Chapter 1

Introduction

1.1 Overview

The parasitic blood fluke *Schistosoma mansoni* has a complex and fascinating life cycle split between snail and human hosts. The parasites dwell in the bloodstream of the mammalian host, associated with particular niches during their early migration, development, and reproduction. Further, the parasites are in constant contact with host tissues at their site of residence, including the endothelial lining of blood vessels and circulating cells of the blood (Bloch, 1980; Crabtree and Wilson, 1986a). Within this environment, individual parasites can survive for more than a decade, demonstrating that they are well adapted to the mammalian host, having evolved strategies to evade host defence mechanisms (Kusel et al., 2007). Moreover, these are successful parasites infecting and reinfecting millions of people globally (Gryseels et al., 2006). Previous work has shown that blood flukes employ multiple strategies to ensure their survival in the bloodstream (reviewed in Cai et al., 2016; Kusel et al., 2007; Wilson, 2012); however, further understanding of these mechanisms is an on-going process. This introductory chapter provides background, and sources of inspiration leading to the work in this thesis. It covers general background of blood flukes, in particular *S. mansoni*, including the disease (schistosomiasis) that it causes and the parasite life cycle. Aspects of the life of the parasites within the bloodstream are discussed, including interaction interfaces, migration paths during their development, acquisition and roles of molecules obtained from host environment, and evasion and modulation of defence mechanisms of their hosts. In addition, the use of genomics and transcriptomics in *S. mansoni* research is discussed in the context of host-parasite interactions. This thesis work aims to add insight into the molecular interactions between parasites and their hosts based on both *in vivo* and *in vitro* approaches using transcriptomes as the main tool of analysis.

1.2 Introduction to schistosomes

The three most common species that infect humans are *S. mansoni*, *Schistosoma japonicum*, and *Schistosoma haematobium*. The parasites belong to the class Trematoda and possess features associated with this group - complex life cycles requiring mollusc and mammalian hosts, flat bodies, and conspicuous suckers. However, they are unique from other members of the group in that members of this genus are dioecious with distinct sexual dimorphism genetically determined by Z and W chromosomes (females are heterogametic with a ZW karyotype), whereas other members of the group are hermaphroditic (Basch, 1991).

The parasites are the causative agent of a Neglected Tropical Disease (NTD) called schistosomiasis. Over 200 million people worldwide are infected with *Schistosoma* species (Gryseels et al., 2006). These are largely largely restricted to Africa, Middle East, South America, and Asia, due to the geographical distribution of the snail intermediate host, whose survival is largely determined by the climate (McCreesh et al., 2015). Recently however, cases of *S. haematobium* infections have been reported in Corsica (France) in individuals who had not been to an endemic area (Berry *et al*., 2014; Boissier *et al*., 2015). Parasitic infections are rarely fatal, instead the parasites produce chronic infections, surviving for a long time in humans and causing long-term morbidity (Gryseels *et al*., 2006).

1.2.1 Schistosome life cycle

The life cycle of *S. mansoni* is split between snail and mammalian host (Figure 1.1). Its natural mammalian hosts are humans and chimps, which is in contrast to *S. japonicum* where bovine and other mammalian hosts are also susceptible. Experimental models of *S. mansoni* are commonly laboratory mice, rats, and hamsters. Each schistosome species is carried by a specific snail species where the parasite asexually reproduces (Gryseels et al., 2006).

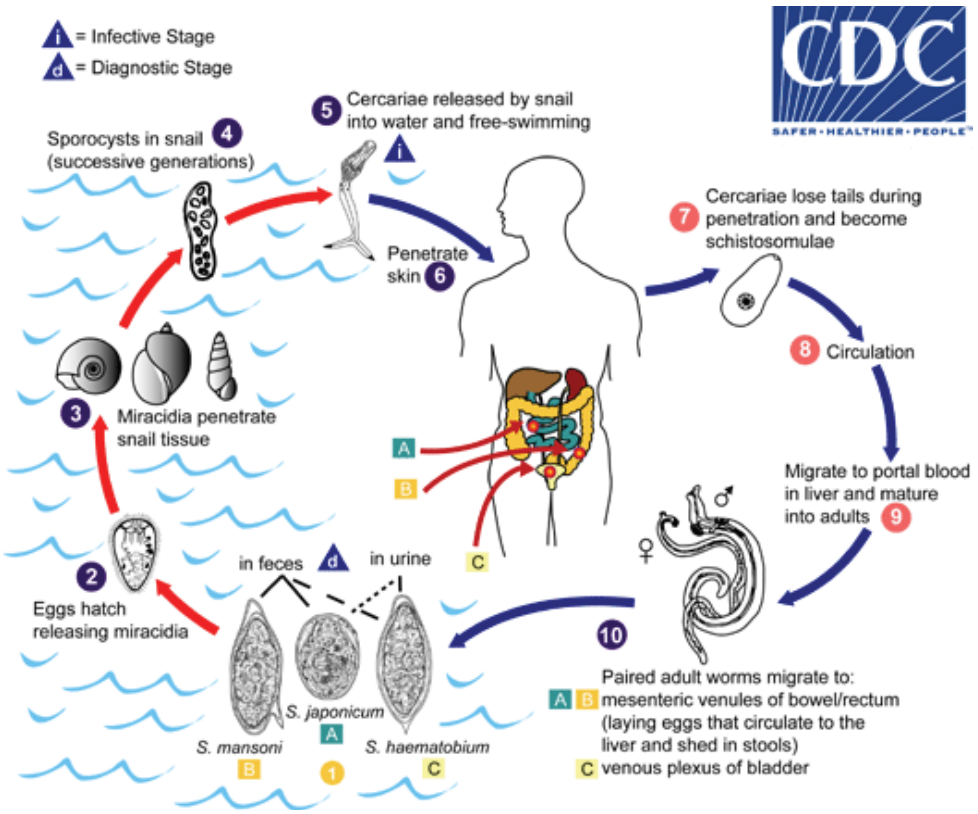

Figure 1.1 Life cycle of* Schistosoma *spp.

Life cycle of three main *Schistosoma* species that infect human. Diagram reproduced from Centers for Disease Control and Prevention (2012).

1.2.1.1 Intramolluscan stages

Snail hosts of *S. mansoni* belong to the group *Biomphalaria*, whereas snail hosts for *S. japonicum* and *S. haematobium* are *Oncomelania*, and *Bulinus*, respectively. This specificity to snail hosts limits the geographical distribution of each parasite species (Colley *et al*., 2014). Once eggs are deposited in water (from, for example, contamination of water with faecal waste from infected individuals), immediately miracidia hatch from eggs. The free-swimming miracidia are phototropic and propel themselves with cilia plates, following chemical cues, towards snails to penetrate (Haas *et al*., 1995). Once in the snail, a miracidium sheds its cilia plate and becomes a mother (primary) sporocyst; this starts to asexually reproduce in the snail. Daughter (secondary) sporocysts later bud off from the mother sporocyst and multiply into further daughter sporocysts. After four weeks, cercariae develop inside daughter sporocysts and leave the snail seeking a mammalian host (Basch, 1991). The shedding of cercariae from snails is stimulated by light and can follow circadian pattern of human behaviour (Lu *et al*., 2009).

1.2.1.2 Intramammalian stages

Upon leaving the snail hosts, cercariae swim upward following light and a thermal gradient, and are affected by chemical cues directing them to suitable mammalian hosts, all relying on cercariae energy reserves (Ghandour and Ibrahim, 1978). Using cues on mammalian skin, such as linoleic acid, cercariae are stimulated to release the content of acetabular glands which is at the head of the cercariae and contain proteases such as cercarial elastase, assisting the cercariae in penetration through the skin of their host (Haeberlein and Haas, 2008; Ingram et al., 2012; McKerrow and Salter, 2002). During penetration, cercariae lose their tails and become schistosomules. Skin excision (Miller and Wilson, 1978), radioisotope tracking (Georgi *et al*., 1982), and histological experiments (Wheater and Wilson, 1979) have shown that the parasites remain in the skin for around 3-4 days before finding and entering a blood vessel. Molecules from the host blood might serve as cues for finding blood vessels. An *in vitro* experiment where cercariae were stimulated to penetrate into agar gel (using linoleic acid) demonstrated that the resulting schistosomules navigated toward human serum, D-glucose, L-arginine, fibronectin, and bradykinin (Grabe and Haas, 2004; Haas *et al*., 2002).

Schistomules enter blood vessels using secretions from head gland (Kusel *et al*., 2007). One of the main components secreted from the head gland is likely to be a protein product of micro-exon gene (MEG)-3 (DeMarco *et al*., 2010). Once in the bloodstream, schistomules are swept by the circulation reaching multiple sites including the heart, the lung, and the liver where they develop into adults (Wilson, 2009). After the parasites develop into adult forms, in *S. mansoni and S. japonicum*, males and females pair and migrate toward mesenteric venules where egg-laying occurs (Wilson, 2009; Wilson *et al*., 2016). In contrast, pairs of adult *S. haematobium* migrate towards venous plexus for egg-laying. The pairing leads to changes in both the male and the female, but most spectacularly, the changes and development of the female reproductive organs and maturity only occur after pairing with a male (LoVerde and Chen, 1991). The size of adult worms varies depending on host type, but generally is around ~10mm in humans (Cheever, 1968) and ~7 mm in mice (Basch, 1981; Clegg, 1965a), with females being slightly (~1mm) longer than males (Basch, 1981; Cheever, 1968; Clegg, 1965). A pair of *S. mansoni* adult worms lays approximately 300 eggs per day and can live for over a decade (Chabasse *et al*., 1985;

Colley *et al*., 2014; LoVerde and Chen, 1991; Pellegrino and Coelho, 1978; Warren *et al*., 1974). Such a long life and continuous massive egg production would require ability to repair tissue damage, to compensate cellular aging, as well as to maintain proliferating germ cells (Collins et al., 2013; Wendt and Collins, 2016). The processes appear to involve a population of proliferative neoblast-like cells which differentiate into various somatic tissues such as tegemental cell body, intestinal lining cells, and muscle layers (Collins *et al*., 2013).

