

***Anopheles/Plasmodium* interactions at
the ookinete-to-oocyst developmental transition**

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DECLARATION OF OWN WORK

I certify that this thesis and the research to which it refers is the product of my own work and that any ideas or quotations from the work of other people, published or otherwise, are fully acknowledged in accordance with standard referencing practices and with scientific collaborators clearly listed.

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May 2011

ABSTRACT

The ookinete to oocyst developmental transition of the *Plasmodium* parasite represents a major population bottleneck in the malaria life cycle. This suggests that it could be a target for intervention strategies, such as transmission blocking vaccines, provided essential parasite target molecules can be identified. A recent microarray analysis has identified a large number of transcripts differentially expressed during the parasite's developmental transitions. Genes differentially regulated during the ookinete-to-oocyst transition may determine the development of the parasite within the mosquito host, as well as, participating directly in parasite/mosquito interactions. Yet, the function of the majority of such molecules is largely unknown.

This PhD thesis aims to identify and functionally characterise genes putatively involved in ookinete development and/or the interactions between the parasite and the mosquito host in the model system *Plasmodium berghei*. Thirty three proteins likely to be implicated in the parasite's interaction with the mosquito immune system and local epithelial response were identified based on their expression pattern and predicted structural features. Generation of knock-out mutants through targeted gene disruption by homologous recombination was the first step towards functional characterization of these candidates. Successful mutants were assessed for their ability to complete their sexual sporogonic development, as well as, their impact on mosquito immunity following infection of *Anopheles* mosquitoes of various immune backgrounds. Interestingly, two of the successful mutants were hampered in their ability to undergo normal differentiation during ookinete development while the third one's ability to invade the mosquito midgut epithelium was impaired. The inability to invade implies a potential interaction of this gene product with mosquito midgut ligands. Eventually malaria transmission through *Anopheles* mosquitoes was affected in all three mutants. Moreover, challenging of a mosquito protein *LRIM1*, a major parasite antagonist, also revealed potential involvement of the three mutants in mosquito/parasite immune response pathways. Genetic crosses with parasite lines deficient in the production of either male or female fertile gametes demonstrated in the case of two mutants that, this defect in ookinete development is sex dependent, thus underlining the critical importance of maternal and/or paternal control during the first few hours of parasite development in the mosquito.

DEDICATION

Στην οικογενεια μου που ειναι παντα εκει..

Στους δασκαλους μου που με πηγαινουν πιο περα...

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ABBREVIATIONS

A

ABS	Asexual Blood Stage
AC	Apical Complex
AMA	Apical Membrane Antigen
ANKA	Anvers/Kasapa
ANOVA	Analysis Of Variance
AP	Apelata
Api	Apicoplast
APL	<i>Anopheles Plasmodium</i> -responsive Leucine-rich
APR	Apical Polar Ring
APO	Apolipoprotein
AT	Adenine-Thymine
ATP	Adenosine TriPhosphate

B

B.C.	Before Christ
BF	Bright Field
bp	base pair
BSA	Bovine Serum Albumin

C

C3	Complement protein 3
C57BL/6	C57 Black 6
CAP	Capsule Protein
CCp	limulus Coagulation factor C domain-containing proteins
CDPK	Calcium Dependent Protein Kinase
cDNA	complementary DNA
CelTOS	Cell-Traversal protein for Ookinetes and Sporozoites
CF11	Cellulose fibre powder 11
cGMP	cyclic Guanosine MonoPhosphate
Chitinase	CHIT
Chr	Chromatin
CLB	Coelenterazine Loading Buffer
CSP	CircumSporozoite Protein
CTL	C-Type Lectin
CTRP	Circumsporozoite and TRAP Related Protein

D

DAPI	4',6-diamidino-2-phenylindole
DDT	DichloroDiphenylTrichloroethane

DHFR/TS	DiHydroFolate Reductase-Thymidilate Synthase
DIC	Differential Interference contrast
DMSO	DiMethyl SulfOxide
DNA	DeoxyriboNucleic Acid
DOZI	Development Of Zygote Inhibited
dsRNA	double stranded RNA
E	
ECP	Egress Cysteine Protease
EDTA	Ethylene Diamine Tetraacetic Acid
eef	eukaryotic elongation factor
EGF	epidermal growth factor (EGF)
eGFP	enhanced Green Fluorescent Protein
eIF2 α	eukaryotic initiation factor 2 α
ER	Endoplasmic reticulum
F	
F	Forward
FCS	Foetal Calf Serum
G	
Gc	Gametocyte
GC	Guanylate cyclase
GCS	Generative Cell Specific
GIG	Gene implicated in gametocytogenesis
Gm	Gamete
Gm	Gametocyte
GTP/GDP	Guanosine Tri/DiPhosphate
H	
Hepes	4-(2-HydroxyEthyl)-1-Piperazine Ethane Sulfonic acid
HMGP	High Mobility Group Proteins
HSP	Heat Shock Protein
I	
IFA	ImmunoFluorescence Assay
IM	Intra-Muscular
IMC	Inner Membrane Complex
INT	Integration
IP	Intraperitoneal
RNAi	RNA interference
K	
KD	Knock-Down
kD	kiloDalton
KO	Knock out
L	

LAP	LCCL/lectin adhesive- like protein
LB	Luria broth
LCCL	Limulus Clotting factor C, Coch-5b2 and
LRIM	Leucine Rich-Repeat Immune gene
M	
MACPF	Membrane Attack Complex Perforin
MAEBL	Merozoite AMA1/Erythrocyte Binding Ligand-like protein
MAOP	Membrane-Attack Ookinete Protein
MAP	Mitogen-Activated Protein kinase
MDV	Male Development gene
Mg	Milligram
µg	microgram
MISFIT	Male-Inherited Sporulation Factor Important for Transmission
mL	milliLiter
µL	microLiter
Mn	Micronemes
mRNA	messenger RNA
MS	Mass spectroscopy
MSP	Merozoite Surface Protein
MTIP	Myosin A Tail domain Interacting Protein
MTOC	MicroTubule Organising Centre
MTRAP	Merozoite Thrombospondin-Related Adhesive Protein
MudPIT	Multidimensional Protein Identification Technology
N	
n	nuclear genome content
N	Nucleus
ng-	nanogram
Nek	NIMA related kinase
NIMA	Never-in-mitosis/Aspergillus
NLS	Nuclear Localisation Signal
NOS	Nitric Oxide Synthase
NTSbl.	Non-Triton Soluble fraction
O	
Ocy	Oocyst
Ook	Ookinete
OPI	Ontology Based Pattern Identification
ORF	Open Reading Frame
P	
PAGE	PolyAcrylamide Gel Electrophoresis
PBS	Phosphate-Buffered Saline
PBS-PI	PBS-Protease-Inhibitors

pBS	pBluescript
PCR	Polymerase Chain Reaction
PDE	PhosphoDiEsterase
PEG	Proteins of Early Gametocyte
PfEMP	P. falciparum erythrocyte membrane protein
PFGE	Pulsed Field Gel Electrophoresis
PH	PhenylHydrazinium chloride
P.I.	Post infection
PKG	cGMP-dependent Protein Kinase
PPLP	Plasmodium Perforin-Like Protein
PV	Parasitophorous vacuole
Q	
QRT-PCR	Quantitative Real- Time PCR
R	
R	Reverse
RBC	Red Blood Cell
RNA	Ribonucleic acid
rRNA	ribosomal RNA
RPMI	Roswell Park Memorial Institute
RT	Room Temperature
RT-PCR	Reverse Transcriptase PCR
S	
Sbl	Soluble fraction
SDS	Sodium Dodecyl Sulphate
SEM	Standard Error of the Mean
SERCA	Sarcoplasmic Endoplasmic Reticulum Calcium
Serpin	Serine protease inhibitor
SGS	Salivary Gland Specific
SM1	Salivary gland and Midgut binding peptide1
SNARE	Soluble NSF (N-ethylmaleimide-sensitive factor) Attachment protein
SOAP	Secreted Ookinete Adhesive Protein
SpMt	Subpellicular Microtubules
SR	Scavenger Receptor
SP	Signal Peptide
SPECT	Sporozoite microneme Protein Essential for Cell
Spz	Sporozoite
SRPN	Serpin
(S)SH	(Suppressive) Subtractive Hybridisation
SUB	Subtilisin
T	

TE	Tris-EDTA
TEM	Transmission Electron Microscopy
TEP	Thioester-containing protein
TF	Transcription Factor
TSbl.	Triton soluble fraction;
TBE	Tris-borate-EDTA
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline Tween-20
TBV	Transmission blocking Vaccine <i>Toxoplasma gondii</i> DiHydroFolate Reductase-Thymidilate
TgDHFR/TS	Synthase
TM	TransMembrane
Tm	Template Melting temperature
TO	Theiler's Original
took	transforming ookinete (took)
TPx	2 Cys Peroxiredoxin
TRAP	Thrombospondin-related adhesive protein
tRNA	transferRNA
TUB	TUBulin
U	
UTR	UnTranslated Region
W	
WARP	von Willebrand Factor A domain-Related Protein
WASP	Wiskott–Aldrich Syndrome Protein
WB	Western Blot
WD40	40 AA Trp-Asp (W-D) motif
WHO	World Health Organisation
WT	Wild Type
X	
XA	Xanthourenic acid

1. INTRODUCTION

1.1. MALARIA

1.1.1. History: An ancient disease

"As one who has the shivering of the quartan so near,
that he has his nails already pale
And trembles all, still keeping the shade,
Such I became when those words were uttered."

The Inferno, Dante (1265-1321)

Throughout history, malaria has been well documented in a series of writings long before Dante's masterpiece, early since 2500BC. Its typical clinical manifestations of extremely high fever, often following a distinctive every-other or every-third-day pattern, accompanied by rigor, common malaise and severe anaemia (White, 2008) has found mentions in the most ancient Roman (Horace), Greek (Galen, Hippocrates), Chinese (The Canon of Medicine), Indian (Atharva Veda, Charaka Samhita) and Egyptian (cuneiform script) manuscripts to the more recent Shakespearean plays (The Tempest).

However, it was only in the 1880s that Charles Louis Alphonse Laveran, a French army surgeon stationed in Algeria, noticed parasites in the blood of a patient suffering from malaria (Laveran, 1880). Five years later Camillo Golgi, an Italian neurohistologist observed the characteristic pattern of every-other or every-third-day fever of the disease, that coincided with the release of parasites into the bloodstream and gave evidence for more than one form of malaria (i.e. attributed to more than one species). Nearly, twenty years later, in 1897, a British officer in the Indian Medical Service, Ronald Ross, demonstrated that malaria parasites could be transmitted from infected patients to mosquitoes necessitating a sporogonic cycle (the time interval during which the parasite developed in the mosquito) (Ross, 1897). Parallel work lead by Giovanni Batista Grassi established that specific strains

of *anopheline* infected mosquitoes could transmit the disease to a healthy individual, completing in this way the developmental cycle of parasite transmission (Grassi, 1898).

Following these observations, we now share the knowledge that malaria is a vector-borne infectious disease caused by protozoan parasites, of the genus *Plasmodium* - named by the Italian scientists Ettore Marchiafava and Angelo Celli who spent many years studying its morphology and biological cycle. Five species of the *Plasmodium* parasite can infect humans; *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae*, *Plasmodium knowlesi* and *Plasmodium falciparum*, among which, the latter causes the most severe forms of the disease that can lead to multiple organ dysfunction and finally death (World Health Organization; Malaria, 2009).

1.1.2. Present reality: a contemporary problem

The World Health Organization (WHO) estimates an annual malaria burden of 247 million cases worldwide (Snow, 2005). Notably, 86% of cases occur in Africa, where temperature and rainfall are most suitable for the development of the malaria-causing *Plasmodium* parasites in *Anopheles* mosquitoes. The disease caused nearly one million deaths in 2006, out of which 85% were in African children under the age of 5 years (Aregawi et al., 2008). Pregnant women were also of higher risk and sensitivity, malaria posing a great risk of premature delivery and infant mortality (Miller & Greenwood, 2002). Even though the majority of cases are that of uncomplicated disease, 1-2% can be fatal making the general socioeconomic burden of the disease profound. Taking into account poverty, economic policy, tropical location and life expectancy, countries suffering from malaria grow 1.3% less per person per year, compared to disease-free countries. On the other hand a 10% reduction in malaria is associated with an estimated annual economic growth of 0.3% (Miller & Greenwood, 2002).

In addition, as a result of poor infrastructure and low socioeconomic standards, prophylactic measurements and treatment are not readily or affordably available in endemic countries. Despite current efforts, malaria is still among the diseases that suffer the “10/90 divide” as to medical research funding, i.e. less than 10% of research funds are spent on the diseases that account for 90% of the global burden of disease (Lee & Mills, 2000).

1.1.3. Overview of malaria life cycle and pathogenesis mechanisms

The *Plasmodium* life cycle comprises numerous transitions and stages throughout its developmental cycle. Those include various stages of intracellular replications, as well as, extracellular migration and invasion patterns. Intriguingly, the parasite requires both a vertebrate host and an invertebrate vector, to complete its developmental cycle (Greenwood et al., 2008) (Figure 1.1).

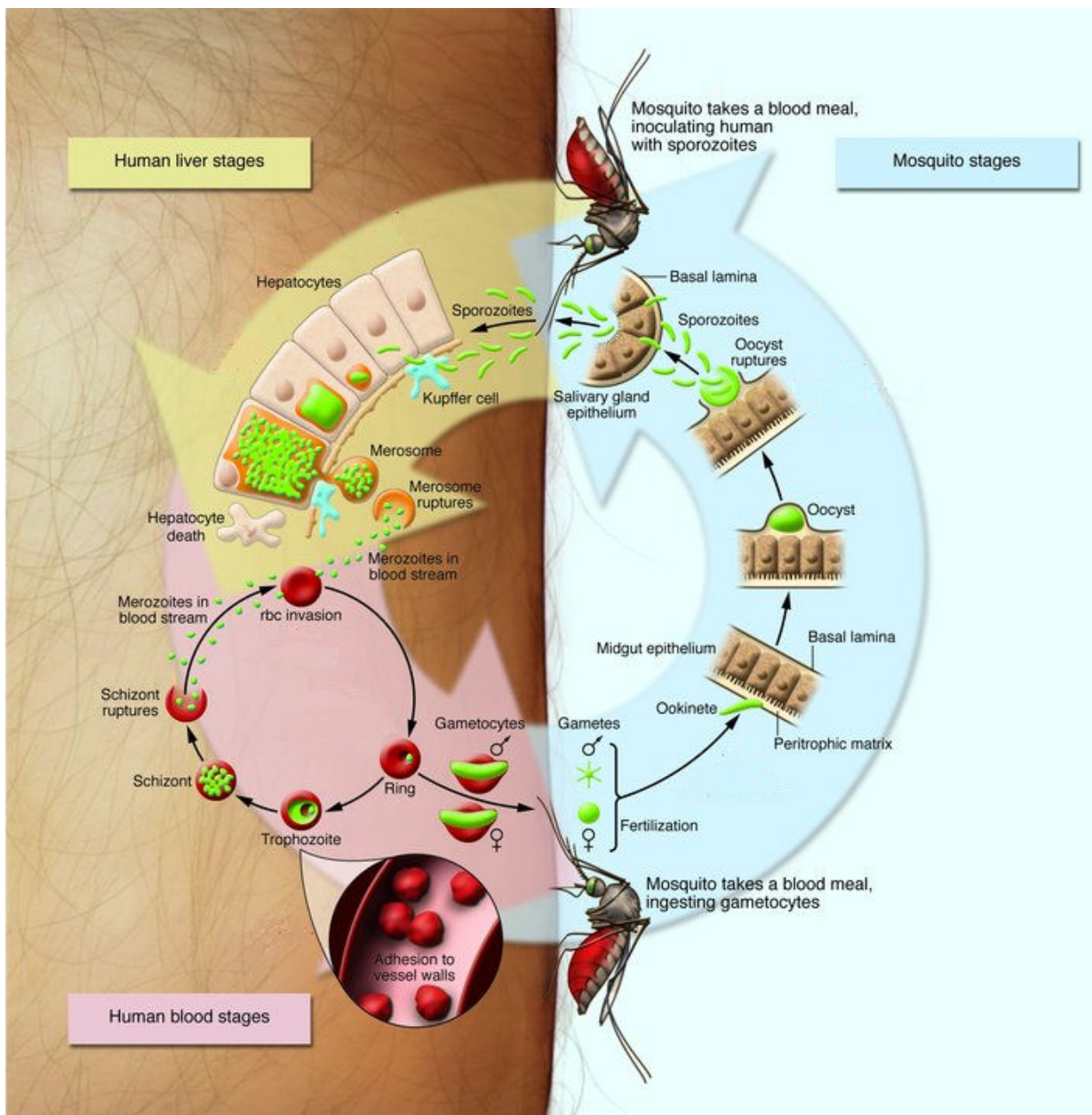


Figure 1.1. Overview of *P. falciparum* life cycle. The malaria parasite enters a vertebrate host during blood meal uptake from an infected female mosquito. The infective sporozoites travel through the dermis and enter the blood stream, till they finally reach the liver. After actively entering the liver through Kupfer cells, they subsequently migrate through several hepatocytes, till they arrest intracellularly where they will develop into exoerythrocytic schizonts. Several thousand merozoites will be released into the blood stream, from one single schizont and commence a cycle of red blood cell invasion and intracellular replication. The merozoites enter the red blood cells by an active process, where they transform into the young ring stage, which then matures into the trophozoite. The trophozoite gives rise to the blood-stage schizont, which ruptures to release a new set of merozoites into the bloodstream and continue the asexual cycle. However, a subset of trophozoites transforms into the male and female gametocytes which constitute the sexual progenitor cells. Gametocytes are essential for transmission and enter the mosquito host during uptake of an infected blood meal by a female mosquito. Following this, and within the mosquito midgut, the gametocytes become activated, leading to the generation of mature female or male gametes. Fertilisation then occurs, giving rise to the zygote. 22-24h later, the zygote matures to transform into the motile ookinete, which will escape the bolus by traversing the midgut epithelium. In the sub-epithelial space the ookinete transforms into the sessile oocyst. Within the maturing oocyst, thousands of sporozoites develop, which are eventually released and make their way to the mosquito salivary glands, from where they can be transmitted to the next vertebrate host, during subsequent blood meals; thus, completing the malaria transmission cycle. Figure adapted with modifications from *Greenwood et al 2008* (Greenwood et al., 2008)

Plasmodium falciparum is responsible for the majority of cases of severe malaria, while the other four human malaria species typically cause febrile but non-fatal disease (Pasvol, 2005). Several factors have been associated with *P. falciparum*'s increased virulence and pathogenicity; nevertheless, a great deal is yet to be uncovered (Pasvol, 2001).

One of the well established factors, include *P. falciparum*'s great redundancy in its cell-to-cell interaction machinery. Interestingly, unlike its orthologous counterpart *P.vivax*, *P. falciparum* displays the ability to invade cells using a variety of ligand-receptor combinations. Furthermore, it shows the ability to invade not only young immature red blood cells (reticulocytes) – like *P.vivax* – but any kind of red blood cell. It is clear that these two factors contribute a great deal to *P. falciparum*'s tendency to create higher parasitaemia levels, resulting in worse clinical outcomes (Miller & Greenwood, 2002).

Mechanisms of cytoadherence and subsequent sequestration also play a major role in *P. falciparum*'s pathogenicity. Parasitized red blood cells adhere to the vascular epithelium facilitated by a single parasite encoded protein with multiple adhesion domains, displayed on the surface of infected RBCs (*P. falciparum* erythrocyte membrane protein 1 - PfEMP1). This results to peripheral sequestration of parasites, which protects them from removal from the circulation as they pass through the spleen, as well as, oxidant damage as they pass through the lungs (Miller & Greenwood, 2002). PfEMP1 also binds to complement receptor-1 resulting in clustering of unparasitized red cells around parasitized ones (rosetting), contributing to micro vascular obstruction. Interestingly, PfEMP1 is encoded by the multigene *var* family, which are subject to clonal antigenic variation. The latter, enables the parasite to evade the host immune response and provide no permanent or slow developing immunity to patients; thus, establishing chronic malaria infections.

However, exactly how adhesion and/or sequestration are related to clinical pathology is poorly understood. It has recently been proposed that damage to the host endothelium and organs may be caused by obstruction to the blood flow and localized or systemic production of pro-inflammatory cytokines (Heddini, 2002).

1.1.4. Clinical manifestations and diagnosis

The clinical manifestations and outcome of any malaria infection are determined by a combination of parasite, host and socio geographical factors. However, their exact and precise role often remains unclear.

In general, in the vertebrate host, liver stage infection is completely asymptomatic and all malaria associated pathology stems from the asexual blood stage cycle of invasion and replication (Matuschewski, 2006). Uncomplicated malaria usually presents with non-specific symptoms of fever, headache, sweats, chills, rigors and joint pain (Bell & Winstanley, 2004). This ambiguity of symptoms can severely impair prompt diagnosis and result in lethal disease (Bell et al, 2006). Symptoms of severe malaria can vary as well, depending on patient factors (e.g. age, genetic background, pregnancy, previous exposure) and regional malaria endemicity (e.g. parasite drug-susceptibility etc) (Miller & Greenwood, 2002) . Patients with severe disease typically present with cerebral malaria, severe anaemia, metabolic acidosis, respiratory, renal and liver failure (Pasvol, 2005). Notably, severe disease has been hypothesized to be related to sequestration to the respective organs (Weatherall, 2002).

Apart from clinical findings, microscopic examination of thick (unfixed drop of blood) and thin (methanol-fixed monolayer) blood smears remains the gold standard in malaria diagnosis (Pasvol, 2005). More recently antigen capture tests have been developed but their role in diagnosis remains in second line (Craig et al., 2002). Most importantly, however, microscopic facilities are scarce in many malaria endemic areas and even though more cost-effective antibody-based rapid diagnostic tests are now available, they are still a long way away from actually being applied in endemic countries (Bell et al., 2006).

1.2. MALARIA MANAGEMENT AND CONTROL

1.2.1. Efforts to control and eradicate malaria

The Global Malaria Eradication Programme was launched by the WHO in 1955 (WHO, 1999) and depended on two key milestones: vector control and host treatment. Implementation of these tools had a substantial impact in some areas, particularly where transmission rates were low, such as India and Sri Lanka (WHO, 1999). The same was accomplished mainly in Western Europe and the USA, as a result of financial development and public health measures in the developed world (Mabaso et al., 2004). Despite these successes, the campaign foundered due to the emergence of regimen-resistant *Plasmodium* parasites and *Anopheles* mosquitoes. It is obvious, that the complex dual life cycle in the development of the parasite, impairs definite control or complete eradication of the disease. Nevertheless, this very complexity opens a window of opportunity to new global therapeutic and control strategies that address the two key players in the game of malaria: that of the vertebrate host and of the invertebrate vector.

1.2.2. Addressing the vertebrate host

1.2.2.1. Drug administration – Host treatment

Currently, the use of antimalarial drugs for both treatment and prevention is variable. Prophylaxis of travellers, the endemic population of high transmission areas and high risk individuals i.e. pregnant women has been widely established (White, 2008).

Main regimens include quinolines, antifolates and artemisin, acting in a wide range of parasite metabolic processes. For example, quinolines are hypothesized to inhibit haemoglobin detoxification (Egan et al., 2007), antifolates prevent nucleic acid synthesis (Hyde, 2007), while artemisin has been implicated in interference of protein transport, processing and mitochondrial function (Golenser et al., 2006; White, 2008).

Unfortunately, most of these drugs have succumbed to global resistance by *P.falciparum* and increasing resistance by *P.vivax*. Unlike bacteria, *Plasmodium* spp. do not have transferable resistance mechanisms but they are eukaryotes and can acquire or lose polygenic resistance mechanisms during meiosis. Resistance arises readily to several drugs because a single point mutation confers resistance and per-parasite mutation frequencies are high. For example, mutations in *pfmdr1* (*Plasmodium falciparum* multi drug resistance) and *pfCRT* (*Plasmodium falciparum* chloroquine resistance transporter) genes (Mita et al., 2009; Nsoby et al., 2007), associated with transporter membrane proteins, are attributable for resistance to quinoline. Similarly, progressive acquisition of mutations in the dihydrofolate reductase (DHFR) or dihydropterate synthetase (DHPS) genes (Mita et al., 2009) is responsible for resistance to antifolates.

1.2.2.2. Vaccine development – Host protection

Current vaccine development strategies fall into two categories reflecting parasite development in the vertebrate host: pre-erythrocytic, liver stage vaccines and erythrocytic, blood-stage vaccines. It is worth noting that these vaccines might not be able to confer total immunity, nonetheless, may still act on reducing parasite load and decreasing morbidity and mortality (Matuschewski, 2006; Matuschewski & Mueller, 2007).

Pre-erythrocytic liver stage vaccines target the sporozoites and the infected hepatocytes. Anti sporozoite inhibitory antibodies that can potentially impair parasite motility or migration, and IFN γ -secreting effector T cells that target infected hepatocytes could be useful. However, in such attempts it is imperative to take into consideration that one single sporozoite can generate thousands of merozoites that can ultimately lead to a blood stage infection (Matuschewski, 2006). Protective immunity by injection of irradiated, live attenuated sporozoites has been achieved in both rodent models (Nussenzweig et al., 1967), as well as human volunteers (Hoffman et al., 2002), and currently remains the gold standard on malaria vaccines. Nevertheless, safety and practical concerns including selection of resistant strains, instability, varying efficiency and lack of high-throughput production, preservation and distribution, hamper a broad use application (Matuschewski, 2006). Recently, injection of genetic live attenuated sporozoites, by reverse genetics, has proven to elicit protracted sterile protection and overcome the above concerns (Mueller et al., 2005; van Dijk et al., 2005). A

more cost effective solution lies in subunit vaccines such as RTS,S which have shown encouraging results (Alonso et al., 2005; Alonso et al., 2004).

The milestones for erythrocytic blood-stage vaccine generation include antibody-mediated inhibition of a) merozoites and b) cytoadhesion by infected erythrocytes. These approaches aim to reduce asexual parasite load and accelerate the immunological memories of naive individuals, compromising disease severity (Matuschewski & Mueller, 2007). Clinical trials targeting hepatocytic development and invasion ability of merozoites have commenced with promising results (Druilhe et al., 2005). Nevertheless, the polymorphic nature of merozoites antigens that does not allow establishment of long term immune memory against any of the merozoite surface protein members, remains a long term obstacle in this effort (Matuschewski, 2006). Targeting cyto-adherence by anti-*Pf*EMP1 antibodies represents another choice (Avril et al., 2006).

1.2.3. Addressing the invertebrate vector

1.2.3.1. Strategies

The main strategies in tackling the problem of malaria from the vector's side can be summed up first in reduction or avoidance of host-vector contact and second in manipulation/elimination of the vector.

Bed nets have been used traditionally to protect people from the nuisance caused by nocturnally biting insects, such as mosquitoes (Lindsay & Gibson, 1988). Today insecticide treated nets coated with pyrethroids are an important tool in malaria control. Several studies have confirmed their efficacy in reducing malaria related morbidity and mortality (Takken, 2002). Nevertheless, in order for the measure to attain maximum efficiency, broad application and re-treatment of nets is necessary. These policies seem to fail in poor endemic regions, thus, represent major issues of concern (Curtis et al., 2006). Currently, no solid data of insecticide resistance directly related to use of treated bed nets have been presented, however, evidence of shifts in hosts and time or site of biting have been noticed (Takken, 2002).

In the 1930s the introduction of effective household spraying of residual insecticides, including the “wonder compound” dichloro-diphenyl-trichloroethane (DDT), together with chloroquine,

formed the cornerstones of the ill-fated, although very successful, global control campaign of the 1950s and 1960s (Zucker, 1996). Rapid emergence of DDT resistant mosquitoes, as well as, environmental concerns and controversial reports of it being a human health hazard (Rogan & Chen, 2005), resulted soon in its abandonment as a potential weapon against malaria (Mabaso et al., 2004).

Prior to DDT, environmental interference (e.g. draining marshes) and larvicidal methods were the main methods for controlling mosquito host populations. These efforts have now been implemented by biological control including the use of larvivorous predators (e.g. *Gambusia*), pathogens, e.g. bacteria (*Bacillus thuringiensis israelensis*), viruses (Baculovirus) and fungi (*Beauveria*). In addition, interest in larvicidal methods has also started to grow (Bruce-Chwatt, 1987). Nevertheless, as mentioned before, interference with mosquito breeding behaviour is also amenable to evolutionary pressure, thus can lead to consequent resistance in the prospect of mosquito behavioural change (Pates & Curtis, 2005).

The progress of genetic engineering as well as our knowledge of the vector's genome have also paved the way of new methods of vector control including the use of lethal homing endonucleases (Burt, 2003; Windbichler et al., 2007; Windbichler et al., 2008), the introduction of cytoplasmic incompatibility induced by the endosymbiont *Wolbachia* (Sinkins and Gould, 2006) or genetic dominant-lethal technologies (Thomas, 2000). Nevertheless, potential attempts to replace a vector population by parasite refractory mosquitoes still faces many challenges (Curtis et al., 1999).

1.2.4. Transmission blocking approach

So far, it has become more than clear that preventative or therapeutic measures are severely hampered by two interlinked factors; the availability of few new effective compounds and the rapid inevitable emergence of resistance to the compounds already available (Egan & Kaschula, 2007). On this basis, a new approach on blocking malaria transmission rather than individual targeting (Miller & Greenwood, 2002) was put forward, under the broader scheme of combinational strategy against malaria (Bruce-Chwatt, 1987).

1.2.4.1. Malaria Transmission

In 1910 Ross (Ross, 1910) defined a mathematical model to the transmission of malaria, later expanded by newer formulations (Macdonald et al., 1968; Smith et al., 2007) as following:

$$Ro = ma^2bc / r\mu$$

Where Ro is the basic reproduction ratio representing the potential number of secondary cases of malaria originating from one primary case, assuming that the population is and remains fully susceptible. b is the proportion of infected bites on human that produce an infection, c is the proportion of infected bites on mosquitoes that produce an infection, $1/r$ defines the average duration of the malaria infection, m is the number of female mosquitoes per human host; a is the rate of biting on man by a single mosquito (number of bites per unit time) μ is the per capita mortality rate for mosquitoes ($1/\mu$ is the average life time of a mosquito)

It is evident that the reproductive ratio is a key indication of transmission intensity. In brief, if $Ro > 1$ the population infected by the parasite increases whereas if $Ro < 1$ that population gradually declines. Therefore, in order to achieve disease control, it remains crucial to reduce Ro . Classical treatment of malaria has very little impact on Ro , since usually gametocyte numbers are not suppressed (Peatey et al., 2009), even though duration of infection can be shortened ($1/r$) (Blandin et al., 2004; Jaramillo-Gutierrez et al., 2009; Osta et al., 2004).

A new approach has recently come forward: targeting the infectivity of the human to the mosquito (c). Complement, phagocytic cells and antibody mediated immunity seem to be still present and active in the mosquito midgut following a blood meal of the vector (Grotendorst et al., 1986; Sinden & Smalley, 1976). Thus, an “altruistic” vaccine that may not protect the individual itself, however, would block transmission by attacking the parasite within its mosquito host.

1.2.4.2. Addressing the parasite within the invertebrate vector

A series of advantages have been described in favour of targeting the parasite within its vector. (Kaslow, 1997). Firstly, of the thousands of parasites that are ingested by the mosquito, only a few survive to form oocysts and this occurs only in a small fraction of the mosquito population. Even less get to be transmitted back to the human host. Invasion stages of the parasite (midgut invasion by the

ookinete, sporozoite invasion of the salivary glands and hepatocytes), by definition represent major bottlenecks in parasite development, hence the best targets for attack.

Moreover, it has been well established that vaccine efficacy is dependent on the exposure time of the parasite to one's chosen "fight mechanism" (Anderson et al., 1989). Current vaccines targeting the surface of the blood stage merozoite only enjoy a few minutes of exposure per cycle, in contrast to current transmission-blocking vaccine targets (Malkin et al., 2005) that are facilitated by the extracellular life of parasite for even 24 hours.

Finally, the parasite has developed plenty of antigenic diversity in order to overcome the adaptive immune systems of the vertebrate hosts, especially during blood stage/sporozyte stages. To date, no evidence of a mosquito adaptive immune system has been described, while molecules expressed on the surface of the ookinete in the mosquito midgut appear not to be polymorphic (Kaslow et al., 1989; Richards et al., 2006).

For all the above reasons, transmission blocking vaccines and/or transmission blocking drugs, complementary to individual protection/treatment seems to be the way forward to malaria control. Of note, transmission blocking vaccine implementation inhibits the spread of drug and vaccine resistance by inhibiting transmission of resistant parasite populations (Carter, 2001; Matuschewski, 2006).

1.2.4.3. Transmission Blocking Vaccines (TBV) and Transmission Blocking Drugs (TBD)

The principle of TBV is the vaccination of individuals with mosquito-stage parasite proteins, using the human adaptive immune response to generate antibodies. These antibodies are subsequently transferred to the mosquito midgut during uptake of a blood meal (Dinglasan et al., 2008), bind parasite surface components and finally facilitate antibody- or complement-mediated killing, abolishing transmission (Saul, 2007). Mechanisms of TBV action seem to fall into various groups depending on the expression pattern of the molecules they target e.g. pre-fertilisation gametes seem to be affected by an antibody-mediated response resulting in blocked fertilisation (Rener et al., 1980), sentization of gametes resistant to complement to complement-mediated lysis (Grotendorst et al., 1986), post fertilisation inhibition of ookinete maturation (Ranawaka, Alejo-Blanco, & Sinden, 1994). Currently, antibodies against a number of *P. falciparum* proteins present on activated gametocytes seem to successfully block transmission (Saul, 2007) (Kaslow et al., 1989; Malkin et al.,

2005; Saxena et al., 2004). Several more gametocyte or ookinete candidate targets are under investigation for their transmission blocking potential, including WARP, chitinase, HAP2 and CTRP (see below for detailed description of these molecules) while recently it has been suggested that even mosquito proteins can be recruited to raise transmission blocking antibodies (Bousema et al., 2006; Dinglasan et al., 2008).

The same underlying principle as in TBV, is valid for TBD, and would ideally target gametocytes in humans. However, such kind of approach can be hampered by the fact that gametocytes are cell-cycle arrested in humans, thus metabolically inactive (Lang et al., 1998). Even though, one could therefore target the early stages of intra - mosquito development, several problems are present e.g. symptomatic infections are not necessarily responsible for the bulk of transmission and population infectivity is very variable, hence highlighting issues such as which groups of the human population should be treated.

However, TBV encounter several difficulties in being established as a major intervention strategy against malaria. Firstly, the antigens are never naturally expressed in the human host, hence, no natural boosting is achieved and thereby antibody responses may be short-lived. Secondly, a very significant portion of the population would have to be vaccinated to ensure an epidemiological break in transmission (Carter, 2001; Sauerwein, 2007; Saul, 2007). The latter varies depending on the transmission setting since the R_0 can vary significantly in different geographic areas (Smith et al., 2007). Perhaps, combining a TBV component with a pre-erythrocytic and / or blood stage vaccine would enhance both its efficiency, as well as, its appeal in endemic communities (Aide et al., 2007; Saul, 2007).

Nevertheless, it is clear that any successful transmission blocking strategy requires a thorough understanding of parasite development in the mosquito.

1.3. PARASITE DEVELOPMENTAL BIOLOGY

To facilitate comprehension with regards to the focus of this thesis, the *Plasmodium* life cycle will be subdivided into three phases centred around ookinete-to-oocyst developmental transition: (i) parasite sexual development and fertilisation, (ii) ookinete-to-oocyst developmental transition (iii) parasite mature sporogonic development and transmission to the vertebrate host.

1.3.1. Parasite sexual development and fertilisation

1.3.1.1. *Plasmodium* sex determination and differentiation - Gametocytogenesis

Sexual development is essential for the successful completion of malaria transmission, in accordance with all other *Apicomplexa* (Smith et al., 2002). In *Plasmodium* the sexual phase of development is already initiated in the infected vertebrate host, when a sub-set of asexual blood-stage parasites escape the continuous cycle of invasion and replication and commit to the production of either male or female gametocytes. This commitment occurs one asexual developmental cycle ahead of initiation of sexual development (Bruce et al., 1990), i.e. all merozoites deriving from a single sexually committed schizont are pre-determined to all develop into male or all female gametocytes (Silvestrini et al., 2000). Typically, a sex ratio of one female to every three male gametocytes is observed (later one female gametocyte gives rise to one female gamete contrary to eight male gametes for the male one), however, the female to male sex ratio can also be highly variable. Timing of sexual differentiation seems to differ between species varying from 10 days in *P. falciparum* to 28 days in *P. berghei* (Waters & Janse, 2004).

1.3.1.2. Gametocytogenesis is regulated by a complex set of environmental and genetic components

The distinct biological events governing gametocytogenesis are yet to be defined (Alano, 2007). A number of genetic factors linked to sexual commitment and differentiation have been identified. The inability to undergo gametocytogenesis has been frequently linked to the loss of cytoadherence, which in turn is associated with a lack of *PfEMP-1* expression (Alano et al., 1995; Day et al., 1993; Kemp et al., 1992). Loss of coding members of the *PfEMP-1* gene family has been genetically linked to a subtelomeric deletion of chromosome 9 (Barnes et al., 1994; Day, et al., 1993). The same region is also bearing the *P. falciparum* gene implicated in gametocytogenesis (*Pfgig*) targeted deletion of which, results in a significant decrease in the number of gametocytes produced (Gardiner et al., 2005). Moreover, in *P. berghei*, targeted deletion of *Tpx-1* (a member of the antioxidant enzyme family of peroxiredoxins) has been reported to be responsible for up to 60% less production of gametocytes (Yano et al., 2006). Recent data have also shown that loss of a gene named the *P. falciparum* male development gene – 1 (*Pfmdv-1*) (also described as *Pfpeg-3*) (Furuya et al., 2005), results in arrest in early stages of gametocytogenesis (Lal et al., 2008). Another gene, *Pfs16* seems also to be crucial for the completion of gametocytogenesis (Kongkasuriyachai et al., 2004). Similarly, *Pfg27* (*Pfg27/25*),-an abundant protein of early stage gametocytes- seems to be important for cell integrity and contributes to successful gametocytogenesis (Lobo et al.,1999; Lobo et al, 1994; Olivieri et al., 2009). A novel subtelomeric gene family, participating in early gametocyte differentiation genes, has also been described (Eksi et al., 2005).

In the case of environmental stimuli, high parasitaemia, depletion of fresh RBC, TNF- α levels, antibodies, intra and inter specific competition and the presence of chloroquine increase the rate of gametocyte differentiation *in vitro* (Bruce et al., 1990; Buckling et al., 1999; Talman et al., 2004). However, drug treatment does not appear to influence gametocyte sex ratio (Talman et al., 2004). This comes in contrast with the observation that host-derived hormones, anaemia and presence of multiple parasite strains in mixed infections, result in a less female biased gametocyte population (Dixon et al., 2008; Talman et al., 2004). Both sexual commitment and gender allocation seem to be evolutionarily

optimised to ensure fertilisation under different environmental variables and finally ensure transmission in the short term (Paul, 2003).

1.3.1.3. Dissecting gametocyte biology

During maturation, the gametocyte remains enclosed within the parasitophorous vacuole (PV) of its host erythrocyte, and obtains nutrients through haemoglobin digestion and import from the extracellular environment, like asexual stages. As development proceeds male and female gametocytes become more distinct and start to exhibit an increasingly different metabolic profile to asexual parasites (Lang-Unnasch & Murphy, 1998). Interestingly, however, both mature female and male gametocytes are arrested in G₀ in the blood stream (Sinden, 1998) and despite the appearance of a nuclear spindle (Sinden & Smalley, 1979) they seem to bear a genomic value between haploid and diploid (Janse et al., 1986). This excess of haploid value has been proposed to be more of a result of evolutionary selective gene amplification, rather than genome amplification, since clear proof of the latter has not been reported (Janse et al., 1989; Janse et al., 1986).

Gametocytes are sexually dimorphic, the male being terminally differentiated (as a male gamete precursor) while the female cell endures and undergoes further development after fertilisation. This is mirrored in the fact that, contrary to the male gametocyte, the female is enriched with abundant protein and energy production particles and machinery including, membrane bound vesicles known as osmophilic bodies, mitochondria, apicoplasts (Api), extensive endoplasmic reticulum (ER) and ribosomes. These organelles together with mRNA accumulate in the cytoplasm, leaving little room for the female nucleus (Sinden, 1998).

In contrast, the male gametocyte nucleus is enlarged and the mature male gametocyte cytoplasm is largely devoid of ER and contains few mitochondria, osmophilic bodies and apicoplasts (Sinden, 1998). Its genome takes upon a highly organised state, allowing the kinetochores of each chromosome to be attached, (through a nuclear pore) to the Microtubule Organising Centre (MTOC) situated on the cytoplasmic face of the nuclear envelope.

1.3.1.4. Gametogenesis and Fertilisation

Plasmodium female and male gametocytes gain access to the mosquito midgut following uptake within the blood meal from an infected vertebrate host (Figure 1.1). During this transition, the gametocytes get exposed to an increase in pH (Billker et al., 2000) , a drop in temperature and xanthuric acid (XA) (Arai et al., 2001; Billker et al., 1998; Billker et al., 1997). As a consequence, a signal transduction cascade is initiated, resulting in the release of calcium into the cytoplasm of the gametocytes, triggering their maturation into gametes (Billker et al., 2004; Kawamoto et al., 1990). Nevertheless, to date the exact components of such a gametocytogenesis-promoting signalling cascade remain unknown, and only a number of potential signalling components proven to play essential roles during gametogenesis and post-fertilisation development have been identified (Alano, 2007). Secondary to gametocytes' activation, the osmophilic bodies are trafficked to the parasite surface where their content is released into the PV. As a result, first the PV and second the RBC which host them, break and the activated gametocyte is released into the mosquito midgut (de Koning-Ward et al., 2008; Sinden, 1982; Sinden, 1998). From that point on, male and female gametocytes follow a totally different path to gametogenesis.

Following the completion of three rounds of DNA replication, male gamete maturation culminates with the release of eight motile haploid flagellated microgametes (Janse et al., 1986; Sinden, 1998) (Figure 1.2). This is facilitated by the formation of the axoneme (the cytoskeletal core of the flagellum) which, upon flagellum activation, pulls the haploid genome into the developing microgamete (Sinden, 1998). In this event, a calcium dependent protein kinase (*cdp4*) (Billker et al., 2004) and a mitogen activated protein kinase (*Pbmap2*) (Tewari et al., 2005) have been found to play significant regulatory role in the rodent malaria parasite *P. berghei*. The former, controls the initiation of genome replication upon activation (Billker et al., 2004), while the onset of cytokinesis and microgamete release (Khan et al., 2005; Tewari et al., 2005) is regulated by the latter. Apart from that, cGMP dependent signalling cascade components including a phosphodiesterase (*pde-δ*) (Taylor et al., 2008) and a cGMP dependent protein kinase (*pkg*) (McRobert et al., 2008) have also been characterized. Recently, a paternally inherited formin, *misfit* has been suggested to play a putative role in the regulation of mitotic spindle formation during male gametogenesis. Nevertheless,

following targeted disruption of this protein, developmental arrest ensues much later during ookinete-to-oocyst transformation (Bushell et al., 2009).

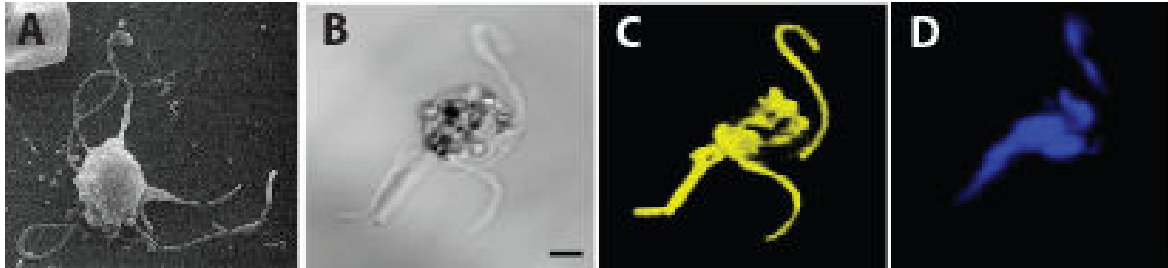


Figure 1.2. Male gametocytogenesis A. Scanning electron micrograph of an exflagellating *P. yoelii* male gametocyte upon activation. **B-D** Immunofluorescence images of the exflagellating *P. berghei* male gametocyte. Images show bright field acquisition (B), anti-Tubulin (TUB) antibody staining in yellow (C) and DAPI staining of DNA in blue (D). Images adapted from Sinden et al, 1978 (A) and Bushell et al., 2009 (B-D)

Unlike male gametogenesis, the activated female (macro) gamete does not undergo DNA replication while it significantly enlarges in size (Sinden, 1998). Osmophilic bodies facilitate its emergence; a process known to be significantly dependent on *Pfg377* in *P. falciparum* and *P. berghei* (Alano et al., 1995; de Koning-Ward et al., 2008; Khan et al., 2005). In recent years a number of genes families have been described to play a role in this process. Interestingly, their function requires female specific inheritance including the *P. berghei* limulus clotting factor C, Coch-5b2 and LgII (*LCCL*) / lectin adhesive-like protein (*Pblap*) family (Claudianos et al., 2002; Raine et al., 2007; Trueman et al., 2004). In *P. falciparum* in which the family finds clear orthologues (*Pfcp*) (Delrieu et al., 2002; Pradel et al., 2004), the proteins are expressed on the surface of both female and male gametocytes, and seem to decline rapidly after fertilisation (Pradel, 2007). Interestingly, loss of one member results in the loss of the whole family, and this finding attributed to the detection of large protein complexes of the family (Simon et al., 2009). Nevertheless, targeted disruption of *Pfcp* / *Pblap* members does not prevent fertilisation, ookinete formation or oocyst development (Claudianos et al., 2002; Pradel et al., 2004; Pradel et al., 2006; Raine et al., 2007; Trueman et al., 2004). Intriguingly, macrogamete maturation is associated with the translation of a subset of translationally repressed mRNA transcripts, including *p25/28* macrogamete surface protein. In *P. berghei*, *p25/28*

can be detected early following activation, and later becomes the predominant zygote and ookinete surface protein. This is not the case for other transcripts that are also subject to translational repression in the female gametocyte, whose translation seems to be delayed until after fertilisation (Mair et al., 2006).

During fertilisation, fusion of the plasma membrane of the two cells (female and male) occurs, with the male nucleus and its axoneme entering the macrogamete cytoplasm (Sinden et al., 1985). Adhesion during the initial stages of fertilisation has been recently suggested to be governed by gamete surface molecules of the 6-Cys repeat motif family; *p47* and *p48/45*. Mutants lacking gamete surface molecules *p47* or *p48/45* produce infertile male or female gametes, respectively. Thus, these molecules may be involved in male-female recognition, adhesion or presentation of accessory molecules necessary for fertilisation (Khan et al., 2005; van Dijk et al., 2001).

1.3.2. Ookinete-to-oocyst developmental transition

1.3.2.1. Ookinete development

Following fertilisation of the mature female gamete (Aikawa et al., 1984), the resulting diploid zygote matures into the tetraploid motile ookinete. The latter process is a result of meiosis, which occurs in the absence of nuclear division or cytokinesis (Sinden et al., 1985), to result in a tetraploid genome. In the zygote, two never in mitosis *Aspergillus* (*NIMA*)-related kinases, *nek2* and *nek4*, are essential for DNA replication and subsequent ookinete maturation (Khan et al., 2005; Reininger et al., 2005; Reininger et al., 2009). The initially spherical zygote gradually elongates to grow an apical protrusion (retort stage), eventually transforming within the next 12-24 hours into the ookinete. Interestingly in *P. berghei*, *mdv-1* appears to play a role during zygote development, since upon gametocyte activation it has been found to mobilise to the leading edge of this very apical protrusion in the retort stage (Lal et al., 2008). In parallel, the generation of an extensive network of subpellicular microtubules and the related microtubule organising centre (MTOC), the apical ring, takes place within the maturing ookinete. Anterior secretory organelles known as micronemes are also synthesised (Canning & Sinden, 1973).

Notably, during ookinete formation a significant degree of *de novo* protein synthesis occurs. Expression of some transcripts is ookinete specific, while for others (e.g. *p25/p28*) prior synthesis in the female gametocyte and translational repression has occurred (Hall et al., 2005; Mair et al., 2006). Taken together, these processes recruit the ookinete motility and invasive machinery which will ensure its successful escape from the bolus and transversal of midgut epithelium.

1.3.2.2. Ookinete biology

The ookinete is a polarised banana-shaped cell that shares similarities with other zoite stages of the *Plasmodium* life cycle and of other *Apicomplexa* (Figure 1.3A). It has an inner membrane complex (IMC) that lies underneath the plasma membrane and which runs along the length of the cell. The IMC links to the apical complex, that includes a polar ring, collar, microtubule organizing centre and micronemes. Micronemes represent ookinete's specialised secretory organelles that

release both secreted and membrane-anchored proteins. Micronemal proteins have been shown to be involved in host cell invasion of *Apicomplexa*; in *Toxoplasma*, micronemal proteins are released upon cell invasion and proteins known to be involved in cell invasion in *Plasmodium* and other *Apicomplexa* have been localised to the micronemes, such as the transmembrane protein Circumsporozoite and TRAP (thrombospondin related anonymous protein) Related Protein (*ctrp*) (Dessens et al., 1999) and the secreted *Plasmodium* Perforin-like proteins (*pplp*) (Kadota et al., 2004).

1.3.2.3. Ookinete motility and invasion machinery

In *Apicomplexa* the molecular machinery required for invasion and motility is tightly linked. Its strict conservation is mirrored by the fact that all three zoite stages (merozoite, sporozoite and ookinete) of the parasite that involve cell invasion are dependent on apical discharge of secretory vesicles to ensure successful motility and thus, invasion (Baum et al., 2006). This was further highlighted by the study of a TRAP protein family, members of which are found along the surface of all three invasive stages. Characteristically, targeted disruption of any of its members in the respective parasite developmental stage renders *Plasmodium* immotile and unable, to invade the midgut epithelium in the case of the ookinete (*ctrp* – ookinete surface protein) (Dessens et al., 1999; Yuda et al., 1999) or the salivary glands in the case of the sporozoite (TRAP) (Kappe et al., 1999; Sultan et al., 1997) or potentially the RBC for merozoites (MTRAP) (Baum et al., 2006).

In contrast to the apical complex of the other invasive stages (merozoites and sporozoites) that contain four types of secretory vesicles: micronemes, rhoptries, dense granules and exonemes (Baum et al., 2008), ookinete functionality appears to be exclusively dependent on micronemal secretion (Li et al., 2004). Microneme vesicles are synthesised and their cargo has already been loaded in the Golgi apparatus (Lal et al., 2009; Schrevel et al., 2008). Data from another invasive stage, the merozoite indicate that pre-loaded vesicles are trafficked along the subpellicular microtubules to the apical complex at the merozoite apical pole (Bannister et al., 2003), where similarly in the ookinete accumulate in the apical complex (Lal et al., 2009). Their content is released on the parasite surface or in the environment (Li et al., 2004), perhaps secondary to calcium mediated signalling (Carruthers et al., 1999; Carruthers & Sibley, 1999). Two of the cell signalling molecules involved in ookinete motility have recently been identified including *cdpk3* (Ishino et al., 2006; Siden-Kiamos et al., 2006)

and a known signalling component guanylate cyclase, $GC\beta$ (Moon et al., 2009). The absence of *cdpk3* or $GC\beta$ significantly reduces ookinete motility and thereby its ability to access the midgut epithelium, resulting in an arrest on the apical surface of the epithelial barrier and/or transmission blockage (Hirai et al., 2006; Moon et al., 2009). The similarity of the intracellular signalling pathways to that of gametogenesis is evident, pinpointing the conserved nature of many parasite mechanisms. A simplified model of ookinete motility machinery is described in Figure 1.3B.

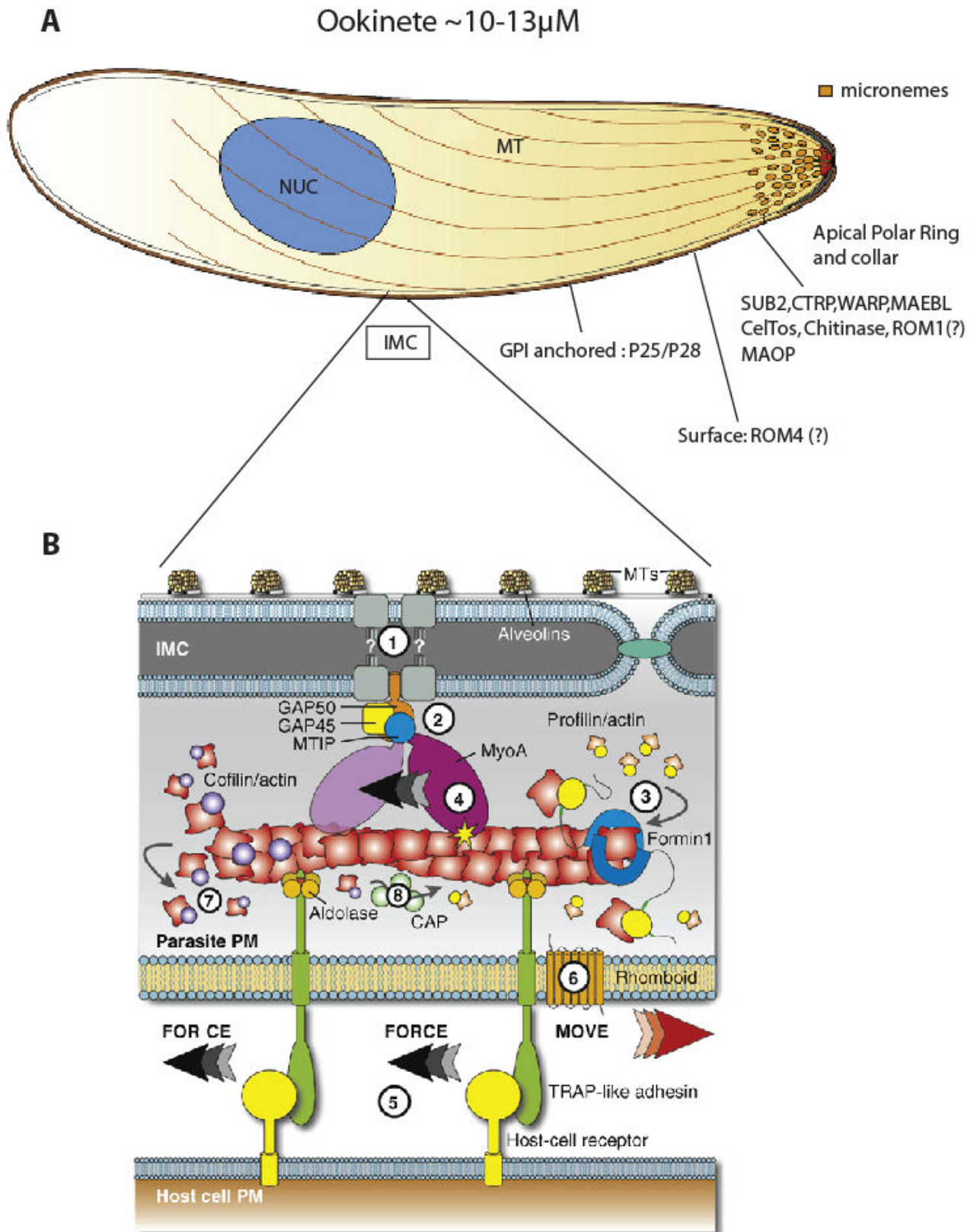


Figure 1.3. Current models of ookinete morphology and motility. A. Cell morphology of the *Plasmodium* ookinete. Schematic of the invasive ookinete stage of *P. falciparum* parasite showing conserved organelles [micronemes (orange)] that are central to motility and invasion. Other structures shown are the nucleus (NUC), inner membrane complex (IMC), sub-pellicular microtubules (MT) and tubulin-rich apical polar rings (APR) with collar. Indicatively, proteins that localize to the secretory organelles and parasite surface and those specifically anchored with a glycosylphosphatidylinositol (GPI)-anchor are listed. The localization of proteins marked with a bracketed question mark remains speculative. **B. Current model for the gliding motor complex.** The force for *Plasmodium* motility and invasion is generated by an actomyosin motor located between the plasma membrane (PM) and the Inner Membrane Complex (IMC) at the apical end of the parasite (Kappe et al., 1999). Myosin is anchored in the IMC through its placement in cholesterol-rich, detergent resistant membranes (Johnson et al., 2007) (1). Myosin interacts with the inner membrane complex through MTIP (Bergman et al., 2003) and the gliding associated proteins (GAP) 45 and 50 (Baum et al., 2006; Baum et al., 2008; Gaskins et al., 2004) (2). Initiation of motility or invasion probably occurs after an intracellular signal (possibly involving calcium) resulting in Formin1 (Baum et al., 2008) and profilin actin polymerization (Plattner et al., 2008) (3) Myosin A, drives ATP-dependent barbed end directed force which through F-actin transient scaffolds (Schmitz et al., 2005) propels the secreted TRAP-adhesin – which in turn binds to it through aldolase (Buscaglia et al., 2003) - backwards through the fluid outer plasma membrane of the parasite (Herm-Gotz et al., 2002) (4-5). By passing the transient filamentous actin scaffolds back onto another MyoA molecule, the parasite glides forward with a “push and grab” mechanism (Schuler & Matuschewski, 2006), through attachment to the host-cell surface. Release from the substrate allows forward movement and subsequent reattachment is orchestrated by rhomboid mediated or SUB2 TRAP cleavage (Baum et al., 2008) (6), which leads to F-actin depolymerization and recycling (Baker et al., 2006; Baum et al., 2006; Brossier et al., 2005; Harris et al., 2005; O'Donnell et al., 2006) (7–8). Figures adapted and modified from Baum et al 2008 (Baum et al., 2008)

1.3.2.4. Ookinete midgut invasion

In order for the ookinete to reach the midgut epithelium, first it has to cross the chitin-rich peritrophic matrix; (Dinglasan et al., 2009) a process which is eventually achieved by the expression of an ookinete specific chitinase (*chit1*) (Dessens et al., 2001). Secondary to that and its attachment to the apical side of the midgut epithelium, disruption of the midgut epithelium cell membrane occurs. A number of *Plasmodium* surface or secreted proteins are known to mediate the invasion process, including members of the conserved *pplp* family, unified by a signature membrane-attack complex and perforin (*macpf*)-related domain. *P. berghei* ookinetes express three genes of the *pplp* gene family; *pplp3*, *pplp4* and *pplp5* (Ecker et al., 2007; Kadota et al., 2004; Kaiser et al., 2004; Raibaud et al., 2006), the former of which *pplp3*, is located to the micronemes supporting the secretion of *pplps* during parasite cell invasion (Kadota et al., 2004; Kaiser et al., 2004). *Applp3* and *Applp5* ookinetes are able to attach to the midgut epithelium but are unable to enter the cytoplasm (Ecker et al., 2007; Kadota et al., 2004), indicating that *pplp3* and *pplp5* (also described as membrane attack ookinete protein, MAOP) are essential during membrane disruption and ookinete midgut invasion.

