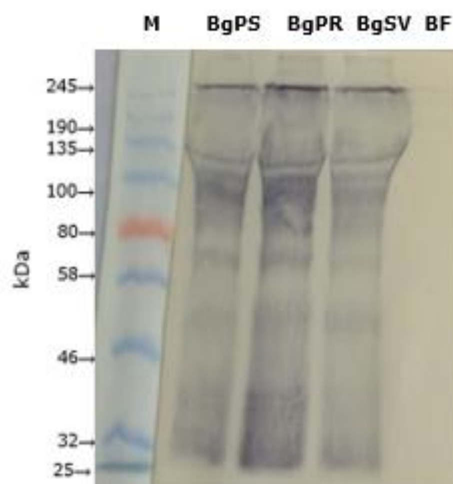

Figure 6.2: Western Immunoblot of *B. glabrata* plasma proteins probed with anti-SEA, 1025Z. Each sample load contained 10 µg of plasma proteins from highly susceptible (BgPS), susceptible (BgPR) and resistant (BgSV) strains. Lane M, molecular weight standards; lane BF, buffer (negative control).

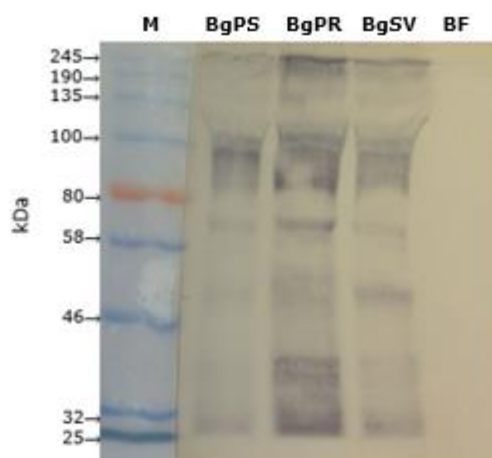

Figure 6.3: Western Immunoblot of *B. glabrata* plasma proteins probed with anti-SEA, BR84. Each lane contains 10 µg of plasma proteins from highly susceptible (BgPS), susceptible (BgPR) and resistant (BgSV) strains. Lane M, molecular weight standards; lane BF, buffer (negative control).

To further investigate the nature of glycoproteins, cross-reactivity of *B. glabrata* plasma was tested with a rabbit anti-KLH immune sera, on the premise that the presence of fucosylated glycan antigenic epitopes are expressed commonly in *S. mansoni* glycoconjugates and KLH (Hamilton *et al*., 1999; Kantelhardt *et al*., 2002). Results obtained showed similar pattern of cross-reactivity of plasma from the three strains, suggesting presence of fucosylated epitopes (Figure 6.4). Also, there was no clear difference in expressed intensity of cross-reactivity with KLH across the three strains as occurred with the polyspecific anti-SEA anti-sera, 1025Z and BR84. In both cases of the latter (Figures 6.2 and 6.3 above), more intensely stained bands were produced.

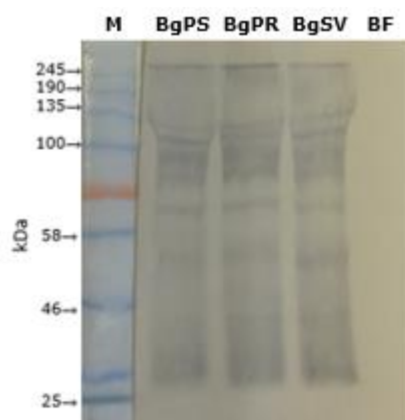

Figure 6.4: Western Immunoblot of *B. glabrata* plasma proteins probed with anti-KLH, BR14. Each lane contains 10 µg of plasma proteins from highly susceptible (BgPS), susceptible (BgPR) and resistant (BgSV) strains. Lane M, molecular weight standards; lane BF, buffer (negative control).

6.3.4 Effect of periodate oxidation on *B. glabrata* glycoproteins

The nature of cross-reacting epitopes was further determined. The plasma glycoproteins were captured on blotted nitrocellulose membranes and periodate oxidation was performed as described in section 6.2.11. Results of $NaIO_4$-treatment and non-treatment of electro-blotted membranes containing plasma glycoproteins of BgPS, BgPR and BgSV strains of *B. glabrata* are shown in Figure 6.5. While $NaIO_4$-untreated blots remained unchanged after Western immunoblotting, the treated membranes showed partial reduction in intensity of protein bands observed in the protein bands of ≥80 kDa, indicating partial periodate-sensitivity of the electrophoresed proteins. However, bands less than 80 kDa showed enhanced intensity.

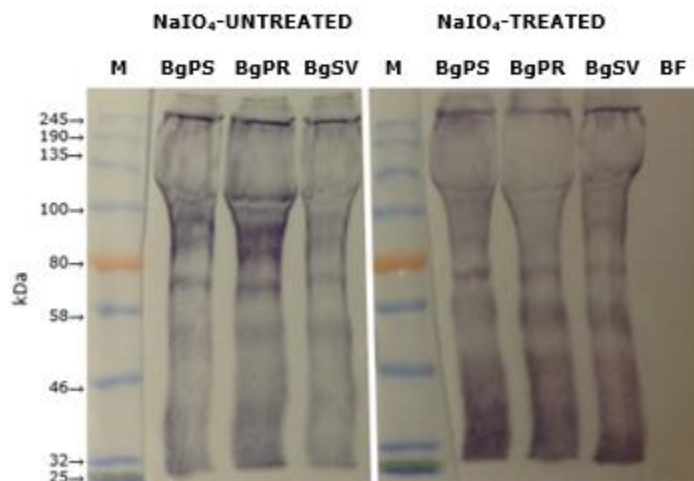

Figure 6.5: Sodium metaperiodate (NaIO4) untreated and treated immunoblots of *B. glabrata* plasma proteins. NaIO4-treated (right) and NaIO4-untreated (left) blot probed with anti-SEA, 1025Z. Ten micrograms of plasma sample was applied in each lane as indicated for BgPS (highly susceptible), BgPR (susceptible) and BgSV (resistant). Lanes M (molecular weight standards) and BF (buffer/negative control).

6.3.5 Shared plasma glycoproteins of *B. glabrata* and *S. mansoni*

To determine if any of the proteins could be matched with corresponding glycoconjugates of parasite origin, plasma of the unexposed snails were compared with protein profile of soluble egg antigens (SEA) of *S. mansoni* on SDS-PAGE and Western immunoblotted membranes. Plasma profiles of the snails were similar as observed in SDS-PAGE and a protein band of about the 90-100 kDa mark was observed in SEA (Figure 6.6).

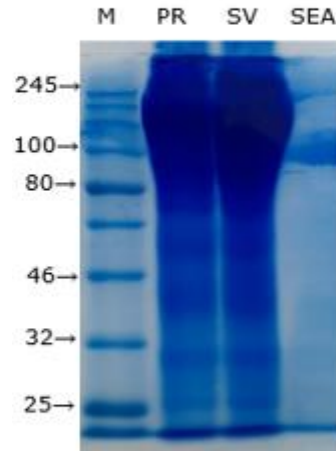

Figure 6.6: Comparison of plasma proteins of *B. glabrata* susceptible (BgPR) and resistant (BgSV) snails with antigenic glycotopes of *S. mansoni* soluble egg antigens (SEA) on Coomassie-blue stained SDS-PAGE gel. Lanes indicate Molecular weight marker (M), plasma samples of BgPR (PR) & BgSV (SV) and *S. mansoni* soluble egg antigen (SEA).

Two distinct cross-reactive protein bands of about 90 kDa and 100 kDa were observed in electro-transferred plasma samples of the snails with the membranes probed with anti-SEA 1025Z. Both bands were not clearly observed on Coomassie-blue stained gels of SDS-PAGE. Also, the distinct 100 kDa cross-reactive band of SEA appeared to correspond to one of the two bands that displayed antigenicity towards anti-schistosome poly-specific antisera in both the BgPR and BgSV plasma samples. Treatment of the blots with $NaIO_4$ to test if cross-reacitvity was due to glycan epitopes was performed. The results showed that ≈33 kDa protein of SEA was not affected by $NaIO_4$ treatment ($NaIO_4$-resistant) while the ≈100 kDa protein appeared to be partially $NaIO_4$-sensitive (Figure 6.7). In the untreated blot,

however, the region surrounding the ≈33 kDa band showed an unexpected, enhanced expression of cross-reactivity.

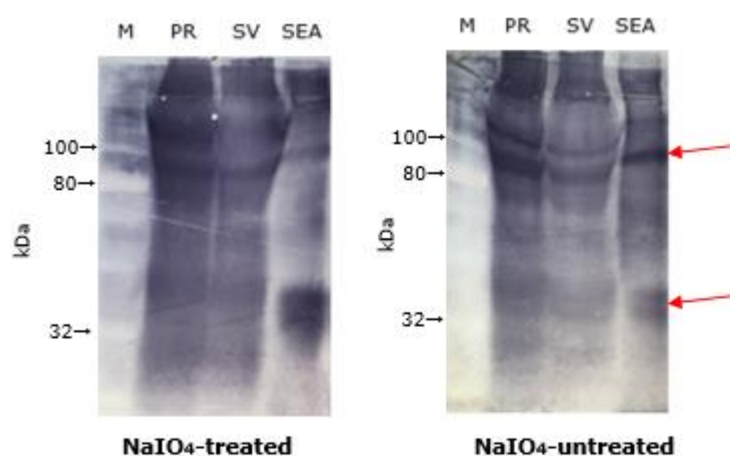

Figure 6.7: Comparison of sodium meta-periodate ($NaIO_4$)-treated and untreated electro-transferred plasma proteins of susceptible (BgPR) and resistant (BgSV) *B. glabrata* strains with antigenic glycotopes of *S. mansoni* soluble egg antigens (SEA). Blots were probed with anti-SEA 1025Z. Left blot was incubated in 10 mM sodium meta-periodate ($NaIO_4$) in 50 mM sodium acetate buffer; right blot was incubated in 50 mM sodium acetate buffer only. Lanes indicate molecular weight marker (M), plasma samples BgPR (PR) & BgSV (SV) and *S. mansoni* soluble egg antigen (SEA). Red arrows indicate ≈33 kDa and ≈100 kda proteins of *S. mansoni* SEA (putatively identified as glycoproteins, kappa-5 and IPSE/alpha-1 proteins).