1.2.2 Schistosomiasis

1.2.2.1 Pathology

The infections consist of two phases: acute and chronic schistosomiasis. Acute schistosomiasis is known as Katayama syndrome and occurs before the egg-laying stage. This seems to be more common in individuals outside an endemic area (Colley *et al*., 2014). The symptoms can include fever, muscular pain, fatigue, diarrhoea and abdominal pain (Colley *et al*., 2014; Vale *et al*., 2017). The most frequent pathology of schistosomiasis results from chronic disease and is caused by eggs released from adult worms (Colley *et al*., 2014; Vale *et al*., 2017). For *S. mansoni* and *S. japonicum*, the eggs traverse the blood vessel wall, and enter into the lumen of the intestine to be excreted into the environment with faeces (Gryseels et al., 2006). However, about half of the eggs remain in the bloodstream and are carried with the bloodflow (Loeffler et al., 2002). Paired adult worms of *S. mansoni* and *S. japonicum* reside in the mesenteric veins, close to the intestine, and the bloodflow carries eggs through the portal vein to the liver, where the eggs can become trapped in liver tissue. This leads to liver inflammation, fibrosis and the formation of granulomas around the eggs. Over time, more eggs are deposited in the liver, disrupting blood flow across the liver, as well as normal liver function, eventually leading to other symptoms such as liver and spleen enlargement, or pulmonary hypertension (Colley *et al*., 2014; Vale *et al*., 2017). Adult *S. haematobium* dwell in the venous plexus draining the urogenital organs, and the eggs are normally released in urine. The traversing and lodging of eggs in the urogenital organs can lead to hematouria (bloody urine), cervical schistosomiasis, infertility, an increased risk of HIV transmission. Furthermore, the infections with *S. haematobium* are associated with squamous cell carcinoma of the bladder (Colley *et al*., 2014; Vale *et al*., 2017). Eggs can also lodge in other tissue,

such as in the lung (Cheever, 1968) and brain (Rose *et al*., 2014), disrupting the organ functions.

1.2.2.2 Immunology of schistosomiasis

During the early phase of infection, i.e. before eggs start to be laid, there is a low level inflammatory response with the production of cytokines that are characteristic of type-1 helper T cells (Th-1), such as tumour-necrosis factor alpha (TNF-α), interferon gamma, and interleukin-2 (IL-2) dominating (Hams *et al*., 2013; Pearce and MacDonald, 2002). However, a balanced Th-1/Th-2 response has also been suggested (Colley *et al*., 2014). Once eggs start to be laid, a type-2 T helper cell (Th-2) response dominates. The switch to Th-2, particularly the expression of IL-4, appears to be important for survival of the host by preventing inflammatory damage from eggs trapped in the internal organs (Colley *et al*., 2014; Hams *et al*., 2013; Pearce and MacDonald, 2002). The switch to a Th-2 response is induced by egg secretions and the main component of which is Omega-1 (Everts *et al*., 2009; Schramm *et al*., 2003). Th-2 cells can appear before egg-laying and this is thought to be a preventative mechanism to ensure a Th-2 polarisation at a later stage (de Oliveira Fraga *et al*., 2010a, 2010b). Alternatively, the early Th-2 responses may be beneficial for the parasite development, as a Th-2 cytokine IL-4 is involved in regulation of CD4+ immune cells that promote parasite development (Riner *et al*., 2013).

1.2.2.3 Diagnosis and treatment

The gold standard diagnostic analysis for schistosomiasis is still the identification of eggs in stool (for *S. mansoni* and *S. japonicum*) or urine (for *S. haematobium*) (Colley et al., 2014). Dominant secreted proteoglycan and other antigens from the parasites may also be used as diagnostic markers (Hamilton *et al*., 1998; Nash and Deelder, 1985). The severity of the pathology can be determined by imaging techniques such as ultrasound and CT-scans (Skelly, 2013). Currently, the main line of treatment is the use of a single drug, praziquantel, which is administered as a single dose, or in two doses separated by ~14 days to treat juvenile parasites which were not killed during the first dose (King *et al*., 2011). The drug is relatively inexpensive, with mild side effects, and generally efficacious (Cioli *et al*., 2014; Vale *et al*., 2017). However, cases of potential resistance or reduced responsiveness to drug treatment have also been reported (Crellen *et al*., 2016). Furthermore, the host immune system is slow to

develop resistance to reinfection which is common in endemic areas (Colley *et al*., 2014). Therefore, research is desperately needed to discover novel targets for drugs and vaccines.

1.2.2.4 Control and prevention

Current attempts to control schistosomiasis include mass drug administration programs (Secor, 2015). Behavioural changes, education, sanitation, infrastructure development, and control of the intermediate host are also important because the infection is caused by contact with contaminated water (Secor, 2014, 2015). Vaccine development is an on-going and challenging effort (Tebeje *et al*., 2016). The most successful 'vaccine' so far is composed of irradiated cercariae which leads to protection rates of over 80% in primate hosts (Bickle, 2009). However, such a method is not scalable because a large number of parasites and hosts are required to maintain the parasite production. Furthermore, introducing live cercariae to humans may not be socially acceptable. Proteins present on parasite tegument have been tested as potential vaccine targets. An example of these is tetraspanin-2 (TSP-2), which has entered phase I clinical trial (Cai *et al*., 2016; Tebeje *et al*., 2016).

1.3 *S. mansoni* life in bloodstream

This thesis concerns blood-dwelling intramammalian stages of *S. mansoni*, and particularly the interactions between the parasite and its host. In this section, the biology of *S. mansoni* intramammalian developmental stages is discussed in detail, aiming to describe key aspects of host-parasite interactions, including multiple mechanisms evolved by the parasite to interact (or interfere) with the host environment in order to thrive in the bloodstream.

1.3.1 Host-parasite interactions: the interfaces

While living in the bloodstream, the parasite interacts with the host environment to obtain nutrients, evades the host immune responses, migrates to seek a mate and find an egg-laying site. As *S. mansoni* is coated with a tegument and feeds on blood, the tegumental surface and lining of the gut form the interface with which the parasite interacts with its environment (Skelly and Wilson, 2006; Skelly *et al*., 2014). Further, excretory/secretory products (ES) are released by all developmental stages of the parasite and are relevant in parasite interactions with the host as well as with other

parasite (Cao *et al*., 2016; El Ridi and Tallima, 2009; Nowacki *et al*., 2015; Sotillo *et al*., 2016; Zhu *et al*., 2016).

1.3.1.1 Tegument

Early works focusing on the *S. mansoni* tegument (Hockley and Mclaren, 1973) described how the cercaria coating is replaced by an intramammalian tegument within 3 hours of skin penetration. A cercaria is covered with a thick glycocalyx layer that protects the parasite from osmotic pressure of fresh water (reviewed in Cai *et al*., 2016). Upon penetration, the cercaria loses its glycocalyx and the resulting schistosomule becomes covered with a double bilayer membrane, or with multiple membrane layers, with glycocalyx remains in some regions (Hockley and Mclaren, 1973). Proteins, carbohydrate, and glycoproteins, and host biomolecules, are found in the new tegument of the parasite, and are thought to be important for early immune evasion (reviewed in Kusel *et al*., 2007; Skelly and Wilson, 2006). The tegument was first known as “heptalaminate” for its appearance as seven layers on the electron microscopy (Hockley and Mclaren, 1973). Later, it was reported to be tightly opposed double bilayers lying over a syncitial cytoplasm (Mclaren and Hockley, 1977). There are cytoskeletal structures underneath the inner bilayer, and underneath this is a muscle layer (Skelly and Wilson, 2006). Cell bodies are embedded beneath the muscle tissue layer and connected to the syncitial cytoplasm by passing multilaminate vesicle into the tegumental layer and releasing their component to become part of the outer membrane, which is known as the membranocalyx (Skelly and Shoemaker, 2001; Wilson and Barnes, 1977) (Figure 1.2).Through this process, the tegument is constantly shed (tegument turnover) and the rate of turnover can be affected by binding of host molecules (reviewed in Van Hellemond *et al*., 2006). The schistosome tegument consists of pits, formed as invaginations of inner and outer membrane layers (Hockley and Mclaren, 1973). The parasite body is coated with spines and sensory papillae that contain nerve ending for sensing the external environment (Gustafsson, 1987). The pattern of coverage changes during development; for example, the mid-body spine and sensory papillae disappear in the lung stage (4-7 day post-infection) and appear again at day 10 and throughout adult stages (Crabtree and Wilson, 1980).

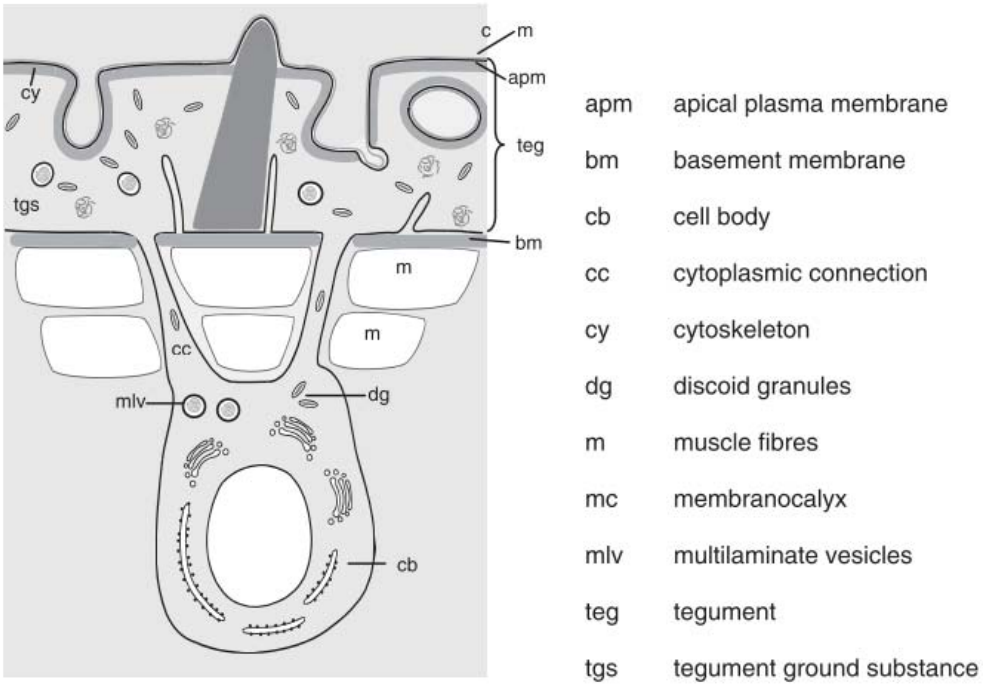

Figure 1.2 The schistosome tegument

Schematic representation of schistosome tegument. Diagram reproduced from Skelly and Wilson (2006).

