Genetic determinants of selectivity of erythrocyte invasion in the human malaria parasite *Plasmodium falciparum*

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Abstract

The aim of this study was to investigate the genetic basis of selectivity in invasion of the red blood cells by the human malaria parasite *Plasmodium falciparum*. Multiple invasions of a single host red blood cell by more than one merozoite, which can be described or assessed in terms of the selectivity index (SI), has been reported to be related to the severity of malaria disease. In this study, selectivity index, defined as the ratio of the number of multiply-infected red cells observed to that expected from random invasion, as modelled by a Poisson distribution was determined for certain clones of *P.falciparum*. SI was measured under static and shaking culturing conditions for *P. falciparum* clones 3D7 and HB3 and 18 progeny clones derived from a genetic cross between these two parasite clones. *P. falciparum* clone 3D7 was found to have a significantly lower SI than HB3 under both static and shaking culture conditions. There was no relationship between SI and days in continuous culture for clone 3D7 under shaking and static conditions; the phenotype therefore appears to be stable over time. The genetic basis of the difference in selectivity index between *P. falciparum* clones 3D7 and HB3 was investigated in progeny clones from a cross between these two clones, to ascertain the inheritance pattern of the phenotype. Under static conditions, ten progeny clones had a selectivity index lower than either parent, one progeny clone had higher selectivity index than both parent, and six progeny clones had selectivity index intermediate between the parents . Under shaking conditions, fifteen progeny clones were observed to have a selectivity index lower than either parent. These observations suggest the involvement of more than one parasite gene in selectivity index. A Quantitative Trait Locus (QTL) analysis was performed in order to identify genomic regions influencing SI in the progeny clones. The highest LOD score of 5.06 was obtained for a QTL on chromosome 13 for SI measured in parasites cultured under shaking conditions. This QTL denoted, *PF_SI_1*, extends for approximately 100kb on chromosome 13 and contains 19 open reading frames. This finding indicates the presence of a gene or genes on chromosome 13 that influence the parasite's selection of erythrocytes for invasion.

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Author’s declaration

I declare that the thesis does not include work forming part of a thesis presented successfully for another degree.

I declare that the thesis represents my own work except where acknowledged to others.

Signed.....

Sultan Alghamdi

May, 2015

Abbreviations

A	adenine
ACTs	artemisinin-based combination therapies
AE1	anion exchange protein 1
AFLP	amplified fragment length polymorphism
AMA	apical membrane antigen
CDPKs	calcium-dependent protein kinases
cM	centimorgan
CSA	chondroitin sulphate A
DBL	duffy binding like
DBPs	duffy antigen binding proteins
DBP	duffy binding protein
DARC	duffy antigen/ receptor for chemokines
EBA	erythrocyte-binding antigen
EBL	erythrocyte binding like
EBP	erythrocyte binding protein
GAP45	glideosome-associated protein 45
GPI	glycosylphosphatidylinositol
GYP	glycophorins
GYPA	glycophorin A
GYPC	glycophorin C
ICAM-1	intercellular adhesion molecule 1
LLINs	long-lasting insecticidal nets
LOD	log of odds
mAbs	monoclonal antibodies
Mar	marker
MIC	microneme proteins
MSP	merozoite surface proteins
MTIP	myosin A tail domain-interacting protein
NBP1	normocyte binding protein 1
P. falciparum	Plasmodium falciparum
PfAMA	P. falciparum apical membrane antigen
PfEMP	P. falciparum erythrocyte membrane protein
PfRBH	P. falciparum reticulocyte binding homologue

PfRH	P. falciparum rhoptry protein homologue
PfRBP	P. falciparum reticulocyte-binding protein
PvDBP	P.vivax duffy binding proteins
PkDBP	P.knowlesi duffy binding proteins
PMR	parasite multiplication rate
QTL	quantitative treat locus
RBCs	red blood cells
RBL	reticulocyte binding like
RBP	reticulocyte binding proteins
SI	selectivity index
SNP	single nucleotide polymorphism
T	thymine

1 General Introduction

Malaria remains a major public health problem especially in the tropical and sub-tropical countries of the world. The disease is caused by parasites which are members of the phylum Apicomplexa. This phylum of intracellular parasites includes parasites that can infect humans and animals, such as *Toxoplasma*, *Theileria*, *Eimeria*, *Babesia*, *Cryptosporidium* and *Plasmodium*. Some parasites of the phylum Apicomplexa are able to pass directly between vertebrate hosts (*Toxoplasma*, *Eimeria* and *Cryptosporidium*). Others, like *Plasmodium*, need an arthropod vector to transmit the parasite to the vertebrate host (Cowman and Crabb, 2006).

All Apicomplexa have a common mechanism of host-cell entry but each individual species has a unique set of ligand-receptor interactions. Apicomplexans share a common specialized apical complex (for which the group is named) which is critical to the invasion process. Some members of the Apicomplexa can invade many different host cells and tissues but malaria parasites are limited in the number of cell types and tissues they invade.

There are many species of *Plasmodium* that can infect animals: humans, other mammals, birds and lizards (Cowman and Crabb, 2006; Snow *et al*., 2005). In humans, malaria can be caused by five species of *Plasmodium*: *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi*. The most virulent among these is *P. falciparum* (CDC, 2010).

1.1 Burden of malaria, distribution of disease and causative species of parasite

According to the 2012 World Malaria Report, there were about 219 million cases of malaria in 2010 resulting in around 660 000 deaths (World Health Organization, 2012). The report further indicated that Africa received most of the cases due to the disease and about 90% of all the reported malaria deaths occurred in this continent. Malaria mortality rates fell by 26% around the world between 2000 and 2010; with estimated 1.1 million cases of malaria attributable deaths averted globally, which has been attributed to the scale-up

of intervention measures such as the introduction of long-lasting insecticidal nets (LLINs) and quality-assured artemisinin-based combination therapies (ACTs).

P. falciparum has a wide geographical distribution and is found in the tropical parts of the world as well as subtropical areas. *P. vivax* is commonly found in Asia, some areas of Africa as well as in Latin America. *P. ovale* is found mostly in Africa, particularly in the western areas, and rarely in the west pacific islands. *P. malariae* has a similar distribution to *P. falciparum* (Collins and Jeffery, 2007).

A zoonotic malaria caused by *Plasmodium knowlesi* is found in Malaysia and other regions of south East Asia (Vythilingam *et al.*, 2008; Singh *et al.*, 2004(Singh et al., 2004; Vythilingam et al., 2008). This parasite is found in long-tailed and pig-tailed macaques, *Macaca fascicularis* and *Macaca nemestrina* respectively (Vythilingam *et al.*, 2008; Singh *et al.*, 2004). Transmission of *P. knowlesi* is currently thought to be from monkey to mosquito to man and that people infected with this parasite, such as Forest workers, must have been in close proximity to macaques (White, 2008).

The risk of complications and death due to of *P. falciparum* is much higher in Africa than in South East Asia and the western Pacific (Snow *et al.*, 2005). This is may be due to better access to medical care, treatment and protection against malaria in South East Asia and the western Pacific (Snow *et al.*, 2005; Miller *et al.*, 2002b).

The malaria parasite's life cycle shown in figure 1.1 is complicated, consisting of three distinct cycles: the sporogonic, the exo-erythrocytic and the erythrocytic cycles. The sporogonic cycle occurs in the digestive tract of the mosquito, the exo-erythrocytic cycle occurs in the liver and erythrocytic cycle occurs in the erythrocytes. Malaria in humans is transmitted by the female *Anopheles* mosquito which injects sporozoites into the blood stream of the vertebrate host during a blood meal.

1.2 *Plasmodium falciparum* life cycle

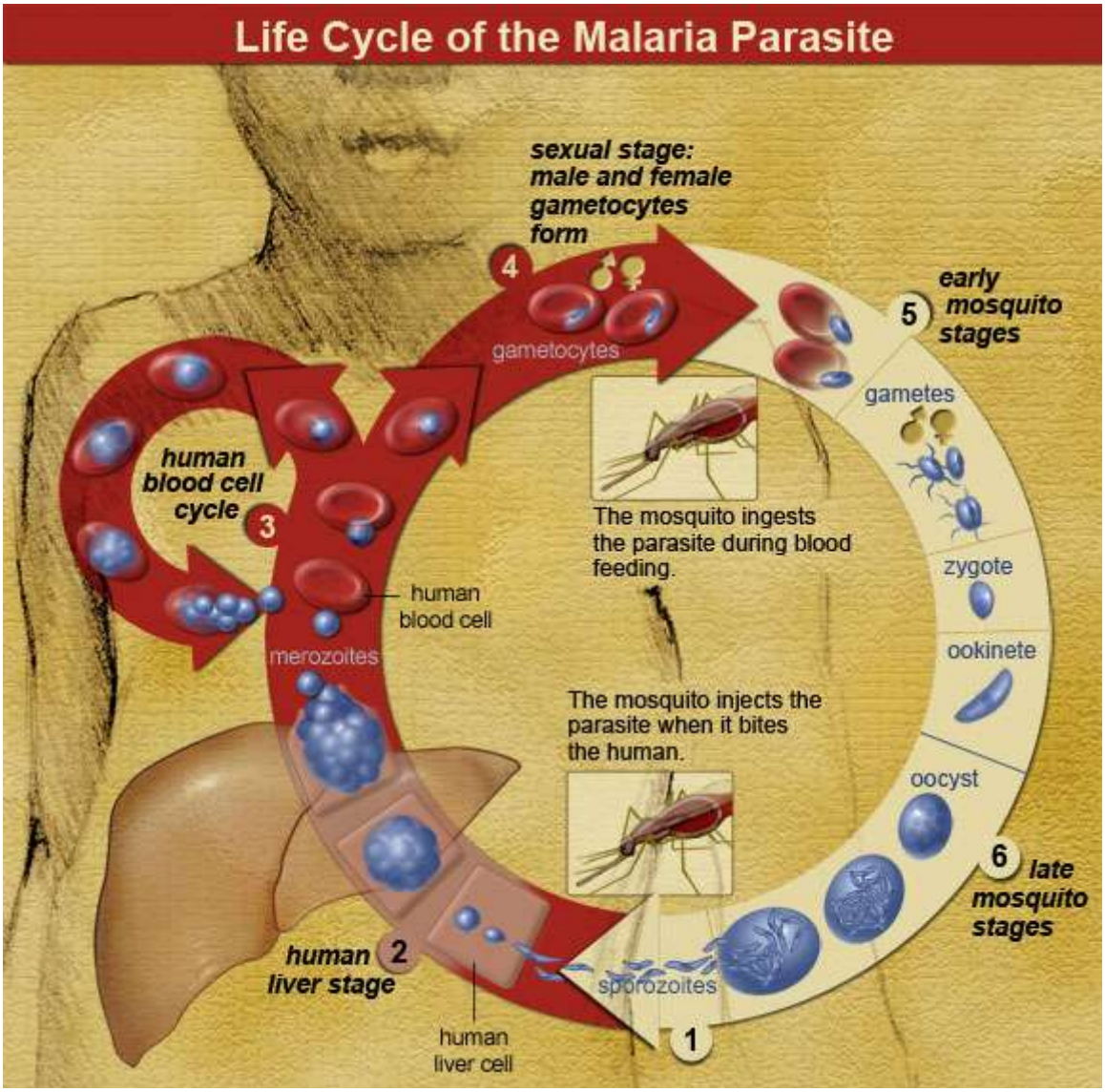

Figure 1.1 Life cycle of *Plasmodium falciparum*. Reproduced with permission from NIAID. Source: http://www.niaid.nih.gov/topics/malaria/pages/lifecycle.aspx

Some of the sporozoites are destroyed by macrophages and some find their way into the lymphatic system (Vaughan *et al.*, 2008). The majority, however, find their way into the blood and are transported to the liver where they penetrate a few hepatocytes within a few hours. Exo-erythrocytic schizogony occurs within the hepatocytes with each sporozoite multiplying into 10000–30000 merozoites, depending on the parasite species (Amino *et al.*, 2008; Jones and Good, 2006). The liver stage takes 5–16 days to complete (Collins and Jeffery, 2007; Tuteja, 2007). The liver stage is referred to as the pre-patent phase and is characterized by little pathology of the liver and no clinical symptoms.

The intra-hepatic merozoites remain enveloped in a host-cell derived membrane called the merosome. The merosome is thought to exit the liver cell intact and only ruptures on entering the bloodstream (Silvie *et al.*, 2008). In *P. falciparum* infection, the merozoites do not re-infect hepatocytes.

In *P. vivax* and *P. ovale* infections, the sporozoites in the hepatocytes may go into dormancy (a stage known as the hypnozoite) and only reactivate after a few months, or even years after primary infection. This explains the phenomenon of relapse in malaria caused by these parasites (Collins *et al.*, 2007).

Erythrocytes are the primary targets of the merozoites and it is the erythrocytic stage of the parasite life cycle that is responsible for most of the clinical symptoms associated with the disease. The merozoites recognize, attach to, and invade the erythrocytes by complex receptor-ligand interactions between parasite membrane proteins and erythrocyte membrane proteins. Greenwood and colleagues suggested that the time taken to complete these interactions is so rapid that within 60 seconds of their release into the bloodstream, the merozoites disappear completely (Greenwood *et al.*, 2008). Following merozoite invasion, the parasite undergoes erythrocytic schizogony.

The erythrocytic stage of the parasite has two easily distinguishable phases: the trophic phase and the replicative phase. In the trophic phase, the parasite is enclosed in an intracellular 'ring' and develops into a trophozoite. This stage is marked by high metabolic activity and increase in size of the 'ring' (Haldar and Mohandas, 2007; Cowman and Crabb, 2006). The parasite breaks down haemoglobin into constituent amino acids and haem. The amino acids are the substrates for parasite protein synthesis. Haem which is toxic to the parasite is polymerized into haemozoin (malaria pigment) which is stored in food vacuoles (Miller *et al.*, 2002a). The trophozoites then develop into schizonts, each containing 8–32 merozoites (Tuteja, 2007; Greenwood *et al.*, 2002). The end of this phase is marked by the lysis of the infected cell and the release of merozoites, that in turn infect new red blood cells and the cycle is repeated. For *P. knowlesi*, the cycle takes 24 hours to complete, and 48 hours for *P.*

falciparum and *P. vivax* and 72 hours for *P. malariae*. In *P. falciparum* infections, multiple parasites are commonly seen in a single erythrocyte.

Sometimes instead of schizogony, the parasites will reproduce sexually, and develop into microgametocytes (male gametocytes) or macrogametocytes (female gametetocytes). During gametogenesis, these micro- or macro-gametocytes metamorphosize into micro- or macro-gametes. This may only occur after the gametocytes have been ingested by a mosquito. After ingestion, the microgametocyte undergoes divisions; the resulting eight nuclei become associated with thrashing flagella (this process is called exflagellation). The highly motile micro-gametes fuse with macro-gametes to form a zygote, which then develops into an ookinete. On reaching the space between the epithelial cells and the basal lamina of the mosquito gut, the ookinete develops into an oocyst. Asexual sporogony occurs in the oocyst to produce several sporozoites which are released into the haemocoel from where they migrate to the salivary glands. Sporozoites can be seen in the salivary glands of the mosquito 8–18 days after the ingestion of merozoites. When the infected mosquito bites a susceptible person, the sporozoites are inoculated and the cycle is repeated (Bousema and Drakeley, 2011).

1.3 Pathogenesis of malaria

The non-specific symptoms of malaria include fever, headache, abdominal pain, diarrhoea, nausea, vomiting, anorexia, and pruritus; these are similar to common viral and bacterial infections. Malaria is characterised by cyclical fevers and chills linked to the intraerythrocytic cycle. In a small number of cases, the disease can develop to severe or complicated malaria. The most common clinical symptoms of severe malaria are high fever, progressive anaemia, organ failure, and unconsciousness (coma) (Miller *et al.*, 1994). The factors that lead to severe malaria are not well understood but the host immune system, environmental factors and parasite properties may all play a role (Miller *et al.*, 2002b).

Cerebral malaria is one form of severe malaria found only in *P. falciparum* infections. It is characterised as an unrousable coma and has a high fatality rate. There are a number of theories explaining the causes of cerebral malaria. Sequestration of parasites in the capillaries of the brain probably contributes to

the development of cerebral malaria. This possibly involve cytokines such as interferon gamma and TNF alpha, platelets, and/or the localised release of nitric oxide (Schofield and Grau, 2005; Hunt and Grau, 2003; Schofield and Grau, 2005). Metabolic acidosis has been identified as a pathophysiological marker (mechanism of the pathology) of cerebral malaria or severe malaria. Lactic acidosis is caused by increased production of lactic acid by parasites or decreased clearance of the lactic acid by the liver or both (English et al., 1997; Marsh et al., 1995).

Severe anaemia is defined as haemoglobin concentration below 5 g/dL, where the normal haemoglobin concentration is 12-18 g/dL (Lamikanra *et al.*, 2007; Weatherall *et al.*, 2002). Both *P. falciparum* and *P. vivax* can induce anaemia, but the severe malarial anaemia caused by *P. falciparum* results in a third of deaths associated with the disease (Haldar and Mohandas, 2009). Severe anaemia may come from several mechanisms such as acute haemolysis of uninfected RBCs, dyserythropoiesis(it is any defect of RBC production which is considered through morphologic changes of the nuclei and cytoplasm in the bone morrow; it is a defective development of erythrocytes), and the interaction of malaria infection with other parasite infections and with nutritional deficiencies (Newton *et al.*, 1997). Hyperparasitaemia is defined as high parasite count above 5% and is a form of severe falciparum malaria. High parasitaemia and the presence of schizonts of *P. falciparum* in the peripheral blood are associated with a higher mortality (World Health Organization, 2009).

1.4 Selectivity of parasites for erythrocytes

In human malaria, multiple invasions of RBCs are a hallmark of *P. falciparum* infection (Pasvol et al., 1980; Pasvol et al., 1982b; Clough et al., 1998). The extent of erythrocyte preference that an individual parasite has is described by the selectivity index (SI). SI is calculated as the ratio of number of multiply-infected red cells observed (i.e. with more than one parasite present within a single RBC) to that expected from random invasion, as modelled by a Poisson distribution for a given parasitaemia (Simpson *et al.*, 1999). Higher SI values indicate a greater selectivity of parasites for red blood cells and therefore more multiply-invaded cells than expected. Low SI values indicate a relative absence of selectivity by the parasite for red cell, such that parasites do

not discriminate between RBCs, and multiple invasion events do not occur more frequently than expected by chance (or as modelled by a Poisson distribution). High SI values on the other hand suggest that parasites are able to distinguish the RBCs and that some RBCs are selected for invasion more than others. The group of Chotivanich reported that severe malaria caused by *P. falciparum* showed reduced selectivity in vivo in RBC invasion than uncomplicated malaria (Chotivanich *et al.*, 2000). Studies of *P. falciparum*-infected patients in Thailand found higher SI values in patients with uncomplicated malaria compared to severe malaria (Simpson *et al.*, 1999). Selectivity index (SI) was also found to be inversely proportional to plasma lactate concentration, a marker of disease severity, and was found to be lower at higher parasitaemia. Therefore, parasites from patients with more severe disease seem to be less selective for the red blood cells they invade, whereas those from mild disease show a higher preference for some red cells than others (and thus there are more multiply-infected cells than expected from random invasion processes). The geometric mean SI was significantly higher in *P. vivax* infections than in *P. falciparum* infections at comparable parasitaemias; this finding is probably explained by the preference of *P. vivax* for reticulocytes and young erythrocytes (Simpson et al., 1999; Triglia et al., 2001).

However, studies in two different sites in Africa identified no significant difference in parasite multiplication rate (PMR) of *P. falciparum* infections in patients with severe malaria in comparison with isolates from patients with uncomplicated malaria (Deans *et al.*, 2006). This finding was in contrast to previous studies performed in Thailand which suggested an association between severity and PMR (Chotivanich *et al.*, 2000; Simpson *et al.*, 1999).

As a consequence of parasite invasion, the morphology of the erythrocyte may change, making it more or less susceptible for attachment and subsequent invasion by other parasites. One of the major structural changes that is likely to occur may be linked to the creation of the parasitophorus vacuole membrane (PMV). The creation of this membrane could lead to a reduction in the surface area of infected red cell. As to whether the 'shrinking of the RBC' surface area favours the entry of more parasites into the infected cell remain questionable. It is also possible that two merozoite could stick to each and co-invade an

erythrocyte. Studies needed to be carried out in order to unravel the exact mechanism of multiple RBC invasions.

There are several factors which make multiple invasions more common than expected by chance, such as an unequal susceptibility of the available red blood cells. Plasmodium falciparum merozoite invasion of erythrocytes is assumed to be a random process. Thus it presupposes that the process would follow a Poisson distribution. However, this is not the observation. Several factors could influence this process, making multiple invasions more common than predicted by chance alone. Though these factors have not been fully investigated a lot of speculations have been made. In 1999, Simpson and colleagues suggested that there would be an increase in the proportion of multiple invaded red cells as a consequence of reduction in the pool of susceptible erythrocytes (Simpson et al., 1999). This is possible in situations where circulating RBCs are not equally prone to invasion or their distribution in areas of schizont sequestration is not uniform(Simpson *et al.*, 1999).

1.5 Parasite multiplication rate and the severity of *falciparum* malaria

The number of successfully invaded RBCs per cycle determines the parasite multiplication rate. The growth rate is a function of cycle time which, taken from invasion of a merozoite to release of new merozoites, is 48 hrs for *P. falciparum*. The parasite multiplication rate is determined by the number of viable merozoites produced per schizont, the time taken for parasites to complete the intraerythrocytic cycle, and the efficiency with which the new merozoites are able to invade new RBCs. However the association of parasite multiplication rate with disease severity is unclear (Chotivanich *et al.*, 2000; Simpson *et al.*, 1999).

Parasite virulence factors such as ability to form rosettes and to mediate clumping of platelets may play a major role in parasite virulence in sub-Saharan Africa (Doumbo *et al.*, 2009), but PMR and RBC selectivity have been suggested to be more important in Asia (Pain *et al.*, 2001; Kun *et al.*, 1998; Rowe *et al.*, 1995). The phenomenon of rosetting in malaria infection is the agglutination of nonparasitized erythrocytes around erythrocytes containing trophozoites and

schizonts of *P. falciparum*. In other words it is the binding of Malaria-infected red blood cells to uninfected red blood cells to form clumps. The rosettes can obstruct flow in small blood vessels and lead to tissue damage from hypoxia and severe malaria disease (Chotivanich *et al.*, 1998).

1.6 Sequestration and cytoadherence of *P. falciparum*

When a malaria parasite invades an erythrocyte, it modifies the surface of cell, so that the trophozoite and schizont stages of asexual parasites can adhere to the endothelium of blood capillaries (known as sequestration or cytoadherence). This protects the parasites from destruction by the spleen as they do not circulate in the peripheral blood. The ring forms of *P. falciparum* are the only asexual stage found in circulating blood (Chen *et al.*, 2000; Langreth and Peterson, 1985; Luse and Miller, 1971). Erythrocytes infected with *P. falciparum* parasites are able to bind to several host epithelial cell molecules including ICAM-1, VCAM, CD36, and thrombospondin and chondroitin sulphate A. Two host receptors, CD36 and chondroitin sulphate A (CSA) have been implicated in cytoadherence (Cooke et al., 1994; Ho and White, 1999).

A single parasite protein, *P. falciparum* erythrocyte membrane protein-1 (PfEMP1), mediates parasite binding to all of the different receptors (Chen et al., 2000; Newbold et al., 1997; Baruch, 1999). PfEMP1 exists in multiple copies within the parasite genome, with each individual parasite having around 50 different PfEMP1 genes, although only one is expressed within a single parasite (Miller et al., 1994; Joergensen et al., 2010). Parasites can sequester in different organs such as the lung, brain, heart, kidney, placenta and subcutaneous tissues. Each parasite can bind to variable number of host receptors by expression of different PfEMP1 variants, and this variability in adhesion affects the tissue distribution and parasite pathogenesis (Beeson et al., 1999; Chen et al., 2000; Newbold et al., 1997). Cerebral malaria is associated with sequestration of the parasites in the brain (Newbold *et al.*, 1997). The differences in parasite adhesion in this type of malaria are explained on the basis of involvement and over expression of intercellular adhesion molecule 1 (ICAM-1) in brain endothelium (Ockenhouse et al., 1991; Turner et al., 1994).

The ability of certain alleles of PfEMP1 to mediate binding to chondroitin sulphate A (CSA) is important in pregnancy in malaria (PAM) (Bockhorst *et al.*, 2007; Fried and Duffy, 1996). CSA is not highly expressed on any tissue in the body except for the placenta, and parasites expressing CSA-binding PfEMP1 would be at a disadvantage in a non-pregnant individual. However in the first pregnancy, parasites expressing specific CSA-binding PfEMP1 variants are able to bind in the placenta and can induce severe malaria. However antibodies to the variant of PfEMP1 are generated during infection, that prevent the infected erythrocytes adhering to CSA, and these may protect the mother and the foetus from placental malaria in a subsequent pregnancy (Fried *et al.*, 1998).