6.3.6 Tissue protein profile of infected and uninfected snails.

Furthermore, SDS-PAGE analysis to determine differences between protein profiles of susceptible (infected, shedding cercariae) and resistant (uninfected, not shedding cercariae) showed that protein profiles of tissue homogenates from these snails were similar. However, a protein band of ≈25 kDa appeared to be more expressed (as observed visually by the

intensity of protein band staining) in uninfected BgPR and BgSV snails. (Figures 6.7).

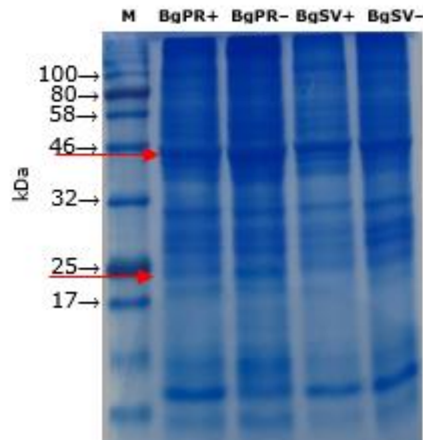

Figure 6.7: Coomassie blue-stained SDS-PAGE gel of *B. glabrata* tissue proteins of exposed infected (+) and uninfected (–) BgPR and BgSV strains. Each lane contained 10 µg of proteins. Red arrows indicate ≈25kDa and ≈46kDa protein bands excised for mass spectrometry.

Further analyses of proteins by Western immunoblotting showed intense cross-reactivity of proteins above 25kDa. Proteins of lower molecular weight (less than 25kDa) however appeared non cross-reactive (Figure 6.8). On the electro-blotted membrane, some proteins appeared to show differential cross-reactivity but were not clearly visible on the gel (e.g., a protein about the 32kDa mark in the infected BgPR (BgPR+) snail and a dimer-like protein just below the 32kDa in the uninfected BgPR (BgPR–) snail. These proteins were however not consistent in repeated immunoblots. Hence, for further analysis, with mass spectrometry (MS), two bands within the cross-reactive range (≈25kDa and ≈46kDa) were selected based on consistency of

expression on SDS-PAGE gel (Figure 6.7) and cross-reactivity pattern with parasite antigen (Figure 6.8).

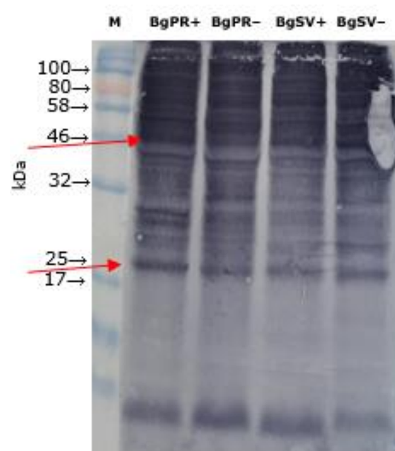

Figure 6.8: Western immunoblot of *B. glabrata* tissue proteins probed with anti-SEA, 1025Z. Each lane contained 10 µg of proteins from exposed infected (+) and uninfected (−) BgPR and BgSV strains of *B. glabrata*. Red arrows indicate ≈25kDa and ≈46kDa cross-reactive proteins corresponding to expressed proteins on SDS-PAGE gel shown in Figure 6.7.

The ≈25kDa protein band earlier mentioned appeared consistent in banding pattern on SDS-PAGE gels showing enhanced expression (visually determined by higher staining intensity on SDS-PAGE) in uninfected (not shedding cercariae) snails. However, there was no difference in its cross-reactivity pattern with parasite antigens when compared between uninfected and infected snails. The choice of the ≈46kDa molecular weight protein band was based on its highly intense expression compared to other proteins within the higher molecular weight region as observed on SDS-PAGE gel. Given its enhanced expression and partial cross-reactivity,

the likelihood of it being glycosylated was of interest since glycosylated proteins are considered relevant in immune recognition.

Bands corresponding to the ≈25kDa and ≈46kDa proteins were excised from repeated SDS-PAGE gels and purified as described in sub-section 6.2.7.4. The purified protein samples were re-electrophoresed, immunoblotted and probed with anti-SEAs. The results confirmed that both protein samples were cross-reactive with anti-SEA 1025Z (Figure 6.9).

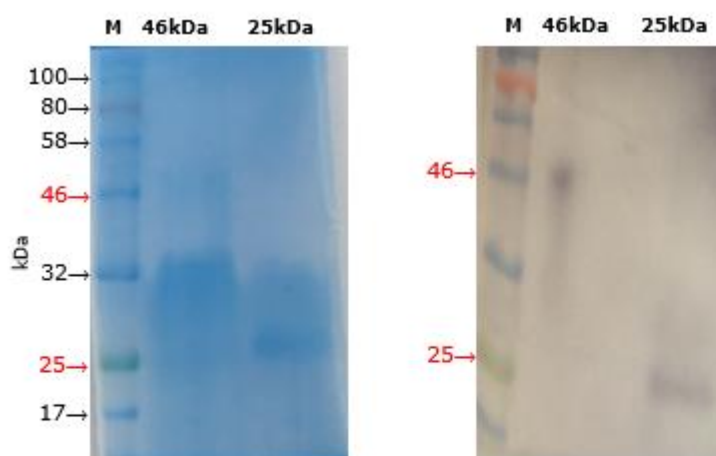

Figure 6.9: SDS-PAGE (left) and corresponding Western immunoblot (right) of purified proteins (≈25kDa and ≈46kDa) from tissue homogenates of unexposed *B. glabrata* probed with anti-SEA 1025Z.

6.3.7 MS identification of purified ≈25kDa protein as glutathione-S-transferase (GST) Mu 1.

Mass spectrometry analysis of the purified ≈25kDa protein band revealed a significant match with the *B. glabrata* predicted glutathione-S-transferase Mu1-like protein (gi|908496008; NCBI Accession number XP_013097111).

This protein is made up of 217 amino acid residues and has a monoisotopic mass (M_r) of 25243 Daltons (Da). Prediction of glycosylation sites using the GlycoEP software (Chauhan *et al*., 2013) indicated one N-linked and one O-linked glycosylated site in the identified GST Mu 1 of *B. glabrata* (summary of results are shown in Tables S6A & S6B of Appendix 9).

A blast search (Table 6.3) for its homology to some species relevant in schistosome transmission studies revealed that percentage identity of *B. glabrata* GST Mu1 shared with those of parasite, mouse and human origin ranged from 29% identity with *S. mansoni* GST to 45% with human GST. The proteins showed significant similarity based on expectation values (E-value), inferring homology of these proteins (E-values less than 0.01) which could also suggest functional similarity (Table 6.3).

Table 6.3: NCBI blast output of *B. glabrata* GST Mu1 protein homology search of transmission-related species.

Description	Max. score	Total score	Query cover	E value	Identity (%)	Accession number
PREDICTED: glutathione S-transferase Mu 1-like [*Biomphalaria glabrata*]	448	448	100%	9.00E-167	100%	XP_013097111.1
glutathione S-transferase M1 [*Homo sapiens*]	199	199	98%	2.00E-68	45%	AAV38750.1
glutathione S-transferase Mu 1 [*Mus musculus*]	198	198	99%	4.00E-68	43%	NP_034488.1
glutathione S-transferase, GST [*Schistosoma mansoni*, Peptide,	168	168	99%	3.00E-56	42%	AAB21173.1
glutathione S-transferase 26 kDa [*Schistosoma mansoni*]	145	145	99%	7.00E-48	39%	XP_018652834.1
glutathione S-transferase 26 kDa [*Schistosoma mansoni*]	145	145	99%	7.00E-48	39%	CCD80234.1
26 Kd glutathione-S-transferase, Sm26, putative [*Schistosoma mansoni*]	144	144	99%	3.00E-47	36%	CCD59592.1
Glutathione S-transferase 28 kDa (GST 28) (GST class-mu), putative [*Schistosoma mansoni*]	63.9	80.1	92%	2.00E-16	29%	CCD60449.1
Sm28 GST [*Schistosoma mansoni*]	63.9	80.1	92%	2.00E-16	29%	AAC60508.1

6.3.8 MS identification of purified ≈46kDa protein as partial haemoglobin

The purified ≈46 kDa protein was putatively identified by mass spectrometry as a subunit of *B. glabrata* haemoglobin (partial haemoglobin of *B. glabrata*; accession number CAH23231). Inference was made based on the Mascot search output of the highest scoring protein family and the similarity between the sequences after a fragment based alignment of matched peptide sequences of 474 amino acid residues (74%). (monoisotopic mass, 53472 Da). The molecular weight difference (≈7000 Da i.e., 7 kDa) suggests the 46 kDa protein was a reduced form of the haemoglobin molecule. Glycosylation prediction revealed no N-linked glycosylated site but three O-linked, potential glycosylated sites were detected. Summary of results are shown in Tables S6C & S6D of Appendix 9).