The tegument was originally thought to be an inert layer of membranocalyx offering protection from host antibody binding and hence antigen-mediated parasite killing, but later studies showed that there are proteins in the inner and outer layers, and in the space between them (Kusel *et al*., 2007; Wilson, 2012). With advances in proteomics, multiple tegument proteins have been identified using various methods (e.g. enzymatic shaving, biotin labelling, freeze-fracture, detergent) with some providing information on protein localisation on the tegument double bilayers and their abundance (Braschi and Wilson, 2006; Braschi *et al*., 2006a; Castro-Borges *et al*., 2011a, 2011b; Sotillo *et al*., 2015). These proteins include enzymes, transporters of glucose and amino acids, aquaporins, receptors, structural proteins, proteins of host origin, and proteins of unknown function (Braschi and Wilson, 2006; Braschi *et al*., 2006a; Castro-Borges *et al*., 2011a, 2011b; Sotillo *et al*., 2015). The tegument also consists of carbohydrates and lipids, particularly sphingomyelin and cholesterol (Kusel *et al*., 2007). Detailed information on the tegumental structure and components is covered by Skelly and Wilson (2006). Selected tegumental proteins with relevance to host-parasite interactions are explored in later sections.

1.3.1.2 Lining of the oesophagus and gut

The gut of the parasite is a blind-ended elongated tube lined with a layer of epithelium called gastrodermis (Basch, 1991). Surrounding the gut is a smooth muscle layer for peristalsis to move substances along the lumen. The gut lining is similar to the tegumental lining in that it is a syncitium layer of membrane. However, the gut lining has nuclei and "biosynthetic machinery" in the syncitial cytoplasm; whereas, the tegumental lining is linked to the cell body by cytoplasmic connections (reviewed in Skelly *et al*., 2014). Studies of the gut physiology, particularly the gastrodermis, have been challenging because of the inaccessibility of the organ (Gobert et al., 2009a). However, recent advances in laser microdissection have allowed the removal of the gastrodermis of *S. mansoni* and *S. japonicum* and led to finding of peptidase transcripts including transcripts encoding cathepsins, as well as transcripts encoding proteins related to lipid uptake, and antioxidant enzymes (Gobert *et al*., 2009a; Nawaratna *et al*., 2011). In addition to the gastrodermis, digestive enzymes are also secreted from a posterior oesophageal gland (Nawaratna et al., 2014) and the lysis of host erythrocytes is seen in the oesophagus (Li *et al*., 2013). Leukocytes are also retained in the posterior oesophagus possibly to prevent its oxidative damage to the gut (Li *et al*., 2013).

1.3.1.3 Excretory/secretory products

ES of parasites include substances from multiple sources. In *Schistosoma* species, ES could be a mixture of substrates secreted from oesophageal glands, gut regurgitation (or "vomitus"), tegumental secretion, or shed tegument. As schistosomes feed on blood, haem molecules are released from red blood cells. Free haems are converted to hemozoin and regurgitated into host blood as part of the vomitus. Vomitus from the parasite gut is a mixture of digestive waste and other proteins such as stress and tegumental proteins (Hall *et al*., 2011). The parasites may not be able to selectively retain the stress and tegumental proteins while regurgitating digestive waste, or their secretion maybe functional. In addition, *S. mansoni* ES contain exosome vesicles which carry miRNA and multiple proteins (Nowacki *et al*., 2015; Sotillo *et al*., 2016). Exosomes secreted from parasitic nematodes can modulate host immune responses (Buck *et al*., 2014), suggesting that exosomes secreted from *S. mansoni* may also have roles in host-parasite interactions (Nowacki *et al*., 2015; Sotillo *et al*., 2016).

1.3.2 Motility, behaviour, and homeostasis: the nervous system

The nervous system can be involved in host-parasite interactions, parasite-parasite interactions, as well as internal signalling within the parasite (Collins et al., 2010; Ribeiro and Patocka, 2013). Given the lack of an endocrine system in schistosomes, the nervous system and neuronal signalling are important for controlling motility, which is crucial for intramammalian migration, as well as other biological processes, such as sensing environment, behaviour, feeding, digestion, and excretion of waste (Ribeiro and Patocka, 2013). Moreover, sensing stimuli from the external environment could be relevant to activation of responses and guiding the behaviour of the parasite in processes such as lung, liver, and mesenteric migration, and mating between males and females (Kusel *et al*., 2007).

The layout of the central nervous system in *S. mansoni* is similar to that of other flatworms. Paired cephalic ganglia connect the nerve cord that runs through the body longitudinally, with transverse connections ("transverse commissures") along the length forming the appearance of a "ladder" (Collins *et al*., 2011). Neuronal signalling involves neurotransmitters, transporters, and GPCR signalling (reviewed in Ribeiro and Patocka, 2013). Neurotransmitters used by *S. mansoni* are of various groups - acetylcholine, glutamate, biogenic amine (e.g. serotonin (5-HT), histamine, dopamine, and noradrenaline), and neuropeptides (reviewed in Ribeiro and Geary, 2010). The involvement of neurotransmitters and neuronal signalling in the motility of *S. mansoni* has been shown by RNAi of serotonin receptors affecting motility in 8-day schistosomules and adults (Patocka *et al*., 2014), and RNAi of acetylcholine GPCR leading to reduced motility in schistosomules (MacDonald *et al*., 2015). In addition, irradiated *S. mansoni* cannot migrate through the lung capillary network and a gene expression study shows that down-regulation of genes related to neuromuscular signalling may be involved (Dillon *et al*., 2008). Neuropeptides, also known as peptide hormones, are involved in developmental control and tissue differentiation in free-living flatworms such as planaria (Collins *et al*., 2010). *S. mansoni* may similarly rely on neuropeptide signalling (Collins *et al*., 2010). Attraction between male and female *S. mansoni* is also likelay to involve sensory organs that connect to the nervous systems to alter behaviour and motility (reviewed in Kusel *et al*., 2007). Neuronal signalling is therefore likely to be a major component in many aspects of parasite biology.

1.3.3 Migration route and development

Upon entering the bloodstream, schistosomules are carried with blood circulation to multiple tissues from lung, heart, systemic organs, to liver, and migrate against the blood flow to the mesenteric venules for egg-laying (reviewed in Wilson, 2009). The encountering with various host tissues may act as signal for metabolic changes and development in the parasite (Kusel *et al*., 2007). Studies aiming to identify the migration route of the parasite have incoporated a range of techniques such as organ mincing to recover parasites from tissues, histology, and isotope tracking (e.g. Georgi *et al*., 1982; Miller and Wilson, 1978, 1980; Wheater and Wilson, 1979). Isotope tracking provides improved accuracy because it does not rely on parasites migrating out of tissues, or on investigators detecting parasites among a background of host tissues (Wilson, 2009). The technique involves incubating snail hosts with [^{75}Se]-methionine (a gamma-emitting isotope) to label cercariae and trace the parasites in pressed organs (Georgi *et al*., 1982). The path and timing of the migration has then been extensively described. However, only circumstantial evidence exists for the mechanisms that lead to the observed patterns of migration (Wilson, 2009).

1.3.3.1 Lung migration

Once schistosomules enter capillaries, it is thought that the parasites are carried by the blood circulation to enter the heart (right atrium) and then enter the lung capillary bed via the pulmonary arteries. Schistosomules can be found in the lung from day 4 and the number of parasites detected in the lung increases over time until it peaks around day 6 post-infection for *S. mansoni* (Clegg, 1965a; Georgi et al., 1986). During the lung migration, the schistosomules elongate to fit within narrow lung capillaries and are in very close contact with the lung capillary wall, prolonging the migration (Crabtree and Wilson, 1986a). The body length increases by 2-3 fold, relative to the skin stage (Clegg, 1965a; Wilson et al., 1978), but no cell division has been reported during this stage (Clegg, 1965a; Lawson and Wilson, 1980). The signals that trigger the morphological changes of schistosomule from the skin stage to the elongated lung stage are unclear, but it has been postulated that host proteins might be involved in this process (reviewed in Kusel *et al*., 2007). Alternatively, physical space in the lung and/or changes in the oxygen pressure might also have an effect.

In addition to elongation of the body, the parasites might also actively dilate blood vessels to allow the migration through the lung capillaries (Carvalho *et al*., 1998), and a use of lubricant has been proposed (Crabtree and Wilson, 1986a). Furthermore, the tegument in the lung stage lacks midbody spines, and the absence could provide room for contraction of the body helping with migration (Crabtree and Wilson, 1980). The spines are however present around the anterior and posterior ends of the parasites, and can cause damage to lung endothelial cell membranes (Crabtree and Wilson, 1986a) that may result in inflammation at a later stage (Crabtree and Wilson, 1986b). In contrast to the notion of prolonged lung migration, *in vivo* microscopy by Bloch (1980), observed schistosomule migration in anesthetised and surgically opened mice, and reported objects of schistosomule size passing through lung capillary without being detained. However, as the author commented, parasites in deeper layers of lung capillary may still have been detained and missed by this technique (Bloch, 1980).