In addition, the major ookinete surface proteins *p25* and *p28* (previously denoted *Pbs21* in *P. berghei*), which are also conserved across *Plasmodium spp.*, seem to play a crucial role in ookinete development and midgut invasion. Even though targeted disruption of either of the two demonstrated no significant phenotype – indicating their functionally abundant nature- interestingly, ookinetes lacking both *pb25* and *pb28* displayed increased sensitivity to proteases in the mosquito midgut, significant defects in traversing the midgut epithelium and reduced ability to transform into oocysts (Tomas et al., 2001). Of note, the *p25/p28* proteins encode three or four epidermal growth factor (EGF) domains, the latter related to various receptor-ligand interactions, in eukaryotes (Tomas et al., 2001).

von Willebrand factor A domain-related protein (*warp*) (Yuda et al., 2001), and secreted ookinete adhesive protein (*soap*), (Dessens et al., 2003) have also been found to be important for midgut invasion. Moreover, a recent study (Ecker et al., 2008) has identified a number of putative secreted ookinete proteins (*psops*), which also play a role at the stage of midgut invasion, utilizing prior protein expression patterns (Hall et al., 2005). Among the genes identified, *psops 2*, *7* and *9* all played roles in the traversal of the midgut barrier, with *psop7* and *9* to be essential for transmission.

1.3.2.5. Ookinete interactions with the mosquito host

In order for the parasite to successfully complete its journey within the mosquito host, it seems to be dependent on a variety of direct or indirect molecular interactions with components of its mosquito host. This fact has been recently highlighted by the identification and characterization of genes that seem to play a significant protective or facilitating role upon parasite development.

First, midgut invasion itself appears to be mediated by a kind of specific receptor-ligand interaction recognition motif including protein-protein, protein-carbohydrate but also protein-lipid interactions, without which parasite invasion is significantly impaired if not totally abolished. In the first case, binding of a phage display-derived peptide, salivary gland and midgut peptide-1 (SM-1) to the apical side of the mosquito midgut epithelium inhibits invasion of *P. berghei* ookinetes (Ghosh et al., 2001; Ito et al., 2002). Oligosaccharides coating the microvilli of the apical side of the mosquito epithelium, midgut glycans or lectins are also essential for *P. falciparum*, *P. berghei* or *P. gallinaceum* to complete successful midgut invasion (Dinglasan et al., 2003; Dinglasan et al., 2007; Zieler et al., 2000; Zieler et al., 1999). Notably, inhibition of *A. gambiae* glycosaminoglycan chain synthesis reduces midgut chondroitin sulfate levels and leads to significantly reduced *P. falciparum* ookinete midgut invasion (Dinglasan et al., 2007). Modification of midgut epithelial cell membranes lipid content has also been associated with impairment of midgut invasion, in the case of introduction of phospholipase A2 (PLA2) to *A. stephensi* (Moreira et al., 2002; Zieler et al., 2001).

Second, agonistic interactions have also exerted their role in protection of the parasite against mosquito immune responses. Melanotic encapsulation (melanisation) represents a wide spread mechanism of pathogen killing and / or disposal in all arthropods, including insects; and is mainly ruled by a serine protease signalling cascade. Interestingly, the latter is subjected to tight regulation by positive (pro-phenoloxdiase activating factors - PPAFs) as well as negative control components (serine protease inhibitors – serpins), a fragile balance that determines the outcome (Cerenius et al., 2008). The knock down (KD) of an *A. gambiae* serpin, *SRPN2*, or any of two members of the C-type lectin family, *CTL4* or *CTLMA2* result in extensive *P. berghei* ookinete melanisation and lysis, up to complete refractoriness in the case of *CTL4* (Michel et al., 2005; Osta et al., 2004). In the same terms, the KD of the mosquito apolipoprotein precursor gene, *APOI/II (RFABG)* leads to a reduction in oocyst formation both in *P. berghei* (Vlachou et al., 2005), as well as *P. falciparum* (Mendes et al., 2008). A third component of the apolipoprotein complex *APOIII*, even though it does not affect *P.*

berghei or *P. falciparum* development in susceptible mosquitoes, still seems to play indicating an inhibitory regulatory role in melanisation, since its silencing significantly increases the number of melanised ookinetes in L3-5 mosquitoes (Mendes et al., 2008).

The parasite suffers significant losses in numbers while the ookinete penetrates the midgut barrier (Alavi et al., 2003; Sinden et al., 2004) (Figure 1.4.), a fact partly attributed to the activity of the mosquito innate immune response. Interestingly, a number of mosquito genes seem to be differentially regulated during midgut invasion, indicating a systematic response of the vector against parasite infection and subsequent epithelial barrier damage (Christophides et al., 2002; Dimopoulos et al., 2002; Holt et al., 2002). This shift in transcriptional activity involves genes implicated in cell adhesion and extracellular-matrix remodelling, actin / microtubule cytoskeleton dynamics, apoptosis and immunity.

First, a number of genes have been identified to be associated with positive regulation of actin polymerisation, including *CIBULOUT* and Wiskott-Aldrich syndrome protein (*wasp*) (Vlachou et al., 2005). *wasp* silencing reveals significant anti-parasitic activity during *P. berghei* (Vlachou et al., 2005), as well as *P. falciparum* midgut invasion (Mendes et al., 2008), suggesting the existence of a conserved actin-based mechanism regulating parasite transmission during midgut invasion. On top of that, the *P. berghei* ookinete has been postulated to modify the host cell cytoskeleton – putatively through the secretion of a known subtilisin-like serine protease, *PbSub2* (Han et al., 2000).

Two members of the leucine rich-repeat immune gene family, *LRIM1* and *APLC1*, and the thioester containing complement-like protein *TEP1*, play a pivotal role in determining the outcome following *A. gambiae* infection with *P. berghei* (Blandin et al., 2004; Osta et al., 2004) as well as refractoriness of the non-malaria vector species *Anopheles quadriannulatus* (Habtewold et al., 2008). Silencing of either *LRIM1* or *TEP1* through RNAi results in a significant increase in oocyst numbers in the mosquito midgut, to a degree that can restore susceptibility in the genetically-selected refractory strain of *A. gambiae*, L3-5 (Collins et al., 1986). *TEP1* is a homologue of the mammalian complement protein 3 (C3), so it has been speculated that its function exerts in opsonising *P. berghei* ookinetes, hence mediating their killing by lysis in a postulated *A. gambiae* complement-like pathway (Blandin et al., 2004; Blandin et al., 2008). *LRIM 1* and *APLC1* exist as a multimeric protein complex in the mosquito hemolymph and directly interact with *TEP1*, activating or stabilizing it, thus facilitating its binding to the ookinete surface (Fraiture et al., 2009; Povelones et al., 2009). Several

other genes had been identified and speculated in the past to determine parasite development in the mosquito host including the antibacterial peptide defensin, a putative Gram-negative bacteria-binding protein (GNBP) and IGALE20, a galactose-specific lectins (Dimopoulos et al., 1997; Richman et al., 1997), however, no significant impact on parasite development was observed following their silencing, implying their secondary if any role in mosquito immune response (Blandin et al., 2002).

From what is described above, it has become evident that midgut invasion represents a critical point in parasite development in the mosquito host, since it constitutes a major bottleneck during sexual and sporogonic development. *Anopheles* immune responses are the processes mostly attributable for these losses, occurring in both refractory as well as, susceptible mosquito strains. Hence, one can wonder why and what it is that leads to that sequence of events that provides eventual transmission blockage in some cases but not others. Three hypotheses have been so far considered to explain this only partially effective immune response (Han et al., 2002). First, since the reproductive fitness of the mosquito has been observed to be affected by elicited immune responses, it is quite possible that in evolutionary terms, the vector has actively adapted to restrain its immune response in cost of heavier parasite infections and non absolutely refractory outcomes (Ahmed & Hurd, 2006; Hurd et al., 2005). Secondly, as the redundancy of *p25/p28* has shown us in combination with the double knock out (KO) increased protease sensitivity (Siden-Kiamos et al., 2000; Tomas et al., 2001) it is possible the parasite has developed protective mechanisms allowing them to withstand the immune response elicited. Last, it has been hypothesized that the timing of invasion and exit may be crucial to parasite survival (Han et al., 2000). According to this model, namely the Time Bomb Model theory, *P. berghei* ookinete midgut invasion triggers among other immune genes the transcriptional activation of a parasite toxic nitric oxide synthase (NOS) in both *A. stephensi* and *A. gambiae* (Dimopoulos et al., 2000; Luckhart et al., 1998). Since the invasion process is accompanied by such dramatic expressional changes, extensive cytoskeletal remodelling and significant morphological changes associated with programmed cell death, the ookinete finds itself in a hostile environment which it must rapidly transverse and exit in order to survive. Therefore it seems reasonable that ookinetes that made it early enough, successfully will proceed to the next developmental stage, contrary to the ones that were caught in the middle of “the detonation of the time-bomb” (Han et al., 2000).

1.3.2.6. Ookinete journey within the midgut epithelium

While a *P. berghei* ookinete invades midgut epithelia, invaded cells seem to undergo programmed cell death and finally become apoptotic, as a result of extensive damage. This process is characterized by extensive DNA fragmentation, a loss of microvilli and protrusion of the invaded cells towards the midgut lumen, where they are finally expelled. Neighbouring cells seem to facilitate this process, in a parallel effort to restore the epithelial barrier (Han et al., 2000; Vlachou et al., 2004). The route to the sub-epithelial space where it will eventually transform into the oocyst, involves invasion of multiple cells and various types of intracellular locomotion, including stationary rotation, translocational spiralling (rotational motility in conjunction with directional changes and translocation) and straight-segment motility (Vlachou et al., 2004). Interestingly, navigation across the cytoplasm of the invaded midgut epithelial cells is mediated by a shared ookinete and sporozoite protein termed cell-traversal protein for ookinetes and sporozoites (*CellTos*) (Kariu et al., 2006). *Δceltos* ookinetes even though they successfully disrupt the midgut epithelial barrier and enter the cell lumen, fail to translocate further, similarly to *Δceltos* sporozoite for the hepatocytes, suggesting that common underlying mechanisms characterize parasite invasive stages.

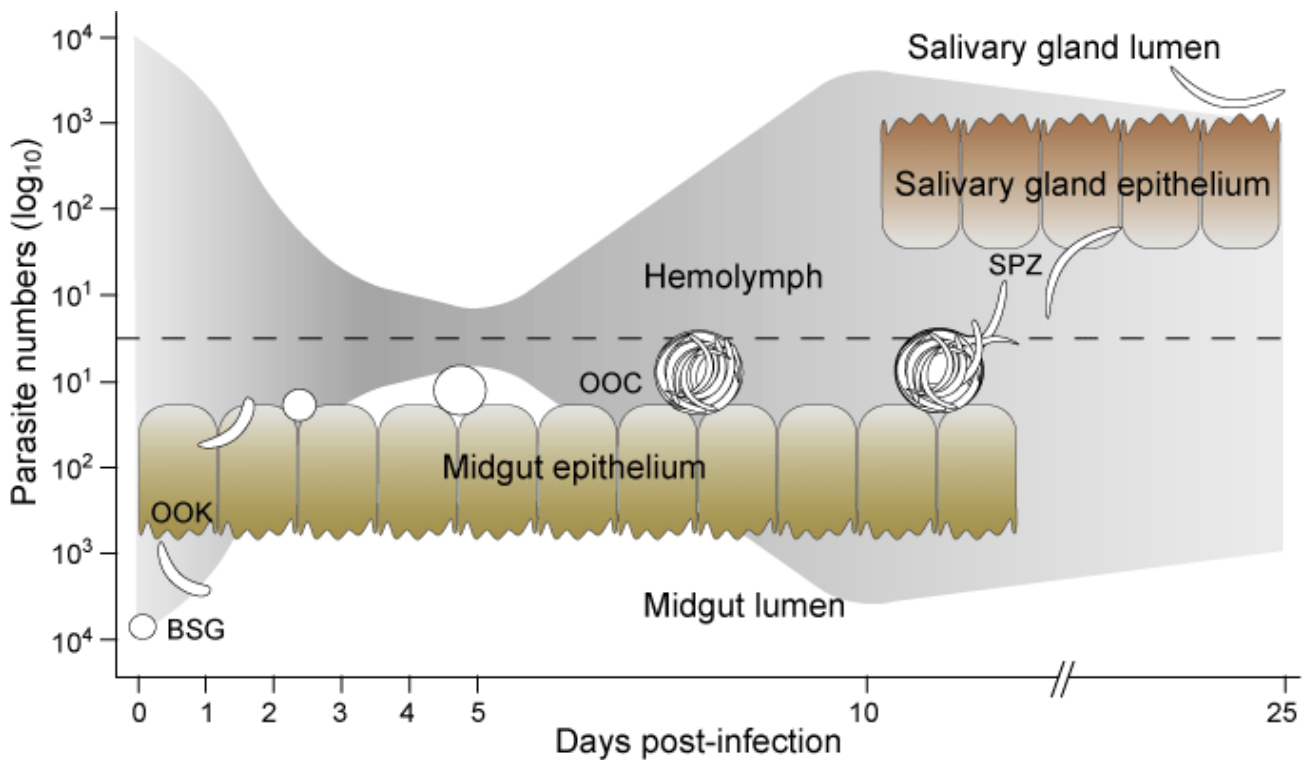


Figure 1.4. *P. berghei* transmission dynamics through its anopheline vector. Parasite numbers fluctuate throughout their development in the mosquito host. 24 hours following an uptake of an infectious blood meal from an anopheline mosquito, ookinetes invade the mosquito midgut epithelium. Midgut invasion represents a major bottleneck for parasite development, since secondary to that; it reaches its lowest numbers coinciding with early oocyst development. Subsequent oocyst sporulation and egress of thousands of sporozoites again increase parasite load, only to fall again during salivary gland invasion. Figure; a gift from G. Christophides.

1.3.2.7. Ookinete-to-oocyst transformation

The *P. berghei* ookinete traverses and exits the mosquito midgut epithelial cell, until it reaches the sub-epithelial space where it encounters the basal lamina and finally transforms into the oocyst. It has been suggested that actual contact of the ookinete with structural elements of the basal lamina - consisting of laminin, collagen IV, entactin and perlecan - appears to act as a trigger for the ookinete to oocyst transformation (Weathersby, 1954). Data for the latter hypothesis come from studies confirming the formation of oocysts on the basal lamina of the malpighian tubules and the fat body (Weathersby, 1954), when ookinetes are injected directly in the haemocoel. Laminin may play a crucial role considering that *ctrp* (Mahairaki et al., 2005), *p25* and *p28* (Arrighi & Hurd, 2002; Vlachou et al., 2001) and *soap* (Dessens et al., 2003) have been shown to bear mosquito laminin binding capacity, while its silencing results in a reduction in oocyst numbers (Arrighi et al., 2005). These findings come in stark contrast with *in vitro* studies where ookinete-oocyst transformation has been reported, in the absence of any basal lamina components. In this system, adequate environmental concentration of bicarbonate acts as the trigger, while a defined range of nutrients are critical to ensure completion of the transformation (Carter et al., 2007). The true role of the interaction between the basal-lamina and the transforming ookinete is still to be elucidated. Recently, a paternally inherited factor (*misfit*) has been described, targeted disruption of which appears to arrest parasite development during ookinete to oocyst transformation, nevertheless it rather seems that the latter phenotype comes as a result of accumulated defects early since the gametocyte stage, rather than absence of a specific trigger at this stage (Bushell et al., 2009).

Morphologically, the so far motile banana-shaped ookinete loses its characteristic subpellicular microtubules, apical complex and pellicle (Canning & Sinden, 1973). An intermediate form, the transforming ookinete (took), has been described at this stage where a hump is formed at the convex outer side of the crescent-shaped ookinete and the protrusions (former apical and posterior tips) are gradually absorbed, to round up to the sessile oocyst. In parallel, the ookinete double-membrane pellicle is sequentially replaced by the single-membrane plasmalemma of the oocyst (Carter et al., 2007), which is surrounded by a protinaceous capsule (Sinden & Strong, 1978; Vanderberg & Rhodin, 1967), later ensuring the oocyst's survival and successful maturation.

1.3.3. Mature sporogonic development and transmission to the vertebrate host

1.3.3.1. Oocyst maturation and Sporogonic development

Oocyst maturation does not remain free of hostile mosquito immune responses. Its survival is largely dependent on the development of a capsule. The protein constitution of the capsule is unknown, but mosquito-derived laminin has been found on the outer surface and within the capsule structure (Nacer et al., 2008). This process has led to the hypothesis that the ookinete becomes coated in laminin during its passage through the midgut epithelium and as it transforms into the oocyst, in an effort to escape from the mosquito immune response (Arrighi et al., 2005; Nacer et al., 2008). Currently, only one *Plasmodium* capsule-specific protein has been identified (*PbCAP380*), in the absence of which normal number of oocysts are formed but are gradually eliminated, leading to transmission blockage (Srinivasan et al., 2008) (Figure 1.5A).

The developing oocyst undergoes multiple syncytial nuclear divisions resulting in a multinucleated parasite which is gradually growing in size. In parallel to this, the plasma membrane of the oocyst is folded inwards so as to form cervices which will eventually span across the entire oocyst, and partition the cytoplasm into compartments termed sporoblasts (Sinden & Strong, 1978). Out from these sporoblasts, developing sporozoites bud off, so that in the end, the mature oocyst contains hundreds or thousands of haploid sporozoites (Sinden & Strong, 1978; Vanderberg & Rhodin, 1967) (Figure 1.5B & 1.6).

Interestingly, so far only a limited number of *Plasmodium* protein families have been identified as required for oocyst maturation or the production of competent sporozoites. The best characterised gene known to be essential for sporozoite formation is *csp*, a major (midgut, haemolymph, as well as, salivary gland) sporozoite surface protein conserved among *Plasmodium* spp. (Nagasawa et al., 1988; Nagasawa et al., 1987). Targeted disruption of *csp* or even attenuation of *csp* expression blocks the cytokinesis event that completes the budding process by which sporozoites are formed, in *P. berghei*, leading to development of oocysts void of sporozoites (or partially defective in the event of diminishing expression levels) (Menard et al., 1997; Thathy et al., 2002). Apparently, *csp* function seems to be also critically dependent on its glycosylphosphatidylinositol (GPI) anchor (Wang et al., 2005) except for its expression levels (Thathy et al., 2002).

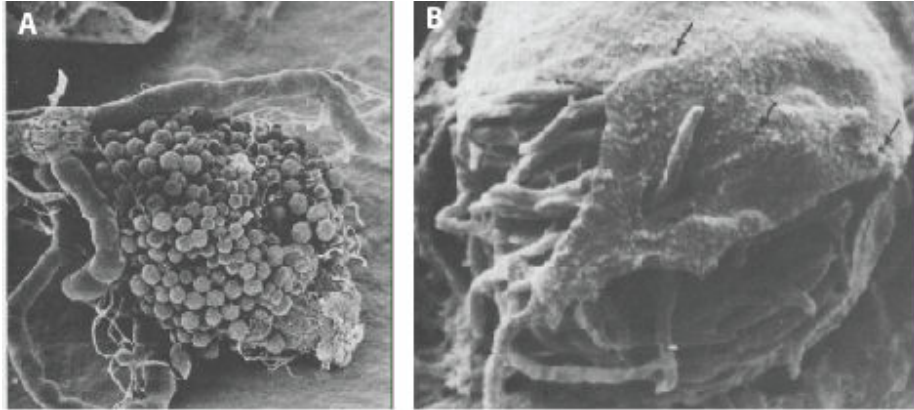


Figure 1.5 Scanning electron micrographs of *P. falciparum* oocysts. A. *A. gambiae* midguts infected with six day old oocysts **B.** Excystation of sporozoites from day 8 oocysts. Arrows indicate pores in the cyst wall through which sporozoites egress. Pictures adapted from Sinden and Strong, 1978.

Female specific inherited members of the *P. berghei* limulus clotting factor C, Coch-5b2 and LgII (LCCL) / lectin adhesive-like protein (*PbLAP*) family. *PbLAP*1-6 (Claudianos et al., 2002; Raine et al., 2007; Trueman et al., 2004) have also been found to play a role in sporozoite development. Intriguingly, their targeted disruption leads to defective sporulation if at all (Claudianos et al., 2002; Ecker et al., 2008; Pradel et al., 2004; Raine et al., 2007; Scholz et al., 2008; Trueman et al., 2004), even though their protein expression appears to peak during gametocyte stages. Similarly, for another two molecules recently identified, *Δasp* and *Δpsop13* oocysts largely fail to sporulate and display a degenerate, morphology also leading to transmission blockage (Ecker et al., 2008). Moreover, a membrane skeleton component, the inner membrane complex protein (IMC1a) is also implicated in sporozoite development. Its targeted disruption generates sporozoites of abnormal shape, which display reduced motility and infectivity (Khater et al., 2004).

1.3.3.2. Sporozoite maturation and salivary gland invasion

In order for the sporozoites to migrate to the mosquito salivary glands, they must be released into the haemocoel, since, till that point they are confined within the oocyst capsule. Sporozoite excystment is an active process (Sinden, 1974), which in *P. berghei* is dependent on an oocyst stage-specific protease, the Egression Cystein Protease (*ecp*) (Aly & Matuschewski, 2005), as well as a positively charged region of *Pbcsp* (Wang et al., 2005a). Following their egression the sporozoites make their way through the haemocoel, to the salivary glands. Even though this process still remains poorly understood, existing hypotheses include passive transportation through haemocoel circulation (Hillyer et al., 2007; Rodriguez & Hernandez-Hernandez, 2004), as well as, chemotactic driven locomotion that requires more active orientation on behalf of the sporozoite (Akaki & Dvorak, 2005).

One can compare the invasion of the salivary gland epithelium by the sporozoite to the midgut wall penetration by the ookinete in a previous stage. Several characteristics, including sporozoite motility and invasive ability, suggest the existence of a conserved common underlying mechanism shared by the invasive stages of *Plasmodium* (Figure 1.6). Salivary gland invasion poses a natural bottleneck in parasite development with similar to ookinete midgut invasion. Sporozoites that fail to invade are rapidly eliminated by the mosquito immune response (Hillyer et al., 2007). However, unlike the ookinete, the sporozoite does not seem to be harmful to the cells it enters, since no apoptosis of the invaded salivary gland epithelial cells has been observed (Pimenta et al., 1994). Still, studies suggest that the recognition and initial binding of the salivary gland epithelium and the invasion of the midgut epithelium wall share common ligands (Ghosh et al., 2009).

At this stage three key proteins have been identified to play a crucial role for the invading sporozoite. First, TRAP (the founding member of the TRAP protein family), which is expressed on the surface of sporozoites, in similarity to *ctrp* in ookinetes. Disruption mutants of TRAP render the sporozoites non-motile and as a result cannot enter the salivary glands (Kappe et al., 1999; Sultan et al., 1997), the same way *ctrp* loss affects ookinete ability to invade the midgut. Second, the region II domain of sporozoite's major surface antigen, *csp* is essential for sporozoite motility and salivary gland invasion (Tewari et al., 2002). *ctrp* is secreted at the apical end of the sporozoite and translocated to its posterior end (Stewart & Vanderberg, 1991), promoting forward motion of the parasite, similar to the

ookinete. Third, another sporozoite surface protein S6, has been recently identified to play an important role in motility and invasion (Steinbuechel & Matuschewski, 2009).

As previously mentioned despite the similarities between sporozoite salivary gland and ookinete midgut invasion, the sporozoite does not seem to be equally harmful. As a result, the mosquito immune response elicited by this process is kept to a minor level even though it is present. The latter fact is reflected on the observation that a limited number of mosquito genes are differentially regulated during salivary gland invasion (Dixit et al., 2009; Rosinski-Chupin et al., 2007) and even a lower amount of proteins are identified (Choumet et al., 2007), compared to its ookinete analogue (Vlachou et al., 2005).

Among the so far identified immune factors that govern salivary gland invasion and contribute to vector anti-parasitic responses, a member of the serpin family, *SRPN6*, has been characterized (Pinto et al., 2008). The very same protein has also been identified as an antagonistic marker of *P. falciparum* and *P. berghei* ookinete midgut invasion (Abraham et al., 2005). Similarly SM-1 peptide, which inhibits the invasive ability of the ookinete, also blocks salivary gland invasion (Ghosh et al., 2001).

A number of components, however, facilitate entry of the parasite in the salivary glands. These components act in a receptor-ligand way of interaction that is shared between midgut and salivary glands. The *A. gambiae* salivary protein, saglin, has been recently reported as a SM-1 receptor as well as a TRAP ligand (Ghosh et al., 2009; Okulate et al., 2007). Another sporozoite surface protein which is essential for salivary gland attachment and / or recognition is a paralogue of the merozoite erythrocyte binding ligand, MAEBL. As a result of its differential splicing, MAEBL gives rise to two ORFs, one of which is required for salivary gland entry (Saenz et al., 2008). CSP has also been implicated in the binding of salivary gland epithelium (Sidjanski et al, 1997). A mosquito protein family, the so called salivary gland specific (SGS) proteins, have also been implicated in sporozoite invasion. Antibodies against *AaSGS1* inhibit *Plasmodium gallinaceum* sporozoite salivary gland invasion in *Aedes aegypti* while putative interactions with CSP and TRAP have been suggested (Korochkina et al., 2006).

The passive transfer of the infective sporozoites from the mosquito salivary gland lumen, during a blood meal, to its next host completes the transmission cycle of *Plasmodium*. Nevertheless, in order

for the sporozoite to successfully infect its next host, it has to undergo significant developmental changes so that it achieves invasion of vertebrate cells, in this case hepatocytes. This process is reflected in the differential gene regulation as well as protein expression between oocyst and salivary gland sporozoites (Lasonder et al., 2008; Matuschewski et al., 2002; Mikolajczak et al., 2008).

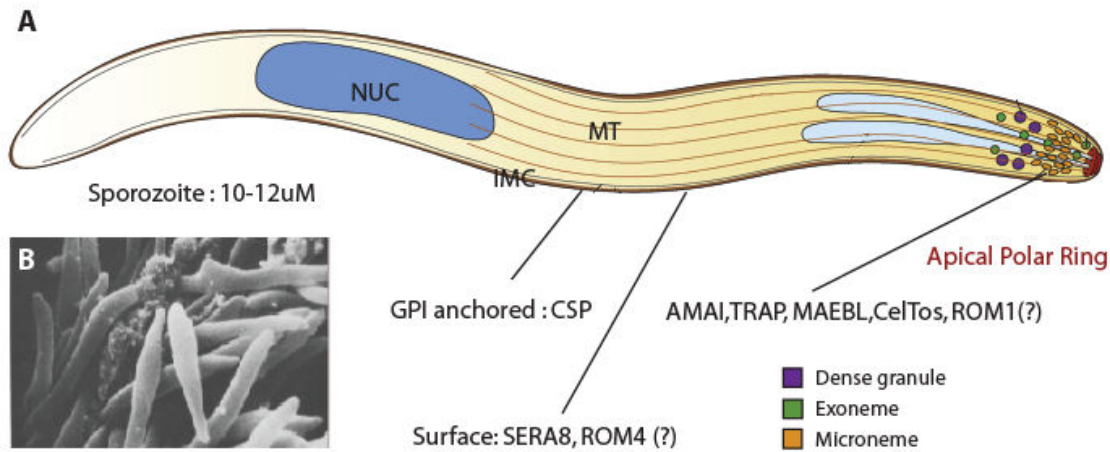


Figure 1.6. The sporozoite. A. Cell morphology of the *Plasmodium* sporozoite Schematic of the invasive sporozoite stage of *P. falciparum* parasite showing conserved organelles [micronemes (orange), dense granules (violet), exonemes (green)] that are central to motility and invasion. Other structures shown are the nucleus (NUC), inner membrane complex (IMC), sub-pellicular microtubules (MT) and tubulin-rich apical polar rings (APR). Indicatively, proteins that localize to the secretory organelles and parasite surface and those specifically anchored with a glycosylphosphatidylinositol (GPI)-anchor are listed. The localization of proteins marked with a bracketed question mark remains speculative (Figure adapted with modifications from Baum et al 2008 (Baum et al., 2008) **B. Scanning electron micrographs of *P. falciparum* immature sporozoites attached to sporoblasts** (Image adapted from (Sinden and Strong, 1978)

1.4. PLASMODIUM GENETICS AND GENOMICS

Recent years have seen the publication of genome sequences of several *Plasmodium* species (Carlton et al., 2008; Carlton et al., 2002; Gardner et al., 2002; Hall et al., 2005; Mourier et al., 2008), including the human malaria parasites *P. falciparum* (Gardner, et al., 2002) and *P. vivax* (Carlton, et al., 2008). This holds great promise in view of the recent publication of the human genome (Lander et al., 2001; McPherson et al., 2001; Venter et al., 2001) and *Anopheles* vector (Holt et al., 2002), as to further accelerate the elucidation of mechanisms that characterize malaria biology, transmission and finally disease eradication or control.

The *P. falciparum* genome spans 22.8 Mbp on 14 chromosomes for a predicted 5,268 genes (Waters & Janse, 2004). The *P. berghei* genome similarly bears 23 Mbp, the same number of chromosomes and around 5,864 genes (Waters & Janse, 2004). Interestingly, both species have a rich A+T genome composition, while 90% accounts for introns and intergenic regions (Gardner et al., 2002; Waters & Janse, 2004). Malaria parasites are obligate sexual organisms and thus can undergo meiosis and associated recombination, providing the ability to perform genetic crosses (Walliker et al., 1987) and linkage analysis. Chloroquine resistance gene (see above) is a striking example of such ability (Su et al., 1997).

In silico analysis of the genome itself has brought deep insights into parasite biology, including the ability to infer homology searches to other organisms, predict protein domain function and structure and last identification of novel regulatory or functional pathways. Examples include metabolic maps (Ginsburg, 2006), regulatory kinases (Ward et al., 2004), or protein localisation patterns (Marti et al., 2004). It is evident that such datasets have limitless and invaluable potential as to the information extensive study and insidious analysis can reveal.

Moreover, such information, except for allowing more in depth understanding of parasite development, has also allowed for further comparative genomic studies while genome-wide surveys have been conducted both at the level of the transcriptome (Abraham et al., 2004; Ben Mamoun et al., 2001; Dessens et al., 2000; Hall, et al., 2005; Hayward et al., 2000; Kappe et al., 2001; Le Roch et al., 2004; Matuschewski et al., 2002; Raibaud et al., 2006; Silvestrini et al., 2005; Srinivasan et al., 2004; Vontas et al., 2005; Xu et al., 2005) and the proteome (Florens et al., 2002; Hall et al., 2005;

Khan et al., 2005; Lal et al., 2009; Lasonder et al., 2002; Le Roch et al., 2004). These studies have shed new light on *Plasmodium* gene regulation during parasite development in host and vector, in view of recent similar analyses in the mosquito host (Abraham, et al., 2004; Christophides et al., 2004; Dana et al., 2005; Dimopoulos et al., 2000; Dimopoulos et al., 2002; Koutsos et al., 2007; Meister et al., 2005; Srinivasan et al., 2004; Vlachou et al., 2005; Xu et al., 2005; Zdobnov et al., 2002). Many parasite processes were examined among which stage specific expression (Patankar et al., 2001; Young et al., 2005), interaction networks (LaCount et al., 2005), organellar proteomics (Sam-Yellowe et al., 2004), antigenic (Doolan et al., 2003) or metabolic profiling (Daily et al., 2007) and drug treatment dynamics (Prieto et al., 2008). Nevertheless, many fields including gene regulation (discussed further ahead) have still remained a big puzzle for the community.

Last, the development of methods allowing the genetic manipulation of the parasite (van Dijk et al., 1995; Wu et al., 1996) and its mosquito host (Blandin et al., 2002; Catteruccia et al., 2000) or the introduction of new selectable markers (Fidock & Wellems, 1997), has allowed targeted functional analysis of parasite genes in conjunction with its mosquito host. More recently, technologies such as inducible expression of transgenes (Meissner et al., 2005), hit and run strategies and new selection systems (Braks et al., 2006; Duraisingh et al., 2002) have found application. Together these technical advances have facilitated our understanding of parasite and vector molecular biology, and lead the current approach to the identification and application of novel malaria control methods.

1.5. RODENT MODELS OF MALARIA

The development and establishment of continuous, *in vitro* culturing system of human malaria parasite *P. falciparum* (Trager & Jensen, 1976) has been of fundamental significance to the ongoing efforts of understanding the molecular mechanisms that underpin basic parasite biology, development and transmission of malaria. However, work with the human pathogen *P. falciparum* is restricted by a serious number of legal and ethical constraints relating to health and safety, as well as, primate research itself.

As a result many studies have focused and lead on the employment of a number of *Plasmodium* model organisms, which can safely and more easily facilitate our understanding of malaria biology, based on the remarkably similar and conserved biology between ortholog *Plasmodium spp.*. The

latter reflects in both their development, as well as, morphology along the different developmental stages (gametocyte excluded). Metabolic pathways, invasion mechanisms, genome organisation and surface molecules seem equally conserved thus, validating the use of ortholog *Plasmodium* model organisms as a tool for dissecting malaria biology (Waters & Janse, 2004).

Different *Plasmodium spp.* species infect a range of mammals, birds and reptiles. To date, several natural occurring species of murine rodent malaria have been reported, including *Plasmodium berghei*, *Plasmodium chabaudi*, *Plasmodium yoelii* and *Plasmodium vinckei*. The different murine rodent models are suitable for different aspects of malaria research i.e. *P. chabaudi* is mainly used to study drug resistance and antigenic variation due to its ability to establish persistent non-lethal murine malaria, *P. yoelii* is extensively used for blood and liver stage vaccine studies etc. (Waters & Janse, 2004).

For this project we utilised *P. berghei* in order to study the malaria transmission life cycle as well as its interactions with the mosquito host. *P. berghei* constitutes an excellent rodent model system, since it provides efficient and reliable murine and mosquito malaria infections. Moreover the production of sporogonic stages in the rodent model system is much easier (for example transfection efficiency in the *P. berghei* model system is significantly higher due to its 24 hour asexual cycle and the possibility of harvesting free merozoites) and less hazardous (non-human) (Waters & Janse, 2004). In addition the biology of *P. berghei* and *P. falciparum*, as well as, a great degree of their interactions with the mosquito host (Mendes et al., 2008) are quite conserved (Prugnolle et al., 2008; Waters & Janse, 2004).

Nevertheless, the two species appear to be quite phylogenetically divergent (Perkins & Schall, 2002), while dynamics of the various mechanisms have often been brought into question due to lack of evolutionary adaptation (Boete, 2005). Differences between the two species do exist and mostly focus in the size, gametocyte biology and developmental time span of parasite replication stages (Waters & Janse, 2004). Differences in interactions with the mosquito host have also been observed (Tahar et al., 2002). Therefore, the need to confirm observations deriving from the model system *P. berghei* with *P. falciparum* in the field remains pivotal.

1.6. AIMS AND OBJECTIVES

The aim of this PhD project was to characterize parasite genes that were differentially expressed during the ookinete-to-oocyst transition stage, and to investigate their roles in this critical *Plasmodium* developmental stage. Notably this stage represents a major bottleneck in parasite's development in the mosquito, hence a pivotal step in successful transmission. Of particular interest were genes that could potentially be involved in ookinete formation and its interactions with the mosquito host.

Selection of genes was accomplished through analysis of microarray data that had been previously produced in the host lab, and revealed various expressional patterns throughout *Plasmodium* development in the mosquito host. The working hypothesis is that *Plasmodium* genes specifically up- or down-regulated during the ookinete-to-oocyst transition stage are likely to be implicated in parasite differentiation and interaction with the mosquito. Genes of interest were taken forward for further functional characterization and understanding of their roles in the ookinete-to-oocyst transition through targeted disruption and generation of KO mutants, as well as, antibody mediated analysis. To investigate potential interactions of parasite genes with the mosquito immune system, research was also carried out in diverse mosquito genetic backgrounds that differ with respect to well-known immunity genes affecting parasite survival, e.g. in *LRIMI* (parasite antagonist) and *CTL4* (parasite agonist) knockdown mosquitoes (Osta et al., 2004).

The overall goal with this project is to increase our understanding of the molecular processes by which *Plasmodium* completes its life cycle in the mosquito host, but might also identify novel targets towards malaria transmission blocking.

2. EXPERIMENTAL METHODS

2.1. BIOINFORMATICS

2.1.2. Gene sequence retrieval and analysis

P. berghei sequences and other *Plasmodium* spp. orthologous sequences were retrieved from the NCBI nucleotide database, the Sanger centre web interface (<http://www.sanger.ac.uk/DataSearch/blast.shtml>) or PlasmoDB (<http://plasmodb.org/plasmo/>) (Bahl et al., 2003). Nucleotide and protein BLAST searches were conducted using the BLASTN and BLASTP tools available through PlasmoDB and NCBI (<http://www.ncbi.nlm.nih.gov/blast/>). Verification of signal peptide predictions (SP), transmembrane (TMs), nuclear localization signal (NLS) or other functional domains were performed using SignalP Server v 3.0 (Bendtsen et al., 2004), ConPredII (Arai et al., 2004), Predict NLS Online tool from the Rost lab (Cokol, Nair, & Rost, 2000), and last SMART database (Letunic et al., 2006; Schultz et al., 1998) and InterproScan (Quevillon et al., 2005) respectively. Multiple sequence alignments were conducted using ClustalW (Thompson et al., 2002) run through, and visualised by Bioedit Sequence Alignment Editor v.7.0.5.3

2.2. PARASITE MAINTANANCE, CULTIVATION AND PURIFICATION

2.2.1. Parasite strains

The parasite strain used for transcriptional profiling by QRT-PCR was the *P. berghei* ANKA clone 259c12. The 259c12 clone is a transgenic parasite line in which the expression of green fluorescent protein (GFP) is under control of native elongation factor- α loci (*ef-1 α*) promoter, resulting in constitutive expression of GFP throughout the parasite lifecycle (Franke-Fayard et al., 2004). For the generation of transgenic parasites by targeted gene disruption, a second GFP reference line was used, the *P. berghei* ANKA clone 507 (Janse et al., 2006). In similarity with the 259c12 line, GFP expression

is under control of the *eef-1a* promoter and the expression cassette was introduced into the genome by homologous crossover into the type D small subunit (*dssu*) ribosomal RNA locus of *P. berghei*. However, the 259c12 line was generated by using the *Toxoplasma gondii* dihydrofolate reductase-thymidylate synthase (*TgDHFR/TS*) pyrimethamine based selection system (Waters et al., 1997). In contrast, the 507 clone was selected for in the absence of a drug resistance marker, by the use of flow-cytometry (Janse et al., 2006). This crucial advancement in selection technology allows for subsequent gene KO by the use of the established *TgDHFR/TS* pyrimethamine selection system. Consequently, it allows for the generation of transgenic parasite in a GFP background, assisting phenotypic analysis. For generation of targeting constructs by sub-cloning, genomic DNA from the *P. berghei* ANKA 2.34 (Wild-type, WT) clone was used.

2.2.2. Parasite maintenance

P. berghei was maintained in Theiler's Original (TO) mice (Harlan, UK) or Charles River CD1 mice according to standard procedures (Sinden et al., 2002). Parasitaemia was monitored by the numbering of blood stage parasites in methanol fixed and DiffQuick (Dade Behring; according to manufacturer's instructions) or Giemsa (Fluka) stained tail blood smears. Giemsa solution was prepared by a 1:5 dilution in Giemsa buffer (0.7% (w/v) anhydrous KH_2PO_4 , 1% (w/v) anhydrous Na_2HPO_4) and staining was performed for 15 min at room temperature. Mice were infected by intraperitoneal (IP) injection of 100-200 μL *P. berghei* infected blood. For infections where a high parasitaemia / gametocytaemia was desirable, reticulocyte proliferation was stimulated by intra peritoneal (IP) injection of phenylhydrazinium chloride (PH); (6 mg/mL stock (Sigma) solution in Phosphate Buffer Saline (PBS); 10X stock solution of 0.2 M phosphate, 1.5 M NaCl, pH 7.4) 2-3 days prior to *P. berghei* infection. Blood samples used for passage or other experimental procedure were collected after cardiac puncture (10-20U/ml heparinised syringe, Sigma) on day 3-5 of infection from anaesthetised mice [intramuscular (IM) injection of 0.1ml/20g body weight of Rompun (Bayer), Vetlar (Pharmacia-Upjohn) and Phosphate Buffer Saline (PBS, Sigma) in proportion of 1:2:3]. All animal procedures were performed according to UK Home Office project licence agreements. Parasite stocks were maintained at -80°C in 90% v/v Alsever's solution (Sigma) and 10% v/v glycerol, prepared with infected blood at 1:2 ratio of blood to freezing solution. For long term storage

the samples were transferred to liquid nitrogen. Upon thawing the stocks were immediately injected into mice.

2.2.3. Purification of mixed blood-stage parasites

White blood cells were removed from infected blood by passage over CF11 cellulose columns (Whatman) pre-calibrated with PBS, from which the purified blood was collected following elution with PBS. Parasites were harvested by centrifugation at 1500 g for 10 min prior to 30 min incubation in ice-cold 0.17M NH₄Cl (Sigma) to facilitate red blood cell lysis. Parasites were subsequently pelleted by 10 min centrifugation at 1500 g, supernatant removed and pellet washed in PBS.

2.2.4. Ookinete and zygote in vitro cultivation

1 mL of infected mouse blood was collected from terminally anaesthetised mice with a parasitaemia of >10 %. The blood was immediately transferred to a tissue culture flask containing 30 mL 80% (v/v) ookinete culture medium (RPMI1640 (Sigma), 25mM Hepes (Gibco), 2mM L-glutamine (Sigma), 0.2% (w/v) sodium bicarbonate (Sigma), 50 U / mL penicillin, 0.05 mg / mL streptomycin (PenStrep; Gibco), 50 mg / mL hypoxanthine (Sigma), 100 µM xanthine acid (pH 7.4, Aldridge) and 20% (v/v) foetal bovine serum (FBS, Gibco). Cultures were incubated at 19°C for 8 hours for zygote harvest and 24 hours for mature ookinete production.

2.2.5. Ookinete and zygote purification

Ookinete and zygote purifications were facilitated by utilizing sheep anti-mouse IgG Dynal Dynabeads (Invitrogen) coated in an anti-P28 mouse monoclonal antibody (13.1) as previously described (Siden-Kiamos et al., 2000). All washing and separation steps were conducted using a magnetic separation rack (Magna Rack™, Invitrogen). Briefly, 13.1 coated Dynabeads were prepared by washing the beads four times in PBS prior to incubation with the 13.1 antibody (1/50 in PBS, antibody a kind gift by O. Billker) for 30 min at room temperature on a rotating rack. Unbound antibody was washed off as above and the 13.1 coated Dynabeads were resuspended in PBS (equivalent to the volume of storage buffer the aliquot of beads were originally kept in) and stored at

4°C until use. Ookinete or zygote cultures were harvested by centrifugation at 500g for 10 min at room temperature. The resulting pellets were resuspended in their own supernatant prior to addition of 10 µL coated beads followed by 5 min incubation on a rotating rack at room temperature. Following 1 min incubation on the Magna Rack™, the supernatant was collected. The ookinetes / zygotes bound to the beads were washed with 5 mL ookinete medium twice, the wash medium was transferred to the supernatant and another 10 µL of 13.1 coated Dynabeads was added. The procedure above was subsequently repeated, however, this time discarding the resulting supernatant and wash medium. Purified ookinetes / zygotes were pooled and pelleted at 500g for 5 min. A small sub-fraction of the pellets was mixed with 1 µL Foetal Calf Serum (FCS, Gibco) smeared onto glass slides, methanol fixed and Giemsa stained prior to microscopic observation to check the quality of the preparation. Pellets were stored at -20°C for Western Blot analysis, -80°C for RT-PCR or directly prepared for immunofluorescence assays.

2.2.6. Gametocyte purification

For preparation of pure viable gametocytes, sulphadiazine treatment was used in conjunction with Nycodenz (Axis-Shield PoC) density gradient purification as previously described (Billker et al., 2004). In short, *P. berghei* infected mice (parasitaemia ideally <10%) were treated with sulphadiazine (20 mg / L, administered in drinking water, Sigma) on day 3 post-passage. Infected blood was collected on day 5 post-passage and diluted in 5 mL Coelenterazine Loading Buffer (CLB), (20 mM Hepes, 20 mM Glucose, 4 mM Sodium bicarbonate, 1 mM EDTA (all chemicals from Sigma) and 0.1% bovine serum albumin (BSA, Sigma) prepared in PBS, pH 7.25. Following passage over CF11 columns pre-calibrated with CLB (elution in 5 mL CLB) the blood was harvested at 500g for 5 min at room temperature. Resulting pellets were resuspended in 3 mL of their own supernatant and loaded onto a 5 mL 48% Nycodenz gradient cushion (52% CLB v/v and 48% v/v Nycodenz stock solution (27.6% w/v Nycodenz (Life Technologies) made up in Nycodenz buffer (5.0 mM Tris, 3.0 mM KCl, 0.3 mM EDTA Na₂Ca, pH 7.2) (Sigma). Following low acceleration and de-acceleration centrifugation at 1000g for 10 min at room temperature, purified gametocytes were collected from the gradient interface and washed in CLB twice. Gametocytes were then either harvested immediately (nonactivated) or activated by incubation in ookinete culture medium at 19°C. Gametocyte viability was tested before and after purification by microscopic observations of

exflagellation in ookinete medium. Quality control by Giemsa-staining and sample storage were performed as for purified ookinete preparations.

2.3. MOSQUITO INFECTIONS AND MICROINJECTIONS

2.3.1. Mosquito infections

Anopheles gambiae Yaoundè mosquitoes (supplied by Dr Tibebu Habtewold and Sala Katarzyna) and *Anopheles stephensi* sda500 (supplied by Ken Baker and Mark Tunnicliff) were cultivated according to standard protocol (Sinden, 1997). Adult (3-7 days post emergence) females were infected with *P.berghei* by feeding on anaesthetized mice with a parasitaemia of 7-10% for 15-30 minutes, out of light in 19°C. After 46-48 hours, unfed females were removed so as to avoid interference in the quality of the infection.

Alternatively, mosquitoes were infected by ookinete membrane feeds. Ookinete culture volumes corresponding to 4×10^5 ookinetes (equivalent to 1600 ookinetes per mosquito as mosquitoes feed approximately 2 μ L infected blood) were centrifuged for 5 min at 500 g at room temperature. Pelleted ookinetes were resuspended in 0.5 mL naïve blood and the ookinete suspensions were loaded onto membrane feeders heated to 37°C and covered in thinly stretched Parafilm (Pechiney Plastic Packaging Company, VWR) to allow mosquitoes to probe and access the ookinetes. Mosquitoes were allowed to feed for 15-30 minutes in the dark at 19°C and unfed females were removed 48 hours after the blood meal so to not interfere with the quality of infection.

2.3.2. Microinjection of dsRNA for RNAi-mediated mosquito gene silencing

Double stranded RNA (dsRNA) for RNAi mediated knock-down (KD) of *A. gambiae* *CTLA* and *LRIMI* were prepared from PCR or plasmid template (Osta et al., 2004) using the T7 Mega script kit (Ambion Inc.) according to manufacturer's instructions. 69nL of 3 mg / mL of purified (Qiagen RNAeasy spin column kit) dsRNA was injected into the thorax of 1-2 day old *A. gambiae* mosquitoes, using glass capillary needles and the Nanoject II microinjection system (Drummond Scientific Company). The injected mosquitoes were allowed to recover and were subsequently infected with *P. berghei* by direct feeds at day 5 (Blandin et al., 2002).

2.4. TISSUE HARVESTING AND PROCESSING

2.4.1. Mosquito midgut dissections

P. berghei infected *A. stephensi* or *A. gambiae* midguts were dissected in PBS under a dissection microscope to remove the carcass, foregut, ovaries and malpighian tubules. Midguts were subsequently processed for counting of oocysts / melanised ookinetes, imaging or RNA / protein extraction accordingly. Similarly, salivary glands of female mosquitoes were dissected from days 18-22, homogenized and sporozoites were counted or pelleted and processed for RNA / protein extraction accordingly.

2.4.2. Harvest of *P. berghei* infected midguts for transcriptional analysis

Thirty to fifty midguts were isolated from *P. berghei* infected *A. gambiae* by dissection at separate time points, each representing a distinct stage of parasite sexual development. The time points were; 1-3 hours (T1; mixed asexual and sexual blood stages), 22-26 hours (T2; invading ookinetes), 48 hours (T3; ookinete-oocyst transformation), 5 days (T4; young oocyst), 10 days (T5; mid-mature oocyst) and 13-15 days (T6; mature oocyst - sporozoite release) post-infection (PI). Midgut dissections were performed on ice in PBS and samples were directly frozen in liquid nitrogen prior to storage at -80°C.

2.4.3. Total RNA extractions

Immediately upon thawing, tissues or cells were immersed in Trizol® reagent (Invitrogen), mechanically homogenised and total RNA was isolated according to manufacturer's instructions under RNase free conditions. RNA quality was assessed by standard gel electrophoresis using a Tris / Borate / EDTA (TBE) (Sambrook, 2001) buffer and denaturing Loading Buffer II (Ambion). Total RNA amount was quantified in 1µL sample volume using the nucleic acid application of 81 NanoDrop® ND-1000 Spectrophotometer (Thermo Scientific). RNA samples were stored at -80°C.

2.4.4. Protein sample preparation for Western Blot analysis

Tissues or cells were immersed in reducing protein loading buffer (Sambrook, 2001), mechanically homogenised, boiled for 5 min, briefly spun down and stored at -20°C.

2.4.5. Genomic DNA isolation

P. berghei genomic DNA was prepared from purified mixed blood stage parasites using the Promega Wizard Genomic DNA extraction kit according to manufacturer's instructions.

2.5. TRANSCRIPTIONAL PROFILING

2.5.1. Preparation of qRT-PCR and RT-PCR templates

Using the RNA collected and prepared as described above, 4µg of total RNA for each sample pool was DNase treated (DNaseI;New England Biolabs) for 15 min at 37°C to remove any traces of genomic DNA contamination. The DNase treated RNA was purified using RNAeasy columns prior to reverse transcription of mRNA (1µg total RNA was purified using a combination of random hexamers and T7 primers (TaqMan reverse Transcription Reagents Kit;Applied Biosystems).

2.5.2. Quantitative Real-Time PCR (QRT-PCR) procedure

Transcriptional profiles of selected genes were generated from the produced cDNA by QRT-PCR using gene specific primers and the SYBR-Green detection and amplification reagent (Applied Biosystems) in conjunction with ABI PRISM 7000 sequence detector (Applied Biosystems) as previously described (Vlachou et al., 2005).

Primers were designed using the web-interface of Primer3 (Rozen & Skaletsky, 2000) according to the following standards: optimum primer length 18-22bp, GC-content 20-80%, T_m 58-60°C and an amplicon length of 70-140bp. Primer concentrations were optimised (Table 2.1) and products run on a 2% agarose gel to ensure specificity. All reactions were run in duplicates and final transcriptional profile was brought up by the average of a minimum of two independent biological experiments. Last, for the analysis, GFP was used as an internal control, so as to provide a reference for the fluctuation in parasite population numbers during its development, hence all values were normalised against respective GFP expression.

2.5.3. Reverse Transcription (RT-PCR) procedure

Gene specific RT-PCR primers were manually designed with a T_m of 52-60°C and an amplicon length of 0.1-1.0 Kb. (Table 2.2). Semi-quantitative RT-PCR was performed using gene specific primers and GoTaq Green DNA polymerase master mix (Promega). PCR products were amplified by a denaturation step of 5 min at 94°C followed by 25, 30 or 35 cycles comprising of 45 seconds at 94°C (denaturation), 45 seconds at 52°C (primer annealing) and 1 min at 62°C (elongation) and concluded by a final elongation step of 5 min at 62°C. RT-PCR products were analysed using standard gel electrophoresis run (1-2% agarose gel accordingly) with TBE buffer (Sambrook, 2001)

Table 2.1. Primer sequences and optimised concentrations for QRT-PCR.