6.4 Discussion

In this study, plasma and aqueous tissue extracts of *B. glabrata* snails known to be susceptible and/or resistant to *S. mansoni* were investigated for variations in their protein profiles and general glycosylation patterns. Only slight differences were found between snail strains in their immune-reactivity but shared epitopes were found between parasite egg antigens and snail plasma proteins. Both glycan and peptide epitopes might be involved in cross-reactivity.

Differences between pigmented and non-pigmented snail isolates might contribute to results and could be investigated further.

6.4.1 Similarity of plasma protein profiles

Protein banding patterns were similar for plasma samples of the three *B. glabrata* strains (BgPR, BgSV and BgPS) as seen in the Coomassie blue-stained SDS-PAGE gels. Similar plasma profiles have also been reported by other authors working with susceptible and resistant snail strains (Yoshino *et al*., 2012). Also, although there were only slight differences in the immune-reactivity as revealed by Western blot analyses, the presence of diffuse bands suggested the presence of shared glycosylated proteins and this was generally attributed to the heterogeneity of carbohydrate epitopes (Varki, 1999). These observed patterns are in line with the findings in a previous study of *B. glabrata* plasma showing that profiles for whole plasma and plasma fractions (<100 kDa and >100 kDa) of susceptible and resistant snails were similar (Yoshino *et al*., 2012; Zelck *et al*., 1995).

The cross-reactivity between schistosome antigenic determinants and snail plasma proteins in this study may indicate presence of shared epitopes between schistosome egg antigens and snail plasma proteins. The glycanic nature of the epitopes was however not clearly evident since there was only little difference between periodate-treated and non-treated immunoblots. Variations in sensitivity of carbohydrate moieties to periodate treatment have been reported since four decades (Coligan & Todd, 1975). Results obtained showed that some epitopes that may be responsible for cross-reactivity of *B. glabrata* plasma proteins with parasite antigens may be

periodate-resistant. It was observed that reactivity was not significantly reduced in periodate-treated blots except between the 80-120kDa region. This may indicate presence of glycan epitopes containing fucose elements in this region, since mannose, galactose and N-acetylgalactosamine are not completely affected by periodate oxidation reaction (Gong *et al*., 2015). The study thus suggests partial involvement of glycanic epitopes in cross-reactivity observed.

Also, life cycle stages of the parasite have been demonstrated to produce several carbohydrate structures which are highly fucosylated and specific to the parasite (Lehr *et al*., 2007; Staudacher *et al*., 2009). Dot blot screening and Western blots using snail-derived molecules cross-reacting with parasite antigenic components produced from different stages of *S. mansoni* indicated specificity between interacting epitopes. Results reported here were obtained using polyspecific antisera and this could be responsible for a greater number of commonly-recognized molecules. While specificity may be increased by using monoclonal antibodies which have been demonstrated to be more sensitive than polyclonal antisera when used in immunoassays, the use of polyclonal antibodies are beneficial for detection of several cross-reacting antigens that may be present in samples (Shalit *et al*., 1985).

Western blot detection of cross-reacting glycoproteins using anti-KLH (BR14) as probe was also showed similar protein banding pattern for all three snail strains. However, based on the intensity of banding patterns, it was observed that the anti-KLH probe exhibited a reduced immuno-reactivity compared to both anti-SEA probes. This may be due to recognition of a single carbohydrate motif, the Fuc ($\alpha1 \rightarrow 3$) GalNAc, as it

has been shown to contain this terminal fucose (Geyer *et al*., 2005; Kantelhardt *et al*., 2002). As expected, more intense staining was obtained when anti-SEA probes were applied, indicating presence of a larger number of glycotopes that could be involved in mediating observed immuno-reactivity. Biochemical- and immunochemical-based methods have often been applied to detect similar proteins that differ in structure of attached glycans (Barletta *et al*., 1998).

Furthermore, while serological cross-reactivity was similar with anti-KLH probed-samples across the three snail haemolymph samples, it differed between susceptible and resistant strains probed with anti-SEAs BR84 and 1025Z. This difference was expressed by the more intense binding pattern observed for the susceptible Puerto Rican snail isolate, BgPR in comparison to the resistant BgSV strain. While this was consistent with previous studies (Lehr *et al*., 2007, 2010), findings from the current work interestingly suggests that this may not be the case across strains since the susceptible BgPS strain showed similar expression of antigenic recognition to that of the resistant snail isolate. The reason for this is not quite clear. Pigmentation and susceptibility in *Biomphalaria glabrata* snails are known to be controlled and inherited separately as in the mid-1980s a study of these two phenotypes showed no linkage between the susceptibility and pigmentation loci (Mulvey & Woodruff, 1985). Given the limited number of strains used, more research using different pigmented and non-pigmented (susceptible and resistant) may be required to determine glycan association in host-parasite compatibility.

Since this pattern was observed on application of two different anti-SEA antibody probes, one logical explanation might be that the pigmented and

albino snails may differ in expression of cross-reacting glycoproteins. Most studies comparing susceptible and resistant snails use resistant, pigmented snails and susceptible albino snails. While this may be useful in identifying molecules present or absent or those expressed differently between the snails, it also begs the question as to the possible effect of colour pigmentation on these differences. Future studies may thus focus on comparisons within-strains and then subsequent similarity and variation studies between strains in relation to pigmentation status.

6.4.2 Putative identification GST Mu 1

Peptide identification following protein digestion often involves the use of well-established methods and for shotgun proteomics experiments, the nano-scale liquid chromatography-electrospray ionization-tandem mass spectrometry (nLC-ESI-MS/MS) is not uncommon (Stone *et al*., 1998; Y. Yang *et al*., 2007). Investigating proteins of immune relevance to *B. glabrata* using this high throughput technique may be important in the discovery of interesting components of the snail proteome which remains yet to be fully elucidated.

The gel-purified protein estimated to be approximately 25kDa was identified by MS as GST mu 1. This finding is relevant to discovery of defence molecules in *B. glabrata* and to our knowledge it presents for the first time evidence that this enzyme could be involved in snail defense. There was a marked difference in the intensity of the protein bands, signifying increased expression of this isoenzyme in exposed, uninfected BgPR and BgSV snails. Increased/differential expression of this protein may indicate its involvement in cytoprotection or portray its immunological tendencies.

A recent study also found genes encoding GST in *B. glabrata* amoebocyte-producing organ showed variation in expression on exposure to a lipopolysaccharide and fucoidan, a mock glycan molecule which expresses fucosylated carbohydrate epitopes similar to those obtained from sporocysts of *S. mansoni*. However, the genes were different from the GST-encoding gene involved in the detoxification of niclosamide, a commonly used molluscicide (Zhang *et al*., 2016). While information on characteristics of non-mammalian GSTs is relatively sparse, identification of novel classes of GST have led to identification of similar isoforms in mammals. This has inadvertently led to discoveries of additional functions for GSTs in mammals. For example, GST mu isoforms have been associated with immunological functions in humans (Tsuchida *et al*., 1990).

Most GSTs are cytosolic enzymes generally known for detoxification of endogenous and exogenous xenobiotics. Also, they possess peroxidase and isomerase activities and protect cells against cell death induced by hydrogen peroxide. They constitute a ubiquitous and highly versatile superfamily whose members are comparable to other stress-related proteins (Sheehan *et al*., 2001).

The purified GST mu 1 identified here was slightly cross-reactive with one of the polyspecific anti-sera raised against SEA, 1025Z indicating recognition of a shared epitope. Glycosylation prediction indicated the protein, GST mu1, had of two potential sites for attachment of glycan moieties. The presence of glycans could be responsible for the slight cross-reactivity observed on immunoblots and also suggests epitopes shared with parasite. Glycosylation of GSTs have been demonstrated and is thought to be the cause of microheterogeneity observable within each class

of GST (Kuzmich *et al*., 1991). These results thus further emphasize the complexity the processes involved in the interaction of an invertebrate host with pathogens.

6.4.3 Haemoglobin involvement host-parasite cross-reactivity.

The present study showed that the cross-reactivity observed in plasma and tissue proteins may be partly due to haemoglobin which is known to be glycosylated. The molecular weight difference between the investigated band and the haemoglobin subunit (approximately 7 kDa) was attributed to the treatment of samples for SDS-PAGE under reducing and boiled conditions which could have resulted in the partial breakdown of subunits. The 46 kDa protein investigated in this study could therefore part of the two globin domains of a partial haemoglobin polypeptide (≈53 kDa) previously reported with other haemoglobin isomers in *B. glabrata.*

The presence of these isomers have led to suggestions that haemoglobin and haemoglobin subunits may be the major component of plasma proteins of *B. glabrata* (Lieb *et al*., 2001). Both SDS-PAGE and MS analyses indicated abundance of high molecular weight haemoglobin in the sample. High abundance of haemoglobin in *B. glabrata* snails has also been reported by other authors (Gong *et al*., 2015; Wu *et al*., 2017). Involvement of haemoglobin in larval binding, thereby providing a protective cover for parasite and enabling its ability to evade immune recognition has been suggested (Spray & Granath Jr., 1988; Yoshino, 1983). The presence of N- and O- linked glycosylation sites could also suggest shared glycan epitopes.

Further studies are needed that will enable the identification of less abundant proteins that may be involved in immune recognition in the snails.

One of the limitations of this study was the non-separation of the haemoglobin fraction from other plasma proteins. Using the appropriate molecular weight cut-off filters could have probably aided identification of low molecular weight and other proteins that could have been masked by the haemoglobin. The gel-purification technique followed by MS analysis was helped to overcome this problem and improved the identification process of samples. Also, visual quantitation of intensity of staining may not adequately measure difference in expression levels in gels. However, since the main purpose was to determine cross-reacting epitopes, the application of equal amounts of proteins in each gel electrophoresis was sufficient to visually detect differences in cross-reactivity and choose proteins to be excised for mass spectrometry. Each result of SDS-PAGE and immunoblotting was repeated at least thrice for validation.