1.3.3.2 Lung as a parasite attrition site

The migration in the lung capillaries is a challenge for the schistosomules (reviewed in Wilson *et al*., 2016). Radioisotope tracking showed that over 85% of the parasites can be counted in the lung, and only half of the parasites that reach the lung can be counted in the liver (Georgi et al., 1986). The number of parasites reaching the liver was similar to the number of adult parasites recovered by perfusion, suggesting that the attrition happened during or after the lung migration (Georgi *et al*., 1982). More evidence for parasite attrition in the lung came from histological examinations, reporting schistosomules accumulating in alveoli over time and none returning to blood vessels (Crabtree and Wilson, 1986a). The observation for infection in mice immunised with irradiated cercariae was similar, but with more host immune cells reaching the lung (Crabtree and Wilson, 1986b; Dean and Mangold, 1992). Similarly, in schistosome infections of rats, macrophages could be seen surrounding schistosomules in the lung vasculature in both primary and secondary infection, with fewer cases in primary infection (Bentley *et al*., 1981). Large inflammatory foci with macrophages and granulocytes were also found but this was not associated with the presence of schistosomules (Bentley *et al*., 1981). It is therefore clear that parasites are eliminated in the alveoli although the cause of death is not clear. Despite the inflammation around the lung tissue or around the parasites, most parasites in the alveoli were intact or appeared to die from autophagy (Crabtree and Wilson, 1986b;

Mastin *et al*., 1985). The accumulation of immune cells, instead of killing the parasites, is thought to either disrupt blood vessels, inducing them to become leaky and enabling the parasites to migrate into alveoli, or act as plugs that block migration (Dean and Mangold, 1992; Wilson, 2009). The departure of the parasites from the bloodstream may lead to eventual parasite death in alveoli. However, it is suggested that the parasites coud be passed up the trachea and swallowed into the host gut where they were digested or passed out to the environment alive (Dean and Mangold, 1992).

It is intriguing that, although the lung schistosomules were surrounded by immune cells and inflammation in damaged lung tissues, the parasites seem to be resistant to the immune-mediate killing (Bentley *et al*., 1981; Crabtree and Wilson, 1986b; Mastin *et al*., 1985). This is consistent with observations that lung schistosomules are resistant to cytotoxic killing (e.g. Clegg and Smither, 1971; McLaren and Terry, 1982). Furthermore, accumulation of inflammatory immune cells was observed after the peak time of schistosomules migration through the lung, and the inflammatory foci were not always associated with the parasites. Therefore, the lung inflammatory responses could be towards damaged lung tissues instead of towards the parasites (Burke *et al*., 2011; Crabtree and Wilson, 1986b; Mastin *et al*., 1985). The mechanisms that protect lung schistosomules from inflammatory damage is unclear, although acquisition of host antigens for immunological camouflage may be involved (Clegg and Smither, 1971; McLaren and Terry, 1982). Furthermore, gene expression profiling using microarrays of *in vitro*-cultivated and *in vivo* lung schistosomules showed that genes responding to stress (hsp70) and other immunomodulation-related genes were upregulated in the *in vivo* lung schistosomules, suggesting that the host environment may also have a role in activation of immune evasion strategies in the parasite (Chai *et al*., 2006).

1.3.3.3 From the lung to the liver

From the lung, parasites are carried with systemic circulation and remain within blood vessels (Bloch, 1980; Wheater and Wilson, 1979) until they arrive at the liver (Bloch, 1980; Wilson, 2009). Schistosomules are found in liver from day 8 (Clegg, 1965a) and all schistosomules reach the hepatic portal system by day 21 (Georgi *et al*., 1986; Wilson *et al*., 1986). Appearance of schistosomules in other organs during this time also supports the hypothesis that migration between lung and liver is not an active,

direct passage (Wheater and Wilson, 1979; Wilson, 2009). A histological study of infected mice confirms systemic circulation of parasites, as schistosomules were found in the pulmonary veins, cerebral blood vessels, and in myocardium (Wheater and Wilson, 1979). Parasites are distributed to systemic organs according to the proportion of cardiac output that supplies the organs (Wilson *et al*., 1986). Sinusoidal networks may act as filters once the parasites grow large enough to be trapped within the portal system (reviewed in Wilson, 2009).

1.3.3.4 Liver schistosomules

Liver schistosomules are in 'interlobular portal venules' which is part of the intrahepatic radicle of the portal vein and are not found in the sinusoid. Furthermore, they are found to reside in blood vessel just large enough for their diameter (Bentley *et al*., 1981; Bloch, 1980). By residing in blood vessels, the parasites appear to reduce blood flow reaching the sinusoid adjacent to their portal triad, and hepatocytes become vacuolate, and develop midzonal necrosis (Bloch, 1980). Microscopic hemorrhage, damage, and fibrosis of liver are also observed before the egg laying stage (Bloch, 1980). This shows that the schistosomules also cause damage to the host liver, in addition to the damage of lung endothelial cells during the lung migration. Similarly to the lung stage, in schistosome infections of rats, eosinophils, mast cells, and mononuclear cells are seen infiltrating hepatic tissue surrounding the portal venule where schistosomules reside, but these cells were not observed bound on to schistosomules (Bentley *et al*., 1981).

Once in the liver, elongated lung schistosomules contract again into the size of skin schistosomules (Wilson, 2009). Although arrival in the liver may be a passive process, the liver environment could provide the parasites with signals to grow (reviewed in Kusel *et al*., 2007). Increased cell division was observed when schistosomules from 20 days post-infection were cultured with portal blood from humans or susceptible rodent hosts (Draz et al., 2008; Shaker et al., 1998). The portal-blood-derived molecules, have not been identified but are less than 50 kDa (Shaker *et al*., 1998, 2011). Mitosis and developmental changes start with the schistosomule body and gut beginning to extend (Clegg, 1965). First the gut appear as two fork ends, and by day 15 the posterior ends of the two forks join (Clegg, 1965a). Development is asynchronous both *in vivo* and *in vitro* (Basch, 1981; Clegg, 1965a). After 21 days

post-infection, reproductive organs start to form in both males and females. Mating can be observed from day 28 (Clegg, 1965a). Egg shell protein synthesis starts at day 30, vitelline tissues then develop in females, and oviposition is observed at day 35 (Clegg, 1965a).

1.3.3.5 Migration paths for egg laying

S. mansoni and *S. japonicum* dwell in mesenteric veins as a site for egg laying. In contrast, *S. haematobium* prefers the venus plexus near the bladder (Gryseels et al., 2006). This distinct migration path specific to different species suggests that some specific interactions might be happening. Although the chemical cues of the site determination is unknown, it is generally thought that male worms determine the site of migration for egg laying (Gryseels *et al*., 2006; Huyse *et al*., 2009; Webster *et al*., 2013) and that pairing occurs in portal veins before the migration towards mesenteric vein (Standen, 1953). However, it is worth noting that females in single-sex infections can also be found in mesenteric veins (Zanotti *et al*., 1982) and that unpaired females can be found in the mesenteric circulation (Bloch, 1980). Furthermore, the number of female worms found in the mesenteric veins increases over time (Zanotti *et al*., 1982) suggesting that the parasites migrate to the site after they mature. Transcriptomes of unpaired females are more similar to those of males than to those of mature females (Lu *et al*., 2016). This similarity in transcriptomes may also lead to similar behaviours of unpaired females migrating as males.

1.3.3.6 Imaging technology

Studies of *S. mansoni* migration pattern were advanced by the use of radioisotope tracking (Wilson, 2009). To study mechanisms that determine the migration site, similar experiments would need to be repeated with modified parasites (such as parasites with a candidate gene knocked down using RNAi). However, [^{75}-Se]-methionine - a key substrate that was used to establish the current understanding on the migration - was withdrawn “around 1990” (Wilson, 2009). Therefore, a new imaging technique will be required to study effects of genes and signaling pathway on the migration inside the mammalian host. Detection of fluorescent trace emitted from parasites was able to show location of foci of adult parasites *in vivo* (reviewed in Skelly, 2013). This technique works by injecting fluorochrome ProSense 680 into infected mice. The fluorochrome is activated by cathepsin in the parasites, releasing

fluorescent signals which can be detected and imaged using Fluorescence Molecular Tomography (*in vivo* imaging). In addition, radioactive tagged glucose allows *in vivo* visualisation of adult stages (Salem *et al*., 2010). However, these techniques have not been demonstrated for early stage schistosomules.

1.3.4 Metabolic requirements and acquisition

The bloodstream environment is usually described as hostile for blood-dwelling parasites (e.g. Berriman et al., 2009; Cook et al., 2004; Wendt and Collins, 2016). However, schistosomes have evolved mechanisms to not only avoid host defence mechanisms but also to take advantage of bloodstream components as essential input for their development and reproduction (Kusel et al., 2007). *S. mansoni* requires multiple substrates from the host including glucose, amino acids, fatty acids, sterol, nucleotides, vitamins, iron, and other ions (Skelly et al., 2014). Some of these are acquired through tegument transporters or receptors, while some are obtained from blood feeding and likely to be absorbed through the gut surface (Kusel *et al*., 2007; Skelly *et al*., 2014).

1.3.4.1 Sugar and other substances absorbed through tegument

Schistosomes consume a large amount of glucose, approximately two fifth of their dry weight per hour in adults (Clegg, 1965a). *S. mansoni* adults mainly rely on anaerobic respiration to obtain energy from glucose (Da'dara *et al*., 2012; Tielens and van den Bergh, 1987). However, aerobic respiration through Krebs cycle can occur in the presence of oxygen (van Oordt *et al*., 1985). Ensuring constant supply of glucose, *S. mansoni* adults store glycogen in their body, and the degradation can be readily triggered by limited external glucose (Tielens and van den Bergh, 1987). It has been suggested that glucose requirement may explain the wandering behaviour of adult pairs in the mesenteric vein (Pellegrino and Coelho, 1978); parasites wander to new areas of glucose supply after their bodies have inhibited the blood flow and limited the glucose availability in their immediate surrounding (Tielens and van den Bergh, 1987).

S. mansoni obtains glucose (and other sugars) through transporters in their tegument (Skelly et al., 2014). Two of the sugar transporters in *S. mansoni* have been tested by RNAi and shown to transport glucose as well as other hexoses (Skelly *et al*., 1994).