1.7 Invasion of red blood cells by *Plasmodium falciparum*

1.7.1 General outline of P. falciparum invasion of erythrocytes

After emerging from hepatocytes, merozoites quickly recognise, attach and enter erythrocytes. The initial contact between the merozoites and RBCs is a fundamental step, during which the parasites differentiate between erythrocytes suitable for invasion and other cell types. The primary attachment of the merozoite occurs at any point on the surface of the red blood cell (Figure 1.2) (Cowman and Crabb, 2006; Miller et al., 2013; Miller et al., 2013). The parasite then re-orientates to direct the apical end of the merozoite towards the erythrocyte membrane allowing a much closer interaction (Bei and Duraisingh, 2012; Cowman and Crabb, 2006; Gunalan et al., 2013). In order for the merozoite to enter the cell, a tight junction is formed between the parasite and host membrane. The tight junction shifts from the apical end of the merozoite in series of events controlled by an actin-myosin motor system (Bei and Duraisingh, 2012; Gunalan et al., 2013; Keeley and Soldati, 2004). As the parasite enters the host cell, it creates a parasitophorous vacuole with a membrane created from the invaginated erythrocyte membrane. This vacuole ensures a suitable

environment for parasite development (Cowman and Crabb, 2006).

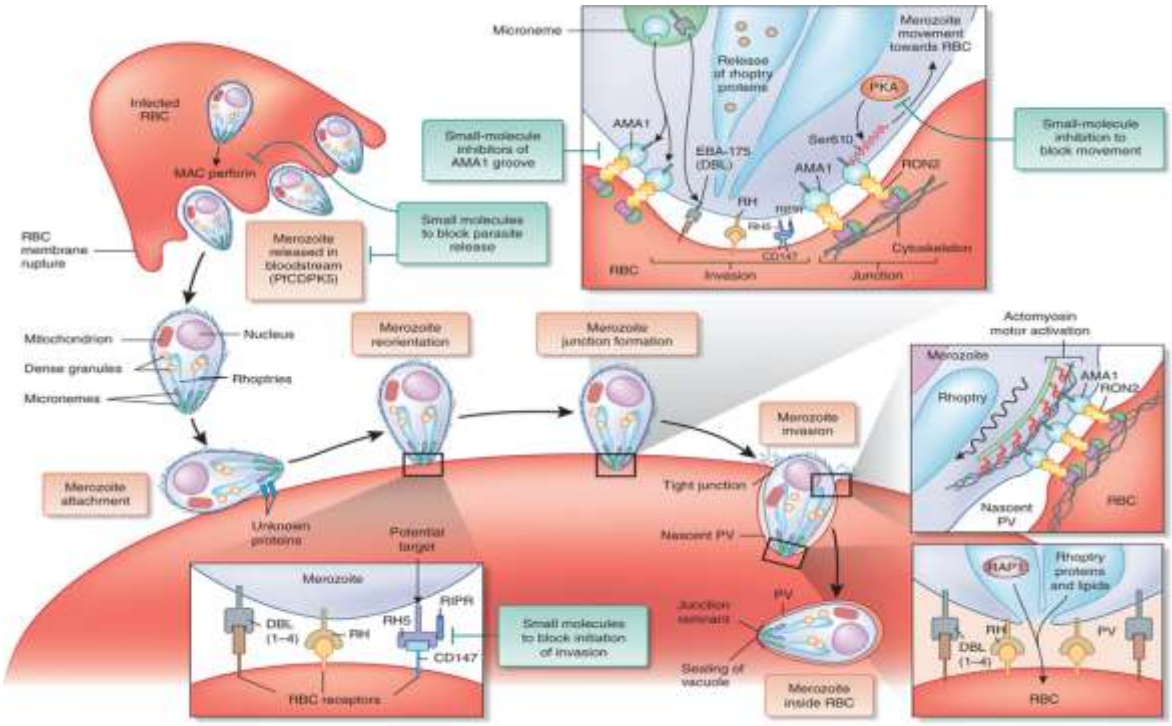

Figure 1.2 The procedure of merozoite invasion of the erythrocyte. Figure is reproduced with permission from Miller L. H. et al., 2013 (License number 3593141378385).Each step is illustrated from merozoite attachment to reorientation to merozoite junction formation and finally to merozoite invasion where it enters the RBC.

1.7.2Primary merozoite attachment

It takes one minute for the merozoite to identify, attach and enter the erythrocyte (Gunalan *et al.*, 2013; Persson, 2010). After the initial contact with the merozoite, the infected erythrocyte then undergoes echinocytosis after 36 seconds, i.e dehydrated form of morphology; this is followed by the recovery to the normal shape which takes 5-11 minutes (Gunalan *et al.*, 2013).

The primary contact between the merozoite and the erythrocyte is dependent on merozoite surface proteins such as MSP-1 (Persson, 2010). There are currently nine glycosylphosphatidylinositol (GPI) anchored membrane proteins known to be present on the surface of the merozoite (Sanders *et al.*, 2005). MSP-1 is the most abundant protein on the surface and is thought to play a major role in parasite survival, early recognition and attachment of invasion events (Cowman and Crabb, 2006; O'Donnell *et al.*, 2000). MSP-1 is produced as a large (195 kDa) protein in the merozoite. Fragments of 83 kDa, 42 kDa, 38 kDa

and 30 kDa are produced by proteolytic processing during schizogony or after merozoite release. The four fragments remain bound as a non-covalent complex at the merozoite surface (Blackman and Holder, 1992; Blackman *et al.*, 1991; Blackman *et al.*, 1990). The C-terminal 42 kDa fragment is attached to the plasma membrane of the merozoite by a glycophosphatidyl inositol (GPI) anchor. The 42 kDa fragment is further processed into a 33 kDa and a 19 kDa fragment, which remain bound to the merozoite plasma membrane during invasion.

Erythrocyte-binding antigen 175 (EBA-175) is a *P. falciparum* ligand for merozoite invasion into human erythrocytes that binds to glycophorin A in a sialic acid-dependent manner. It is been shown that W2mef, a strain of *P. falciparum* depends on sialic acid for invasion of erythrocytes, whereas 3D7 is sialic acid-independent (Dolan *et al.*, 1994; Dolan *et al.*, 1990; Sim *et al.*, 1990). Full length MSP-1 binds to erythrocytes in sialic acid-dependent manner (Goel *et al.*, 2003). It has been shown that antibodies against MSP-1_{19} inhibit erythrocyte invasion, and immunization with recombinant MSP-1_{19} protected mice and monkeys from malaria infection (Persson, 2010; Rotman *et al.*, 1999; Blackman *et al.*, 1990). Recently, Moss and colleagues looked at MSP1 processing by investigating inhibition ability of parasite growth by antibodies prepared from rabbits immunized with recombinant proteins of AMA1 and MSP1_{19} (Moss *et al.*, 2012).It was observed that inhibition of growth and invasion is not only caused by MSP1 processing inhibition but may involve several other members of the MSP family such as MSP-2, MSP-4, MSP-5, MSP-10, MSP-12, Pf38, Pf92 and Pf113 (Persson, 2010). It remains to be determined if these proteins have a role in the initial recognition and interaction between erythrocytes and merozoites (Cowman and Crabb, 2006).

Wilson and colleagues evaluated the functional antibodies to MSP-1_{19} and the importance of MSP-1_{19} as a target of acquired immunity (Wilson *et al.*, 2011). In a longitudinal cohort study involving 206 children conducted in Papua New Guinea, the growth inhibitory antibodies against asexual-stage parasites and IgG to recombinant MSP-1_{19} in plasma samples were measured. The group concluded that MSP-1_{19} is not a major target of growth inhibitory antibodies and suggested that the protective effects of antibodies to MSP-1_{19} may instead be

mediated by other mechanisms. They also thought that antibodies to MSP-1_{19} could act as a marker of protective immunity.

1.7.3 Reorientation of the merozoite

P. falciparum merozoites invasion of erythrocytes is complex and involves many steps of specific interactions between the parasites and erythrocytes. In brief, there is an initial merozoite attachment to the RBC followed by apical reorientation of the merozoite and then formation of an irreversible tight junction. The final stage involves the entry of the parasite into the host cell.

The second phase of invasion involves reorientation of the merozoite after the initial binding to the RBC surface (Bei and Duraisingh, 2012; Gunalan et al., 2013; Farrow et al., 2011). This is an important part in the process of invasion and at this point the merozoite is positioned such that its tip points in the direction to the surface of the erythrocyte, ready for invasion (Farrow *et al.*, 2011).

P. falciparum apical membrane antigen 1 (PfAMA-1) is thought to be involved in the reorientation step, since antibodies specific to PfAMA-1 did not prevent initial attachment but stopped reorientation of the merozoite (Mitchell *et al.*, 2004). PfAMA-1 is located in the rhoptries of mature merozoites in the late stage schizont (Chitnis, 2001), and is translocated to the merozoite surface before invasion takes place. Antibodies against AMA-1 inhibit erythrocyte invasion, and immunization with purified AMA-1 provides protection against malaria in mice (Anders *et al.*, 1998; Peterson *et al.*, 1989).

PfAMA-1 plays a key link between the weak initial contact associated with MSPs and the irreversible tight junction formed via microneme proteins. Recently it has been shown in *Toxoplasma gondii* (another member of the phylum Apicomplexa) that the homologue of AMA-1, TgAMA-1, interacts with one other protein (at least), known as RON4. Similar proteins to RON4 have been discovered in *P. falciparum* (Alexander *et al.*, 2005; Lebrun *et al.*, 2005). In *T. gondii* tachyzoites, several proteins of the neck of the rhoptries (RON2, RON4, RON5 and RON8) have been shown to form a complex with proteins originating from two post-Golgi specialized secretory organelles, and together with proteins

released from micronemes (TgAMA-1), they form a moving junction. TgAMA-1and RON4 have been previously localized to the moving junction during invasion (Alexander et al., 2005; Besteiro et al., 2009).

Srinivasan and colleagues demonstrated that an interaction between RON2 and AMA1 is crucial for the formation of the tight junction (Srinivasan *et al.*, 2011). Using antibodies that bound near the hydrophobic pocket of AMA1 and AMA 1 mutation that occurs, the group were able to identify the binding site for RON2 on AMA1. Specific residues in the RON2 that are involved in the binding to the AMA1 pocket were also identified. Overall, they showed that binding of RON2 to the hydrophobic pocket of AMA1 is a necessary step that commits the merozoites to erythrocyte invasion. The group also demonstrated that immunization with AMA1-RON2 peptide complex (RON2L) resulted in induction of protection against *P.yoeli* infection and that protection was mediated by antibodies (Srinivasan *et al.*, 2014).

Small molecules that bind to AMA1 were identified from screening of a library containing over 21000 compounds (Srinivasan *et al.*, 2013), and one was shown to completely inhibit parasite invasion of RBC, by blocking the formation of AMA1-RON complex required for junction formation. This discovery is important as it confirms the importance of the AMA1-RON complex in erythrocyte invasion. A combination of this inhibitor with existing antimalarials could constitute a perfect novel antimalarial drug.

1.7.4Merozoite invasion of the erythrocyte

After the parasite re-orientates, the invasion process is activated by direct interaction of parasite ligands at the apical end of the merozoite with erythrocyte receptors. A tight junction is formed between the merozoite and erythrocyte surface, which appears as an electron-dense thickened area under the erythrocyte membrane at the point of contact with the merozoite (Aikawa et al., 1978; Farrow et al., 2011). Formation of the junction is followed by parasite invasion of the cell. The merozoite drags the plasma membrane of the erythrocyte over its surface during invasion thus leading to the creation of a parasitophorous vacuole within the RBC.

Two substrates of *P. falciparum* Calcium-dependent protein kinases (CDPKs) have been identified as components of the motor complex that generates the force required by the parasite to actively invade host cells. These are the Myosin A tail domain-interacting protein (MTIP) and Glideosome-associated protein 45 (GAP45).The invasion motor complex is highly conserved and distinctive to Apicomplexa (Farrow *et al.*, 2011).

However, it has recently been shown that *Toxoplasma gondii* can invade a host cell in the absence of motor protein Myosin A (Egarter *et al.*, 2014), and the authors suggested a different mechanism for invasion depending on osmotic forces, which are formed in the parasite cytosol, and are transformed into gliding motility (Egarter *et al.*, 2014).

1.7.5Alternative Pathways of invasion

Experimentally, enzymatic modification of the erythrocyte surface makes RBCs less susceptible to invasion by some parasite strains. Erythrocytes can be treated with three enzymes which have differential effects on the glycophorins (Okoyeh *et al.*, 1999). Neuraminidase treatment cleaves sialic acid residues. Trypsin and chymotrypsin also cleave sialoglycoproteins in the membrane, but trypsin cleaves at lysine and arginine residues, whereas chymotrypsin cleaves at large hydrophobic residues such as tryptophan, tyrosine and phenylalanine. These different enzymes have been used to define different invasion pathways for *P. falciparum in vitro* (Miller *et al.*, 1977). It has been reported that red blood cells treated with neuraminidase or trypsin had reduced susceptibility to *P. knowlesi* and *P. falciparum* (Miller *et al.*, 1977). However when treated with chymotrypsin, invasion by *P. knowlesi* and not *P. falciparum* is blocked. This observation suggests that the two parasite species interact differently with receptors on the surface of the erythrocytes.

Species-specific pathways of *P. falciparum* invasion determined by erythrocyte binding protein polymorphisms are discussed in the following sections.

1.7.6 Molecular aspects of parasite invasion

1.7.6.1 The DBL-EBP family

Merozoite proteins reported to be involved in invasion of the erythrocyte includes some members of the Duffy Binding Like - Erythrocyte Binding-Protein (DBL-EBP) family. These proteins bind with high affinity to glycophorins on the surface of erythrocytes. Five members of the DBL- EBP family have been identified in the *P. falciparum* genome (Miller *et al.*, 2002a; Triglia *et al.*, 2005). The DBL-EBP family includes the *P. vivax*/*P.knowlesi* Duffy antigen binding proteins (DBPs) and the *P. falciparum* erythrocyte binding antigen 175 (EBA-175) (Adams *et al.*, 1992; Sim *et al.*, 1992; Adams *et al.*, 1990; Aikawa, & Miller, 1990).The *P. falciparum* DBL proteins are orthologues of the DBL protein identified in *P. vivax* and in the simian malaria *P. knowlesi* that bind the Duffy blood group protein present on the RBCs surface so as to facilitate parasite invasion (Miller et al., 2002b; Miller et al., 2002a; Miller et al., 1975; Mason et al., 1977). Although *P. falciparum* is independent of the Duffy blood group antigen on erythrocyte invasion, and can invade Duffy-negative erythrocytes, the parasite has a number of proteins that share a similar cysteine rich binding domain of other parasites species such as *P. vivax*/*P. knowlesi* DBP. These proteins are called the Duffy binding-like (DBL) superfamily (Adams *et al.*, 2001) and are believed to be involved in erythrocyte binding.

The EBPs (erythrocyte binding proteins) are present in the micronemes, located in the apical (invasive) end of merozoites. The initial interaction of *Plasmodium* merozoites with the erythrocyte results in the release of EBPs from the micronemes onto the merozoite surface (Carruthers and Sibley, 1999; Chitnis, 2001). It has been shown in *Toxoplasma gondii* that the release of microneme proteins such as MIC2 is regulated by the release of cytoplasmic Ca^{+2} (Starnes *et al.*, 2006). The release of MIC2 onto the apical surface of *T. gondii* tachyzoites was induced by Ca^{+2} ionophores such as A23187, but chelating of intracellular Ca^{+2} prevented release of MIC2 (Lovett and Sibley, 2003).

1.7.6.1.1 P.vivax and P.knowlesi Duffy binding proteins (PvDBP and PkDBP)

The Duffy antigen, named after the person in whom antibodies to Fy^a (one of the Duffy antigens) were first found, is located on the surface of RBC

(Chaudhuri *et al.*, 1993). *P. vivax* was thought to invade only erythrocytes expressing the Duffy blood group antigen (DARC) (Storti-Melo *et al.*, 2009).As such, *P. vivax* infections were noted to be rare in areas of the world where the majority of people have no Duffy antigen (Duffy negative), such as West Africa. The ligand on the *P. vivax* merozoite which mediates this selectivity was identified as PvDBP (Hans *et al.*, 2005; Tran *et al.*, 2005). It is 140KDa protein which mediates the interaction with DARC for invasion of human erythrocytes (Hans *et al.*, 2005).

The simian malaria *P. knowlesi* also depends on the Duffy blood group antigen for invasion (Miller *et al.*, 1975; Miller *et al.*, 1976).When *P. knowlesi* merozoites tried to invade Duffy-negative human erythrocytes, initial attachment and apical reorientation was seen to occur normally, but the junction was not formed, suggesting a role for the Duffy blood group antigen in junction formation (Miller *et al.*, 1979). A 135kDa protein, Duffy Binding Protein (DBP), was identified in *P. knowlesi*, and shown to bind the human Duffy blood group antigen, as well as that in rhesus monkeys, whereas the 140kDa homologue in *P. vivax* was shown only to bind human Duffy blood group antigen (Adams et al., 1990; Chitnis and Miller, 1994; Wertheimer and Barnwell, 1989; Haynes et al., 1988). However, recent studies demonstrated *P. vivax* and *P. knowlesi* infections in Duffy-negative populations (Ryan *et al.*, 2006). In fact, *P. knowlesi* was shown to invade chymotrypsin-treated rhesus erythrocytes (chymotrypsin treatment removes the Duffy antigen from the red blood cell surface) indicating that *P. knowlesi* can use a Duffy-independent pathway for invasion, similar to the glycophorin A-independent invasion pathway for some strains of *P. falciparum* (Haynes *et al.*, 1988).

1.7.6.1.2 The EBL family in P. falciparum

The *P. falciparum* EBLs are located in the sub- telomeric regions of different chromosomes (Gardner *et al.*, 2002). The proteins consist of structured domains, including the F region (region II) which is subdivided into two related domains, F1 and F2, involved in receptor binding (Adams et al., 2001; Gilberger et al., 2003b; Mayer et al., 2001). A cysteine-rich domain near the N-terminus has been shown to mediate the binding of EBA-175 to its receptor and it is likely that similar domains exist in EBA-140 and EBA-181 (Mayor A. *et al.*, 2005). The

DBL proteins also have cytoplasmic domains that are not linked to the actin-myosin motor system (Gilberger *et al.*, 2003a).

1.7.6.1.2.1 EBA-175

EBA-175 is a 175 kDa protein encoded by a single gene on chromosome 7 (Mayer *et al.*, 2001). Antibodies against EBA-175 caused the inhibition of RBC binding and invasion, providing proof of its role in invasion and RBC binding (Narum et al., 2002; Jakobsen et al., 1998; Jones et al., 2001). The involvement of EBA-175 in invasion is believed to be through the pathway of sialic acid-dependence (Adams *et al.*, 2001). Genetic disruption of the gene for EBA-175 prevented invasion of strain W2MeF of *P. falciparum* (Persson, 2010; Reed *et al.*, 2000). However, disruption of PfEBA-175 in strain Dd2/NM had no effect on its growth rate or invasion; it is recognized that RBC invasion by this strain of *P. falciparum* follows the sialic acid- independent pathway (Nery *et al.*, 2006; Kaneko *et al.*, 2000).

1.7.6.1.2.2 EBA-140

The gene of EBA-140 is located on chromosome 13 and it codes for a 140 kDa protein (Gilberger *et al.*, 2003b). Like EBA-175, antibodies against EBA-140 were shown to inhibit erythrocyte binding and invasion (Jakobsen et al., 1998; Jones et al., 2001; Narum et al., 2002). However where EBA-175 appears to interact with sialic acid residues on glycophorin A, EBA-140 is believed to interact with glycophorin C (GYPC, Gerbich Antigen Blood Group) on the RBC membrane, and this interaction mediates *P. falciparum* invasion pathway into human RBCs (Gilberger *et al.*, 2003).The evidence for the specificity of EBA-140 to GYPC is that it does not bind to GYPC in Gerbich-negative erythrocytes, and *P. falciparum* cannot invade such cells using this invasion pathway (Maier *et al.*, 2003). A single amino acid change in EBA140 has been shown to produce different erythrocyte binding. Indeed, 'Polymorphisms in the binding domains of EBA-140 have been suggested to alter their respective receptor specificities' (Maier *et al.*, 2009).

1.7.6.1.2.3 EBA-181

EBA-181 is a 181 kDa protein produced in the late schizont stage and located in the micronemes. The gene for EBA-181 is on chromosome 1. EBA-181 has 37% similarity to EBA-140. The protein binds to an uncharacterized

erythrocytic sialoglycoprotein (Gilberger et al., 2003a; Mayer et al., 2004). A single amino acid change in EBA-181 was shown to alter host (RBC) receptor recognition (Gilberger *et al.*, 2003).

1.7.6.1.2.4 EBA-165

EBA-165 is considered an un-translated pseudogene located on chromosome 4 contains multiple mutations. To date, no protein product has been seen to be transcribed by this gene (Triglia *et al.*, 2001).

1.7.6.1.2.5 EBL-1

P. falciparum protein EBL-1 is a 304kDa protein encoded by a gene of chromosome 13 (Adams *et al.*, 2001; Peterson and Wellems, 2000). Glycophorin B on the RBC surface is known to be the receptor for EBL-1 (Mayer *et al.*, 2009). In 2012, Li and co-workers identified a precise region of EBL-1 of *P. falciparum* that inhibits merozoite invasion when binding to host receptor glycophorin B in human erythrocytes (Li *et al.*, 2012).

1.7.6.2 The RH or reticulocyte binding like (RBL) family

The PfRH proteins are homologues of the Py235 family of rhoptry proteins in the rodent malaria parasite *P. yoelii* (Ogun *et al.*, 2011; Preiser *et al.*, 2002) . The proteins are thought to be involved in determining the specificity of host cell invasion. The parasite antigen (Py235) recognised by these mAbs (Monoclonal antibodies) had a molecular mass of 235 kDa (Gruner *et al.*, 2004).

P. vivax, which has a preference for reticulocyte invasion (Cowman and Crabb, 2006), expresses two homologues of the Py235 family (PvRBP1 and PvRBP2), which are thought to be responsible for the preferential invasion of reticulocytes over normocytes (Galinski *et al.*, 1992). The PvRBP proteins are located in the apical end of the merozoite. PvRBP-1 and 2 may form a complex that mediates adhesion and recognition of the reticulocyte (McCutchan et al., 1984; Okenu et al., 2005). The receptor on the reticulocyte for RBPs is unknown (Oh and and Chishti, 2005). Invasion of reticulocytes by *P. vivax* requires both recognition of reticulocytes (mediated by reticulocyte binding proteins RBP) and interaction with the Duffy blood group antigen, mediated by the Duffy binding protein DBP (Galinski and Barnwell, 1996).

1.7.6.2.1 P. falciparum RH family

The *P. falciparum* reticulocyte-binding protein homologue (PfRh or PfRBL) family are known to be involved in the invasion process. Six members of the RH family were identified in *P. falciparum*, the rhoptry protein homologue 1 (PfRH1) also known as normocyte binding protein 1(NBP1) (Gunalan *et al.*, 2013; Rayner *et al.*, 2001), the reticulocyte-binding protein homologues 2a and 2b (PfRBP-2a and 2b) (Gunalan et al., 2013; Rayner et al., 2000; Triglia et al., 2001), the rhoptry protein homologue 3 (PfRH3) which may be a pseudogene (Gunalan *et al.*, 2011; Taylor *et al.*, 2001), the reticulocyte binding homologue 4 (PfRBH4) (Gunalan et al., 2013; Tham et al., 2012; Nery et al., 2006; Kaneko et al., 2002; Tham et al., 2012) and the reticulocyte-binding protein homologue 5 (PfRH5) (Cowman and Crabb, 2006; Gunalan et al., 2013). These can be refered as PfRH1, PfRH2a, PfRH2b, PfRH3, PfRH4 and PfRH5 respectively. The PfRh proteins are thought to be important in merozoite invasion, but with one exception (PfRH5) they do not appear to be essential, as the genes can be disrupted in different *P. falciparum* without any obvious effect on the growth rate of the blood stages (Duraisingh et al., 2003b; Stubbs et al., 2005; Duraisingh et al., 2003a).

1.7.6.2.1.1 PfRH1

PfRH1 has been shown to bind directly to sialic acid residues on the erythrocyte, and antibodies against this protein inhibit merozoite invasion (Rayner *et al.*, 2001). The gene for PfRH1 is located on chromosome 4 (Rayner *et al.*, 2001). The PfRH1 binding region contains a coiled coil motif at the C-terminal end possibly involved in protein multimerization and the presence of a trimeric complex in the recombinant protein (Rayner *et al.*, 2001). Rayner and colleagues identified a region of about 300 amino-acids (the erythrocyte-binding domain) that retains the binding properties reported for the full length PfRH1 protein (Rayner *et al.*, 2001).