Table 2.1 QRT-PCR primers		
Name	Sequence	Optimal Primer Concentration
CS Forward	GAATTCGTTAAACAGATCAGGGATAGTA	900nM
CS Reverse	TTATACCAGAACCACATGTTACGTTACA	300nM
CTRP Forward	TGCAATGATGTTTGTGGTGATT	900nM
CTRP Reverse	TGGTGATACATTTCTGGTCTTATTCTT	100nM
GFP Forward	CCTGTCCTTTTACCAGACAACCA	900nM
GFPReverse	GGTCTCTCTTTTCGTTGGGATCT	300nM
PB000317.00.0 Forward	TGACAATTCTATGCTGGTTCG	900nM
PB000317.00.0 Reverse	GGGTAGGGGATGGTGTGTCATA	300nM
PB000467.02.0 Forward	CATCGAATTTAAAGGGCACCTA	300nM
PB000467.02.0 Reverse	AATGCTTTTTGAAGGTCATTAGTT	900nM
PB000489.03.0 Forward	TGCTGGAATAACACCACCAG	900nM
PB000489.03.0 Reverse	GCTGTCATCACGTTTCTTTTCA	900nM
PB000508.03.0 Forward	ATATTGGAGGCCAAGTTCCA	900nM
PB000508.03.0 Reverse	AGTATCGGATGGTCCAGCAA	900nM
PB000551.00.0 Forward	TGTTGACACACAAGGTGGTGA	900nM
PB000551.00.0 Reverse	TCGTTTTCTTGAGCATCATC	300nM
PB000653.00.0 Forward	TGGAACTCGAGAAACAGCAT	900nM
PB000653.00.0 Reverse	TGTGCAATTAGGGCATAACACA	900nM
PB000712.03.0 Forward	TTACCATATATTGGAAGGCAAGA	300nM
PB000712.03.0 Reverse	TTAAAGAAGCGGCTGTGTTATG	900nM
PB000747.03.0 Forward	TATTGGCCCGAGCTCTAAAA	300nM
PB000747.03.0 Reverse	CACCCACCAGTCAATCCTTC	900nM
PB000773.02.0 Forward	TTCTTTCAAGAAAAAGCCAAAA	900nM
PB000773.02.1 Reverse	AAAATTTGCGATGCTAAAGATG	900nM
PB000808.02.0 Forward	TGGCTTCGGAAGAAGGAAT	900nM
PB000808.02.0 Reverse	GCCTTTTGACTGCTTCCATT	300nM
PB000846.01.0 Forward	AGACAAGGAAAGGGGCTCAC	300nM
PB000846.01.0 Reverse	TCCCCAATTTTTGATGTTTCA	900nM
PB000874.00.0 Forward	CCAGTTGGAGGCCATGTAAT	900nM
PB000874.00.0 Reverse	TGCATTCTATTTGCGGAAGG	300nM
PB000893.01.0 Forward	AAGCCAGAGGTTGTATTTGCAT	900nM
PB000893.01.0 Reverse	TAGCATGGTTTCGATGATATGG	300nM

Table 2.1 QRT-PCR primers *continued*

Name	Sequence	Optimal Primer Concentration
PB000964.01.0 Forward	TTTTAAATTTTACCCCGACGAA	300nM
PB000964.01.0 Reverse	TTCCATTGTTTTATGCGAATC	100nM
PB001039.00.0 Forward	CGAAAGCGAATCAAATTGTTTAT	300nM
PB001039.00.0 Reverse	TTGTTCTGGGGATACTTCAAAA	900nM
PB001180.00.0 Forward	TATTCAGTAGGGGATGGGAATG	900nM
PB001180.00.0 Reverse	TAACTTTTGACATGTCGGTTCG	900nM
PB001229.02.0 Forward	CGAGCAGTGGGAGCAGTAAT	100nM
PB001229.02.0 Reverse	TTGCAACTTCTGGAAATGGTT	900nM
PB001289 Forward	GATGTAACACCGAATGAGTCCA	300nM
PB001289 Reverse	CAGAACCACCTTCGTCACAATA	900nM
PB001312.02.0 Forward	TGACTGGAACCTCTGGGTTG	300nM
PB001312.02.0 Reverse	CTCCCATACCGCCAAAATA	900nM
PB001343.02.0 Forward	GCACAAGGGTCGTTTGAAGT	300nM
PB001343.02.0 Reverse	GCTGGGGTATTATCGTGCAT	300nM
PB001363.02.0 Forward	TAGAAAATGGGGAAATGATTGG	900nM
PB001363.02.0 Reverse	AATGGCTCGTCAAATGTTTCAT	300nM
PB001632.02.0 Forward	TAACGTCTCTTGGGGTTATTGG	900nM
PB001632.02.0 Reverse	ACTTCCAGTTTCCATGATTTCG	900nM
PB103082.00.0 Forward	TATTCCTTGGGCCGATTATG	300nM
PB103082.00.0 Reverse	TGCACTATATCACTGCATTCCA	900nM
PB104218.00.0 Forward	GGGGTCGACTCTATAGGCAA	900nM
PB104218.00.0 Reverse	GCTACAATTGCCGATTCCAT	900nM
PB105834.00.0 Forward	GCGATCGGTTTTAGAAAACATT	300nM
PB105834.00.0 Reverse	AGGGAGAGTTGGTTCCATTACT	900nM
PB105866.00.0 Forward	TGACCCTGATCTTCCAAATCTT	900nM
PB105866.00.0 Reverse	CATCGATTTCTGATGCAAAGAG	100nM
PB108456.00.0 Forward	GATCGTATGCTCGACCCATC	300nM
PB108456.00.0 Reverse	GAGTTTTCCCACTCCCAACA	900nM
PB300759.00.0 Forward	ATAGGCCATAGGGGTTCTGG	900nM
PB300759.00.0 Reverse	GCCATGCATCTAATTCAATACC	300nM
PB300785.00.0 Forward	GATGAAGGATGGGCATGG	300nM
PB300785.00.0 Reverse	TGTTCTCCAAAGCCTTTTTCG	900nM
PB301371.00.0 Forward	CCAATATCCACACATCCACA	300nM
PB301371.00.0 Reverse	GTTGAGAGGTTTGCCACGTT	900nM
PB301555.00.0 Forward	TTTGATATGTTGTTTCGAAAGG	100nM
PB301555.00.0 Reverse	AAGGTTTGTCTTCTCGCAAAC	300nM
Pbs21 Forward	AATGCACAGGTACAGGAGAACTAAAT	900nM
Pbs21 Reverse	CACACTCATAATGTTTTCCAGTCAATT	900nM

Table 2.1 QRT-PCR primers *continued*

Name	Sequence	Optimal Primer Concentration
PB000254.00.0 F	CGAAAAATGGCAAGAAAGGA	900nM
PB000254.00.0 R	CGATTGTTTGTTCCTTC	300nM
PB001039.00.0 QRT F 2	TGGAATTATAAAGCACCAGGA	300nM
PB001039.00.0 QRT R2	CAGGGGCATATTTGATTTG	900nM
PB000551.00.0 QRT R 2	TCCTGTCCATCTTTTGTTC	100nM
PB000551.00.0 QRT F 2	CGTTGGAGAAATACCAAATG	100nM
PB301555.00.0 QRT F2	CTGAAAATCTTGAAACACAAAA	900nM
PB301555.00.0 QRT R2	AATTTTCCACCTTTTCGATTG	900nM
PB000251.01.0 QRT F	ATCATGGGACGAATTACCACA	900nM
PB000251.01.0 QRT R	GAACCTGCTGGAAAGGCTACT	300nM
PB000679.03.0 QRT F	AACTCGGCTTCCAGAAGAAAG	900nM
PB000679.03.0 QRT R	TGGATCGGAACACATACAACA	300nM
PB000111.00.0 QRT F	AAAGAAGAAATGTATTCGAAGC	300nM
PB000111.00.0 QRT R	TCTTTTCACTTTTCCACAAAT	900nM
PB000692.01.0 QRT F	CCAAATGGGAAATCCAAATAC	900nM
PB000692.01.0 QRT R	CAATTTCTTCGGATACCTGCAT	100nM
PB000918.03.0 QRT F	TGCTCATGAAATGGATATGGA	900nM
PB000918.03.0 QRT R	AATTATGGGGTCCATCCTCAC	300nM
PB000882.03.0 QRT F	CACCATATCCAAACACACCAA	900nM
PB000882.03.0 QRT R	TCCCTTACATGGTTAAGCTCTG	100nM

Table 2.2. Primer sequences for RT-PCR. Forward (F), Reverse (R).

Table 2.2 RT-PCR Primers	
Name	Sequences
PB001343.02.0 F	ATACGGGAATCCATCAACCA
PB001343.02.0 R	ACTTCAAACGACCCTTGTGC
PB001312.02.0 F	GCATTGGATAAAATGTGACTGG
PB001312.02.0 R	TGCATGAGAATGATCTGCATC
PB000489.03.0 F	TCAAGATGAAGCAAAAATGGA
PB000489.03.0 R	TGCCTTGGTTTTATTTGTTTTC
PB000846.01.0 F	CAAACACCCCGTTCAATTTT
PB000846.01.0 R	TTCTCCATCCTGAGCATTGTT
PB000874.00.0 F	TGCGGAAAACTCAAGTGTG
PB000874.00.0 R	TGCATTCTATTTGCGGAAGG
PB000508.03.0 F	GAAAGCGAATTCAACGGATT
PB000508.03.0 R	AGTATCGGATGGTCCAGCAA
PB001289 F	GACAATCCAAGGCGATTCAT
PB001289 R	GCAATATCAGCGTCATCAGAA
PB001180.00.0 F	CAAATGCGAGTCTAGAATGGTT
PB001180.00.0 R	TAACTTTTGACATGTCGGTTCG
Pbs21 F	CACTTGCGAGCCAATTGTTA
Pbs21 R	ATTTGATGGCGTTTCGCTAC
AMA I F	CCGGTGATCAGTCAGTGAGA
AMA I R	AACCATCAACCCAAATTCCA
PB000950.01.0 F	ACTCGGATGAATTCCACTACAATAA
PB000950.01.0 R	GGGATATATTTCCATAATTGAGC
PB000026.01.0 F	ACCATATTGCACCGATAGAAAATTA
PB000026.01.0 R	ATACCTACATTTGAGCACACAACAA
PB300785.00.0 F	GAAAACATTTCTCACAAAAACACAAC
PB300785.00.0 R	CTTCTTCTTCATCGTTATCATTTCC
PB001511.02.0 F	TTGTGGTATCTGTACAGGGGATAAT
PB001511.02.0 R	TCCAATATTTGTTAATAGCATCC
PB000329.03.0 F	GATACTAGCCATTATTGTTTTAG
PB000329.03.0 R	TATCCTATAAGCTGTTGATATTC
PB000407.00.0 F	AGAAGGTATTCTATGTTATGCTA
PB000407.00.0 R	CCACTATTACTATTTTCAATGTC
PB000752.02.0 F	TTCATAGTAATAGGAATAGCAGT
PB000752.02.0 R	CTTAAAGTCACCAAACATTC
PB001052.02.0 F	CAATGTAGATATGATTAGGTTCT
PB001052.02.0 R	GTATACATATATCAGCAATCCAT
PB001363.02.0 F	GATATTGGCAAGACAGTGGATT
PB001363.02.0 R	CAACATCAACCCTTAGGAAC
PB000808.02.0 F	GTTCTATAGGAGCAAATTTTGTTGAG
PB000808.02.0 R	ATTTTTCCTTAGAAGAGATATTCACG
PB000254.00.0 F RT	GGGATAAGGAAACATGCATAGA
PB000254.00.0 R RT	AAACTCGATTTTGTCTTGGTTG
chit RT-PCR F	GCCCGCCAGATGTAATTATA
chit RT-PCR R	TGCCAAATTCCTACACCATCG

2.6. GENERATION OF TRANSGENIC PARASITES

2.6.1. Molecular cloning: Construction of vectors for parasite transgenesis

2.6.1.1. Construction of vectors for targeted gene disruption

P. berghei sequences for selected genes were retrieved from the NCBI nucleotide database and the Sanger centre web interface. Upstream and downstream target sequences (500-1000 bp of the 5' UTR and 3'UTR of respective ORF) for disruption through double homologous recombination were amplified from *P. berghei* ANKA clone 2.34 genomic DNA (collected as described above) by PCR (Amplitaq, BD) by using gene specific primers with restriction enzyme site overhangs (Table 2.3) The PCR program entailed: 5 min at 94°C followed by 35 cycles of 45 sec at 94°C, 45 sec at 52°C and 2 min at 68°C.

PCR products were consequently each separately cloned into a pGEM®-T Easy Vector System according to manufacturer's instructions and standard protocols (Sambrook, 2001) Clones containing the correct inserts were digested by using the appropriate, respective restriction enzymes (Table 2.3), identified through standard diagnostic digests and gel extracted by use of QIAquick Gel Extraction Kit (QIAGEN). Inserts were then being cloned into the pBS-DHFR vector in which polylinker sites flank a *tgdhfr-ts* pyrimethamine resistance cassette (Dessens et al., 1999). Upstream and downstream targeting sequences were cloned by using the appropriate, respective restriction enzymes. All cloning procedures were performed according to manufacturer's instructions and standard protocols. Clones containing the correct inserts were identified through standard diagnostic digests. The resulting vectors for targeted disruption were further analysed by PCR to confirm the correct orientation and positioning of the inserts. The primers used for the upstream target sequence were 5' prime of homology forward primers in combination with the TgDHFR-TS 5'UTR reverse primers. For the downstream target sequence, the TgDHFR-TS 3'UTR forward primer was used in combination with 3' prime of homology reverse primers (Table 2.3.).

2.6.1.2. Construction of vectors for c-terminal tagging of PB001289

Fusion of PB001289 to a c-terminal MYC tag by replacing 1.5 kb of the most 3' terminal portion of the endogenous PB001289 locus with a tagged counterpart. 1.5 kb corresponding to the most 3' terminal portion of PB001289 was amplified by PCR and cloned in-frame with a c-terminal MYC tag held in the pDR0007 tagging vector (kind gift from D. J. Raine), which also carries a *TgDHFR/TS* selection cassette. Following transfection, introduction of tagging vectors into the PB001289 locus would be facilitated by single homologous recombination.

2.6.2. Preparation of DNA for transfections

The disruption vectors were prepared for transfection using the high speed MidiPrep plasmid purification kit (Qiagen). Prior to transfection, the constructs were linearised by double restriction enzyme digests at sites flanking the targeting construct using *ApaI* and *BamHI* or *ApaI* and *XbaI* (Table 2.3). Linear constructs were purified by ethanol precipitation (Sambrook, 2001) and 1-5 µg of DNA in a volume of 5-10 µl was utilised for each transfection. The PB001289 tagging constructs were linearised by single restriction enzyme digests using *EcoRV*, cutting in the centre of the 1.5Kb homology region.

2.6.3. Generation of transgenic parasites - Transfection procedures

The procedure was performed according to Janse et al 2006 (Janse et al., 2006). Briefly, blood was collected on day 1-2 of infection (parasitaemia less than 5%) from three anaesthetised mice infected with *P. berghei* ANKA clone 507 to serve as a source of blood-stage parasites for the transfections. The blood was transferred to 120 ml of 75% (v/v) schizont culture medium RPMI1640 pH 7.2 (RPMI1640, 24mM L-glutamine, 25mM HEPES, 0.2% (w/v) NaHCO_3 (Gibco), 50 U / ml penicillin, 50 µg / ml streptomycin) and 25% (v/v) FBS. The culture was gassed with 5% CO_2 , 5% O_2 and 90% N_2 and incubated overnight shaking gently at 37°C. Prior to starting transfections, the parasite quality and quantity was examined on a Giemsa stained smear of the culture (0.5 ml aliquot pelleted and thinly smeared). Parasites were purified on a 15.2% (v/v) Nycodenz (Life technologies) / PBS density

gradient (30 min at 300 g) and those localizing at the interface between the resulting two layers were collected, washed in 20 ml culture medium, centrifuged at 300 g for 8 min and supernatant was discarded. The pellet was re-suspended in schizont culture media, split into 1 ml aliquots and spun down for 1 min at 200 g. The supernatant was discarded and the pellet re-suspended in 100µl AMAXA supplemented nucleobuffer (AMAXA) containing the DNA which is to be transfected (prepared as outlined above). The DNA/ buffer/ parasite solution was transferred to an electroporation cuvette (AMAXA) and electroporation was performed according to manufacturer's instructions (Protocol U33; AMAXA). 150 µl of uninfected mouse blood (obtained from a phenylhydrazine treated mouse on day 2-3 after treatment; 125 mg / kg body weight) was added to the electroporated parasites. The parasites were incubated at 37°C for 15 min to allow invasion of merozoites into erythrocytes prior to injection of the suspension intra-peritoneally into a mouse.

2.6.4. Selection of transgenic parasites

Two rounds of selection of pyrimethamine-resistant parasites were performed by administration of 0.07 mg / pyrimethamine (Sigma) in the drinking water of transgenic injected mice.

2.6.5. Limited dilution cloning of transgenic parasites

Clonal transgenic parasite populations were obtained by limited dilution cloning as previously described (Waters et al., 1997) Briefly, blood-stage parasites were enumerated in fixed and stained tail blood smears from infected mice and diluted in RPM1 medium to a final concentration of 20 parasites / mL, prior to IP injection (200µL) into TO mice.

Table 2.3. Primer sequences for generation of transgenic parasite lines. Where appropriate, target restriction sites are shown as underlined. The appropriate restriction enzymes are indicated, a, b and c, d denote forward (F) and reverse (R) primers of upstream and downstream region of homology respectively.

Table 2.3 Cloning Primers		
Name	Primer Sequence 5'-3'	Restriction Enzyme
PB000254.00.0 a	TT- <u>GGGCCC</u> -GAATAAGTGAGTTAAAAGAGAAATTAACAA	Apa I
PB000254.00.0 b	CC- <u>AAGCTT</u> -CTTTACAACAGCACAACCGTTTTAT	Hind III
PB000254.00.0 c	T- <u>GAATTC</u> -AATAGGAGAAGAGAACATCCCAGAT	EcoR I
PB000254.00.0 d	TT- <u>GGATCC</u> -TAATCAACTGGAGCTTTTTGGTAAC	BamH I
PB000329.03.0 a	TT- <u>GGGCCC</u> -ATATTTTTTCATAATAATGTGACC	Apa I
PB000329.03.0 b	CC- <u>AAGCTT</u> -GTTTTACGACTAAATTAACAT	Hind III
PB000329.03.0 c	T- <u>GAATTC</u> -GGTTATATTACAAGGTATTTATT	EcoR I
PB000329.03.0 d	TT- <u>GGATCC</u> -TATTACATTTTTAAGGATTTGAG	BamH I
PB000489.03.0 a	TT- <u>GGGCCC</u> -TGATGATGAAGCTATTGGTAAGTGA	Apa I
PB000489.03.0 b	CC- <u>AAGCTT</u> -ATGGTAATGCATCAATATCCTCTAA	Hind III
PB000489.03.0 c	T- <u>GAATTC</u> -TGCATGCATATCTTTTTATTTTGA	EcoR I
PB000489.03.0 d	GG- <u>GGATCC</u> -ATGTTAAAATAGCCATGCAAATGAT	BamH I
PB000508.03.0 a	TT- <u>GGGCCC</u> -ATTACAATTTGATATAGCCTTGCA	Apa I
PB000508.03.0 b	CC- <u>AAGCTT</u> -TGTTTGAAAAATCGAAAAATAATAAGG	Hind III
PB000508.03.0 c	T- <u>GAATTC</u> -GCAATATAAGAGTAAGGAGCCGATT	EcoR I
PB000508.03.0 d	TT- <u>GGATCC</u> -TAAAGACGTTCTAACAGCATGAGTG	BamH I
PB000747.03.0 a	TT- <u>GGGCCC</u> -TTGGATTGGAGACAATAACAAT	Apa I
PB000747.03.0 b	CC- <u>AAGCTT</u> -CTCATCACAATTTACATTTCCATCT	Hind III
PB000747.03.0 c	T- <u>GAATTC</u> -TTACCAGATAATTTCTTTACATGCAC	EcoR I
PB000747.03.0 d	TT- <u>GGATCC</u> -GCATAAATTATAAATGATTGGGAAAT	BamH I
PB000808.02.0 a 1	TT- <u>GGGCCC</u> -GTTCTATAGGAGCAAATTTGTTGAG	Apa I
PB000808.02.0 a 2	TT- <u>GGGCCC</u> -AGAATAAGAAGTACAGCGATAA	Apa I
PB000808.02.0 b 1	CC- <u>AAGCTT</u> -ATTTTTCTTAGAAGAGATATTCACG	Hind III
PB000808.02.0 b 2	CC- <u>AAGCTT</u> -TTTTCTTAGAAGAGATATTCAC	Hind III
PB000808.02.0 c 1	T- <u>GAATTC</u> -GTATGATCATTTAACCAAAGCAGAC	EcoR I
PB000808.02.0 c 2	T- <u>GAATTC</u> -AAGTACAAGAGAAGACTTTAC	EcoR I
PB000808.02.0 d 1	TT- <u>GGATCC</u> -CTCTTAGTTGCTAGCTTTTCATAGG	BamH I
PB000808.02.0 d 2	TT- <u>GGATCC</u> -ACTCTTAGTTGCTAGCTTTT	BamH I
PB000846.01.0 a	TT- <u>GGGCCC</u> -GTATATTATTTGGCTTATACGTCAGCAA	Apa I
PB000846.01.0 b	CC- <u>AAGCTT</u> -CAACCATATTTAGTATTATCCGTATATTCA	Hind III
PB000846.01.0 c	T- <u>GAATTC</u> -TCTTTATTTGACTTCATTTAGTGATTTGC	EcoR I
PB000846.01.0 d	GG- <u>GGATCC</u> -CCATCATAATTGTTTTACCATATATATTCAC	BamH I
PB000874.00.0 a	TT- <u>GGGCCC</u> -GCATGCCTTTCATAATAAAGTATAAACAAC	Apa I
PB000874.00.0 b	CC- <u>AAGCTT</u> -GAAAAATGAAATAATAGCGATTAGACAGAC	Hind III
PB000874.00.0 c	T- <u>GAATTC</u> -GTAATGTTGGATATAAAGGCTGATCAATTA	EcoR I
PB000874.00.0 d	GG- <u>TCTAGA</u> -CATCAGTTTTATCACCATTTATTTTTGT	Xba I
PB001052.02.0 a	TT- <u>GGGCCC</u> -ATTCATAAAGGAAATATAGATAACA	Apa I
PB001052.02.0 b	CC- <u>AAGCTT</u> -AAATATTTAGAATGGCTGTAAC	Hind III
PB001052.02.0 c	T- <u>GAATTC</u> -TAGTCCATCTATTATATTTTACT	EcoR I
PB001052.02.0 d	TT- <u>GGATCC</u> -ATATACTTGTTAACTGTTTGAGTT	BamH I

2.3 Cloning Primers <i>continued ...</i>		
Name	Primer Sequence 5'-3'	Restriction Enzyme
PB001180.00.0 a	TT-GGGCCC-CTGTTGCTTTTGTGGCC	Apa I
PB001180.00.0 b	CC-AAGCTT-TTTGGTATGGTTGCTCATTTATTTA	Hind III
PB001180.00.0 c	T-GAATTC-TCCAATTAATTTCAAGGATCGAAATTC	EcoR I
PB001180.00.0 d	TT-GGATCC-ACTATACCATGAATTAACCCGATTG	BamH I
PB001229.02.0 a	TT-GGGCCC-GGTTACGCTGAACAGACATT	Apa I
PB001229.02.0 b	CC-AAGCTT-TTCCTCGTTTATTACATTGTTCTTC	Hind III
PB001229.02.0 c	T-GAATTC-TTCGAACCCCTACTAATAATACCAGTT	EcoR I
PB001229.02.0 d	TT-GGATCC-ATCTGTGCCTATTTATACTTGGAGTT	BamH I
PB001312.02.0 a	TT-GGGCCC-AACACCATCGCAAAGGATAA	Apa I
PB001312.02.0 b	CC-AAGCTT-GCATTGAGCAAGGCGTAT	Hind III
PB001312.02.0 c	T-GAATTC-TTAGGAGGAATGGACCAATTT	EcoR I
PB001312.02.0 d	GG-GGATCC-TGAGGTTCTCTTAGCTAGCTCGT	BamH I
PB001343.02.0 a	TT-GGGCCC-TTTTATGAAAACATTTTCTACATTC	Apa I
PB001343.02.0 a 2	TT-GGGCCC-TATGAAAACATTTTCTACATTCT	Apa I
PB001343.02.0 b	CC-AAGCTT-TGAATTAATACTCTCCAATTTTGT	Hind III
PB001343.02.0 b 2	CC-AAGCTT-TACTAATCTCTCCAATTTTGTAT	Hind III
PB001343.02.0 c	T-GAATTC-AAGCGAATGTATATCCAGGTTAATA	EcoR I
PB001343.02.0 c 2	T-GAATTC-TAGAAGATTTCTGAATAACTGTA	EcoR I
PB001343.02.0 d	GG-GGATCC-CTATGGATTTCCTGATTTTCATTTTC	BamH I
PB001343.02.0 d 2	TT-GGATCC-TATTTGTTTTCTTTGTTTCTATAC	BamH I
PB001363.02.0 a 1	TT-GGGCCC-TCAAAGGTTTGAGTATCGAATTG	Apa I
PB001363.02.0 a 2	TT-GGGCCC-TCTACTATTCACATTGTTGAGA	Apa I
PB001363.02.0 b 1	CC-AAGCTT-AATTTCCATTACCCCTTCCAATCAT	Hind III
PB001363.02.0 b 2	CC-AAGCTT-GATATCCCTCATTCTCTTTTA	Hind III
PB001363.02.0 c 1	T-GAATTC-GTGAAAGGTATTTTTTTTCAGAAAATTGC	EcoR I
PB001363.02.0 c 2	T-GAATTC-GATATAAGGATATTGGGGATTA	EcoR I
PB001363.02.0 d 1	TT-GGATCC-GCAGTCTAACGCAGGTTAAATAAA	BamH I
PB001363.02.0 d 2	TT-GGATCC-GTTTGCAGACAGAAAATTA	BamH I
PB001511.02.0 a	TT-GGGCCC-AGTGTATATTCACACAAACG	Apa I
PB001511.02.0 b	TTT-AAGCTT-ATAATCAGGATCAACTACATTC	Hind III
PB001511.02.0 c	T-GAATTC-GAAATCTTGCTATTTTGTGA	EcoR I
PB001511.02.0 d	T-GGATCC-TTGTATGCCATTATCAAAAG	BamH I
PB001289 a	TT-GGGCCC-CGTTGTAAAATTGTCAAACAAA	Apa I
PB001289 b	CC-AAGCTT-ACACCCTTTGAAACATATTATCATCA	Hind III
PB001289 d	TT-GGATCC-GTTTTCCGAAGTAAATTCATTAT	BamH I
PB001289 c	T-GAATTC-ACTTAAAGATGGGGTTAATTCAAAGA	EcoR I
PB000251.01.0 a	TT-GGGCCC-GAAAGCTCACCCATGTTTATATTGT	Apa I
PB000251.01.0 b	CC-AAGCTT-ACAATTTTATTGAACTAGGGGTCA	Hind III
PB000251.01.0 c	T-GAATTC-ATGCTATCAGGTTATCAACCCAGT	EcoR I
PB000251.01.0 d	TT-GGATCC-AATTGTCCACGAAAACACTGTA	BamH I
TgDHFR 3'UTR F	TCGTGGGCTACGTCCCGCAC	
TgDHFR 5'UTR R	GATGTGTTATGTGATTAATTCATACAC	
PB001289 mycF	TT-GGTACC-GTTCTGATGAAGATATAGTGAGTA	KpnI
PB001289 mycR	TT-GGGCCC-TCTAGAATATTTTCTTGAATATTTT	Apa I

2.7. GENOTYPIC ANALYSIS

Genomic DNA was prepared from the transfected parasite populations following purification from infected mouse blood as outlined above. To determine whether parasites surviving the second round of drug selection carried integrated disruption constructs, diagnostic PCRs were performed in conjunction with Pulse Field Gel Electrophoresis (PFGE). Assessment of purity of the dilution cloned *Δpb1289*, *Δpb1180* and *Δpb508* were conducted by PCR and Southern Blot analysis

2.7.1. PCR- based analysis of transfectants and clonal transgenic lines

The parasite genomic DNA was extracted (either from mixed post transfection population or clonal transgenic population following limited dilution cloning) using the Promega Wizard Genomic DNA extraction kit according to manufacturer's instructions. To determine whether parasites surviving the second round of drug selection carried integrated disruption constructs, diagnostic PCRs were performed by using the forward integration/reverse integration primers, situated upstream/downstream of the target site for homologous recombination, in combination with the TgDHFR-TS 5'UTR reverse/TgDHFR 3'UTR reverse forward primer depending on the gene (Table 2.3). This primer positioning ensures that a product can only be obtained if integration taken place. In combination to that, presence or absence of the respective wild type locus was verified by the INT F primer in combination with a primer (WT R), (Table 2.4) located within the region of the WT locus, which should have been excised by homologous recombination. With this primer positioning, a product can only be obtained if the WT locus is left intact.

Table 2.4. Primer sequences for genotypic analysis of transgenic parasite lines. Integration (Int), wild type (W), Forward (F) and reverse (R) is shown

2.4 Genotypic analysis primers	
Name	Sequence
PB000489.03.0 INT R	TCGTGTTTTCTATAAGACGAGTCC
PB000489.03.0 WT R	TGGTGGTGTTATTCCAGCAG
PB000846.01.0 INT F	CAACGACACTAGCCTTTAAAAATGT
PB000846.01.0 WT R	GGAAATATGTAGCAAATTGGAAATG
PB001343.02.0 INT R	TGCGCACATATATAATACAGTATGCTACTTTTACA
PB001343.02.0 WT R	AGCCAAATCGCATTGATTGATCAGATAGACTT
PB001312.02.0 INT F	ATCGAATTAATCCACGATCAAT
PB001312.02.0 WT R	CAGTATCAACCCAAGAGTTCCAGTCACA
PB00874.00.0 INT F	GGGGATTCACCTATGATGATTTAT
PB00874.00.0 WT R	GAGCAGTAACCGCTAAAGTATGGCACAC
PB000508.00.0 INT F	TGCCAATCTAATGCTTTTTGGTTTAC
PB000508.03.0 WT R	CACAACATAACCCCTTTCGCGT
PB1289 INT F	AGCGTCTAGTAGTTTGAGCTAGCTA
PB001289 WT R	GCAATATCAGCGTCATCAGAA
PB001180.00.0 INT F	TCGTTATTTTATCATTGATAATTTTACTATC
PB001180.00.0 WT R1	AGGTGAATTATTTGGTCCATTCA
PB001180.00.0 WT R2	ACCATTCTAGACTCGCATTGTT
PB000808.02.0 INT F	TGCATATAATGGAAAACACAGC
PB000808.02.0 WT R	TCATTTCCCAAACCCATAGAC
PB001229.02.0 INT F	TGCACATATATAACAATCAAGTGGTG
PB001229.02.0 WT R	TCCCCCTGTTAGATTATCATTGTT
PB001363.02.0 INT F	CACTAATAATAATAATGCCGATGC
PB001363.02.0 WT R	TTTTGATGGTGGTATGACAATTT
PB000254.00.0 Int F	AACAAGACGTTACCCCTGT
PB000254.00.0 WT R	ATAGGAAGCGCCTGTGGTCG
MYC INT R 1	TGTTCCAGATCCTCTTCTGAGATG
PB001289 mycINT F	GATGTAACACCGAATGAGTCCA

2.7.2. Pulse field gel electrophoresis (PFGE) analysis of transfectants

2.7.2.1. Whole chromosome preparations

Whole chromosome preparations were prepared from fresh *P. berghei* mixed blood stage parasite populations purified as outline above and immediately transferred into agarose plugs as previously described (Reininger et al., 2005). Briefly, purified parasites were resuspended in TNE buffer (10 mM Tris HCl pH 7.5, 100 mM NaCl, 5 mM EDTA) and 2% (w/v) low melting agarose (Sigma) in TNE was added at a 1:1 ratio. The resulting 1% agarose solution was transferred to plug moulds and allowed to set on ice, prior to digestion in 5 mL Sarkosyl buffer (3 mL TNE, 1 mL 0.5 M EDTA pH 8.0, 1 mL 10% (v/v) Sarkosyl) with 400µg/mL proteinase K, for 72 hours, shaking at 37°C. Plugs were stored in Tris EDTA (TE) buffer at 4°C (Sambrook, 2001).

2.7.2.2. PFGE

Whole chromosome pulse field gel electrophoresis was performed on 1% (w/v) agarose gels using a 0.5x TBE buffer for 70 hrs at 14°C, utilising the LKB Bromma 2015 Pulsaphor Plus system (switch time 60-500 sec; 98V). All blots were hybridised against a probe recognising the *tgdhfr-ts* cassette. The *tgdhfr-ts* probe fragment was obtained by *Hind III* and *EcoRV* double restriction digest of the pBS-*TgDHFR-TS* vector to release the cassette.

2.7.2.3. Southern blot analysis of *Δpb1289*, *Δpb 508*, *Δpb 1180*

For Southern blot analysis of the dilution cloned transgenic lines, genomic DNA was digested over-night with *EcoRI* (*Δpb508*) or *EcoRV* (*Δpb1289*, *Δpb1180*) (New England Biolabs). The insertion of the disruption-cassette results in a 3-4 Kb size-increase of the disrupted *Δpb508*, *Δpb1289*, *Δpb1180* locus compared to the respective WT locus. The blot was hybridized against a

PCR-generated probe recognizing the 5' prime upstream region of homology for all *Pb508*, *Pb1180* and *Pb1289*. Gel electrophoresis of digested DNA was performed for approximately 6-8 hours (80V) on large 0.8% agarose gels prepared with Tris-Acetate (TAE) buffer (Sambrook, 2001).

2.7.2.4. Radioactive detection of target loci

Gels were incubated with E1510 Ethidium bromide (Sigma), (50 µL of a 10 mg/mL stock per 1 L of 0.5x TBE or TAE) on a rocker at room temperature for 30 min and subsequently imaged and photographed next to a ruler for size reference. Stained gels were depurinated for 15 min in 0.25M HCl and subsequently denatured in 0.5 M NaOH and 1.5 M NaCl for 25 min. The DNA was transferred to HybondN+ membrane (Amersham Biosciences) by standard Southern blotting overnight (Sambrook, 2001). The following day the membrane was quickly rinsed in 3X sodium chloride sodium citrate (20x SSC stock prepared with 17.53% (w/v) NaCl, 8.82% (w/v) sodium citrate, pH 7) prior to cross-linkage in a CL-1000 Ultraviolet Crosslinker (UVP; 100 µJ/cm² for 1,500 sec). Probe fragments were labelled with dATP (P32-α), (Amersham Biosciences) at 37°C for 30 min utilising the High Prime DNA Labelling Kit (Roche). Labelled probes were purified using the ProbeQuant G-50 Micro columns (Amersham Biosciences). All procedures were carried out in a designated radioactivity work area and performed according to manufacturer's instructions.

Membrane pre-hybridisation was conducted at 55°C for 30 min in 50 mL of hybridisation buffer (0.35 M Na₂HPO₄ pH7, 4% (w/v) sodium dodecyl sulfate (SDS), 1% (w/v) bovine serum albumin). The probe was denatured at 100°C for 5 min and cooled on ice prior to addition of the probe to the pre-warmed hybridisation buffer and membrane. Hybridisation was allowed to proceed over-night at 55°C in a rotating hybridisation oven. The membrane was washed under increased stringency in 100 mL 3 XSSC with 0.1% (w/v) SDS followed by a second wash in 100 mL 2 X SSC 0.1% (w/v) SDS. Washes were conducted at 55°C in a rotating hybridisation oven for 25 min each. The membrane was then wrapped in Saran wrap (SaranBrands, Dow Chemical Corporation) and radioactivity was monitored using a Geiger Scintillation Counter. If high activity was detected across the membrane, a third wash in the high stringency buffer was conducted. The membrane was exposed onto phosphor imaging screens (>2 hours). Exposed screens were developed

using a FujiFilm FLA-5000 phosphor imager analyzer. The positions of the bands were visualised using the Aida352 software.

2.8. PHENOTYPIC ANALYSIS

2.8.1. Exflagellation assays

Exflagellation assays were performed by mixing equal parts of parasite infected tail blood with ookinete culture medium. Following 10 min incubation at room temperature, exflagellation was observed at 40X magnification in a standard haemocytometer (Hausser Scientific, USA). The number of exflagellation centres and total number of RBC were counted (proportion of exflagellating male gametocytes) and were directly compared to the total male gametocytaemia (total proportion of male gametocytes) as determined by Giemsa stained tail-blood smears, processed in parallel.

2.8.2. Ookinete imaging

In vitro cultured ookinetes were mounted on a microscope slide and covered by a Vaseline-sealed cover slip prior to immediate imaging.

2.8.3. Ookinete conversion assays

Macrogamete to ookinete conversion assays were performed in on a haemocytometer in ookinete medium, utilizing a Cy3 conjugated P28 (13.1) monoclonal antibody (kind gift by O. Billker) as previously described (Billker et al., 2004). Briefly, the ookinete culture samples (0.5 mL) were harvested at 500g for 5 min, the resulting pellets were resuspended in 0.5 mL Cy3 13.1 diluted to 1:500 in ookinete medium and incubated on ice for 10 min. The conversion ratio was calculated from the total number of Cy3 positive ookinetes (crescent-shaped) divided by the total number of Cy3 positive macrogametes (spherical).

2.8.4. Ookinete invasion assays

Invaded ookinetes were visualised by their melanotic encapsulation in midguts of *A.gambiae* dsCTL4 KD mosquitoes, as previously described (Bushell et al., 2009) and with gene silencing carried out as outlined above. Enumeration was carried out at day7 post-infection by light microscopy.

2.8.5. Ookinete motility assays

Ookinete cultures were added to an equal volume of Matrigel™ (BD) on ice, mixed thoroughly, dropped onto a slide, covered with a Vaseline-rimmed cover slip, and sealed with nail varnish. The Matrigel™ was then allowed to set at room temperature for at least 30 minutes. After identifying a field containing ookinetes, time-lapse videos (1 frame every 5 seconds, for 10 minutes) were taken of ookinetes using the differential interference contrast settings with a 63x objective lens on a Leica DMR fluorescence microscope and a Zeiss Axiocam HRc camera controlled by the Axiovision (Zeiss) software package. Speed of motility of individual ookinetes was measured by multiplying the number of body lengths moved by the length of the ookinete during the 10 minute video, divided by 10. Multiple independent slides and cultures were used for each parasite line. Video processing and annotations was carried out using the Axiovision or Axiovision LE (Zeiss) software.

2.8.6. Mosquito midgut sample preparations for enumeration and imaging

Following mosquito infection and dissection as described above, midguts were fixed in 4% formaldehyde (v/v) (16% methanol free, ultra pure stock diluted in PBS, Polysciences Inc.) for 20 min at room temperature and washed three times for ten minutes each in PBS, prior to mounting in Vectashield® (VectorLabs) on glass slides under sealed cover-slips. Slides were stored in the dark at 4°C until processing.

2.8.7. Oocyst imaging and enumeration

P. berghei infected midguts were obtained from *A. stephensi* or *A. Gambiae* mosquitoes at 3, 10 or 15 days post-infection. Oocysts were enumerated at X10 (fluorescence microscopy; Pbc507 GFP

reference lines) or X40 (light microscopy; Pb2.34 WT lines). Imaging was performed at X10 and X63 magnification using light and fluorescence microscopy. Counting was conducted with the aim of using a minimum of 50 infected midguts per parasite genotype.

2.8.8. Sporozoite enumeration

Midgut and salivary gland sporozoite numbers were calculated from homogenates of *P. berghei* infected *A. stephensi* midguts or salivary glands, assayed in three batches of ten midguts / salivary glands on day 21 post-infection. Dissected salivary glands or midguts were immediately transferred to a 0.1mL tissue homogenizer (Jencons England, VWR) using a Hamilton syringe (Hamilton Company Ltd.), mechanically homogenized and loaded onto a haemocytometer. The final sample volume was carefully measured using a Hamilton syringe, allowing for calculation of the total number of sporozoites per 10 mosquitoes.

2.8.9. Transmission to mice

Sporozoite infectivity was assayed by bite-back experiments, where *P. berghei* infected mosquitoes (day 18 and 21 post-infection) were allowed to feed on anaesthetised C57BL/6 mice for 15 min. The mice were then allowed to recover and parasitaemia was monitored on day 5, 7, 10 and 14 following the recipient of potentially infective sporozoites. Infected mice were immediately culled upon detection of blood-stage parasites on Giemsa stained tail blood smears, or if remaining uninfected, at the endpoint of the experiment (day 14). Directly after the bite-back, the number of successfully fed mosquitoes was counted.

2.8.10. Genetic crosses

Genetic crosses between different transgenic KO parasite strains were carried out by infected mice with different combinations of KO parasites. *A. stephensi* mosquitoes were subsequently infected by membrane feeding on ookinetes cultivated *in vitro* using parasites from the co-infected mice (Raine et al., 2007). The $\Delta pbs47$ and $\Delta p48/45$ lines were kindly provided by O.Bilker, previously gifted from A. Waters and C. Janse

2.9. IMMUNODETECTION METHODOLOGY

2.9.1. Western blot analysis

All purified parasite or infected midgut samples were boiled for 5 min in SDS sample loading buffer. Protein fractionation was performed under reducing conditions on 8% *Sodium Dodecyl Sulfate (SDS) PolyAcrylamide Gel Electrophoresis (PAGE)* gels, according to standard methods (Sambrook, 2001) using the Bio-Rad Mini-PROTEAN II Electrophoresis Cell system, (Bio-Rad Laboratories Ltd.). The gel, Hybond-P polyvinylidene difluoride (PVDF) membrane (Amersham, GE Healthcare) and Whatman filter papers (Whatman) were briefly soaked in transfer buffer (80% 25 mM Tris base, 150 mM glycine (v/v), 20% (v/v) methanol) prior to semi-dry transfer at constant voltage (15 V) for 30 min (Trans-Blot SD Semi-Dry Transfer Cell, Bio-Rad Laboratories Ltd.). Following transfer, membranes were washed briefly in PBS-Tween (0.1%), (v/v), (Fluka) prior to blocking in 5% (w/v) Marvel dried skimmed milk powder (Premier International Foods Ltd UK) in PBSTween (0.1%) for 1 hour in room temperature.

All secondary and primary antibodies were diluted in 5% Milk PBS-Tween (0.1%). Primary antibodies were incubated on a rocker at 4°C over-night. Secondary antibodies were incubated for 1 hour on a rocker at room temperature. Membranes were washed with PBS-Tween (0.1%) three times for 10 minutes each, and following secondary antibody incubation. Blots were developed by enhanced chemiluminescence (ECL) using the ECL Plus Western Blotting Detection Reagents (Amersham, GE Healthcare).

2.9.2. Immuno-fluorescence assays (IFA)

Purified and pelleted ookinetes were re-suspended in RPMI: FCS (1:1) smeared onto glass slides (2 µL /slide) and rapidly air-dried prior to fixation in 4% formaldehyde (PBS) for 10 min at room temperature. Samples were washed once with TBS and permeabilised with 0.2% (v/v) Triton X-100 in PBS for 5 min at RT. Regions of interest were circled with a PapPen (liquid blocker, Daido Sangyo Co. Ltd., <http://www.ogcorp.co.jp/en/group/daido.html>). Unless otherwise indicated, all following steps were carried out at RT, and all washes were 5 min. Slides were washed three times in TBS, quenched in 0.1% (w/v) sodium borohydride in TBS for 5 min, rinsed in TBS and then blocked

for 45-60 min in blocking buffer [10% (v/v) goat serum (Jackson Immuno Research), 1% (w/v) BSA, 0.02% (w/v) NaN₃ in PBS]. After one wash in TBS slides were incubated overnight at 4°C in a wet chamber with primary antibody, in dilutions as described below. The next day, slides were washed 3 times in TBS, incubated with Alexa Fluor Abs as below in 1% (w/v) BSA in TBS; [Molecular Probes, Invitrogen] for 45 min in the dark in a wet chamber, and subsequently washed three times in TBS.

2.9.3. Immunoprecipitation

For each immunoprecipitation, ookinete medium deriving from equal number of ookinetes for each set, was collected and diluted into a total volume of 50ml of ookinete medium. Tubes were mixed overnight at 4°C, after Pb1289IgG35 antibody was crosslinked to protein A sepharose. The sepharose beads were then pelleted by brief centrifugation after which the supernatant was removed. The beads were washed with 1mL of lysis buffer (PBS with 1% Triton X-100). After the wash they were resuspended in 1mL of lysis buffer and transferred to a fresh tube. After one final wash, the beads were extracted with 10µL of 5x NR sample buffer for 5 min at room temperature followed by the addition of 40µL of lysis buffer. The samples were heated to 100°C for 5 and then analyzed by western blot.

2.9.4. Antibodies

Purified, polyclonal anti-Pb1289 IgG (IgG35 and IgG36), against Pb1289 peptide targets EP080783 (Pb1289-35) and EP080784 (Pb1289-36) was obtained from the pooled sera of two immunised rabbits and supplied by Eurogentec. P28 was detected with the 13.1-Cy3 Mab (1:500) (Winger et al., 1988). For anti- α -TUBULIN I (TUB) detection, a mouse monoclonal antibody against *Trypanosoma brucei* alpha-tubulin (tat1), was used at 1:1000 for immunofluorescence and at 1:10000 for western blot analysis. The secondary antibodies used for IFA were ALEXA FLUOR 488 goat anti-rabbit IgG, ALEXA FLUOR 647 goat anti-mouse or ALEXA FLUOR 647 goat anti-rabbit (all at 1:1500, Molecular probes). For western blot analysis, horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (1:15000) or goat anti-mouse IgG (1:10000) (Promega) was used.

2.10. MICROSCOPY

Cells or tissue were mounted in VECTASHIELD Mounting Medium with or without DAPI (Vector Labs).

2.10.1. Light and fluorescence microscopy

Parasites were imaged using a Leica DMT fluorescence microscope and images captured using a Zeiss AxioCam HRc camera coupled to Zeiss Axiovision40 version 4.6.1.0 software. Post-processing of images was performed using ImageJ x.38.

2.10.2. Confocal microscopy

For IFA, visualization was achieved on a Leica SP5 confocal microscope. Images were background-corrected and noise-filtered with the Leica LAS AF software (Leica Microsystems). Additional image adjustments were performed with the Adobe Photoshop CS2 (Adobe) software package.

2.11. STATISTICAL ANALYSIS

For statistical analysis numbers of oocysts were logtransformed [$\log_{10} (n+1)$] so that their distribution resembles a normal distribution and arithmetic mean and standard error of log-transformed data was calculated. 95% confidence intervals were defined by adding and subtracting respectively the amount of 1.96 x standard error of log-transformed data to the mean. Data in tables are presented as initial arithmetic and backtransformed geometric means of log-transformed data, while median value of number of oocysts is depicted in graphs. P values and F values were calculated using One-way Anova (GenStat program, VSN International, UK). Statistical analysis of ookinete counts (conversion ratio and motility assays) was performed using a two-tailed, unpaired Student T-test of equal variances, graphs representing arithmetic mean \pm standard error of the mean.

3. RESULTS

3.1. Selection and Transcriptional analysis of candidate genes

3.1.1. Introduction

Since the publication of both the *Plasmodium* (Gardner et al., 2002) and *Anopheles* (Holt et al., 2002) genome sequences, a various number of parasite transcriptional (Abraham et al., 2004; Ben Mamoun et al., 2001; Bozdech et al., 2003; Bozdech et al., 2003; Daily et al., 2005; Dessens et al., 2000; Hall et al., 2005; Le Roch et al., 2003; Matuschewski et al., 2002; Sacci et al., 2005; Silvestrini et al., 2005; Srinivasan et al., 2004; Vontas et al., 2005) and proteome studies (Florens et al., 2002; Hall et al., 2005; Khan et al., 2005; Lal et al., 2009; Lasonder et al., 2008; Le Roch et al., 2004; Sam-Yellowe et al., 2004) have followed, paving the way to the development of a vast field of functional genomic research. These studies have compared gene expression between different parasite life cycle stages and have demonstrated that the timing of expression for the majority of genes correlates with a known physiological demand during their developmental cycle. (Bozdech et al., 2003; Le Roch et al., 2003). Various gene expression patterns as illustrated by their later functional proteomic analysis have been identified involving, a) house-keeping i.e. expressed in all stages; b) host/vector-interactions i.e. expressed in either the vertebrate host or mosquito host c) specific stage specific action, i.e. related to invasion (e.g. invasive organellar and motor proteins), replication or sexual development (DNA replication machinery in replicative stages), antigenic variation etc. Notably, functionally related genes seem to share common expression profiles, and transcriptional regulation appears to play a significant role in these processes.

Establishment of malarial infection requires successful completion of ookinete development and invasion of mosquito midgut. Importantly the ookinete-to-oocyst developmental transition represents a major bottleneck in the parasite's life cycle since the parasite loses 90% of its initial population at this stage. Characteristically, it is accompanied by a large scale transcriptional switch in clusters of genes, which mirrors the ookinete's proteome requirements pre and post midgut invasion. So far, few transcriptome and proteome projects have specifically focused on the ookinete stage of development. Transcriptional studies include data deriving from Suppressive Subtractive Hybridisation (SSH) of *in*

vitro cultivated parasites (Dessens et al., 2000; Raibaud et al., 2006), *in vivo* Subtractive Hybridisation (SH) of *Plasmodium* ookinetes (Abraham et al., 2004), as well as microarray studies focusing on ookinete invasion, parasite-mosquito interaction and early oocyst development (Vontas et al., 2005; Xu et al., 2005). This information has been complemented with proteome based surveys (Hall et al., 2005; Lal et al., 2009) identifying gene products putatively involved in these processes. Nevertheless, little is known as to the precise mechanisms that underpin ookinete development and its interactions with the mosquito host. Recently, an *in vivo* transcriptional study (Vlachou group – unpublished) has revealed clusters of genes that are differentially regulated throughout parasite’s development in the vector. Utilization of these transcriptional patterns can successfully facilitate the discovery of key elements in parasite development and its interaction with the *Anopheles* vector. The work presented in this chapter represents an effort to broaden our understanding of the ookinete-to-oocyst developmental transition by identifying and characterizing genes putatively involved in ookinete development and parasite-mosquito interactions.

3.1.2. Selection of 113 *Plasmodium berghei* genes based on their developmental expression profile in the mosquito host

This thesis was based on a transcriptional survey of *P. berghei* sexual and sporogonic development in *A. gambiae* that was recently carried out in the host laboratory using a near full genome oligonucleotide microarray. The study was performed on samples collected *in vivo* at distinctive, stage-specific time points corresponding to: mixed asexual and sexual stages (1-3 hours post-infection (PI)), ookinete midgut invasion (22-26 hours PI), early oocyst (48h PI), mid-oocyst (5d PI), mature oocyst (10 d PI) and sporozoite release into the haemocoel (13-15 days PI). The microarray analysis identified 810 *P. berghei* genes that were differentially regulated during the parasite developmental migration through *Anopheles*. Cluster of genes that were differentially regulated during the ookinete-to-oocyst transition were of particular interest.

Special focus was given to genes specifically up regulated at the ookinete and/or early oocyst developmental stage, showing stronger expression in those stages in comparison to all others. Gene clusters showing specific up regulation 24 hours and/or 5 days post infection (PI) were prioritised. Genes with a similar expression pattern to that of already characterised genes that play a role in parasite development in the mosquito host, at the ookinete to oocyst developmental transition such as *Pbs21* and *Pbs25* were also selected (Dessens, et al., 1999; Yuda, et al., 1999). In total, 113 genes were selected, manually re-annotated and subjected to certain bioinformatic filters described below to optimize gene selection.

Firstly, since proteins involved in the interactions of the parasite with its environment are likely to be secreted or localised to the surface, we used a bioinformatic filter to retain and thus, prioritize genes whose products were predicted to be secreted (presence of signal peptide;SP) or membrane-bound (presence of transmembrane domains;TM) or that had GPI (glycosylphosphatidylinositol) anchor motifs.

Secondly, protein domains commonly implicated in parasite/host interaction e.g. adhesion-related domains like EGF-like domains, thrombospondin-like domains, scavenger receptor domains etc, were also sought for (Appendix Table 1). Genes that appeared to hold functional domains that made an “interaction role” unlikely i.e. heat shock related, oxidative stress, ribosomal, DNA related were excluded. Genes without any predicted domains or other features were also included in further

analysis. This was chosen because (a) the *Plasmodium* genome is not fully annotated, therefore domains/features that could be of interest currently remain hidden; (b) these genes exhibited an expression profile of potential interest; and c) there is always the possibility that the respective proteins might contain domains or carry out functions unique to *Plasmodium*, thus, unidentifiable with current methods. Notably, due to incomplete *P. berghei* gene models in several cases, genes were also considered if their orthologues were predicted to meet our criteria. Selected genes, sequence updates of which, revealed new domains of no interest were excluded in the course of time (see ‡ in Appendix Table 1).

Thirdly, since our work was based on the model system *P. berghei*, genes showing clear orthology to genes of human malaria parasites primarily *P. falciparum* (and/or *P. vivax*) were prioritised (Appendix Table 1). Candidates with no annotated ortholog in the above species were not considered further.

Finally, even though our selection was primarily based on differential transcriptional profile deriving from microarray data, final selection was also complemented by global literature. This included previous transcriptome and proteome studies in *P. berghei* and *P. falciparum*; hits found in this ookinete-to-ooocyst transition stage selected for respective candidates, while hits found at other parasite developmental stages deprioritized genes (Appendix Table 2). At the same time this approach helped us select against proteins involved in basic house-keeping functions, or exclusively expressed in the asexual blood stages (ABS). This exclusion should reduce the risk of producing a knock out (KO) that is lethal in the blood stages and thus would not allow analysis in the mosquito stages.

In parallel to this, genes were also examined as to how feasible it would be to knock them out by targeted disruption. Gene sequence analysis was carried out, to determine how easy it would be to design disruption vectors. Duplicates (i.e. two genes predicted from the same open reading frame (ORF) and orthologues or partial gene models belonging to the same gene were unified. Genes that showed any kind of overlap with other genes or were closely spaced to neighboring genes were excluded so as to ensure the specificity of our knock-out. Lastly, we excluded genes that have already been or are being characterized by others.

This primary selection, as described above resulted in 33 candidate genes for further study. Figure 3.1 summarizes the selection filters applied in the data set of selected genes.

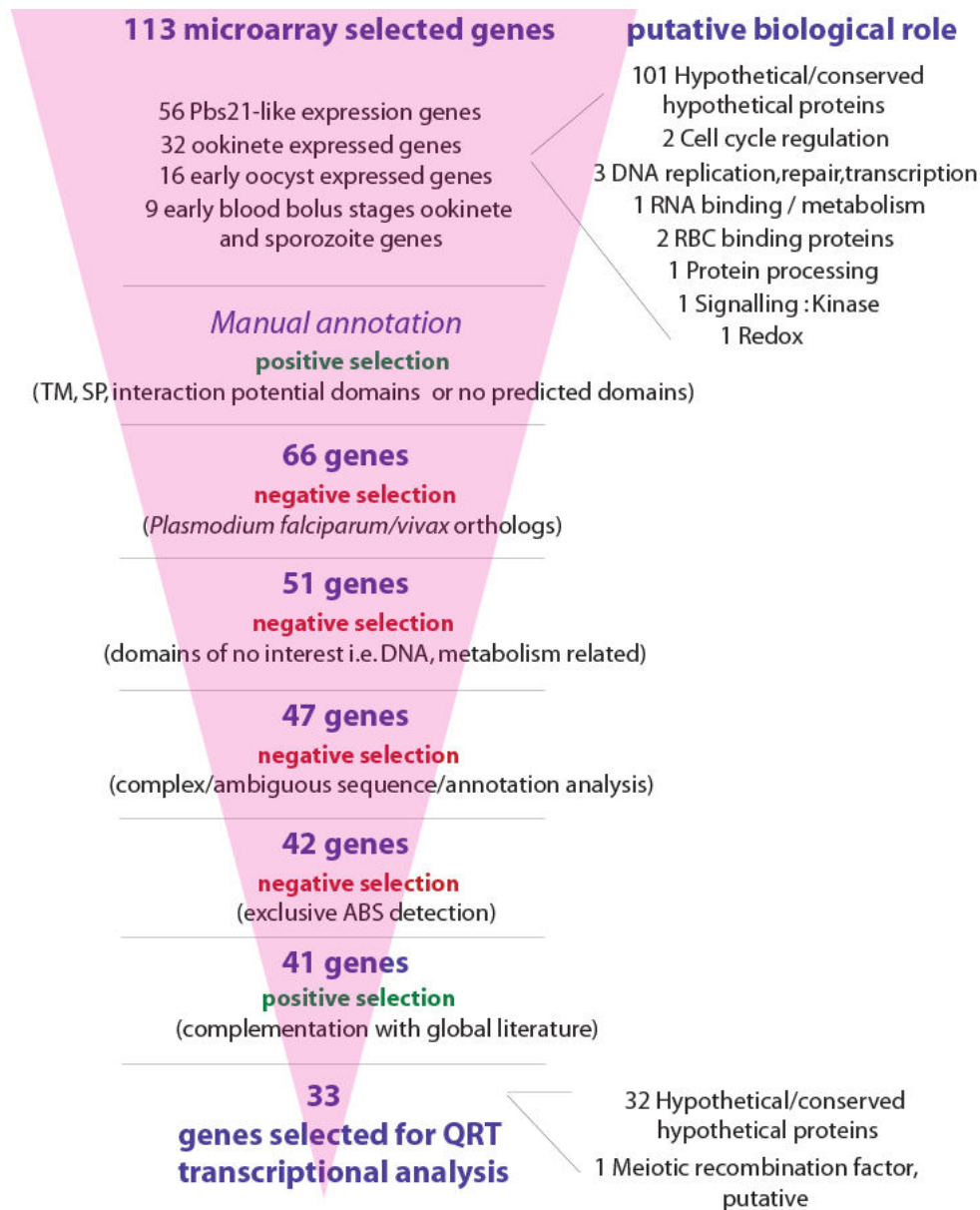


Figure. 3.1. Summary of candidate selection from the microarray transcriptional analysis

The figure outlines the selection screen applied to the *P. berghei* life-cycle microarray transcriptional analysis derived data-set, demonstrating the selection criteria applied and the number of genes passing each filter. The putative biological role of genes, included in this analysis is also indicated on the right of the flow diagram.