Although strong reactivity of polyclonal antibodies and metaperiodate-treatment of blots indicated the presence of shared epitopes some of which could be glycanic in nature, patterns of cross-reacting of host and parasite proteins varied in intensity between snails. This pattern could not be specifically associated with the susceptible snails as previously reported (Lehr *et al*, 2010). In the latter report, cross-reactivity between snail haemolymph and parasite glycoconjugates were more enhanced in susceptible than resistant snail strains. The susceptible snails used in the latter study were albino while the resistant snails were pigmented. In the

present study, a third pigmented strain was included for comparison. Instead, this data indicated that this differential expression may also be dependent on other traits such as pigmentation in the snails. In either case, antigenicity in *B. glabrata* snails may involve periodate-sensitive as well as periodate-resistant carbohydrate epitopes. Further studies involving comparison of cross-reacting proteins within *B. glabrata* strains may be useful in identifying markers of immune relevance rather than strain-associated markers.

In conclusion, the above comparative assessment of *B. glabrata* plasma and tissue proteins and/or glycoproteins shared with schistosome egg antigens shows possible heterogenous associations between the snail and the parasite as indicated by the variations in binding intensities of proteins with different anti-sera. Also glycans shared may be strain specific as shown for larval stages of the parasite. This is consistent with the notion that schistosomes exhibit a wide diversity of glycans at different stages of their life cycle. Although it is not fully apparent how carbohydrate moieties may initiate or prevent parasite recognition by the host, these results show the presence of antigenic epitopes that may be differentially expressed in snails and which may influence recognition or non-recognition by cell receptors in the snails.

CHAPTER 7

GENERAL DISCUSSION AND CONCLUSION

The main aim of this research was to determine the differences between susceptible and resistant *B. glabrata* snails and was focused characterising molecules associated with immune defence in the snails. The study had four objectives. The first, which was an important prerequisite to the others was to determine susceptibility and infectivity patterns of the snails in host-parasite infection experiments. The second was to map the existing populations against other *B. glabrata* references in order to clarify their phylogeographical connections. The third was to determine the *sod1* alleles segregating in these snail populations and the fourth was to identify and characterise differentially expressed *B. glabrata* glycoproteins cross-reacting with *S. mansoni* glycoconjugates.

7.1 Host-parasite co-evolutionary dynamics

The general concept of local adaptation between host and parasite stems from complex interactive forces often resulting from natural selection. Host and parasite populations may exert various degrees of selective pressure on one another leading to differences in compatibility between both species. From the extensive study of host-parasite relationship presented in chapter three, it was clear that all *B. glabrata* snails are potentially susceptible to *S. mansoni* infection irrespective of the origin of the parasite. It was shown that *S. mansoni* strains from different continents can infect snails of another

continent. The levels of susceptibility and/infectivity however varied between various hosts and parasite strains. Variations in host snail susceptibility and parasite infectivity have also been demonstrated by previous authors (Bech *et al*., 2010; Theron *et al*., 2008, 2014) and may be partly due to geographical and strain variations.

It was also clear from our data that *B. glabrata* snails and *S. mansoni* from the same locality (sympatric combinations) may not always be co-adapted and hence do not always form compatible host-parasite pairs. This was evident from passages of sympatric combinations of host and parasites tested, e.g. Bg-Cmp snails and the Sm-Cmp parasite. A possible explanation is that parasites and host from similar localities could become so co-adapted that they develop some level of tolerance to each other. Introduction of allopatric parasite could therefore trigger a defence mechanism in which the allopatric parasite completely overwhelms the host defences leading to increased patency among allopatric snail-schistosome pairs than sympatric pairs.

The present study showed an oscillatory pattern in snail susceptibility to parasite over the period of study. This pattern is similar to the time-lagged dependent changes in susceptibility/infectivity of host-parasite combinations described by previous authors (Morand *et al*., 1996). The latter authors demonstrated dynamic polymorphisms in which parasite and host allele frequencies changed with a time lag. Due to this lag, higher degrees of compatibility between allopatric combinations in comparison to sympatric combinations become possible (Morand *et al*., 1996). These frequency-dependent selection models generate complex patterns of

parasite performance on sympatric and allopatric populations (Kaltz & Shykoff, 1998).

The second objective of this study was to establish the phylogenetic link between the existing snail strains and other previously published strains. It was inferred from the 16S rRNA that the existing snail strains, BgPR, BgPS and BgSV were of the same panmictic population and confirmed the snails were mainly of Brazilian ancestry. In addition, the results clearly showed the relationship between the present laboratory strains and previously reported field and laboratory strains. Furthermore, the phylogeny of the BgPS strain was inferred to be partly Caribbean (one of the parental stocks from which this hybrid was developed was unknown). Defining laboratory populations prior to experimental studies has been suggested as a way to ensure uniformity of results during experimental manipulations (Mulvey & Bandoni, 1994). Our data showed that snails from other studies can be compared based on the neutral 16S rRNA gene and this could improve comparison of snails used by various researchers as well as improving result interpretations. As expected, the number of haplotypes found was not representative of the diversity of the snails. This further confirmed that neutral markers cannot be used to define adaptive variations. The variability reflected in host susceptibility thus can only be measured using adaptive markers. Our data is however limited and cannot be used to determine genetic processes such as gene flow within the populations. The work reported in chapter five was thus focused on further quantitative genetic experiments that could provide more insight into the adaptive potentials of the laboratory populations using the *sod1* gene.

Relationship between host phenotypes and genotypes

Given the rapid changes observed between cycles of parasite passages in some of the allopatric as well as sympatric pairs, it was unclear how the level of compatibility of the BgPR snails for instance, remained stable for multiple years of passages. This interesting trend, in spite of repeated bottlenecks is similar to reports by other authors for a sympatric pair of snail and parasite (Theron *et al*., 2014). One explanation for this is that the genotype frequencies in the population remained fairly constant and the substantial threshold of a truly bottlenecking event had not been reached. Once this threshold was reached, it caused a change in the proportion of infected or uninfected individuals in populations resulting in a frequency-dependent selection pattern. This agrees with a matching allele model that stems from changes in allele frequency that could have caused the oscillatory pattern of host susceptibility to parasites as found in the longitudinal infection data (chapter three results).

The third objective was to determine variability in resistant and susceptible snail populations based on the *sod1* gene. An important finding in this study was the extensive polymorphism of the *sod1* gene. In contrast to previous findings that revealed four alleles at this gene locus (Goodall *et al*., 2006), this study found additional 34 alleles most of which were rare. This difference in alleles was mainly attributed to strain differences. Phylogeographical analysis (chapter four results) indicated that the *B. glabrata* used for the present study were different from the 13-16-R1 strains used by Goodall *et al*, 2006. Although some of the snails shared

similar 16S rRNA haplotypes with the 13-16-R-1 resistant snails (based on the cluster patterns of the mitochondrial 16S rRNA gene haplotypes), this was consistent with the hybrid nature of the strain (i.e., 13-16-R1).

Furthermore, genetic drift following repeated laboratory bottlenecks could lead to changes in allele frequencies which may eventually lead to loss of rare alleles. For host-parasite coevolution to lead to an ongoing advantage for rare genotypes, parasites should infect specific host genotypes and hosts should resist specific parasite genotypes. The most prominent genetics capturing such specificity are matching-allele models (Luijckx *et al.*, 2013). The effect of the rare alleles are not clear. We could, however speculate that if these alleles reduce host fitness, then selection forces would maintain them at low frequency. The diversity of the *sod1* gene based on the number of haplotypes and the conspicuously high frequency of one haplotype, H5 (especially in the BgSV snails which represent a predominantly resistant population) could also be a sign of balancing selection in the population.

Individual possessing haplotype H5 had a reduced risk of infection and a larger proportion of resistant individuals were either homozygotes for this haplotype (i.e., H5H5) or heterozygotes, having this allele and the previously published resistant allele B (i.e., H2H5). It is possible that possessing the H2 haplotype in the homozygote state has lethal effects, hence the low numbers of homozygotes observed with this haplotype in our study and the previous study by Goodall *et al*, 2006. It was statistically shown that haplotype H5 conferred resistance, however, it is also possible that H5 is less lethal and thus could be acting to reduce possible fitness

costs of having the H2 (previously reported resistance allele). Future studies to compare these two alleles as well as other alleles of the *sod1* gene in field populations would be an important step in determining the use of this gene or any other gene as a marker for resistance or susceptibility in *B. glabrata* snails.

Relationship between host immune defence and compatibility phenotypes

A major determinant of compatibility between interacting host-parasite combinations of *B. glabrata* and *S. mansoni* is the immune defence of the snails. An important hypothesis which many authors seem to agree with is that schistosome sporocysts evade elimination by haemocytes through molecular mimicry which may involves proteins and carbohydrates (glycans). The sixth chapter therefore was focused on identifying glycoproteins (that could be involved in *B. glabrata* defence against *S. mansoni)* by their differential cross-reactivity with *S. mansoni* antigens.