Plasmodium falciparum protein	Binding to RBC			
	Chymotypsin sensitive	Trypsin sensitive	Neuraminidase sensitive	Identity of RBC protein
EBA-175	R	S	S	GlyA
EBL-1	S	R	S	GlyB
EBA-140	R	S	S	GlyC
EBA-181	S	R	S	E
Rh1	S	R	S	W
Rh2a	R	R	S	Z
RH2b	S	S	S	Z
Rh4	S	S	R	CR1
Rh5	S	S	S	BSG
Pf92	S	S	S	X

Table 1.1 Host-parasite interactions involved in erythrocyte invasion. GlyA, glycophorin A; GlyB, glycophorin B; GlyC, glycophorin C; CR1, complement receptor 1; BSG, Basigin; EBA, erythrocyte binding antigen; EBL, erythrocyte binding like; Rh, reticulocyte binding protein homolog; Pf92, *Plasmodium falciparum* 92 ; S, sensitive; R, resistant. E, W, Z and X are unknown receptors (Bei *et al*, 2012; Obando-Martinez *et al*, 2010; Sanders *et al*, 2007; Sanders *et al*, 2005;Tham *et al*, 2011).

1.7.6.2.1.2 PfRH2a and PfRH2b

Type-I transmembrane rhoptry proteins are encoded by the PfRh2a and PfRh2b genes and can be readily deleted by either single or double crossover recombination, indicating that they are not essential for the invasion process (Duraisingh *et al.*, 2003b).They diverge only in their C-terminal regions that correspond to a portion of the ectodomain, a transmembrane domain and a short cytoplasmic tail (De Simone *et al.*, 2009). PfRH2a is a 370 kDa protein encoded by a gene on chromosome 13 (Gunalan *et al.*, 2013). The amino terminal regions of PfRH2a and PfRH2b are highly similar (Hayton *et al.*, 2008). PfRH2b is a 383 kDa protein encoded on chromosome 13. PfRH2a and PfRH2b are localized to the rhoptries of *P. falciparum* (Gunalan *et al.*, 2013; Taylor *et al.*, 2002). Binding of PfRH2a and PfRH2b to erythrocytes has not been demonstrated, but invasion in some parasite strains can be inhibited by antibodies against these proteins (Persson, 2010; Ocampo *et al.*, 2004; Triglia *et al.*, 2001).

1.7.6.2.1.3 PfRH4

The sialic acid-independent invasion pathway is dependent on the expression of *P. falciparum* reticulocyte binding protein-like homologue 4 (PfRh4), as disruption of the gene abolishes the ability of parasites to switch to this pathway (Tham *et al.*, 2009). PfRh4 has been shown to be present as an

invasion ligand in culture supernatants as a 160-kDa proteolytic fragment (Tham *et al.*, 2009). It was further indicated that PfRh4 binds to the surfaces of erythrocytes through recognition of an erythrocyte receptor that is neuraminidase resistant but trypsin and chymotrypsin sensitive. Serum antibodies from malaria-exposed individuals show reactivity against the binding domain of PfRh4 (Tham *et al.*, 2009). Human affinity-purified antibodies to the binding region of PfRh4 effectively inhibited erythrocyte invasion by *P. falciparum* merozoites (Reiling *et al.*, 2012). Invasion by the parasite clone, Dd2, requires sialic acid on the erythrocyte surface; Dd2/NM is a variant selected for its ability to invade neuraminidase-treated erythrocytes that lack sialic acid. However,PfRH4 is uniquely up-regulated in Dd2/NM compared with Dd2, indicating that it may be a parasite receptor involved in invasion (Gaur *et al.*, 2007). The erythrocyte binding of PfRH4 and the invasion of neuraminidase treated erythrocyte are blocked by $RRH4_{30}$ (a recombinant 30-kDa protein to a conserved region of PfRH4).However, both PfRH4 and part of PfRH4 ($RRH4_{30}$) bind efficiently to neuraminidase-treated erythrocytes. It has been shown that two genes on chromosome 4, PfRH4 and a pseudogene, PEBL (EBA-165), are highly up regulated in the Dd2 (NM) clones when compared to Dd2 (Gaur *et al.*, 2006).

1.7.6.2.1.4 PfRh5

The PfRH5 protein is 63kDa, making it the smallest member of the RH family (Rodriguez *et al.*, 2008). The gene is encoded on chromosome 7 (Hayton *et al.*, 2008). It has regions homologous to the N-terminal regions of PfRH2a and PfRH2b, but lacks regions corresponding to the C-terminal regions of PfRH2a and PfRH2b, including a predicted transmembrane domain characteristic of other family members (Figure 1.3).

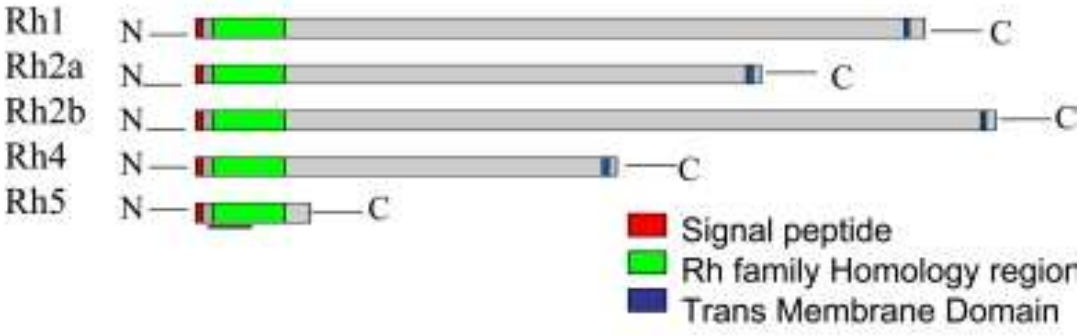

Figure 1.3 Structural representations of the members of the PfRH family. The location of the signal peptide is shown in red, the region of homology among the various RH ligands in green and the trans-membrane region at the C-termini of the proteins in dark blue. Figure reproduced with permission from Rodriguez et al., 2008.

PfRH5 has been shown to exhibit erythrocyte binding activity (Chen *et al.*, 2011; Sahar T. *et al.*, 2011). Unlike the other PfRH proteins, attempts to disrupt the PfRH5 gene have not been successful (Cowman and Crabb, 2006; Hayton et al., 2008). PfRH5 may be essential for the ability of merozoites to recognize and enter various human and non-human erythrocytes. Co-localisation studies using antibodies to PfRH5 suggest an apical location, probably the rhoptries (Gunalan *et al.*, 2013; Hayton *et al.*, 2008). In 2008, Rodriguez and colleagues demonstrated that PfRH5 binds to the surface of erythrocytes and that its target is a sialic-acid-independent receptor (Rodriguez *et al.*, 2008).

Basigin is recognized as the erythrocyte receptor of PfRh5, and shown to be necessary for the pathogen invasion of erythrocytes (Muramatsu *et al.*, 2012).

1.8 Host genetic factors affecting parasite invasion: polymorphisms of the erythrocyte surface

Human erythrocytes have many surface membrane proteins that form the basis of the many blood groups (Avent and Reid, 2000; O'Neill and Reddy, 2011). Three groups of polymorphic surface proteins are thought to be important in invasion by malaria parasites: the glycophorins, the Duffy blood group antigen and Band 3.

Glycophorins are membrane bound glycoproteins with a single transmembrane alpha helix. They are heavily glycosylated with oligosaccharides attached to 16 sites on the extracellular portion of the polypeptide chain. Band 3 consists of 929 amino acids (95 kDa protein) and has alpha helices that cross the membrane 14 times. It functions as a transporter exchanging bicarbonate ions for chloride ions. Band 3 also helps to define the cell shape of the erythrocyte, through interactions of the cytoplasmic domain of the protein with spectin and ankyrin bridge. The Duffy blood group antigen (also known as the Duffy-Antigen Chemokine Receptor, DARC) is a 75kDa transmembrane protein that spans the RBC membrane seven times, with an extracellular N-terminal domain and a cytoplasmic C-terminal domain (VanBuskirk *et al.*, 2004).

1.8.1Glycophorins

The glycophorins(GYP) are major integral membrane proteins that are blood group antigenic determinants and are also known to be ligands for viruses, bacteria, and parasites (Cartron and Rahuel, 1992). Five glycophorins, known as GPA, GPB, GPC, GPD, and GPE, have been fully identified (Cartron and Rahuel, 1992). Glycophorins have been shown to play an important role in RBCs invasion by *P. falciparum*. Experimentally enzymatic modification of glycophorin structure makes RBCs less susceptible to invasion by some parasite strains (Okoyeh *et al.*, 1999). In addition, erythrocytes with natural changes or defects in glycophorins are less susceptible to invasion by *P. falciparum* than normal erythrocytes (Okoyeh *et al.*, 1999).

1.8.1.1 Glycophorin A

Glycophorin A is encoded by the human gene *GYPA* on the long arm of chromosome 4. It is responsible for the MNS blood group system in humans (Hadley *et al.*, 1987). In addition to the M or N antigens, there are variant phenotypes including the deletion variant Ena, (Wright b antigen, Wrb). Glycophorin A is required on the red blood cell surface for efficient invasion by *P. falciparum* merozoites (Hadley *et al.*, 1987). Invasion into the En (a-) erythrocytes, which lack glycophorin A, is significantly reduced but not abolished (Miller et al., 1977; Pasvol et al., 1982b). Glycophorin A is bound specifically by EBA-175. This binding was shown to be abolished following neuraminidase

treatment of the erythrocytes, which removes sialic acid residues (Camus and Hadley, 1985). En (a-) erythrocytes were not bound by EBA-175 (Sim *et al.*, 1994).

Some strains of *P. falciparum* are able to use alternative invasion pathways other than those relying on interactions with sialic acid residues of glycophorin A, as demonstrated by their ability to grow in erythrocytes treated with enzymes such as neuraminidase, or in glycophorin A or sialic acid-deficient erythrocytes (Miller et al., 1977; Mitchell et al., 1986; Okoyeh et al., 1999; Pasvol et al., 1982b). For example, *P. falciparum* clones HB3 and 7G8 are able to invade neuraminidase-treated human erythrocytes, implying that that they can use sialic acid-independent invasion pathways (Doran *et al.*, 1994). Other cloned parasite lines such as Dd2 and FCR3 are completely dependent on sialic acid residues for invasion but invade trypsin-treated erythrocytes that have lost glycophorin A. Dd2 and FCR3 can use sialic acid residues present on both glycophorin A as well as trypsin-resistant glycophorin B as receptors for invasion (Doran *et al.*, 1994).

Recently the group of Baldwin used phage display technology to study the interaction between the amino-terminus of MSP1 and RBC glycophorin A (Baldwin *et al.*, 2015). Their observations suggest an essential role of the MSP1-GPA-Band 3 complex during the initial adhesion phase of malaria parasite invasion of red blood cells.

1.8.1.2 Glycophorin B

Glycophorin B is encoded by the human gene *GYPB* on the long arm of chromosome 4. It is responsible for the SS blood group system in humans (Storry et al., 2000). Glycophorin B contains only O-glycosylation sites, which are linked via serine or threonine (Wilson *et al.*, 1991). Erythrocytes of the S-s-U phenotype, common in pygmies in African countries, lack glycophorin B. Such erythrocytes were found to be partially resistant to invasion by *P. falciparum* (Facer, 1983; Pasvol *et al.*, 1982a)

Glycophorin B is believed to be the receptor for EBL-1, a member of the Duffy-binding-like erythrocyte-binding protein (DBL-EBP) receptor family (Mayer

et al., 2009). Glycophorin B, erythrocytes adsorbed EBL-1 from the *P. falciparum* culture supernatants whereas glycophorin B-null erythrocytes did not (Mayer *et al.*, 2009).

1.8.1.3 Glycophorin C

Glycophorin C is encoded by *GYPC* on the long arm of chromosome 2. Together with glycophorin D, GYPC encodes the Gerbich (Ge) antigen on the red blood cell, of which there are 4 alleles, Ge-1 to Ge-4. GYPC has a role in maintaining red blood cell shape and membrane properties.

A deficiency in glycophorin C (Leach, Gerbich or Yus blood groups) reduces invasion by *P. falciparum* (Pasvol *et al.*, 1984). Where Leach erythrocytes have no GYPC, Gerbich and Yus erythrocytes have truncated GYPC (and also no GYPD, which is encoded by the same gene with an alternative start site) (Colin, 1995). The Gerbich phenotype is found at high frequency in malaria endemic regions of Papua New Guinea (GYPCDex3) (Booth and McLoughlin, 1972; Cartron et al., 1993; Serjeantson et al., 1994). Individuals with a Gerbich-negative phenotype were found to have lower rates of infection compared to Gerbich-positive individuals form the same area (Serjeantson *et al* 1994).

It has been reported that glycophorin C (GPC) is the binding site for PfEBP-2 (baebl, EBA-140) which is lately recognized as a binding ligand of *P. falciparum* (Lobo *et al.*, 2003). It was also demonstrated that cells lacking glycophorin C do not bind PfEBP-2 (Lobo *et al.*, 2003).

1.8.2 Duffy blood group antigen

The Duffy blood group antigen is a 38-kD glycoprotein with seven transmembrane domains and an extracellular N- terminus (Chaudhuri *et al.*, 1993). The Duffy blood group antigen is a chemokine receptor, binding a family of chemokines such as IL-8 (Chaudhuri *et al.*, 1993; Horuk *et al.*, 1993). The Duffy antigen is encoded by the FY gene on chromosome 1, with two serologically distinct forms determined by alleles FY*A and FY*B. Each of these alleles exists in a mutant, unexpressed or null form as $FY*A^{null}$ and $FY*B^{null}$ (Zimmerman *et al.*, 1999). Individuals are Duffy-negative because the two

alleles are mutant and so no expression of the Duffy protein occurs. Duffy negativity is particularly common in West and central Africa; 95% to more than 99% of the population are Duffy antigen negative, decaying to a frequency of 70% in east Africa and 50-60% in the Arabian peninsula (Cavalii-Sforza, 1994). Infection with *P. vivax* was observed to be very rare in regions with high Duffy negativity, suggesting that the Duffy negative state protected against *P. vivax* infection. It was subsequently discovered that merozoites of *P. vivax* depend completely on the Duffy antigen for RBCs invasion (Arevalo-Herrera *et al.*, 2005; Chitnis and Miller, 1994). The simian malaria *P. knowlesi* is also dependent on the human Duffy blood group antigen for invasion of human erythrocytes (Miller *et al.*, 1975).

The Duffy antigen is not used as a receptor for invasion by *P. falciparum* which can invade Duffy-negative erythrocytes (Friedman et al., 1984; Miller et al., 1977). Chymotrypsin treatment removes the Duffy antigen from the red blood cell surface. *P. knowlesi* can invade chymotrypsin-treated rhesus erythrocytes indicating that it can use Duffy independent pathway for invasion similar to the glycophorin A-independent invasion pathway for some strains of *P. falciparum* (Haynes *et al.*, 1988).

1.8.3 The anion exchange protein 1 (AE1) or band 3

The AE1 is a major membrane protein of RBCs and has several functions such as maintaining the shape of the cell. The structure of AE1 changes as the RBC ages, and thus is thought to play a role in the recognition of and destruction of old RBCs by the spleen (Low et al., 1985; Kay et al., 1990).

A deletion of the gene for AE1 causes hereditary ovalocytosis, which is common in Papua New Guinea (Jarolim *et al.*, 1991). Protection against severe malaria can be provided by ovalocytosis (Allen *et al.*, 1999; Serjeantson *et al.*, 1977) It has been shown that erythroglycan, a carbohydrate component of band 3 transmembrane protein, inhibits invasion effectively (Friedman *et al.*, 1985). The lactosamine chains of erythroglycan contributed much of the inhibitory activity. Polylactosamine isolated from the erythrocyte protein Band 3 has been reported to inhibit invasion of red blood cells by *P. falciparum in vitro*; this suggests that polylactosamine plays a role in adhesion of the parasite (Dhume *et*

al., 1994). More recently, it has been reported that Band 3 is a host receptor binding merozoite surface protein 1 during the *P. falciparum* invasion (Goel *et al.*, 2003).

1.9 Environmental factors affecting parasite invasion

1.9.1 The effect of erythrocyte age on P. falciparum invasion rate

Red blood cells are formed in the bone marrow. Stem cells in the red bone marrow are called hemocytoblasts. The formation of a red blood cell from hemocytoblast takes about 2 days and the body turn around about two million RBCs every second. Mature RBC circulates for 100-120days. When they become old or damaged, the vast majority of red blood cells are removed from circulation by the spleen, liver, and lymph nodes (Mebius and Kraal, 2005).

P. falciparum can invade erythrocytes of any age, including younger blood cells such as reticulocytes (Mons, 1990). It has been previously assumed that the ideal conditions for culturing *P. falciparum* were accomplished by using RBCs stored for at most 2 weeks (Capps and Jensen, 1983; Kim et al., 2007). Early experiments suggested that the malaria parasites grew as well in culture with old RBCs as in freshly isolated RBCs (Jensen and Trager, 1977). These results were surprising as fresh blood is more likely to be able to supply the parasite with comfortable environment and nutrients than older RBCs (Fairlamb et al., 1985; Ono and Nakabayashi, 1990).

It is also known that the membrane of RBC becomes more fragile when RBCs are stored; this may lead to an unsuitable environment for completion of schizogony (Schuster, 2002). Recently, it has been suggested that the rate of malaria multiplication *in vitro* was higher in cultures prepared with fresh RBCs than in cultures with old RBCs, when the parasitaemia was lower than 8% (Kim *et al.*, 2007).

1.10 Molecular genetics of *Plasmodium falciparum* malaria

The genome of *P. falciparum* published in 2002 revealed the presence of 5484 predicted genes, half of which (57%) are considered hypothetical (of

unknown function), with no similarity of sequence to categorized genes in other species to allow assignment of potential function (Gardner *et al.*,2002). This is a 23-megabase nuclear genome, the 14 chromosomes ranging in size from 0.643 to 3.29 Mb. The genome is highly A+T-rich: overall is about 80.6%(Gardner *et al.*, 2002).

1.10.1 Experimental genetic crosses in P. falciparum

Analysis of the progeny resulting from genetic crosses between two parasite strains with different phenotypic characteristics allows the genetic causes of natural phenotypic variation to be determined. To date, three crosses have been successfully carried out and published. These are crosses between the parasite clones 3D7 and HB3 (Walliker *et al*, 1987), between HB3 and DD2 (Wellems *et al*, 1990) and between GB4 and 7G8 (Hayton *et al*, 2008). The first experimental genetic cross with *P. falciparum* was performed in 1985 by Walliker and colleagues (Walliker *et al*. 1987). Recombinant progeny clones obtained from the cross were genetically different from either parent, and their phenotypes were different from their parents. Many recombinant progeny were obtained from the original cross, some of which were used in this study. The procedure for carrying the experimental cross is shown in figure 1.4.

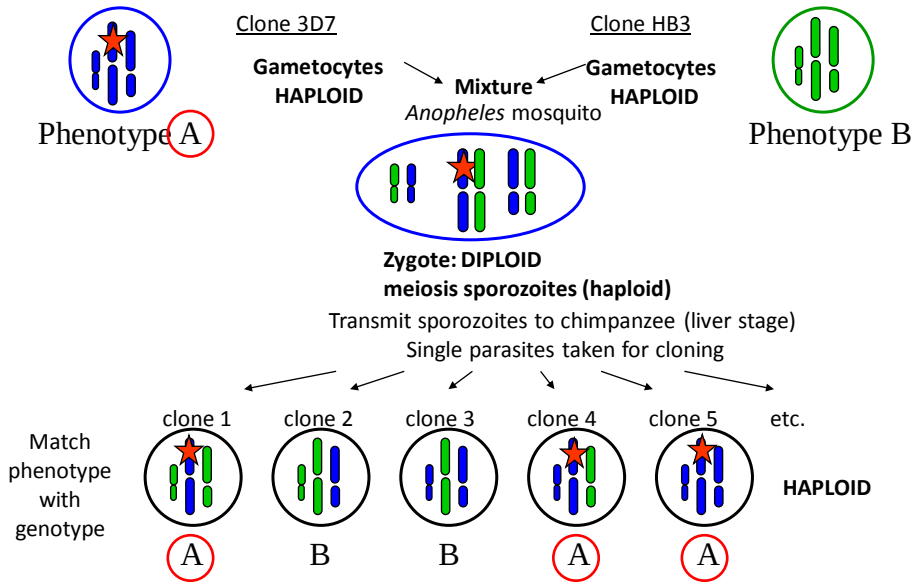

Figure 1.4 Experimental genetic cross between clone 3D7 and clone HB3.

1.11 Quantitative trait locus (QTL) analysis

Linkage analysis refers to a statistical method employed to identify areas of a genome of an organism that influence a specific phenotype of interest. Generally, a linkage analysis begins with an experimental cross, involving two parents with different phenotypes, followed by phenotypic and genotypic analysis of their progeny using a detailed genetic map to identify the genomic regions inherited from each parent (reviewed by Ranford-Cartwright and Mwangi, 2012). A linkage map shows the position of its known genes relative to each other in terms of recombination frequency; genes that are located next to each other on a chromosome are less likely to be separated onto different chromatids during the process of meiotic crossover. With the haploid nature of *Plasmodium* at the blood stage, in addition to the high recombination rates during the sexual stage of its life cycle, genomic regions of interest can be mapped to short stretches of DNA with high precision using linkage analysis (Su *et al.*, 1999; Su *et al.*, 1997).

In 1987, Walliker and colleagues created an experimental cross between two parental clones of *Plasmodium* parasite, clones 3D7 and HB3 (Walliker *et al.*

1987). They showed that the recombinant progeny were genetically different from either parent, and their phenotypes were different from their parents. Many recombinant progeny were obtained from the original cross, some of which were used in this study.

A Quantitative trait Locus is a stretch of DNA containing the genes that underlie a quantitative trait. In other words, a QTL is a genetic locus that contributes to the variation of a quantitative trait (Broman and Sen, 2009). QTL analysis is a statistical method that links phenotypic data and genotypic data and is used to explain the genetic basis of variation in complex traits (Lynch & Walsh, 1998; Falconer & Mackay, 1996). QTL mapping requires polymorphic markers spread through the genome, and microsatellite, Amplified fragment length polymorphism (AFLP) markers or Single nucleotide polymorphisms (SNPs) markers are the most commonly used. A methodology and programme to perform QTL analysis (R/QTL) was developed by Broman and Sen (2009) using the R statistical software platform. The steps involved in QTL analysis with this programme are detailed in chapter 2 of this thesis.The outcome using the program is discussed in detailed in chapter 4 (section 4.6) of this thesis.

1.11.1 LOD scores

LOD Scores (Log of Odds) are a statistical assessment that is used for linkage analysis in eukaryotes such as human, animal and plants. The LOD Score was developed by Morton (1955) and it relates the obtained data of two linked loci with the possibility of accidentally getting the same data. The evidence for the presence of QTL is indicated by large LOD scores (Morton, 1955). Strachan and Read (1999) described the method in detail and it works as follows: firstly, establishment of a pedigree. Secondly, estimation of recombination frequencies has to be made (Strachan and Read, 1999). Third, LOD score calculation is then performed for each estimate; the highest LOD score of an estimate favours the best estimate (Broman and Sen, 2009). The evidence for considering linkage is when the LOD score exceeds 3.0 as it indicates that the linkage did not happen by chance; linkage is definitely excluded when LOD score is less than 2.0.

2 Materials and Methods

2.1 Preparation of culture media

All the parasites used in the experiments described in this thesis were cultured in RPMI 1640 medium supplemented with 5.94g/L HEPES (Sigma) and 50mg/L hypoxanthine (Sigma) (incomplete medium) to which 0.21% NaHCO3 (w/v) and 10% heat-inactivated normal human serum (group AB) were added (complete medium). Heat-inactivation of complement present in the human serum was performed at 56^{o}C for 1 hour prior to use.

2.2 Processing of red blood cells for parasite culture

Fresh human whole blood used for the parasite cultures was obtained from the Glasgow and West of Scotland Blood Transfusion Service (any blood group). Prior to use, the blood was washed to remove the plasma, buffy coat and anticoagulant by centrifugation at 630 *x* g for 10 minutes. The supernatant was then removed and the pellet was re-suspended in an equal volume of incomplete RPMI 1640. The washing was repeated twice more, and then the erythrocytes were finally re-suspended in an equal volume of incomplete RPMI 1640 (50% haematocrit).

2.3 Culturing of malaria parasites

Human erythrocytes infected with *P. falciparum* were maintained in continuous culture using a modification of the standard method originally described by the groups of Trager and of Haynes (Trager and Jensen, 1976; Haynes et al., 1976). Parasites were grown in a 5% suspension of human erythrocytes in complete RPMI 1640 medium, maintained at 37 °C under a gas mixture of 1% O_2, 3% CO_2, and 96% N_2. Changing of the medium was done daily and the culture was kept below 8% parasitaemia at all times.