Their silencing by RNAi led to a deleterious effect on the parasites especially when the parasites were subjected to *in vivo* environment or when they were maintained *in vitro* in a low glucose medium (Krautz-peterson *et al*., 2010). One of the transporters, SGTP4, is expressed on the outer layer of tegument, while the other one, SGTP1, is expressed in the inner layer, as well as in muscle (Jiang *et al*., 1996; Zhong *et al*., 1995). Such localisation would allow the sequential uptake of glucose from the bloodstream to pass through the outer layer of the tegument, into the interlayer space, and through the inner tegument layer into the parasite body (Da'dara et al., 2012; Skelly et al., 2014). The sugar transporters are expressed early after the skin penetration before the parasites reach the liver and start developing functional gut and oral sucker, suggesting the requirement of glucose even before the peak of development in the liver (Skelly and Shoemaker, 1996). Intriguingly, the *S. mansoni* genome also encodes two other sugar transporters, SGTP2 and SGTP3 (Berriman et al., 2009). However, biophysical modelling predictions suggest that the products encoded by these genes are not functional glucose transporters (Cabezas-Cruz *et al*., 2015), and previous characterisation has also confirmed the absence of glucose transport function in SGTP2 (Skelly *et al*., 1994).

Other substances acquired through the tegument include purine nucleotides and ions such as calcium, sodium, phosphorus, and copper (Skelly et al., 2014). Transporters of these ions and their associated ATPase have not been characterised and functionally tested but they have been described in tegumental proteomics (reviewed in Da'dara *et al*., 2012; Skelly and Wilson, 2006; Skelly *et al*., 2014). *S. mansoni* lacks pathways for *de novo* synthesis of purine nucleotides and needs to obtain purines from the host (Dovey *et al*., 1984). The process of acquisition may involve hydrolysis of phospho-nucleotide and diffusion through the tegumental surface (Levy and Read, 1975). It has subsequently been demonstrated that alkaline phosphatase and ATP diphosphohydrolase on the tegument can cleave nucleotide phosphate (e.g. ATP and ADP) into de-phosphorylated form (e.g. AMP), which can be transported across the membrane (Da'dara *et al*., 2014).

1.3.4.2 Amino acid - through tegument and gut

During the development as well as maintenance of egg-laying and survival in adults, *S. mansoni* feeds on host blood and obtains essential nutrients and iron from ingested

erythrocytes (reviewed in Kusel *et al*., 2007; Skelly *et al*., 2014). Haemoglobin is a major source of protein and iron. In addition, plasma proteins, especially albumin, are also a source of amino acids (Delcroix *et al*., 2006; Hall *et al*., 2011). Proteomic studies of worm vomitus (Hall *et al*., 2011) and gene expression profiling of microdissected gastrodermis (Gobert *et al*., 2009a; Nawaratna *et al*., 2011) have revealed that schistosomes have an array of proteases in their guts to digest host erythrocytes and obtain nutrients (Gobert et al., 2009a; Hall et al., 2011; Nawaratna et al., 2011). The proteases involved in digestion of blood work as a cascade consisting of cathepsins (D, B1, L1, and C) and metallo-aminopeptidase (Delcroix *et al*., 2006). Suppression of these cathepsins (e.g. D and B1) affect parasite growth and digestion (Skelly *et al*., 2014). The expression of genes encoding proteins used for blood feeding – such as cathepsins and iron transport proteins – increases *in vitro* when schistosomules are fed erythrocytes, suggesting that these processes are stimulated by blood feeding (Gobert *et al*., 2010).

At least five amino acid transport systems have been suggested for *S. mansoni* adults (Asch and Read, 1975). So far, one system of amino acid transporter has been identified and it has been located on the tegument surface (Braschi *et al*., 2006b; Skelly *et al*., 1999) and in the gut lining (Skelly *et al*., 2014). The transporter is known as SPRM1 (schistosome permease 1) and it transports a range of amino acids (phenylalanine, arginine, lysine, alanine, glutamine, histidine, tryptophan, and leucine) (Krautz-Peterson *et al*., 2007). Other amino acid transporter systems have not yet been identified.

1.3.4.3 Iron - through gut and tegument

Blood feeding not only provides a source of amino acids for *S. mansoni*, but also a source of iron which the parasite requires for development and reproduction (Clemens and Basch, 1989; Glanfield *et al*., 2007; Jones *et al*., 2007). In addition, the competition between the host and the pathogen to sequester iron is one of the determinants of infection success (reviewed in Glanfield *et al*., 2007). In the liver, mammalian hepatocytes retain iron (by expression of hemopexin and hepcidin) competing with invading bacteria (reviewed in Zhou *et al*., 2016). Given that *S. mansoni* development is associated with liver and iron is required for parasite growth,

the parasites must have an efficient method for obtaining iron. However, the processes of iron uptake is not well defined.

It has been suggested that iron is taken up from blood feeding given that there isexcess of iron in the haem which results from digested haemoglobin (Glanfield et al., 2007). However, obtaining iron from haem requires haem oxygenase which has not been identified in *S. mansoni* and not found in the genome (reviewed in Glanfield *et al*., 2007; Skelly *et al*., 2014). In additon, growth of early schistosomules is stimulated by presence of iron, suggesting that iron uptake happens before blood feeding starts (Clemens and Basch, 1989). This early sequestration of iron may also help the parasite evade the host immune responses by competing for iron with immune cells. For this purpose, the sequestration should take place as soon as the parasite enter the bloodstream. The system for uptake of iron through the tegument has been suggested to use a divalent metal transporter (DMT) (Clemens and Basch, 1989; Smyth *et al*., 2006). Such a transporter has been identified on the tegumental surface of *S. mansoni* and displays the ability to transport Fe^{2+} (Smyth *et al*., 2006). In host blood, ferric ions (Fe^{3+}) are bound to transferrin. The Fe^{3+} would be reduced to Fe^{2+} which could be transported through DMT and stored in parasite's ferritin (reviewed in Glanfield *et al*., 2007). Ferritin stores iron in a non-toxic form, which otherwise could lead to oxidative damage of surrounding tissues (Munro, 1990). Two types of ferritin are described in *S. mansoni*: ferritin-1 is expressed in reproductive organs, and ferritin-2 is expressed in somatic cells (Schüssler *et al*., 1996). Interestingly, ferritin genes in "higher animals" possess upstream sequences in their mRNA and translation into ferritin protein is regulated by the level of iron in the environment (Munro, 1990; Schüssler et al., 1996). In contrast, *S. mansoni* ferritins do not have such upstream sequences and it appears that *S. mansoni* ferritin levels are regulated using different, yet to be identified, mechanisms (Schüssler *et al*., 1996). Using a mechanism different from that used by the host may be beneficial in competition for iron.

1.3.4.4 Lipid through gut

S. mansoni is coated with a double lipid bilayer consisting of cholesterol as one of the major components (Skelly and Wilson, 2006). However, the parasite cannot produce fatty acid or sterol *de novo*, but can use complex lipid if provided with fatty acid

(Meyer et al., 1970). Therefore, the starting molecules need to be obtained from their host (Meyer *et al*., 1970). So far there is no evidence of lipid uptake through the tegument. Low density lipoprotein (LDL) has been shown to bind to the tegument but the uptake of LDL through tegument has not been reported and a receptor for LDL has not been found (reviewed in Skelly *et al*., 2014). It is thought that lipid uptake happens in the gut using Niemann Pick type C2 protein (NPC2) and saposin (reviewed in Skelly *et al*., 2014). In eukaryotic cells, NPC2 is involved in traffic of cholesterol, sterol, and glycolipids and a homologue is reported in the *S. mansoni* gut vomitus (Hall *et al*., 2011). In addition, saposin that may work as a lipid-binding and interacting protein is found in the gut vomitus and is expressed in the gastrodermis (Don *et al*., 2008; Hall *et al*., 2011; Nawaratna *et al*., 2011). The role of saposins in *S. mansoni* has not been determined, but assuming similarity with other systems, they may be involved in lipid binding and uptake in the gut (reviewed in Skelly *et al*., 2014).

1.3.4.5 Hormone receptors and host molecules stimulating parasite development

In addition to nutrients, essential ions, and nucleotide that the parasite acquires from the host, other unknown host factors may display essential roles for the development and reproduction of schistosomes. As mentioned previously, a protein from portal blood can stimulate growth of schistosomules (Shaker *et al*., 1998, 2011). In addition, *S. mansoni* develops more slowly *in vitro* than *in vivo* and appears to be 20–40% smaller (Basch, 1981; Clegg, 1965a). Despite some *in vitro* culture conditions supporting the adult parasites, eggs laid by these *in vitro*-cultured worms are not fertile (Basch and Humbert, 1981). The presence of host molecules stimulates downstream effects that can lead to increase nutrient uptake, or trigger developmental progress (Hernandez et al., 2004; Saule et al., 2002, 2005).

The *S. mansoni* genome encodes genes that are homologous to receptors of host hormones such as host insulin receptor (SmIR1 and SmIR2) (Verjovski-Almeida *et al*., 2003) and transforming growth factor-beta (TGF-β) receptor (Beall and Pearce, 2001). SmIR1 and SmIR2 bind to insulin (Khayath *et al*., 2007) and affect regulation of glucose uptake in the parasite (Ahier *et al*., 2008). Extra supplies of insulin in infected mice during liver stage development (day 14 - day 21 post-infection) results in increased worm burden and size (Saule *et al*., 2005). Knocking down similar

receptors in *S. japonicum* resulted in a negative impact on growth and reproduction of adult worms (You *et al*., 2015), together suggesting that the hormone may help with both growth and survival of the parasite. For TGF-β, SmSK1 was identified as a receptor which activates SmSmad2 (Beall and Pearce, 2001). This in turn leads to gene expression of the gynaecophoral canal protein (GCP) (Osman *et al*., 2006).