Glycan involvement in parasite evasion of host defence has been suggested as one of the basis for differences between susceptible and resistant snails (van Die & Cummings, 2010; van Diepen *et al*., 2012; Yang *et al*., 2017). Results showed similarity in SDS-PAGE protein profiles of both susceptible and resistant snails, however, there were slight variations in protein banding patterns and intensity of haemolymph and tissue glycoproteins cross-reacting with *S. mansoni* glycoconjugates. Glycan epitopes shared between snail hosts and parasites may be differentially expressed in susceptible and resistant snails. This carbohydrate-based form of molecular

mimicry has been demonstrated by several authors (Lehr *et al*., 2008; Yoshino *et al*., 2013)

Previously, other authors have demonstrated higher expression of serologically cross-reacting fucosylated carbohydrate epitopes and larger number of glycans for susceptible BgPR snails compared to the resistant BgBS-90 snails (Lehr *et al*., 2007). While the current findings agree with the latter, in terms of differential expression between the snails, a contrary opinion is derived from these results. This is because the serological cross-reactivity profiles obtained for an highly susceptible strain (BgPS) revealed an interesting twist, raising another question, does pigmentation of snail have an influence on glycan expression? Both the BgPS and the BgSV snails are pigmented snails while the BgPR is an albino snail. The present findings are based on expression as observed by SDS-PAGE and Western blotting and so limited in thoroughly determining the specific epitopes involved. Further research employing HPLC and MS may be required to determine if expression is solely associated with compatibility phenotypes (susceptibility or resistance) or by pigmentation.

Also, intense cross-reactivity of high molecular weight proteins and identification of the purified 46kDa protein as haemoglobin which is heavily glycosylated may suggest its involvement in glycan mimicry. The involvement of haemoglobin in larval binding has been previously suggested (Yoshino, 1983). Another molecule identified in this study is the GST mu 1, a 25kDa protein. In *B. glabrata*, altered expression of genes encoding GST classes of protein and other enzymes involved in degradation and detoxification have been demonstrated in response to injection of

substances mimicking *S. mansoni* glycans (Zhang *et al*., 2016). The putatively identified GST-mu 1 appears to be glycosylated and its differential expression in *B. glabrata* snails suggests possible role in host-parasite interaction processes. The GST-mu 1 of *B. glabrata* and its role in relation to other immune associated proteins requires further investigation. Variability in expression of defence molecules relative to one another, could be a key factor in determining success in each snail-schistosome encounter. Previously, identified interacting molecules of the parasite, *S. mansoni* polymorphic mucins (SmPoMucs) and those of the host, *B. glabrata* FREPs (Mitta *et al*., 2012) could be a possible stabilizing factor. As such, variability inherent in the host molecule, would provide the host with a vast system that matches and counters the variability within parasite molecules. This counteraction between host and parasite has also been shown for oxidant and antioxidant production during host-parasite encounters (Mone *et al*., 2010).

In the light of the findings obtained in the present study, claims that certain laboratory populations of snails are completely resistant and remain unchanged over time is questionable. While we agree that *B. glabrata* snails could remain stable at some point during laboratory maintenance, we argue that *B. glabrata* snails cannot remain stable through long periods of laboratory culture. It is more likely that laboratory maintained snails change over time. There are however several limitations that should be noted some of which are associated with both the host and parasite population. Laboratory populations used are bottlenecked and some of the assumptions of HWE are more likely to be violated as demonstrated by this study. *Biomphalaria glabrata* snails are hermaphrodites and are capable of self-

fertilisation even though they preferentially cross-fertilize. Hence the flow of resistance genes may considerably vary in each cycle or generation of snail. Lewis *et al*, 2003 suggested that HWE that may not be met in interbreeding populations of *B. glabrata* may include non-random mating differences in reproductive capabilities such as fecundity, differences in survival in relation to genotypes as well other life-history traits (Lewis *et al*., 2003). Going by the findings of this research, it is likely that homogeneity of snail populations are not the norm and suggestions of producing homogenous population of snails may not be realistically achievable. The future of genetic control of vector populations might thus be in favour of cloned snails.

Recommendations and future work

The set aims of this research have been achieved, however due to constraints of time and finance, further research to collect, identify and characterise *sod1* gene in field obtained *B. glabrata* for comparison with laboratory snails could not be carried out. It would be interesting to study and further characterise field samples based on the *sod1* gene.

Also, some proteins of interest such as the 90 kDa protein in the snail plasma remains to be fully elucidated. In addition, there were several cross-reactive proteins above 25 kDa that appeared to have been masked by haemoglobin. Since these proteins are found within the molecular weight range for *B. glabrata* haemoglobin, separation and purification methods that would remove these heavy molecules prior to MS analysis are required. Thus, separation of purer molecules for MS could have been better achieved

using 2-D SDS-PAGE which separates molecules based on both their molecular weight and isoelectric points (pI).

In addition, because of the abundance and high molecular weight of haemoglobin, separation of the heavy protein fractions e.g., by size exclusion chromatography, possibly followed by lectin-based affinity chromatography could greatly improve the separation and purification process of proteins of low abundance. Given the heterogeneity of *B. glabrata* populations which could lead to differences in results obtained in various studies, developing a standard system define laboratory stocks of snails is also recommended. This may aid better interpretation of results obtained using various laboratory strains of *B. glabrata* and reduce the complexity that has surrounded the various aspects of this snail-trematode interaction for many decades.

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APPENDICES

Appendix 1

CTAB DNA Extraction Protocol

1) For each tissue slice, grind the tissue quickly with glass beads and add immediately, 500 µL CTAB solution into each tube

2) Add 10 µL of a 20 mg/mL proteinase K solution

3) Add 0.2% **ß**mercaptoethanol (0.2% of 500 µL = 1µL)
Note: Step 3 in the fume hood

CTAB solution:
Tris HCL 100 mM pH 8 (10 mL of 1M stock solution)
EDTA 20 mM pH 8 (4 mL of 0.5M stock solution)
NaCl 1.4M 28 mL of 5M stock solution)
CTAB 2% (2 g for 100 mL)

CTAB = Hexadecyltrimethylammonium bromide

4) Incubate the tissue at 55°C till the tissue is completely digested (usually 2 to 3 hours)

5) Add 500 µL Chloroform-Isoamyl Alcohol (24:1)
Mix (invert tubes) 5 min
Spin 5 min at 13 000 rpm
Remove the aqueous layer (~400 µL) and put in correspondingly labelled fresh tubes.

6) Repeat step 5
*** For many tissues a single Chloroform-Isoamyl Alcohol step (step 6) is sufficient**

7) Add 2.5 volumes (1 mL for 400 µl) ethanol 95% (icecold) + 1/10 volume (4 µL for 400 µL) 3M NaAc
Place for 2 hours at -20°C at least (alternatively overnight and/or -80°C)
Spin 10-15 min at 13 000 rpm
Remove the ethanol

8) Wash the pellet with 500 µL ethanol 70% (cold)
Spin 5 min at 13 000 rpm

9) Dry the pellet (air dry or 15 min. maximum at 45°C)

10) Re-suspend in 200 µL Tris pH 8.0 (elution buffer)

(11) Boil for 10 minutes at 95°C, then cool to room temperature and put in the fridge for about 30 minutes.

(12) Store in -20°C freezer.

Appendix 2

Chemicals / reagents were purchased from Sigma-Aldrich company, United Kingdom, except otherwise stated.

Anaesthetisation of mice

Ket/Dom mixture

- Ketamine comes as 10 mL with 100 mg/ml.
- Domitor comes as 10 mL with 1 mg/ml.
- Antisedan comes as 10 mL with 5mg/ml.

1. Put 0.75 mL ketamine plus 1.0 mL Domitor plus 8.25 mL isotonic saline to make up 10 mL of sterile solution.
2. Use 1 mL/100 g mouse for anaesthetisation.
3. Therefore 0.25 mL will anaesthetise a 25 g mouse.
4. And 0.20 mL will anaesthetise a 20 g mouse.

Reversal with Antisedan

1. Put 0.2 mL Antisedan plus 9.8 mL saline together.
2. 0.25 mL will reverse a 25 g mouse.
3. 0.20 mL will reverse a 20 g mouse.

Appendix 3

Protocol for preparation of parasite egg antigens

Schistosoma mansoni soluble egg antigens (SEA) prepared using the method described by Doenhoff *et al.* (1988) summarized as follows:

- Each mouse was injected with 1.5 mg of hydrocortisone acetate (three days before infected mice were perfused for adult worm recovery) to prevent granuloma formation (Lucas *et al*., 1980).
- After perfusion, livers and intestines were removed from the culled animals and macerated in 3 volumes of 1.8% saline solution using a regular food blender.
- The mixture was then incubated for 2 hours at 30°C with porcine pancreatic trypsin in a 1% solution (1g/100mL homogenate).
- The resultant solution was then purified by filtering sequentially through sieves with decreasing mesh sizes 600, 300 and 150 µM.

- The filtrate obtained from the 150 μM sieve was rinsed and allowed to sediment in 1.8% saline solution. This sedimentation process was repeated till a filtrate free of tissue aggregates was obtained.
- Excess supernatant was decanted after the final sedimentation step and the egg pellets were stored at –20°C.
- To complete the egg antigen preparation process, stored pellets were thawed, homogenized in 3x volume of isotonic saline solution, and then centrifuged at 4000 x g at room temperature.
- The supernatant obtained was stored as aliquots of 1 mg/mL protein concentration at –80°C.

<u>Appendix 4</u>

Procedure for production of rabbit polyspecific antisera

- Homogenates of the different parasite stages are first emulsified in complete Freund's adjuvant
- Once a week, injections of 1 mL are given in 0.1 mL doses either intramuscularly in the hind legs or subcutaneously at varied positions on the back, injected into rabbits (Dunne *et al.*, 1986; Modha *et al.*, 1988).
- The rabbit's antibody response is tested qualitatively by immune-electrophoresis till considered strong enough, then the rabbit is bled alternately from each ear weekly. When about at least 30-40 mL of serum is obtained, the rabbit is then euthanized using a lethal dose of pentobarbitone anaesthetic and cardio-exsanguination is performed.
- After separation from blood, the pooled sera is stored at –80°C in 5 mL aliquots.