2.4 Making and staining of thin smears for evaluation of parasitaemia by microscopy

A thin blood film was prepared on a clean microscope slide and allowed to air dry. The film was fixed by brief immersion in absolute methanol. After allowing the alcohol to completely evaporate, the blood film was stained in a jar containing freshly prepared 5% Giemsa stain for 40 minutes. After staining, the slide was removed, rinsed with tap water, air-dried and examined under the light microscope at 1000x magnification. Parasitaemia was determined by counting both uninfected and parasite-infected erythrocytes in twenty five microscopic fields and expressing the parasite-infected erythrocytes as a percentage of the total erythrocytes.

2.5 Plasmion enrichment of culture

Parasite-enriched cultures used for all the experiments described in this study were obtained using the Plasmion enrichment method (Lelievre *et al.*, 2005). Late trophozoites and schizonts exhibit a slower sedimentation rate through a gelatin solution (Jensen, 1978), thus allowing their separation from earlier parasite stages and from uninfected erythrocytes. In this method, 5 to 25mL of parasite culture with a good proportion of late stage parasites at a parasitaemia of approximately 3-6% was initially centrifuged at 232 × g for 10 minutes and the supernatant discarded. Three volumes of RPMI 1640 were added to the pellet followed by an equal volume of Plasmion (Laboratoire Fresenius Kabi, France). The suspension was incubated at 37 ^{o}C for 30 minutes. Finally, the mixture was centrifuged at 232 × g for 4 minutes followed by the removal of the supernatant and re-suspension of the pellet in 10 volumes of RPMI 1640 (Lelievre *et al.*, 2005). The resulting material consisted of late trophozoites and schizonts enriched approximately 10-13-fold.

2.6 Determination of the selectivity index of *P. falciparum*

The relationship between parasitaemia and selectivity index was investigated, under shaking and static culture conditions. A Plasmion-enriched culture of *P. falciparum* clone 3D7 (section 2.5) was diluted to obtain initial

parasitaemia levels of 0.1%, 0.2%, 0.6%, 1% and 2% in 25cm^2 flasks in a total volume of 6ml each. 3ml of this was placed into each of two 25cm^2 flasks, one of which was incubated in a shaking incubator (set at 120 rpm) for 18 hours at 37ºC whilst the other flask was kept static in an incubator for 18 hours at 37ºC.All flasks were set up in triplicate and 3 independent experiments were performed. The diluted cultures were incubated simultaneously at 37ºC for 18 hours. Smears were made as previously described and the number of red blood cells with 1,2,3 etc parasites was determined. The selectivity index at each starting parasitaemia was calculated using the formula described in section 2.6.1 below.

The selectivity index of clones 3D7 and HB3 of *P. falciparum* was then determined in a similar manner. Cultures of clones 3D7 and HB3 clones were enriched using the Plasmion method (section 2.5). The parasitaemia for each clone was adjusted to 1% in a 6ml volume by dilution with uninfected blood. 3ml of this was placed into each of two 25cm^2 flasks, one of which was incubated in a shaking incubator (set at 120 rpm) for 18 hours at 37ºC whilst the other flask was kept static in an incubator for 18 hours at 37ºC. After incubation, the parasitaemia in each flask was determined by Giemsa staining. Selectivity index for each clone at the set condition was calculated using the formula in section 2.6.1 below. All flasks were set up in triplicate and three independent experiments were performed for this investigation.

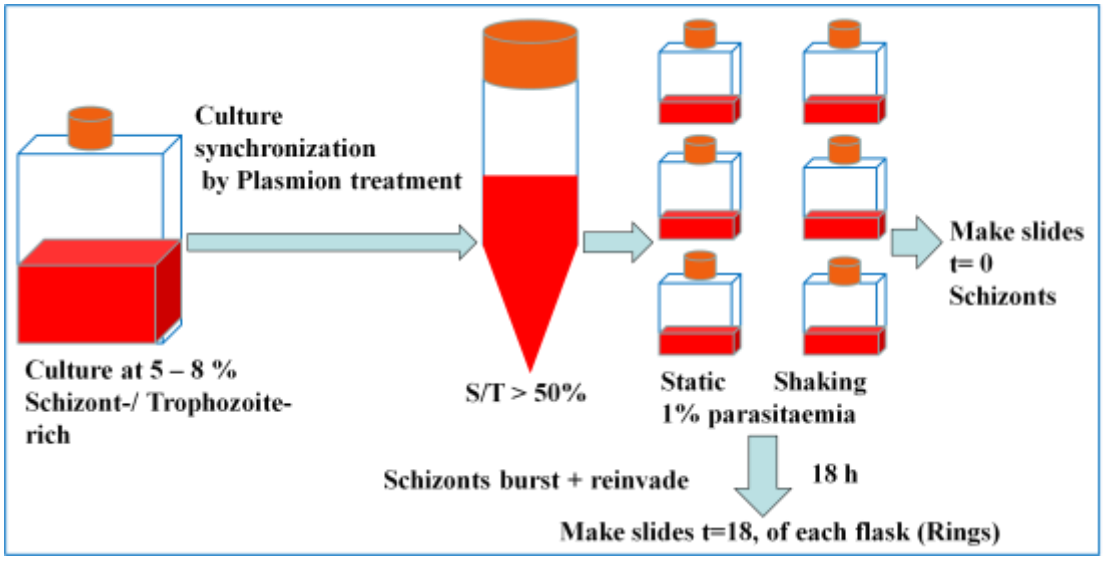

Figure 2.1. Schematic diagram summarising the procedure to measure SI under shaking and static culture conditions. S=Schizont, T=Trophozoite. Plasmion enriched schizont cultures of 3D7 and HB3 diluted to 1% parasitaemia.

To investigate if selectivity index varied between parasites that had been grown in continuous culture for different periods of time, 3D7 cultures were maintained in continuous culture and selectivity index was measured at 13, 20, 37, 51 and 77 days post-thawing from liquid nitrogen, using the methods described in section 2.6

2.6.1 Calculation of Selectivity Index

Parasitaemia was determined by counting both uninfected and parasite-infected erythrocytes in at least ten microscopic fields and by expressing the parasite-infected erythrocytes as a percentage of the total number of erythrocytes (section 2.4).

The number of cells expected to have one or more parasites for a given parasitaemia, assuming random invasion, was calculated from a poisson distribution ($e^{-\mu}\ \mu^x/x!$), where μ = parasitaemia, x= the number of parasites within one cell (Simpson *et al*, 1999). For example, at a parasitaemia of 5% (μ = 0.05), the probability of an RBC having one parasite (x=1) is calculated as

$e^{-0.05}\ 0.05^1/1! = 0.0476$

The expected number of RBC that would have a single RBC is calculated as the [probability of having one parasite/ probability of having any parasites] multiplied by the number of parasitised cells counted (Simpson *et al*, 1999).

In the example given, if 100 parasitised RBC were counted, 97.5 would be expected to contain a single parasite ([0.0476/(1-0.951) x 100]. By substituting the equation with values of x from 2 onwards, the predicted number of red blood cells with 1 to 4 parasites can be calculated as shown in table 2.1.

RBC with X parasite	Predicted no. out of 100 parasitised RBC
1	97.5
2	2.44
3	0.04
4	0.00051

Table 2.1. The number of red blood cells with one to four parasites, at a parasitaemia of 0.05%, as predicted from a poisson distribution, within 100 parasitised erythrocytes.

Selectivity Index (SI) is defined as the ratio of multiply infected cells that were observed to those estimated using the Poisson equation (Simpson *et al* 1999). In the example, if the observed numbers of RBC (out of 100 parasitised cells counted) with a single infection was 97, and 3 RBC had infections with more than one parasite, then SI can be calculated as:

$$\mathrm{SI} = \frac{\text{Observed number of multiply-infected RBC(O)}}{\text{Expected number of multiply-infected RBC(E)}} = \frac{3}{100-97.5} = \frac{3}{2.5} = 1.2$$

The SI of 1.2 indicates that the parasite in this example did not have significantly different numbers of multiply invaded cells compared to that expected from a random (Poisson) distribution.

The selectivity index for each clone for each experiment was measured using six flasks, three for static conditions and three for shaking conditions. Each experiment was repeated at least three times, giving a total of 9 flasks for each clone for each of the two culture conditions (shaking and static). Twenty five fields (approx 3000 RBC) on each slide were examined.

2.7 Statistical Analysis

2.7.1 Comparison of Selectivity Index between parental and progeny clones

The selectivity index obtained for each progeny clone was compared to each of the two parent clones, to assess if the phenotype was parental or non-parental. Each progeny clone was also compared to each of the other progeny clones to assess how many different non-parental phenotypes existed in the cross.

For each parasite clone, variation in SI obtained across the three replicates (3 flasks per replicate) for each clone and treatment was assessed using ANOVA. Statistically similar replicates were pooled to give an overall mean and SEM.

For each progeny clone, the SI obtained under static and shaking culture conditions were compared using t-tests. T-tests were also used to compare each

progeny clone with each of the two parental clones. P values for significance were adjusted for multiple comparisons using a Bonferroni correction. All statistical analyses were performed using Excel.

2.7.2 Analysis of changes in selectivity index over time in continuous culture

An analysis was performed to assess if SI changed during continuous culture of clone 3D7. The difference in SI over time was analysed by ANOVA performed using Excel.

2.8 Quantitative Trait Loci (QTL) analyses

To investigate possible quantitative trait loci linked to SI under shaking and static conditions, a QTL analysis was performed using the statistical package R/qtl (Broman and Sen, 2009). There are three steps in the analysis: (i) establishment of a genetic map of the cross using genomic markers; (ii) association of the inheritance of the markers with the phenotype, and (iii) location of the QTL on the genetic and physical map (Broman and Sen, 2009).The steps are described in detail in chapter 4.

3 Phenotypic analysis of selectivity index *in vitro* in parasites cultured under static and shaking conditions

3.1 Introduction

An important feature of an infection with *Plasmodium falciparum* is the multiple invasions of erythrocytes. These multiply-invaded cells are commonly observed in malaria infections where parasitaemia is high. The multiple invasions of erythrocytes has been described as partly responsible for the severe effect of the disease (Pasvol et al., 1980; Pasvol et al., 1982b; Clough et al., 1998). If multiple invasions of erythrocytes occur randomly, it would be expected to follow a Poisson distribution (Simpson *et al.*, 1999). A positive deviation away from random invasion (indicating more multiple invasions than expected under a poisson model) suggests selectivity of the parasite for a smaller population of erythrocytes within the blood. The reason for invasion of these specific erythrocytes is unknown.

Selectivity index (SI) is the term used to describe an individual parasite's preference for erythrocytes. Selectivity index is defined as the ratio of number of multiply-infected red cells observed (i.e. with more than one parasite present within a single RBC) to that expected from random invasion, as modelled by a Poisson distribution for a given parasitaemia (Simpson *et al.*, 1999).

Increasing SI values are associated with a greater selectivity of parasites for red cells, meaning more multiply-invaded cells than expected. A low SI associated with an infection suggests a relative absence of selectivity by the parasite for red cell, such that parasites do not discriminate between RBCs, and multiple invasion events do not occur more frequently than expected by chance (or as modelled by a Poisson distribution). High SI values on the other hand suggest that parasites are able to distinguish between RBCs and that some RBCs are selected for invasion more than others. Chotivanich and colleagues reported that severe malaria caused by *P. falciparum* in adult Thai patients showed reduced selectivity *in vivo* in RBC invasion compared to infections causing uncomplicated malaria; they examined the parasite factors by measuring differences in RBC selectivity and the parasites' reproduction capacity when

isolated from both patients, with severe and uncomplicated malaria *in vitro* (Chotivanich *et al.*, 2000). They concluded that there was an inverse relationship between SI and disease severity. However Deans and colleagues found no significant association between the SI and malaria severity in children in Mali and Kenya (Deans *et al* 2006), and they suggested that there could be differences in the virulence mechansisms between *P. falciparum* from sub-Saharan Africa and Asia.

Various factors could influence the parasite's selection of erythrocytes for invasion. Selectivity Index differences observed between infections in different people could be influenced by the genotype of the infecting parasite, which is the topic of this thesis. The blood group (including minor red cell variants) of the host could also influence SI, although there is no published data on this hypothesis. It has been reported that blood group O may be protective against severe malaria, while blood group A patients' were more susceptible to severe malaria, but the mechanism is believed to be due to a difference in rosetting and not to SI (Rowe *et al.*, 2007). Due to the limited information on the relation between SI and blood group the influence of ABO on SI in *P. falciparum* infection was determined in this research.

Rosetting is a phenomenon which occurs in *Plasmodium* infection, whereby an infected erythrocyte adheres to two or more non-infected erythrocytes, forming a clump of red cells surrounding the parasitised cell (Chotivanich *et al.*, 1998). This adhesion appears to be mediated by a subset of *var* genes that encode variants of PfEMP1, binding to an erythocyte protein called complement receptor 1 (CR1) (Rowe *et al.*, 2007). Rosetting is likely to increase SI because invasion of red cells within the rosette is more likely than invasion of non-adhered circulating red cells.

Within an infection, host antigens will be invariant, but red cell age could be a factor influencing invasion preference, since within a human host the red cell population will vary from erythrocytes newly emerged from the bone marrow to cells of up to 120 days old. RBC aging, senescence and *in vivo* death signalling pathways have been reviewed by Antonelou and colleagues (Antonelou *et al.*, 2010). RBCs were reported to go through a range of continuous metabolic

and physical damages as they age, including membrane vesiculation (Willekens *et al.*, 2003), haemoglobin (Hb) modifications and progressive failure of both cellular homeostasis and antioxidant defences. An increase in RBC density (Piomelli and Seaman, 1993), the nonenzymatic glycation of Hb (Bosch *et al.*, 1992) and the deamidation of some proteins (Lutz *et al.*, 1992; Mueller *et al.*, 1987) have been widely used as sensitive RBC age markers. Oxidation, phosphorylation, and aggregation of RBCs have all been implicated in the regulation of RBCs homeostasis and lifespan of the RBC (Antonelou *et al.*, 2010). In this study erythrocytes were always from material drawn within the last 7 days from healthy donors; red cell age has not been assessed as a factor contributing to differences in SI.

As reported in this chapter, the effect of growing parasites under shaking and static conditions was investigated separately. Growing parasites under static conditions was chosen to mimic rosette formation, where merozoites emerging from the schizont are more likely to invade the red cells immediately surrounding them. Shaking culture conditions, where red cells are kept in suspension, was chosen to mimic normal blood flow conditions in the absence of rosetting.

Previous research has shown a relationship between level of parasitaemia and SI, with lower SI recorded in patients with higher parasitaemia (Simpson *et al.*, 1999) whilst a study by Deans and colleagues in Africa did not find any relationship (Deans *et al.*, 2006). The effect of parasitaemia on SI measurements *in vitro* will also be presented in this chapter.

3.2 Aims

The aims of the research described in this chapter were:

- To investigate the effect of level of parasitaemia on selectivity index *in vitro;*
- To ascertain the effect of growing parasites under static and shaking conditions on selectivity index;
- To investigate the stability of SI for a specific clone over extended periods of time in continuous culture;

- To ascertain the relationship between SI and blood group (ABO)
- To measure SI in the parents and progeny of a genetic cross, to investigate the influence of parasite genetic differences on selectivity index.

3.3 Materials and Methods

3.3.1 Effect of parasitaemia on Selectivity Index

The relationship between parasitaemia and selectivity index was investigated, under shaking and static culture conditions. A culture of of *P. falciparum* clone 3D7 was synchronised using the previously described sorbitol method (Lambros and Vanderberg, 1979) and the schizont-stage parasites concentrated using the Plasmion method (Lelievre *et al.*, 2005), as described in section 2.5. The culture was diluted to obtain initial parasitaemia levels of 0.1%, 0.2%, 0.6%, 1% and 2% in a total volume of 6ml. Three millilitre of each culture was placed into each of two 25cm^2 flasks. One of the flasks was incubated in a shaking incubator (set at 120 rpm) for 18 hours at 37ºC whilst the other flask was kept static in an incubator for 18 hours at 37ºC. All flasks were set up in triplicate and 3 independent experiments were performed. Smears were made as previously described (section 2.4) and the selectivity index at each starting parasitaemia was calculated using the formula described in section 2.6.1.

3.3.2 The effect of growing parasites under static and shaking conditions on selectivity index

Identical experiments were set up for cultures that were then grown in a shaking incubator or as static cultures as described in section 3.3.1 above. For a given starting parasitaemia, the SI obtained for static and shaking cultures were compared, for each clone, using Student's t-test.

3.3.3 The stability of SI for a specific clone over extended periods of time in continuous culture.

The variation in SI obtained from parasites that had been in continuous culture for different periods of time was investigated using parent clone 3D7, under both shaking and static culture conditions. SI was measured at five different occasions on days 14, 21, 35, 56, and 77 for the same culture over a time period up to 77 days of continuous culture from the date of thawing parasites from nitrogen storage.Changes in selectivity index during the continous culture were ascertained using Excel.

3.3.4 Influence of genetic differences on selectivity Index

The effect of genetic differences between parasites on SI was investigated using *P. falciparum* clones 3D7 and HB3. Cultures of clones 3D7 and HB3 clones were enriched for schizonts as previously described using the Plasmion method (section 2.5). The parasitaemia for each clone was adjusted to 1% in a 6ml volume by dilution with uninfected blood (the starting parasitaemia used for the determination of SI was selected based on the outcomes of the experiments described in section 3.3.1 above). 3ml of the culture was placed into each of two 25cm^2 flasks, one of which was incubated in a shaking incubator (set at 120 rpm) for 18 hours at 37ºC whilst the other flask was kept static in an incubator for 18 hours at 37ºC. After incubation, the parasitaemia in each flask was determined by microscopy after Giemsa staining. Selectivity index for each clone at the set condition was calculated as usual (section 2.6.1). All flasks were set up in triplicate and three independent experiments were performed for this investigation. The significance of any difference in SI between the two parent clones was tested using Student's t-test.

SI was then determined in 18 progeny clones from the genetic cross between clones 3D7 and HB3 (Walliker *et al.,* 1997). The progeny clones were selected from those available because they were known to be genotypically different from one another and to be recombinants (Ranford-Cartwright and Mwangi, unpublished data). SI was determined under both shaking and static conditions, as described in section 3.3.1, with nine replicate flasks per clone and culture condition, and three experimental replicates. The significance of any

difference in SI between each progeny clone and either of the two parent clones was tested using the multiple t-tests.

3.4 Results

3.4.1 Effect of parasitaemia on selectivity index

SI measurements descreased as parasitaemia increased under both static and shaking culture conditions (Figure 3.1). The SI obtained for cultures started at a parasitaemia of 0.1% was significantly different to cultures started at 1% and 2% for both static (P=0.001) and shaking (P=0.001) culture conditions (Figure 3.1). For cultures incubated under both static or shaking conditions, the difference in SI between the starting parasitaemias and 2% parasitaemia were observed to be statistically significantly (P<0.001 in all cases).

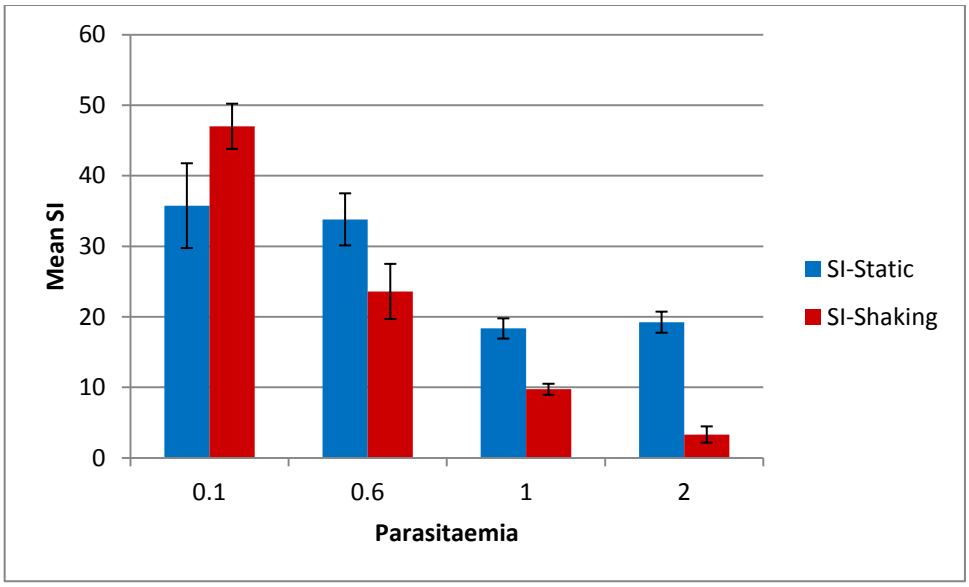

Figure 3.1 Selectivity index for parasite clone 3D7 at different starting parasitaemias of 0.1, 0.6, 1 and 2%, grown in a shaking incubator (red bars) or as static cultures (blue bars). The mean SI was calculated from three flasks. Error bars are the standard error of the mean.

From a preliminary analysis of the outcome of this experiment a decision was made to use a starting parasitaemia of 1% for the remaining experiments because of the following: (i) there was a clear difference of SI under static and shaking conditions, (ii) the final parasitaemias obtained at a starting parasitaemia of 0.1% or 0.6% were very low, and it took a long time to count sufficient numbers of infected cells to calculate SI, and (iii) a starting

parasitaemia of 2% resulted in a very high parasitaemia and death of some parasites.

3.4.2 Effect of culture conditions (static and shaking cultures) on Selectivity index

Further investigation of the effect of culture conditions on SI were performed at a starting parasitaemia of 1%. The SI obtained in three replicated experiments with three flasks of each parent clone in each experiment was compared for cultures grown in static conditions and under agitation. The results are shown in Figure 3.2.

Higher SI values were observed for both parasite clones incubated under static conditions compared to when they were agitated (shaking) (t-tests for 3D7: $P=2.91x10^{-19}$; for HB3, $P=7.85x10^{-12}$). HB3 appears to have higher SI under both static and shaking culturing conditions (Figure 3.2).

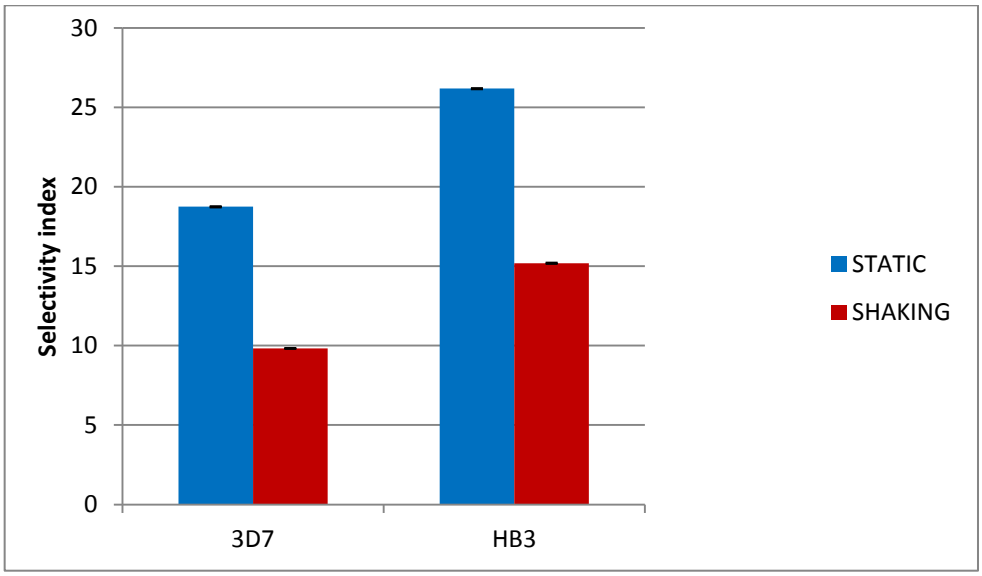

Figure 3.2 Comparison of SI of *P. falciparum* clones 3D7 and HB3 under static (blue bar) and shaking (red bar) culturing conditions. The mean SI was calculated from three flasks. Error bars are the standard error of the mean.

3.4.3 Effect of erythrocyte blood group on selectivity index

The erythrocytes used in each experiment were from different donors (fresh blood is obtained weekly from the Blood Transfusion Service). For clone 3D7, the SI was measured on thee replicate as this clone was always included as a control. The effect of ABO blood group on the SI is shown in Figure 3.3. The results show that ABO blood group has no effect on SI obtained for a given parasite clone (ANOVA, P = 0.87; shaking culture; P =0.84; static cultures).