In addition to host hormones and growth factors, *S. mansoni* has integrated in to the mammalian host environment to such an extent that components of the host immune system have become essential for development (Davies *et al*., 1980). In immunosuppressed mice, male and female development of *S. mansoni* was delayed (Harrison and Doenhoff, 1983). Later, it was shown that, CD4+ lymphocytes, IL-7, and IL-2 are required for parasite development in the portal system and egg production (Blank *et al*., 2006; Davies *et al*., 2001; Hernandez *et al*., 2004). Such effects may also intertwine with the positive effect of IL-7 supplement, as well as thyroxine (T4), on the development of schistosomes (Saule *et al*., 2002). More recently, it has been shown that the effect of CD4+ cells involves stimulation from other innate immune cells such as macrophages and monocytes (Lamb *et al*., 2010). The effect on parasite development was shown to, at least in part, work through regulation of such immune cells by IL-4 (a Th-2 cytokine) which might explain appearance of Th-2 cytokine profiles before egg-laying (Riner *et al*., 2013).

Together, the roles of host molecules demonstrate intricate connections between the parasite and host environment, including the regulation of the host immune system for their development. Further it suggest how the parasites might benefit from the infiltration of some immune cells in the liver tissue outside the blood vessel where the worm resides (as described by Bentley *et al*., 1981), as well as a role of liver residential macrophages (Kupffer cells) in development of the parasites.

1.3.5 Immune evasion

Adult pairs of *S. mansoni* may live in the mammalian host over years with no sign of immune-mediated killing of the worms (Keating *et al*., 2006; Kusel *et al*., 2007; Pearce and MacDonald, 2002). Therefore, some mechanisms must have evolved to allow the evasion of the host immune response. During the migration, immune cells are in close proximity to the parasite but do not appear to induce any damage or

killing (Bentley *et al*., 1981; Crabtree and Wilson, 1986a). Early stages of *S. mansoni* are susceptible for immune killing by the complement cascade (Clegg and Smithers, 1972; Marikovsky et al., 1990). In particular, it has been shown that cercariae were killed by complement *in vitro* and the glycocalyx coating of the cercariae contributes to this killing (Marikovsky et al., 1990). Soon after the parasite penetrates the skin and lose their coating, schistosomules become resistant to complement killing (Clegg and Smithers, 1972; Kusel *et al*., 2007; Marikovsky *et al*., 1990; Wilson, 2012).

1.3.5.1 Camouflage

One of the very first proposed mechanisms of immune evasion involved the role of the tegument (Clegg and Smither, 1971). Host proteins are found on the parasite tegumental surface, leading to a proposed mechanism of immune evasion whereby the parasites camouflage underneath the host antigens (Clegg and Smither, 1971). This hypothesis suggested that *S. mansoni* is able to absorb host blood group antigens among other mammalian antigens on to their surface (Clegg and Smither, 1971; Goldring *et al*., 1976; Smithers *et al*., 1969). When worms were transferred from mice (hence coated with mouse antigen) to monkeys that had been immunised against mouse antigen, the worms were killed by the antigen-mediated immune responses (Clegg *et al*., 1970; Smithers *et al*., 1969). Similar findings were also described for schistosomules co-cultured with different human blood groups and tested in monkey immunised against the appropriate human blood group antigen (Goldring *et al*., 1976). Interestingly, when the lung stage parasites were transferred, the parasites were resistant to the antibody-mediated killing. However, the resistance was not observed when 15 days old parasites were used for the transfer (Clegg and Smither, 1971). *In vitro* observations are not fully consistent with these *in vivo* experiments. In a study where *S. mansoni* were recovered from mice and cultured with eosinophils and antibody against mouse protein, the lung stage was resistant to the antibody-mediated killing and, in contrast to *in vivo* observations, day-14 and day-21 worms were also resistant *in vitro* (McLaren and Terry, 1982). Only adults and larger liver developmental stages were killed by this process (McLaren and Terry, 1982). Despite the contradictions, both studies agree that the parasites are coated with host antigens, and further show that the lung stage might possess additional protective mechanisms against cytotoxic killing.

The camouflage strategy alone, however, could not suffice for immune evasion. The parasite tegument is constantly shed and renewed; therefore, phases where the parasites are not completely covered by host antigen will exist. In addition, the uptake of nutrients through the tegument may require some of the parasite transporters to be exposed to the host environment. Furthermore, activities of antibody-dependent cell-mediated cytotoxicity (ADCC) and complement are reduced in mice infected with schistosomes (Attallah *et al*., 1987), suggesting other immune-modulation strategies in schistosome infections.

1.3.5.2 Tegument complement inhibition

The complement system, or complement cascade, forms a part of immune defence in the mammalian host which *S. mansoni* needs to evade. The complement system consists of serum and surface proteins working together as protease activation cascades, and inhibitors exist at multiple levels (Markiewski et al., 2007). Molecules involved in the complement cascades are illustrated in Figure 1.3. The cascades ultimately result in the formation of membrane attack complexes (MAC) on the surface of invading pathogens, leading to the elimination of pathogens. In addition to MAC formation, an activated complement component (C3b) binds to pathogen surfaces, opsonizing them for attack by other immune cells (Abbas et al., 2014).

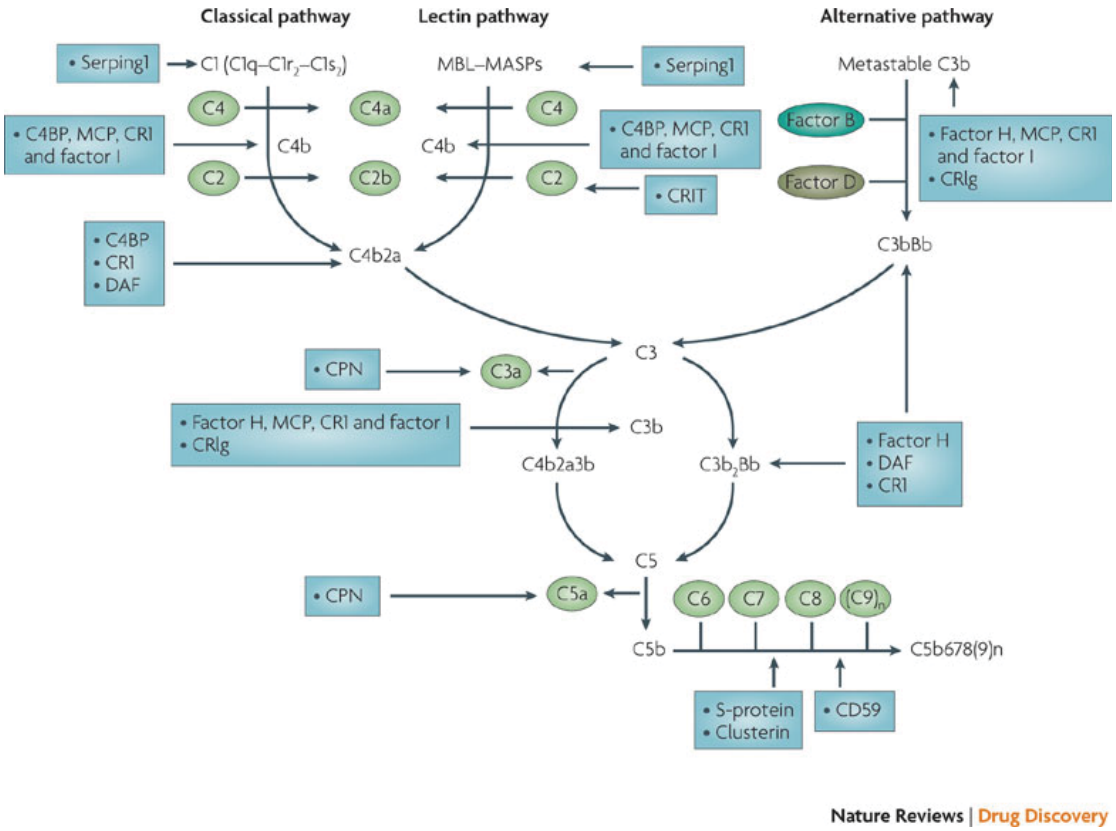

Figure 1.3 Coagulation and complement cascade.

Complement cascades consist of three pathways. The classical pathway is activated by an antigen-antibody complex interacting with complement component 1 (C1 subunits). The lectin pathway is activated by detection of carbohydrate on pathogen surfaces. The alternative pathway starts with the spontaneous activation of complement component 3 (C3), which forms C3b on pathogen surface. The three pathways converge at the formation of C3 and C5 convertase, leading to release of anaphylatoxin C3a and C5a that induce inflammatory responses. To the C5 convertase, other components (C6-C9) convene and form membrane attack complex which produces a pore on invading cells and kills them. Binding of C3b opsonized the surface for targeting by phagocytic cells. Represented in blue boxes are inhibitors of complement cascades. Diagram reproduced from Wagner and Frank (2010).

Schistosomes become resistant to complement killing soon after they transform into schistosomules (Marikovsky et al., 1990); however, this inhibition of complement seems to be reversed when parasites are treated with trypsin suggesting that some proteins on the tegument are involved in the resistance to complement killing (Marikovsky *et al*., 1990). Paramyosin (also known as schistosome complement inhibitory protein-1, or SCIP-1) has been identified as one of these tegumental proteins and it functions as an inhibitor of complement C9. The parasite paramyosin

also cross reacts with antibody against human CD59 which is also a complement C9 inhibitor (Deng *et al*., 2003; Parizade *et al*., 1994). In addition to inhibiting complement cascade, paramyosin is also one of the main receptors for host immunoglobulin binding (Loukas *et al*., 2001). More recently, proteomic work showed that other host proteins also bind to parasite tegument but receptors have not been identified (Braschi *et al*., 2006b, 2006a; Wu *et al*., 2015).