Appendix 5

SDS-PAGE

Solutions/Mixtures and buffers:

10x Running Buffer, 1 litre

- 30 g Tris (Tris Ultrapure, Invitrogen, # 15504-020, Lot 1222263).
- 144 g Glycine (#G8898-1kg, Lot: 055K0188).
- 10 g SDS (Lauryl Sulfate, # L-4509, Lot: 102K0203).
- Distilled water.

1. Dissolve ingredients in ~800 mL distilled water
2. Stir to dissolve
3. Add distilled water to final volume of 1 litre
4. Store at RT
5. Use 1 in 10 dilution

1.5 M Tris HCl pH 8.8 @ 25°C, 100 mL

- Tris Ultrapure (Invitrogen, # 15504-020, Lot 1222263).

1. Dissolve 18.17 g in 80 mL distilled water.
2. Cool to RT for adjusting pH (pH of Tris is temperature dependent).
3. Use specific pH electrode for use with Tris.
4. Adjust to pH 8.8 with concentrated HCl.
5. Adjust final volume to 100 mL with distilled water.
6. Autoclave, store at RT.

0.5 M Tris HCl, pH 6.8 @ 25°C

- Tris Ultrapure (Invitrogen, # 15504-020, Lot 1222263).

1. Dissolve 3.0 g in 80 mL distilled water.
2. Put in oven to RT for adjusting pH (pH of Tris is temperature dependent).
3. Use specific pH electrode for use with Tris.
4. Adjust pH 6.8 with concentrated HCl.
5. Adjust final volume to 100 mL with distilled water.
6. Autoclave, store at RT.

10% SDS, 50 mL (Lauryl Sulfate, Sigma, # L-4509, Lot: 102K0203)

An anionic detergent which denatures secondary and non-disulfide-linked-tertiary structures and applies a negative charge to each protein in proportion to its mass.

1. Dissolve 5 g SDS (wear face mask) in ~40 mL distilled water.
2. Stir to dissolve, takes a long time (best overnight or heat to around 68°C).
3. Add distilled water to final volume of 50 mL.
4. Don’t autoclave, store at RT.

4x Laemmli buffer (sample loading buffer) (Maizels, RM), 10 mL

- 250 mM 1 M Tris pH 6.8 2.5 mL

- 8% SDS 0.8 g
- 40% glycerol 4 mL
  (Sigma, #G-5150, Lot 28H093315)
- Bromophenolblue 8.2 mg
  (Sigma, #B802-5G, Lot 044K3700)
- β-mercaptoethanol 1 mL
  (Sigma, # M7-154-25, Lot 104K1060)
- Distilled water 2.5 mL

1. Dissolve SDS in Tris solution and distilled water.
2. Add remaining ingredients and mix.
3. Aliquot in 500 µL and store at -20°C.

β-mercaptoethanol denatures proteins further by reducing the disulfide linkages (hence its presence in reducing loading buffer and absent in non-reducing loading buffer).

Molecular weight marker

ColorPlus Prestained Protein marker (7 - 175 kDa). New England Biolabs Inc.

- Lot: 0251308, Cas: 4043-96-3. P7709S.

Coomassie blue for SDS-PAGE

- ProtoBlueSafe (National Diagnostics, Hessle UK, #ELR-722-001D (from Fisher).
- Use 1 volume ethanol (100%) and 9 volumes ProtoBlueSafe freshly prepared.
- Store at RT.

Destaining solution

- If stained with ProtoBlueSafe only distilled water is used to destain.

PageBlue Protein staining solution

- Thermo Scientific, 3747N, Meridian road, Rockford IL, 61101, USA.
- Lot: 00106194.

SimplyBlue SafeStain

- Lot: 1452689, Cat: LC6060.
- Invitrogen, Carlsbad, CA 92008.7606037200.

Gel drying solution

- Distilled water with 2% (v/v) glycerol.
- Store at RT.
- If stained with ProtoBlueSafe, gel can’t be kept for longer than 20 minutes in standard gel drying solutions containing >20% ethanol.

Water saturated n-Butanol

- Cas: #BT-105, Lot: 27H5023.
- 50 mL Butanol.
- 5 mL distilled water.
- Store at RT.

30% Acrylamide / bis-Acrylamide Solution

- Lot: SLBD2629V, Pcode: 1001361989.
- Store at 4°C.

TEMED

- Cas: 110-18-9, Lot: AO334088. Acros Organics, New Jersey, USA.
- Initiates together with APS the polymerisation of acrylamide gels.
- Store at RT.

100% ethanol

- Riedel-de Haën, #32221.

10% Ammonium persulphate (APS)

- BDH Chemicals Ltd Poole, UK, #27195, Lot 5797970B (general purpose reagent).
- Catalyses polymerisation of acrylamide gels.
- Prepare 1 mL.
- Aliquot in 200 µL and store at -20°C.

Na_2HPO_4 buffer (500 mL)

1. Dissolve 1.425 g of Na_2HPO_4 in 500 mL distilled water.
2. Put on magnetic stirrer to completely dissolve.
3. Adjust pH to 7.5 using 1M HCl.

10X PBS (1 litre)

- A stock solution of 1 litre is usually made.

1. Add 800 mL of ultrapure water to a 2 litre beaker.
2. Place on a magnetic stirrer and stir.
3. Add the following to the stirring water:

- 80 g NaCl
- 2 g KCl
- 7.62 g Na_2HPO_4 (Disodium hydrogen orthophosphate).
- 2.4 g KH_2PO_4 (Potassium dihydrogen orthophosphate).

4. Stir the mixture until the solution become clear.
5. Adjust pH to 7.4.
6. Top up the solution to 1 litre with distilled water.

To make 1X PBS, take 100 mL of 10X PBS and dilute in 900mL distilled water.

Extraction buffer (0.05M Tris-HCl, pH 7.4) 100 mL

- For the aqueous extraction of tissue proteins.

1. Dissolve 600 mg of Tris in 80 mL of distilled water.
2. Stir on a magnetic stirrer to dissolve.
3. Adjust pH to 7.4 using 0.1M HCl.
4. Top up the solution to 100 mL with distilled water.
5. Store at RT.

Sodium acetate buffer (0.05M) pH, 4.5 (1000 mL)

- Lot: 057K0014, Cas: 127-09-3.
- For the dilution of sodium m-periodate and esterase substrates.

1. Dissolve 4.10 g of sodium acetate in 800 mL of distilled water.
2. Stir on a magnetic stirrer to dissolve.
3. Adjust pH to 4.5 using 0.1 M HCl.
4. Top up the solution to 1000 mL with distilled water.
5. Store at RT.

Sodium m-periodate

- Lot: 18H0763, Cas: 7790-28-5.

PBST buffer (0.2% Tween-20 in 1X PBS) (500 mL)

- Tween-20, Cas: 9005-64-5, Lot: SLBC7100V, Pcode: 1001549482
- For washing worms and blocking buffer.

1. Add 1 mL of Tween 20 in 490 mL of 1X PBS solution.
2. Stir on a magnetic stirrer to dissolve.
3. Store at RT.

Blocking buffer (1% Albumin Bovine Serum (BSA) in PBST)

- BSA: Lot: 096K1205, Cas: 9048-46-8.

1. Dissolve 20 mg of BSA in 2 mL PBST.
2. Place on a roller to dissolve.

Protein assay reagents

- Bio-rad D_C protein assay reagent A, Cat: 500-1113, 210000828.
- Bio-rad D_C protein assay reagent B, Cat: 500-0114, 210000710. 2000 Alfred Nobel. Dr., Hercules, CA 94547.

Appendix 6

Biorad minigel system set-up requirements

The BIORAD minigel system (Bio-Rad Laboratories, Inc., Hercules, California, USA) set up for a single gel required .

- Two clean glass plates
- Two spacers,
- One comb,
- n-butanol,
- deionised water (dH_2O),
- 1.5M Tris HCl (pH 8.8)
- 0.5M Tris HCl (pH 6.8),

- 30% polyacrylamide solution
- 10% ammonium per sulphate (APS) solution
- N,N,N',N'-tetramethylethane-1,2-diamine (TEMED).

1. Wash, dry and decontaminate the glass plates, spacers and combs by wiping with 70% ethanol.
2. Set up equipment following manufacturer's guide. Check for leakages in equipment set up using deionised water. When leakages have been ruled out, discard water and dry off traces with filter papers. Place set up on the bench ready for gel casting.
3. Prepare resolving and stacking gels by sequentially adding the components required for each gel (Tables S1a and S1b).

Table S1a: BIORAD minigel SDS system components for 2 thin resolving gels (0.8mm thick; 8% and 12%)

Stock solutions and reagents	Separating gel (8%) mL	Separating gel (12%) mL
1.5M Tris HCl; pH 8.8	2.5	2.5
10% SDS	0.1	0.1
30% Acrylamide	2.7	3.9
H_2O	4.7	3.5
10% APS	0.05	0.05
TEMED	0.005	0.005
Final Volume (mL)	10	10

Table S1b: BIORAD Minigel SDS system components for 2 thin stacking gels (0.8mm thick)

Stock solutions and reagents	4.5% mL	Final concentration
0.5M Tris; pH 6.8	1.25	375mM
10% SDS	0.05	0.1%
30% Acrylamide	0.75	4.5%
H_2O	2.95	

10% APS	0.025	0.05%
TEMED	0.0025	0.05%
Final Volume	5	Final

Appendix 7

Western Blotting: Wet-Blot Method

General Materials / Reagents

- Chill 1x transfer buffer at 4 °C, improves heat dissipation.
- BIORAD Mini Trans-Blot Electrophoretic Transfer Cell.
- Whatman 3 mm filter paper.
- 0.2 µm Hybond-C-Extra nitrocellulose membrane, Lot: 84561, Cat: RPN303E. Amersham Biosciences, UK Ltd.
- Anti-rabbit IgG peroxidase conjugate (Sigma, #A-0545).
- 4-Chloro-1-naphthol, Lot: 031M1726V, Cas: 604-44-4.
- Hydrogen peroxide, Lot: SZBE0710V, Cas: 7722-84-1.