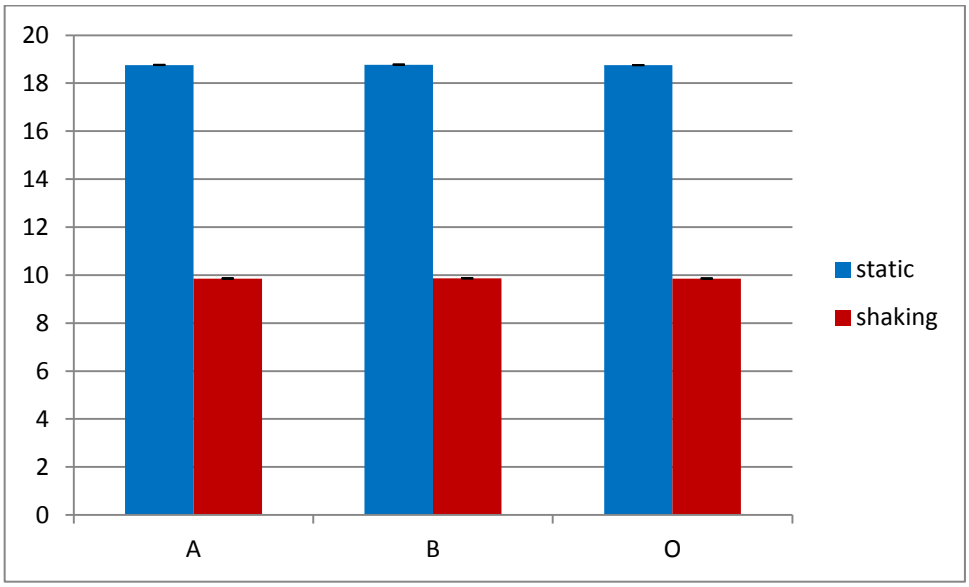

Figure 3.3 Effect of ABO blood group on SI for clone 3D7. The mean SI for clone 3D7 for each blood group (ABO) is shown. Error bars represent the SEM. SI was measured in separate experiments for blood group O, blood group A, and blood group B. Insufficient experiments were performed using blood group AB.

3.4.4 The stability of SI for a specific clone over extended periods of time in continuous culture.

The variation in SI obtained from parasites that had been in continuous culture for different periods of time was investigated using parent clone 3D7, under both shaking and static culture conditions.

SI was measured at five different occasions for the same culture over a time period up to 77 days of continuous culture from the date of thawing parasites from nitrogen storage. The results are shown in figure 3.4.

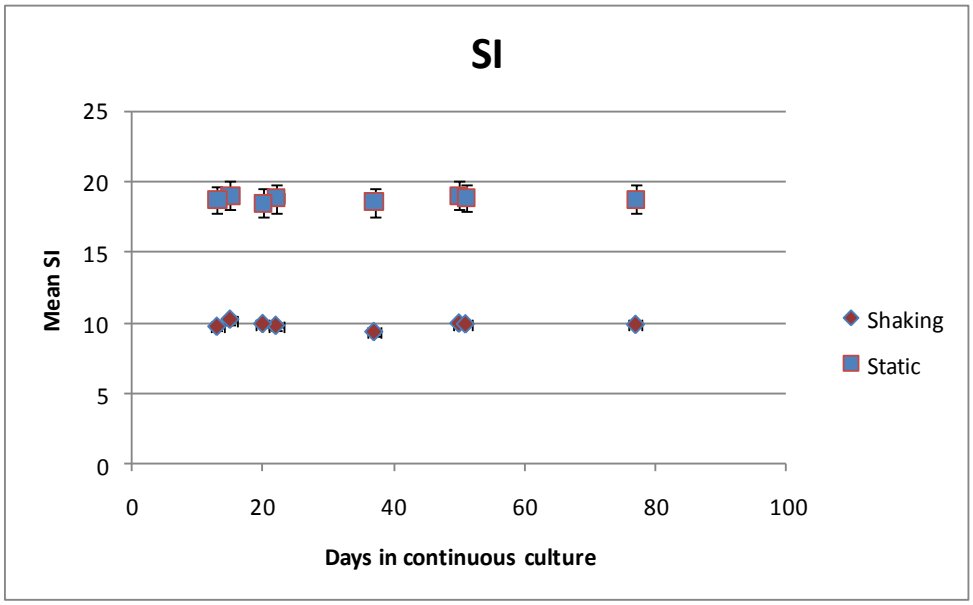

Figure 3.4. Relationship between mean selectivity index and days in continuous culture under shaking and static conditions for clone 3D7 kept in continuous culture for 77 days. Each symbol represents the mean SI for 9 flasks. Error bars are the standard error of the mean.

The results show that SI for one parasite clone did not change over a period of 77 days in continuous culture (Figure 3.4). There was no significant difference in the SI obtained for clone 3D7 at the 5 time points (ANOVA, P = 0.84; shaking culture; P =0.77; static cultures).

3.4.5 Variation in Selectivity index obtained with parasite clones 3D7 and HB3 under static and shaking culture conditions

The SI value for HB3 was significantly higher than that of 3D7 under both static and shaking conditions (Figure 3.2) (t-test- shaking conditions: $P=3.45x10^{-14}$; static conditions: $P=3.25x10^{-09}$).

3.4.6 Variation in Selectivity index obtained with progeny clones, under static and shaking culture conditions

To investigate the inheritance of the difference between 3D7 and HB3, SI was measured for eighteen progeny clones under static and shaking culture conditions. The outcome of the experiments are summarised in Figure 3.5, Tables 3.1 and Table 3.2.

In most cases, the SI of the progeny clones tested was significantly different to either parent under shaking and static culture conditions, after

Bonferroni correction for multiple comparisons (Table 3.2). The selectivity indices of parent clones and progeny clones were always higher under static conditions than under shaking conditions (Figure 3.5).

P-values				
	STATIC		SHAKING	
Progeny clone	compared to 3D7	compared to HB3	compared to 3D7	compared to HB3
X12	$2.82X10^{-12}$	$5.43X10^{-11}$	$8.17X10^{-14}$	$1.53X10^{-18}$
XP5	$2.43X10^{-07}$	$2.26X10^{-10}$	$1.10X10^{-16}$	$3.87X10^{-16}$
X5	$4.27X10^{-14}$	$7.50X10^{-10}$	$6.28X10^{-10}$	$1.38X10^{-12}$
X30	$3.65X10^{-12}$	$3.77X10^{-08}$	$2.35X10^{-10}$	$9.32X10^{-13}$
XP9	$5.03X10^{-12}$	$1.02X10^{-09}$	0.00018	$3.11X10^{-11}$
X10	$1.79X10^{-05}$	$3.59X10^{-11}$	**0.38**	$4.50X10^{-14}$
X6	$3.50X10^{-11}$	$5.77X10^{-10}$	$2.25X10^{-09}$	$1.26X10^{-11}$
X4	$4.31X10^{-16}$	**0.0018**	$1.98X10^{-11}$	**0.008**
X2	$3.77X10^{-12}$	$5.81X10^{-10}$	$8.1X10^{-09}$	$3.49X10^{-11}$
X33	$4.49X10^{-19}$	**0.0016**	$2.4X10^{-09}$	$1.3X10^{-11}$
XP24	$1.79X10^{-13}$	$4.39X10^{-07}$	**0.6504**	$9.9X10^{-11}$
X50	$5.4X10^{-13}$	$3.15X10^{-10}$	$3.36X10^{-10}$	$5.74X10^{-10}$
XP3	$2.7X10^{-09}$	$2.55X10^{-09}$	$6.17X10^{-08}$	$6.04X10^{-11}$
XP4	$6.1X10^{-17}$	$5.29X10^{-05}$	$3.89X10^{-05}$	$2.39X10^{-11}$
XP52	0.000103	$2.32X10^{-08}$	$6.52X10^{-08}$	$4.11X10^{-11}$
X11	$6.2X10^{-10}$	$1.18X10^{-07}$	**0.634**	$5.97X10^{-10}$
X44	$4.6X10^{-22}$	$5.03X10^{-05}$	0.000155	$2.49X10^{-10}$
X39	$1.1X10^{-13}$	$1.76X10^{-10}$	$1.94X10^{-09}$	$2.29X10^{-11}$

Table 3.1. Statistical comparison of SI for each progeny clone with that of the two parents. The mean values were compared between shaking and static cultures for each progeny using t-tests, and the P values are shown. Non significant P values are shown in bold text. Bonferroni corrections for multiple comparisons were applied so that the adjusted significance level was 0.0007.

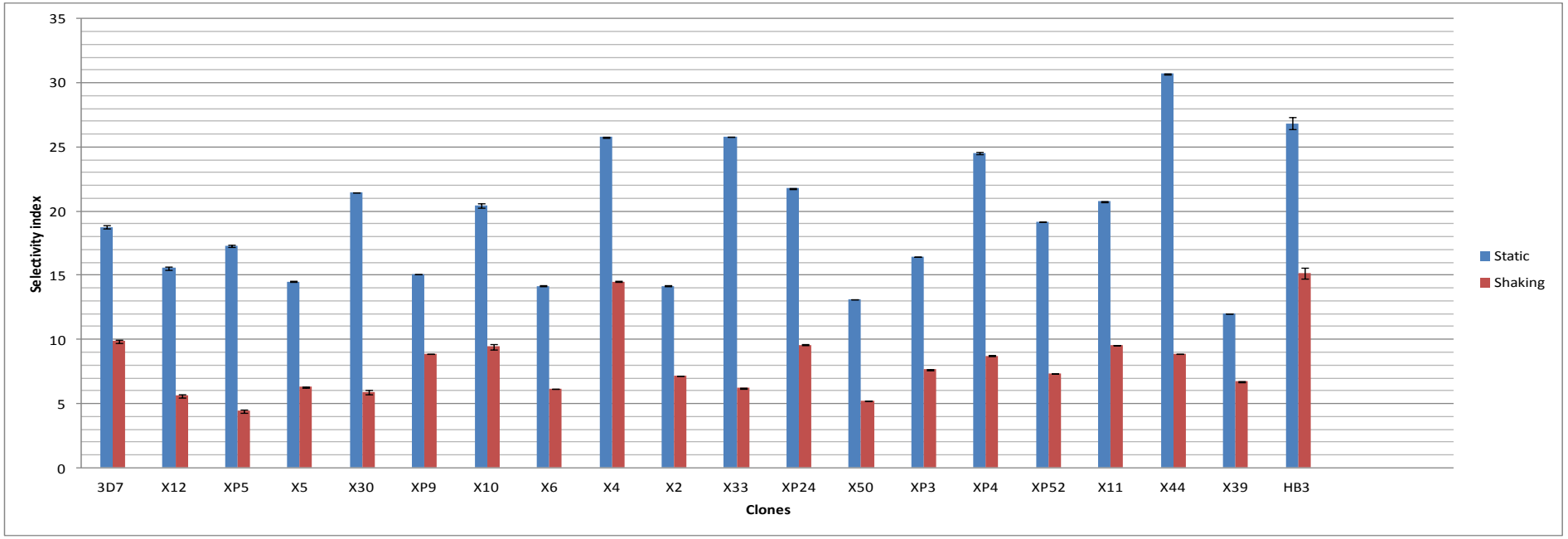

Figure 3.5. Selectivity indices of parent clones 3D7 and HB3, and those of eighteen progeny clones, under static and shaking culture conditions are shown. The bars represent the mean of 9 flasks. Error bars = standard error of the mean.

Under shaking culture conditions, four progeny clones out of the eighteen had parental phenotypes. Three progeny clones, X10, XP24 and X11 had an SI that was not statistically different from parent clone 3D7 (P=0.38(X10), P=0.65(XP24) P=0.63(X11)), and clone X4 was not significantly different to parent clone HB3 (P=0.008) (Table 3.1). All other clones were significantly different to either parent clone.

Under static culture conditions, two progeny clones were parental in phenotype: both X33 and X4 had SI values not statistically different to parent clone HB3 (P=0.0016 (X33) and P=0.0018 (X4).

The results obtained were summarised as to parental and non-parental types and are shown in table 3.2. Under shaking conditions, the fifteen progeny clones with non-parental phenotypes had SI measurements below that of either parent. Under static culture conditions, ten progeny clones had lower SI than either parent, and five had SI that were intermediate between the two parents.

Comparisons of SI between the progeny clones revealed that under static conditions (Appendix 1), most clones had statistically different SI to one another. After correction of the threshold for significance for multiple comparisons (threshold for significance is P=0.0007), nine pairs of progeny clones had statistically indistinguishable values of SI: XP9 and X12 (P=0.005), X10 and X30 (P=0.002), X6 and X2 (P= 0.91), X33 and X4 (P = 0.80), X12 and XP9 (P=0.005), XP24 and X30 (P=0.05), XP24 and X10 (P=0.003), XP52 and X10 (P=0.0005), X11 and X10 (P=0.24).

Under shaking culture conditions (Appendix 1), twenty one pairs of progeny were statistically similar (threshold for significance P=0.0007): X33 and X6 (P=0.42), X30 and X12 (P=0.05), X6 and X12 (P=0.0015), X33 and X12 (P=0.0014), X5 and X6 (P=0.2), X33 and X5 (P=0.04), XP9 and X10 (P=0.0085), XP24 and X10 (P=0.5), X50 and X12 (P=0.02), X50 and XP5 (P=0.001), XP4 and XP9 (P=0.002), XP4 and X10 (P=0.003), X11 and X10 (P=0.5), X11 and XP24 (P=0.98), X44 and XP9 (P=0.84), X44 and X10 (P=0.009) and X44 and XP4 (P=0.002).

Progeny clone	Shaking culture conditions	Static culture conditions
X12	Non parental (lower than either parent)	Non parental (lower than either parent)
XP5	Non parental (lower than either parent)	Non parental (lower than either parent)
X5	Non parental (lower than either parent)	Non parental (lower than either parent)
X30	Non parental (lower than either parent)	Non parental (Intermediate)
XP9	Non parental (lower than either parent)	Non parental (lower than either parent)
X10	3D7 like	Non parental (Intermediate)
X6	Non parental (lower than either parent)	Non parental (lower than either parent)
X4	HB3 like	HB3 like
X2	Non parental (lower than either parent)	Non parental (lower than either parent)
X33	Non parental (lower than either parent)	HB3 like
XP24	3D7 like	Non parental (Intermediate)
X50	Non parental (lower than either parent)	Non parental (lower than either parent)
XP3	Non parental (lower than either parent)	Non parental (lower than either parent)
XP4	Non parental (lower than either parent)	Non parental (Intermediate)
XP52	Non parental (lower than either parent)	Non parental (Intermediate)
X11	3D7 like	Non parental (Intermediate)
X44	Non parental (lower than either parent)	Non parental (Higher than either parent)
X39	Non parental (lower than either parent)	Non parental (lower than either parent)

Table 3.2 Classification of SI of progeny clones under static & shaking conditions. SI for each progeny clone was compared to each parent using t-test, with Bonferroni correction for multiple comparisons.

3.4.7Effect of parasitaemia on Selectivity index in the progeny clones

Similar to what was investigated for the parental clones the relationship between the SI and parasitaemia was assessed for the progeny clones. The experiment was performed with progeny clones starting at 1% parasitaemia. Because of difference in growth rate in different progeny clones, presumably as a result of different invasion efficiencies and/or merozoites produced per schizont, there was some variation in the parasitaemia observed 18h after incubation of mature schizonts with uninfected erythrocytes (Figures 3.6 and 3 7).

There was an inverse relationship between parasitaemia and SI (Figures 3.6 and 3.7). Under both static (Figure 3.6) and shaking (Figure 3.7) conditions, the higher the final parasitaemia, the lower the selectivity index measured. Final parasitaemia under shaking conditions was always higher than under static conditions, as expected given the higher efficiency of invasion with shaking cultures.

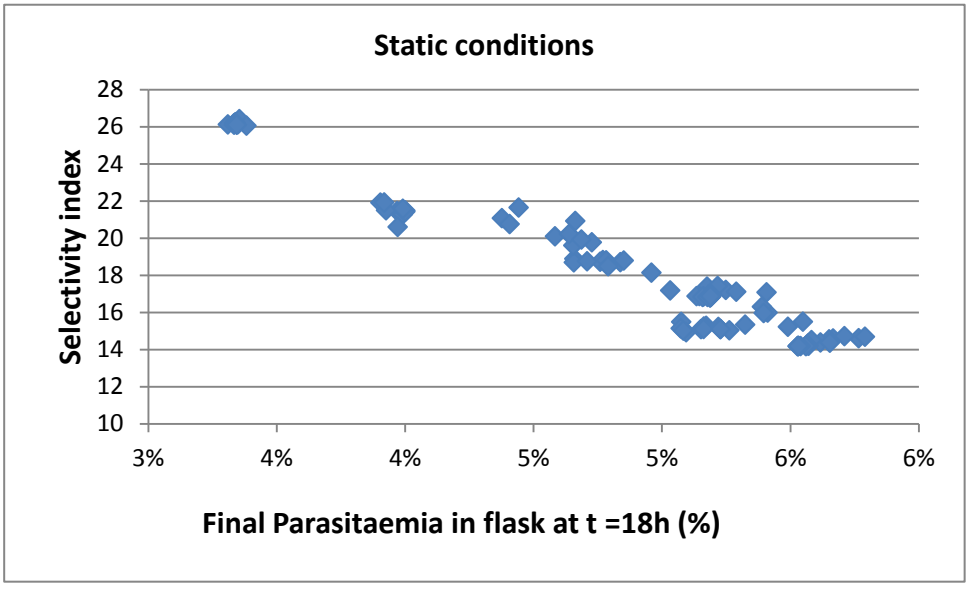

Figure 3.6. The relationship between Selectivity index and parasitaemia under static culture conditions for the 18 progeny clones. Each experiment was run in triplicate, and the individual experiment results are shown on the graph.

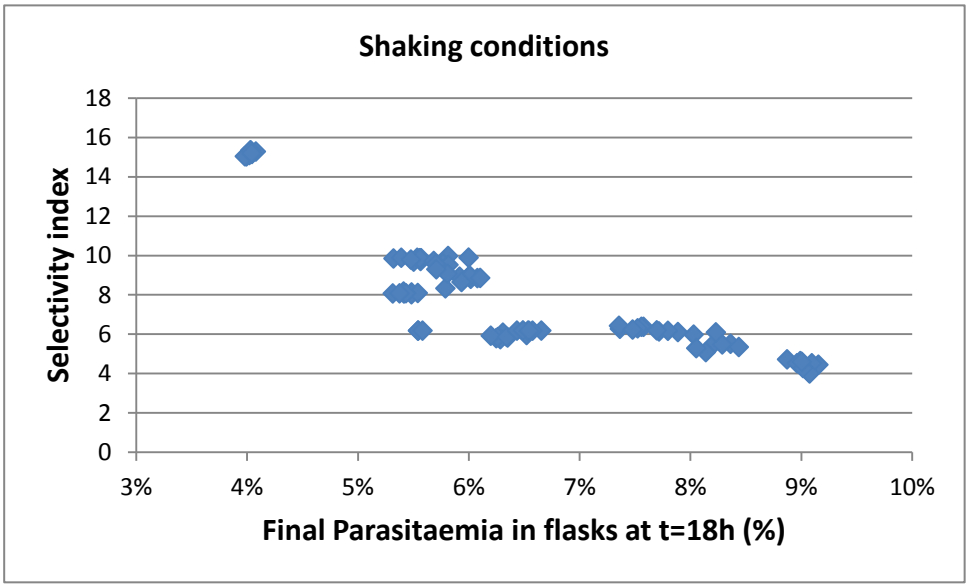

Figure 3.7. The relationship between Selectivity index and parasitaemia under static culture conditions for the 18 progeny clones. Each experiment was run in triplicate.

3.5 Discussion

The first aim of this chapter was to establish the variation in selectivity index caused by differences in the conditions of culture, such as agitation of cultures, starting parasitaemia and time that the parasites had been in continuous culture.

As had been reported in the literature for *in vivo* infections and *in vitro* cultures (Chotivanich *et al.*, 2000; Simpson *et al.*, 1999), selectivity index was inversely proportional to parasitaemia (Figure 3.1) over the range tested.

Cultures grown with agitation to keep the erythrocytes in suspension (shaking cultures) produced a lower SI than those grown under static conditions. This is not surprising because in static cultures merozoites, which are not motile, will invade the red cells around the bursting schizont, and it is likely that several merozoites will attach to and invade the same red cell, whereas in a shaking cultures, merozoite from a burst schizont will circulate more freely and come into contact with more red cells in suspension. It is known that growth rate is increased in shaking cultures, and that there are fewer multiple invasion events in shaking cultures (Riback *et al.*, 2013). A static culture may mimic the effect of rosetting, where uninfected red cells cluster around an infected schizont,

although previous studies have found no association of rosetting and SI *in vivo* (Deans *et al.*, 2006; Clough *et al.*, 1998).

Selectivity index of a given clone was found to be a stable phenotype that did not vary with the time the parasite had been in continuous culture, either under static or shaking conditions.

Having established the environmental factors influencing SI *in vitro*, a standard parasitaemia of 1% was selected as the starting parasitaemia to measure SI.

The second aim of this chapter was to establish the variation in SI in two parasite clones, 3D7 and HB3, and the inheritance pattern of SI in progeny clones from a genetic cross between 3D7 and HB3 (Walliker *et al.*, 1987). The two parent clones have significantly different SI under both static and shaking culture conditions, with the SI for HB3 higher than that of 3D7 under both culture conditions, suggesting that 3D7 parasites have a lower selectivity for red cells than HB3.

The inheritance of the SI was complex. Under shaking culture conditions, only 4 of the 18 progeny clones examined had SI statistically similar to either of the two parents, and the remaining progeny clones had SI values that were lower than either of the two parents. A similar result was observed under static conditions, with only two parasite clones displaying a parental-type SI, ten exhibiting SI below either parent and five having SI values that were intermediate in value between the two parents.

Since the *Plasmodium* parasite is haploid in the blood stages, if SI was under the influence of a single locus in the genome, progeny clones would be expected to display only 3D7 or HB3 phenotypes, with no intermediates or values higher or lower than the SI seen in the parent clones. The result observed therefore suggests that SI is inherited as a complex or multigenic trait, with contributions from multiple unlinked genes that have been inherited independently in the progeny clones.

A further observation is that progeny clones that displayed parental SI values under shaking conditions could exhibit non-parental SI under static conditions (e.g. clone X10, XP24, X11), or non-parental SI under shaking conditions and parental under static (e.g. clone X33). This suggests that selectivity index is influenced by different genes under the two culture conditions.

In conclusion, the results presented in this chapter indicate that SI is a stable phenotype in a parasite clone at a given starting parasitaemia, that is unaffected by time in culture or blood group of the erythrocytes, but that different parasite genotypes exhibit significantly different SI. This suggests a genetic component of control of SI that is likely to be inherited in a complex manner with contributions from multiple genetic loci in the parasite.

The next two chapters describe the identification of such loci using quantitative trait locus analysis, which allows the examination of the contribution of multiple loci to a given phenotype.

4 Quantitative trait locus analysis to identify genomic regions of the parasites potentially responsible for the selectivity index phenotype

4.1 Introduction

In this chapter, the techniques of quantitative trait locus (QTL) analysis in addition to other information (such as phenotype information and genetic maps) were brought together to map the selectivity index trait of the progeny clones from the cross described previously (between 3D7 and HB3) to particular regions of the genome. This was performed for parasites cultured under shaking or static culturing conditions.

QTL analysis is a statistical method that links phenotypic data and genotypic data which could explain the genetic basis of variation in complex traits (Falconer, 1996; Lynch M and Walsh B, 1998). QTLs are stretches of DNA containing or linked to the genes that underlie a quantitative trait.

Mapping regions of the genome that contain genes involved in specifying a quantitative trait is usually performed using molecular tags such as Amplified fragment length polymorphism (AFLP) or Single nucleotide polymorphism (SNPs). In this chapter, SNP data from whole genome sequencing of the parent and progeny clones have been used to generate genetic maps of the progeny.

Variation in the phenotype may occur as a consequence of changes in the environment together with variation in the phenotype. The process can be summarised as following:

$$\Delta P=\Delta G+\Delta E$$

Where, ΔP=phenotypic variation ΔG=genotypic variation, ΔE=environmental changes.

It must be emphasied that performing QTL allows the genotypic variation to be defined. Further discussion on this relationship is found in the next section (section 4.2) of this thesis.

4.2 Identification of genetic traits by linkage analysis

The sexual stage of the malaria parasite, *P. falciparum*, occurs in the mosquito. During this stage fertilisation of haploid gametes takes place resulting in parasites with diploid chromosomes, followed by meiotic segregation and the restoration of haploid genome. An asexual stage follows with the parasites multiplying by mitotic cell division and differentiating into sporozoites which are haploid. The sporozoites can exhibit new combinations of alleles which may be different from that of their parents (recombinants).