3.1.3. *In vivo* developmental transcriptional analysis of 33 selected candidates by QRT PCR

3.1.3.1. Assessment of experimental design and sample quality

Thirty three candidate genes (Table 3.1) were selected for transcriptional analysis by QRT-PCR. This step not only would validate our previous selection upon the microarray derived expressional profile, but also provide new insights in the potential developmental stage they might be implicated.

The study was performed on *in vivo* samples collected at distinct, stage-specific time points corresponding to: mixed asexual and sexual stages (1-3 hours PI), ookinete midgut invasion (22-26 hours PI), early oocyst (48h PI), mid-oocyst (5 d PI), mature oocyst (10 d PI) and sporozoite release into the haemocoel (13-15 days PI). This experimental design is identical to the one used during microarray transcriptional analysis, upon which candidate genes were selected.

Parasite numbers naturally oscillate as the parasite migrates through its mosquito host (Sinden, 2002) while parasite dynamics also varies between infections in a density dependent manner (Sinden et al., 2007) (Figure 1.4). Notably, parasite numbers rapidly decline following uptake of the blood meal by the mosquito. Very low numbers are observed post midgut invasion, reflecting the selective pressure posed by the increased requirements of midgut invasion, the midgut hostile environment and the vector immune response. During oocyst development, when parasite replication and subsequent sporulation takes place, a gradual increase is again observed. As a result, these continuous changes in parasite numbers require normalisation of transcriptional data, in regards to parasite population numbers, and this normalisation must be specific to each sample and each time-point investigated. Thus, we used *eGFP* expression, driven by the *eef1-α* promoter in the transgenic *PbGFP_{con}* parasite line for normalisation purposes (Franke-Fayard, et al., 2004).

Prior to QRT-PCR transcriptional profiling of selected candidates, the RNA samples collected were subjected to quality control, assaying 1) three *P. berghei* genes with known transcriptional profile and 2) GFP expression as an index of parasite transmission dynamics. The control genes used in this study were; *ctrp*, *Pbs21* and *csp* (expression profiles and biological roles described in detail in the Introduction chapter). As previously shown (J. Thompson & Sinden, 1994), *Pbs21*, transcripts

are at their peak levels 1 hour after infection (mixed sexual and asexual blood stages) , remain elevated during ookinete midgut invasion (22-26 hours) and they disappear below the threshold of detection at day five post-infection In contrast, *ctrp* transcripts are abundantly detected at the ookinete stage (24hours), where a sharp peak in transcriptional activity occurs, followed by rapid transcript depletion (Dessens, et al., 1999). For *csp*, transcriptional activity peaks at the last time point considered in this study at day 13-15 day post-infection, which corresponds to the mature oocyst ready to release fully formed sporozoites into the mosquito haemocoel. *eGFP* is constitutively expressed throughout parasite development in the mosquito host, thus, its levels correspond to parasite numbers at the respective developmental stage. Lower, transcript levels are observed during early oocyst development after which a gradual increase is noted (Figure 3.2)

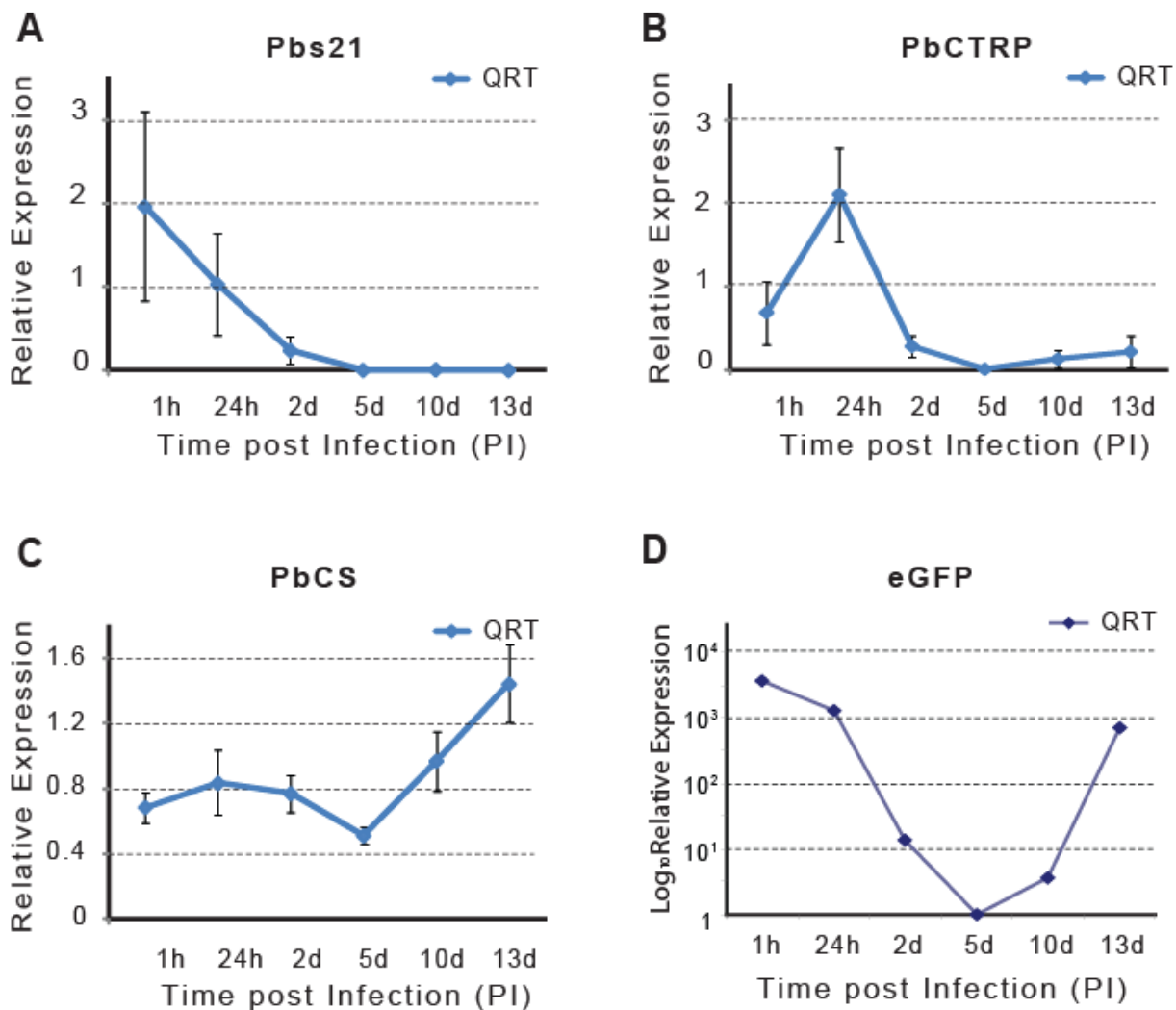


Figure 3.2. QRT-PCR results for *P. berghei* control genes and eGFP expression

The figure displays the results for the QRT-PCR based analysis of transcription levels of *Pbs21* (A), *ctrp* (B), *csp* (C) and *eGFP* (D), as the *P. berghei* infection proceeds in the mosquito *A. gambiae* between 1 hour to 13-15 days post-infection. Transcript levels were measured by QRT-PCR in arbitrary units of the average values derived from five separate biological experiments, in which each time point was run in duplicates, and relative expression presented. Of note in all 5 separate biological replicates, the same pattern consistently to the average presented here, was observed. *eGFP* expression levels were used to normalise QRT-PCR data. Standard error bars indicate standard error of the mean (SEM).

Table 3.1 Selected *Plasmodium* Genes for validation of transcriptional profile

Plasmodium Transcription Programmes in the mosquito (unpublished)	Putative Function	Orthologs				TM & SP					Predicted Domains
		Py	Pc	Pv	Pf	Pb	Py	Pc	Pv	Pf	
<i>Pbs21-like</i>											
PB001039.00.0	conserved hypothetical protein	PY02477	PCAS_061130	PVX_084645	PF14_0360	0	0	0	0	0	Translation initiation factor eIF-2 β
PB103082.00.0/ PB000518.01.0 PB301555.00.0	conserved hypothetical protein	PY00824	PC402123.00.0	PVX_000565	MAL7P1.86	0	0	0	0	0	
PB000893.01.0	hypothetical protein	PY0478	PC000844.02.0	Pv100615	MAL12P1.319	0	0	0	0	0	
PB000551.00.0	Fibrillarin putative	PC000051.00.0	Pv086075	PF14_0068		0	0	0	0	0	Fibrillarin \ddagger
PB000489.03.0	conserved hypothetical protein	PY00558	PC301025.00.0	Pv082885	PF13_0215	0	0	0	0	0	
PB000846.01.0	conserved hypothetical protein	PY05078	PC000582.00.0	Pv114000	MAL6P1.254	TM	0	0	0	0	
PB000874.00.0	conserved hypothetical protein	PY06380	PC001349.02.0	Pv095235	PFC0886w	TM	0	0		0	
PB001229.02.0	meiotic recombination protein	PY05593	PC000403.01.0	Pv089570	MAL8P1.76	TM	TM	TM	TM	TM	DNA-binding
PB001312.02.0	conserved hypothetical protein	PY02610	PC300029.00.0	Pv111025	PF11_0451	TM	0	TM	0	TM	Sec1
	conserved hypothetical protein	PY00792	PC300839.00.0	Pv117795	PF14_0510	TM+SP	TM	0	0	0	CS
<i>Ookinete</i>											
PB000317.00.0	hypothetical protein	PY07130	PC000114.03.0		PF08_0008	TM+SP	SP	TM		TM+SP	
PB000773.02.0	conserved hypothetical protein	PY01743	PC000912.01.0	Pv091195	PF11_0115	0	0	0	0	0	
PB108456.00.0/ PB000759.02.0/ PB000760.02.0/ PB300784.00.0	hypothetical protein	PY02606	PC000151.04.0 PC000265.02.0 PC000279.01.0 PC000442.00.0 PC302392.00.0	Pv093645	MAL8P1.154	TM	TM	0	0	0	
PB300785.00.0	conserved hypothetical protein	PY02798	PC000772.00.0	Pv122365	PF13_058	0	0	0	0	0	RNA recognition motif \ddagger
PB000254.00.0	conserved hypothetical protein	PY02271	PCAS_031550	PVX_00270	PFB0765w	0	0	0	0	0	AAA-filament
PB000508.03.0	conserved hypothetical protein		PC000587.00.0	Pv093500	PF08_0119	0	0	0	0	0	
PB000808.02.0	conserved hypothetical protein	PY02678	PC301893.00.0	Pv122785	MAL13P1.103	TM	0	0	0	0	
PB001180.00.0	conserved hypothetical protein	PY05386	PC000070.04.0	Pv084290	PFLO260c	0	0	0	0	0	
PB001289	conserved hypothetical protein	PY04330	PC001277.02.0	Pv097925	PFE0360c	SP	SP	SP	SP	SP	
PB001343.02.0	conserved hypothetical protein	PY03245	PC000012.05.0	Pv114125	PFF0975c	TM+SP	TM	TM	TM	TM+SP	
PB001363.02.0	conserved hypothetical protein	PY04768		Pv081495	PFA0270c	0	0	0	0	0	
<i>Oocyst</i>											
PB000111.00.0/ PB000180.00.0	conserved hypothetical protein	PY00092	PCAS_021040	PVX_081700	PF07_0116	TM	TM	TM	TM	TM	
PB000679.03.0	conserved hypothetical protein	PY06608	PCAS_102610	PVX_08563	PF14_0162	0	0	0	0	0	
PB000692.01.0/ PB000112.00.0	conserved hypothetical protein	PY04558	PCAS_135850	PVX_082940		0	0	0	0	0	
PB000882.03.0	conserved hypothetical protein	PY01913	PCAS_131030	PVX_11833	PF14_0410	0	0	0	0	0	
PB000918.03.0/ PB3007005.00.0/ PB108513.00.0	conserved hypothetical protein	PY00591	PCAS_136310	PVX_083180	MAL13P1.228	0	0	0	0	0	
PB000467.02.0	conserved hypothetical protein	PY02327	PC000158.00.0	Pv114375	PFF1230c	SP	SP	SP	SP	SP	
PB000712.03.0	conserved hypothetical protein	PY04401	PC000473.04.0 PC001173.02.0	Pv091480	PF11_0176	TM	TM	TM	TM	TM	
PB001632.02.0	cell differentiation protein rcd1	PY05128	PC000437.04.0	Pv097940	PFE0375w	0	0	0	0	0	Rcd1
PB301371.00.0	conserved hypothetical protein	PY01290	PC000355.00.0 PC000104.02.0	Pv081690	PF07_114	0	0	0	0	0	Prenyltransferase \ddagger
PB000747.03.0	conserved hypothetical protein	PY07199	PC000935.01.0	Pv094585	PF10_0076	TM	TM	TM	TM	0	
<i>Early blood biolus stages, ookinete+Sporozoite</i>											
PB000251.01.0	conserved hypothetical protein	PY00455	PCAS_100610	PVX_00081	PFD0425w	SP	SP	SP	SP	SP	
PB300759.00.0	Hypothetical protein	PY03220	PC000414.04.0	Pv086115	PF14_0060	0	0	0	0	0	GDPD \ddagger

Table 3.1. Selected *Plasmodium* Genes for validation of Transcription profile

Genes are grouped according to their expression profile in *Plasmodium* transcription programmes (Vlachou group, unpublished). Gene ID, putative function, Orthologs, Transmembrane domains (TM), Signal peptides (SP) and Predicted functional domains are shown. In green genes selected for further functional analysis and engineering of knock out constructs. Pb; *Plasmodium berghei*, Pf; *Plasmodium falciparum*, Py; *Plasmodium yoelii*, Pv; *Plasmodium vivax*, Pc; *Plasmodium chabaudi*, †; domains revealed in later annotations of PlasmoDB

3.1.3.2. QRT-PCR Transcriptional profiling of selected candidates

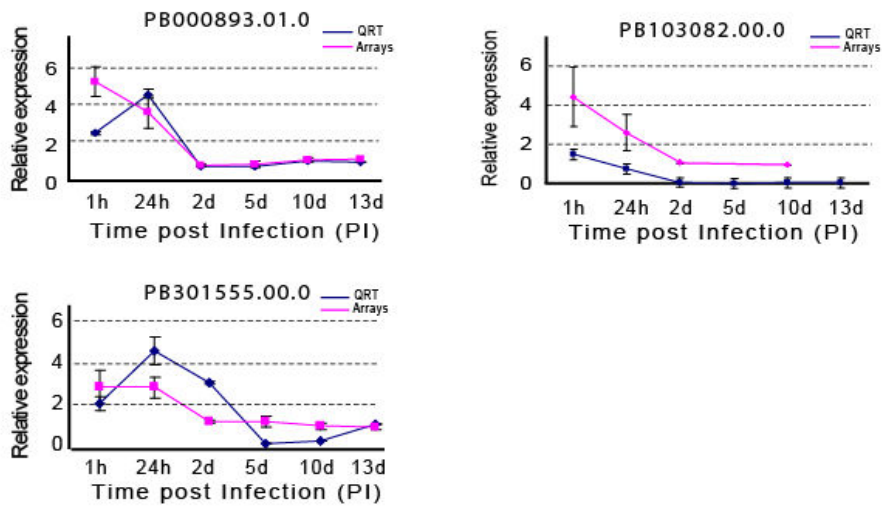
Selected genes were subjected to QRT-PCR transcriptional analysis on *in vivo* samples collected as described above. In line with microarray expression selection criteria, candidate genes were divided based on their transcriptional profile into four categories: A) genes exhibiting a transcription pattern similar to Pbs21 (10 genes) i.e. transcription peaks 1 hour after infection (mixed sexual and asexual blood stages) and remains elevated during ookinete midgut invasion (22-26 hours) (Figures 3.3Ai, 3.3Aii and 3.4A), B) ookinete expressed (11 genes) i.e. a sharp peak in transcriptional activity occurs 24 hours post infection followed by rapid transcript depletion (Figures 3.3B and 3.4B), C) early oocyst expressed genes (10 genes) i.e. transcript levels peak at or show a sudden increase in transcription activity between 24 and 48 hours post infection and (Figures 3.3Ci and Cii) D) genes expressed in blood bolus, ookinete and sporozoite development (2 genes)(Figure 3.3D).

The microarray study utilized two oligonucleotide probes per gene, while only one pair of primers was designed and used in the QRT-PCR study, which were designed independently. The potential bias introduced by the fact that oligonucleotide-primers anneal to different sites was assessed for two genes (PB001039.00.0 and PB000551.00.0) (Figure 3.3Aii). For PB000551.00.0 two different pairs of QRT primers were used, which anneal at the same site as the two different microarray oligonucleotide probes. In the case of PB001039.00.0 the sites of the two microarray probes coincided, thus the second pair of QRT-PCR primers was designed at a different site.

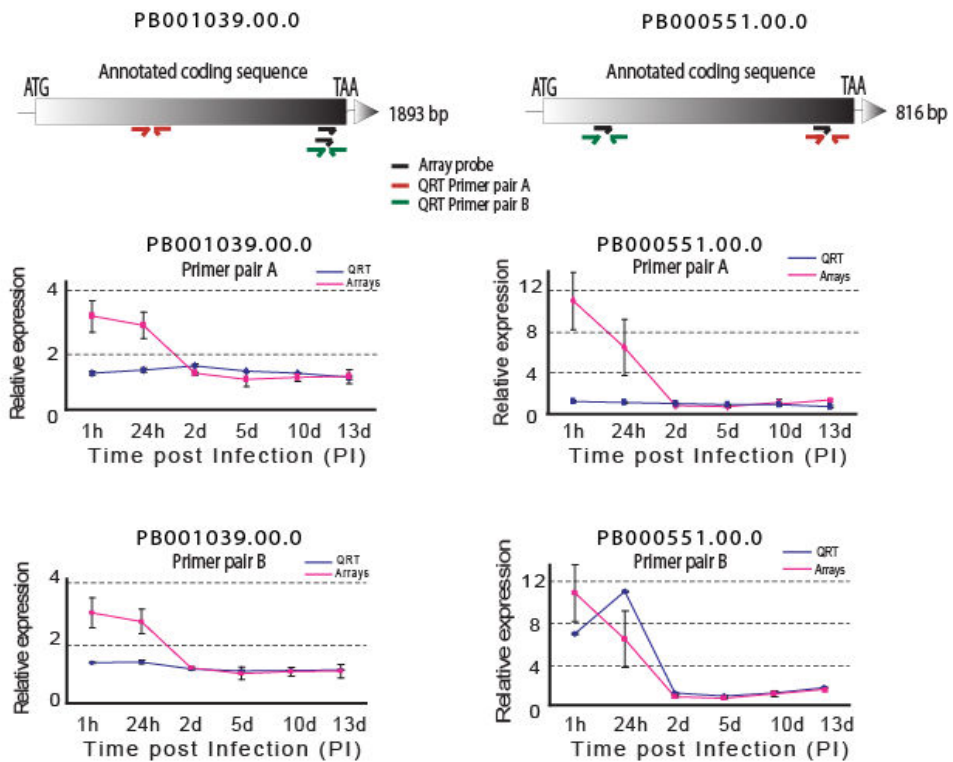
The correlation of microarray and QRT-PCR based transcriptional analysis of candidate genes was assessed, depending on the transcription levels and trends (ascending or descending) of the candidate genes. In total, 33 genes were assessed by QRT-PCR, 19 of which showed good correlation

with the microarray transcriptional data, and were thus selected for downstream analysis. The potential discrepancies between QRT-PCR and microarray transcriptional analysis are discussed in section 4.1.2.

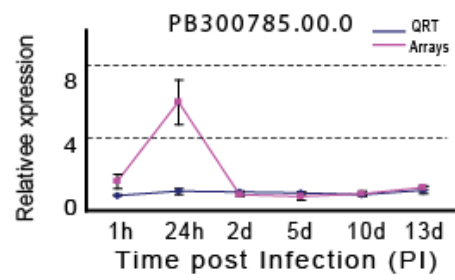
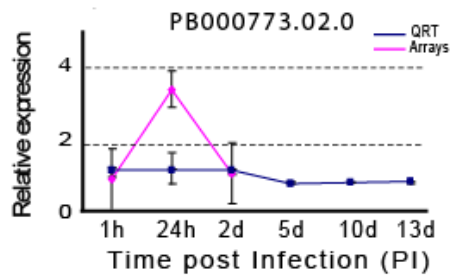
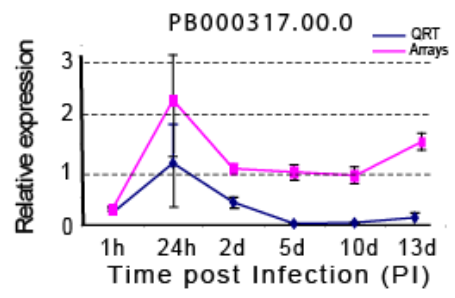
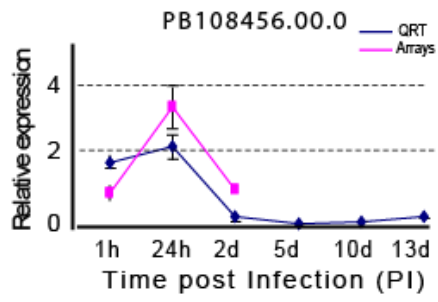
A i. Pbs21-like expression genes



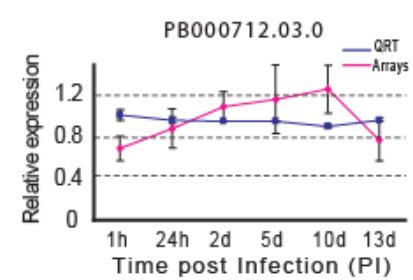
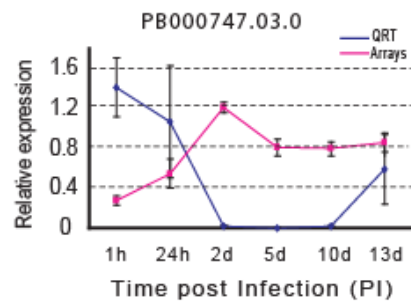
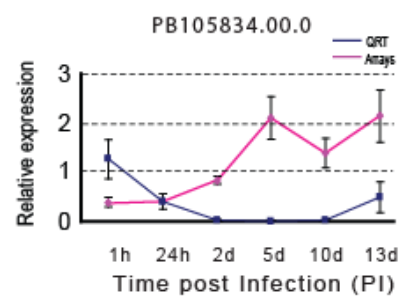
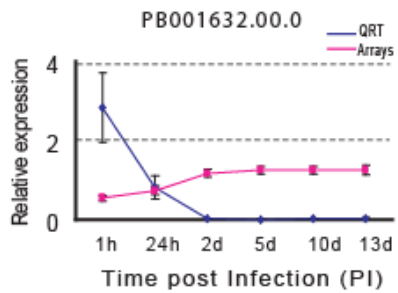
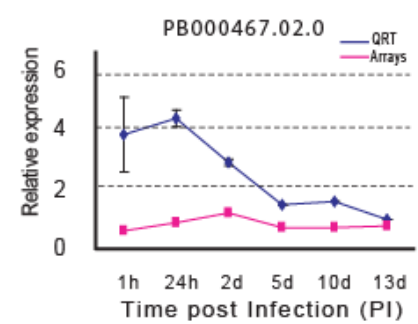
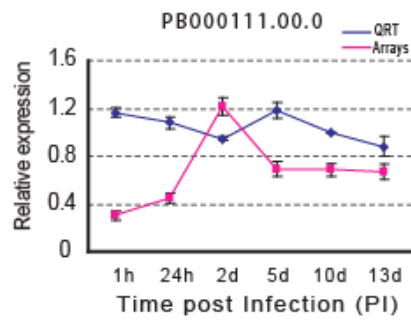
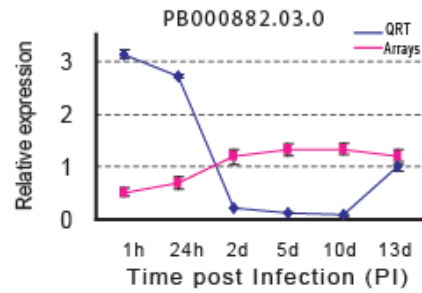
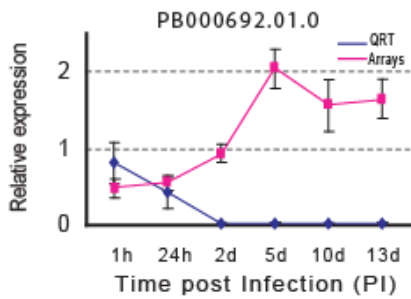
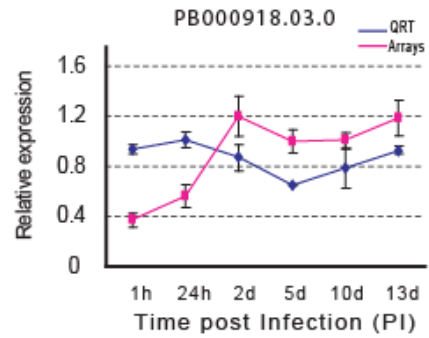
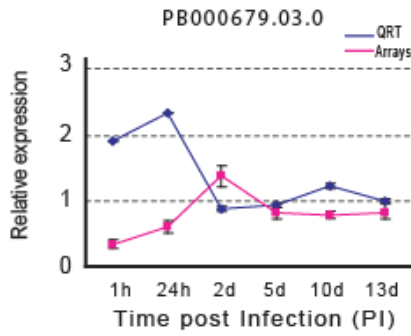
A ii. Pbs21-like expression genes



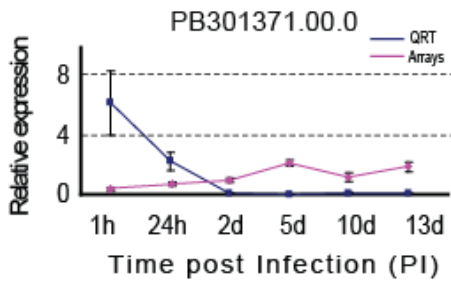
B. Ookinete expressed genes



C i. Early oocyst expressed genes



C.ii Early oocyst expressed genes



D. Early blood bolus, Ookinete and Sporozoite developmental stages

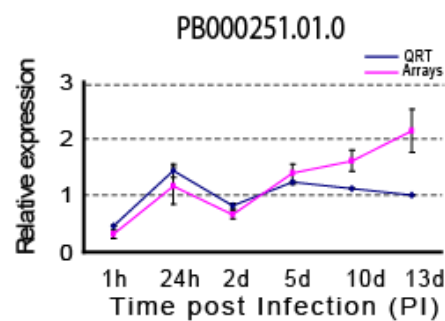
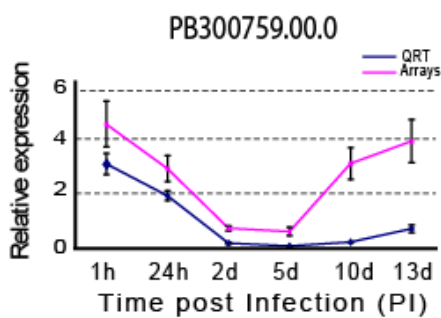


Figure 3.3. *In vivo* transcriptional analysis of candidate genes

Graphs showing QRT-PCR and microarray transcriptional profile of Pbs21-like expression candidate genes (Ai and Aii), ookinete expressed genes (B), early oocyst expressed genes (Ci and Cii), and early blood bolus, ookinete and sporozoite developmental stages expressed genes (D). In pink and blue gene expression according to microarray and QRT analysis are respectively depicted. Horizontal axis represents the respective time post infection, RNA samples were collected, while the vertical axis represents the relative expression normalized against GFP expression, as described above. Values represent the average of three biological replicates. Figure Aii additionally represents differential QRT expression analysis, using two pairs of QRT primers, annealing of which on the respective gene coding sequence is shown on the left panel. In black, red and green, annealing sites of array oligo probes, QRT primer pair A and QRT primer pair B is shown respectively.

3.1.4. Selection of 12 candidate genes for further functional analysis

Following QRT-PCR transcriptional analysis, a second round of selection was carried out. Genes confirming microarray based expression profile (19 genes) were selected against those whose transcriptional pattern conflicted with the QRT derived data (14 genes).

New sequence analysis was performed on these 19 selected genes based on the PlasmoDB 5.4 release.

A selection screen against genes that encoded functional domains which were not within our interest was performed. In particular, PB000893.01.0 and PB300759.00.0 were excluded, since PlasmoDB 5.4 release revealed functional domains pertaining to small nuclear ribonucleoproteins (fibrillar) and glycerol metabolic processes (glycerophosphoryl diester phosphodiesterase) respectively.

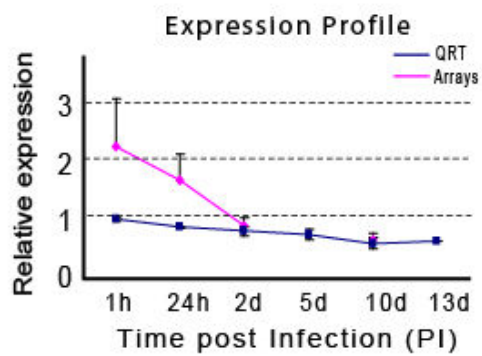
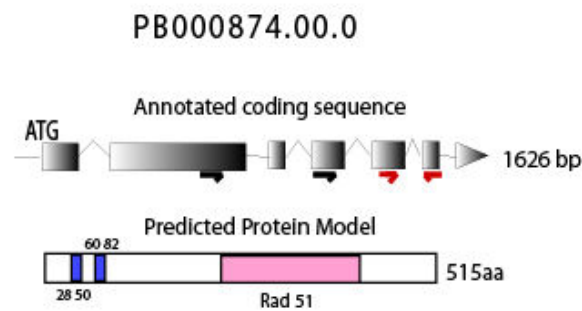
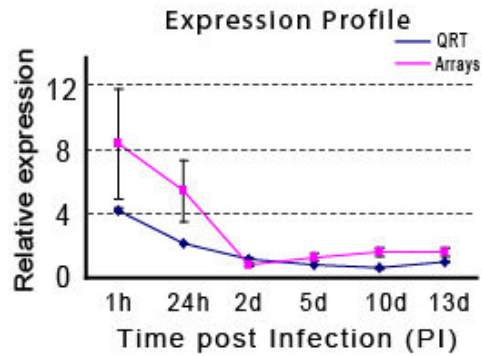
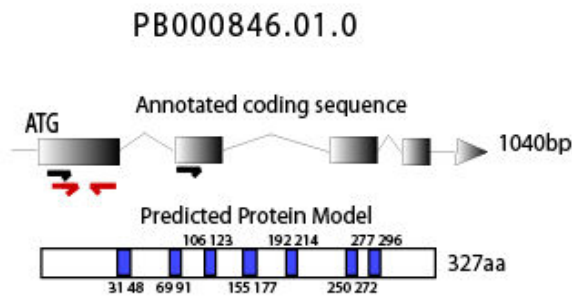
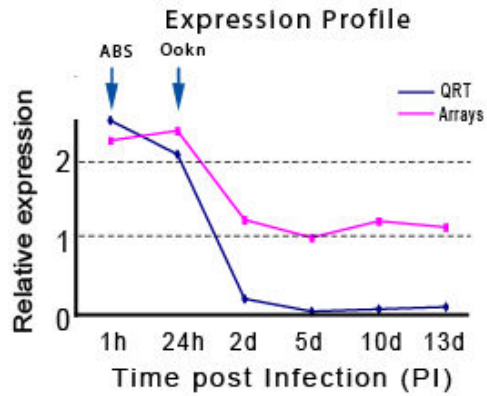
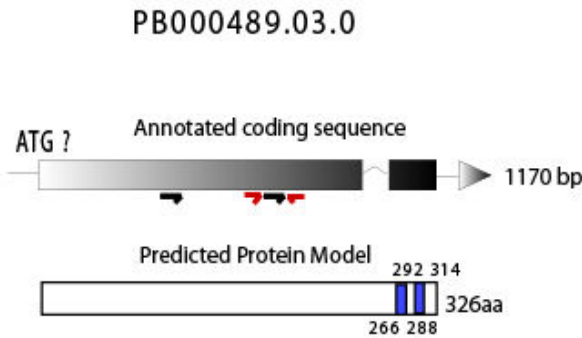
Genes, whose reannotation rendered the design of potential future specific targeting impossible or unsure, were also selected against. Specifically, Pb103082.00.0, Pb301555.00.0 and Pb108456.00.0 were excluded from further study due to their close neighbouring with other genes (Pb103082.00.0 with PB000518.01.0) or doubtful contig sequence (Pb301555.00.0 and Pb108456.00.0).

Lastly, Pb000317.00.0 (sharing an ORF with PB000129.01.0) and Pb000251.01.0 had recently been characterized and were also excluded (Ecker et al., 2008; Lasonder et al., 2008).

As a result, 12 genes were put forward for further functional characterization through gene targeting approach. Figure 3.4, Appendix Tables 1 and 2 and Table 3.1 summarize the transcriptional, proteome and structural characteristics of selected candidates.

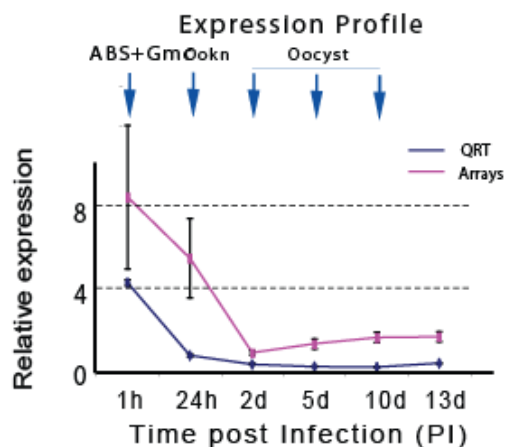
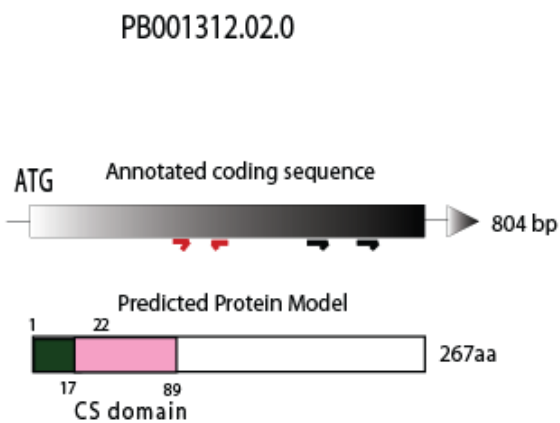
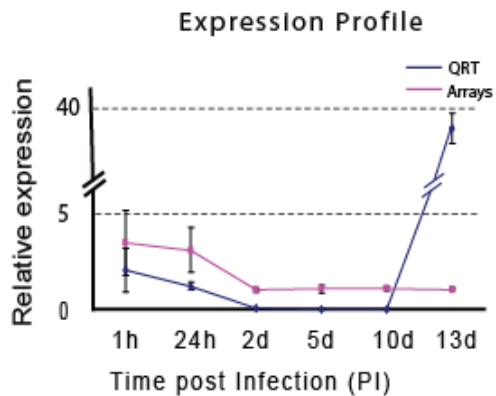
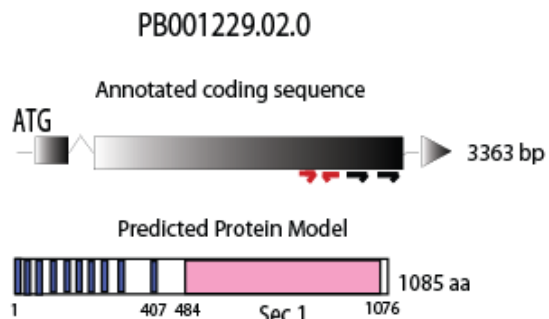
Briefly, our selection included five genes displaying a Pbs21-like expression profile and seven genes specifically and highly expressed during the ookinete developmental stage. Selection included 11 conserved hypothetical proteins and a meiotic recombination factor (PB000874.00.0). Among those, 8 genes (PB000489.03.0, PB000846.01.0, PB001343.02.0, PB000808.02.0, PB001312.02.0, PB000874.00.0, PB001229.02.0 and PB001289) displayed the presence of secretory signals (SP) or transmembrane domains (TM). PB001229.02.0 coded for a Sec I, while PB000874.00.0 for a Rad51 domain. Finally, unknown domains were predicted for PB000508.03.0, PB001180.00.0 and PB001363.02.0. The potential significance of these findings upon the putative role of selected candidates is discussed in section 4.1.4.

A i.Pbs21-like expression selected genes

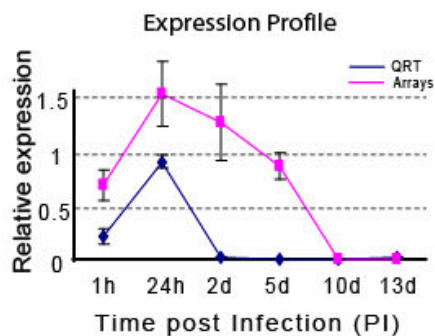
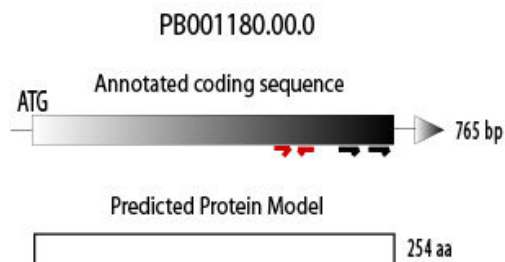
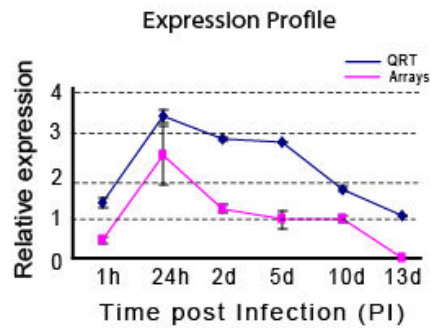
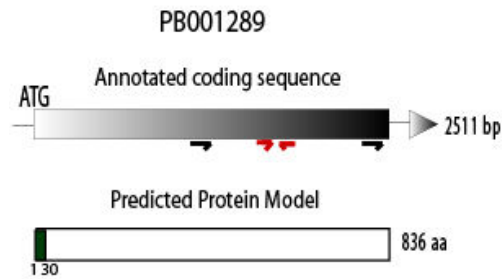
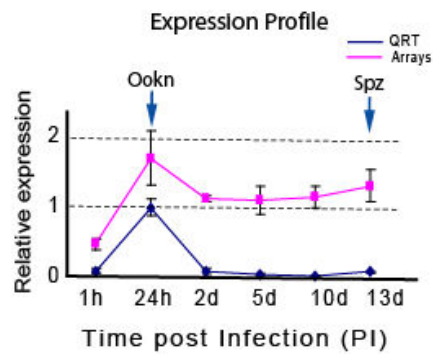
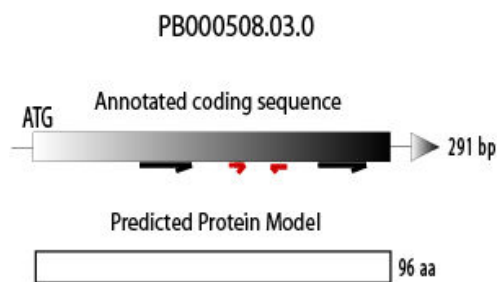
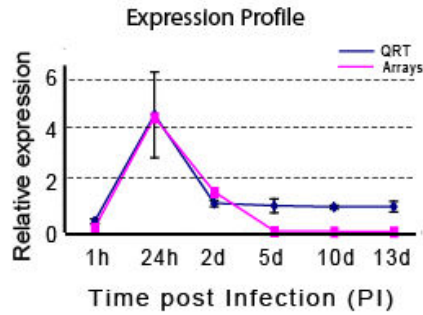
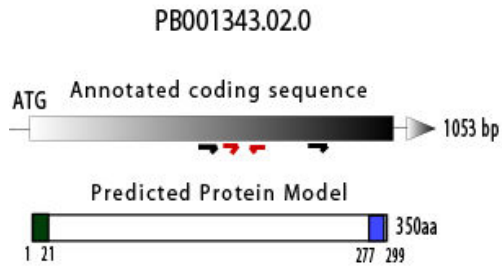


- QRT primer ■ TM ■ signal peptide
- Array probe ■ domains ↓ Protein Expression

A ii. Pbs21-like expression selected genes



Bi.Ookinete expressed selected genes



Bii.Ookinete expressed selected genes

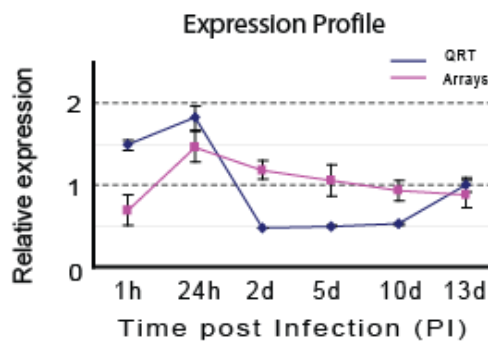
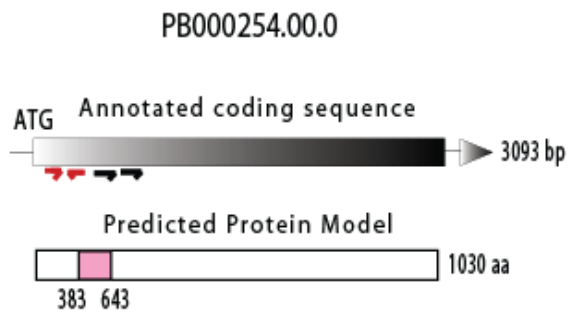
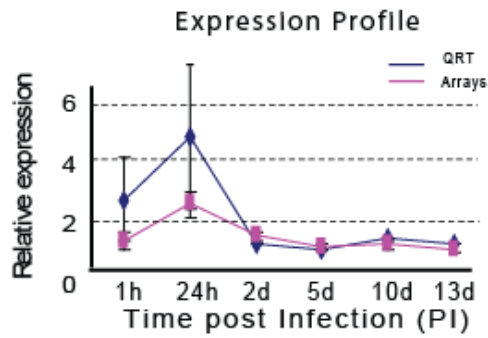
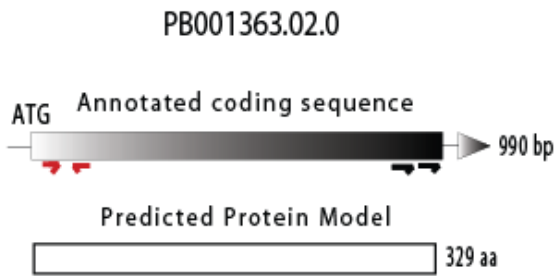
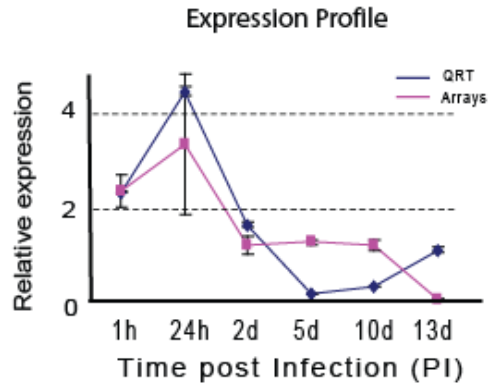
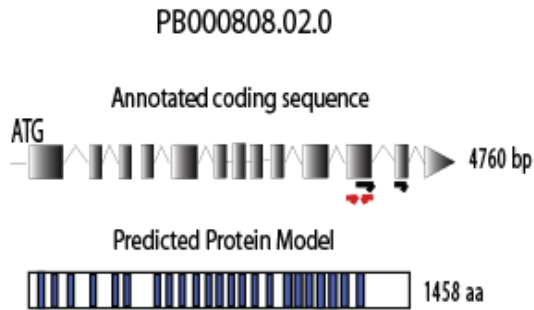


Figure 3.4. Gene model and *in vivo* transcriptional analysis of selected candidate genes

Graphs representing annotated coding sequences and predicted protein models for selected genes (left panel), as well as QRT and microarray transcriptional profile (right panel) of Pbs21 like expression candidate genes (Ai and Aii), and ookinete expressed genes (Bi and Bii). Annotated coding sequence and predicted protein model have been designed for each gene. Transmembrane domains (TM), signal peptides (SP), functional domains, start codon (ATG), size in base pairs (bp) or amino acids (aa), QRT primer and array probe annealing sites have been indicated accordingly (in red and black arrows). Protein expression according to proteome data available (Appendix Table 2) is also presented (in blue vertical arrows indicating ABS; asexual blood stages, Ookn; ookinete; Gm; Gametocytes/Gametes). Graphs represent expression profiling according to microarray data (pink line) and QRT-PCR (blue line). Horizontal axis represents the respective time post infection that RNA samples were collected, while the vertical axis the relative expression normalized against GFP expression, as described above.

3.1.5. *In vitro* developmental transcriptional profiling of 12 selected candidates

The aforementioned *in vivo* transcriptional studies (microarray and QRT), revealed parasite gene expression within the mosquito host, but could not provide firm conclusions regarding the parasite stage-specificity of transcripts present in the mixed parasite population within the mosquito bolus. Presence of transcripts 1-24 hours post infection could reflect both sexual stage transcription as well as asexual stage contamination. In order to elucidate the exact developmental stage that these transcriptional changes occur, we performed *in vitro* transcriptional profiling of the 12 selected candidate genes (Figure 3.5). Enriched purified clusters of parasites of distinct developmental stages, were utilized, including blood stages of the gametocyte non producing ANKA 2.33 parasite strain. In addition to better understanding of candidate parasite gene regulation, these experiments would also allow selection against genes showing exclusive asexual blood stage expression.

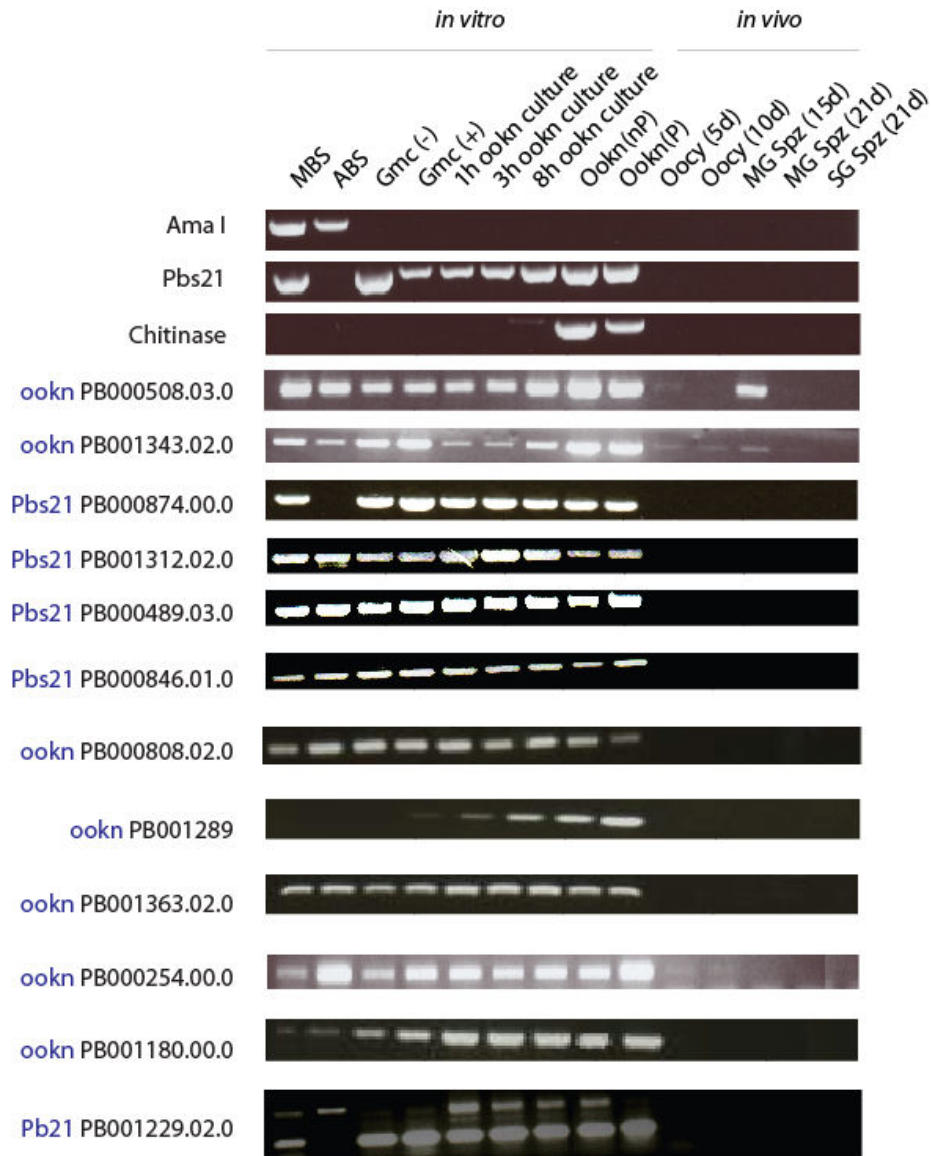


Figure 3.5. *In vitro* developmental transcriptional profiling of 12 selected candidates

RT-PCR-based analysis (32 cycles) of 12 candidate genes in *Pbc507WT* parasite populations. Primers specific for candidate genes detect an approximately 0.5 Kb band in *Pbc507WT* parasite populations (See Table 2.2 for primers). Primers amplifying *Pbs21* (sexual stage-specific transcript), *Ama1* (blood stage-specific transcript) and chitinase (ookinete specific transcript), were utilised as stage-specific and loading controls. The samples analysed were purified (P), non-purified (nP), activated (+) and non-activated (-) asexual blood stages (ABS), mixed blood stages (MBS) gametocytes (Gc) and ookinetes (Ook). Study was complemented with *in vivo* 5 and 10 day (d) oocyst, 15 and 21day midgut sporozoite and day 21 salivary gland sporozoite samples deriving from *A.stephensi* mosquitoes. In blue the transcriptional profile as revealed by QRT-PCR is indicated.

In vitro RT-PCR analysis revealed abundant transcripts for almost all genes throughout their development from asexual blood stages to ookinete formation. Notably PB000508.03.0 also exhibited expression during late sporulation, on day 15 post infection, fully confirming the QRT-PCR and microarray data. In contrast PB001289 transcripts appear to accumulate post fertilisation where they reach their expression peak at the ookinete stage. Interestingly, PB000874.00.0 showed an expression profile very similar that of Pbs21 (absent from asexual stages). Two different sets of transcripts were detected for PB001229.02.0, of which the larger was also found in asexual blood stages. The significance of these findings is discussed in section 4.1.3.

3.1.6. Summary

This chapter has outlined the following:

- **Selection of 113 genes based on their developmental transcriptional profile in the mosquito host, as explored by a previously conducted microarray study**
- **Selected candidates were manually annotated, gone through bioinformatic analysis and their expressional profile was complemented with previous expression data**
- **Following bioinformatic analysis 33 genes were selected for validation of their *in vivo* transcriptional expression by QRT-PCR.**
- **QRT-PCR analysis put forward 12 genes for in-depth transcriptional analysis using enriched parasite stages *in vitro***
- **Finally, 12 genes were finally selected for further functional analysis through targeted gene disruption**

3.2. Generation and basic phenotypic analysis of disruption mutant parasites

3.2.1. Introduction

Despite the availability of functional genomic data that allows for a genome-wide approach to answer questions relating to gene temporal and spatial expression, little is known to date as to the functions of the vast majority of the 5,864 predicted ORFs encoded by the *P. berghei* genome. In fact, the functional characterisation of the *Plasmodium* genome still remains in a very early stage despite its relatively small size. The generation of the first *P. berghei* and *P. falciparum* KO lines, that took place more than a decade ago (Crabb et al., 1997; Dessens et al., 1999; Menard & Janse, 1997; Sultan et al., 1997), set the foundations for reverse genetic studies. In recent years improvements in transfection technologies (Janse et al., 2006) allowed medium throughput gene targeting in both *P. berghei* (Ecker et al., 2008) and *P. falciparum* (Maier et al., 2008). Nonetheless, to date, only around 40 genes that are expressed during the mosquito stages of the *P. berghei* life cycle have been functionally characterised (reviewed and extended from (Ecker et al., 2008)). This can be attributed to a number of issues that were encountered in this study and will be discussed further below.

Gene targeting studies have identified a number of proteins expressed in the ookinete and potentially implicated in parasite-host interactions, involving membrane-bound (e.g. the major ookinete surface proteins *Pbs25* and *Pbs28*) (Tomas et al., 2001) or secreted proteins. The latter include micronemal molecules (e.g. *ctrp* (Dessens, et al., 1999; Yuda, et al., 1999), *CellTOS* (Kariu et al., 2006), chitinase (Dessens et al., 2001) and *soap* (Dessens et al., 2003), as well as, *laps* (Raine et al., 2007) and *pplp* family members (Ecker et al., 2007; Kadota et al., 2004)). However, only a proportionally small number of these disruption mutants hinder malaria transmission, the majority of which being implicated in ookinete molecular motor machinery (e.g. *ctrp*) (Dessens et al., 1999). This observation suggests that significant functional redundancy characterizes the ookinete to oocyst developmental transition. Little is known about molecular pathways involved in the interactions with the mosquito host during ookinete maturation and the ookinete to oocyst developmental transition.

The complex molecular interplay between vector and parasite that determines the outcome of parasite's development within the vector is yet to be understood.

P. berghei gene disruptions are achieved preferentially by double homologous recombination between a linear gene-targeting cassette and the wild type locus. As a result, complete and stable (in contrast to selection of single homologous recombination) removal of the gene of interest from the genomic region is achieved. All gene disruptions in this study were attempted using double homologous recombination, as described below.