SOLUTIONS / BUFFERS

10x Transfer buffer – 1 litre

250 mM Tris base 30 g
1.92 mM glycine 144 g
Top up with ddH_2O to 1 litre

1x Transfer buffer – 1 litre

100 mL 10x transfer buffer
100 mL methanol 10%
Top up with ddH_2O to 1 litre
Adjust pH to 8.3 is mostly around 8.2, but check
5 mL 20% SDS 0.1% (improves transfer of larger proteins, but reduces binding efficiency of proteins

10x TBS – 1 litre

500 mM Tris base 60.00 g
1.5 M NaCl 87.66 g
Top up with ddH_2O to 1 litre
Adjust pH to 7.5

TBST – 1 litre

1x 10 x TBS 100 mL
0.5% Tween20 5 mL
Top up with ddH_2O to 1 litre

Blocking buffer – 50 mL

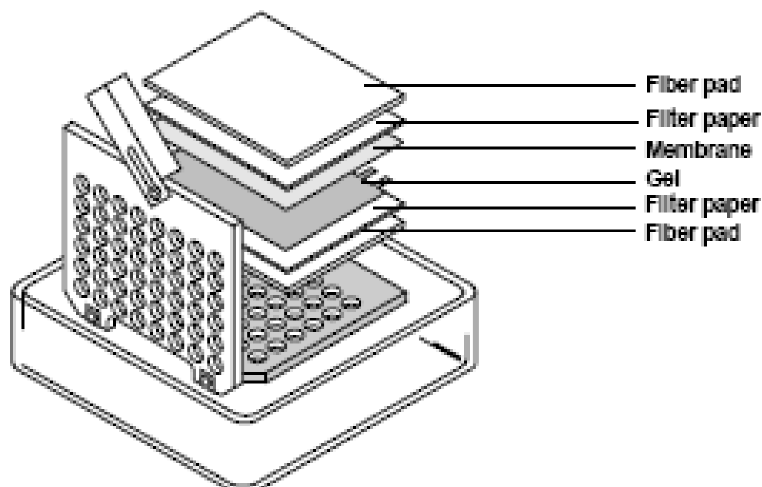
50 mL TBST
2.5 g skimmed milk powder 5 %

Labelling buffer – 50 mL

50 mL TBST
250 mg skimmed milk powder 0.5 %

METHOD:

- Run SDS PAGE gel as normal, load 1-10 µg of protein or up to 30 µg of complex antigen mixture per well.
- Equilibrate the SDS gel in 1x transfer buffer for 15 minutes.
- Cut the membrane and the filter paper to the dimension of the gel.
- Wear gloves when handling the membrane.
- Soak 2 fibre pads and 2 sheets filter paper in 1x transfer buffer.
- Prepare the gel sandwich
    - Place the cassette, with the grey side down, on a clean surface.
    - Place two pre-wetted fibre pads on the grey side of the cassette
    - Place a sheet of filter paper on the fibre pad.
    - Place the equilibrated gel on the filter paper and remove any air bubbles with a glass pipette.
    - Place the pre-wetted membrane (PVDF membranes have to be activated) on the gel and remove again any air bubbles.
    - Complete the sandwich by placing a piece of filter paper on the membrane.
    - Add the last two fibre pads.



- Close cassette firmly, being careful not to move the gel and filter paper sandwich.

- Lock the cassette closed with the white latch.
- Place cassette in module, grey side of cassette facing black side of module.
- Add frozen Bio-ice cooling unit (if available), place in tank and completely fill the tank with refrigerated transfer buffer.
- Add a standard stir bar the help maintain even buffer temperature and ion distribution, set speed as fast as possible.
- Blot at 4°C using 70 V for 2 hours.
- Recover membrane (and remove strip containing marker proteins, stain as usual in coomassie blue).
- Steps below not necessary if pre-stained markers are used, show successful transfer of protein.
- Check successful protein transfer by incubating membrane in Ponceau solution.
- Block the membrane for 2 hours at RT or at 4°C overnight in blocking buffer during gentle agitation.
- Rinse once with TBST.
- Dilute first antibody to working concentration in labelling buffer, polyclonal antisera are normally diluted 1:100 to 1:500.
- Use 3-5 mL of diluted antibody and incubate for 2 hours at RT or overnight at 4 °C during gentle agitation.
- Rinse 3x in labelling buffer, 10 minutes each.
- Dilute the peroxidase-conjugated antibody 1:1000 in labelling buffer and incubate for 2 hours at RT.
- Rinse 3x in labelling buffer, 10 minutes each with a final wash in TBST only.
- Incubate in a solution of 4-chloro 1-naphthol:
- Dissolve 20 mg 4-chloro-1-napthol in 4 mL ethanol (solution very sensitive to the light). Heat 20 mL of 1xTBS for 20 seconds in the microwave and add to the solution. Add 10 µL 30% hydrogen peroxide immediately before developing blots.
- Agitate gently for around 15 min, more or less depending on how well the reaction develops.
- Rinse in water and dry.

Appendix 8

Mass spectrometry protocol

(As performed by BSRC Mass Spectrometry and Proteomics Facility, University of St. Andrews St. Andrews, Fife).

A. In-Gel Digestion

The excised gel slice or chunk containing purified protein was cut into 1 mm cubes. These were then subjected to in-gel digestion, using a ProGest Investigator in-gel digestion robot (Genomic Solutions, Ann Arbor, MI) using standard protocols (Shevchenko *et al*., 1996). Briefly the gel cubes were de-stained by washing with acetonitrile and subjected to reduction and alkylation before digestion with trypsin

at 37°C. The peptides were extracted with 10% formic acid and concentrated down to 20 μL using a SpeedVac (ThermoSavant).

B. nLC-ESI MSMS and Data Analysis on the TripleTOF 5600

After in-gel digest with trypsin, the peptides were injected on an Acclaim PepMap 100 C18 trap and an Acclaim PepMap RSLC C18 column (ThermoFisher Scientific), using a nanoLC Ultra 2D plus loading pump and nanoLC as-2 autosampler (Eksigent).

For a single gel slice, the peptides were eluted with a gradient of increasing acetonitrile, containing 0.1 % formic acid (5-40% acetonitrile in 6 min, 40-95 % in a further 2.5 min, followed by 95% acetonitrile to clean the column, before re-equilibration to 5% acetonitrile).

Thereafter, the eluate was sprayed into a TripleTOF 5600 electrospray tandem mass spectrometer (ABSciex, Foster City, CA) and analysed in Information Dependent Acquisition (IDA) mode, performing 250 msec of MS followed by 100 msec MSMS analyses on the 20 most intense peaks.

Appendix 9

Supplementary results CH.5

Haplotype	H1	H2	H5	H6	H7	H18	H19	H27	H28	H29	H30	H31	H33	H34	H35	H36
H1 (A)	0															
H2 (B)	0	1														
H5	1	22	43													
H6	0	1	2	1												
H7	0	0	1	0	0											
H18	0	0	0	0	0	1										
H19	0	0	0	0	0	0	1									
H27	0	0	0	0	0	0	0	0								
H28	0	0	0	0	0	0	0	1	0							
H29	0	0	0	0	0	0	0	0	0	0						
H30	0	0	0	0	0	0	0	0	0	1	0					
H31	0	0	0	0	0	0	3	0	0	0	0	0				
H33	0	0	0	0	0	0	0	0	0	0	0	0	0			
H34	0	0	0	0	0	0	0	0	0	0	0	0	1	0		
H35	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
H36	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0

Figure S5A: Genotype matrix for BgSV snails. Previously published *sod1* haplotype designations are shown in parenthesis.

Haplotype	H1	H2	H4	H5	H6	H7	H8	H11	H15
H1 (A)	0								
H2 (B)	0	0							
H4 (D)	1	0	0						
H5	0	1	0	1					
H6	0	0	0	0	2				
H7	0	2	0	0	0	0			
H8	1	0	0	0	0	0	0		
H11	0	0	0	0	0	0	0	0	
H15	0	0	0	0	0	0	0	4	0

Figure S5B: Genotype matrix for BgPS snails. Previously published *sod1* haplotype designations are shown in parenthesis.