The complement cascade is also inhibited at an early step. C3 and C4 were found on the outer layer of parasite tegument (Braschi *et al*., 2006b, 2006a; Castro-Borges *et al*., 2011a); however, the small amount of C3 suggests that complement activation, which results in amplification of C3, does not occur (Castro-Borges *et al*., 2011a). The inhibition of C3 and C4 activation has been partially illustrated (Da'dara et al., 2016; Fatima et al., 1991; Pearce et al., 1990); *S. mansoni* can recruit decay-accelerating factor (DAF) (70- kDa glycoprotein, inhibitor of C3b activation) from host erythrocytes onto their tegument and prevent activation of complement through C3b (Fatima *et al*., 1991; Pearce *et al*., 1990). Furthermore, when human serum was incubated with adult *S. mansoni*, multiple complement activating proteins, including C3 and C4, were degraded. The resulting fragments could derive from the involvement of factor I (complement inhibitor enzyme) and its cofactor, factor H. However, these proteins have not been identified in *S. mansoni* (Da'dara et al., 2016a). Binding of C3 to parasite tegument also stimulates membrane synthesis and shedding of the tegument outer layer, which might be another mechanism to prevent completion of complement cascade activation (Silva *et al*., 1993).

1.3.5.3 Modulation of immune responses through secretion

Another mechanism of protection from immune attack comes from the ES from the parasites. Complement receptor-related protein y (Crry, or CR1) inactivates C3-derived fragment and is found in the proteome of adult extracts (Braschi and Wilson, 2006; Braschi *et al*., 2006b). One of the major secreted antigens described in the vomitus (circulating anodic antigen, CAA) is able to bind complement C1q, suggesting that CAA prevents antibody-mediated complement attack in the parasite gut (van Dam *et al*., 1993). Apart from complement inhibition, superoxide dismutase, peroxidase, and thioredoxin are found in *S. mansoni* vomitome (Hall *et al*., 2011) and might be involved in protecting worms from oxidative stress (Gobert *et al*., 2009b). In

addition, in *S. japonicum*, thioredoxin peroxidase from 14 days old *ex vivo* schistosomules inhibits the expression of MHCII and CD80 genes in lipopolysaccharide-activated macrophage (LPS-activated macrophage), leading to reduced inflammation (Cao *et al*., 2015). During skin invasion, anti-inflammatory protein, Sm16, is secreted from schistosomules and induces production of host antinflammatory cytokines (Ramaswamy *et al*., 1995), and the protein is also expressed in other stages suggesting its function beyond skin invasion stage (Rao and Ramaswamy, 2000).

ES are also involved in the interaction with endothelial cells. However, the responses of endothelial cells to ES from lung schistosomules are different from the responses to adult parasites (Oliveira et al., 2011; Trottein et al., 1999a, 1999b). Lung schistosomule ES decrease the permeability of the endothelium lining (Trottein *et al*., 1999a), and reduce leukocyte adhesive protein VCAM and E-selectin, causing fewer leukocytes to bind to the treated endothelial cells (Trottein *et al*., 1999b). The key immunomodulatory molecule appears to be prostaglandin D2 (Angeli *et al*., 2001, 2001). In contrast, the permeability of the endothelium lining and the interactions with leukocytes are increased when ES collected from adult worms are employed (Oliveira *et al*., 2011), suggesting that the parasite interacts with host endothelial cells differently during its developmental stages.

1.3.5.4 Coagulation cascades

The coagulation process consists of platelet activation and formation of blood clots. Blood clots can form via extrinsic and intrinsic pathways. Extrinsic pathways are activated by tissue factors exposed when endothelial cells are damaged, and by tissue factors expressed on immune cells and on endothelial cell surfaces. The intrinsic pathway is activated when the factor 12 bind to collagen or a negatively charged surface. The accumulation of factor 12 can lead to auto-activation and activation of coagulation cascade (Figure 1.4).

Coagulation and innate immune responses are closely related. Multiple components in the coagulation and complement cascades activate components in the other pathways. For example, complement component C3 fragment (C3a) activates platelets and enhances platelet aggregation (reviewed in Esmon *et al*., 2011; and Markiewski *et al*., 2007); thrombin, which cleaves fibrinogen chains leading to formation of fibrin clot,

can also cleave C3 and C5 (reviewed in Esmon *et al*., 2011; and Markiewski *et al*., 2007). Given that schistosomes can inhibit complement cascades, it is likely that coagulation might be also affected. Coagulation is a well-known protective mechanism to prevent the spread of pathogens from the site of entry in the host (Markiewski et al., 2007). It is, however, less understood how coagulation affects macroparasites like parasitic worms. Presumably, blood coagulation hinders parasite migration during early stages, and blood clots might be deleterious for blood-feeding (reviewed in Mebius *et al*., 2013).

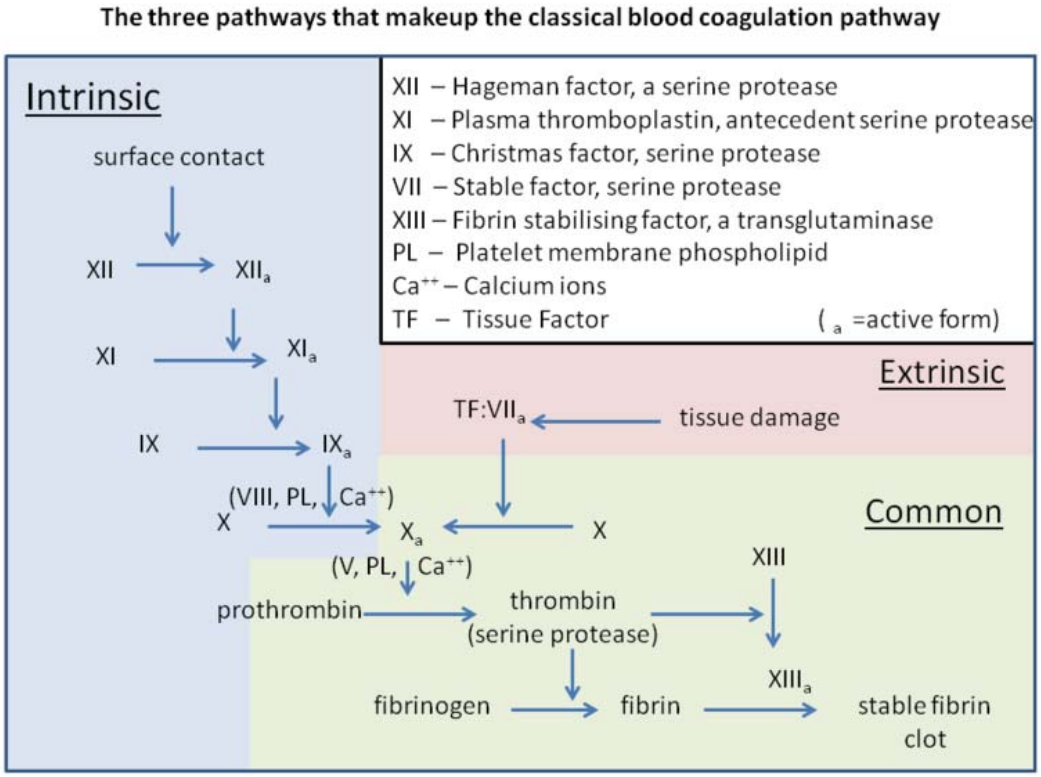

Figure 1.4 Coagulation pathway

Both intrinsic and extrinsic pathways lead to cascades of protease activation which join at factor 10 (FX, shown as X). The coagulation pathway finishes with conversion of fibrinogen to fibrin which form blood clots. Platelet activation step is not included in the diagram. Diagram reproduced from Graham Beards, via Wikimedia Commons (2012).

1.3.5.5 S. mansoni interference with coagulation

S. mansoni resides in blood vessels just about the size of its body (Bentley *et al*., 1981; Bloch, 1980). This is expected to interrupt blood flow and cause damage to the endothelium, which should trigger blood coagulation cascades. However, blood

clotting is not observed around migrating parasites (reviewed in Da'dara and Skelly, 2011; and Mebius *et al*., 2013). Interactions of *S. mansoni* with coagulation cascades ranges from prevention of cascade activation to degradation of blood clots once the clots have been formed (Mebius *et al*., 2013). Vasodilators may be involved in prevention of coagulation by reducing the interruption of blood flow, and thereby preventing damage of host endothelium (Mebius *et al*., 2013). For example, *S. mansoni* adults express a tegumental enzyme with kallikrein-like activity (sK1) which can lead to production of bradykinin and cause vasodilation (Carvalho *et al*., 1998). Interestingly, bradykinin also stimulates inflammation and activation of neutrophils and mast cells (Hofman *et al*., 2016). Therefore, it seems that a fine balance between prevention of coagulation and regulation of inflammation is required for the *S. mansoni* survival in bloodstream.

In addition to vasodilators, a heparin-like protein is found in the tegumental proteome of *S. mansoni* although its function remains to be investigated (Castro-Borges *et al*., 2011b). The coagulation cascades can also be activated by ADP through platelet activation (Woulfe et al., 2001). The enzyme ATP-diphosphohydrolase on the parasite tegument, which assists hydrolysis of ATP and ADP to AMP for nucleotide uptake, may also have additional role in preventing activation of coagulation cascade by reducing availability of ADP (Da'dara *et al*., 2014; Mebius *et al*., 2013; Vasconcelos *et al*., 1993). Furthermore, a Kunitz type protease inhibitor, which is located in the tegument of adult worms and also secreted, inhibits a coagulation factor shared between both intrinsic and extrinsic pathways, factor 10a (Ranasinghe et al., 2015a). To prevent the formation of blood clots, secreted *S. mansoni* protein Sm22.6 inhibits protease activity of thrombin - an enzyme that cleaves fibrinogen chains for the formation of insoluble fibrin blood clot (Lin and He, 2006). Lastly, once the fibrin clots have been formed, increasing clot degradation (a process known as fibrinolysis) could be a counteraction strategy. Annexin in the tegument, which previously was thought to have structural function (reviewed in Wilson, 2012), was demonstrated in *S. bovis* to bind to plasminogen and enhance its conversion into plasmin (fibrinolysis enzyme). This could therefore lead to an increase in fibrinolysis rate and eventual reduction in coagulation (de la Torre-Escudero *et al*., 2012).