If a single polymorphic gene, present in both parents, is responsible for an observed phenotype, then inheritance of the phenotype should be parental only, i.e. like 3D7 or HB3. Any deviation from the parental-like phenotypes may be attributable to environmental factors only. Most phenotypic traits are governed by several genetic loci in addition to any influence by the environment (Fisher and DeFries, 2002). Lynch and Walsh (1998) define quantitative traits as ''the hereditary phenotypes which are the result of the interaction between genome (multiple genes) and environmental factors". Traits which manifest as continuous variation require specialised methods such as Quantitative trait locus (QTL) analysis, for the localisation of contributing loci and an estimation of the genetic contributions of such loci to the trait. Continuous variation is the combined effect of many genes (known as polygenic inheritance) and is often significantly affected by environmental influences. Quantitative trait loci may be described as stretches of DNA containing genes that control a quantitative trait. Multiple genes could participate in the variation in phenotype, which is not explained by simple analysis of a single gene from the parents. Phenotype measurements can thus be higher, lower or intermediate to that of the parents. Knowledge of the number of QTLs responsible for variation in the phenotypic trait will provide an idea of the genetic constitution of the trait.

Mapping regions of the genome that contain genes involved in specifying a quantitative trait is done with the aid of multiple markers throughout the genome, defined using a variety of molecular methods such as Amplified Fragment length Polymorphism (AFLP) or Single Nucleotide Polymorphisms (SNPs). Analysis of QTL involves the use of statistical methods to establish the relationship between phenotypic data and genotypic data which provide the

genetic basis of variation in complex traits (Broman and Sen, 2009; Lynch and Walsh, 1998). The analysis permits the linkage of complex phenotypes to specific regions of chromosomes. Using this analysis, the interaction, number, as well as the precise location of these regions can be identified.

4.3 Aims

The selectivity index of the independent recombinants under static/shaking conditions was determined and discussed in chapter three. In this chapter linkage analysis was carried out to identify genomic regions of the parasites potentially responsible for the selectivity index phenotype.

4.4 Methodology

4.4.1 Phenotypic measurement

Data from the selectivity index experiments with cultures under shaking or static culture condition were obtained as described in chapter three. Each progeny clone was defined by a single SI measurement (a mean of at least 3 replicate experiments) for static culture conditions, and a separate single mean SI value for shaking cultures.

4.4.2 Genotyping

Fifty five independent recombinants were identified from over one hundred progeny clones of the 3D7 x HB3 cross using 9 polymorphic markers on different chromosomes (unpublished data, Baton and Ranford-Cartwright). Eighteen of these recombinants were selected for phenotyping and genetic mapping. Affymetrix molecular inversion probes (MIP) SNP arrays containing 10,000 SNP markers (Takala-Harrison *et al.*, 2013) were used for the mapping of the parents and progeny clones. The analysis was performed by the NIH/NIAID Research Technologies Branch, Rocky Mountain Labs Technology section, USA, through collaboration with Xin-Xhuan Su, Functional Genomics Section, Laboratory of Malaria and Vector Research, NIAID, NIH. 36% (2,870 of the markers) were found to be polymorphic between 3D7 and HB3 (Ranford-Cartwright and Mwangi, 2012).

In addition, all the progeny clones analysed were sequenced, using next-generation Illumina sequencing technology, through collaboration with Prof. Dominic Kwiatkowski, Wellcome Trust Sanger Institute, Hinxton UK (manuscript in preparation). This gave a much larger number of SNPs between 3D7 and HB3. Using a combination of these data, a genetic map including all available markers was generated which was used for the QTL analysis described in this part of the study.

4.4.3 Quantitative trait loci analysis:

The method used for QTL analysis is as previously described by Broman & Sen, using a single-QTL model to detect regions of the genome that correspond to phenotype and the pattern of inheritance (Broman and Sen, 2009). The statistical significance of each genomic region including the intervals of the genomic region of interest was then ascertained appropriately as described below.

4.4.4 Procedure for QTL analysis:

4.4.4.1 Establishment of a genetic map

Analysis was performed using the package R/qtl version 2.15.2 (Broman *et al*. 2003) and was carried out in the R statistical package (R version 2.15.2 [R Core Team, 2013]).

Firstly, a genetic map was generated using the SNP data from microarrays and sequencing. Genotype (SNP) and phenotype data for each clone were collated in an excel file saved in a comma-separated (csv) format, and imported into the R/qtl programme. The data were analysed as a backcross to account for the haploid nature of the data (the version of the programme used did not allow for haploid genomes).

Duplicate markers were defined as those markers that are always inherited together in the progeny and present within the same genomic region. They may not be markers at exactly the same locus but they have not been

separated by recombination in any progeny clone. Their presence does not aid the linkage investigation and increases the calculation times, so they were identified using the command "findDupMarkers" and removed.

The genetic map was then produced from the marker data for the 18 progeny clones and two parent clones. A genetic map illustrates the recombination frequency (in centimorgans (cM)), where markers sited 1 cM apart implies that 1% of progeny would have a recombination event between the two markers. The order of the markers was checked by calculating the recombination fractions for each pair of markers (r) and the logarithm of odds (LOD) score for the test of r=1/2. The LOD score compares the probability of obtaining the test data if the two loci are linked to the probability of obtaining the test data if the two loci are not linked. The reason for using 0.5 as a factor in the calculation of LOD is that any alleles that are completely unlinked have a 50% chance of recombination, due to independent assortment. The inter-marker distances of the genetic map were re-estimated from the genetic data assuming a genotyping error rate of 0.1%. Expanded areas of the genetic map could indicate misplaced markers; the "ripple" function was used on each chromosome (with a sliding window of 5 to compare alternative marker orders) and the order resulting from the lowest number of recombination events was selected. Genotyping errors were identified and removed by calculating the error LOD scores of Lincoln and Lander (1992); large LOD scores (>4) were considered likely to indicate genotyping errors.

4.4.5 Identification of QTL associated with SI

4.4.5.1 QTL mapping using a single QTL model

QTLs were mapped using a single-QTL model using the EM (expectation-maximisation) algorithm for the phenotypes from shaking and static culture conditions separately. A LOD score above 3 was considered as significant. A secondary scan was performed controlling for the primary QTL, in order to identify any additional QTL contributing to the phenotype.

4.4.5.2 Localisation of QTL with Interval Estimates and within genome sequence

The location of any QTL with significant LOD scores was estimated using both LOD support intervals and Bayesian credible intervals (95%). These methods provide a maximum likelihood estimate of the location of QTL (on the cM map). The command "refineqtl" was used to improve the localisation of QTL.

The position of QTL within the genome sequence (kb location) was achieved by identifying the flanking markers within the 3D7 genome sequence. This allows the estimation of the beginning and the end of the QTL region.

4.4.5.3 Estimation of QTL Effect

The effect of a QTL is the difference in the phenotype averages among the QTL genotype groups (Broman and Sen, 2009). A QTL effect can also be denoted as the proportion of phenotypic variance which is explained by the QTL (Broman and Sen, 2009). The command "effectplot" was used for the estimation of QTL effects for the nearest marker. If multiple QTL were discovered, any additive or interactive effects were investigated using the command "fitqtl" to obtain estimates of their relative contributions to the differences in phenotype.

4.4.6 Identification and bioinformatic analysis with QTL of genes controlling selectivity index

The number of open reading frames (genes) within each QTL was identified from the full sequence of the 3D7 genome available on www.plasmodb.org (version 9.3). Data on the expression profile of each gene were then examined to select likely candidate genes that could be responsible for selectivity of red cell invasion. The expression profile data for different stages of the life cycle were obtained from PlasmoDB.org. Evidence of expression was considered from proteomics data (Silvestrini *et al.*, 2010; Florens *et al.*, 2002) and transcriptomics including microarray and RNAseq (Lopez-Barragan *et al.*, 2011; Otto *et al.*, 2010; Bartfai *et al.*, 2010; Llinas *et al.*, 2006; Bozdech *et al.*, 2003; Le Roch *et al.*, 2003).

The polymorphisms in the coding sequence between 3D7 and HB3 were assessed using data on SNPs from the Broad Institute, deposited in plasmodb.org v9.3. SNPs were defined as synonymous or non-synonymous within coding regions. SNPs within introns were also noted.

Predictions of transmembrane domains were also analysed using data on Plasmodb.org. A protein mediating selectivity of invasion is likely to be on the surface of the merozoite, and to be anchored in the merozoite membrane. A gene encoding a protein with a trans-membrane domain or other evidence of merozoite surface location would therefore be a good candidate for inclusion in a priority list of candidates for selectivity of RBC.

4.5 Results

4.5.1 Generation of a genetic map of the progeny clones used in the analysis of selectivity index

The initial genotyping data for the 18 progeny clones and two parent clones studied contained 7882 polymorphic sites. The number of markers varied over the fourteen chromosomes from 1148 markers on chromosome 14 to 165 markers on chromosome one. Removal of duplicate markers reduced the number of informative markers in the map to 1486, with a reasonably even distribution over the 14 chromosomes of the parasite genome, as shown in Figure 4.1.

The initial cM map generated from these markers had large differences between physical and genetic length, suggesting that some markers were incorrectly placed. The marker number was further reduced following removal of markers with high error LOD scores (likely genotyping errors). Certain chromosomes, e.g. 7, appeared to be long relative to their physical length, which suggests that some markers were incorrectly sited along the chromosome.

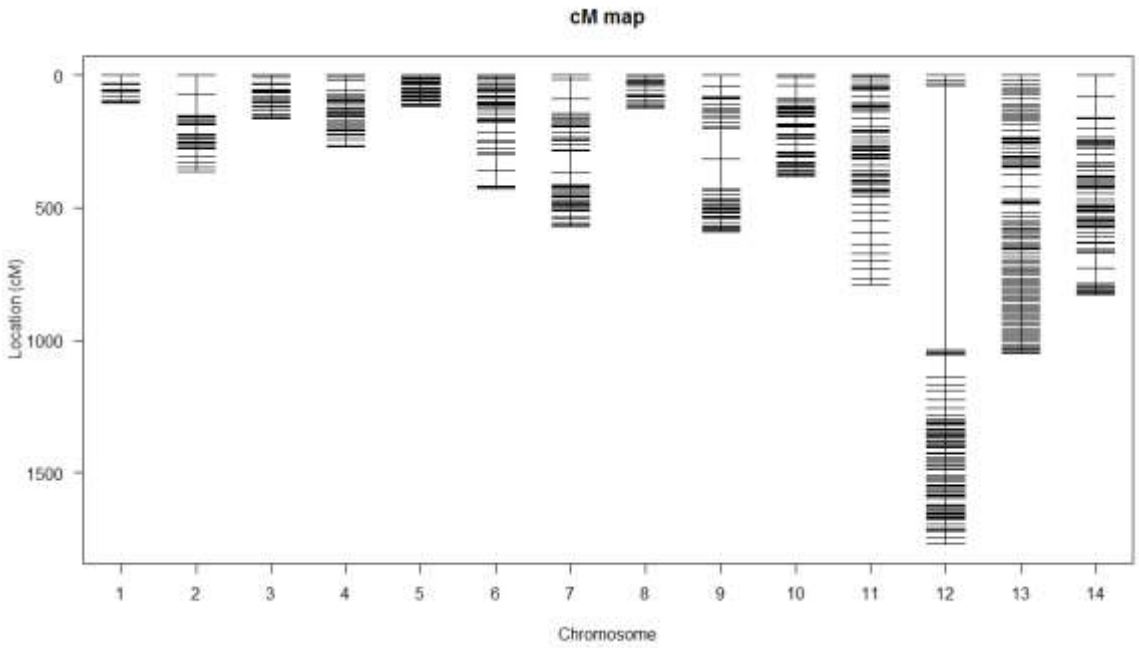

Figure 4.1. Initial genetic map (cM map) of progeny clones used in the analysis of selectivity index, based on a total number of 1486 markers. Each horizontal line represents a marker showing its location in cM. This map has not had genotyping errors removed, nor has marker order been modified.

Analysis of the centimorgan map showed large spaces on some of the chromosomes of *P. falciparum* especially that of chromosome 12. Chromosome 13 also looked expanded relative to its physical length. Marker order was then corrected using the ripple function on chromosomes 7, 12, 13 and 14. The final map used for QTL analysis is shown in Figure 4.2. The final genetic map contained 991 markers.

There was no indication of any problem with marker location according to the recombination fractions for markers within this map (Figure 4.3).The lower right triangle shows no red or yellow colouration that would have suggested linked markers or markers placed on the wrong chromosome. The genetic map shown in Figure 4.2 was therefore used to investigate linkage to the phenotype.

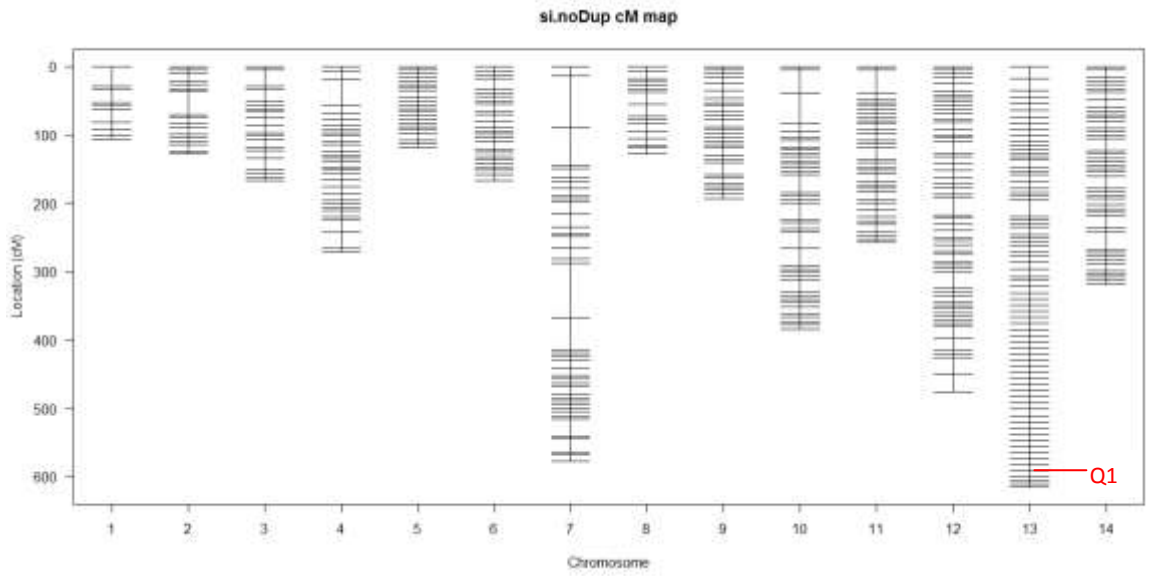

Figure 4.2 Final genetic map (cM map) following correction of marker order, removal of duplicate markers and genotyping errors. of progeny clones. Each horizontal line indicates a marker, which is characterised by the chromosome that it lies on and its genetic location in cM.

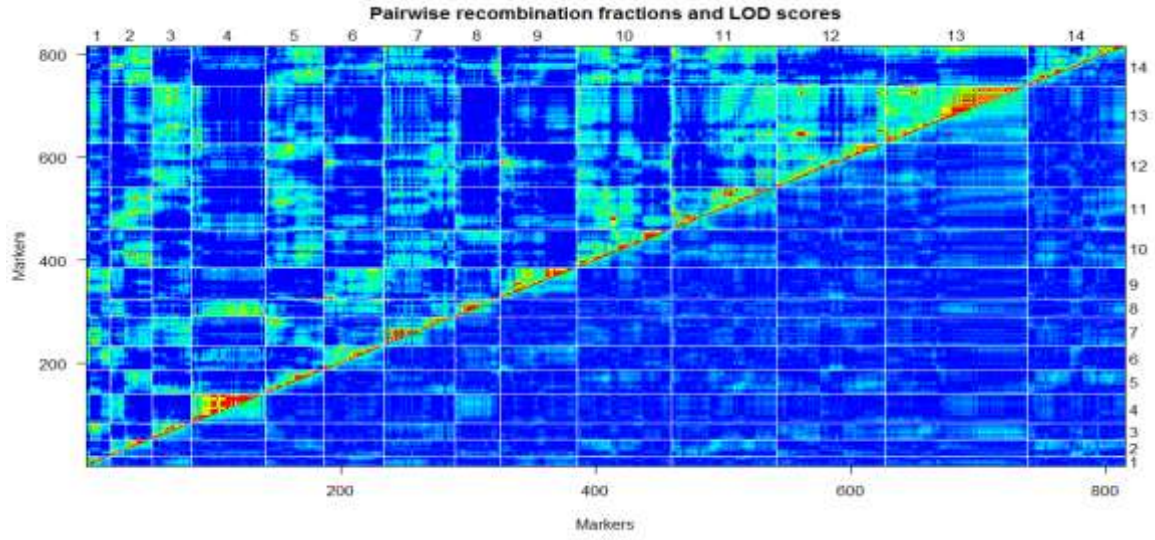

Figure 4.3 Plot of pairwise recombination fractions and LOD scores for all pairs of markers for the corrected genetic map. The estimated recombination fractions are shown in the upper-left triangle and the LOD scores in the lower- right triangle. Red in the figure indicates linked markers (large LOD score or small recombination fraction) and blue indicates no linkage (small LOD score or large recombination fraction).

4.5.2 Single-QTL genome scan.

The results of the single-QTL genome scan using the SCANONE function in R/qtl, performed for selectivity index under static conditions, are shown in figure 4.4. Results are shown as a LOD score for each marker, plotted against marker position. A LOD score above 3 is considered to be significant (Starkey and Elaswarapu, 2011). No significant LOD scores were observed. The largest LOD score, though not significant, was observed on chromosome 6.

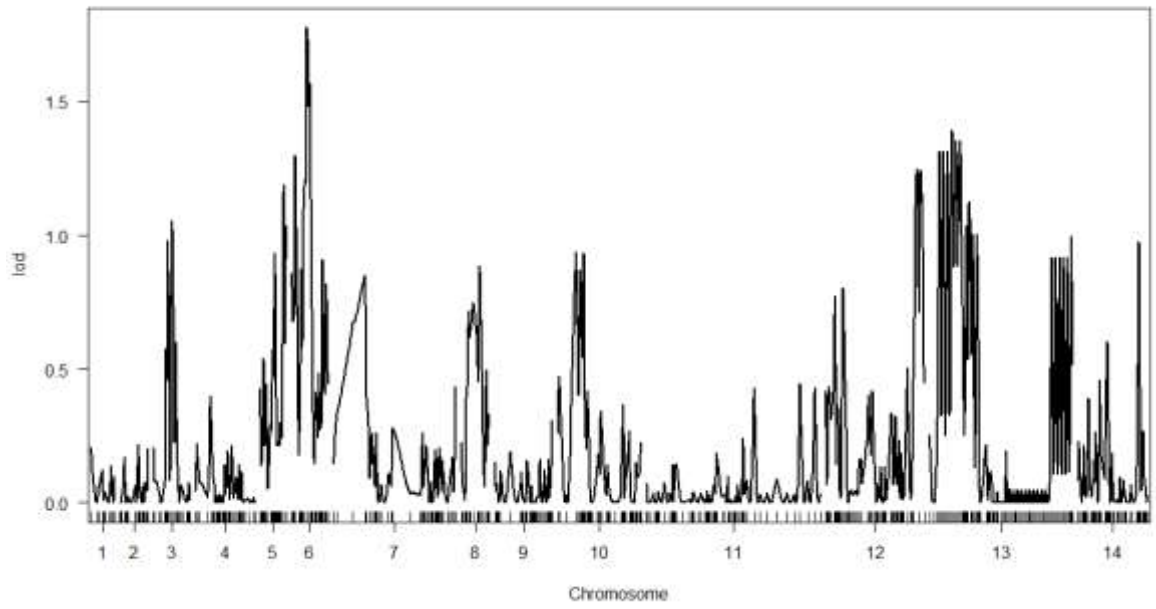

Figure 4.4 Single-QTL scan for the phenotype of selectivity index under static culture conditions. LOD scores for each marker are displayed on the Y-axis against chromosomes on the X-axis.

Following similar steps as described for static condition, the QTL analysis was performed for SI under shaking condition. The outcome is shown in Figure 4.5. The largest significant LOD score, of about 5.0, was observed on chromosome 13, at an approximate position of 638cM in the genetic map (Table 4.1) The QTL on chromosome 13 was named *PF_SI_1*. The location of the QTL on chromosome 13 is shown in Figure 4.2 as Q1. There is a second potential QTL on chromosome 12, LOD= 3.04 but this has not been analysed further.

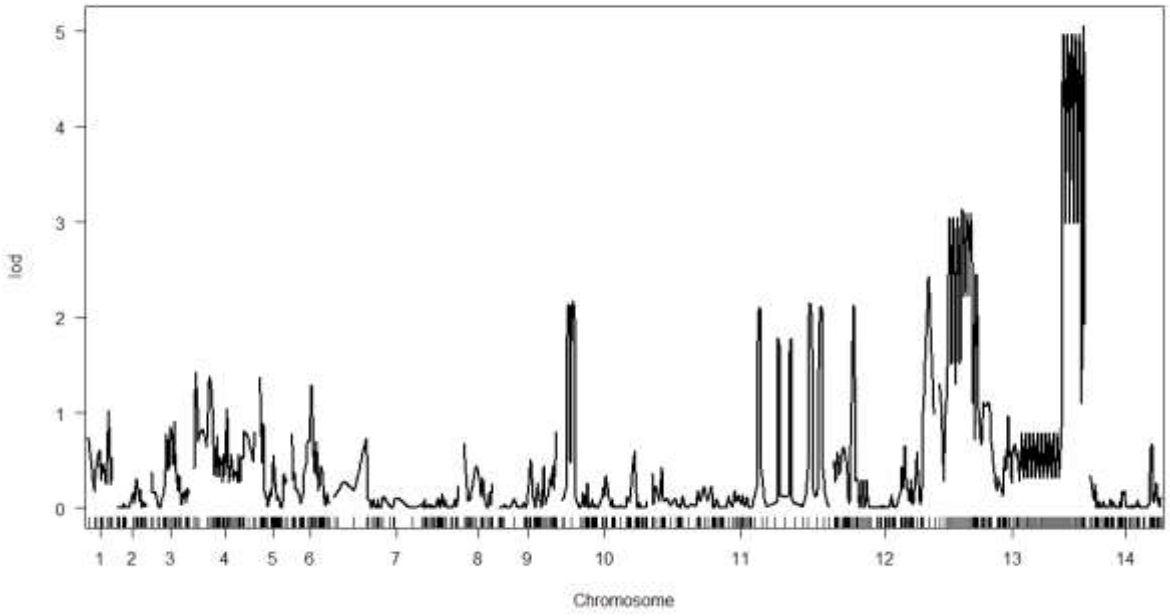

Figure 4.5 Single-QTL scan for the phenotype of selectivity index under shaking culture conditions. LOD scores are shown on the Y-axis and chromosome numbers on the X-axis.

Marker name	Chromosome	Position (cM)	LOD score
SNP_10K_13_2597245	13	638	5.06

Table 4.1 Summary of LOD scores from single-QTL analysis showing a significant QTL with a LOD above the significance threshold of LOD = 3.

4.5.3 Secondary scan to identify additional QTL

Two-dimensional, two-QTL scans lead to detection of interacting loci or to separation of pairs of linked QTL. Analysis was accomplished with Scantwo, similar to Scanone (Broman 2012). After controlling for the effect of the QTL Pf_SI_1 on chromosome 13 the secondary scan did not detect any further significant QTL with LOD score >2, including the second potential QTL on chromosome 12.

4.5.4 Interval Estimates of QTL Location and location of QTL within the Genome Sequence

The location of the QTL denoted Pf_SI_1 was refined further using the command “refineQTL”. Confidence intervals were obtained from using both LOD intervals and Bayesian intervals, which allowed identification of the flanking

markers (Table 4.2). These were then identified in the genome sequence and thus the physical location and size of the QTL in kb was established.

Method	Marker	Position (cM)	LOD score	Closest physical marker	Location in kb on chromosome 13
LOD intervals	c13.loc516	543.987	4.903074	SNP_10K_13_2511971	2511.971
	c13.loc516	581.987	7.035183	SNP_10K_13_2558776	2558.776
	c13.loc516	641.987	4.933892	SNP_10K_13_2610842	2610.842
Bayesian intervals	c13.loc516	545.987	6.502662	SNP_10K_13_2511971	2511.971
	c13.loc516	581.987	7.035183	SNP_10K_13_2558776	2558.776
	c13.loc516	639.987	6.451202	SNP_10K_13_2599956	2599.956

Table 4.2. Maximum likelihood estimates of the (PF_SI_1) QTL location. 95% Bayesian credible intervals (Bayesian intervals) were employed for calculating the intervals as well as a 1.5 LOD support interval (LOD). The positions of markers which are flanking as well as central markers for the QTL are shown on the genetic map in cM. The QTL on chromosome 13 spanned 100kb from ~2593.564 to ~2611kb (LOD interval estimate) or ~2600kb (Bayesian interval estimate).