Haplotype	H2	H5	H6	H7	H9	H10	H11	H12	H13	H14	H16	H17	H20	H21	H22	H23	H24	H25	H26	H27	H32	H37	H38
H2 (B)	0																						
H5	3	4																					
H6	0	0	1																				
H7	8	2	0	0																			
H9	1	2	1	0	8																		
H10	0	0	1	0	0	0																	
H11	0	0	0	0	0	0	0																
H12	0	0	0	0	0	0	1	0															
H13	0	0	0	0	0	0	0	0	0														
H14	0	0	0	0	0	0	0	0	1	0													
H16	0	0	0	0	0	0	0	0	0	0	0												
H17	0	0	0	0	0	0	0	0	0	0	1	0											
H20	0	1	0	0	0	0	0	0	0	0	0	0	0										
H21	0	0	0	0	0	0	0	0	0	0	0	0	0	0									
H22	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0								
H23	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0							
H24	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0						
H25	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0					
H26	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				
H27	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0			
H32	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		
H37	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
H38	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Figure S5C: Genotype matrix for BgPR snails. Published *sod1* haplotype designations are shown in parenthesis

Table S6A: Summarised MASCOT data-search output for 25kDa purified protein of *B. glabrata*

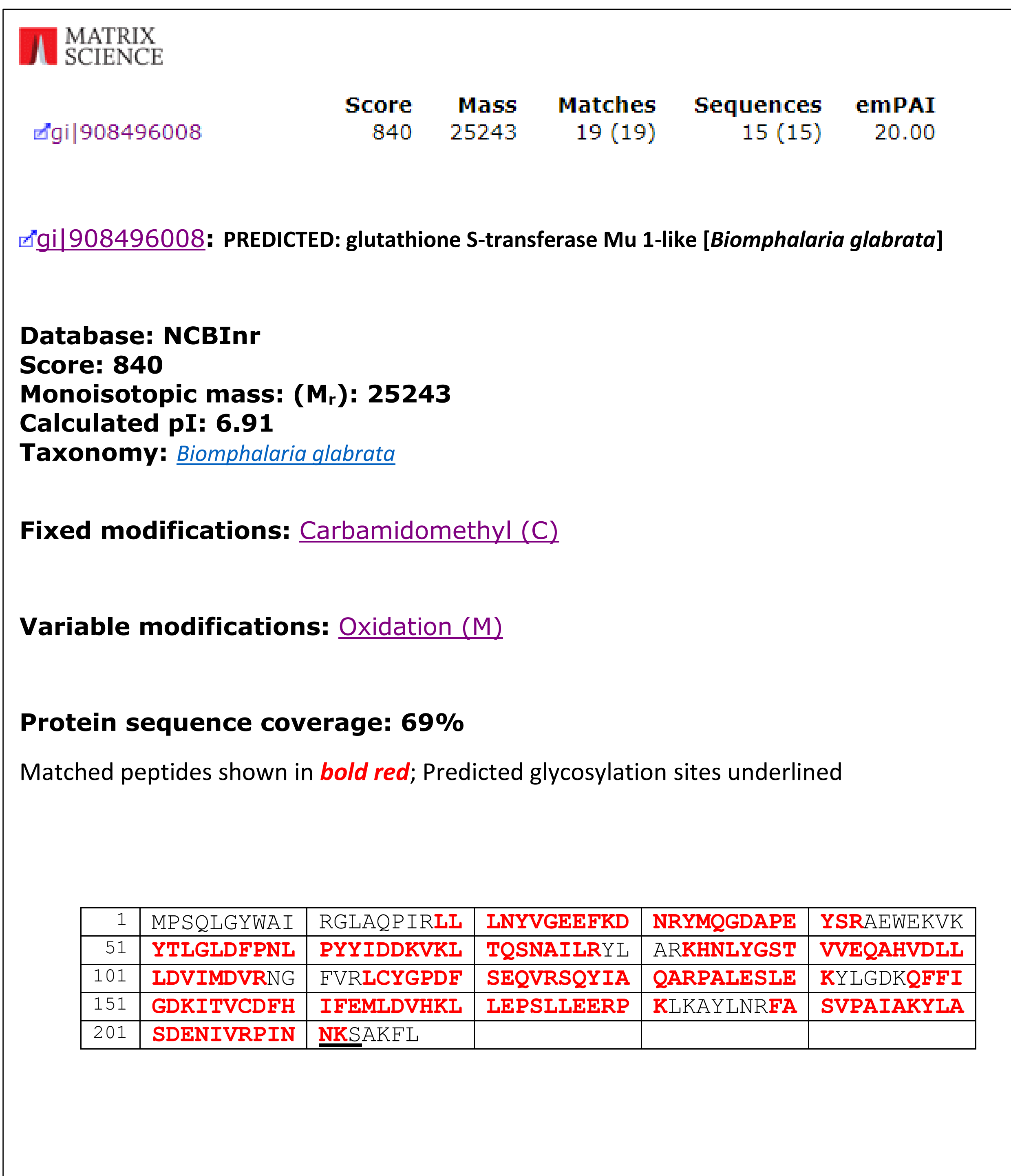


Table S6B: Predicted N-linked glycosylated sites for GST Mu 1 of *B. glabrata*. Potential glycosylated N-linked sites are indicated in red/green font

Protein sequence	Position	Residue	Score	Prediction summary
MPSQLGYWAIRGLAQPIRLLLNYVGEEFKDNR YMQGDAPEYSRAEWEKVKYTLGLDFPNLPYYI DDKVKLTQSNAILRYLARKHNLYGSTVVEQA HVDLLLDVIMDVRNGFVRLCYGPDFSEQVRS QYIAQARPALESLEKYLGDKQFFIGDKITVCDF HIFEMLDVHKLLEPSLLEERPKLKAYLNRFASV PAIAKYLASDENIVRPINNKSAKFL	22	NYV	-1.1348855	Non-glycosylated
	31	NRY	-1.0832779	Non-glycosylated
	59	NLP	-1.1210939	Non-glycosylated
	74	NAI	-0.94452865	Non-glycosylated
	85	NLY	-1.2558224	Non-glycosylated
	109	NGF	-0.67352569	Non-glycosylated
	187	NRF	-1.011378	Non-glycosylated
	204	NIV	-1.3936532	Non-glycosylated
	210	NNK	-1.230174	Non-glycosylated
	211	**NKS**	**0.41309454**	**Potential Glycosylated**

Table S6C: Predicted O-linked glycosylated sites for GST Mu 1 of *B. glabrata*. Potential glycosylated N-linked sites are indicated in red/green font

Protein sequence	Position	Residue	Score	Prediction summary
MPSQLGYWAIRGLAQPIRLLLNYVGEEFKDNR YMQGDAPEYSRAEWEKVKYTLGLDFPNLPYYI DDKVKLTQSNAILRYLARKHNLYGSTVVEQA HVDLLLDVIMDVRNGFVRLCYGPDFSEQVRS QYIAQARPALESLEKYLGDKQFFIGDKITVCDF HIFEMLDVHKLLEPSLLEERPKLKAYLNRFASV PAIAKYLASDENIVRPINNKSAKFL	**3**	**S**	**0.045789366**	**Potential Glycosylated**
	42	S	-0.19008823	Non-glycosylated
	52	T	-0.2201466	Non-glycosylated
	71	T	-0.40480241	Non-glycosylated
	73	S	-0.38353033	Non-glycosylated
	89	S	-0.54317107	Non-glycosylated
	90	T	-0.36986029	Non-glycosylated
	121	S	-0.3934635	Non-glycosylated
	126	S	-0.48227497	Non-glycosylated
	138	S	-0.45717244	Non-glycosylated
	155	T	-0.27970969	Non-glycosylated
	174	S	-0.66917804	Non-glycosylated
	191	S	-0.44181229	Non-glycosylated
	201	S	-0.43884472	Non-glycosylated
	213	S	-0.15418383	Non-glycosylated

Table S6D: Predicted O-linked glycosylated sites for partial haemoglobin, *B. glabrata*. Potential glycosylated O-linked sites are indicated in red/green font.

Protein sequence	Position	Residue	Score	Prediction summary
GKLQANLERLTDVHLHFVPSVGPEFFGPLQKNI HTFIEQALGVGADSDEPKAWTDLIGAFNKVLND HAIQHIGLSETDRRALDSSWKRLTAGENGVQK AGVNLVLWFFNNIPNMRERFTKFDANQADDAL RADPEFQKQVNVIVGGLKSFLDSVNDPIALQAN MDRVAEAHLSMDPVVGVPYFSALSQNIHRFIEI SLGVTADSDESQAWTDLLAGFTRVVRNRAVLR KVSDSDKSAFVSSWNELIRKAASRRNAGVNLV LWLFNNVPNMRNHFTKFNGNQPDAALRNDQEF LNQVDRIAGGLESLVKNVNNPARFLDALERLSS AHLNMKPSIGLEYFGPLQQNIHTYIESALGVAAG SDEANAWTDVFGAFNEILKYSSVEKIGLS	11	T	-0.31295652	Non-glycosylated
	20	S	-0.33154088	Non-glycosylated
	35	T	-0.3164759	Non-glycosylated
	47	S	-0.39948206	Non-glycosylated
	54	T	-0.13370094	Non-glycosylated
	75	S	-0.39795185	Non-glycosylated
	77	T	-0.2652392	Non-glycosylated
	84	S	-0.30482725	Non-glycosylated
	85	S	-0.32727247	Non-glycosylated
	90	T	-0.11974682	Non-glycosylated
	119	T	-0.34105087	Non-glycosylated
	149	S	-0.6448632	Non-glycosylated
	153	S	-0.36008248	Non-glycosylated
	173	S	-0.15701323	Non-glycosylated
	184	S	-0.31625298	Non-glycosylated
	187	S	-0.34574739	Non-glycosylated
	197	S	-0.33555375	Non-glycosylated
	201	T	-0.1133943	Non-glycosylated
	204	S	-0.47621495	Non-glycosylated
	207	S	-0.36594912	Non-glycosylated
	211	T	-0.077241908	Non-glycosylated
	218	T	-0.13718096	Non-glycosylated
	231	S	-0.27287871	Non-glycosylated
	233	S	-0.52119575	Non-glycosylated
	236	S	-0.50344374	Non-glycosylated
	240	S	-0.0080743	Non-glycosylated
	241	S	-0.33972141	Non-glycosylated
	251	S	-0.51077523	Non-glycosylated
	275	T	-0.16456361	Non-glycosylated
	305	S	-0.58301775	Non-glycosylated
	324	S	-0.31257343	Non-glycosylated
	325	S	-0.33976088	Non-glycosylated
	333	S	-0.43703603	Non-glycosylated
	348	T	-0.26580332	Non-glycosylated
	352	S	-0.48817696	Non-glycosylated
	367	T	**0.056910477**	**Potential Glycosylated**
	380	S	-0.38175839	Non-glycosylated
	381	S	-0.33769978	Non-glycosylated
	388	S	**0.054149732**	**Potential Glycosylated**