3.2.2. Generation of transgenic parasite lines

3.2.2.1. Generation of disruption constructs for 12 selected genes

Regions of 500-1000bp up- and downstream of each gene locus of interest were amplified (Figure 3.6A) and subsequently cloned into a plasmid vector encompassing a genetically modified *T. gondii* dihydrofolate reductase/thymidilate synthase (*TgDHFR/TS*) cassette (Figure 3.6B). The *TgDHFR/TS* gene in its resistant configuration confers resistance to the antimalarial drug pyrimethamine, against which, transgenic parasites are to be eventually selected (Figure 3.6). A total of 15 gene disruption constructs were generated (two for PB001343.02.0, PB000808.02.0 and PB001363.02.0 respectively) (Table 3.2). Of those, disruption constructs against the complete open reading frame (ORF) were designed for PB000508.03.0 and PB000846.01.0 (complete KO). For the rest of the genes, constructs were designed to excise only a part of the respective ORF (partial KO) (Table 3.2). This design of complete and partial KO vectors was determined by sequence analysis of each gene. For genes whose manual annotation ensured that potential gene disruption of the whole ORF would not interfere with the expression of other genes, complete KO vectors were designed. For the rest, regions closely (~200bp) spaced to the 5 or 3'UTR of neighbouring genes were spared and partial KO vectors were designed.

3.2.2.2. Generation of disruption mutants for 12 genes

Following generation of disruption constructs, vectors were linearized and electroporated into schizonts (immature blood stage forms of the parasites susceptible to electroporation). Anticipated double homologous recombination between the linear gene-targeting cassette and the WT locus ensures integration of the resistance cassette and deletion of the respective WT gene locus. Potential transgenic parasites (following successful integration), were later isolated, following two rounds of pyrimethamine selection (Figures 3.6 C, D and E).

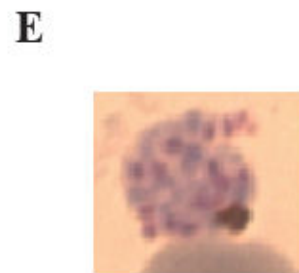
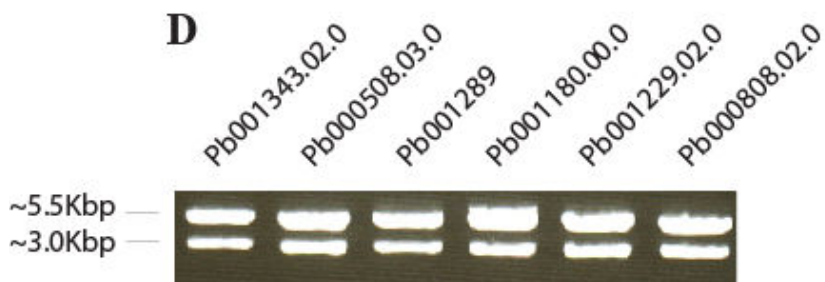
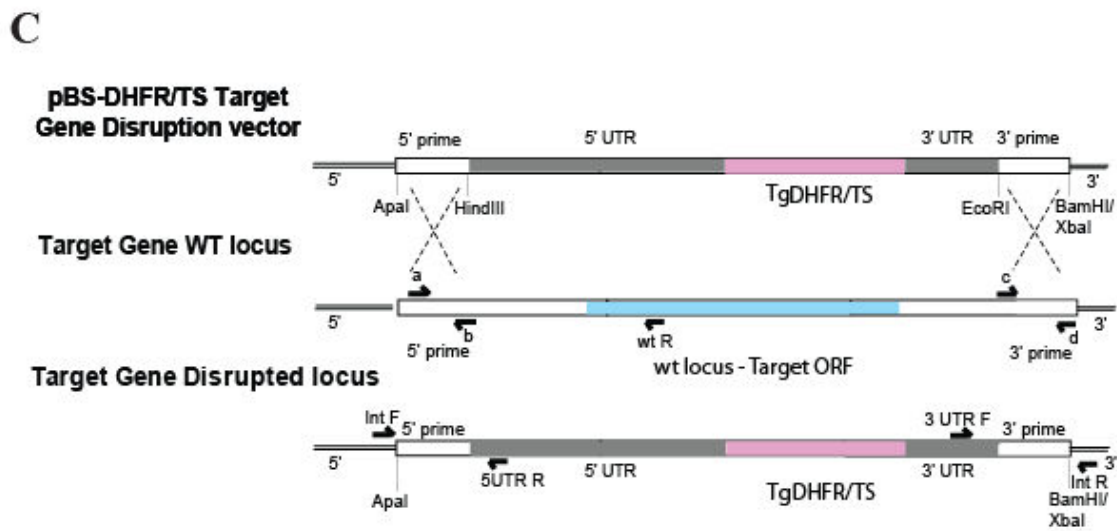
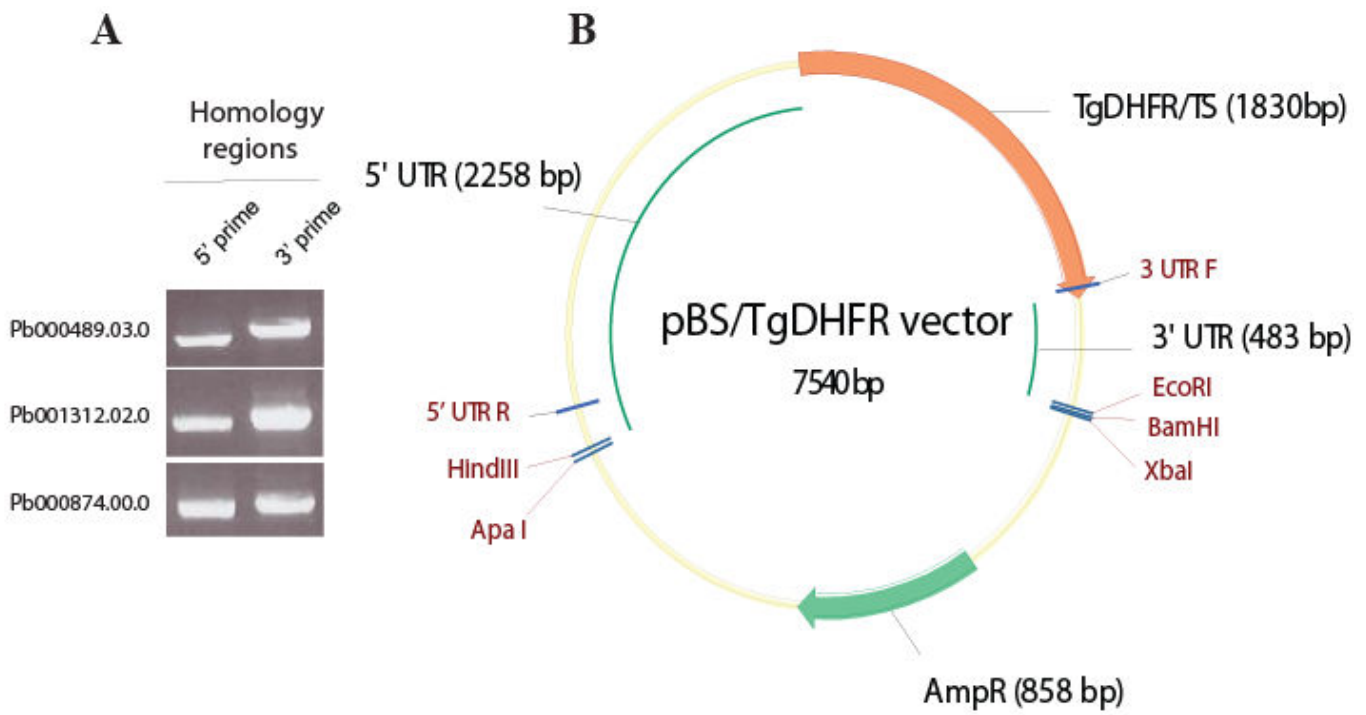


Figure 3.6. Generation of transgenic parasite lines

A. Amplification of regions of homology. 500-1000bp regions, up- and downstream the respective gene locus of interest were amplified. Three genes are presented indicatively

B. pBluescript –DHFR/TS cloning vector. Regions of homology were then cloned into the pBluescript –DHFR/TS (a modified *Toxoplasma gondii* dihydrofolate reductase / thymidilate synthase cassette which provides the trait for positive selection) vector, on specific restriction sites (Apa I - Hind III for 5' prime and BamHI- EcoRI/XbaI for 3' prime).

C. Schematic representation of gene targeted disruption of the selected *Plasmodium* candidates. Upstream region of homology (5' prime) and downstream region of homology (3' prime) target sequences (ranging from 500-1000 bp of the 5' UTR and 3'UTR of respective ORF) have been amplified (using a,b and c,d respective pairs of primers) and cloned into a pBluescript –DHFR cloning vector and will serve as regions of homology for future double homologous recombination as presented in C top and middle graph. Successful integration and gene disruption were later confirmed by diagnostic PCR, using the following pairs of primers Int F (Integration forward) and 5'UTR R (5'UTR reverse) or 3'UTR F and Int R (Integration reverse) respectively (C bottom graph).

D. Linearization of disruption constructs. Disruption constructs were linearized prior to transfection according to protocol. Six genes are presented indicatively

E. Purified schizont. Following purification of mature schizonts according to protocol, DNA was electroporated into parasites later propagated into mice.

3.2.2.3. Genotypic analysis of transfectant population

To identify successful integration of the gene targeting construct, gDNA from potentially resistant parasites following two rounds of pyrimethamine selection was initially analyzed by diagnostic PCR. A combination of a primer upstream of the 5' prime (or downstream of 3 prime in the case of PB001343.02.0), and a primer within the *TgDHFR/TS* cassette was used to amplify gene-targeting constructs that had integrated into the right position in the genome (Figure 3.6 C). The same upstream (or downstream) primer was used together with a primer binding in the genomic region that is replaced by the *TgDHFR/TS* cassette to amplify the WT allele (Figure 3.6 C). All gene-targeting constructs were transfected four times each, or until integration of the disruption construct into the chromosomal target locus could be detected. Attempts to generate a transgenic line were abandoned if integration had not been detected within four independent transfection batches. The results are summarized in Table 3.2. and an indicative example of genotypic analysis for all genes is shown in Figure 3.7. Briefly, 19 rounds of transfections were performed in total, out of which integration was achieved for 5 of the 12 genes attempted. In all other cases either episomal contamination that conferred resistance or no live parasites were detectable following a round of drug selection. Surprisingly, in the case of PB000846.01.0, a number of transfections revealed a population of seemingly WT parasites surviving two rounds of drug selection and thus being clearly resistant to pyrimethamine.

No correlation was observed between successful integration and features of regions of homology or excised domain as those described in Table 3.2. Additionally, no correlation was found between successful integration events and gene expression during asexual blood stage development. The significance of these findings is discussed in section 4.2.1.2.

Henceforth, parasite WT and KO lines of respective genes will be indicated as Pbx and Δ pbx respectively, where x corresponds to the last three or four numerical digits of the gene accession number. i.e. Pb846 and Δ pb846 for PB000846.01.0, Pb1343 and Δ pb1343 for PB001343.02.0 etc. In italics genes are indicated while roman characters indicate proteins.

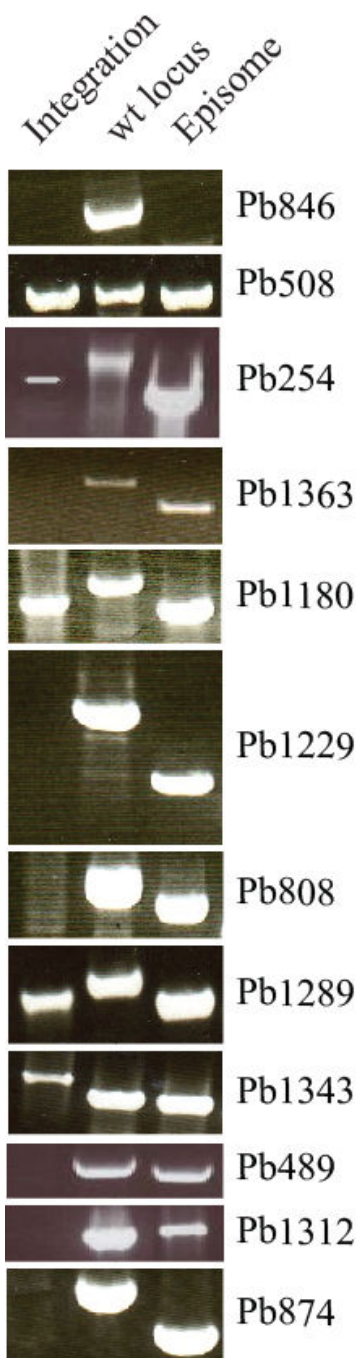


Figure 3.7. Genotypic analysis of transfectant population.

Genomic DNA was collected from parasites surviving two rounds of pyrimethamine selection and genotyped by diagnostic PCR. Indicative results deriving from various rounds of transfections are shown. Bands represent presence of integration, WT locus or episome as amplified by specific pairs of primers (see figure) In total integration was observed for 5 candidate genes. WT parasites that had survived drug selection, possibly through episomal contamination were almost always observed. In one case (Pb846) several numbers of transfections revealed presence of WT parasite in absence of integration or episomal contamination, suggesting development of native resistance.

3.2.2.4. Genotypic analysis of 5 successful mutant parasite strains

Following detection of potential integration by PCR, successful mutant populations were assessed for successful integration in the right genomic loci by southern blot analysis of their separated chromosomes by pulse field gel electrophoresis (PFGE), as predicted by *P. berghei*/*P. falciparum* synteny maps (Kooij et al., 2005) and exemplified in Figure 3.8. Even though not precise, radio band intensity of the parasite mutant population can give an indication of the proportion of successfully transgenic parasites in the mixed post transfectant population. Significant degree of integration was observed for $\Delta pb508$ (chromosome 12) and $\Delta pb1180$ (chromosome 6) but not for the other three populations (Figure 3.8). Nevertheless, due to technical difficulties that resulted in a gel of poor visual quality, absence of PFGE integration signal in the presence of a positive PCR integration was considered inconclusive and all 5 successful mutant parasite populations were set to be dilution cloned.

GENE ID	5' prime		3 prime		Excised sequence		<i>In vivo</i> expression	ABS expression		Transfection				Dilution Cloning
	size(bp)	AT (%)	size(bp)	AT (%)	size(bp)	KO		Transcript	Protein	1	2	3	4	
PB001229.02.0	519	74	842	76	2280	PARTIAL	Pbs21-like	√	√	E	N	E	N	
PB000846.01.0	546	84	553	80	1337	COMPLETE	Pbs21-like	√		WT	WT	E	E	
PB000874.00.0	737	80	532	70	1183	PARTIAL	Pbs21-like			E	N	N	E	
PB000489.03.0	538	79	683	78	795	PARTIAL	Pbs21-like	√	√	E	E	N	N	
PB001312.02.0	635	79	521	75	596	PARTIAL	Pbs21-like	√	√	E	N	E	N	
PB000254.00.0	534	83	509	75	1956	PARTIAL	Ookinete	√		I				<i>pending</i>
PB001363.02.0 #1	556	77	572	75	510	PARTIAL	Ookinete	√		E	E	N	N	
PB001363.02.0 #2	556	75	530	73	588	PARTIAL	Ookinete		E	N	E	N		
PB000508.03.0	521	83	526	83	471	COMPLETE	Ookinete	√		I				√
PB000808.02.0 #1	681	78	539	74	3295	PARTIAL	Ookinete	√	√	E	N	E	N	
PB000808.02.0 #2	867	77	714	75	3124	PARTIAL	Ookinete			E	N	E	N	
PB001180.00.0	691	80	628	77	938	PARTIAL	Ookinete	√		E	I			√
PB001289	591	78	535	79	2327	PARTIAL	Ookinete		√	E	I			√
PB001343.02.0 #1	517	78	538	77	471	PARTIAL	Ookinete	√		I				<i>pending</i>
PB001343.02.0 #2	519	78	637	78	505	PARTIAL	Ookinete		E	E	E	E		

Table 3.2. Generation of disruption mutants for 12 genes. The list of candidate genes selected for functional characterization is presented. Genes and information of 5' and 3' prime regions of homology of the respective disruption constructs are listed, including size in base pairs (bp), AT richness (%) and resulting KO. *In vivo* transcriptional expression as initially indicated by microarray data is shown in 5th large column (ABS expression). The latter size of excised sequence in base pairs (bp) and type of KO is shown. Information on presence (√) of transcript according to our study (Figure) and protein hits in asexual blood stages (ABS), (Appendix Table 2) is also included. Four series of transfections for each construct, following two rounds of drug selection, resulted in, no detection of live parasites (N), episomal contamination (E) and wild type (WT) resistant parasite and finally successful integration (I) for 5 of them. Dilution cloning of 3 was completed as indicated (√) while for the rest this was not completed.

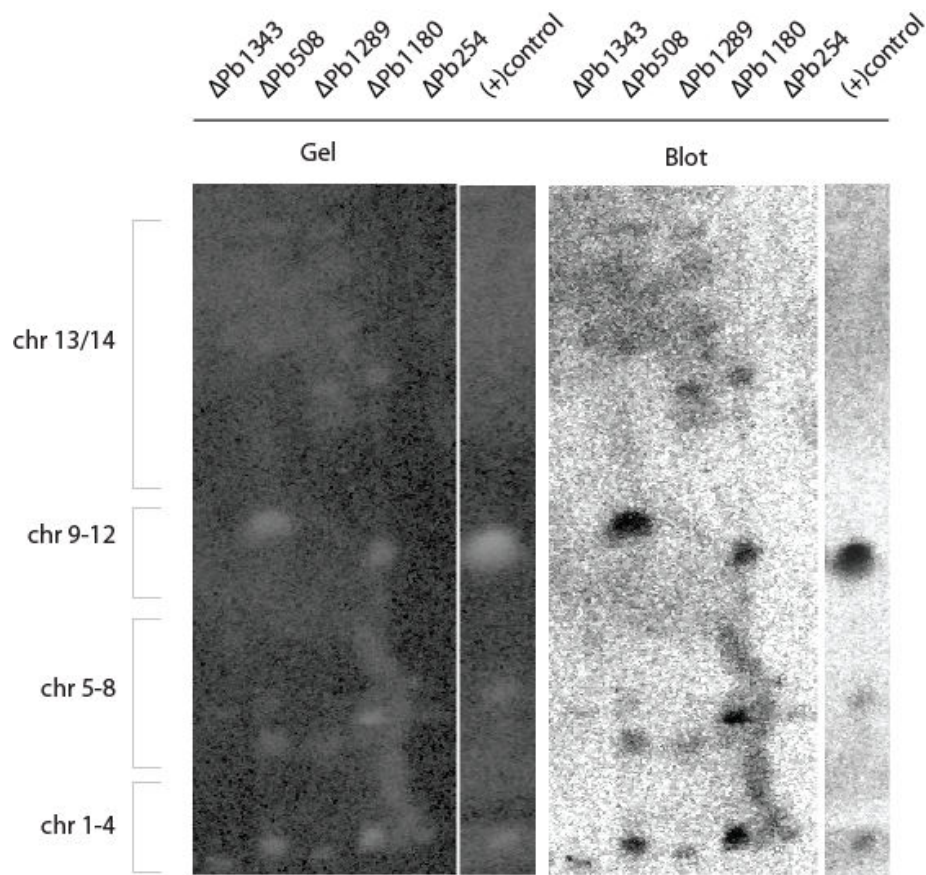


Figure 3.8. Genotypic analysis of successful mutant parasite population

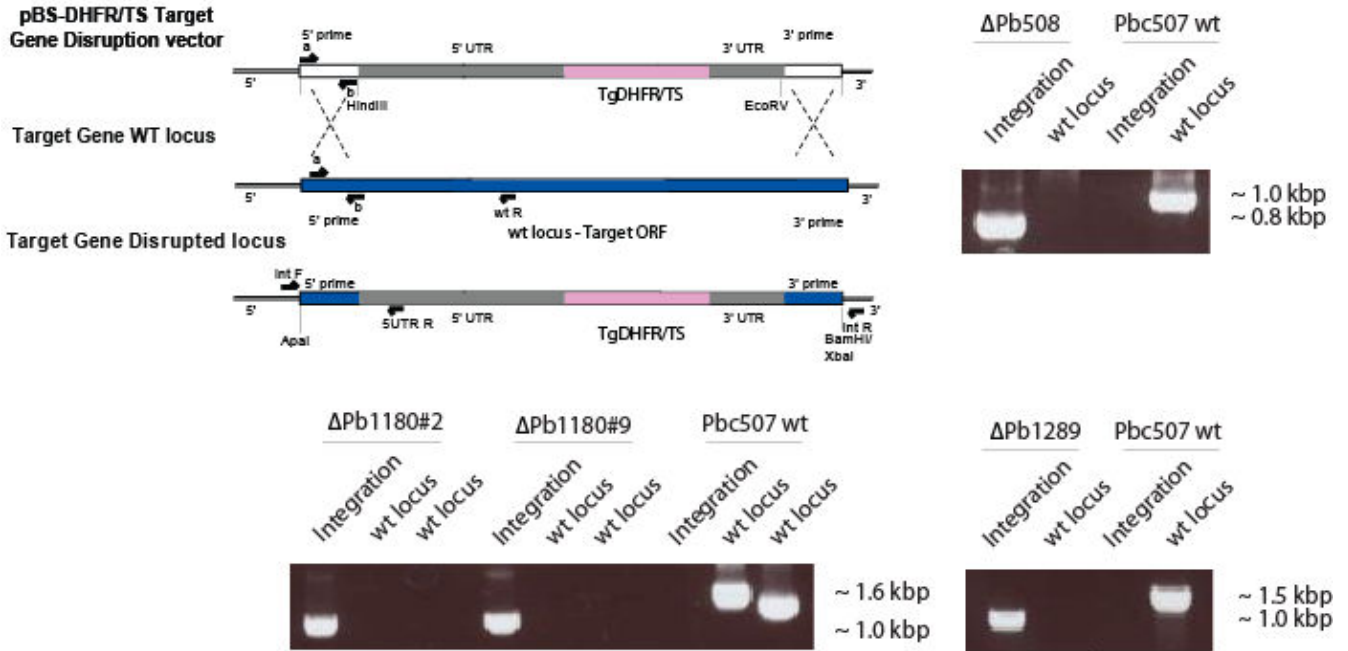
PFGE analysis and the respective blot probed with a *TgDHFR/TS* fragment. Contrary to PCR data, no integration for *Δpb1343* and *Δpb1289* populations in chromosome 11 was observed by PFGE, neither for *Δpb254* in chromosome 3. Integration for *Δpb508* and *Δpb1180* is indicated by a hybridisation signal on chromosomes 12 and 6 respectively. A signal obtained around chromosomes 13/14 was observed as a slowly migrating smear and is indicative of episomes, which in *P. berghei* are maintained as circular concatamers of 9-15 plasmid copies (Williamson et al., 2002). A gel of poor visual quality was produced due to technical difficulties hence, for reference purpose, a positive control, which exhibits a strong hybridisation signal on chromosome 10, has been included. The band in the region of chromosome 7 is the result of the hybridisation of the *TgDHFR/TS* probe to the native *P. berghei* DHFR locus.

3.2.2.5. Dilution Cloning and Genotypic analysis of 3 successful clonal mutants

Following drug selection and diagnostic selection of integrations, mutant parasite populations were not pure; some of the parasites had the integration but the rest did not (Figure 3.7). In order to obtain clean mutant clones successive dilutions of this mixed population (limited dilution cloning) were performed. Limited dilution cloning of successful mutant parasite populations rendered clonal populations for three out of five transgenic parasites i.e. $\Delta pb508$, $\Delta pb1289$ and $\Delta pb1180$. For $\Delta pb508$ and $\Delta pb1289$, only one clone was obtained, while for $\Delta pb1180$ two clones (#2 and #9) were isolated. Four attempts of dilution cloning for $\Delta pb1343$ and $\Delta pb254$ populations, failed to produce a clean mutant clone, hence these populations were not pursued further.

Clonal populations of $\Delta pb508$, $\Delta pb1289$ and $\Delta pb1180$ were genotyped for the presence of integration and absence of WT locus with PCR (Figure 3.9A). Successful integration of *TgDHFR/TS* cassette is demonstrated by the detection of the integration locus in all four clones or three mutants, which also lack the respective WT locus. Southern blot analysis of genomic DNA was also performed to confirm successful and appropriate integration (Figure 3.9B). Genomic DNA was digested according to described methods (EcoRI/EcoRV) and subsequently subjected to hybridisation against a PCR-derived probe corresponding to the respective gene's upstream region of homology. The Southern blot analysis reveals a 3-4 kb shift to a higher molecular weight as a result of the insertion of the *TgDHFR/TS* resistance cassette in the gene's WT locus. The WT locus is absent from all four clonal lines.

A Diagnostic PCR of clonal mutants



B Southern blot analysis of clonal mutants

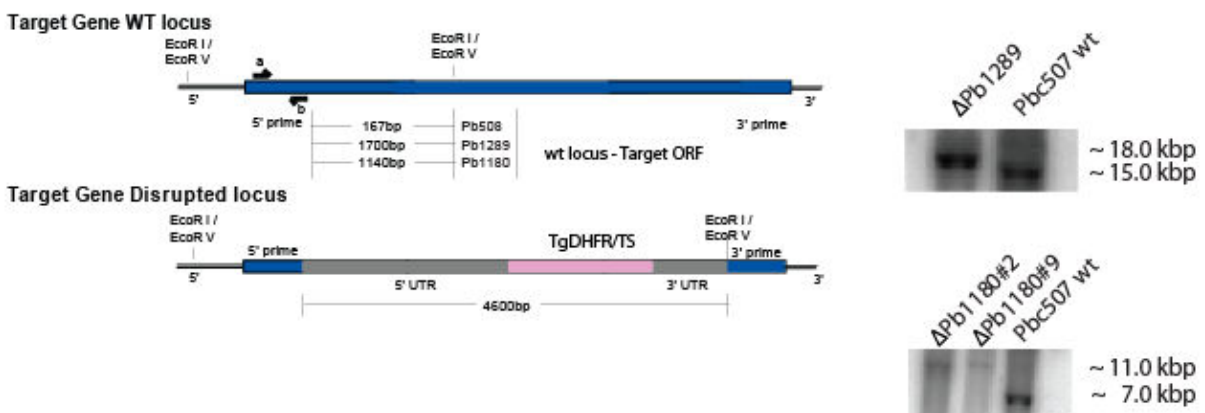


Figure 3.9. Genotypic analysis of 3 successful clonal mutants A. Diagnostic PCR analysis performed on the successful mutants' clonal parasite populations. Successful integration of *TgDHFR/TS* cassette is demonstrated by the detection of the integration locus in all four clones or three mutants, which also lack the respective WT locus. Two pairs of WT reverse primers were used for *Pb1180*. B. Southern blot analysis of successful mutants' clonal parasite populations. The Southern blot analysis reveals a 3-4 kb shift to a higher molecular weight as a result of the insertion of the *TgDHFR/TS* resistance cassette in the gene's WT locus. The WT locus is absent from all four clonal lines.

3.2.3. Phenotypic analysis of the 3 successful mutants

3.2.3.1. *Δpb508*, *Δpb1289* and *Δpb1180* show normal rate of asexual blood growth and gametocytaemia

Parasite development in the mosquito host follows a complex cycle of invasion and replication in the vertebrate host. Successful asexual development and gametocytogenesis in the vertebrate host is a prerequisite for successful parasite development in the mosquito host. In order to assess asexual and sexual blood stage growth ability of *Δpb508*, *Δpb1289* and *Δpb1180*, mice were infected with the respective mutants using equivalent passage number and initial parasitaemia of injecting blood. Growth of asexual blood stages and gametocytaemia from tail blood smears were subsequently monitored using light microscopy. Asexual and sexual blood stage growth of all mutants were comparable to those of *Pbc507*WT (Figure 3.10 A and B).

3.2.3.2. *Δpb508*, *Δpb1289* and *Δpb1180* show a normal rates of male gametocytes that exflagellated to levels comparable to that of WT

Successful male gametogenesis is crucial for subsequent fertilisation. In order to assess whether mutant male gametogenesis occurs normally or is impaired, mutant male gametocytes were activated by exposure to 19°C for 10-20 minutes until exflagellation was observed. Exflagellating parasites were then counted using a haemocytometer, under light microscopy. *Δpb1289* male gametocytes formed and exflagellated at a rate comparable to that of the WT (Figure 3.10 C). Even though a lower rate of exflagellation was observed for *Δpb508* and *Δpb1180*, compared to WT, the differences were statistically not significant (p=0.2633 and p=0.3083 respectively).

3.2.3.3. $\Delta pb508$ & $\Delta pb1180$ form decreased number of ookinetes

Mutants were then assessed for their ability to form ookinetes. Mosquito midguts were homogenized, smeared, stained and observed under light microscopy 24h PI with respective mutants. In order to better quantify ookinetes, mutants were allowed to activate, fertilize and form ookinetes *in vitro*. After 22-24 hours, ookinete cultures were stained with anti *Pbs21* antibody that specifically marks macrogametes and ookinetes. Ookinetes were enumerated based on their staining and morphology and their proportion against all *Pbs21* positive parasites was obtained (ookinete conversion ratio). All mutants were able to form ookinetes of normal morphology *in vivo* and *in vitro* (Figure 3.11). However, $\Delta pb508$ & $\Delta pb1180$ were significantly impaired in their ability to successfully form normal numbers of ookinetes, reaching only 14-18% of WT (Figure 3.10). On the other hand, $\Delta pb1289$ gametocyte to ookinete conversion ratio was comparable to WT, even though slightly lower. However, this difference was not statistically significant ($p=0.09$). All mutants exhibited normal distribution of *Pbs21* (Paton et al., 1993).

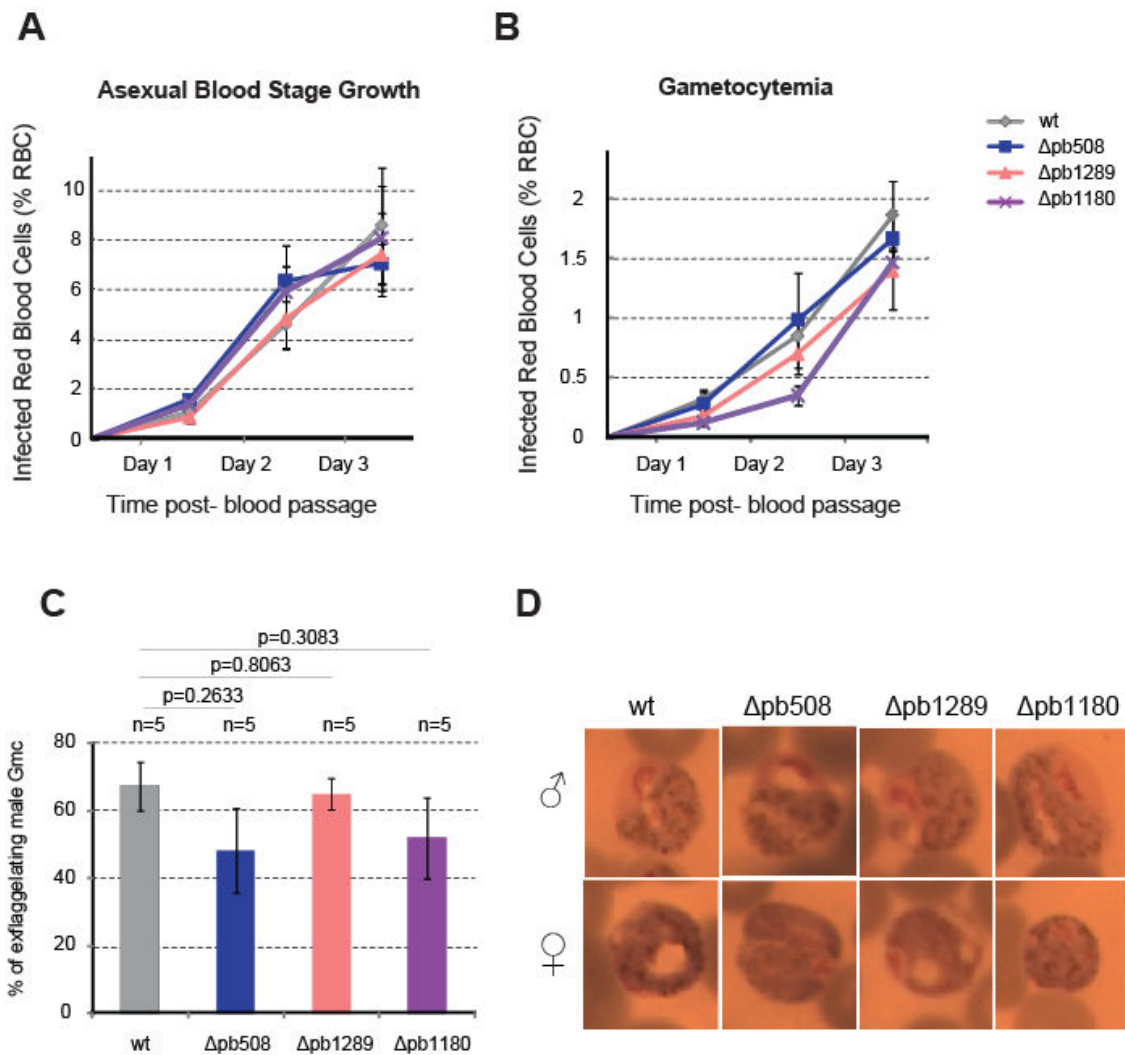


Figure 3.10. *Δpb508*, *Δpb1289* and *Δpb1180* asexual and sexual blood stage growth and exflagellation assays. **(A)** Asexual blood stage growth and **(B)** gametocyte production of the *Δpb508*, *Δpb1289* and *Δpb1180* mutants is comparable to that of WT. **(C)** *Δpb508*, *Δpb1289*, *Δpb1180* male gametocytes exflagellate at a rate comparable to that of WT and **(D)** Giemsa staining of WT, *Δpb508*, *Δpb1289* and *Δpb1180* gametocytes revealed the typical staining pattern for *P. berghei* gametocytes. (P values were calculated by a two-tailed, unpaired Student T-test of equal variances. Error bars indicate standard error of the mean, n indicates number of biological replicates).

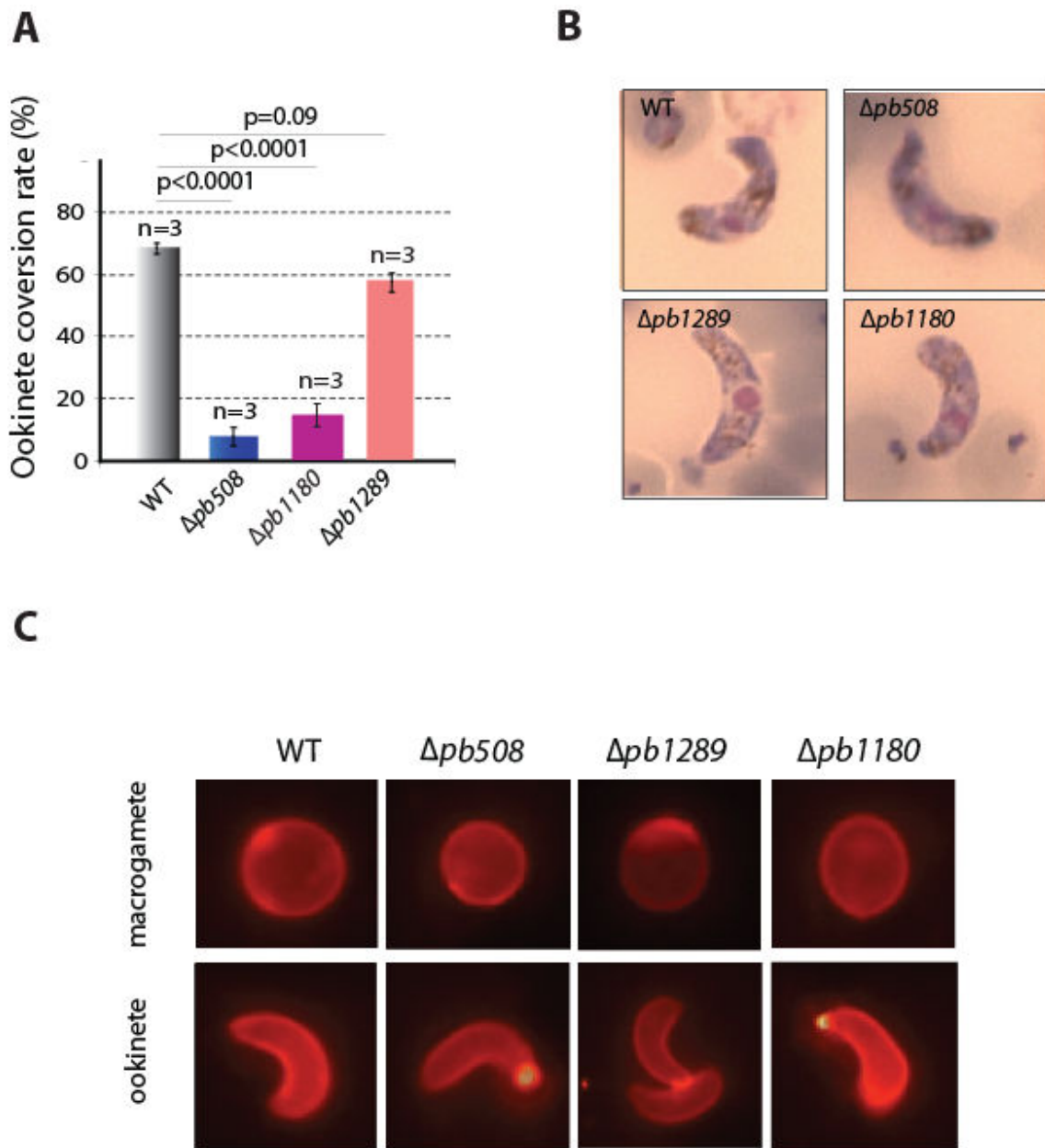


Figure 3.11. *Apb508*, *Apb1289* and *Apb1180* ookinete formation. (A) *Apb508* and *Apb1180* exhibit a lower gametocyte-to-ookinete conversion ratio comparing to WT, while *Apb1289* ability to form ookinetes is not impaired (B) Giemsa staining of WT, *Apb508*, *Apb1289* and *Apb1180* *in vivo* formed ookinetes reveals normal morphology. (C) *Apb508*, *Apb1289*, *Apb1180* macrogametes and ookinetes demonstrate normal distribution of *Pbs21*

3.2.3.4. *Apb508* & *Apb1180* ookinetes successfully invade the mosquito midgut

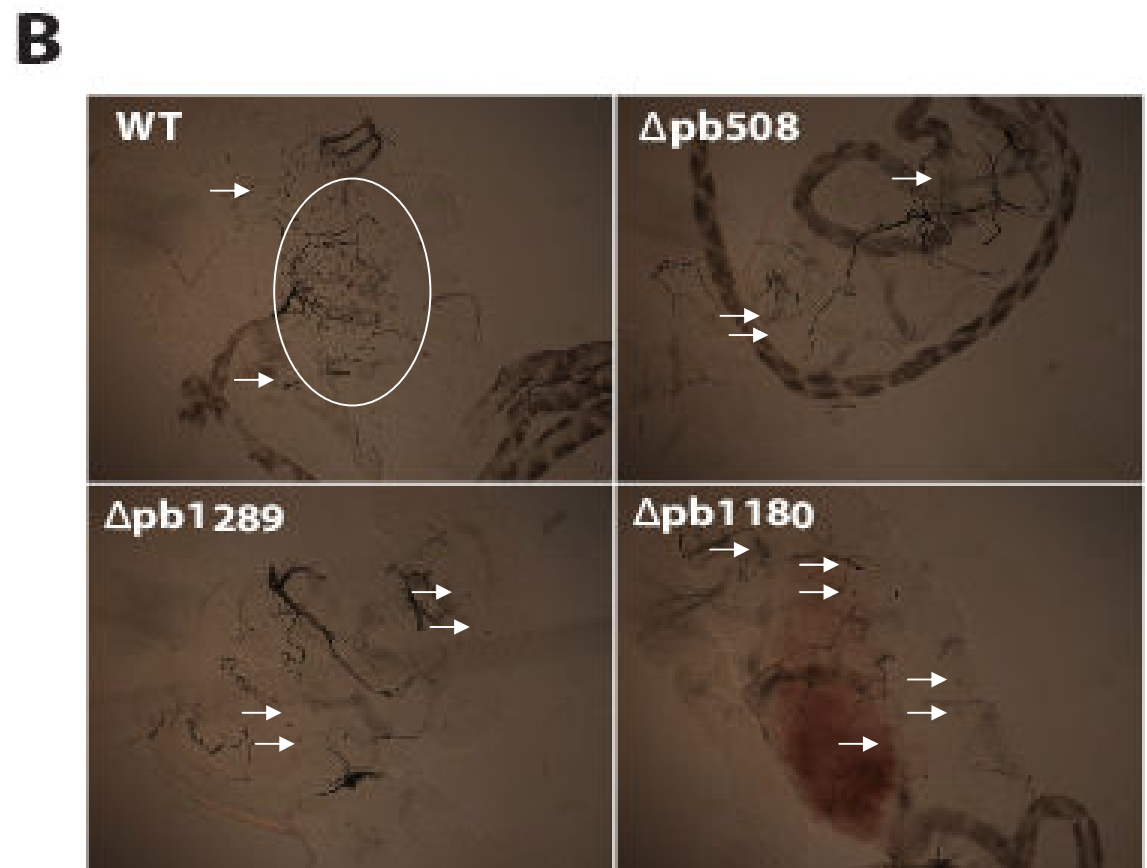
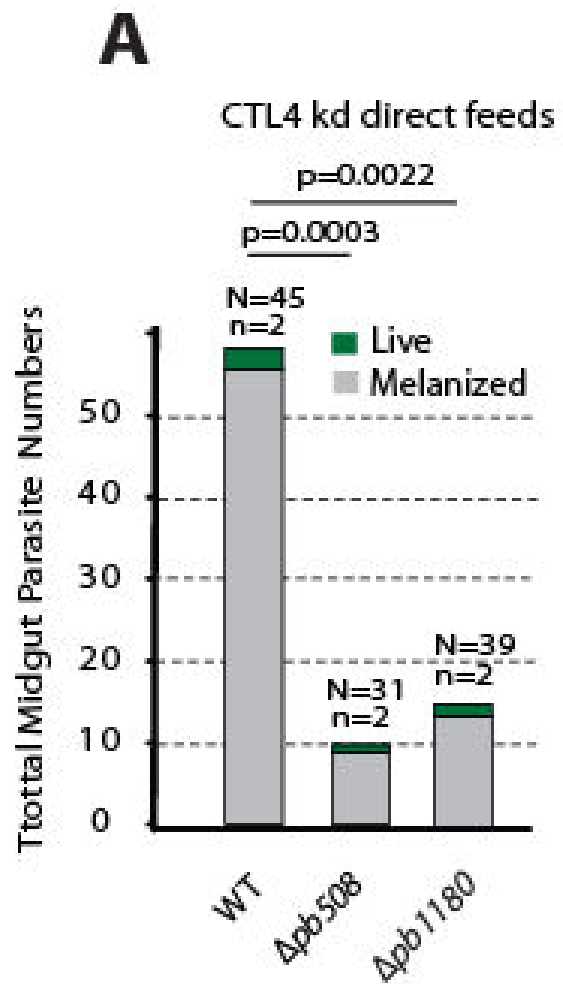
In order to investigate whether mutant ookinetes can invade the midgut epithelium, we used *A. gambiae* C-type lectin 4 (*CTL4*) knockdown (KD) and L3-5 strain mosquitoes. *CTL4* is an inhibitor of melanization, and its depletion by RNAi causes mosquitoes to melanize ookinetes soon after they reach the basal sub-epithelial space, where they encounter haemolymph components that are essential for melanization (Osta et al., 2004). In the same manner L3-5 strain refractory mosquitoes demonstrate increased melanization ability, allowing visualization of melanized parasites that have crossed the midgut epithelium by light microscopy via their melanotic encapsulation as they traverse the midgut barrier.

As shown in Table 3.3 and Figures 3.12 A and B *Apb508* & *Apb1180* parasites could be detected in *CTL4* KD mosquitoes; however, their numbers were much lower compared to WT parasites, reflecting their prior developmental defect in ookinete formation (Figure 3.12A). The number of *Apb508* live parasites in control *LacZ* double stranded (ds) RNA injected mosquitoes (mean=1.92) was lower than the number of melanised parasites in the *CTL4* KD mosquitoes (mean= 3.28), suggesting that the few ookinetes that do invade may be more sensitive to the mosquito immune reactions ($p=0.0044$). The same did not apply to *Apb1180* where the number of melanised parasites in *CTL4* KD mosquitoes was similar to the number of live parasites in the control *LacZ* dsRNA injected mosquitoes, suggesting that those few KO ookinetes that do invade develop successfully. Even though, a lower number of melanized ookinetes was consistent with a previously lower number of formed ookinetes following fertilization, a membrane feed of equal number of ookinetes for all WT, *Apb508* & *Apb1180* strains would be pivotal for invasion ability to be clearly assessed.

3.2.3.5. *Apb1289* ookinetes are impaired in their ability to invade the mosquito midgut

Significantly lower numbers of *Apb1289* parasites (both melanised as well as live) were observed in both *CTL4* KD as well as L3-5 refractory strain mosquitoes despite that this parasite forms normal numbers of ookinetes in the midgut bolus (Figure 3.12B, C, D). This finding implied that *Apb1289* ookinetes are impaired in their ability to invade the mosquito midgut. To ensure that this reduction was not attributed to variation in gametocyte numbers, ookinetes were cultured *in vitro* and fed at

known numbers to mosquitoes via a membrane feeding apparatus. This experimental setup allows ookinetes to develop in the absence of mosquito factors and invade the midgut prior to formation of the peritrophic matrix (PM) (Dessens et al., 2001). Therefore, it could also reveal if the phenotype is attributed to a potential deficiency in PM penetration. However, $\Delta pb1289$ ookinete membrane feeding failed to rescue midgut invasion, suggesting that $\Delta pb1289$ phenotype is due to ookinete failure to invade the mosquito midgut (Figure 3.12 E, Table 3.3).



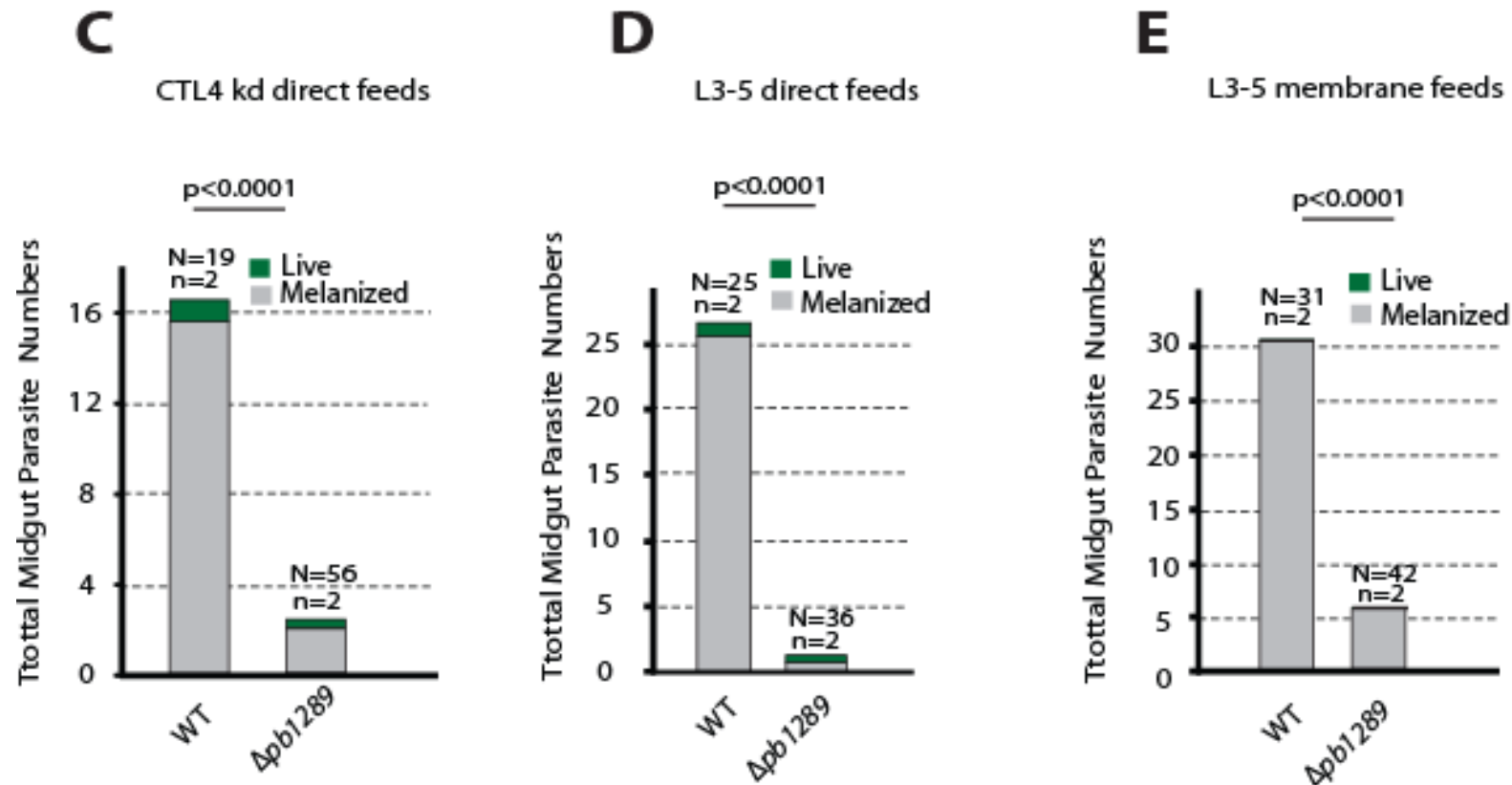


Figure 3.12. Midgut invasion assays. $\Delta pb508$ and $\Delta pb1180$ ookinetes successfully invade the midgut epithelium (A) albeit in lower numbers reflecting their prior developmental defect. Light microscopy (B) facilitated visualization and enumeration of encapsulated ookinetes (white arrows and circle). $\Delta pb1289$ ookinetes fail to cross the midgut epithelium, in both *CTL4* KD (B and C) and L3-5 refractory mosquitoes (D). In the latter case, membrane feeds of known number of ookinetes do not rescue $\Delta pb1289$ invasion defect (E) (p values calculated for the total number of parasites according to one way ANOVA following data normalization, N: numbers of midguts; n: number of biological replicates)

3.2.3.6. *Δpb1289* ookinete motility is not impaired compared to WT

On the basis that *Δpb1289* ookinetes fail to invade the mosquito midgut and considering that the ookinete's invasion machinery is linked to its motility (Baum et al., 2006), a motility assay was performed. Ookinetes' translocation *in vitro* was monitored, and their translocation speed was assessed. *CTRP* mutant parasites were used as a control. This experiment would enable to determine whether the mutant phenotype is attributed to defective motility prior to invasion. *Δpb1289* mutant and WT ookinetes exhibited no significant variation as to their gliding motile behavior, since both translocated comparably *in vitro*. This finding implies that Pb1289 exerts its function during midgut invasion (Figure 3.13).

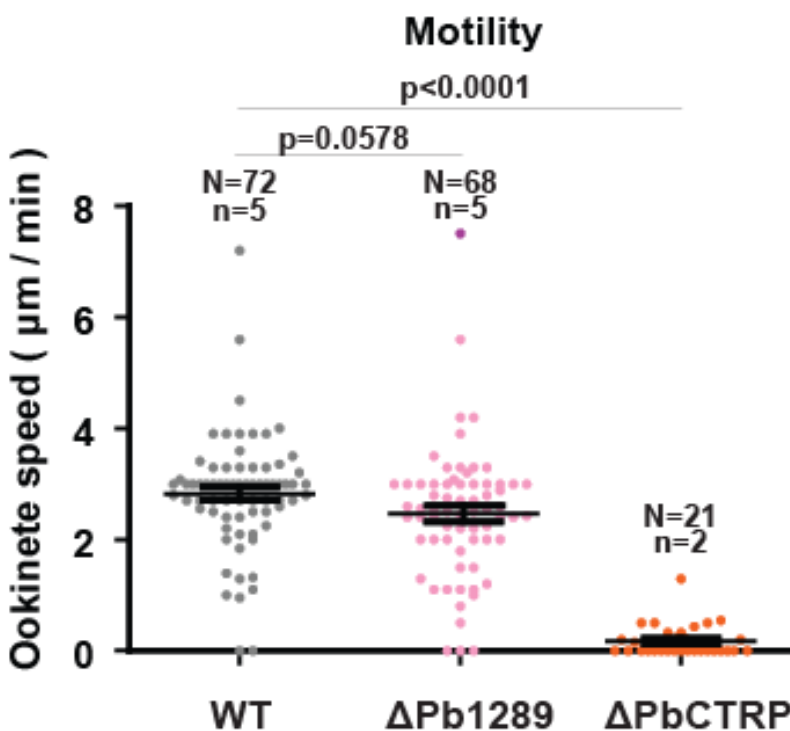


Figure 3.13. *Δpb1289* Motility assay WT, *Δpb1289* and *ΔpbCTRP* ookinetes were allowed to translocate on Matrigel™ prepared slides and their speed was calculated. *Δpb1289* ookinetes' gliding motility is not significantly impaired compared to WT. *ΔpbCTRP* was used as a control. *ΔpbCTRP* ookinetes fail to translocate normally, thus cannot invade the midgut epithelium. (*p* values calculated according to student's t-test, N: numbers of ookinetes; n: number of biological replicates; horizontal and error bars indicate arithmetic mean and standard error of the mean respectively)

Table 3.3. Effect of *Pb508*, *Pb1289*, *Pb1180* disruption on ookinete invasion.

Time point	Parasite	# of exp	# of midguts	# of live parasite midguts	Prevalence (%)	Parasite density		Analysis of variance (one-way)			
						Arithmetic mean	Geometric mean	Parasite range	P value	F value	Fold difference
Direct feeds											
Day 5	WT										
	<i>CTL4</i>	2	45	11	93%	65.8	40.7	0-210			
	<i>LacZ</i>	2	49	35	83%	11.8	6.17	0-78			
	<i>Δpb508</i>										
	<i>CTL4</i>	2	31	4	64%	11.39	3.678	0-54	0.0003	17.24	-11.06
	<i>LacZ</i>	2	42	15	40%	1.214	0.706	0-8			
	<i>Δpb1180</i>										
	<i>CTL4</i>	2	39	12	69%	19.9	4.32	0-334	0.0022	1.675	-9.4
	<i>LacZ</i>	2	42	29	80%	6.04	3.54	0-30			
Day 5	WT										
	<i>CTL4</i>	2	19	6	84%	18.1	7.19	0-91			
	<i>LacZ</i>	2	42	30	73%	13.4	4.68	0-119			
	<i>Δpb1289</i>										
	<i>CTL4</i>	2	56	16	33%	2.36	1.00	0-22	<0.0001	22.07	-7.1
	<i>LacZ</i>	2	36	20	58%	6.30	2.58	0-35			
L 3-5 refractory strain direct feeds											
	WT	2	25	12	100%	24.40	15	0-73			
	<i>Δpb1289</i>	2	37	10	59%	1.26	0.83	0-6	<0.0001	221.2	-18.1
L 3-5 refractory strain membrane feeds											
	WT	2	31	9	100%	30.58	23	0-87			
	<i>Δpb1289</i>	2	42	3	59%	5.27	2.3	0-30	<0.0001	9.35	-10

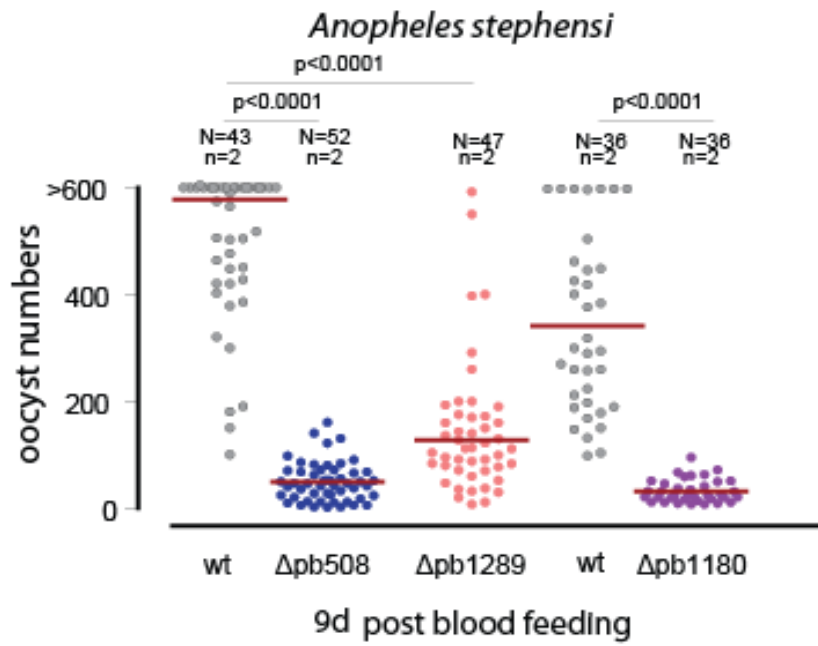
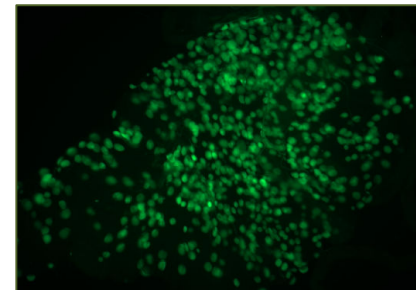
The table reports results from *Δpb508*, *Δpb1289*, *Δpb1180* or WT (Pbc507 WT) parasite infections of *A. gambiae CTL4*, *LacZ* KD and L3-5 refractory

strain mosquitoes. Equal numbers of *Δpb508*, *Δpb1289*, *Δpb1180* or WT infected midguts from independent biological replicates were pooled for each group. The total number of midguts is indicated in the fourth column. Prevalence shows the percentage of midguts with at least one oocyst or melanised parasite. Midguts with zero parasites were also considered for calculation of the arithmetic and geometric (following normalization of data distribution) means of parasite densities (number per midgut). P and F values were calculated using the one way ANOVA analysis of variance, following normalization of data distribution . Fold differences between *Δpb508*, *Δpb1289*, *Δpb1180* and WT oocyst densities were computed using the geometric means.