4.5.5 The phenotypic effect of the QTL on selectivity index

The contribution of the QTL to the phenotype was calculated using the command as previously described (the command in R/qtl), and the percentage of variation in SI under shaking culture conditions explained by the QTL was determined to be 68.8%. The estimated effect that *Pf_SI_1* has on selectivity index was estimated to be 7.5± 1.2. This means that replacing the genomic region of one parent with that from the other parent would give a change in SI of 7.5. Switching the allele from 3D7 to HB3 increases selectivity index.This is illustrated in Figure 4.6.

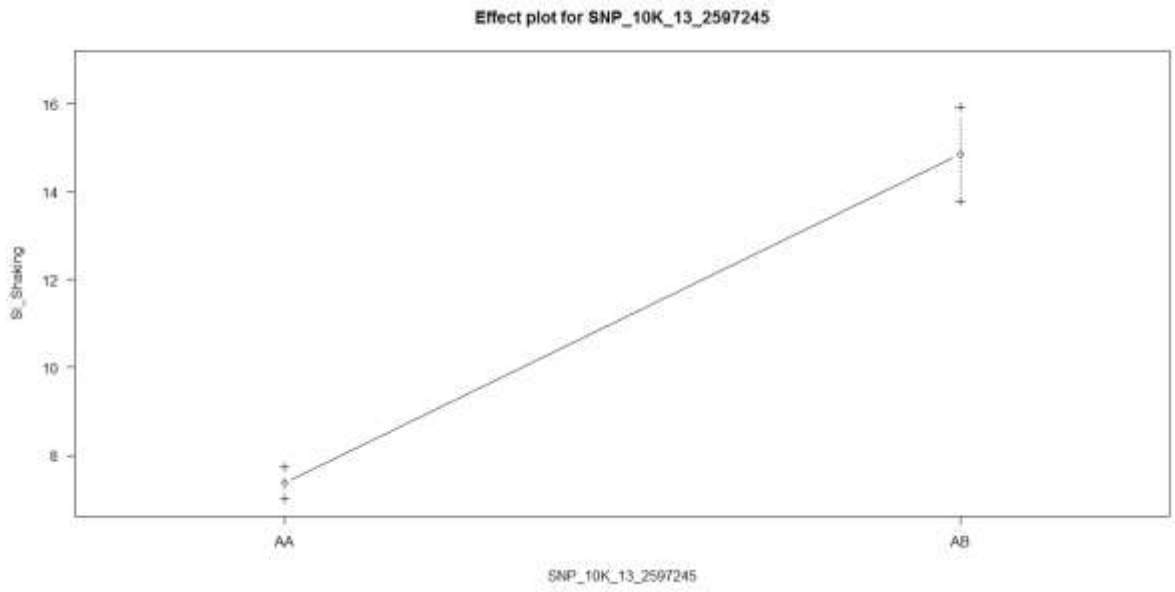

Figure 4.6. Effect plot showing estimated selectivity index under shaking conditions for the 3D7 and for HB3 at SNP_10k_13_2597245 marker (the closest marker to the QTL). The circles represent the mean SI for all progeny clones with the allele of 3D7 (AA) or HB3 (AB). The error bars show ± 1 SE.

The phenotypes of the individual progeny are plotted against the QTL in Figure 4.7. Only two clones have the HB3 version of the QTL (allele B in Figure 4.7) and both exhibit high SI measurements.

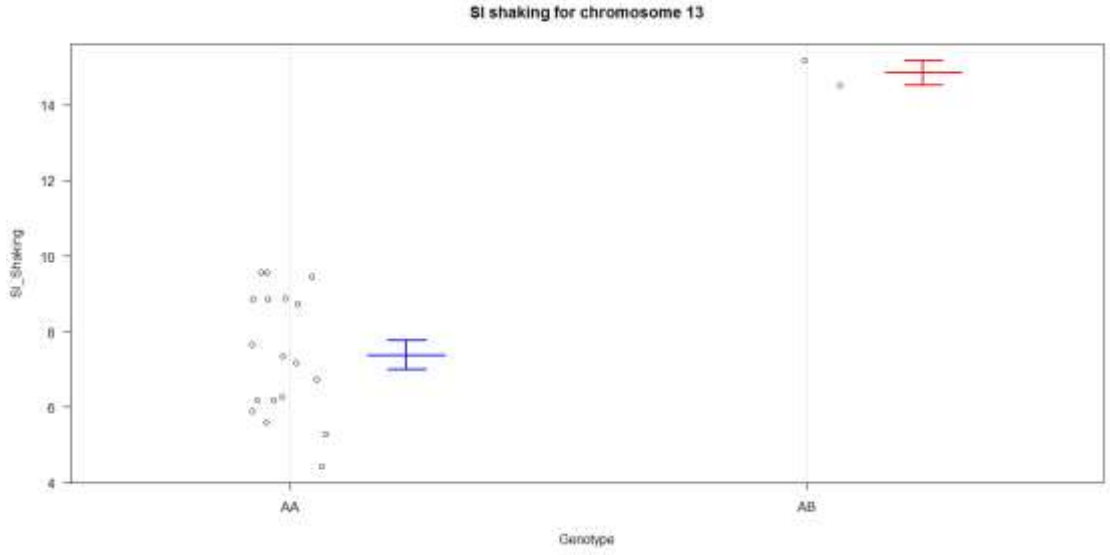

Figure 4.7. Effect plot showing selectivity index under shaking conditions for the 18 progeny clones against their inheritance of the QTL PF-SI_1. . Each circle represents a progeny clone. On the x-axis the 3D7 allele is shown as AA and HB3 as AB. Bars indicate the mean SI over all progeny for each allele, and the error bars show ± 1 SE.

4.5.6 Bioinformatics Analysis of Genes within the QTL

The QTL on chromosome 13 spanned 100kb from ~2593.564 to ~2611kb. From the published genome sequence available for clone 3D7 (www.PlasmoDB.org version 9.3) this region was found to contain 19 open reading frames (Table 4.3 and Figure 4.8). Eleven of the nineteen genes have no putative function assigned.

Gene ID	Previous Gene ID	Gene Function
PF3D7_1362800	MAL13P1.314, MAL13P1.315	conserved *Plasmodium* protein, unknown function
PF3D7_1362900	MAL13P1.316	conserved *Plasmodium* protein, unknown function
PF3D7_1363000	MAL13P1.317	conserved *Plasmodium* protein, unknown function
PF3D7_1363100	PF13_0333	conserved *Plasmodium* protein, unknown function
PF3D7_1363200	PF13_0334	bifunctional polynucleotide phosphatase/kinase (PNKP)
PF3D7_1363300	MAL13P1.318	mitochondrial ribosomal protein L9 precursor, putative
PF3D7_1363400	PF13_0335	polyubiquitin binding protein, putative
PF3D7_1363500	PF13_0336	DNase I-like protein, putative
PF3D7_1363600	PF13_0337	conserved *Plasmodium* protein, unknown function
PF3D7_1363700	MAL13P1.319	*Plasmodium* protein, unknown function
PF3D7_1363800	MAL13P1.320	conserved *Plasmodium* protein, unknown function
PF3D7_1363900	PF13TR006:ncRNA	unspecified product
PF3D7_1364000	MAL13P1.321	conserved *Plasmodium* protein, unknown function
PF3D7_1364100	PF13_0338	cysteine-rich surface protein (Pf92)
PF3D7_1364200	PF13_0339	conserved *Plasmodium* protein, unknown function
PF3D7_1364300	MAL13P1.322	pre-mRNA-splicing factor ATP-dependent RNA helicase PRP16)
PF3D7_1364400	MAL13P1.323	conserved *Plasmodium* protein, unknown function
PF3D7_1364500	PF13_0340	exosome complex exonuclease, putative
PF3D7_1364600	MAL13P1.324	aldo-keto reductase, putative

Table 4.3 Putative genes within the QTL on chromosome 13. Data are taken from Plasmodb.org version 9.3. For each gene the new gene ID as well as the previous gene ID (Plasmodb version 9.3) is shown.

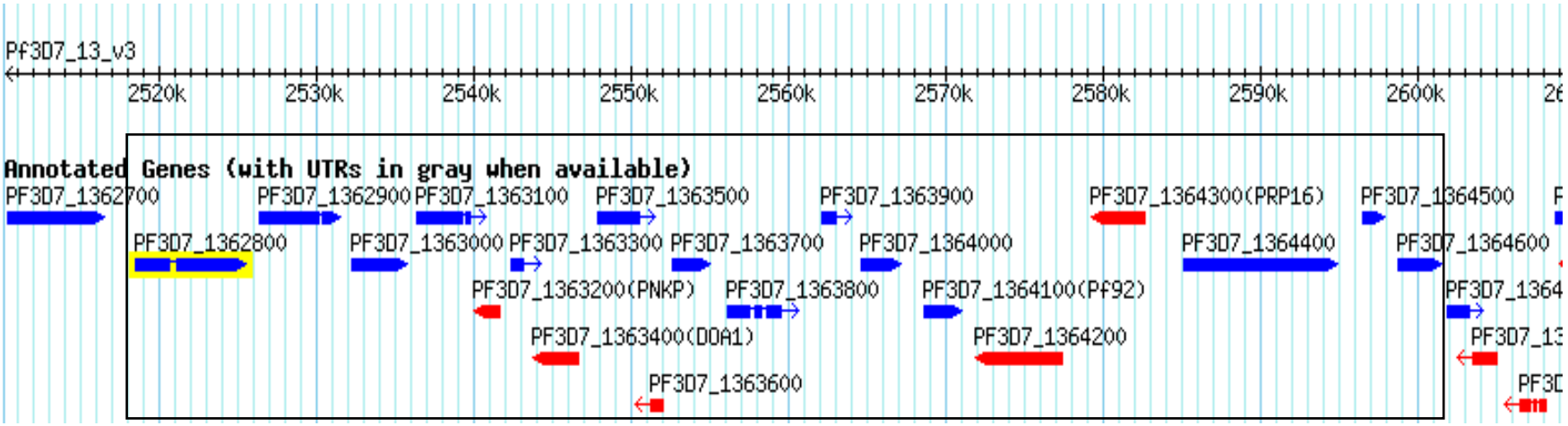

Figure 4.8 Localisation of open reading frames within the QTL on chromosome 13. Data extracted from PlasmoDB.org (v 9.3). The QTL *Pf_SI_1* contains the genes listed in Table 4.3, from *Pf3D7_1362800* to *Pf3D7_1364600*, shown in the boxed area in the figure.

4.5.6.1 Sequence polymorphisms in the candidate genes within the QTL.

In order to explain a difference in the phenotype between the parents 3D7 and HB3, there must be a difference in the coding sequence, or in the 5' and 3' untranslated regions that would affect the level of gene expression. Polymorphisms in the coding region between the 3D7 and HB3 alleles are shown in Table 4.4 Polymorphisms in the 5' and 3' untranslated regions (containing potential promoter/gene control elements) have not been analysed futher.

Gene ID	Number of SNPs in coding region		
	Non synonymous	Synonymous	within introns
PF3D7_1362800	0	1	0
PF3D7_1362900	5	15	0
PF3D7_1363000	1	0	n/a
PF3D7_1363100	1	0	0
PF3D7_1363200	0	0	0
PF3D7_1363300	0	0	0
PF3D7_1363400	0	1	1
PF3D7_1363500	1	0	0
PF3D7_1363600	0	0	n/a
PF3D7_1363700	1	0	n/a
PF3D7_1363800	1	1	1
PF3D7_1363900	0	0	n/a
PF3D7_1364000	0	1	n/a
PF3D7_1364100	5	0	n/a
PF3D7_1364200	1	0	n/a
PF3D7_1364300	0	1	n/a
PF3D7_1364400	6	7	n/a
PF3D7_1364500	0	1	n/a
PF3D7_1364600	1	0	n/a

Table 4.4 Sequence polymorphisms in the candidate genes within the QTL. Genes lacking an intron have n/a in the intron column.

4.5.6.2 Expression data on the candidate genes within the QTL

In order to be a possible candidate gene with an influence on selectivity index, the protein product of the gene is expected to be present during the asexual cycle, and especially in the schizont and merozoite. This was examined for the 19 genes in the QTL by initial analysis of each gene expression data in plasmoDB.org. The expression data for each gene is summarised in Table 4.5. Of the 19 genes, 18 are expressed in the asexual stages with only one gene not expressed at all during the asexual cycle (Pf3D7_1362900). This gene is unlikely to explain differences in selectivity index and can be eliminated as a candidate.

4.5.6.3 Transmembrane domains and predicted surface localisation of the proteins

4 of the 19 genes in the QTL were predicted to encode proteins with transmembrane domains (Table 4.4). Three of these genes have no annotated function (Table 4.4): PF3D7_1363700, PF3D7_1363800 and PF3D7_1364400. PF3D7_1364100 encodes Pf92 which is known to be on the surface of the merozoite (Sanders et al 2005),

4.5.6.4 Prioritising Genes of known function in *Pf_SI_1*

Eight of the nineteen genes in the QTL have been assigned a function, usually by sequence homology to genes of known function in other organisms, as described below. Most of the assigned functions are not linked to RBC invasion, and based on function alone, only one is a strong candidate (*PF3D7_1364100)*.

PF3D7_1363200 encodes a putative bi-functional polynucleotide phosphatase/kinase (PNKP). The gene is expressed in ring and trophozoite stages but expression falls in schizonts. This protein is involved in repair of damaged DNA. There are no polymorphisms in the coding region between 3D7 and HB3. Therefore it is a weak candidate to explain SI.

Gene DB	Genomic location (bp)	Annotated Function	Expression				Protein			TM
			R	T	S	M	T	S	M	
PF3D7_1362800	2,518,487 to 2,525,493	unknown function	Y	Y	Y	Y	Y	Y	N	N
PF3D7_1362900	2,526,360 to 2,531,483	unknown function	N	N	N	N	N	N	N	N
PF3D7_1363000	2,532,201 to 2,535,722	unknown function	Y	N	N	Y	N	N	Y	N
PF3D7_1363100	2,536,315 to 2,539,642	unknown function	Y	Y	Y	Y	Y	Y	N	N
PF3D7_1363200	2,539,948 to 2,541,540	bifunctional polynucleotide phosphatase/kinase (PNKP)	Y	Y	Y	N	Y	Y	N	N
PF3D7_1363300	2,542,284 to 2,543,150	mitochondrial ribosomal protein L9 precursor, putative	Y	Y	Y	Y	N	N	N	N
PF3D7_1363400	2,543,644 to 2,546,483	polyubiquitin binding protein, putative (DOA1)	Y	Y	Y	Y	Y	Y	Y	N
PF3D7_1363500	2,547,764 to 2,550,477	DNase I-like protein, putative	N	Y	Y	N	N	Y	N	N
PF3D7_1363600	2,551,243 to 2,551,926	unknown function	N	Y	Y	Y	N	N	N	N
PF3D7_1363700	2,552,554 to 2,554,926	unknown function	N	Y	Y	N	N	N	N	Y
PF3D7_1363800	2,556,025 to 2,559,547	unknown function	N	N	Y	N	N	N	N	Y
PF3D7_1363900	2,562,118 to 2,562,968	UnspecifiedProduct	Y	Y	Y	N	N	N	N	N
PF3D7_1364000	2,564,625 to 2,567,108	Unknown Function	Y	Y	Y	Y	Y	Y	Y	N
PF3D7_1364100	2,568,556 to 2,570,946	cysteine-rich surface protein (Pf92)	N	Y	Y	Y	N	Y	Y	Y
PF3D7_1364200	2,571,845 to 2,577,286	UnknownFunction	Y	Y	Y	Y	Y	Y	Y	N
PF3D7_1364300	2,579,159 to 2,582,614	pre-mRNA-splicing factor ATP-dependent RNA helicase PRP16 (PRP16)	Y	Y	N	Y	N	N	N	N
PF3D7_1364400	2,585,032 to 2,594,829	UnknownFunction	Y	Y	Y	Y	Y	Y	Y	Y
PF3D7_1364500	2,596,389 to 2,597,822	exosome complex exonuclease, putative	Y	Y	Y	Y	Y	Y	N	N
PF3D7_1364600	2,598,757 to 2,601,399	aldo-keto reductase, putative	N	Y	Y	Y	Y	N	N	N

Table 4.5. Summarised genes within QTL on chromosome 13 and the information of their location, expression and function as shown in plasmodb.org version 9.3. The transcriptomic data and microarray and RNAseq (Le Roch *et al*. 2003; Bozdech *et al*. 2003; Llinás *et al*. 2006; Lopez-Barragán *et al*. 2011; Bártfai, *et al*. 2010; Otto *et al*. 2010) are shown in the columns "Expression ((R(ring)/T(trophozoite)/S(schizont)/M(merozoite))". Proteomic data (Florens *et al* 2002; Silvestrini *et al*. 2010) are shown in the column "protein (T/S/M)". The column "TM" indicates if there is a predicted transmembrane domain.

PF3D7_1363300 encodes a putative mitochondrial ribosomal protein L9 precursor. It is expressed throughout the asexual lifecycle but does not have a TM domain. There is no polymorphism between the coding regions of 3D7 and HB3 alleles. Therefore it is a weak candidate to explain SI.

PF3D7_1363400 encodes a putative polyubiquitin binding protein. It is expressed throughout the asexual lifecycle but the protein does not have a TM domain. There is a single synonymous change and one change within the intron. This is unlikely to affect the function of the protein and therefore it is a weak candidate to explain SI.

PF3D7_1363500 encodes a putative DNase I-like protein. This gene is expressed in trophozoite and schizont stages but the protein does not have a TM domain. There is a single non synonymous change. Therefore it is a possible candidate to explain SI.

PF3D7_1364100 encodes a cysteine-rich surface protein Pf92, a member of a 6-cysteine adhesion protein family containing Pfs48/45 domains, that includes Pf12, Pf38, and Pf41, proteins implicated in merozoite invasion (Sanders *et al.*, 2007; Sanders *et al.*, 2005). The gene is expressed in late trophozoites and schizonts. The protein is present on the surface of merozoites, in detergent resistant membranes (lipid rafts); these have been suggested to be sites of interactions between the RBC and merozoite (Obando-Martinez *et al.*, 2010; Sanders *et al.*, 2005). Pf92 is reported to be involved in invasion (Obando-Martinez *et al* 2010). There are five non-synonymous changes between 3D7 and HB3 in the coding sequences, 5 of which occur in regions implicated in binding to red blood cells (www.PlasmoDB.org version 9.3). The locus is therefore a strong candidate to explain differences in RBC selectivity.

PF3D7_1364300 encodes a putative pre-mRNA-splicing factor ATP-dependent RNA helicase PRP16 (PRP16), and is thus thought to be involved in pre-mRNA splicing. The gene is expressed in the ring, trophozoite and merozoite stages. There is a single synonymous change but this is unlikely to affect the function of the protein and the protein does not have a TM domain. Therefore it is a weak candidate to explain SI.

PF3D7_1364500 codes for a putative exosome complex exonuclease (Anderson and Parker 1998), which is involved in RNA processing and RNA binding. It is expressed throughout the asexual lifecycle but does not have a TM domain. There is a single synonymous change. This is unlikely to affect the function of the protein and therefore it is a weak candidate to explain SI.

PF3D7_1364600 encodes a putative aldo-keto reductase, enzymes which catalyze oxidation-reduction reactions. The gene is expressed in the trophozoite, schizont and merozoite stages but does not have a TM domain. There is a single non synonymous change. Therefore it is a possible candidate to explain SI.

4.5.6.5 Genes of unknown function in *Pf_SI_1*

The remaining eleven genes in the QTL have not been assigned a function, but can be prioritised based on their timing of expression and predicted surface location, and the existence of polymorphisms (non-synonymous) between 3D7 and HB3 alleles.

PF3D7_1362800 is expressed at around 35 hours post invasion. As this gene is mostly expressed at the late schizont stage, it has potential as a candidate gene for selectivity of RBC. However it has no transmembrane domains or signal peptide and there is no evidence that it is a surface protein, and there is only a single synonymous change in the coding region between 3D7 and HB3. The gene is therefore a weak candidate to explain differences in SI between 3D7 and HB3.

PF3D7_1362900 is not expressed in the asexual blood stages (from both transcriptomic and proteomic data) and therefore can be excluded as a candidate for involvement in RBC selectivity.

PF3D7_1363000 is not expressed in the trophozoite or schizont stages, although it is present in ring and merozoite stages, but does not have a TM domain. There is a single non synonymous change. Therefore it is a possible candidate to explain SI.

PF3D7_1363100 It is expressed throughout the asexual lifecycle but does not have a TM domain. There is a single non synonymous change. Therefore it is a possible candidate to explain SI.

PF3D7_1363600 is expressed during the trophozoite, schizont and merozoite stages, but does not have a TM domain. There is no polymorphism between the coding regions of 3D7 and HB3 alleles. Therefore it is a weak candidate to explain SI.

PF3D7_1363700 is expressed in the trophozoite and schizont stages. There is a single non synonymous change and the protein does have a TM domain. It is a possible candidate to explain SI.

PF3D7_1363800 is expressed in the trophozoite and schizont stages. There is a single non synonymous change, a single synonymous change, one change within the intron and the protein does have a TM domain. It is a possible candidate to explain SI.

PF3D7_1363900 is expressed in the ring, trophozoite and schizont stages, but does not have a TM domain. There is no polymorphism between the coding regions of 3D7 and HB3 alleles. Therefore it is a weak candidate to explain SI.

PF3D7_1364000 It is expressed throughout the asexual lifecycle but the protein the protein does not have a TM domain. There is a single synonymous change. Therefore it is a weak candidate to explain SI.

PF3D7_1364200 It is expressed throughout the asexual lifecycle but the protein does not have a TM domain. There is a single non synonymous change. Therefore it is a possible candidate to explain SI.

PF3D7_1364400 It is expressed throughout the asexual lifecycle and the protein does have a TM domain. There are six non synonymous changes and seven synonymous changes. Therefore it is a possible candidate to explain SI.

4.6 Discussion

The loci controlling selectivity index in *P. falciparum* were investigated using a QTL approach, using a genetic map based on 991 SNP markers and 18 progeny clones of the 3D7 x HB3 experimental cross. The analysis successfully identified one QTL with a significant LOD score of 5.6, associated with the SI phenotype in cultures grown under shaking conditions. The QTL is located close to the end of chromosome 13. It contains nineteen open reading frames within a 100 kb region, and explains 67.8% of the variation in SI observed. This locus was named *Pf_SI_1*. No QTL was identified associated with SI in static cultures.

Parasites with the HB3 allele at the QTL have a higher selectivity index (more multiply-invaded cells) compared to those with the 3D7 allele, although both parasites exhibit a higher selectivity of RBC invasion than expected from a random (poisson) distribution.

In order to be considered as a candidate explaining the difference in SI observed, I hypothesise that the gene should meet some or all of the following criteria:

(i) be expressed during the asexual lifecycle, with expression of protein during the schizont and merozoite stages;

(ii) be polymorphic either in the coding sequence (especially non-synonymous changes), or in the 5' and 3'UTR, between the alleles of 3D7 and HB3;

(iii) if a function is assigned, be involved in red cell interactions or binding, or some aspect of the invasion process;

(iv) be expressed on the surface of the merozoite.

There are nineteen genes within the *Pf_SI-1* QTL, eight of which have an annotated function, and eleven are of unknown function. One of the genes *PF3D7_1362900 is* not expressed at all during the asexual stages of the parasite lifecycle, and can therefore be eliminated.

Of the remaining 18 genes, seven can be probably eliminated from their annotated functions and/or lack of polymorphism between 3D7 and HB3 (*PF3D7_1363200:* DNA damage repair; *PF3D7_1363300:* mitochondrial ribosomal protein*; PF3D7_1363400:* polyubiquitin binding protein; *PF3D7_1363500:* DNase I activity; *PF3D7_1364300:* RNA helicase; *PF3D7_1364500:* exosome complex exonuclease; *PF3D7_1364600:* aldo-keto reductase).

Eight of the remaining genes have a low likelihood of explaining SI differences because of a lack of sequence polymorphism, or low expression in schizints and merozoites. *PF3D7_1362800, PF3D7_1362900, PF3D7_1363000, PF3D7_1363100, PF3D7_1363600, PF3D7_1363900, PF3D7_1364000 and PF3D7_1364200.*

The strongest candidate amongst the genes with annoted function is *PF3D7_1364100,* which encodes a cysteine-rich surface protein Pf92, implicated previously in merozoite invasion and RBC binding (Obando-Martinez *et al.*, 2010).

Amongst the genes of unknown function, *PF3D7_1363700, PF3D7_1363800 and PF3D7_1364400* are also possible candidates with expression during schizogony, or protein presence in merozoites, and polymorphisms (non-synonymous changes) in the proteins of 3D7 and HB3.

The location of genes associated with differences in selectivity index in the parasites cultured under shaking conditions has a several implications. Selectivity index and parasite multiplication rate (PMR) are two major factors implicated in malaria virulence. With regards to SI, parasite receptor preference, host RBC polymorphisms, or host merozoite-agglutinating antibodies are some of the factors that may lead to non-random invasion whilst an SI < 1 could occur if invasion by one merozoite rendered an RBC refractory to further invasion.