1.3.6 Section summary

S. mansoni faces multiple challenges during its infections in the mammalian host, and the interactions with the host environment is essential for parasite development and survival. The interactions could be manifested through many interfaces such as tegumental surface, gut lining surface, and multiple sources of ES. The parasites migrate through the blood circulation network to reach suitable sites for development and egg-laying. Throughout their life in the bloodstream, the parasites rely on their host for metabolic requirements that they obtain through the tegument and the gut. Although living in the bloodstream can provide ample metabolic resources, the parasites are also in close contact with circulating leukocytes and other protective systems of the host against pathogens. Multiple strategies have evolved in *S. mansoni* to exploit the molecular host-parasite interactions. Arguably, suppression of inflammation could be beneficial to both *S. mansoni* and its mammalian host because excessive responses to tissue damages and inflammation responses against pathogens may also harm the host. Many aspects of the mechanisms for successful infections have been explored, but they are not fully understood; for example, how the parasites navigate and anchor to suitable sites; how certain metabolites are obtained and become essential for different stages; and what other mechanisms are involved in the interfering with host defence systems. Recent development in genomic information of *S. mansoni* and other parasites now allow more information to be extracted.

1.4 *S. mansoni* genome and transcriptome

1.4.1 Genome and gene annotation

1.4.1.1 Genome and annotation

Studying a genome provides a broad picture of an organism and the mechanisms underlying its biology. Prior to the availability of *Schistosoma* genomes, gene discovery was done using Express Sequence Tags (ESTs) where mRNA fragments were cloned, sequenced and matched to databases of known genes (e.g. Verjovski-Almeida *et al*., 2003). As reviewed by Hoffmann & Dunne (2003), the approach led to improved details of molecular functions of the parasites and supports previous biochemical work. However, ESTs do not usually cover the whole length of genes, are expensive and labour-intensive, and many *S. mansoni* ESTs could not be matched

to known ESTs in databases, likely because of the specificity to parasite clades (Hoffmann and Dunne, 2003). The first *S. mansoni* draft genome was published in 2009 (Berriman *et al*., 2009). Later work on the *S. mansoni* genome dramatically improved its assembly and gene annotations giving an estimated genome size of 364.5 megabases (Protasio *et al*., 2012). This version is used in the analyses of this thesis. Presently, the genome of *S. mansoni* is undergoing further improvements which include incorporating new data from long-read technology to close gaps in the genome, and obtaining full-length transcripts from multiple stages of clonal infections (single-miracidium infection of snails) to aid gene finding. In the new assembly (unpublished; M Berriman, personal communication, 2017) the seven autosomes and single pair of sex chromosomes are close to completion (<300 gaps), with improved accuracy of repetitive regions, and clarification of many genes structures. In addition to the *S. mansoni* genome, genomes of other helminth species have become available as part of the 50 Helminth Genome Initiative (50 HGI, http://www.sanger.ac.uk/science/collaboration/50hgp). Even though many of these genomes have not been extensively curated, the current quality allows comparisons of genes across species, both parasitic and non parasitic, and provide additional information on gene homology which could help researchers infer functions of parasite genes (Howe *et al*., 2016a, 2016b).

Genomic information provides resource for investigating specific groups of genes. In the following sections, three major groups are covered that have potential roles in parasitism or host-parasite interactions. Moreover, these groups present a particualr challenge to annotation efforts (due to their complex structures) and studies of them have substantially benefited from the availability of high quality genome data.

1.4.1.2 S. mansoni genome and GPCRs

Genomic sequences can be mined for members of an entire gene family. An example of a relevant protein family involved in host-parasite interaction is the G-protein coupled receptors (GPCRs). Prior to availability of the genome, the first GPCR characterised in schistosomes was identified from ESTs (Hoffmann et al., 2001). *S. mansoni* GPCRs are likely to be involved in multiple signalling pathways including chemosensory, regulation of physiological and developmental processes, and behavioural responses to stimuli (Chaisson and Hallem, 2012). In addition, GPCRs

are targets of multiple drugs (McVeigh et al., 2012a; Stevens et al., 2012). Multiple effort to mine GPCR-encoding genes from *S. mansoni* genomes have identified over 100 GPCRs (Berriman *et al*., 2009; Campos *et al*., 2014; Protasio *et al*., 2012; Zamanian *et al*., 2011). Some of these have been characterised for roles in parasite motility (e.g. MacDonald *et al*., 2015; Patocka *et al*., 2014). GPCRs for neurotransmitters and neuropeptides may regulate development and behaviours (Collins *et al*., 2010; Ribeiro and Patocka, 2013). Lastly, some GPCRs are localised to the tegument suggesting that their ligands are from external environment (e.g. Taman and Ribeiro, 2011).

1.4.1.3 S. mansoni genome and MEGs

Genomic data provide information on gene structures as well as their sequences. Micro-exon genes (MEGs) were identified in the genome of *S. mansoni* for their unique genic structure consisting of multiple small exons mainly comprising multiples (2-12) of three base pairs flanked at their 5' and 3' ends by longer exons of normal length (Berriman et al., 2009). Over 70 MEGs have been described and are currently grouped into families based on sequence similarity (Berriman et al., 2009; Chalmers et al., 2008; DeMarco et al., 2010).

MEGs have been postulated to have roles in host-parasites interactions owing to their presence being limited to parasitic flatworms (currently found in *Schistosoma* spps and tapeworm *Echinococcus*) (Tsai *et al*., 2013) and their expression patterns (different MEGs are expressed in specific stages of *S. mansoni* life cycle) (Parker-Manuel *et al*., 2011; Wilson, 2012). Moreover, with multiple exons and extensive alternative splicing, it has been proposed that proteins encoded by MEGs may contribute to antigenic polymorphism which could be a strategy for host immune evasion (Cai *et al*., 2016; DeMarco *et al*., 2010; Wilson, 2012). However, currently there is no report of host antibody recognition against MEGs; therefore, it is unlikely that antigenic polymorphism is a protective measure, for the protein products are not antigenic. However, some MEGs may, indeed, have roles in interactions with mammalian hosts (Philippsen et al., 2015; Wilson, 2012). Many MEGs are expressed in the oesophagus where many digestive enzymes are secreted, suggesting that MEGs may have roles in blood feeding or may be released into host environment (Li *et al*., 2014; Nawaratna *et al*., 2014). An example of this is a gene from the MEG-3 group

secreted by skin stage and lung stage schistosomules that has been suggested to interact with endothelial cells (reviewed in Wilson, 2012). Additionally, MEG-14 is expressed in the oesophagus and its protein product binds to inflammatory protein preventing the protein from reaching the parasite gut (Orcia *et al*., 2016). Furthermore, a study of gene orthologues between *Schistosoma* species demonstrated that MEG-1, MEG-9, and MEG-15 may have been under selective pressure reflecting their possible role in host-parasite interactions (Philippsen *et al*., 2015). Many MEGs have not been functionally characterised, and their amino acid sequences do not match any known protein domains (Berriman *et al*., 2009; DeMarco *et al*., 2010). However, some genes may have exonic structure resembling MEGs and also have exons encoding a conserved protein domain. An example of this is the *venom allergen-like 6* gene (VAL6) which contains exons encoding a conserved CAP (*cysteine-rich secetory protein/Antigen-5/Pathogenesis-related 1*) domain adjacent to a long stretch of micro exons (Chalmers et al., 2008). Further investigation on this group of genes may provide insights into parasite-specific features and host-parasite interactions.

1.4.1.4 S. mansoni *genome and hypothetical proteins*

Regarding another parasite-specific feature, approximately 30% of *S. mansoni* genes (~3000 genes) are annotated as hypothetical proteins due to absence of sequence similarity to known proteins and protein domains (Berriman et al., 2009; Protasio et al., 2012). Given their uniqueness to *S. mansoni* (and possibly to other parasitic species), they may explain parasite-specific biology or interactions. Finer details of their requirement and putative functions could be inferred from studying their expression at specific stages or in specific conditions.

1.4.2 Transcriptomes

Prior to the availability of RNA-sequencing (RNA-seq) technology for quantification of gene expression, DNA microarrays were used in many studies of *S. mansoni* and have provided insights into key changes in life stages as well as effects of the environment on parasites, and have been used for identifying drug targets (Chai *et al*., 2006; Fitzpatrick *et al*., 2009; Gobert *et al*., 2009b; Jolly *et al*., 2007; Parker-Manuel *et al*., 2011). As RNA-seq becomes more widely adopted, it has contributed to improvement of genome annotation and provided evidence for gene finding (e.g.

Almeida *et al*., 2012; Anderson *et al*., 2015; Protasio *et al*., 2012) as well as a deeper understanding of parasite biology (e.g. Lu *et al*., 2016; Picard *et al*., 2016; Protasio *et al*., 2013). RNA-seq has multiple benefits over microarrays: first, it allows the inclusion of more genes including genes that are not previously identified at the time of microarray probe design (i.e. RNA-seq is an unbiased approach, not relying on having previous information about the genes); second, it is less prone to resolution problems for genes with low expression levels (signal on microarray cannot be separated from background noises) and genes with high expression level (signal become saturated); and third, microarrays are more prone to technical variations whilst RNA-seq is highly reproducible (Wang *et al*., 2009). Current development of the RNA-seq technology, focussing on sample preparation procedures and sequencing chemistries, is driving down the requirements for input materials, allowing previously less accessible life-cycle stages to be investigated. Furthermore, multiple samples can be pooled into one sequencing run (multiplexing) reducing costs and the technical variation between samples.

1.5 This thesis

In this thesis, I have employed RNA-seq approaches (mRNA only), together with genomic information from available databases, to interrogate host-parasite interactions in *S. mansoni* during intramammalian stages. The next chapter covers generic methods used for this thesis such as parasite maintenance, preparation of samples for RNA-seq, and details of data analysis tools. In chapter 3, I investigate transcriptomic profiles of *in vivo S. mansoni* obtained from infected mice over a timecourse, covering the intramammalian life cycle stages (i.e. lung, liver, and adult stages) allowing exploration of changes associated with key stages of the infection. In chapter 4, I use co-cultured mechanically transformed *S. mansoni* schistosomules *in vitro* with three human-derived cell types to study how the parasite respond, transcriptomically, to different host environments. Then in chapter 5, I use the co-cultured cells to investigate how the parasite influence host environments.