3.2.3.7. $\Delta pb508$, $\Delta pb1289$ and $\Delta pb1180$ ookinetes successfully form oocysts, in both *Anopheles stephensi* and *Anopheles gambiae*

Successful mutants were then assessed for their ability to form oocysts *in vivo* in both *Anopheles stephensi* (Figure 3.14), and *Anopheles gambiae* (Figure 3.15) mosquitoes. Oocysts were observed by fluorescence microscopy and carefully enumerated at days 9 and 15 post blood meal, to monitor for potential defects in oocyst maturation. All mutants successfully formed oocysts in both mosquitoes which were of normal size and shape; however, their numbers were significantly lower than those of WT. These data reflected prior developmental defects in ookinete development for $\Delta pb508$ and $\Delta pb1180$, and midgut invasion for $\Delta pb1289$ respectively. No significant differences were observed in oocyst levels between days 9 and 15 post infection, suggesting that the absence of respective genes does not bear any consequences on oocyst maturation, nor does it render oocysts more vulnerable to mosquito immune responses.

Details of sample sizes, number of biological repeats, absolute oocyst numbers and statistical analysis from all $\Delta pb508$, $\Delta pb1289$ and $\Delta pb1180$ mosquito infections are summarised in Tables 3.4 and 3.5.

A**B***A. stephensi* midguts

wt

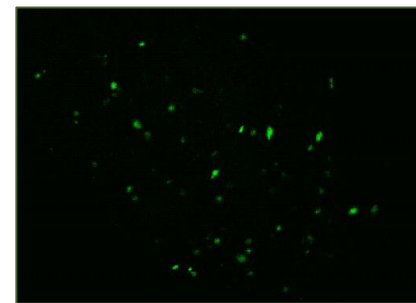
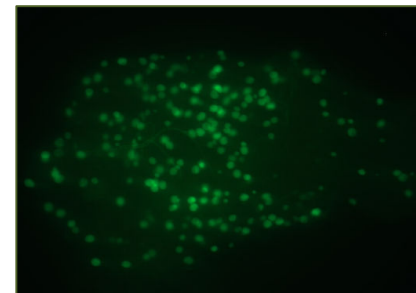
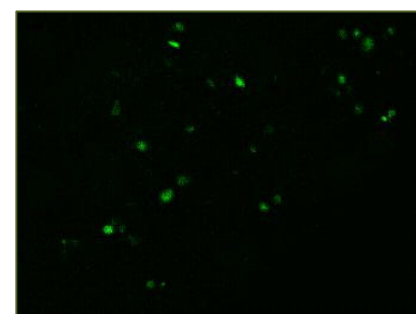
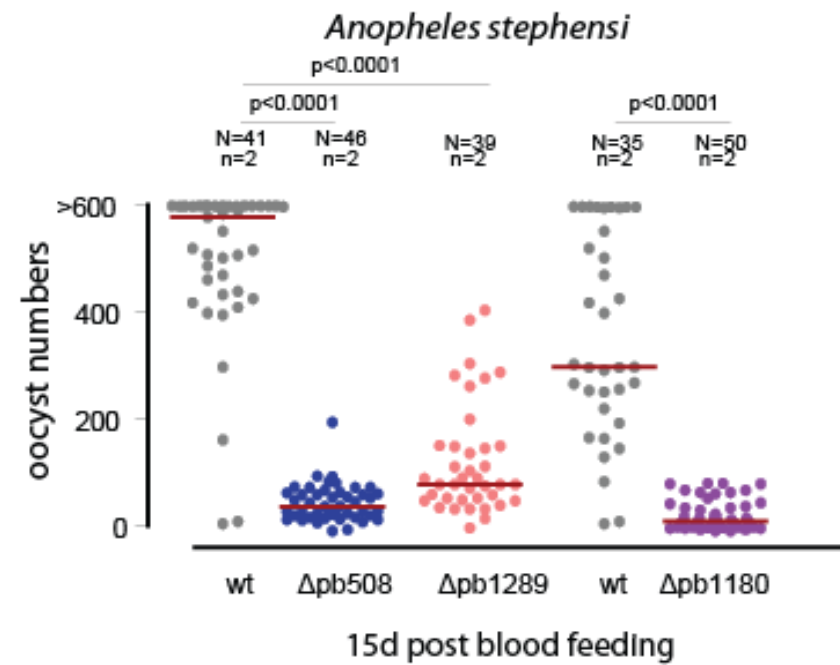
 Δ pb508 Δ pb1289 Δ pb1180

Figure 3.14 *Δpb508*, *Δpb1289* and *Δpb1180* oocyst development in *A. stephensi*. **A.** *Δpb508*, *Δpb1289* and *Δpb1180* oocyst graphs indicating oocyst distribution in *A. stephensi* midguts as this counted on days 9 and 15 post infection. All mutants successfully form oocysts in *A. stephensi*, in numbers significantly lower than WT, mirroring their prior developmental defects. No significant difference was observed between days 9 and 15 in any of the mutants (also see Table 3.4). **B** Fluorescent microscopy images of GFP expressing oocysts (*Pbc507* eGFP reference line) representative of WT, *Δpb508*, *Δpb1289* and *Δpb1180* oocyst density in *A. stephensi* midguts at X10 magnification.

(*p* values as calculated one-way analysis of variance (ANOVA) following normalisation of the distribution of the data, N: numbers of midguts; n: number of biological replicates; horizontal bars indicate the median value)

Table 3.4. Effect of *Pb508*, *Pb1289*, *Pb1180* disruption on oocyst development in *A.stephensi*.

Time point	Parasite	Number of exp	Number of midguts	Oocyst Prevalence (%)	Parasite density		Analysis of variance			
					Arithmetic mean	Geometric mean	Parasite range	P value	F value	Fold difference
<i>A. stephensi</i> infection										
Day 9	WT	2	43	100%	552	508.03	100-902			
	<i>Δpb508</i>	2	52	100%	52.75	38.4	1-160	<0.0001	31.47	-13.2
	<i>Δpb1289</i>	2	47	100%	205.05	129.1	17-602	<0.0001	2.73	-3.93
Day 15	WT	2	36	100%	350.5	319.5	105-902			
	<i>Δpb1180</i>	2	36	100%	27.42	21.61	4-91	<0.0001	95.07	-14.8
	WT	2	41	100%	536.25	460.5	14-799			
	<i>Δpb508</i>	2	46	98%	50.66	39.55	0-200	<0.0001	27.96	-11.6
	<i>Δpb1289</i>	2	39	98%	153.85	126.45	0-401	<0.0001	3.21	-3.64
	<i>Δpb1180</i>	2	50	96%	33.68	29.23	0-88	<0.0001	61.92	-10.24

The table reports results from *Δpb508*, *Δpb1289*, *Δpb1180* or Pbc507 WT parasite infections of *A. stephensi*. Equal numbers of *Δpb508*, *Δpb1289*, *Δpb1180* or Pbc507 WT infected midguts from independent biological replicates (indicated in the third column) were pooled for each group. The total number of midguts is indicated in the fourth column. Prevalence shows the percentage of midguts with at least one oocyst or melanised parasite. Midguts with zero parasites were also considered for calculation of the arithmetic and geometric (following data normalization) means of parasite densities (number per midgut). P and F values were calculated using the one way ANOVA analysis of variance test, following data normalization. Fold differences between *Δpb508*, *Δpb1289*, *Δpb1180* and Pbc507 WT oocyst densities were computed using the geometric means.

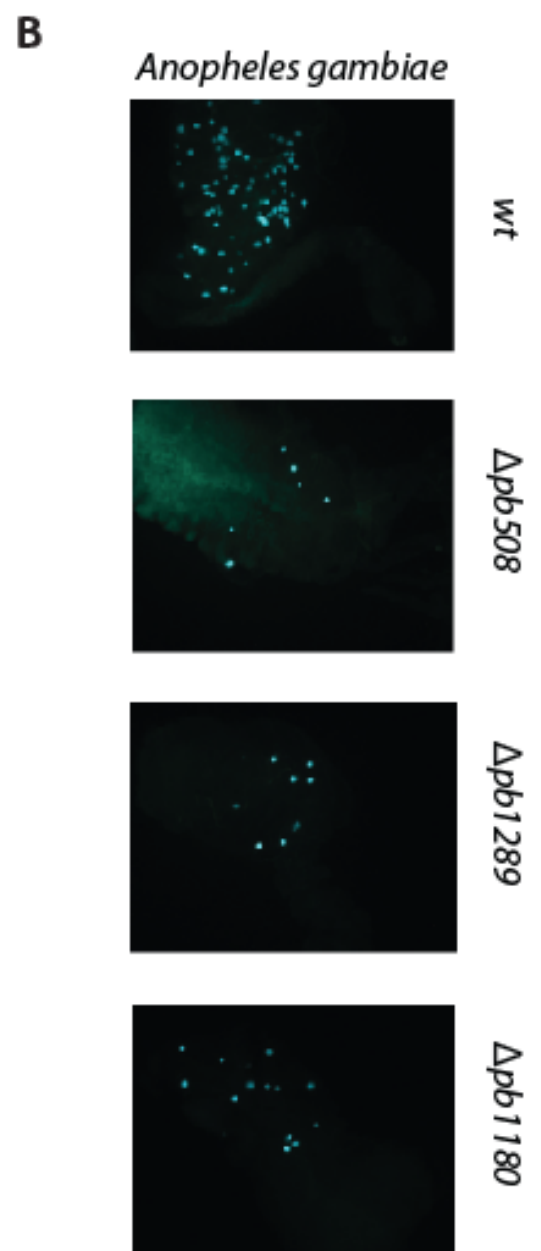
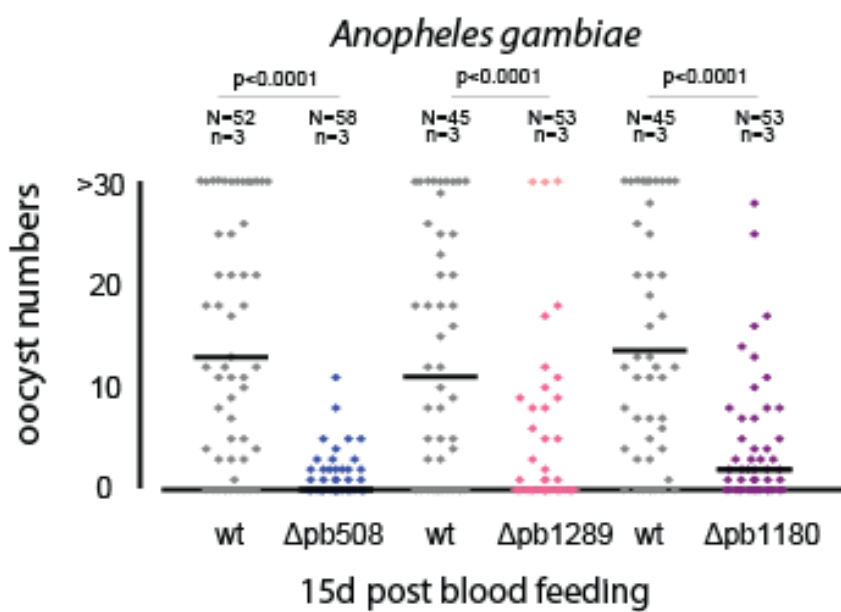
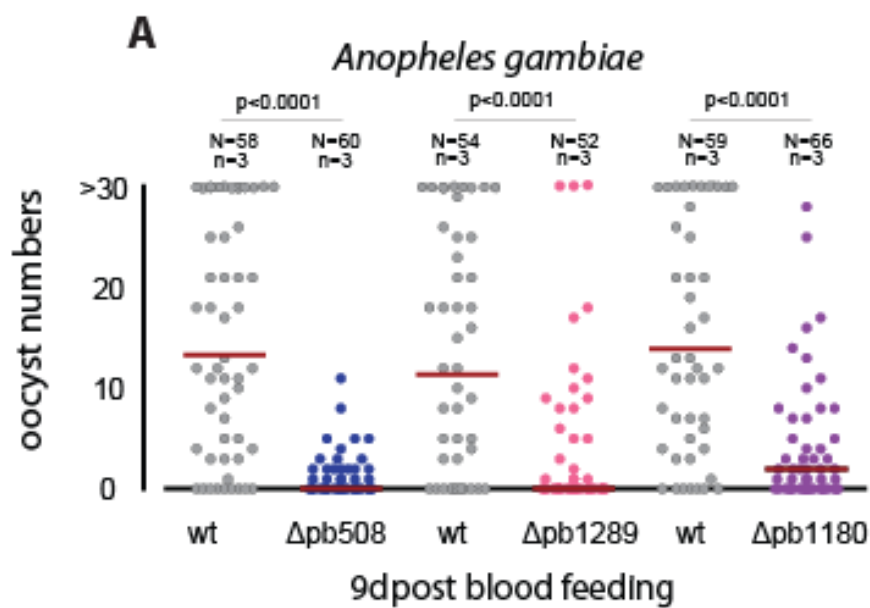


Figure 3.15 $\Delta pb508$, $\Delta pb1289$ and $\Delta pb1180$ oocyst development in *A. gambiae*. **A.** $\Delta pb508$, $\Delta pb1289$ and $\Delta pb1180$ oocyst graphs indicating oocyst distribution in *A. gambiae* midguts as this counted on days 9 and 15 post infection. All mutants successfully form oocysts in *A. gambiae*, in numbers significantly lower to WT, mirroring their prior developmental defects. No significant difference was observed between days 9 and 15 in any of the mutants (also see Table 3.5). **B** Fluorescent microscopy images of GFP expressing oocysts (*Pbc507* eGFP reference line) representative for WT, $\Delta pb508$, $\Delta pb1289$ and $\Delta pb1180$ oocyst density in *A. gambiae* midguts at X10 magnification. (*P* values as calculated one-way analysis of variance (ANOVA) following data normalization, N: numbers of midguts; n: number of biological replicates; horizontal bars indicate the median value)

3.2.3.8. $\Delta pb508$, $\Delta pb1289$ and $\Delta pb1180$ oocysts demonstrate successful nuclear division

$\Delta pb508$, $\Delta pb1289$ and $\Delta pb1180$ oocysts were monitored for their ability to sporulate following their development in the mosquito midgut. Sporulation was observed under differential interference contrast, where neatly arranged arrays of sporulation centres were observed 13-15 days PI. Nuclear staining confirmed the presence of normal nuclear divisions within the maturing oocyst. Indeed, similar to the WT, $\Delta pb508$, $\Delta pb1289$ and $\Delta pb1180$ oocysts were large in size and a large proportion of them had sporulated by day 13-15PI (Figure 3.16).

However, the total mutant sporozoite numbers were significantly lower than the WT, as expected due to their prior developmental defect in ookinete development ($\Delta pb508$, $\Delta pb1180$) or midgut invasion ($\Delta pb1289$) (Table 3.6).

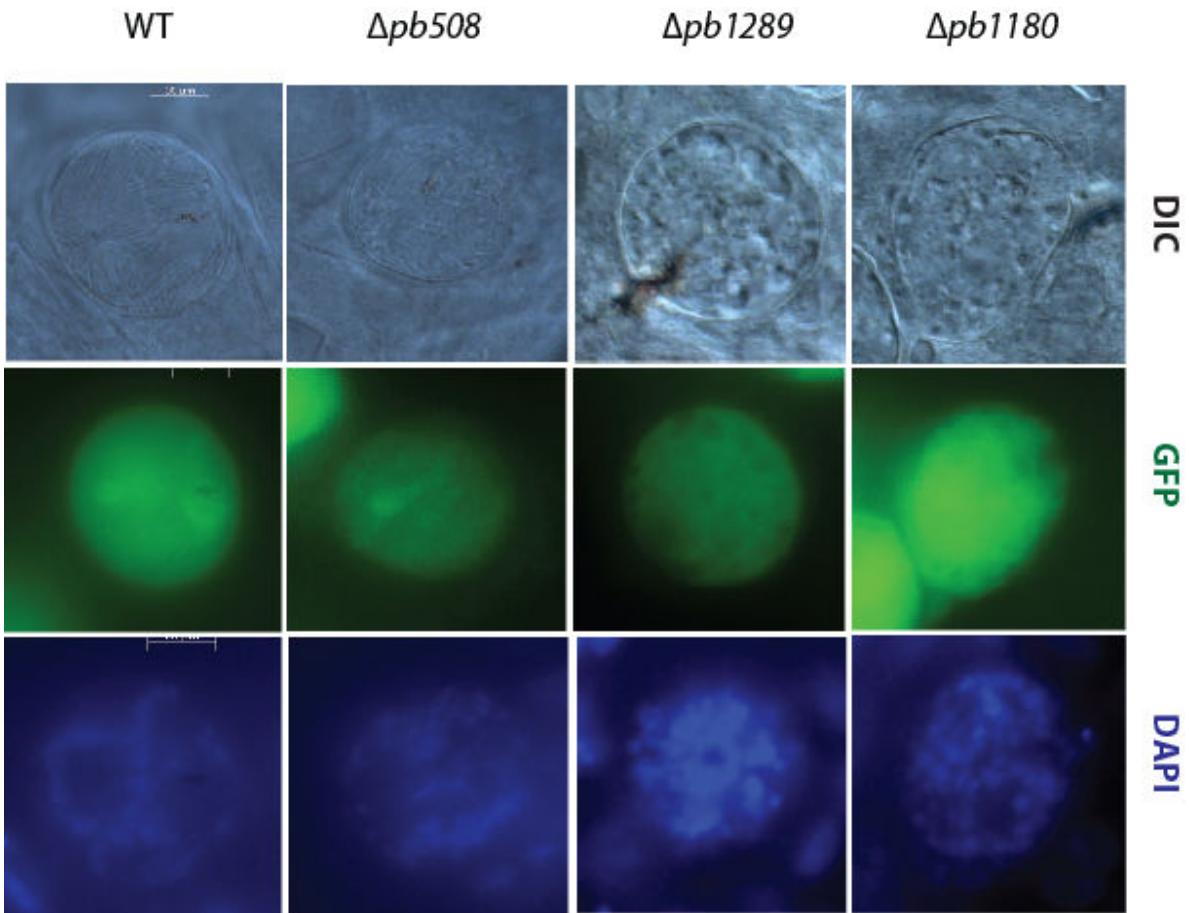


Figure 3.16. *Apb508*, *Apb1289* and *Apb1180* oocysts demonstrate successful nuclear divisions. Fluorescent microscopy images (X63 magnification) of WT, *Apb508*, *Apb1289* and *Apb1180* GFP expressing oocysts. A DNA-staining pattern (DAPI) that is characteristic of mature WT oocysts, where the haploid nuclei of the fully formed sporozoites are distinct, highly organised and aligned is evident in all mutants.

Table 3.5. Effect of *Pb508*, *Pb1289*, *Pb1180* disruption on oocyst development in *A.gambiae*.

Time point	Parasite	Number of exp	Number of midguts	Oocyst Prevalence (%)	Parasite density		Analysis of variance			
					Arithmetic mean	Geometric mean	Parasite range	P value	F value	Fold difference
<i>A. gambiae</i> infection										
Day 9	WT	3	58	80%	23.47	11.00	0-118			
	<i>Δpb508</i>	3	60	36%	1.70	0.72	0-36	<0.0001	123.33	-15.2
	WT	3	54	80%	18.89	8.74	0-8-			
Day 15	<i>Δpb1289</i>	3	52	46%	3.42	1.38	0-21	<0.0001	13.41	-6.3
	WT	3	59	80%	27.46	13.7	0-118			
	<i>Δpb1180</i>	3	66	62%	4.75	2.44	0-42	<0.0001	11.59	-5.6
	WT	3	52	82%	20.92	9.77	0-99			
	<i>Δpb508</i>	3	58	37%	1.14	0.61	0-11	<0.0001	123.1	-16.1
	WT	3	45	75%	17.32	7.96	0-73			
Day 15	<i>Δpb1289</i>	3	43	38%	4.67	1.93	0-35	<0.0001	4.648	-4.12
	WT	3	45	85%	19.9	9.95	0-99			
	<i>Δpb1180</i>	3	58	74%	4.03	2.09	0-28	<0.0001	15.12	-5.8

The table reports results from *Δpb508*, *Δpb1289*, *Δpb1180* or *Pbc507* WT parasite infections of *A.gambiae*. Equal numbers of *Δpb508*, *Δpb1289*, *Δpb1180* or *Pbc507* WT infected midguts from independent biological replicates (indicated in the third column) were pooled for each group. The total number of midguts is indicated in the fourth column. Prevalence shows the percentage of midguts with at least one oocyst or melanised parasite. Midguts with zero parasites were also considered for calculation of the arithmetic and geometric (following data normalization) means of parasite densities (number per midgut P and F values were calculated using the one way ANOVA analysis of variance test, following data normalization. Fold differences between *Δpb508*, *Δpb1289*, *Δpb1180* and *Pbc507* WT oocyst densities were computed using the geometric means

3.2.3.9. *Δpb508*, *Δpb1289* and *Δpb1180* sporozoites successfully egress from the oocyst, migrate through the haemolymph and invade the mosquito salivary glands.

Mutant parasites were then assessed for their ability to reach and invade the mosquito salivary glands. On day 21 PI mosquito salivary glands, were dissected, homogenized and sporozoites were counted using a haemocytometer and light microscopy. Detection of sporozoites in the mosquito salivary glands would require successful egression from the “mother” oocyst, migration through the haemolymph as well as invasion of the salivary gland epithelium. Presence of sporozoites in the salivary glands was observed for all mutants, even though in lower numbers comparing to the WT due to their prior developmental defects. This finding suggests that their ability to egress, migrate and invade the salivary glands had remained to a vast proportion intact (Table 3.6).

3.2.3.10. *Δpb508*, *Δpb1289* and *Δpb1180* parasites successfully transmit onwards following *A.stephensi* bite but not that of *A.gambiae*

The ability of the mutant parasites to be transmitted to a vertebrate host upon a mosquito bite and thus complete parasite’s life cycle was assessed. On days 18-21 post infection mosquitoes were allowed to feed on mice, which were then monitored for consequent infection. Interestingly, even though *Δpb508*, *Δpb1289* and *Δpb1180* parasites were transmitted following bites by *A. stephensi* mosquitoes, transmission appeared to be unsuccessful following *A. gambiae* bites (Table 3.6). The parasite population from the *A. stephensi* bitten and infected mice (output parasite population) was genotypically analysed, to ensure that transmission took place in the absence of the respective gene (*Pb508*, *Pb1289* or *Pb1180*) and exclude potential WT contamination (Figure3.17).

Table 3.6. Effect of *Pb508*, *Pb1289*, *Pb1180* disruption on sporozoite development in *A. stephensi* and *A. gambiae* mosquitoes.

Parasite	Midgut sporozoites		Salivary gland sporozoites		Infectivity to mice	
	Arithmetic Mean	SE	Arithmetic Mean	SE	Day 18	Day 21
<i>A. stephensi</i>						
WT	71753	2192.03	7762	2043.5	2/2	2/2
	57669	4167.33	16324	1045.33	2/2	2/2
	46792	2482.29	5134	1224.02	2/2	2/2
<i>Δpb508</i>	15997	1885.85	1158	65.05	2/2	2/2
	11660	1067.37	7265.5	972.62	1/2	2/2
	9976	1887.26	2845	1267.84	1/2	2/2
<i>Δpb1289</i>	9945	683.77	2719	1871	2/2	2/2
	10710	1307.79	948	2143.59	1/2	2/2
	12386	874.69	1304	3043.38	2/2	2/2
WT	5714	202.2	2630	77.78	2/2	2/2
	6903	795	2856	118	2/2	2/2
	46792	2482.29	5134	1224.02	2/2	2/2
<i>Δpb1180</i>	756	48.7	300	70.7	1/2	2/2
	2343	200	720	123.7	1/2	2/2
	4087	343.3	1651	106.8	2/2	2/2
<i>A. gambiae</i>						
WT	10750	883	1800	70.7	1/2	2/2
	22160	1001	1900	141.42	1/2	2/2
	14100	2050	3200	1484.924	2/2	2/2
<i>Δpb508</i>	1212	79.19	245	35.35	0/2	0/2
	605	208	235	28.28	0/2	0/2
	2450	671.75	65	10.6	0/2	0/2
<i>Δpb1289</i>	7050	1378	450	176.77	0/2	0/2
	14712	1320	650	106.06	0/2	0/2
	5735	470.26	700	282.84	0/2	0/2
<i>Δpb1180</i>	375	94.45	113.5	70.3	0/2	0/2
	1735	456	278	45.60	0/2	0/2
	1505	215.7	400	141.42	0/2	0/2

The table outlines mean Pbc507 WT or $\Delta pb508$, $\Delta pb1289$, $\Delta pb1180$, sporozoite densities in *A. stephensi*/*A. gambiae* midguts and salivary glands. The mean was calculated by quantifying the number of sporozoites in suspensions from three pools of ten homogenised midguts or salivary glands, respectively, at day 15 and 21 post infection. SE represents standard error. Infectivity to mice was assayed by allowing WT or $\Delta pb508$, $\Delta pb1289$, $\Delta pb1180$ infected mosquitoes to feed on two C57BL/6 mice (bite-back) at day 18 and 21 of infection, respectively. Mice were allowed to recover and parasitaemia was assessed at day 5 post-feeding and up to day 14 if infection was not detected.

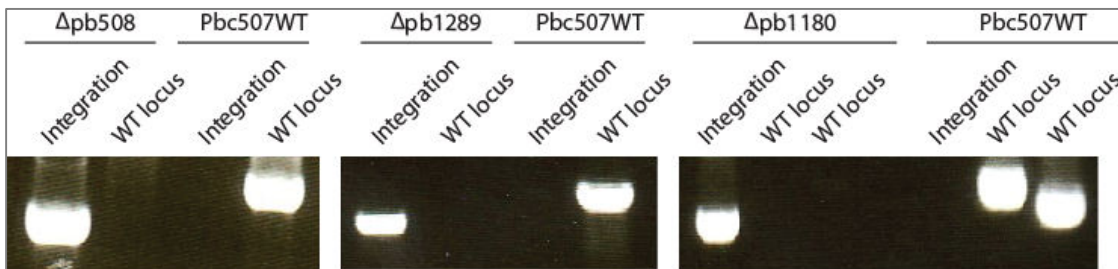


Figure 3.17 Genotypic analysis of output parasite population. Genotypic analysis of output parasite population by diagnostic PCR revealed that transmission of parasites following mosquito bites of naive mice, occurred in absence of the respective gene WT locus, when then mosquito was *A. stephensi*.

3.2.4. Summary

This chapter has outlined the following:

- **Generation of disruption vectors for 12 selected candidates was carried out, later transfected into schizonts.**
- **Transfection procedures for 12 selected candidates, produced five successful disruption mutants as confirmed by genotypic analysis, following drug selection**
- **Three disruption mutants ($\Delta Pb508$, $\Delta Pb1180$ and $\Delta Pb1289$) were dilution cloned and were subsequently put under phenotypic analysis**
- **$\Delta Pb508$ and $\Delta Pb1180$ ability to produce ookinetes *in vitro* was found to be significantly impaired compared to WT**
- **$\Delta Pb1289$ produced normal number of ookinetes, however mutant parasites suffered a defect in invading the mosquito midgut**
- **All three disruption mutants failed to be onwards transmitted to mice hosts by *A.gambiae***

3.3. Analysis of parasite gene function, sexual regulation and immune interactions with the mosquito host

3.3.1. Introduction

The recent completion of the *Plasmodium* genome revealed that some 60% (3208) of all predicted genes encode hypothetical proteins, lacking any functional domain predictions (Gardner et al., 2002). As a result, in depth characterization of selected candidates is mostly directed by phenotypic analysis. Due to time constraints only *Pb1289* was put forward for in depth functional characterization. This gene was chosen because its predicted secretory signal, in conjunction with its role in midgut invasion may suggest a role in parasite/mosquito interactions. Prior to this point however, it was decided to pursue some interesting issues relating to all candidates, and that was their sexual regulation and immune interactions with the mosquito host.

Following fertilisation the diploid zygote undergoes meiosis, not followed by cellular division, thus resulting in the tetraploid ookinete. At this stage the developing zygote is provided with both female and male gene alleles upon which transcription could commence. However, little is known about the regulation of gene expression from the male and female genome post-fertilisation. Pre-synthesized products carried over by the mating gametocytes or potential sex specific expression of genes can determine the outcome of parasite further maturation in the developing zygote.

The concept that mosquito immunity could be an important determinant of the infectivity of *Plasmodium* for the mosquito was put forward almost a century ago (Huff, 1927). This hypothesis was supported by the fact that the parasite undergoes substantial stage-specific losses during its development in the mosquito, while these stage-to-stage specific losses seem to depend on both the vector and the parasite species, supporting the existence of species-limited interactions (Alavi et al., 2003). The publication of the *A. gambiae* genome and comparative genomic analyses with *Drosophila melanogaster* have allowed the identification of immunity-related genes in *Anopheles* (Christophides et al., 2002; Holt et al., 2002; Zdobnov et al., 2002). These studies have demonstrated

that core innate immunity signalling pathways are conserved between the two species, while in combination with further transcriptional analysis (Christophides et al., 2002; Dimopoulos et al., 2002; Oduol et al., 2000; Povelones et al., 2009; Vlachou et al., 2005) have identified core components of innate immunity.

Following this chapter, gene names in roman indicate respective proteins i.e. Pb1289, , while italics indicate genes.

3.3.2. Sexual Regulation of Parasite genes

Phenotypic analysis of the three mutants revealed developmental defects, either during ookinete development ($\Delta pb508$ and $\Delta pb1180$) or during ookinete midgut invasion ($\Delta pb1289$). In order to determine whether *Pb508*, *Pb1180* and *Pb1289* gene function is required during early pre- or post-fertilisation, genetic complementation experiments with sex-specific deficient lines were performed. In case the respective mutant phenotype is rescued after crossing with male and female deficient line, requirement of the gene product post fertilisation is hypothesized, at least after both the male and female genomes are available for transcription. In case of sex specificity rescue, then, the respective gene product has to be present and functional pre-fertilisation or in very early stages following fertilisation, before both genomes (male and female) become available for transcription.

Hence, genetic complementation of transgenic lines with male deficient ($\Delta pb48/45$ display impaired male fertility) (van Dijk et al., 2001) and female deficient ($\Delta pb47$ display impaired female fertility) (Khan et al., 2005; Mair et al., 2006; van Schaijk et al., 2006) lines was carried out. Study of ookinete conversion ratio and oocyst enumeration following fertilization was also performed to investigate potential sex specific effect in parasite maturation. Even though, both $\Delta pb48/45$ and $\Delta pb47$ lines are unable to fertilise *in vitro*, partial activation has been observed *in vivo* (van Dijk et al., 2001), thus experiments were complemented with mosquito ookinete membrane feeds to reveal defects in ookinete invasion.

3.3.2.1. *Pb1180* paternal allele inheritance is required for normal parasite development

A significant difference was observed between $\Delta pb48/45 \times \Delta pb1180$ and $\Delta pb47 \times \Delta pb1180$ genetic crosses, where $\Delta pb47$ appeared to almost rescue $\Delta pb1180$ ookinete conversion ratio, compared to crosses with $\Delta pb48/45$ (Figure 3.18 A). This was also reflected in respective oocyst counts following *A. stephensi* direct infections (Figure 3.18 B and Table 3.7). Significantly higher numbers of oocysts were observed in midguts of $\Delta pb47 \times \Delta pb1180$ infected mosquitoes compared to $\Delta pb48/45 \times \Delta pb1180$ infected mosquitoes. As shown by experiments presented in the previous chapter, a role of *Pb1180* in ookinete development was confirmed. Ookinete membrane feeds of

$\Delta pb47 \times \Delta pb1180$ produced a significantly higher number of oocysts comparing to membrane feeds of $\Delta pb48/45 \times \Delta pb1180$ ookinetes (Figure 3.18 C and Table 3.7).

3.3.2.2. Both paternal and maternal *Pb508* allele are required for normal parasite development

Interestingly, genetic complementation of $\Delta pb508$ with $\Delta pb48/45$ or $\Delta pb47$ lines retained for both genetic crosses a low ookinete conversion ratio (Figure 3.18 A), comparable to that of $\Delta pb508$. This was also mirrored in later oocyst development; where in *A. stephensi* mosquito feeds oocyst numbers remained low (Figure 3.18 B, C). Even though, a difference of borderline significance was noted following direct feeds, this was attributed to *in vivo* cross fertilization leaking of $\Delta pb48/45$ or $\Delta pb47$ lines, rather than phenotype rescue.

3.3.2.3. *Pb1289* is expressed from both male and female genomes

Genetic crosses of $\Delta pb1289$ with $\Delta pb48/45$ or $\Delta pb47$ lines, revealed a comparable number of oocysts in *A. stephensi* mosquito midguts. The difference of borderline significance between the two crosses observed following direct mosquito feeds was not confirmed in an ookinete membrane feeding apparatus, thus is solely attributed to *in vivo* activation of $\Delta pb48/45$ or $\Delta pb47$ mutants (Figure 3.18 B, C and Table 3.7). This finding suggests that, *Pb1289* can be expressed by either the male or the female genomes after they become available for transcription post fertilisation, in line with its invasion related requirement.

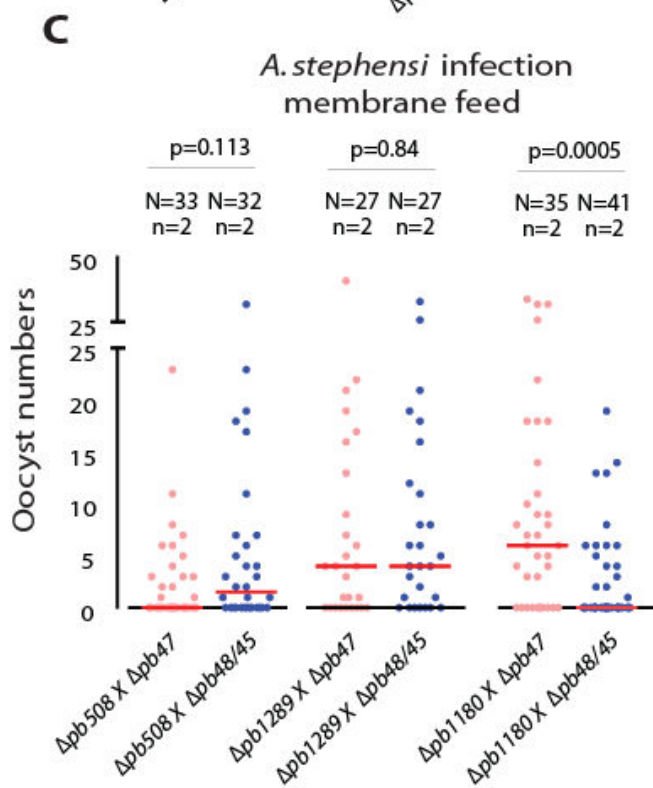
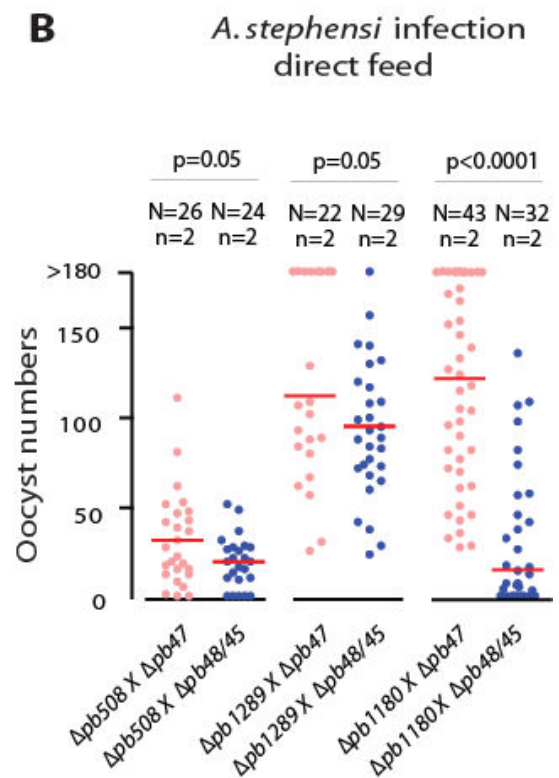
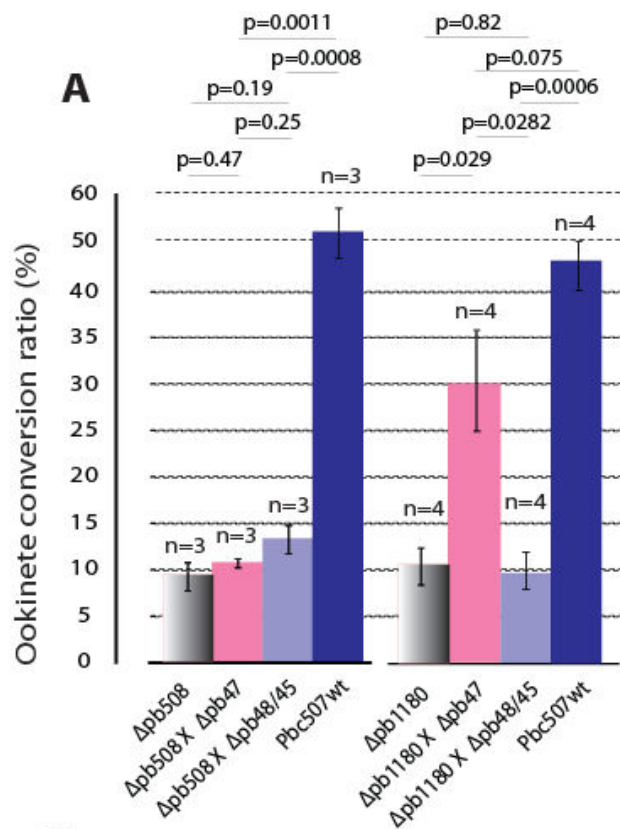


Figure 3.18 *Apb1180*, *Apb1289* and *Apb508* crosses with male and female deficient lines **A** No phenotype rescue is observed following cross of *Apb508* with male of female deficient lines. In contrast, *Apb1180* ookinete conversion ratio is significantly increased when the male allele is provided. **B, C** Following direct and membrane feeds of anopheline mosquitoes, oocyst numbers are restored after genetic crosses with both male and female deficient mutants for *Apb1289*. For *Apb508* and *Apb1180* similarly, ookinete conversion ratio/oocyst numbers are not restored in either case for *Apb508*, but only when the male allele is provided for *Apb1180*. (P values as calculated by one-way ANOVA analysis of variance following data normalization, N: numbers of midguts; n: number of biological replicates; horizontal red bars indicate the median value (B and C), while black error bars indicate the standard error of the mean)

Table 3.7. Oocyst formation in genetic crosses of $\Delta Pb508$, $\Delta Pb1289$ and $\Delta Pb1180$ parasites with male and female deficient mutants.

Time point	Parasite	# of exp	# of midguts	Prevalence (%)	Parasite density		Parasite range	Analysis of variance		Fold difference
					Arithmetic mean	Geometric mean		P value	F value	
<i>A. stephensi</i> infection Direct feed										
Day 8										
	$\Delta pb508x \Delta pb47$	2	26	92	31.0	19.65	0-110	0.05	3.313	1.76
	$\Delta pb508x \Delta pb48$	2	24	83	18.8	10.76	0-51			
	$\Delta pb1289x \Delta pb47$	2	22	100	128.0	107.8	30-262	0.05	2.617	1.27
	$\Delta pb1289x \Delta pb48$	2	29	100	93.5	83.76	28-253			
	$\Delta pb1180x \Delta pb47$	2	43	100	125.5	105.15	35-203	<0.0001	3.310	10.56
	$\Delta pb1180x \Delta pb48$	2	32	71	30.8	9.968	0-135			
<i>A. stephensi</i> infection Membrane feed										
Day 7										
	$\Delta pb508x \Delta pb47$	2	33	45	2.27	1.07	0-23	0.1129	2.985	-1.98
	$\Delta pb508x \Delta pb48$	2	32	59	4.37	2.12	0-32			
	$\Delta pb1289x \Delta pb47$	2	27	70	6.91	2.99	0-41	0.84	1.210	-1.29
	$\Delta pb1289x \Delta pb48$	2	27	74	7.64	3.88	0-33			
	$\Delta pb1180x \Delta pb47$	2	35	71	7.16	3.69	0-34	0.0005	4.59	4.24
	$\Delta pb1180x \Delta pb48$	2	41	53	1.92	0.87	0-19			

The table reports results from $\Delta pb508$, $\Delta pb1289$, $\Delta pb1180$ genetic complementation with $\Delta pb47$ and $\Delta pb48$ and subsequent parasite infections of *A. stephensi*. Columns include number of independent biological replicates as well as total number of infected midguts. Prevalence shows the percentage of midguts with at least one oocyst. Midguts with zero parasites were also considered for calculation of the arithmetic and geometric (following data normalization) means of parasite densities (number per midgut). P and F values for each genetic cross were calculated using one-way ANOVA statistical test following data normalization. Fold differences between different crosses' oocyst densities were computed using the geometric means.

3.3.3. Putative role of mosquito immunity in mutant phenotypes

3.3.3.1. Mosquito immune response is implicated in $\Delta pb508$, $\Delta pb1289$ and $\Delta pb1180$ reduced oocyst numbers

The interplay between parasite development and mosquito immune response is currently not well understood even though its presence is well established in the rodent model systems (Blandin et al., 2004; Osta et al., 2004). An interesting question arising from the study of our mutants was whether mosquito elicited immune response could be held responsible for our mutant phenotypes and if so to what degree. A powerful way to determine this was by performing RNAi experiments against *LRIMI*, a major parasite antagonist followed by mosquito infections with the three mutants. These experiments indicate that silencing of *LRIMI*, can restore mutant parasite numbers to levels comparable to these of WT, indicating that mosquito elicited immune responses may play a role in reduced parasite numbers displayed by all mutants. Nevertheless, even though increased mutant parasite numbers are observed in absence of *LRIMI*, their levels fail to reach anywhere near that of *Pbc507*WT suggesting that none of the mutant' phenotypes can be solely attributed to the mosquito immune response. Rather, a partial contribution of mosquito immunity in combination with parasite specific deficiencies is more likely to be responsible (Figure 3.19).

Sample sizes, number of biological repeats, oocyst numbers and statistical analysis from all *Pbc507* WT, $\Delta pb508$, $\Delta pb1289$ and $\Delta pb1180$ mosquito infections in *LRIMI* kd and control *LacZ* dsRNA treated mosquitoes are summarised in Table 3.8.

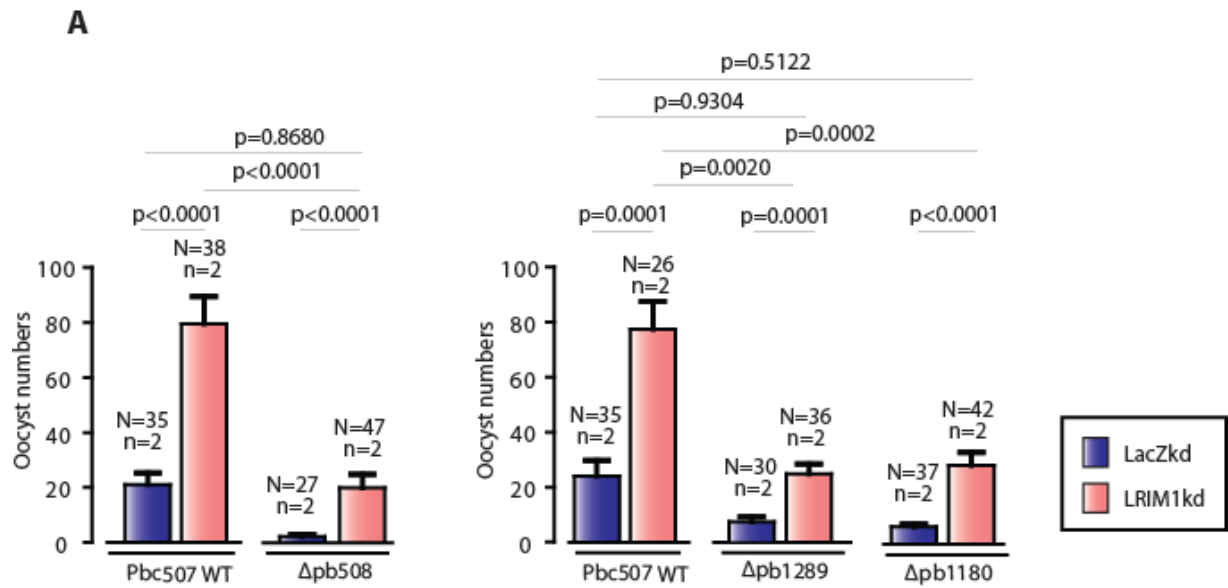


Figure 3.19. Pbc507 WT, *Apb508*, *Apb1289* and *Apb1180* infections of ds*LacZ* and ds*LRIMI* knock-down experiments in *A. gambiae*. Mean oocyst numbers are displayed for *Pbc507*WT and *Apb508*, *Apb1289* and *Apb1180* infections of ds*LacZ* and ds*LRIMI* depleted mosquitoes. (*P* values as calculated one-way ANOVA analysis of variance following data normalization, N: numbers of midguts; n: number of biological replicates; error bars indicate the standard error of the mean).

Table 3.8. Effect of *LRIMI* silencing on *Pbc507* WT, Δ *Pb508*, Δ *Pb1289* and Δ *Pb1180* oocyst development.

Time point	Parasite	Number of exp	Number of midguts	Prevalence (%)	Parasite density		Analysis of variance			
					Arithmetic mean	Geometric mean	Parasite range	P value	F value	Fold difference
<i>A.gambiae</i> infection										
Day 8	<i>Pbc507</i> WT									
	<i>LRIMI</i>	2	35	94%	74.79	54.11	0-250	<0.0001	4.684	5.7
	<i>LacZ</i>	2	38	81%	20.83	9.44	0-118			
	Δ <i>pb-508</i>									
	<i>LRIMI</i>	2	27	59%	16.61	5.99	0-87	<0.0001	18.82	6.5
	<i>LacZ</i>	2	47	36%	2.8	0.92	0-36			
Day 8	<i>Pbc507</i> WT									
	<i>LRIMI</i>	2	35	91%	72.39	50.44	0-250	<0.0001	4.272	4.6
	<i>LacZ</i>	2	26	83%	22.15	10.95	0-103			
	Δ <i>pb-1289</i>									
	<i>LRIMI</i>	2	30	80%	27.66	19.09	0-61	<0.0001	4.654	5.7
	<i>LacZ</i>	2	36	66%	7.61	3.34	0-35			
	Δ <i>pb-1180</i>									
	<i>LRIMI</i>	2	37	94%	28.70	16.97	0-80	<0.0001	14.15	5.2
	<i>LacZ</i>	2	42	71%	6.04	3.26	0-30			

The table reports results from Δ *pb508*, Δ *pb1289*, Δ *pb1180* or *Pbc507* WT parasite infections of *A. gambiae* *LRIMI* KD and *A. gambiae* *LacZ* dsRNA treated. Number of independent biological replicates and total number of midguts are indicated in the second and third column, respectively. Prevalence shows the percentage of midguts with at least one oocyst or live parasite. Midguts with zero parasites were also considered for calculation of the arithmetic and geometric (following data normalization) means of parasite densities (number per midgut). P and F one-way ANOVA values for each *LRIMI* KD vs *LacZ* KD infection were calculated using normalized density data of total parasite numbers. Fold differences between *LRIMI* kd vs *LacZ* dsRNA treated oocyst densities following infections with WT and Δ *pb508*, Δ *pb1289*, Δ *pb1180* were computed using the geometric means.

3.3.4. Functional analysis of Pb1289

3.3.4.1. Pb1289 is a putatively secreted subtilase

As shown during candidate selection Pb1289 is an 836aa length protein encoded by an intronless 2511bp open reading frame. The predicted gene product bears the characteristics of a secreted protein, containing a predicted cleavable amino-terminal signal peptide (1-30aa), and lacks other typical organelle targeting or membrane anchoring signals. BLAST homology searches of the Malaria Genome Project sequence databases identified sequences with high homologies of Pb1289 to proteins of the murine malaria parasites *P. chabaudi* (PCAS_110660) and *P. yoelii* (PY04330), the monkey (and human) malaria parasite *P. knowlesi* (PKH_102560) and human parasites *P. falciparum* (PFE0360c) and *P. vivax* (PVX_097925). These proteins appear to be also encoded by a single exon and constitute structural orthologues. Full-length protein comparisons showed that *Pb1289* is 80% and 70% identical to PY04330 and PCAS_110660, reflecting an orthologous ancestry, while identity with PKH_102560, PFE0360c and PVX_097925 was approximately 27%. Interestingly the presence of a signal peptide is conserved among all orthologs except for *P. knowlesi*.

Alignment of the six predicted gene products revealed the presence of two regions characterized by a variable level of amino acid sequence conservation between all orthologs, namely regions I and II (Appendix 1). Region I coincides with the presence of an aspartic acid subtilisin-active site (VCLIDTGL) as predicted by Interpro for the murine parasites *P. berghei*, *P. chabaudi* and *P. yoelii* putatively indicating the presence of putative subtilase activity. Subtilisins are non-specific serine proteases initially identified in *Bacillus subtilis*. Members of this family appear to have independently and convergently evolved a charge-relay network involving Asp/Ser/His arranged in a catalytic triad. In the human malaria parasites *P. knowlesi* and *P. vivax*, a longer subtilisin-like domain extending downstream and upstream of these conserved residues is also predicted, including region II. No evidence of putative subtilisin domain was detected for *P. falciparum*.

A schematic representation of protein models of Pb1289 orthologs is presented in Figure 3.20.

Pb1289 orthologs

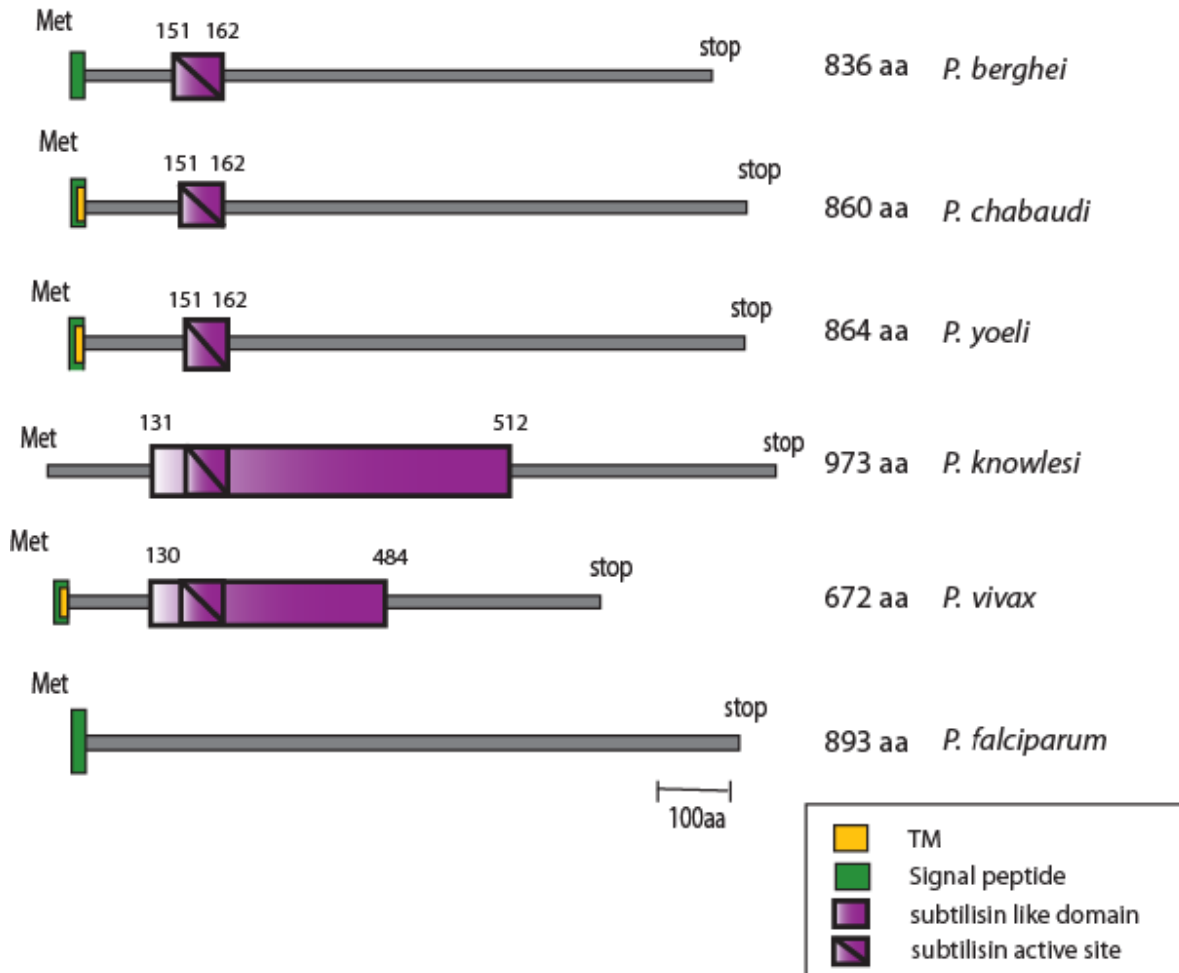


Figure 3. 20. Models of Pb1289 and orthologous proteins in other *Plasmodium* spp. The schematic protein models of *Pb1289* orthologs were generated by *in silico* sequence analysis and reveal that all *Plasmodium* orthologs except for *P. knowlesi* encode a signal peptide (solid green block), in an N-terminal position. Occasional detection of co-existent predicted transmembrane domains (solid yellow block) in *P. chabaudi*, *P. yoelii* and *P. vivax* are likely to serve as endoplasmic reticulum membrane guides, prior to protein release. In addition, all orthologues, with the exception of *P. falciparum* appear to encode for a conserved subtilisin related region (solid and crossed purple block), starting shortly downstream the secretion signal. Orthologs are aligned so that the predicted active site aspartic acid residue of each protein aligns vertically with *Pb1289* (*P. berghei*).