In summary a region on chromosome13 was identified that could be responsible for the differences in selectivity index observed between parasites 3D7 and HB3 grown under shaking culture conditions. This region contained 19 genes. Of the 19 genes in the QTL, 15 could either be eliminated on the grounds

of expression data or unknown function, or were considered less likely because of the timing of expression and lack of sequence polymorphism. The remaining genes are considered to be strong candidates to influence selectivity index.

5 General discussion

Despite our poor understanding of the factors responsible for severe malaria, it is thought that parasite properties, host genetics and environmental determinants all contribute to the aetiology of this condition in malaria infection (Miller *et al.*, 2002). Two properties of the parasite that have been implicated in parasite virulence, and reported in literature, are parasite multiplication rate (PMR) and red blood cell selectivity index (Chotivanich *et al.*, 2000; Simpson *et al.*, 1999;). Selectivity Index (SI), which describes the differential preference of a *P. falciparum* clone for erythrocytes, was the subject of the research presented in this thesis.

The genetic basis, if any, for selectivity index differences between parasites, was not known and was the major topic of the research presented here. Human host genetics and immune responses could of course play a part in selectivity index as well as any variation in the parasite itself. For example, rosetting is associated with severe malaria, and is reduced in blood group O erythrocytes compared to A, B, and AB groups (Rowe *et al.*, 2007). However, little attention has been paid to the involvement of the ABO blood group system in selectivity index.

The aim of this research was to investigate the genetic determinants of selectivity of erythrocyte invasion in *Plasmodium falciparum*. The measurements of selectivity index (SI) were described in chapter 3. The quantitative trait locus analysis, identification of a QTL on chromosome 13 and bioinformatic analyses of genes within the QTL were described in chapter 4.

5.1 Variation in selectivity index in *P. falciparum* clones measured *in vitro*

5.1.1 Influence of parasitaemia on SI

As had been previously reported for *in vivo* infections (Chotivanich *et al.*, 2000) there was an inverse relationship between parasitaemia and SI *in vitro* (figure 3.1). The reason for this observation is not clear; merozoites that discriminate between RBC less (low SI) could simply result in more RBCs being

successfully invaded, leading to an increase in parasitized RBC. A starting parasitaemia of 1% was selected for all experiments to control the influence of parasitaemia on SI measurement.

5.1.2 Influence of culture environment on SI

The Selectivity Index for a given parasite clone was ascertained under static and shaking culturing conditions. Culturing the parasites under static conditions was an attempt to mimic rosetting, where the P. falciparum infected erythrocyte adheres to two or more non-infected red cells. Culturing under shaking conditions was an attempt to mimic non-rosetting parasites. The selectivity index of a given parasite clone in a specific batch of red cells (an experiment) was always significantly lower under shaking culture conditions than when cultures were grown with no agitation (e.g. figure 3.2) . A high SI for static cultures is not surprising because merozoites are not motile, and in a static culture are likely to invade the red cells which they come into immediate contact with. However under shaking culture conditions, a distribution of parasites more similar to a random (poisson) distribution might be expected, since all cells are in suspension. All parasite clones tested, in all experiments, exhibited SI significantly different to random invasion in shaking cultures. This observation implies that parasites are selective in the red cells that they choose to invade and that the process of invasion is not solely dependent on proximity of the red cell and bursting schizont.

5.1.3 Influence of erythrocyte ABO blood group on SI

Selectivity of the same parasite line in erythrocytes of known ABO blood group was briefly investigated. It was observed that blood group has no influence on SI in *P. falciparum* infection under both shaking and static culturing conditions (section *3.4.3*). However since donors of the blood used are all from the West of Scotland, it is possible that blood group variants more common in malaria-endemic countries could influence SI.

5.1.4 Stability of SI over time in culture

SI for a given parasite clone was shown to be a stable phenotype over several months of continuous culture. There was no relationship between

selectivity index and days in continuous culture for 3D7 clone under shaking and static conditions (section 3.4.4).

5.1.5 Variation in SI in different parasite clones

Two parasite clones, 3D7 and HB3, were initially investigated. HB3 parasites always exhibited a significantly higher SI than 3D7 under both static and shaking conditions (figure 3.2). The genetic basis of the difference in selectivity index between *P. falciparum* clones 3D7 and HB3 was therefore investigated in progeny clones from a cross between these two clones, to ascertain the inheritance pattern of this phenotype.

The progeny clones exhibited a wide range of SI values; different progeny clones had SI measurements lower than either parent, higher than both parents, and intermediate between the parents (section 3.4.6). This observation suggests the involvement of more than one parasite gene in selectivity index. There was a difference in the behaviour of some progeny clones under static and shaking conditions, with some clones exhibiting parental phenotypes for one culture condition but non-parental for the other. This suggests that different genes are involved in selectivity index determination under the two culture conditions.

5.2 Identification of *P. falciparum* Quantitative Trait Loci linked to differences in SI

Quantitative Trait Locus analysis of 18 progeny clones revealed a single qtl on chromosome 13 (LOD score 5.06) linked to SI measured in parasites cultured under shaking conditions. This finding indicates the presence of gene(s) on chromosome 13 that may encode for selectivity index. The qtl, named *PF_SI_1*, extends for approximately 100kb and was found to include 19 open reading frames. The QTl explained 68.8% of the observed variation in SI. Based on putative function, timing of gene expression and polymorphisms in the coding sequence between 3D7 and HB3 (all published work available on plasmodb.org), 4 genes were prioritised as suitable candidates for further research.

LOD scores determined for SI measurements from cultures under static conditions were below the significance threshold. Possible reasons for this could

be that the genetic effect is lower in static cultures and the environmental effect is greater, or that there were too many genes acting together and because the sample size is too small it couldn't be detected.

5.2.1 Candidate genes prioritised for future work

Four genes (*PF3D7_1363700, PF3D7_1363800, PF3D7_1364100 and PF3D7_1364400*) were considered the top candidates for future work into their role in RBC selectivity during invasion. *PF3D7_1364100*, encoding a protein denoted Pf92, is a member of the *Pfs48/45* family of putative adhesins with a six-cysteine-like domain (Sanders *et al.*, 2007). Previous research demonstrated that Pf92 localised to the merozoite surface and contains high-activity binding peptides (HABPs) that are able to bind red blood cells (Obando-Martinez *et al* 2010). Using enzyme-treated RBCs and cross-linking assays the natures of the RBC surface receptors for these HABPs were determined. Exploring invasion inhibition and immunofluorescence localization studies the group concluded that Pf92 and Pf113 were involved in RBC invasion and that their adhesion to RBCs is mediated by such HABPs (Obando-Martinez *et al* 2010).

5.3 Future analyses of candidate genes

We do not know how the interaction of a candidate protein, possibly located on the merozoite surface, with an unknown red cell receptor, affects selectivity index. A Selectivity Index greater than 1 could imply that merozoites are choosing certain RBC over others for invasion. HB3 appears to be more selective than 3D7, possibly as a result of polymorphisms in a parasite protein involved in selection of RBC for invasion. How this difference results in a more selective invasion of a subpopulation of red cells is unknown. It is also not known what the differences are between the red cells within the population that makes them more or less attractive for invasion. It is possible that certain parasites select RBC on the basis of differences in the numbers or density of a host receptor on the red cell, or the glycosylation (or other modifications) of these proteins. Alternatively, the age of the red cell could be important in the selection of RBC for invasion; the RBC receptor could change with age, possibly reducing or increasing in density or with changes in glycosylation or other modification.

A major objective of this study was to investigate the genetic basis of selectivity in invasion of the red blood cells by *Plasmodium falciparum*, the human malaria parasite. A significant quantitative trait locus for SI was identified on chromosome 13 in this study. Of the genes within this QTL, Pf92 can be considered to be the strongest putative candidate.

The role of Pf92 as the causative gene underlying the differential SI within the QTL on chromosome 13 will be ascertained or confirmed in future studies. With regard to this, two complimentary approaches could be pursued. In the first instance, transgenic HB3 parasites could be generated that endogenously express the 3D7 Pf92 allele, by a process of allelic exchange. Endogenously expressed genes generally encode native protein which are more often stable.

The recently developed CRISPR/Cas9 technique could be used to confirm if the SI difference between 3D7 and HB3 parasites is due to a SNP in the HB3 allele. CRISPR/Cas9 has been reported as the most efficient and specific for genome editing of P. falciparum (Wagner et al., 2014). Using this technology the Pf92 gene could be modified in one of the two clones of the parasites after which the parasites would be compared. A change, up to 70% in SI, is expected.The rest (30%) if observed could be attributed to environmental effect and other unknown genes

5.3.1 Proof of the role of candidate proteins in red cell binding and invasion

There are several methods commonly used to implicate parasite proteins in the invasion process and/or in binding to RBC.

Antibodies could be raised to recombinant proteins of each of the candidate genes and tested for their ability to block the invasion process. Polyclonal antibodies raised to *Pf92* have already been shown to inhibit *P. falciparum* invasions of RBC (Obando-Martinez *et al.*, 2010). Attempts to knock out *Pf92* have not been successful (Cowman et al., 2012; Sanders et al., 2006) suggesting that the gene is essential.

5.3.2Role of polymorphism in the candidate gene

There are five non-synonymous changes between 3D7 and HB3, within the coding sequences in the *Pf92* gene. A polymorphism which is non-synonymous leads to changes in the amino acid sequence in the protein, which presumably result in a different selectivity of 3D7 and HB3 parasites during red blood cell invasion.

Data obtained from a study by Obando-Martinez and colleagues suggested that *Pf92* and *Pf113* establish an array of specific binding interactions with RBC membrane receptors mediated through the HABPs, which are probably implicated in the parasite's rolling over RBC membrane and establishing multiple points of contact with the target cell (Obando-Martinez *et al.,* 2010). From polymorphism studies by the same group the HABPs showed well-defined secondary structure elements and high conservation among different *P. falciparum* strains. Due to existence of vast antigenic variability among parasite strains, the polymorphism of *Pf92* and *Pf113* HABPs was analyzed in parasite strains isolated from different geographical regions. The data indicate that the *Pf92* and *Pf113* HABPs are highly conserved among parasite strains probably because of their involvement in processes important for parasite survival.

Protein glycosylation is a post-translational process in which sugar is covalently attached to a target protein. Modification of the protein this way is for various reasons (Drickamer and M.E.Taylor, 2006). In a review it was indicated that glycosylation serves in some instances to enhance proper folding of a protein or ensures its stability (Ferris *et al*., 2014). It must be emphasised that glycosylation of protein depends on specific amino acids. It therefore follows that a change in the amino acid sequence may not lead to glycosylation. Therefore should a genetic difference in the genes being referred here results in a change in glycosylation, malfunction of the protein is likely to occur.

5.3.3 Identification of the binding partners for interactions with RBCs

Enzymatic treatment of erythrocytes is commonly used to investigate the the interactions of parasites with red cell surface proteins (reviewed by Perkins 1981) Reduced invasion following treatment of RBC with neuraminidase, which removes sugars, would suggest interaction with glycosylated receptors. The use of antibody that means that the protein will be blocked hence can't bind to a glycosylated receptor on the RBC (or to a sialic acid moiety).

5.3.4 Association of parasite candidate gene polymorphisms with SI in vivo

Selectivity index of P. falciparum isolates is reportedly associated with disease severity in Thailand (Simpson *et al.,* 1999). In that study severe malaria isolates showed random, unrestricted invasion of RBCs (geometric mean SI ~ 1.35), whereas uncomplicated malaria isolates showed restricted, non-random invasion with a higher number of multiple-infected cells (geometric mean SI < 2.31). In the light of these observations, it would be interesting to carry out further studies using clinical isolates. For instance, the polymorphism and/or expression levels of *Pf_SI_1* candidate genes in clinical *P. falciparum* isolates could be ascertained and linked to SI and severity of disease.

With regards to vaccination, the antibodies generated from *Pf92* vaccine are likely to bind to the molecule which will greatly affect parasite selectivity for erythrocytes.There is the possibilty that such binding may decrease selectivity which in turn may increase parasite multiplication rate. This could result in a greater severity of malaria in some parts of the world.

5.4 Conclusion

In this study, selectivity index was determined for *P.falciparum* progeny clones from a genetic cross under static and shaking culturing conditions. A genomic region, QTL *PF_SI_1*, was identified that explains almost 70% of the variation seen in SI in the cross. This is the first evidence of parasite genetic loci involved in the selection of RBC for invasion.

Identification of genes for selectivity index and their expression level should be useful in understanding the molecular mechanism of invasion and the interaction of SI gene products with the erythrocytes. Increased knowledge of invasion and red cell selectivity could aid in the development of vaccines for the disease. It is also possible that such information will allow the development of antimalarial drugs which may target the products of these genes and disrupt their activity, thus preventing parasite invasion of erythrocytes.

Appendix 1

STATIC	3D7	HB3	X12	XP5	X5	X30	XP9	X10	X6	X4	X2	X33	XP24	X50	XP3	XP4	XP52	X11	X44	X39
3D7		$3.25X10^{-09}$	$2.82X10^{-12}$	$2.43X10^{-07}$	$4.27X10^{-14}$	$3.65X10^{-12}$	$5.03X10^{-12}$	$1.79X10^{-05}$	$3.50X10^{-11}$	$4.31X10^{-16}$	$3.77X10^{-12}$	$4.49X10^{-19}$	$1.79X10^{-13}$	$5.4X10^{-13}$	$2.7X10^{-09}$	$6.1X10^{-17}$	0.000103	$6.2X10^{-10}$	$4.6X10^{-22}$	$1.1X10^{-13}$
HB3			$5.43X10^{-11}$	$2.26X10^{-10}$	$7.50X10^{-10}$	$3.77X10^{-08}$	$1.02X10^{-09}$	$3.59X10^{-12}$	$5.77X10^{-10}$	**0.001762**	$5.81X10^{-10}$	**0.001644**	$4.39X10^{-07}$	$3.15X10^{-10}$	$2.55X10^{-09}$	$5.29X10^{-05}$	$2.32X10^{-08}$	$1.18X10^{-07}$	$5.03X10^{-05}$	$1.76X10^{-10}$
X12				$8.82X10^{-08}$	$1.96X10^{-05}$	$7.07X10^{-06}$	**0.004777**	$1.09X10^{-10}$	$5.58X10^{-06}$	$5.42X10^{-15}$	$5.85X10^{-06}$	$9.75X10^{-13}$	$1.24X10^{-13}$	$7.45X10^{-08}$	0.000175	$2.22X10^{-15}$	$4.01X10^{-09}$	$3.13X10^{-11}$	$8.32X10^{-18}$	$4.15X10^{-09}$
XP5					$1.75X10^{-09}$	$8.65X10^{-14}$	$3.84X10^{-08}$	$3.93X10^{-08}$	$8.01X10^{-09}$	$1.93X10^{-14}$	$8.54X10^{-09}$	$2.60X10^{-12}$	$2.28X10^{-12}$	$8.09X10^{-10}$	0.000235	$1.27X10^{-14}$	$3.55X10^{-07}$	$6.43X10^{-10}$	$9.97X10^{-19}$	$1.27X10^{-10}$
X5						$2.34X10^{-13}$	$1.81X10^{-07}$	$1.11X10^{-09}$	0.000112	$1.06X10^{-27}$	$5.63X10^{-05}$	$9.63X10^{-20}$	$7.99X10^{-24}$	$9.85X10^{-11}$	$4.12X10^{-12}$	$5.11X10^{-26}$	$4.11X10^{-17}$	$7.66X10^{-22}$	$2.16X10^{-27}$	$4.04X10^{-14}$
X30							$3.60X10^{-12}$	**0.001955**	$1.11X10^{-11}$	$2.24X10^{-11}$	$1.18X10^{-11}$	$7.67X10^{-10}$	**0.051075**	$4X10^{-12}$	$2.4X10^{-10}$	$2.12X10^{-10}$	$1.18X10^{-07}$	0.000415	$1.49X10^{-15}$	$1.52X10^{-12}$
XP9								$1.23X10^{-08}$	$1.19X10^{-09}$	$7.40X10^{-26}$	$7.9X10^{-13}$	$1.16X10^{-26}$	$2.23X10^{-19}$	$4.76X10^{-18}$	$6.14X10^{-16}$	$3.95X10^{-21}$	$2.01X10^{-22}$	$9.4X10^{-26}$	$1.77X10^{-21}$	$1.18X10^{-21}$
X10									$3.42X10^{-09}$	$2.67X10^{-09}$	$3.48X10^{-09}$	$1.25X10^{-08}$	0.000323	$9.97X10^{-10}$	$1.24X10^{-07}$	$3.2X10^{-08}$	0.000555	**0.242068**	$9.2X10^{-12}$	$3.29X10^{-10}$
X6										$3.74X10^{-17}$	**0.907414**	$3.93X10^{-20}$	$7.35X10^{-15}$	$1.03X10^{-11}$	$2.11X10^{-14}$	$6.72X10^{-16}$	$6.09X10^{-17}$	$3.47X10^{-16}$	$3.8X10^{-17}$	$9.51X10^{-14}$
X4											$2.24X10^{-20}$	**0.798595**	$4.01X10^{-19}$	$2.05X10^{-22}$	$5.78X10^{-21}$	$1.1X10^{-11}$	$2.79X10^{-19}$	$2.82X10^{-22}$	$6.88X10^{-19}$	$1.93X10^{-24}$
X2												$1.37X10^{-34}$	$1.28X10^{-17}$	$6.61X10^{-18}$	$3.29X10^{-23}$	$6.38X10^{-19}$	$1.93X10^{-28}$	$4.9X10^{-22}$	$8.78X10^{-19}$	$3.4X10^{-21}$
X33													$7.95X10^{-15}$	$8.19X10^{-35}$	$9.21X10^{-33}$	$8.76X10^{-10}$	$3.57X10^{-30}$	$5.58X10^{-22}$	$4.86X10^{-14}$	$6.37X10^{-35}$
XP24														$3.81X10^{-18}$	$4.98X10^{-16}$	$1X10^{-16}$	$6.71X10^{-13}$	$4.33X10^{-10}$	$1.81X10^{-24}$	$1.33X10^{-18}$
X50															$1.66X10^{-25}$	$2.65X10^{-19}$	$1.96X10^{-29}$	$2.98X10^{-24}$	$9.44X10^{-21}$	$2.74X10^{-17}$
XP3																$8.4X10^{-18}$	$6.33X10^{-24}$	$5.12X10^{-21}$	$3.56X10^{-18}$	$5.07X10^{-27}$
XP4																	$5.38X10^{-16}$	$2.83X10^{-17}$	$6.96X10^{-22}$	$1.22X10^{-19}$
XP52																		$2.88X10^{-15}$	$6.64X10^{-19}$	$3.7X10^{-30}$
X11																			$7.57X10^{-21}$	$2.06X10^{-26}$
X44																				$5.78X10^{-21}$
X39																				

Table 1 Comparisons of SI between the progeny clones revealed that under static conditions

SHAKING	3D7	HB3	X12	XP5	X5	X30	XP9	X10	X6	X4	X2	X33	XP24	X50	XP3	XP4	XP52	X11	X44	X39
3D7		$3.45X10^{-14}$	$8.17X10^{-14}$	$1.10X10^{-16}$	$6.28X10^{-10}$	$2.35X10^{-10}$	0.00018	**0.38**	$2.25X10^{-09}$	$1.98X10^{-11}$	$8.1X10^{-09}$	$2.4X10^{-09}$	**0.6504**	$3.36X10^{-10}$	$6.17X10^{-08}$	$3.89X10^{-05}$	$6.52X10^{-08}$	**0.634**	0.000155	$1.94X10^{-09}$
HB3			$1.53X10^{-18}$	$3.87X10^{-16}$	$1.38X10^{-12}$	$9.32X10^{-13}$	$3.11X10^{-11}$	$4.50X10^{-14}$	$1.26X10^{-11}$	**0.007729**	$3.49X10^{-11}$	$1.3X10^{-11}$	$9.9X10^{-11}$	$5.74X10^{-10}$	$6.04X10^{-11}$	$2.39X10^{-11}$	$4.11X10^{-11}$	$5.97X10^{-10}$	$2.49X10^{-10}$	$2.29X10^{-11}$
X12				$1.64X10^{-06}$	0.000499	0.049936	$1.01X10^{-09}$	$9.63X10^{-12}$	**0.00151**	$1.21X10^{-13}$	$5.75X10^{-07}$	**0.001394**	$3.34X10^{-11}$	**0.01637**	$5.73X10^{-08}$	$1.49X10^{-09}$	$6.72X10^{-07}$	$1.04X10^{-09}$	$9.71X10^{-10}$	$9.42X10^{-06}$
XP5					$1.62X10^{-09}$	$1.58X10^{-08}$	$2.66X10^{-13}$	$4.26X10^{-12}$	$2.74X10^{-08}$	$6.4X10^{-17}$	$1.6X10^{-10}$	$2.85X10^{-08}$	$7.84X10^{-16}$	$1.32X10^{-03}$	$5.49X10^{-12}$	$3.36X10^{-13}$	$8.11X10^{-11}$	$4.76X10^{-13}$	$2.33X10^{-13}$	$8.03X10^{-10}$
X5						$3.77X10^{-07}$	$9.02X10^{-20}$	$1.71X10^{-08}$	**0.022195**	$3.17X10^{-28}$	$3.75X10^{-12}$	**0.04009**	$1.38X10^{-19}$	$9.3X10^{-12}$	$8X10^{-16}$	$1.09X10^{-19}$	$6.69X10^{-13}$	$3.07X10^{-18}$	$3.73X10^{-20}$	$2.33X10^{-08}$
X30							$9.26X10^{-21}$	$6.15X10^{-09}$	$2.14X10^{-05}$	$1.42X10^{-28}$	$2.40X10^{-14}$	$6.35X10^{-06}$	$2.5X10^{-20}$	$1.24X10^{-09}$	$1.49X10^{-17}$	$9.75X10^{-21}$	$1.18X10^{-14}$	$7.55X10^{-19}$	$3.77X10^{-21}$	$3.34X10^{-12}$
XP9								**0.008593**	$4.86X10^{-13}$	$9.9X10^{-26}$	$4.4X10^{-17}$	$3.14X10^{-15}$	$8.89X10^{-10}$	$8.4X10^{-18}$	$4.14X10^{-15}$	**0.00191**	$5.13X10^{-15}$	$2.4X10^{-10}$	**0.838798**	$2.12X10^{-18}$
X10									$4.87X10^{-08}$	$1.66X10^{-09}$	$8.52X10^{-07}$	$5.1X10^{-08}$	**0.4767**	$5.54X10^{-09}$	$5.41X10^{-06}$	**0.002594**	$1.6X10^{-06}$	**0.472**	**0.009204**	$2.19X10^{-07}$
X6										$1.82X10^{-17}$	$1.31X10^{-10}$	**0.422689**	$4.54X10^{-13}$	$2.47X10^{-12}$	$2.08X10^{-11}$	$3.22X10^{-13}$	$8.77X10^{-12}$	$4.13X10^{-16}$	$1.76X10^{-13}$	$2.6X10^{-08}$
X4											$1.22X10^{-28}$	$2.07X10^{-22}$	$1.98X10^{-21}$	$1.42X10^{-24}$	$1.38X10^{-27}$	$2.35X10^{-26}$	$2.21X10^{-27}$	$1.65X10^{-22}$	$3.12X10^{-26}$	$7.85X10^{-29}$
X2												$1.25X10^{-13}$	$2.07X10^{-16}$	$3.52X10^{-18}$	$4.24X10^{-10}$	$6.64X10^{-17}$	$1.95X10^{-05}$	$2.98X10^{-20}$	$1.93X10^{-18}$	$9.17X10^{-10}$
X33													$2.27X10^{-15}$	$3X10^{-19}$	$4.57X10^{-14}$	$1.4X10^{-16}$	$3.92X10^{-16}$	$9.53X10^{-26}$	$6.28X10^{-17}$	$3.06X10^{-10}$
XP24														$2.05X10^{-16}$	$1.17X10^{-15}$	$7.81X10^{-11}$	$3.16X10^{-15}$	**0.9845**	$9.68X10^{-10}$	$3X10^{-17}$
X50															$1.52X10^{-17}$	$2.42X10^{-19}$	$1.56X10^{-20}$	$1.37X10^{-28}$	$1.23X10^{-19}$	$1.82X10^{-15}$
XP3																$1.46X10^{-14}$	$3.98X10^{-07}$	$4.2X10^{-17}$	$1.55X10^{-15}$	$3.94X10^{-14}$
XP4																	$1.15X10^{-15}$	$1.61X10^{-12}$	**0.001744**	$2.99X10^{-19}$
XP52																		$1.49X10^{-21}$	$2.28X10^{-16}$	$5.11X10^{-12}$
X11																			$1.64X10^{-11}$	$5.54X10^{-21}$
X44																				$7.99X10^{-20}$
X39																				

Table 2 Comparisons of SI between the progeny clones revealed that under shaking conditions

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