3.3.4.2. Epitope tagging of *Pb1289*

In order to gain further insights into Pb1289 protein function two avenues were explored including epitope tagging and antibody generation.

A MYC tag is a polypeptide protein tag derived from the c-MYC gene product that can be added to a protein using recombinant DNA technology. The sub-cloned gene targeting sequence is cloned (in the absence of its TAG stop codon) in-frame with a double MYC-tag epitope held on a gene-targeting vector, which is then introduced into the respective WT locus by single homologous recombination (cloning, transfection and genotypic analysis procedures as described for transgenic parasite generation). A scheme outlining MYC-tagging strategy is shown in Figure 3.21 and described in detail in Materials and Methods. In this study epitope tagging of Pb1289 was attempted in three batches of transfections. However, integration was never observed despite repeated attempts, thus was not eventually pursued.

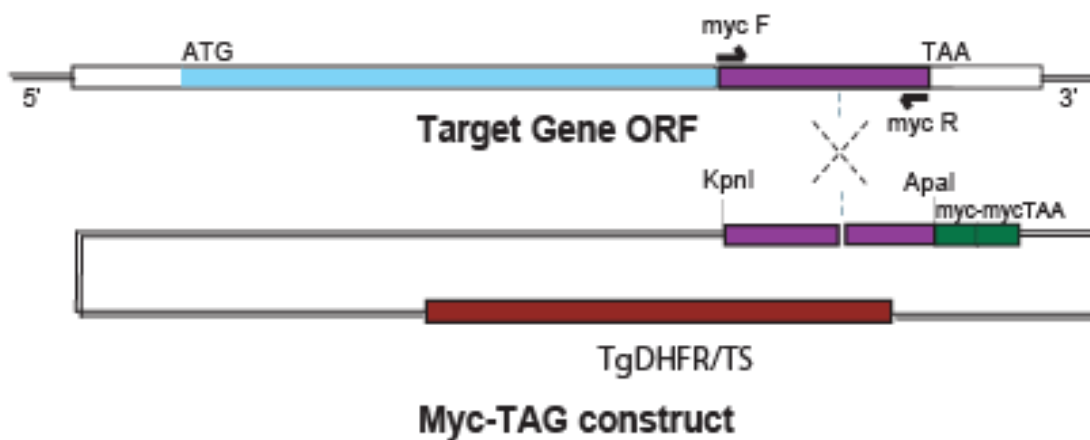


Figure 3.21. Epitope tagging. The gene targeting construct consists of a C-terminal fragment of the gene of interest (amplified by the respective primer pair MYC F and MYC R) in frame with a double c-MYC tag and a central, unique restriction site. Linearised vector integrates into the targeting locus by single homologous recombination, resulting in the tagging of the endogenous gene copy, and duplication of an incomplete copy. Positions of start and stop codons, annealing sites of amplification primers and sequence features are indicated.

3.3.4.3. Pb1289 peptide antibody design, generation and optimization

An array of choices of peptide antibody target sequences was initially provided by Eurogentec. Following Pb1289 multiple alignments with its orthologs, final sequences were selected, based on their degree of conserved amino acids among all orthologs. Rabbit polyclonal antibodies, against C-terminal and N-terminal peptides of Pb1289 (Pb1289IgG35 and Pb1289IgG36) were raised, purified and supplied by Eurogentec.

Distinct purified parasite stages were utilized to initially optimize Ab concentrations on parasite stages selected upon the likelihood of protein expression. *Pb1289* showed accumulating levels of transcription during ookinete development and phenotypic analysis implied that the protein is functional in the ookinete. Purified WT ookinete samples were probed with different concentrations of Pb1289IgG35 and Pb1289IgG36 until a specific signal was observed. *Δpb1289* purified ookinetes were used to control for non specific signal. Pb1289IgG35 was found to perform well in western blots, revealing a specific band of approximately 100kD for *Pb1289* as expected. In contrast, IgG36 was unable to detect the respective protein.

A schematic model outlining design and optimization of the above peptides is shown in Figure 3.22.

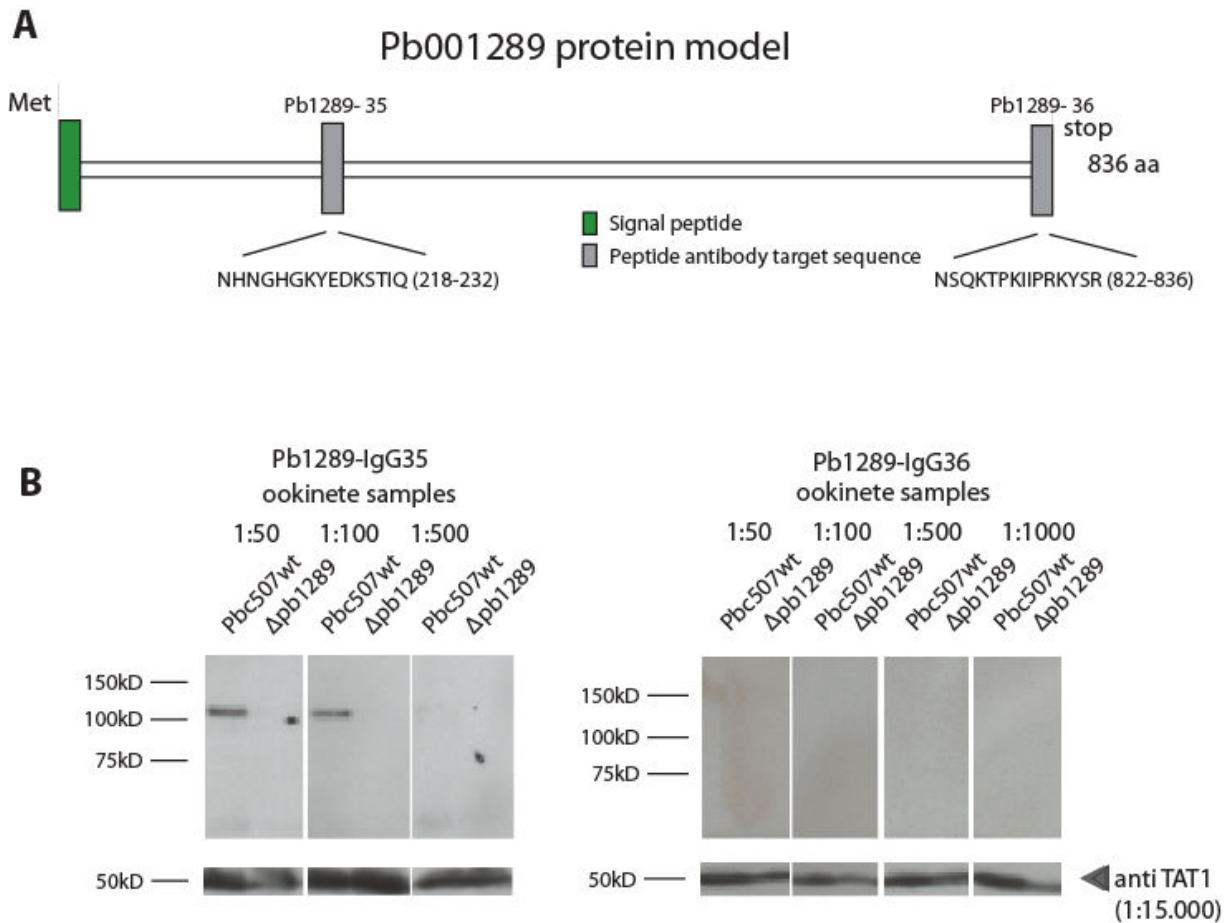


Figure 3.22 Design and optimisation of Pb1289 peptide antibodies (Pb1289IgG35 and Pb1289IgG36) **A.** Schematic model outlining the position and sequences of EP080783 (Pb1289-35) and EP080784 (Pb1289-36) peptide targets, against which, purified, polyclonal Pb1289 Ig s (IgG35 and IgG36) were obtained from the pooled sera of two immunised rabbits and supplied by Eurogentec **B** Western Blot analysis of *Pbc507WT* and Δ *pb1289* purified ookinetes testing and utilising different concentrations of Pb1289IgGs. Pb1289IgG35 clearly detects a protein in the region of the expected 100kD, at the concentration of 1/100, which is present in WT and absent in KO parasites. In contrast, Pb1289IgG36 does not seem to perform satisfactory in Western Blot analysis, in the concentrations tested here. A mouse anti α -Tubulin-1 (TAT1) antibody was used as loading control. All primary antibodies were incubated at 4°C over-night.

3.3.4.5. *Pb1289* protein expression peaks at the ookinete stage of development

In order to gain more information on *Pb1289* protein expression, western blot analysis was performed. For this study purified MBS, gametocytes (inactivated and activated for 20'/2h), and clusters of purified and non purified ookinetes were utilized. *Pb1289* protein product is detected during the ookinete stage of development where a full length ~ 100kD protein can be seen (Figure 3.23A)

3.3.4.6. Putative *Pb1289* cleaved forms are detected at the ookinete stage

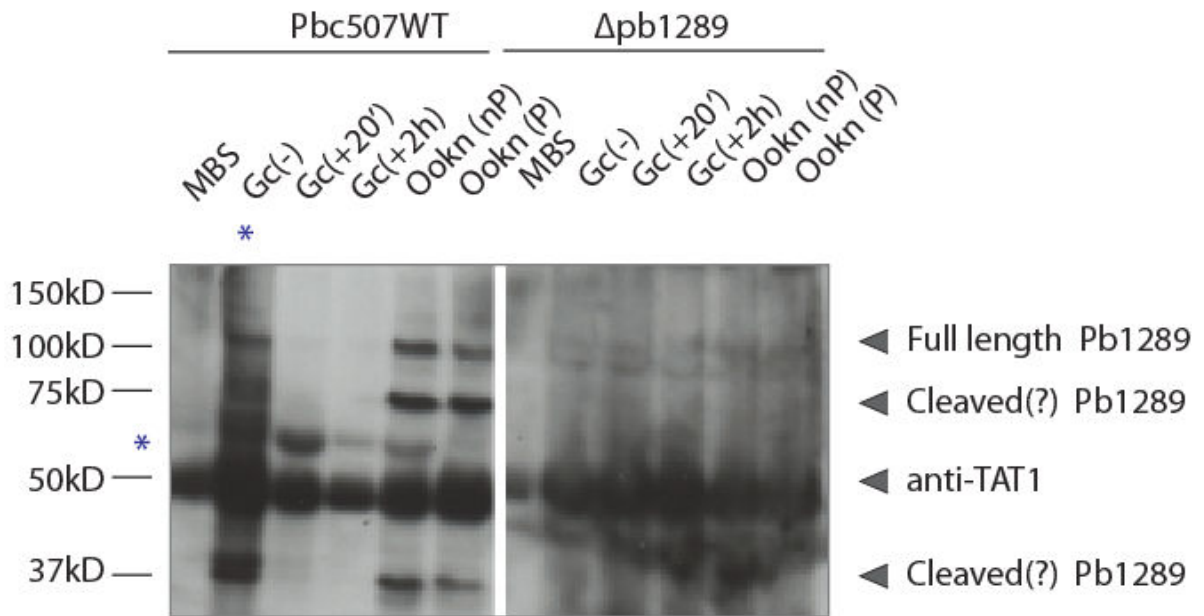
A number of smaller size bands were also detected at the ookinete stage, both in reducing and in non reducing conditions as well as in the presence of protease inhibitors (Figure 3.23B). The latter finding suggests that these could represent cleaved forms of full length protein rather than complexes. Moreover, this cleavage is not attributed to proteolytic processes occurring during sample preparation, but most probably within the ookinete itself.

3.3.4.7. *Pb1289* is localized at the periphery and apical end of the ookinete stage

Acquiring knowledge of a protein's cellular localization is pivotal in achieving better understanding of its potential function. We proceeded with *in vitro* and *in vivo* immunofluorescence (IFA) assays of WT ookinete samples, using the *Pb1289* antibody target sequences. The major ookinete surface protein *Pbs21* was used as a control. Antibody binding was detected by utilizing, fluorophor-labelled secondary antibodies, and observed by confocal microscopy. Staining of *in vitro* 2.34 WT purified ookinetes revealed a strong peripheral distribution of *Pb1289*IgG35 with notable accumulation at the apical end of the parasite (Figure 3.24A). A second set of staining using the *Pbc507*WT and Δ *pb1289* strains confirmed this apical localization; however, peripheral staining was either absent or weak in this experiment (Figure 3.24B). These findings lead to two conclusions. Firstly, *Pb1289* is probably localized at the apical end of the ookinete, where its' major secretory organelle, the microneme is found, in accordance with prediction of a secretory signal. Secondly, one cannot exclude potential surface localization since a variable degree of signal was always observed, which was absent in the appropriate controls.

A

Pb1289IgG35 1:100 / anti-TAT1 1:15.000

**B**

Pb1289IgG35 1:100

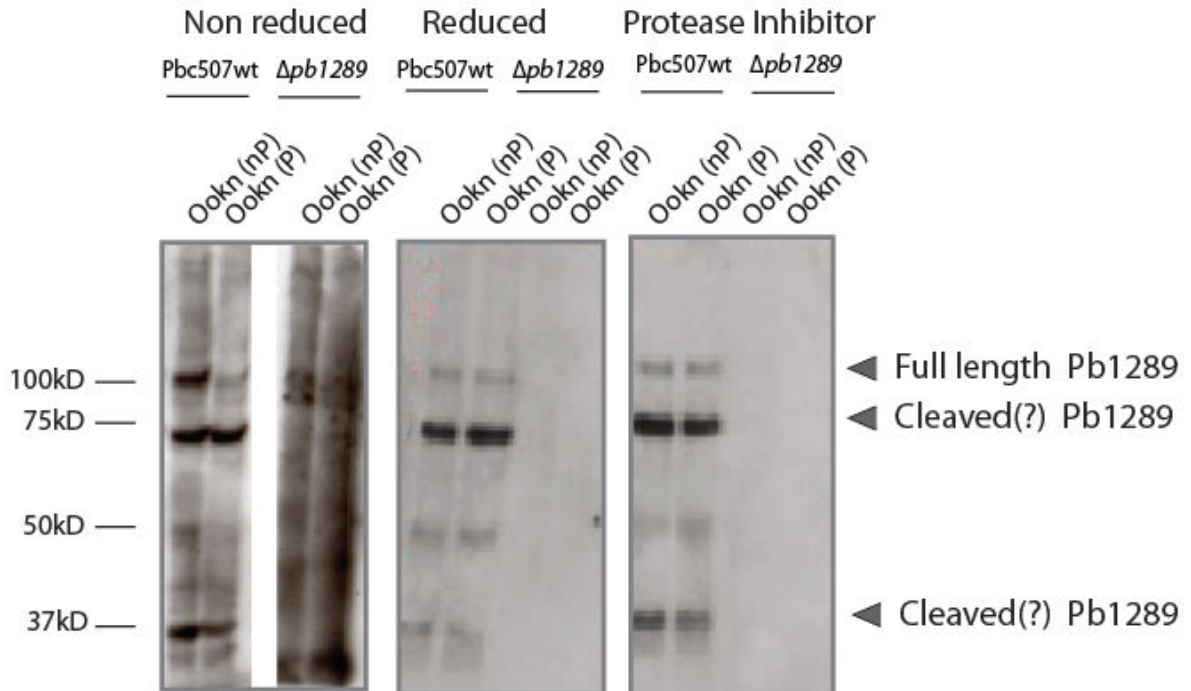


Figure 3.23 Developmental protein expression of *Pb1289* and protein cluster analysis

A. Western blot analysis of *Pbc507* WT and $\Delta pb1289$ purified parasite populations, probing with a rabbit Pb1289IgG35 and anti-tubulin (anti-TAT1) antibody. $\Delta pb1289$ parasites were used as a negative control. A strong signal migrating at ~100kD was detected during ookinete development, representing the expected full length Pb1289 protein as estimated after sequence analysis. Presence of alternate bands at ~75 and 37 kD were also observed. Asterisk (*) indicates background signal as confirmed by single labelling and independent experiments with single parasite stage populations. **B** Western blot analysis of *Pbc507* WT and $\Delta pb1289$ purified (P) and non purified (nP) ookinetes, in absence and presence of reducing agents as well as protease inhibitors. A strong signal migrating at ~75kD is detected, together with weaker signals at ~37 and ~100 kD, mirroring potential protein cleavage.

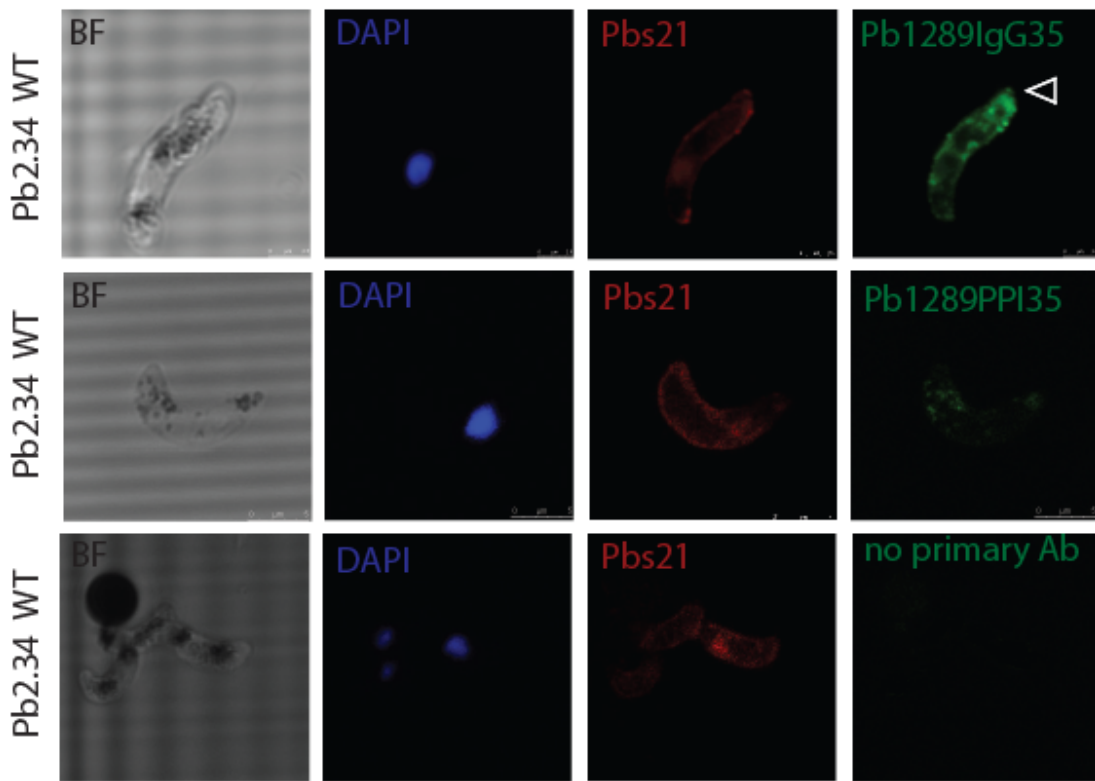
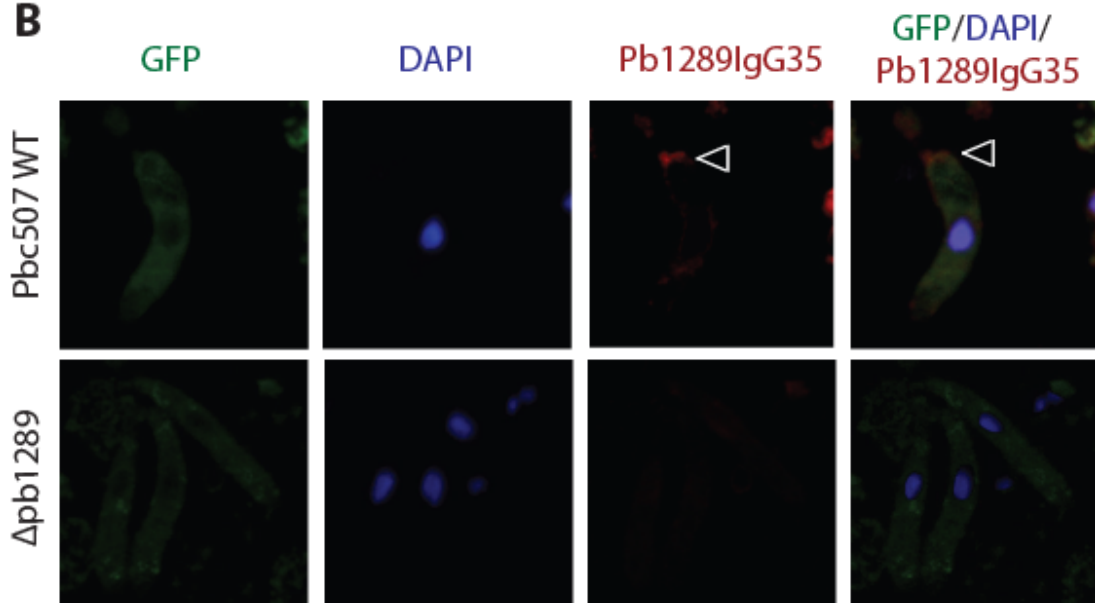
A**B**

Figure 3.24. Pb1289 localisation in purified ookinetes. A. Immunofluorescence images of purified Pb2.34 ANKA WT ookinetes stained with Pbs21 (red), DAPI (blue) and Pb1289 IgG35 or Pb1289PPI35 (green). Bright field visualization is also shown. Figure illustrates peripheral and apical distribution of Pb1289 (white arrow), as confirmed by preimmune serum (Pb1289PPI35) and no primary Ab control staining **B. Immunofluorescence images of purified Pbc507 WT and Δ pb1289 ookinetes.** Parasites were labelled with Pb1289 IgG35 (green) and DAPI (blue) for nuclear DNA detection. Strong apical signal for Pb1289 was detectable (white arrow), in contrast to a much weaker or absent peripheral staining, as confirmed by control staining of Δ pb1289. All images were taken from confocal sections of fixed parasites.

3.3.4.8. Pb1289 is soluble in membrane fractionation experiments

The results from ookinete staining that implied potential membrane localization of Pb1289 prompted us to investigate, whether the protein has indeed a potential membrane-anchoring ability by performing membrane fractionation experiments. Purified *Pbc507*WT and *Apb1289* ookinetes were sonicated prior to membrane extraction in using 1% Triton X-100. The non-soluble component was extracted from the latter fragment in 5% SDS at 100°C. Each fraction was collected by ultracentrifugation and analyzed by western blot. Pb1289 was observed neither in the 1% Triton X-100 fraction, where membrane-bound proteins are expected to locate nor in the Triton X-100 non-soluble fraction, where proteins only extractable by 5% SDS at 100°C are collected. Instead Pb1289 was observed in the cytosolic fraction that is released by sonication, indicating that Pb1289 is not membrane bound (Figure 3.25A).

3.3.4.9. Pb1289 is not secreted *in vitro*

Pb1289 sequence analysis confidently predicts a secretory signal. If this targeting signal results in extracellular secretion of Pb1289 – at least prior to invasion - then, the protein product is expected to be found in the medium within which the developing ookinete is maturing and functioning. To investigate this possibility, *Pbc507*WT and *Apb1289* parasites were allowed to grow *in vitro* for 24h, and following successful ookinete development, the culture medium was collected and *Pb1289* protein immunoprecipitated, utilizing Pb1289IgG35. Western blot analysis of *Pbc507*WT and *Apb1289* ookinete culture medium precipitates was performed (Figure 3.25B). The results revealed the presence of non specific bands of ~150 and ~250kD, in both *Pbc507*WT and *Apb1289*; but no

specific detection of *Pb1289*. This preliminary finding even though suggests that Pb1289 is not secreted *in vitro*, cannot be conclusive as to Pb1289 putatively secretion role.

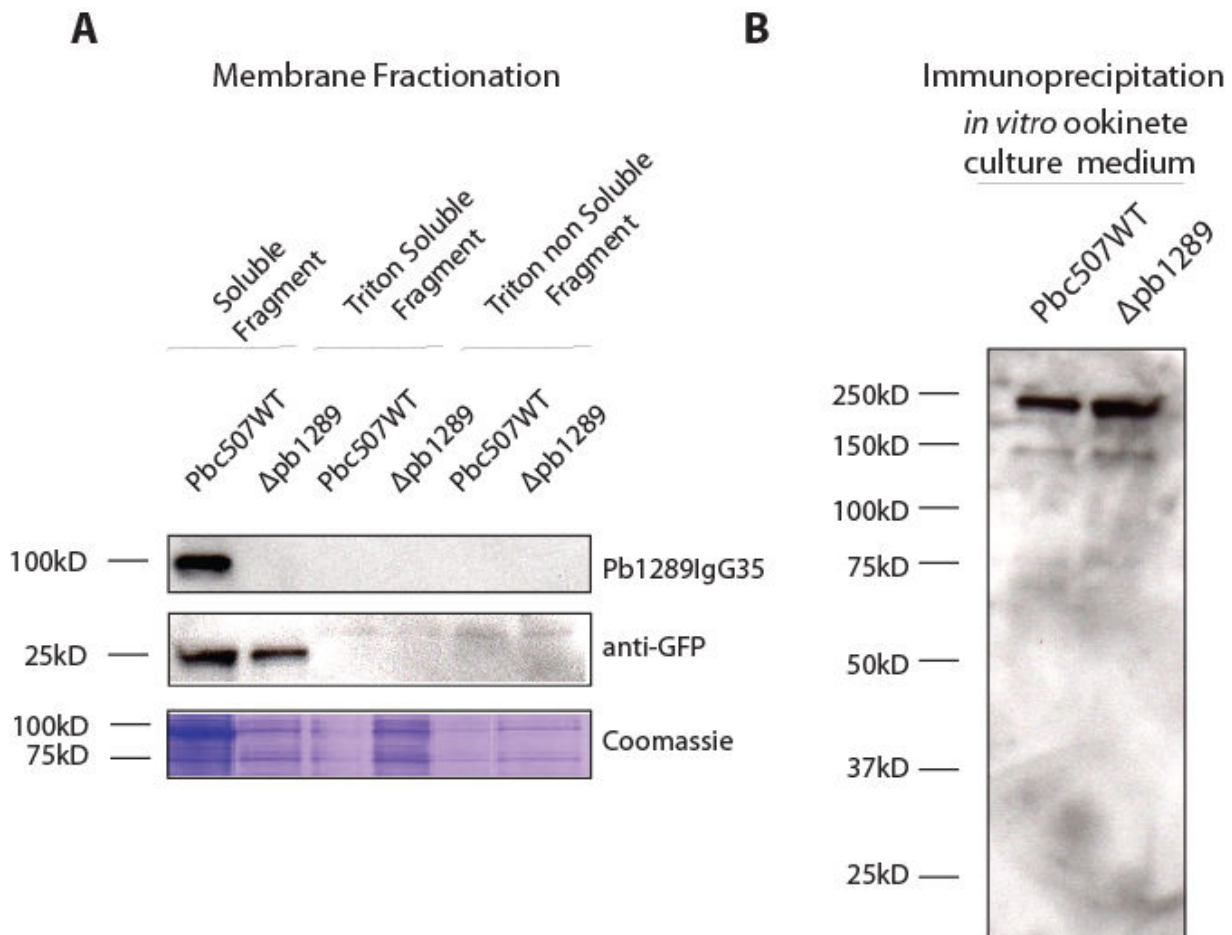


Figure 3.25 Western blot analysis of membrane fractionation and immunoprecipitation experiments derived from *Pbc507WT* and $\Delta pb1289$ *in vitro* ookinete cultures. **A.** Fresh ookinete fractions were collected by ultracentrifugation secondary to sonication (soluble component), extraction in 1% Triton X-100 extraction (Triton soluble fragment) and extraction in 5%SDS at 100°C (Triton non-soluble fragment). All fractions were probed with Pb1289IgG35 and a rabbit anti-GFP antibody serving as a control to the soluble fragment. Coomassie staining was used as a loading control to ensure successful extraction of parasite proteins in all fragments. **B** *In vitro* ookinete culture medium deriving from the same number of *Pbc507WT* and $\Delta pb1289$ ookinetes (used in A) was collected and subjected to immunoprecipitation with Pb1289IgG35 cross linked to protein A sepharose beads. Following beads extraction, samples were subjected to western blot analysis. Presence of Pb1289 in the culture media was not detected instead, non specific bands of ~150 and ~250kD were observed in both *Pbc507WT* and $\Delta pb1289$ samples.

3.3.4.10. *Pb1289* transcriptional expression remains unchanged in *Δpbmisfit* ookinetes

A recent study in the host lab (Bushell et al., 2009), utilising gene disruption of a nuclear formin-like protein *Pbmisfit*, revealed that *Δpbmisfit* ookinetes lack formation of micronemes. Moreover, a transcriptional down-regulation of a number of micronemal genes is observed in *Δpbmisfit* ookinetes, although, the reason for this is unclear. One hypothesis is that defect of microtubule-mediated microneme trafficking is defective in *Δpbmisfit* ookinetes resulting in accumulation of micronemal cargo in Golgi apparatus. This in turn may act as a potential negative feed-back, ultimately leading to further down-regulation of micronemal cargo genes such as *warp* and *chitinase* (Bushell et al., 2009).

In the view of the finding that *Pb1289* is apically localized and plays a role in midgut invasion, we investigated its putative micronemal nature was investigated, by utilising *Δpbmisfit* parasites as a tool to study transcriptional expression of *Pb1289* in *Δpbmisfit* ookinetes. Potential down-regulation of *Pb1289* in this setting would offer a stronger indication (but not a proof) of possible micronemal localization of *Pb1289*. RT – PCR revealed no change in the transcriptional pattern of *Pb1289* in *Δpbmisfit* ookinetes (Figure 3.26).

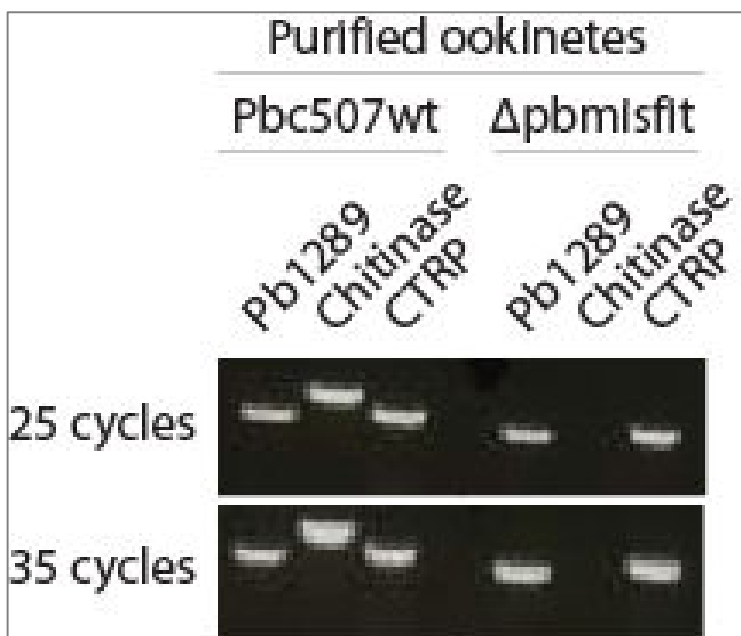


Figure 3.26 *Pb1289* expression in *Δpbmisfit* ookinetes. Gene specific primers were used to amplify *Pb1289*, chitinase and CTRP from cDNA pools obtained from bead purified, in vitro cultivated *Δpbmisfit* and WT ookinetes. Increasing numbers of cycles (25 and 35) were utilized. As expected chitinase expression is affected by loss of *misfit* gene, while CTRP expression remains unchanged despite its common micronemal localization. *Pb1289* expression levels remain unchanged in absence of *misfit*. Amplicon size ranges from 0.5-0.9kb.

3.3.5. Summary

This Chapter has outlined the following:

- *Pb1180* paternal allele inheritance is required for normal parasite development
- *Pb508* both male and female allele inheritance is required for normal parasite development
- *Pb1289* is expressed by both male and female genome
- *Pb508, Pb1289* and *Pb1180* seem to be implicated in eliciting mosquito immune response
- *Pb1289* is a soluble putatively secreted subtilase
- *Pb1289* protein expression peaks at the ookinete stage of development, when it is putatively cleaved
- *Pb1289* is found in the apical end and in the periphery of the ookinete
- *Pb1289* expression is not affected by the absence of *misfit*

4. DISCUSSION

Despite persistent and laborious efforts for malaria eradication over the last century, malaria remains one of the leading infectious agents causing global morbidity and mortality. The inefficiency of current preventive and therapeutic measures that are attributed to *Plasmodium*'s complex developmental cycle urge for a systematic control/therapy approach, reflected in both vector and host based research. Elucidation of the molecular mechanisms underlying the parasite's development and interactions with its vertebrate and invertebrate host will eventually succeed not only in providing deeper understanding of *Plasmodium* biology, but also promote generation of wide application therapeutic or disease control measures.

This thesis has focused on *Plasmodium*'s sexual and sporogonic development in the anopheline mosquito host, in particular on the ookinete-to-oocyst developmental transition. Successful completion of this stage is an absolute pre-requisite in order for the parasite to achieve onwards transmission to the vertebrate host. This stage also represents a major bottleneck in parasite's development in the mosquito host, making it an ideal target for intervention. The aim of this thesis was to identify and characterize genes potentially involved in ookinete-to-oocyst transition, including ookinete development and its interactions with the mosquito host. Overall, the goal of this study was to expand current knowledge and increase our understanding of molecular processes underpinning the parasite's complex development.

4.1. SELECTION AND TRANSCRIPTIONAL ANALYSIS OF CANDIDATE GENES

4.1.1. Selection of 113 candidate genes based on their developmental transcriptional profile in the mosquito host

This study was initially based on a near full (92%) genome, transcriptional microarray analysis of *Plasmodium*'s development in the mosquito host that had been carried out in the host lab (unpublished). In line with previous transcriptional and proteome studies, this study demonstrated

that transcriptional regulation plays a significant role in several different aspects of *Plasmodium* biology, as also reflected in the numerous morphological transformations, immune evasion and drug resistance mechanisms throughout *Plasmodium*'s complex life cycle (Kyes et al., 2007). The timing of expression for the majority of the expression clusters identified in all these studies seems to correlate with a known physiological demand for that process at that time, suggesting a “just-in-time” mode of control. This hypothesis states that genes are activated as their biological function becomes necessary to the parasite, after which the genes are down regulated. Several studies have established this finely tuned and accurate control of mRNA transcription during asexual replication of parasite development when distinct sub-sets of genes are expressed at different stages, as the invading merozoite develops intraerythrocytically via the ring and trophozoite forms to eventually undergo schizogony (Ben Mamoun et al., 2001; Bozdech et al., 2003). Similarly, sexual sporogonic development is characterized by significant changes in transcriptional activity (Hall et al., 2005; Young et al., 2005).

In particular, ookinete differentiation appears to be dependent on at least two different transcriptional strategies (Yuda et al., 2009). First a significant proportion of genes important for zygote maturation and initial ookinete differentiation are already transcribed in the female gametocyte but remain translationally repressed until immediately after fertilisation (Mair et al., 2006). This process is largely under control of the development of zygote inhibited (DOZI) RNA helicase in the absence of which transcripts follow the degradation pathway. Examples of such genes include the major ookinete surface proteins *p25 / p28* and *warp*; transcripts of which are stored, repressed and stabilised by DOZI in the female gametocyte, to be later expressed. The former transcriptional pattern of high expression early in sexual development, even though respective products are later observed, is mirrored in Pbs21-like microarray expression cluster, which was included in the selection of candidate genes. Interestingly, several of our candidates appeared to be regulated by DOZI (as summarized by Appendix Table 2), among which are candidates *Pb1343* and *Pb254*, for which transgenics were generated in the present study. In the absence of DOZI, *Pb1343* appeared to be approximately 3 times down regulated, implying that the respective transcript stretching from MBS to ookinete formation (Figure 3.5) is possibly subjected to translational repression, until the ookinete stage of development is complete, when it is finally translated (Hall et al., 2005).

Second a small set of genes appear to be specifically transcribed post fertilisation, mainly serving midgut invasion (Raibaud et al., 2006; Yuda et al., 2009). Transcription of these genes commences approximately 8 hours post-fertilisation and remains active until invasion of the mosquito midgut is completed. This was reflected in the ookinete specific expression cluster of genes, included in our list of potential candidates, including the known micronemal proteins *warp*, *soap*, *maop*, and *ctrp* (unpublished). This is in line with previous observations that *de novo* ookinete transcription is mostly related to ookinete's invasion/motility machinery as also observed by our study of Pb1289. Specific transcription of *Pb1289* in the maturing ookinete, is consistent with its role in midgut invasion and apical localization.

Even though transcriptional control plays a major role underlying parasite's development, the components of the complex programme of gene regulation that rules the plasticity of *Plasmodium* transcriptome remain largely unexplored (Vlachou et al., 2006). Initial analysis of the sequenced *Plasmodium spp.* genomes reported a lack of known *cis*-regulatory elements and transcription factors, homologous to other eukaryotes (Aravind et al., 2003). This led at first to the hypothesis that regulation of gene expression at the level of transcription was insignificant, compared to the impact of post transcriptional control and post translational modification. However, recent reports have changed this view, identifying several components consistent with the presence of classical eukaryotic transcription machinery in *Plasmodium* (Coleman & Duraisingh, 2008). Several experimental studies using upstream regions in transfection systems to drive expression of reporter genes (Dechering et al., 1999; Yuda et al., 2009) and other studies based on the correlation of mRNA expression and conservation of *cis*-acting sequences among divergent species (van Noort & Huynen, 2006; Wu et al., 2008) have shown that *Plasmodium* promoters do contain *cis*-elements several hundred bases upstream of the transcriptional start site that can either activate or repress transcription. This was also confirmed by Ontology based Pattern Identification (OPI) studies that used a combination of expression data and gene annotations to identify clusters of co-expressed genes (Young, et al., 2005; Young et al., 2008). A number of putative *cis*-elements were found in promoter regions of genes implicated in a range of cellular processes spread across 21 OPI clusters, covering sexual development, antigenic variation, sporozoite development, invasion, DNA replication and protein translation (Young et al., 2005; Young et al., 2008).

Presence of classical mechanisms of eukaryotic transcriptional control are further supported by identification of the Apetala2 (AP2) family; so far the only known gene family predicted to encode

transcription factor candidates in the *Plasmodium* genome, where in the human malaria parasite *P. falciparum*, 26 AP2-related genes are predicted (Balaji et al., 2005). A recent systemic investigation of the predicted AP2 factors, not expressed in asexual blood stages, came to discover AP2-O (AP2 in ookinetes) that is essential for the formation of invasive ookinetes and the expression of ookinete invasion-related genes. AP2-O is specifically transcribed in the female gametocyte and its associated transcript is translationally repressed in a DOZI-dependent manner. AP2-O translation is initiated post-fertilisation in the developing ookinete and its binding to a TAGCTA *cis* element, or four other closely related sequences, directly activates the transcription of all known *P. berghei* midgut invasion genes including *ctrp*, *soap*, *chit1*, *warp*, *p25*, *pbs21*, *maop*, *cdpk3* and two *psop* genes implicated in midgut invasion (Yuda et al., 2009). Nevertheless, interestingly targeted disruption of AP2-O resulted in the down-regulation (>5-fold) of only a small sub-set of 15 genes out of all the genes transcribed in the ookinete. This subset included four known ookinete specific micronemal genes (Dessens et al., 2003; Vinetz et al., 2000; Yuda et al., 1999; Yuda et al., 2001), six genes known to be transcribed in the developing ookinete (Raibaud et al., 2006) and five hypothetical genes, including two *psop* genes involved in midgut invasion (Ecker et al., 2008). Importantly, *Pb1289* was also identified among those 15 genes; a finding that correlates well with our transcriptional and phenotypic data that suggest ookinete specific expression and role in midgut invasion, in line with its N-terminus secretion signal. This study however (Yuda et al., 2009) failed to identify several genes that their transcription was predicted to be regulated by AP2-O, as implied by the presence of the respective *cis*-regulatory elements; expression of *p25* and *p28*, was not detected to be altered in absence of AP2-O, similarly to our candidates *Pb508* and *Pb1180*, despite the presence of TAGCTA *cis* element shortly upstream their ORF. Of these genes, *p25* and *p28* are abundantly transcribed in female gametocytes (Paton et al., 1993). Therefore, pre-synthesized abundant transcripts in previous stage of development could mask the defect of transcription due to AP2-O disruption later in the ookinete stage. Findings, from genetic cross experiments (Chapter 3.3.2) implying that *Pb508* and *Pb1180* respective products are required prior or immediately after fertilisation make it likely that transcripts observed in the ookinete have been priorly synthesized and stored, hence absence of AP2-O would not have an impact on respective transcript levels.

Based upon the hypothesis that *Plasmodium* genes specifically up- or down regulated are likely to be implicated in parasite differentiation and determine the outcome of parasite's interactions with

the mosquito, we utilized a parasite microarray developmental transcriptional analysis already conducted in the lab. We mainly focused on clusters of genes differentially regulated during ookinete-to-oocyst developmental transition. Key time points of this microarray study, upon which we based our selection included among others 24h PI coinciding with completion of ookinete development and midgut invasion, as well as, 48h PI reflecting early oocyst formation. In the future, it may be interesting for *in vivo* studies to include a more extensive time course, in order to gain specific insights into gene expression in distinct stages of the midgut invasion process. Even though, such a detailed study could offer a more comprehensive insight in transcriptional patterns during this developmental transition, such a task could be complicated by the asynchronous parasite development.

Subsequent to initial candidate gene selection, a number of bioinformatic filters were applied to further prioritize genes for functional characterization. To this respect, alignments of orthologous sequences of the *P. berghei*, *P. yoelii*, *P. chabaudi*, *P. vivax*, *P. knowlesi* and *P. falciparum* genes revealed that most *P. berghei* gene models were incomplete, many lacking both the start and stop codon. Gene predictions in *P. berghei* are largely based on the *P. falciparum* protein set thus *P. falciparum* gene builds were used to manually predict the N-termini of *P. berghei* gene models. It should be noted though that even in *P. falciparum*, annotation of N-termini is under constant revision, making many predictions ambiguous. Particularly the most N-terminal exon, which in most known exported proteins contains the signal peptide, is frequently missing from any gene prediction (Bowman et al., 1999; Marti et al., 2004); therefore, such genes could have been missed from our selection. As we sought for proteins involved in the interactions of the parasite with its environment, we prioritized genes that are putatively secreted (SP), localised to the surface (TM), or possessing specific functional domains known to be involved in extracellular protein interactions. Nevertheless, some proteins, such *PfEMP1*, can be secreted from the parasite without a classical signal peptide (Papakrivos et al., 2005) or on the other hand, some signal peptide-containing proteins are not secreted from the cell, but remain and function within organelles, thus inevitably complicating our selection. Similarly, our selection may have inappropriately included or excluded certain candidates, based on their functional domains. Indeed, proteins that would not have passed our filters, with functional domains that make an extracellular function unlikely (such as kinases) have recently been shown to be secreted into the host cell by *T. gondii* (Gilbert et al., 2007; Saeij et al., 2007) and *P. falciparum* (Schneider & Mercereau-Puijalon, 2005).

One of our criteria for candidate selection was clear orthology with human malaria parasite *P.falciparum*/*P.vivax* genes. An important goal of research in this field is to identify ideal targets for transmission blocking interventions, such as drugs and vaccines. Nevertheless, searching for highly conserved protein-candidates, so that model-system simulates in reliable fashion field-strain behaviour, can be misleading. If the main molecules interacting were highly conserved, that would automatically mean that not much parasite/host co evolution has happened. This comes in stark contrast with the enormous selective pressure put on these molecules along the evolutionary host/parasite arms race (Blanc et al., 2005; Shanks & Pyles, 2007). Hence, selecting only for highly conserved molecules bears a risk of actually biasing against them. Yet, where both rodent and human KO parasites were available, the phenotypes are either indistinguishable or similar (Claudianos et al., 2002; Dessens et al., 1999; Dessens et al., 2001; Dorin et al., 2001; Pradel et al., 2004; Raine et al., 2007; Templeton et al., 2000; Tsai et al., 2001; van Dijk et al., 2001; Yuda et al., 1999). Only in very few cases, such as *map2* the phenotypes were different: *map2* is essential for microgametogenesis in *P. berghei* and ABS development in *P. falciparum* (Dorin-Semblat et al., 2007; Khan et al., 2005; Rangarajan et al., 2005; Tewari et al., 2005)

Candidate selection using expression data in combination with bioinformatic analysis is a common approach in identifying molecules, playing an important role in parasite's developmental processes. A similar to our study large scale approach to identify components of ookinete's secretome has been carried out with success. This study, relied on a proteome dataset (Hall et al., 2005), to identify six ookinete secreted proteins important or essential for transmission (Ecker et al., 2008). However, more direct methods could also be envisaged for identifying putatively interacting surface molecules, including: i) cell fractionation and cell plasma membrane isolation, ii) surface protein labelling and affinity purification, or iii) surface protein removal, and identification e.g. by mass spectrometry. In either case, the generation of a cell surface proteome remains a challenging task, due to the difficulty of working with membrane proteins and the unpredictable responses of different cells and plasma membranes to labelling and fractionation. Importantly, successful isolation of surface proteins lies largely on the ability of the cells to remain intact so that contamination with intracellular components is avoided. Recently, an ookinete surface enriched proteome study was performed using ookinete surface-biotinylation and high throughput mass spectrometry analysis of affinity-purified biotinylated proteins (Dr R.Stanway unpublished). It would be interesting to see in

the future whether such approaches could prove more efficient in identifying key interacting parasite molecules.

4.1.2. Transcriptional analysis of 29 selected candidates by QRT PCR

Following candidate gene selection, we proceeded in the validation of gene expression profiles by QRT-PCR. This method was selected because of its high sensitivity, rapid use, and requirement of minimal amounts of input RNA, compared to techniques such as Northern blotting or ribonuclease protection assays. Results were subsequently normalized against *eGFP* expression, driven by the *eef1-a* promoter, in order to level naturally fluctuating parasite numbers. Of note, *eef1-a* is constitutively expressed throughout the *P. berghei* lifecycle, showing a slight but insignificant down regulation in male compared to female gametocytes (Franke-Fayard et al., 2004)

In total, QRT-PCR expression analysis of 19 out of 33 genes (8/10 Pbs21 like, 9/11 ookinete specific, 2/2 early blood bolus, ookinete and late sporogonic development) correlated well with microarray transcriptional analysis. No relation between correlation and degree of gene regulation was observed in contrast to previous reports noting lower correlation for genes exhibiting small degree of regulation, (generally less than 2-fold change) (Etienne et al., 2004; Rajeevan et al., 2001; Wurmbach et al., 2003).

For 14 genes discrepancies between QRT-PCR/microarray data were observed. Variability of both biological (e.g. alternative splicing, presence of polymorphisms) and technical factors (e.g. carry-over of organic / inorganic contaminating factors, varying efficiency of enzymatic procedures, data normalization) regarding both methods can be held responsible for such discrepancies. Different transcriptional activity was observed between the two oligonucleotide probes utilized in the microarrays for *Pb551*. Two pairs of QRT-PCR primers designed upon the annealing sites of the oligonucleotides confirmed this finding, showing a completely different pattern of expression, suggestive of poor annotation or potential alternative splicing (discussed below).

Further in-depth analysis revealed experimental error in array design, for genes *Pb773* and *Pb712*. After manual annotation, their respective microarray oligonucleotide probes were found to anneal on introns. This could be explained by PlasmoDB's reannotated gene models generated after the microarray platform was designed. It is worth noting that, due to the high AT-content of the parasite genome, over 80% of gene prediction, particularly of 5' ends and exon-intron boundaries, is

challenging (Gardner et al., 2002). Comparison between a large cDNA library with predicted gene models revealed that approximately 24% of genes models are inaccurate (Lu et al., 2007). Expression studies demonstrated that many genes have been missed, since many of the detected cDNAs or peptides seemed to match the parasite genome but not any of the predicted gene models (Carlton et al., 2002; Khan et al., 2005; Lasonder et al., 2002; Lu et al., 2007). These discrepancies can prove quite significant in later analyses as is reflected by the fact that 50% of the MS spectra recorded in the sex-specific proteome remained unassigned to genes (Khan et al., 2005).

Intrinsic microarray experimental inconsistencies between individual replicates were observed for genes *Pb467*, *Pb301371* and *Pb300785*. QRT-PCR transcriptional analysis of *Pb1039* and all early oocyst genes (*Pb679*, *Pb918*, *Pb692*, *Pb882*, *Pb111*, *Pb747*, *Pb1632* and *Pb105834*) revealed significant inconsistency. This finding was intriguing since all 8/8 genes peaking their expression during early oocyst development (48h PI) were found to be inconsistent between microarray and QRT transcriptional analysis. A potential explanation could lie within the different normalisation approaches utilized in microarray and QRT-PCR data. Global normalization against an average value versus individual normalization against GFP expression was used for microarray and QRT respectively. As a result this could potentially over or under-estimate gene expression at specific developmental time points. Further investigation is required to resolve the specificity of expression of these genes. Due to time constraints, genes not showing good correlation between transcriptional analysis of QRT and microarray data were not pursued further.

4.1.3. *In vitro* transcriptional analysis

In order to gain more information about the transcriptional profile of selected candidates, *in vitro* transcriptional analysis was carried out. This analysis revealed a temporal stretch of transcripts of the majority of genes, from ABS to ookinete stage of development. This finding complemented the initial Pbs21-like profile and ookinete enriched expression obtained by microarray/QRT analyses.

Interestingly, for *Pb508* mRNA was also detected in day 15 post infection, that timely correlated with midgut sporozoite egress prior salivary gland invasion. This finding is in accordance with previous studies that detect *Pb508* gene-product utilization not only during early but also late sporogony (Hall et al., 2005; Lasonder et al., 2008; Le Roch et al., 2004). As previously discussed, such genes could reflect a common set of conserved mechanisms underlying motility/invasion of

parasite zoite stages' such as the ookinete and the sporozoite (Baum et al., 2006). Such cases include members of the TRAP family involved in ookinete's (Dessens et al., 1999; Yuda et al., 1999) and sporozoite's (Sultan et al., 1997) gliding motility. Recently, the micronemal CelTOS protein, has been shown to be expressed during both stages, being involved in cell-traversal of the mosquito midgut epithelial cells and host Kupffer cells by the ookinete and sporozoite respectively (Kariu et al., 2006). Further investigation into such mechanisms, reflected in similar stage specific transcriptional patterns, could provide new insights into ideal targets for multi level acting transmission blocking vaccines.

Pb1289 transcripts were detected, starting early post fertilisation and peaking during ookinete formation. Contrary to Le Roch et al (Le Roch et al., 2003), who detected *Pb1289* transcripts almost throughout *Plasmodium* life cycle, no transcripts were observed later than the ookinete stage of development in accordance with Raibaud's previous report (Raibaud et al., 2006). This is consistent with our protein and phenotypic analysis which reveal that *Pb1289* is expressed in ookinetes and plays a role in midgut invasion. As previously noted, it appears that even though zygote and initial ookinete formation mainly relies upon the utilization of previously synthesized, stored and translationally repressed maternal transcripts; once a certain stage of zygote/ookinete development has been reached ookinete motility and invasion specific gene transcription take place. During asexual development the same strategy finds its analogue in the transcriptional "delay" of merozoite motility and invasion genes to late erythrocytic schizogony (Bozdech et al., 2003). Nevertheless, *de novo* gene expression in the ookinete has been described for a surprisingly small set of genes involved in ookinete specific functions including motility and invasion. *ctrp* mRNA is detected from 4 hours post gametocyte activation onwards, while the protein is first observed after 10 hours (Dessens et al., 1999; Vlachou et al., 2004; Yuda et al., 1999). Similarly, absence of expression in gametocytes by RT-PCR or northern blot is reported for *chitinase* (Dessens et al., 2001), *soap* (Dessens et al., 2003), and *warp* (Yuda et al., 2001), although contrarily Mair et al. (Mair et al., 2006) detect WARP protein in gametocytes. It would be interesting to determine how far ookinete development can progress in the absence of *de novo* transcription, for example by treating ookinetes with α -amanitin. Alpha amanitin inhibits activity of RNA Pol II, upon which transcription of mRNA relies in *Plasmodium spp.* (Callebaut et al., 2005; Militello et al., 2005).

For *Pb1229* two types of transcripts were detected. First, a product was detected in MBS, ABS, during and following fertilisation (1h/3h/8h ookinete culture) and in non-purified ookinetes,

mirroring respective transcript utilization during ABS. On the other hand, a 200bp shorter transcript was detected in MBS, Gc +/-, and during the course of ookinete development but not in ABS, indicating the presence of sexual development specific transcripts. These findings suggest a different regulatory pathway for *Pb1229* between sexual and asexual parasite development, in line with its exon/intron structure (Figure 3.4 Aii). Alternative splicing, through suppression of canonical splicing junctions and exon skipping, is a common RNA regulatory mechanism among multicellular eukaryotic organisms (Brett et al., 2002; Caceres & Kornblihtt, 2002; Stamm et al., 2005). However, the frequency of alternative splicing dramatically varies among living organisms, reaching 35–65% for human genes (Stamm et al., 2005). In *Plasmodium* despite the large number of introns predicted in the genome, information of how this mechanism affects the protein repertoire only comes from reports (Bracchi-Ricard et al., 2000; Knapp et al., 1991; Muhia et al., 2003; Singh et al., 2004; van Dooren et al., 2002; Volkman et al., 2001) while its prevalence remains unclear. Several key players in regulated RNA processing are readily identifiable in the *P. falciparum* genome, including homologues of the SR protein ASF/SF-2 and the SR-related protein U2AF-65, in accordance with a mechanism of spliceosome assembly on exonic splicing enhancer elements. Separate from creating different isoforms, alternative splicing may also be a mechanism controlling *Plasmodium* gene expression through introduction of early termination codons that lead to nonsense mediated decay (NMD) of such mRNA. Recently, a small-scale systematic analysis of alternative splicing in *P. falciparum* demonstrated that, not only alternative splicing does occur in *Plasmodium* more often than detected (~15%), but also has effects on the domain architectures of the respective gene products, which might result in modifying the cellular localization and function of these products (Iriko et al., 2009). This comes in accordance with the different needs of sexual and asexual development of the malarial parasite, as already discussed.

4.1.4. Selection of 12 genes for further functional analysis

Following transcriptional analysis, candidate gene selection included 11 conserved hypothetical proteins and a meiotic recombination factor (*Pb874*).

Among those, 8 genes (*Pb489*, *Pb846*, *Pb1343*, *Pb808*, *Pb1312*, *Pb874*, *Pb1229* and *Pb1289*) displayed the presence of secretory signals or transmembrane domains, and therefore were potentially implicated in parasite/vector interactions.

In addition, *Pb1229* encoded for a Sec I domain, feature involved in a variety of eukaryotic vesicle transport processes including neurotransmitter release by exocytosis (Halachmi & Lev, 1996). Sec I family members appear to regulate vesicle transport by binding to a t-SNARE from the syntaxin family, and preventing membrane fusion. Interestingly, although Sec1 molecules are essential for neurotransmitter release and other secretory events, their interaction with syntaxin molecules seems to represent a negative regulatory step in secretion (Bracher et al, 2000). This finding makes *Pb1229* an interesting candidate in view of recent findings, highlighting the importance of vesicle trafficking mechanisms for the formation and placement of rhoptries and micronemes (Schrevel et al., 2008).

Among selected candidates a putative meiotic recombination *dmc1* like protein (*Pb874*) was also included, encoding a Rad51 domain related to DNA repair and recombination (Proudfoot & McCulloch, 2005). In general, *dmc1* proteins are essential for homologous recombination during meiosis, playing an important role in generating diversity of genetic information. Notably, meiosis is an event only taking place during parasite's development in the mosquito host, unlike mitotic events occurring all throughout parasite's sexual and asexual development, including microgametogenesis, sporogony, liver-stage (exo-erythrocytic) and erythrocytic schizogony. Following fertilisation between the haploid female and male gametes, the resulting diploid zygote enters meiosis that takes place in the absence of nuclear division and cytokinesis. Surprisingly, the derived tetraploid parasite completes nuclear and cell division only after a week's time when completion of the endomitotic events that characterise sporogony and result in the release of infective sporozoites occur. Interestingly, *Pb874* role in meiosis is consistent with its *in vitro* expression profile that is similar to that of *Pbs21*. *Pbs21* transcripts start appearing in the developing gametocyte for the product to be utilized later during fertilisation that is followed by meiosis. Meiotic recombination within the mosquito host is supported by evidence from both laboratory genetic crosses between different strains of *P. falciparum* as well as field studies. Among the natural parasite population intragenic recombination in the MSP-1 gene is a major example of allelic variation (Kaneko et al., 1997; Tanabe et al., 2000). Amplification of the multi drug resistance gene (*pfmdr1*) on chromosome 5 (Triglia et al., 1991) or spontaneous recombinational rearrangements within a cluster of var genes on chromosome 12 also represent classical examples.

Sequence analysis and manual annotation of *Pb254*, predicted an actin filament domain, a feature most likely involved in parasite's cytoskeleton and contractile apparatus. Parasite zoites stages including the merozoite, sporozoite and ookinete move by gliding motility, a substrate-

dependent motility that occurs without change of cell shape and is unique to the *Apicomplexa*. Gliding motility is powered by the parasite's actomyosin motor and provides the force for host cell invasion (Baum et al., 2006; Baum et al., 2006; Dobrowolski & Sibley, 1996). In view of the motile nature of the ookinete that needs to enter the midgut epithelial cell in order to successfully complete the next stage of development, this gene was an excellent candidate to be potentially implicated in ookinete-to-oocyst developmental transition and interaction with the mosquito host.

Finally, *in silico* analysis predicted no specific structural characteristics for 3 of our selected candidates (*Pb508*, *Pb1180* and *Pb1363*). As already discussed, surface proteins mostly encode an N-terminal signal sequence that targets the protein to the secretory pathway. However, proteins without signal peptides have also been shown to be present on cell surfaces (Nickel, 2003). For example, the variant antigen *PfEMP1* is exported from the parasite to the erythrocyte membrane, although the only hydrophobic domain that could qualify as a signal sequence is located near the C terminus of the polypeptide chain (Baruch et al., 1995). Therefore, taking into concern their excellent transcriptional (*Pb508*, *Pb1180* and *Pb1363*) and proteome profile (*Pb508*), PlasmoDB's constant reannotation, absence of criteria against selection, as well as the possibility that those proteins might contain domains or carry out functions unique to *Plasmodium*, they were included in further analysis via targeted gene disruption.

4.2 GENERATION OF TRANSGENIC PARASITE LINES AND PHENOTYPIC ANALYSIS OF SUCCESSFUL DISRUPTION MUTANTS

4.2.1. Generation of transgenic parasite lines

A common approach for carrying out functional studies lies in reverse genetic technology, which we also used in the current study. It includes functional disruption of the gene of interest by e.g. targeted gene disruption (knockout - KO) or RNAi mediated silencing (knockdown- KD). Such an approach can be either a systematic gene-by-gene effort or a more wide-genome approach as is case of transposon-insertion mutagenesis. So far, almost complete, genome-wide KO, KD or mutant libraries have been generated for model organisms including *Bacillus subtilis* (Kobayashi et al., 2003) *Saccharomyces cerevisiae* (Giaever et al., 2002), *Caenorhabditis elegans* (Kamath et al., 2003), *Pseudomonas aeureginosa* (Jacobs et al., 2003) and the fruit fly *Drosophila melanogaster* (Dietzl et al., 2007), while efforts are currently in progress, relating to a production of a genome wide-KO mouse library (Accili, 2004; Austin et al., 2004). However, such advances in *Plasmodium* are currently hampered by a number of reasons including the absence of RNAi tools (Baum et al., 2009), as well as the practical bottleneck that parasite's propagation into mice poses, including time and cost. Therefore, as has been high lightened by this study and discussed below, a number of issues must be considered before any high throughput KO attempt in *Plasmodium*.

4.2.1.1. Generation of disruption constructs

Conventional construction of gene-targeting constructs by restriction-ligation cloning represents a significant constraint in any high throughput knock out approach, such as the one presented here. However, gene targeting constructs can be constructed directly by PCR, as is performed in yeasts (Giaever et al., 2002) and *Trypanosoma brucei* (Gaud et al, 1997), where long primers (~100 nucleotides) harbour the homology regions. In *P.berghei* though, generation of disruption constructs bearing such short homology regions would be inefficient, since double homologous recombination generally requires longer homology regions. Nonetheless, gene targeting constructs with long homology regions can also be constructed by PCR, and have been successfully used in a number of pathogenic and non-pathogenic fungi (Amberg et al., 1995; Davidson et al., 2002; Krawchuk &

Wahls, 1999; Kuwayama et al., 2002; Noble & Johnson, 2005), *C. elegans* as well as recently in *P.berghei* (Ecker et al, 2006). This method bears significant advantages with respect to speed and cost, since the whole process is amenable to robotic automation, requires only slightly longer primers, removes the hurdle of restriction enzymes, ligases, competent cells etc., and thus, can be completed within a day. It should be noted that generation of gene-targeting constructs described in this thesis was chosen to occur using construction of gene targeting vectors by restriction-ligation cloning, since by the time generation of KO lines commenced limited experience with the PCR method existed. In the future, the galloping field of gene synthesis technology (Khorana et al., 1972) may even overcome the advantages set by the PCR approach. Nonetheless, financial issues remain of concern, since, today gene synthesis costs far exceed sequencing technology cost.

4.2.1.2. Generation of disruption mutants

Gene-targeting constructs made during this study were introduced into *P. berghei* by electroporation of schizonts, grown *in vitro* overnight. Despite the high efficiency of the current electroporation technology (Janse et al., 2006), generation of transgenic parasites still remains a laborious task. Time required to reach optimal level of parasitaemia in schizont quality of the culture, and time required for selection of transfectant population, are only some of the constraints.

A number of parameters which generally thought to influence integration efficiency were considered during generation of KO constructs. First, as pairing of the homologous regions could be unfavourable if they flanked very long or very short genomic regions, the sizes of the genomic regions to be replaced with the selectable marker cassette (4,591 bp) were determined. No significant difference in efficiency of integration depending on the length of the replaced genomic region was observed; regions ranging from 471bp (*Pb508*, *Pb1343* #1) to 2,327 bp (*Pb1289*) were successfully replaced by the selection cassette.

The sizes of homology regions were then compared between the successfully and unsuccessfully disrupted parasites, as recombination efficiency is directly correlated with the length of the homologous regions. Successful gene-targeting can be achieved with relatively short homologous regions, but transfection outcome may improve following increase of the length of these regions (provided enough genomic sequence is contained in the contig). However, no such correlation was

observed in our group of 12 selected candidates. Successful integration occurred with pairs of homology regions varying from 500-600bps (*Pb254*, *Pb508*, *Pb1289*, *Pb1343*#1).

Moreover, the transcriptional state in the ABS could influence the transfection outcome. It has been previously reported that recombination with transcriptionally active loci is more efficient (Krawchuk & Wahls, 1999). On the other hand, genes that are expressed in the ABS may be essential and thus refractory to disruption, making such analysis difficult to assess. Ten out of 12 of our candidates showed experimental evidence of transcript presence during ABS (Figure 3.5), and only 4 out of these 10 were successfully disrupted (*Pb508*, *Pb254*, *Pb1180*, *Pb1343*) (Table 3.2) indicating a putative essential role for the rest of the genes. Moreover, one of the two genes that appeared to be transcriptionally silent during ABS (*Pb1289* and *Pb874*), was successfully disrupted. While these observations are interesting the size of our sample is too small to reach a firm conclusion. A meta-analysis, looking into all targeted disruption studies in *Plasmodium*, including this thesis, could provide a more solid ground for how the various parameters can affect the efficiency of parasite transgenesis.

Interestingly, intrinsic differences in DNA sequence could play a role in recombination efficiency. For example, utilizing two different constructs for *Pb1343* (#1 and #2) led to integration for one (#1) but not the other (#2) despite repeated attempts and increased homology region size that could theoretically enhance integration. AT richness may be an important factor that influences recombination efficiency since AT rich regions are more prone to DNA breakages. In our sample AT% varied from 70-80%, hence, no significant difference was observed to allow comparison.

To sum up, the efficiency of integration for our candidate genes reached 41% (5/12), not considering double constructs for some of them. For those genes that integration was never observed a number of reasons already discussed above could be responsible. Notably 85% (6/7) of those genes appeared to be expressed in ABS, while 71% (5/7) of them had ABS proteome hits (Hall et al., 2005). The latter observation in combination with the inability to knock them out suggests that the respective gene products could be essential, hence not dispensable for parasite blood stage development, during which integration occurs. However, definite proof would at least require a demonstration that the same locus can be modified without disrupting gene function (e.g. by tagging) (Kooij et al., 2005), or ideally by using genetic complementation techniques (i.e. the locus can be disrupted if the gene is provided in parallel as a transgene (Dorin-Semblat et al., 2007)). To date a number of techniques have been applied that allow mutagenesis or the down regulation of expression

to be controlled. Conditional mutagenesis using site-specific recombination in *P. berghei* has been successfully reported (Carvalho et al., 2004). Regulated expression of dominant negative genes, based on tetracycline-inducible transcriptional activation in *P. falciparum*, has also been established and might facilitate generation of conditional mutants for essential genes (Meissner et al., 2005). Finally, a powerful system, acting directly at the protein level has been established in *P. falciparum*, in which an engineered version of human FKBP12 (ddFKBP) is fused to the N or C terminus of a protein, promoting degradation of the fusion protein. In the presence of a ddFKBP ligand, degradation is mitigated, allowing regulation of protein levels in mammalian cells (Armstrong & Goldberg, 2007).

4.2.1.3. Genotypic analysis of transfectant population

Following schizont electroporation, parasite selection and genotypic analysis of pyrimethamine resistant parasite population, three different outcomes were observed: (i) homologous recombination and integration; (ii) maintenance of a circular gene-targeting vector as episome; or (iii) infrequently, none of the above with presence of only WT population.

The first case represents the most desirable outcome. Nonetheless in many cases pyrimethamine-resistant parasites were obtained without integration of the targeting construct. In most of these instances the transfected DNA was retained episomally. Notably, episome-containing parasites were first detected after drug selection, relatively in a later time (day 12-15 post transfection) than parasites where integration had occurred (day 7-11 post transfection). This is in accordance with previous reports that showed that parasites with episomes have a lower growth rate under drug pressure than parasites with an integrated resistance marker due to unequal episome segregation at schizogony (van Dijk et al., 1997; Waters et al., 1997).

Resistance was in some cases observed in the absence of episomes, suggesting it was due to a spontaneously arisen mutation. This has been observed occasionally and cannot be predicted (Janse et al., 2006). Theoretically, non homologous recombination could also be responsible for non detection of integration in presence of resistant WT; nevertheless it is not believed to occur in *P. berghei* (Menard & Janse, 1997; Waters et al., 1997)

4.2.1.4. Limiting Dilution Cloning

As already mentioned transfectant populations represent a mixed population of integrated but also often episomally contaminated WT parasites, which require clonal mutant isolation. Episomes represent a major drawback, as parasites cannot be easily cloned by limiting dilution *in vitro* since the number of mice that can be ethically and financially used is limited (Menard & Janse, 1997). Especially if the ratio of episome contaminated WT to KO parasites is high, (as roughly assessed by PCR and Southern blot analysis), limiting dilution cloning can be extremely difficult. Despite repeated attempts two of our mutants (*Pb254* and *Pb1343* #1) could not be successfully cloned.

Theoretically, it should be possible to eliminate episomes from parasite populations by growing parasites for prolonged periods without drug pressure (van Dijk et al., 1995; Waters et al., 1997). However, this will also select against gametocyte production, and for an increased parasite growth rate. Since sexual differentiation is a prerequisite for successful parasite development in the mosquito host, such an approach is not suitable for our study.

Similarly, episomal contamination could possibly be eliminated by mosquito passage, of a drug resistant mixed population consisting of episome-containing WT and KO, following mosquito feeding. The long period without drug pressure in combination with the enormous amplification in the oocyst and liver stage could select against episomal presence. Defects of KOs in prior stages of development could be rescued by cross-fertilisation with WT (Trueman et al., 2004; Yuda et al., 1999), thus maintaining the integrated genotype. If the ensuing sporozoite and liver stage development are normal, in principle the KO should be recovered from the blood stage infection originating from an infected mosquito bite. Nevertheless, the natural numerical bottlenecks before the oocyst and liver stage of parasite development pose constraints (Sinden & Billingsley, 2001). A large number of mosquitoes and mice would be required, to compensate for loss in parasite numbers, making such an effort inefficient in terms of time or resource.

4.2.2. PHENOTYPIC ANALYSIS OF KNOCK OUT PARASITE LINES

Mutants were assessed for their ability to successfully complete their development in the mosquito host, by allowing anopheline mosquitoes to either directly or membrane feed on infected mouse blood. Subsequent phenotypic analysis of $\Delta pb508$, $\Delta pb1180$ and $\Delta pb1289$ revealed developmental defects for all three successful mutants through their migration in the mosquito host. Before mutant phenotypes are discussed further, a number of common features encountered in this analysis should be noted.

A major limitation of this kind of analysis lies in the fact that, significant defects in early stages of development cannot easily permit detection of potential defects in later ones. For example, developmental stages following ookinete development (*Pb508 and Pb1180*) as well as midgut invasion (*Pb1289*) appear to be unaffected, implying that these genes are not important for parasite development further than the ookinete stage. However, this may only be an artefact of experimental settings. It should be noted that infectivity was studied under optimal transmission conditions -high gametocytaemia and maximal gametocyte infectivity (Dearsly et al., 1990) - and it cannot be ruled out that functional impairments may become apparent under less than ideal circumstances e.g. under stress or in more natural transmission settings. Furthermore, the phenotypic screen assessed only one aspect of parasite development, i.e. parasite numbers, which have overlooked more subtle differences. Reports in yeast have previously demonstrated that some gene deletions even though do not cause an overt mutant phenotype; exert an effect on the intracellular concentration of metabolites (Raamsdonk et al., 2001). Analysis of a KO metabolome can, by comparison with metabolic profiles of KOs of genes of known function, help to reveal the complete function of unknown genes.

Furthermore, even though all of our mutants displayed strong phenotypes, complete blockage was not observed (at least in *A. stephensi*) which could be explained by a significant degree of functional gene redundancy. Other proteins encoded by the parasite genome may be able to compensate for the lack of the respective deleted protein, thus hiding absolute effect of this gene knockout from view using the phenotypic analyses performed in this study. This could, for example, occur by a gene knockout resulting in transcriptional or translational up regulation of an alternative protein/pathway, able to compensate for the function of mutants. Stable switching of genotype and phenotype has been shown before in *Plasmodium* in relation to switching antigenic types (Wahlgren et al., 1999) and pathways of erythrocyte invasion (Dolan et al., 1990; Reed et al., 2000). For

example, in *P. falciparum*, disruption of *map1* leads to the possibly compensatory up regulation of *map2* (Dorin-Semblat et al., 2007). Ookinete-midgut interactions is a well known site of functional redundancy, as also suggested by the observation that mosquito infectivity of *P. berghei* was incompletely blocked even in the presence of two independent transmission blocking molecules, SM1 and α -amino peptidase N antibodies (Dinglasan et al., 2007).

Moreover, while our phenotypic analysis has underscored the importance of the encoded proteins in parasite biology, a reduction in numbers by itself cannot reveal much about the actual protein function. The absence of any known functional domains in candidates *Pb508* and *Pb1180*, even *Pb1289*, makes it difficult to suggest a function for these proteins. This is further illustrated by the fact that several years after their discovery and despite detailed information about their localisation, KO phenotype and/or crystal structure still no definite role can be assigned to proteins like *Pbs25*, *Pbs28*, *soap* or *warp* (Dessens et al., 2003; Tomas et al., 2001; Yuda et al., 2001)

Furthermore, for all three genes transmission was hampered in *A. gambiae* but not in *A. stephensi*. It is unclear if the transmission failure in *A. gambiae* is simply due to lower infection loads or due to an inherent defect in infectivity after passage through these mosquitoes. Although the chance of establishing an infection in the vertebrate host increases with increasing sporozoite numbers, 90% of C57BL/6 mice can already be infected with as little as 100 salivary gland sporozoites (Jaffe et al. 1990). It is also possible that these genes are only necessary for *P. berghei* development in its natural mosquito host *An. gambiae*, as it is well known that co-evolution shapes both parasites and vectors. A recent example of dynamic relationship between parasite and vector is illustrated by *lap1* gene. Even though, previously a complete lack of sporulation has been reported in $\Delta lap1$ infections (Claudianos et al., 2002), some midgut sporozoites have since been observed in *An. stephensi*, but not in *An. gambiae* (Dr A. Ecker PhD thesis 2007). Inoculation of mice with the exact same number of infective sporozoites deriving from either *A. gambiae* or *A. stephensi* is an essential experiment before any conclusion is made. If the same number of sporozoites can still result in no transmission in *A. gambiae*, then a species specific phenotype can be speculated. In the future, it would be also interesting to study the transcriptional profile of infective sporozoites, deriving from different anopheline species that could reveal components of such species-to-species interactions.

Finally, it should be noted that single clones for $\Delta pb508$ and $\Delta pb1289$ were analyzed. It is general notion that two or more independent clones are required to safely conclude that a mutant phenotype is due to the gene disruption *per se* and not due to random mutations introduced by the

transfection procedure. In other organisms, such as bacteria and yeast, this is routinely done by complementation, i.e. the disrupted gene is re-introduced as a transgene. This method as compared to the generation of independent clones allows control for effects of the integration of the gene-targeting cassette on the chromosome structure, or potential removal of gene regulatory sequences together with the gene of interest. However, complementation is often technically difficult in *Plasmodium* due to problems with gene cloning arising from the high AT-content and repetitive nature of many *Plasmodium* genes. A method to control for transfection induced unrelated mutations by restoration of the disrupted locus using negative selection has been described (Braks et al., 2006). Nevertheless, genetic crosses with gamete deficient lines and follow-up phenotypic analysis were carried out for all mutants, which to a certain extent controls for mutations that may have been created and selected for during transfection and cloning, respectively (Goldberg et al., 2010). Therefore, we believe that even in the two cases where single clones were examined the results can lead to reliable conclusions. Experiments towards generation of second clone and independent analysis in order the results described here to be confirmed are also currently under progress

4.2.2.1. Phenotypic analysis of $\Delta pb508$ and $\Delta pb1180$

Phenotypic analysis revealed that very similar phenotypes, for $\Delta pb508$ and $\Delta pb1180$ mutants, resulted in a markedly reduced macrogamete to ookinete conversion ratio. A possible explanation is that this phenotype results from the loss of a pathway that encompasses both proteins, e.g. it would be possible to hypothesize that one is involved in processing of the other. In such case, future epistasis analysis by crossing $\Delta pb508$ and $\Delta pb1180$ with each other could reveal a putative relationship between these proteins. The identification of several proteins involved in the same pathway could facilitate its characterisation, as in the recent example of *lap* family (Raine et al., 2007). An alternative explanation is that similar phenotypic changes observed in the absence of seemingly unrelated genes might be independent and concern pathways that operate in parallel. Finally these phenotypes may not be specific for the primary defect caused by the gene deletion, but may simply be the ultimate result of several “knock-on effects”.

Ookinete development requires a capable number of fertile male and female gametes, successful fertilisation and further maturation of the developing zygote. As a result, a defect in any of these steps could result in a markedly reduced number of ookinetes as observed for $\Delta pb508$ and $\Delta pb1180$. In

the first case of gametocyte activation, successful exflagellation was observed to numbers comparable to these of the WT for both mutants even though notably great variability among independent replicates was recorded. This is in line with previous observations supporting the importance of optimal environmental conditions for male gamete fertility. Even though not quantified, female fertility also appeared unaffected for both mutants. The surface protein Pbs21, was successfully detected on female parasite periphery, as expected following female gametocyte activation and parasite emergence from the red blood cell (Paton et al., 1993). Thus, defect in ookinete formation could be traced in two events: the inability of fertilisation to occur either from defect in one or both gametes, and the inability of the zygote to successfully mature to the motile ookinete, despite successful fertilisation.

The observation that the competence to produce ookinetes is restored by crossing the $\Delta pb1180$ with $\Delta pb47$ that is defective in female gametocytogenesis but not by crossing it with the male defective mutant $\Delta pb48/45$ confirms that female $\Delta pb1180$ parasites are competent for fertilisation, and that the phenotype is likely to be linked to male gametes. It is possible that, the male gamete suffers developmental defects that make it unable to fertilize the otherwise normal macrogamete, which may include recognition, adhesion, or presentation of accessory molecules. Even though successful exflagellation was observed in this study, no male gamete motility or fertilisation assays were performed; thus, *Pb1180* could be involved in either process. During exflagellation, the microgamete detaches from the residual body and is freely motile (Sinden & Croll, 1975). It is not known whether the microgamete meets the macrogamete by coincidence, whether it actively scans the blood meal, or whether it migrates along a gradient of an attractant that is released by the macrogamete. Interestingly, filamentous protrusions of the *P. falciparum* gamete surface have been recently identified, which form immediately upon activation and which appear to establish long-distance contacts between parasites in the mosquito midgut, increasing the chance of parasite mating (Kuehn & Pradel, 2010). Once the microgamete adheres to a macrogamete, fertilisation begins by fusion of the plasma membranes. Male gamete adhesion to a female gamete has been found to require the species-limited surface protein *p48/45* (van Dijk et al., 2001). *p48/45* interacts physically with at least one other gametocyte protein, *p230* (Kumar, 1987) and in *P. falciparum* is required to retain the complex on the cell surface once gametes have emerged from their host cells (Eksi et al., 2006). Of note, potential sex specific function requirement of *Pb1180* in a similar manner does not necessarily

mean sex specific expression, as has previously been reported in the case the *lap* genes (Khan et al., 2005; Scholz et al., 2008). Surprisingly, expression of the male-required *p48/45*–*p230* complex is observed in both male and female gametes (Khan et al., 2005; Kumar, 1987).

Similarly, the finding that crosses of $\Delta pb508$ with $\Delta pb47$ and $\Delta pb48/45$ respectively, fail to restore the mutant phenotype, makes it likely that provision of the respective allele or gene product by both gametes is needed in order for them to initiate and complete fertilization. One possibility for *Pb508* role in sexual development lies during gametes emergence from the host erythrocyte. It has been shown that egress of *Plasmodium* gametes from the host cell erythrocyte is governed by possibly common mechanisms for both male and female gametocytes, in which recently characterized *Pbmdv1/peg3* protein is implicated (Ponzi et al., 2009). It appears that *Pbmdv1/peg3* is involved in parasitophorous vacuole membrane destabilization, prior to red cell membrane lysis in a yet unknown mechanism in which other molecules could also be implicated. As expected, $\Delta Pbmdv1/peg3$ crosses with $\Delta pb47$ and $\Delta pb48/45$ fail to rescue the mutant phenotype, since neither the male nor the female gamete is capable of egressing from the host red cell and fertilizing. It is thus possible that in a similar manner *Pb508* is required by both sexes and could be implicated in the egress of their host erythrocyte. Another possibility could be lying on the process of gamete fusion, for which no widely conserved mechanism of has been identified to date (Chen & Olson, 2005; Primakoff & Myles, 2002; Rubinstein et al., 2006). Thus, it remains unknown for any organism whether adhesion and fusion of gamete membranes is accomplished by a single set of proteins, as happens with fusion of many viruses (White et al., 2008), or if these two processes are associated with distinct sets of proteins. The molecular basis of species specificity of gamete fusion is also not well understood (Ferris et al., 1997; Swanson & Vacquier, 2002; Vieira & Miller, 2006). Two recent studies on *P. berghei* described the identification of the microgamete protein GCS1 (generative cell specific 1), also termed HAP2, which enables gamete fusion (Hirai et al., 2008; Liu et al., 2008). GCS1/HAP2 is a conserved protein of algae and plants, where it is involved in pollen tube guidance and seed formation (Johnson et al., 2004; von Besser et al., 2006), and was also identified in protozoan parasites (Hirai et al., 2008; Liu et al., 2008; Mori et al., 2006). Importantly, conserved GCS1/HAP2 dependent process of membrane fusion follows and is not involved *per se* in the initial binding between the two mating partners, which appears to be mediated by other species-specific adhesion proteins (Liu et al., 2008).

The findings from phenotypic and genetic crosses analysis from $\Delta pb508$ and $\Delta pb1180$ leave open the possibility that respective transcripts/gene products are not required for fertilisation per se. On the contrary, requirement of paternal inheritance for *Pb1180* may be essential after fertilisation has successfully occurred. The same applies for *Pb508*, where both male and female allele may be required for zygote maturation. Studies in zygote to ookinete differentiation has provided genetic evidence that cell cycle progression in the zygote is an essential requirement for ookinete morphogenesis (Janse et al., 1986). It is thus possible that *Pb1180* and *Pb508* are implicated in cell cycle control, misregulation of which could result in abortion of zygote/ookinete maturation. In wild-type parasites, fertilisation is followed by fusion of gamete nuclei and one round of replication, increasing the nuclear DNA content of the zygote to 4N prior to meiosis. Following meiosis four sets of chromosomes are maintained within the nucleus of the ookinete, making this stage tetraploid. It would be pertinent to quantify the amount of DNA in $\Delta pb508$ and $\Delta pb1180$ developing zygotes, to investigate whether cell cycle progression can proceed normally in these mutants. Recently components of this process that fulfil a number of cell cycle-related functions in centrosome separation, mitosis, meiosis, and checkpoint control have been identified in *Plasmodium*, including the NIMA (never in mitosis/*Aspergillus*) related protein kinases (*Neks*) (O'Regan et al., 2007). *Neks* constitute an extended family of eukaryotic mitotic serine/threonine kinases, targeted disruption of family members (*Nek2* and *Nek4*), renders zygotes unable to differentiate into ookinetes. Pre-meiotic genome replication appears to be impaired in $\Delta Nek2$ and $\Delta Nek4$ (Reininger et al., 2005; Reininger et al., 2009). It would be interesting to see whether *Pb508* and *Pb1180* play a role in cell cycle progression. Epistasis experiments using $\Delta Neks$ mutants could offer further insight into the function of *Pb508* and *Pb1180*, including their temporal requirement, during parasite sexual development.

4.2.2.2. Sexual Regulation of $\Delta pb508$ and $\Delta pb1180$

Expression of *Pb1180* appears to occur during the gametocyte stage of parasite development as shown in this study. However, no confirmed differential expression of *Pb1180* in male/female gametocytes or gametes has been previously observed (Khan et al., 2005). Thus the failure of the female *Pb1180* allele to rescue the developmental blockade at the gametocyte to ookinete stage could be due to the female-derived *Pb1180* genes i) not being expressed, (ii) being expressed too late post fertilisation, after its essential function is required. Theoretically, RT-PCR analysis on purified

ookinetes from ookinete cultures of $\Delta pb1180$ (σ) \times $\Delta pb48/45$ (ρ) could reveal if and when female gametocyte/gamete expression takes place. Transcript absence could confirm the first scenario of no female transcription. Interpretation of transcript presence could prove to be more complex as it would be difficult to distinguish whether transcripts derive from expression of the female gene in a $\Delta pb1180$ (σ) \times $\Delta pb48/45$ (ρ) ookinete, or whether it is a result from contamination from unfertilised $\Delta pb48/45$ females, which also might express *Pb1180* genes and inevitably co-purify with ookinetes on a-*Pbs28* magnetic beads. Perhaps the use of *in situ* RNA hybridisation (Thompson & Sinden, 1994) or IFAs (once antibodies become available) and RFP-reporter constructs could overcome stage specificity problems, and be able to determine if and at which stage female *Pb1180* transcription takes place.

Similarly paternal *Pb1180* allele inheritance could be explained with several scenarios including (i) the male gamete contributes *Pb1180* mRNA, exclusively transcribed in the male and translated post-fertilisation (ii) the male gamete directly contributes *Pb1180* protein, or a combination of transcript and protein (iii) no meaningful contribution of transcript or protein is made by the male gamete during fertilisation, but the male *Pb1180* allele is made available for transcription post-fertilisation and (iv) *Pb1180* exerts its role during male gametogenesis. In the latter scenario, the mutant phenotype would be due to the inheritance of a defective male gamete. The first two scenarios would require translational repression of mRNA in the microgametocyte, which has not been demonstrated to date. In sperm cells different types of mRNAs are known to accumulate in the nucleus and are thereby introduced into the zygote (Dadoune et al., 2004). It would be interesting to determine if the microgamete nucleus carries significant amounts of RNA and in this case to characterise this population of RNAs. Several examples of paternal inheritance in *Plasmodium* have been described including genes with male specific functions that act pre-fertilisation (*cdpk4* and *map2*) (Billker et al., 2004; Tewari et al., 2005), during fertilization (*p48/45* and *hap2 / gcs1*), (Liu et al., 2008; van Dijk et al., 2001) as well as post fertilisation (*misfit*) (Bushell et al., 2009).

The inability of $\Delta pb508 \times \Delta pb48/45$ or $\Delta pb508 \times \Delta pb47$ crosses to rescue the mutant phenotypes is difficult to interpret. Potential requirement of inheritance of protein products from both male and female gametes could be likely during fertilisation. For example gamete fusion necessitates the presence of both male and female protein products. The same could apply if both female and male derived proteins (e.g. in a complex) are required very early post fertilisation i.e. to complete fertilisation or initiate zygote development, before the male and female genome become available for transcription. Both processes would be impaired in both $\Delta pb508 \times \Delta pb48/45$ or $\Delta pb508 \times \Delta pb47$

genetic crosses leading to persistence of the mutant phenotype. Alternatively, it could be possible that gene expression from both the female and male genome is required, in order for a critical threshold level of mRNA to be reached for later successful development. The latter scenario is consistent with the phenomenon of haploinsufficiency in diploid organisms, when a dominant loss-of-function phenotype is observed in a heterozygous state. A genome-wide study in yeast has demonstrated that haploinsufficiency is most likely caused by reduced levels of protein, and most commonly seen for essential genes, highly expressed genes, or genes encoding proteins that function in complexes (Deutschbauer et al., 2005).

This study has utilized mutants $\Delta pb48/45$ and $\Delta pb47$ in order to study the male and female contribution to the mutant phenotype. This however, bears the risk of observations that are specific to these two lines. For example if *Pb1180* or *Pb508* are involved in the same molecular cascades as *Pb48/45* / *Pb47*, targeted disruption of one could immediately affect expression or function of the other. For example the disruption of *lap1* also causes loss of *lap2* and *lap4* protein but not transcript (Pradel et al., 2006). Similarly, targeting of the gene encoding the rhoptry associated protein 1 (*rap1*) also abolishes rhoptry localisation of *rap2*, which is instead retained in the ER (Baldi et al., 2000). Genetic crosses with additional mutants, that are also unable to produce either functional female ($\Delta pbnek4$), (Reininger et al., 2005) or male ($\Delta pbmap2$ and $\Delta pbcdpk4$), (Billker et al., 2004; Tewari et al., 2005) gametes will clarify these issues.

4.2.2.3. Mutant phenotypes in immunocompromised mosquitoes

We sought to investigate whether *Pb508*, *Pb1180* and *Pb1289* were implicated in parasite-mosquito immune interactions by silencing *LRIMI*, a major parasite antagonist, and infecting mosquitoes with *Pb508*, *Pb1180* and *Pb1289* mutants respectively. Interestingly, a significant increase in oocyst numbers in *LRIMIKD*, compared to control LacZ dsRNA injected mosquitoes, was observed for all mutants. However, mutant oocyst numbers in *LRIMIKD* mosquitoes did not reach anywhere near the numbers observed for WT parasites. This observation further confirms the existence of intrinsic defects, responsible for the phenotype, rather than a specific interaction between mutant parasites and the mosquito immune system. It is possible that impaired parasites are killed and cleared faster than wild-type parasites and that in the absence of efficient immune system some of these parasites are able to survive and may arrest in development later, thus leading to the observed phenotype. These data correlate well with the hypothesis that mosquito immunity sets the base level at which vector-parasite interactions occur, and that the parasite has to overcome defence mechanisms that are already in place, and finally determine the outcome (Sinden et al., 2004).

Therefore the study of such interactions should be carried out with caution. For example, one problem using *P. berghei/An. gambiae* to model mosquito immunity to *P. falciparum* is that for many reasons, including the strikingly different infection intensities, mosquito immune responses to the rodent and human parasite are both qualitatively and quantitatively different (Dong et al., 2006; Tahar et al., 2002). Consequently, so far most immune effectors that have been identified in the laboratory model, such as *LRIMI*, *CTL4*, *CTLMA4* and *SRPN2*, did not influence *P. falciparum* infections in sympatric infections (Cohuet et al., 2006; Michel et al., 2006). Possible reasons for this include the lower infection intensity, higher temperature (26°C instead of 19°C) and co-evolution of parasite and vector in *P. falciparum* infections compared to *P. berghei* (Dong et al., 2006; Tahar et al., 2002).

4.3. FUNCTIONAL ANALYSIS OF PB001289

Importantly, the work presented in this thesis identified *Pb1289*, a putative secreted protein with a mutant phenotype manifested during the ookinete-to-oocyst transition. Interestingly, its N-terminus secretory signal appears to be highly conserved in all *Plasmodium spp.* except for *P. knowlesi*. The *Plasmodium* trafficking machinery presents various similarities to that of other eukaryotic cells, where proteins targeted to the ER -the initial step of the secretory pathway -carry a signal sequence at their N-terminus (Rapoport et al., 1996). Many of the parasite-secreted polypeptides, including all micronemal proteins, have a typical eukaryotic signal sequence at their N-terminus. Secretion of *Pb1289* in the ookinete culture medium could not be confirmed experimentally, which could be due to small protein amounts or translocation of the protein to the cell surface, possibly in response to stimuli encountered in the midgut.

In addition, further *in silico* analysis for *Pb1289* revealed the prediction of a highly conserved Asp subtilisin-active site related to a subtilisin like domain. Subtilases are an ancient group of serine proteases that are widely dispersed throughout evolution. Even though they seem to be functionally diverse, in the vast majority of cases, they are secreted and function either within the secretory transport system or extracellularly. Subtilases are characterized by the order in which their catalytic triad residues are arranged in their primary sequence (Asp-His-Ser) and the possession of easily recognizable sequence motifs around these residues (Rawlings et al., 2002; Siezen & Leunissen, 1997). Current annotation hampers confident prediction of putative subtilisin role for *Pb1289*. Also only a highly conserved Asp subtilisin active site is being predicted along *Plasmodium spp.* suggesting potential subtilisin like activity. Notably, this feature is not detectable in the *P. falciparum* orthologue.

In *Plasmodium*, three genes encoding products belonging to the superfamily of subtilisin-like serine proteases, or subtilases, have been identified. Two of these genes, *pfsub-1* and *pfsub-2*, and their gene products have already been partially characterized (Blackman et al., 1998; Hackett et al., 1999), whereas the presence of the third, *pfsub-3*, was revealed only by the *P. falciparum* genome project and has only been referred to briefly in literature (Reviewed by Withers-Martinez et al., 2004)). Both *pfsub-1* and *pfsub-2* are expressed in asexual blood stages, however, evidence exists to support *pfsub-2* expression in gametocytes (Florens et al., 2002) and a role in ookinetes (Han & Barillas-Mury, 2002). Interestingly current information on *Plasmodium* subtilases has shown that they are subject to a

mechanism of controlled protease activation in which the enzymatically inactive precursor (zymogen) is converted to an active enzyme only when it reaches an appropriate subcellular compartment. This is reflected in the fact that both *pfsub-1* and *pfsub-2* appear to undergo a two step maturation, via autocatalytic processing, during which the N-terminal is truncated twice, first so as to be targeted to an appropriate post ER compartment and second so that mature and functional protein form is shaped. The finding that *Pb1289* appears to be cleaved in the ookinete, as detected by the differential detection of size bands of ~35, ~75 and ~100kD, further supports its putative subtilisin-like nature. Nevertheless, in order to reach a conclusion about *Pb1289* protein function activity assays are required. This is shown not to be a trivial task, since the essential role of the propeptide in subtilase folding requires that it is included in expression constructs (Ikemura et al., 1987). As such, even minor truncations or modifications of the propeptide can prove to have detrimental effects on expression of active protease, while final expression constructs tend to be very large haltering the cloning process.

We experimentally showed that *Pb1289* transcript and protein expression exclusively peaks in the maturing ookinete. Interestingly, the transcriptional profile of *Pb1289* matches that of other genes encoding well characterised ookinete invasion-related proteins, such as *warp*, *soap*, *chitinase* and *ctrp* (D.Vlachou unpublished). This correlates well with previous studies reporting *Pb1289* to be under transcriptional control of the AP2-O transcription factor. As already discussed above AP2-O specifically binds to a *Pb1289* upstream sequence TAGCTA and regulate *de novo* ookinete specific transcription (Yuda et al., 2009). These findings indicate *Pb1289* involvement in ookinete midgut invasion, as suggested by its concomitant phenotypic analysis. Several genes involved in midgut invasion including *ctrp*, *warp*, *chitinase* have been observed to be *de novo* expressed by the developing ookinete post fertilisation (Raibaud et al., 2006). Nevertheless, several previous transcriptome and proteome surveys excluding a relatively recent SSHS library (Raibaud et al., 2006), extend *Pb1289* expression beyond the ookinete stage of development, including asexual blood stage and gametocytes (Florens et al., 2002; Lasonder et al., 2002). Interestingly *Pb1289* expression in the developing sporozoite has been also reported (Le Roch et al., 2003), but such observation was not confirmed by us or others (Lasonder et al., 2008). Interestingly, genetic crosses of Δ *pbl289* with female and male deficient mutants demonstrated rescue of phenotype by both male and female alleles. This comes in line with previous findings suggesting that invasion- related genes, like *PbPOSH* and *pplp5* (Dr A.Ecker thesis 2007), appear to be expressed from both the male and female genomes within the first 24 hours post fertilisation.

In PFA-fixed *in vitro* cultivated ookinetes, Pb1289 was shown to localise at the ookinete periphery and the apical end. The apical end can be identified morphologically both by shape and position of the nucleus; it is rounder than the posterior end and during ookinete maturation the nucleus migrates from the posterior end to the middle of the cell (Janse et al., 1985). Since the ookinete does not possess rhoptries or dense granules, protein secretion is mainly served via the micronemes, thus apical detection is suggestive of a localisation to the micronemes. However, unequivocal identification would definitely require co-localisation with an apical marker such as *ctrp* or use of immune-electron microscopy.

The putative secretion and micronemal localisation of Pb1289 is consistent phenotypic data clearly showing that $\Delta pb1289$ mutants have a markedly reduced capacity to invade the mosquito midgut. It is well established that *Plasmodium* micronemal proteins are involved in cell invasion (Dubremetz & Schwartzman, 1993). It is also consistent with the likely proteolytic cleavage of Pb1289 as previously shown for other micronemal proteins including *Pbsop2* and *POSH* (Ecker et al., 2008). Nevertheless, a recent study that characterized the micronemal proteome by MudPIT analysis did not identify Pb1289 in the ookinete microneme enriched fraction (Lal et al., 2009), suggesting that the protein if not absent, may be less abundant, developmentally limited or not easily amenable to MudPIT analysis. Alternatively, Pb1289 may be secreted by an as yet unknown pathway as reported for Pxs28 (Blanco et al., 1999). Analysis of permeabilised ookinetes by confocal microscopy showed also significant concentration of Pb1289 at the ookinete periphery. This could be attributable to its high concentration immediately below the ookinete cell surface, in a region that would coincide with the double inner pellicle membranes (IMC). Importantly, a previous study in *P. gallinaceum* ookinetes has identified the presence of pores of 43nm along the IMC of an as yet unknown function (Raibaud et al., 2001). These pores may constitute another pathway for the transport of molecules to and from the cortex, which is independent of the well-described route through the apical micronemal/rhoptry complex (Raibaud et al., 2001). In *Toxoplasma* vesicles packed along the IMC have been shown to form part of the secretory pathway for GRA2 (Mercier et al. 1998). Similarly we cannot dismiss the possibility that this same mechanism may form part of the trafficking network for the export of other proteins such as *Pbs25* and *Pb1289*. This hypothesis correlates with the fact that *Pb1289* expression is not affected in *misfit* null mutant ookinetes which suffer severe microneme-defect resulting in several micronemal proteins to be significantly down-regulated (Bushell et al., 2009). Nevertheless, additional well known micronemal proteins are also not affected by *misfit* disruption including *ctrp* (Dessens et

al., 1999) and *soap* (Dessens et al., 2003), although these proteins are found among the same micronemal population of proteins (Li et al., 2004) that are significantly down regulated in *Δpbmisfit* ookinetes, such as chitinase (Bushell et al., 2009). Perhaps a double export mechanism combining both IMC-pore/vesicle and micronemal export applies to Pb1289.

Unfortunately, repeated attempts of *in vivo* staining for Pb1289 were unsuccessful. However, as previously noted for the protein *maebl*, negative immunofluorescence data may not only be attributable to experimental faults. On the contrary, it could be indicative of the absence of a specific epitope, for example, due to *in vivo* conformation, proteolytic processing, or interactions with other proteins, and not necessarily the absence of the protein *per se*.

Data obtained from phenotypic analysis showed a clear defect during ookinete-to-oocyst developmental transition in *Anopheles* mosquitoes. This reduction, at least in the greatest part, stems from a diminished ability of *Δpb1289* ookinetes to invade the mosquito midgut rather than defects in ookinete motility or ability to escape the blood bolus. This is supported by the mutant's motility that is comparable to that of WT parasites, as well as, our inability to rescue the phenotype using ookinete membrane feeds. *Δpb1289* parasites showed increased losses after ookinete entry of the midgut epithelium., indicating that the impairment of midgut invasion is caused by a reduced ability to enter the epithelial cells. It would be interesting to investigate and confirm at precisely what point ookinetes fail to complete midgut invasion, for example by use of midgut invasion markers such as *Serpin 6* or transmission electron microscopy (Abraham et al., 2005). For example, *Applp5* ookinetes manage to cross the peritrophic matrix and are trapped in the microvilli network, failing to invade the midgut epithelium (Ecker et al., 2007). *ΔPOSH* parasites, on the other hand, fail even to achieve proper attachment to the midgut epithelium (Ecker et al., 2008). The above findings in combination with its putative secretory role leave open the possibility that Pb1289 could be an important player in parasite/vector interactions during midgut invasion.

Invasion requires prior contact between the anterior pole of the zoite and the host cell surface. It goes to completion rapidly, within the order of 20–30 s (Dvorak et al., 1975), is driven by an actinomyosin motor (Baum et al., 2006; Kappe et al., 2004) and in the ookinete is mainly facilitated by the regulated discharge of micronemes (Blackman & Bannister, 2001). Pb1289 lack of adhesion domains, as for e.g. *warp* does not support a potential adhesion-like role during invasion (Buscaglia et al., 2003; Robson et al., 1988; Trottein et al., 1995). On the contrary, prediction of putative subtilisin-like activity makes a proteolytic role more likely. Even though, numerous genes encoding putative

proteases of all known mechanistic classes have been identified in *Plasmodium* (Wu et al., 2003), the vast majority of them appear to be involved in intracellular processes including signal transduction or proteasome formation rather than invasion itself. However, the role of putatively secreted proteases such as Pb1289 has yet to be subjected to detailed experimental analysis. As noted before, *Pfsub1* and *Pfsub2*, are expressed in merozoites, the invasive form of the malaria parasite during blood-stage development (Barale et al., 1999; Blackman et al., 1998; Hackett et al., 1999; Sajid et al., 2000) These and other protease activities have been implicated in the invasion process, as potential mediators for processing the merozoite surface protein 1 (*mSP1*), and modifying the red blood cell surface (Blackman & Holder, 1992; Cooper & Bujard, 1992; McPherson et al., 1993; Roggwiler et al., 1996). In the ookinete stage of development only *Sub2* has been observed to play a role. Interestingly, *Sub2* is expressed by the parasite during invasion of the mosquito host and is secreted into the invaded midgut cells, where it has been hypothesized to modify the cytoskeletal network (Han, et al., 2000). A similar role for Pb1289 is likely, altering host cell cytoskeleton and promoting midgut invasion. Alternatively, it is possible that Pb1289 is implicated in proteolytic cleavage of other adhesive proteins involved in parasite motility and invasion that link the molecular motor to the invaded host cell. Even though *Sub2* has been shown to be implicated in the processing of *mSP1* and *Ama1* in *Plasmodium* merozoites (Harris et al., 2005), the enzymes responsible for cleavage of other transmembrane adhesions have not been yet identified, especially in the ookinete. Recently, a group of rhomboid-like serine proteases has been found in the *Apicomplexa*, a member of which, ROM4, is hypothesized to be responsible for the cleavage of *CTRP* in ookinete invasion of epithelial cells (Baker et al., 2006; Dowse et al., 2005; O'Donnell et al., 2006). It is likely therefore that Pb1289 is localized on the ookinete surface, after secretion and involved in such proteolytic activities. Nevertheless, before a clear establishment of Pb1289 putative proteolytic activity, role and function of Pb1289 during invasion can only be speculative.

Following defect in midgut invasion, a proportionally lower number of oocysts comparing to WT were observed. As shown by successful backbite infections of mice (at least by *A. stephensi*) *Pb1289* seems to be dispensable at the sporozoite stage; oocysts successfully sporulate, sporozoites egress, migrate through the hemolymph and successfully invade mosquito salivary glands and can subsequently establish mouse infections. Hepatocyte infection and liver stage development are not affected as suggested by the establishment of infection in prepatent periods similar to WT. However, bearing into mind the closely linked biology of zoite parasite stages (Baum et al., 2006) and previous

data relating to Pb1289 expression during sporozoite development (Le Roch et al., 2003), it would be interesting to investigate more closely sporozoite development with regards to Pb1289. Sporozoite staining or $\Delta pb1289$ sporozoite motility/invasion assays could explore some of the possibilities of potential Pb1289 conserved role in both parasite invasive stages.

4.4. CONCLUDING REMARKS AND FUTURE DIRECTIONS

This project represents an attempt to elucidate the complex molecular mechanisms between parasite-vector that underlie the ookinete-to-oocyst developmental transition. Taken together this thesis has come to further support expression patterns that govern parasite development in the mosquito host. Data have confirmed that strict and complex transcriptional control is closely linked to the parasite's successful development and transmission. In addition, it has expanded our knowledge in parasite biology, as mirrored by the identification and characterization of three novel genes that are involved in ookinete development and maturation, depletion of which results in severe defects and can subsequently hamper transmission by *A. gambiae* to rodent hosts. Functional and phenotypic analysis of the ookinete specific putatively secreted protease *Pb1289* has also opened new opportunities in studying and understanding parasite-vector interactions during ookinete-to-oocyst developmental transition. Besides follow up experiments described along this chapter, further research should aim to firmly establish whether *Pb1289* is a true secreted protease and identify its interacting partners and exact role during midgut invasion. Similarly, further information on putative function of *Pb508* and *Pb1180* needs to be obtained, so that their role is understood. Unfortunately, this task is not trivial, since lack of bioinformatic data on *Pb508* and *Pb1180* does not allow directed research. Perhaps an initial antibody mediated analysis, in terms of temporal and spatial expression could assist in later more depth investigation. Finally, this project has provided a large list of potential candidate genes for further functional analysis coupled with developmental transcriptional expression, manual bioinformatic annotation and complementation with global literature, as well as two remaining transgenic populations that are to be further explored ($\Delta Pb1343$ and $\Delta Pb254$). Even though, gene targeting with the resources and technologies now available is a valuable tool for understanding parasite biology, in relatively large-scale attempts such as the current study, one must not underestimate the difficulties, especially in times of stringent budget. Therefore, in my opinion, such approaches should be essentially combining the large number of post genomic datasets and studies available, in order for an educated choice of candidates to be made, and the highest degree of efficiency to be obtained.

The study of organisms that are also major human pathogens, particularly when it involves the experimental use of animals, should not only elucidate basic biological principles, but also aim at generating knowledge that could aid disease intervention or treatment. What potential applications exist for the data generated in this thesis? Ookinete development and midgut invasion both represent major population bottlenecks in the malaria life cycle that can successfully be targeted by intervention strategies such as TBV. The functional characterisation of the three newly identified proteins particularly expressed at this part of the life cycle can assist in the identification of new vaccine candidates. Nonetheless, such a task will be implicated by several quantitative and qualitative aspects of parasite life cycle. The redundancy of gene function in *Plasmodium* can render the impact of future intervention quite unpredicted. Although all of our mutants displayed decreased ability of ookinete invasion and oocyst development, full arrest was never observed. Prevalence of mosquitoes with oocysts remained high despite infection intensity being low, while the few but still remaining oocysts were able to sporulate and establish successful infection in rodents. Taking into account that mosquito survival is *Plasmodium* density dependent (Dawes et al., 2009), if an intervention reduces but does not eliminate mosquito infection, mosquito survival increases hence elongating in infection potential. Further constraints in intervention strategies are set by the recent observation that the developmental transition in the vector, despite its single-cell correspondence, is also density dependent i.e. progressively less efficient at increasing densities. Hence, interventions targeting specifically gametocyte, ookinete or oocyst production have different impacts upon parasite transmission at different forces of parasite infection, underlining the need for combinational strategies against malaria infection/transmission (Churcher et al., 2010). Nevertheless, using currently available technology it has been shown that in regions where transmission is low or moderate and mosquitoes mainly feed indoors, it should be possible to reduce parasite prevalence to less than 1% provided a sustained intervention program is achieved (Griffin et al., 2010). Importantly, however, in regions where malaria transmission is high or where mosquitoes rest and bite outside houses, new approaches are definitely required in order for malaria control and elimination to be successfully achieved.(Griffin et al., 2010)

APPENDIX

Appendix Table 1 Candidate genes

Plasmodium Transcription Programmes in the mosquito vector (Vlachou group, unpublished)	PlasmoDB description <i>P.berghei</i>	<i>P.falciparum</i> ortholog	Manual annotation	TM	SP	Features
Pbs21 - like						
PB000001.03.0	Skp1 family protein, putative	MAL13P1.337				Skp1 Family
PB000017.03.0	conserved hypothetical protein	PF11_0109				
PB000019.02.0	conserved hypothetical protein	PF08_0086			✓	RNA recognition motif
PB000089.00.0	conserved hypothetical protein	PF11_0443	PB000295.02.0		✓ ✓	DNAJ domain - heat shock related
PB000142.00.0	hypothetical protein		Ribosomal protein L3 ortholog			
PB000417.00.0						
PB000149.01.0	conserved hypothetical protein	PF07_0066	PB102502.00.0			RNA Binding Domain
PB000250.03.0	Vacuolar ATP synthase subunit D	PF13_0227				V-ATP synthase subunit D
PB000270.03.0						
PB000053.00.0	hypothetical protein	PF07_0029	heat shock protein			
PB001532.02.0						
PB300823.00.0						
PB000311.01.0	conserved hypothetical protein	PFL1485w				Prefoldin manual annotation
PB000314.03.0	conserved hypothetical protein	PF10_0101	retinitis pigmentosa (Py)	✓		
PB000319.03.0	hypothetical protein					Semaphorin domain
PB000325.03.0						
PB000332.02.0	hypothetical protein		PB000175.00.0 sharing ORF			Alpha-haemoglobin stabilising protei
PB000371.00.0	hypothetical protein		Blood stage membrane protein (Pk,Py)			Fibronectin
PB000370.00.0						
PB000434.03.0	helicase, truncated, putative			✓	✓	Helicase
PB000465.02.0	hypothetical protein		PB402192.00.0, PB403066.00.0			
PB300869.00.0						
PB000473.03.0	conserved hypothetical protein	PF14_0708				Phosphatase
PB000536.02.0	conserved hypothetical protein	PFA0420w				ELM 2
PB000752.01.0	conserved hypothetical protein	PF14_0633	Pfemp3 (Py)			
PB000779.00.0	Rhoptry-associated protein 1, putative	PF14_0102				
PB000812.02.0	conserved hypothetical protein	MAL13P1.233	ALBA protein (Pk)			alba like nucleic acid binding
PB000819.00.0	conserved hypothetical protein	PFL1735c				TPR -HAT
PB000840.00.0	conserved hypothetical protein	MAL8P1.95	PB000736.00.0		✓	
PB000856.03.0	conserved hypothetical protein		cathepsin C precursor		✓	Papain family cysteine protease
PB000590.03.0						
PB000866.03.0	conserved hypothetical protein	PF08_0073				
PB000892.02.0	chromatin assembly factor 1 protein	PF13_0149 PF14_0314 PFA0520c				WD40 domain
PB000909.03.0	conserved hypothetical protein	PF14_0324				TPR-Heat Shock Chaperon Binding Motifs
PB000922.00.0	heme binding protein, putative	PFI0885w			✓	Cyt-b6
PB001081.02.0	conserved hypothetical protein	PFL0855c				
PB001198.00.0	conserved hypothetical protein	PFC0465c				PWI domain
PB001232.02.0	hypothetical protein	PF10_0328	DNA binding protein			Bromodomain
PB001306.00.0	hypothetical protein	PFD0885c				
PB100823.00.0	hypothetical protein					
PB405718.00.0						
PB101572.00.0	conserved hypothetical protein	PFD0207c	PB000894.00.0 RanBP protein (Pk)		✓	
PB101678.00.0	hypothetical protein					
PB102173.00.0	conserved hypothetical protein				✓	
PB105908.00.0	hypothetical protein	MAL7P1.320	ribosomal all orthologs			ribosomal
PB106158.00.0	protein gar2, putative	PF13_0165			✓	
PB107035.00.0	hypothetical protein		PB107034.00.0			Ataxin
PB000704.03.0						
PB108856.00.0/ PB108056.00.0	hypothetical protein		332 antigen (Pf)			
PB401579.00.0	hypothetical protein					
PB405164.00.0	hypothetical protein					HSA domain
PB300953.00.0						
PB300508.00.0	ubiquitin-conjugating enzyme					Ubiquitin
PB103734.00.0	hypothetical protein					
PB000801.00.0						
PB000867.00.0	conserved hypothetical protein	PFC1055w			✓	
PB104218.00.0	conserved hypothetical protein				✓	
PB105866.00.0	conserved hypothetical protein				✓	Spoll1
PB102403.00.0						
PB000551.00.0	conserved hypothetical protein	PF13_0215				
PB000893.01.0	fibrillarlin	PF14_0068	PB105969.00.0			Fibrillarlin+
PB103082.00.0	hypothetical protein	MAL7P1.86	PB000518.01.0		✓	
PB301555.00.0						
PB107098.00.0	conserved hypothetical protein	PFL1600c				
PB001039.00.0	conserved hypothetical protein	PF14_0360				translation initiation factor eIF-2+
PB001229.02.0	conserved hypothetical protein	PF10_0331	PB106314.00.0/PB106313.00.0			Sec1
PB000846.01.0	conserved hypothetical protein	PFC0886w			✓	
PB000874.00.0	Meiotic recombination protein dmc1-like protein	MAL8P1.76 PF11_0087			✓	Rad1
PB000489.03.0	conserved hypothetical protein	PFF0835w			✓	
PB001312.02.0	conserved hypothetical protein	PF14_0510			✓	CS

Appendix Table 1 Candidate genes *continued*

Plasmodium Transcription Programmes in the mosquito vector (<i>Vlachou group, unpublished</i>)	PlasmoDB description	<i>P.falciparum</i>	Manual annotation	TM	SP	Features
Ookinete						
PB000096.01.0	conserved hypothetical protein	PF10_0039	membrane complex protein 1c (PY)			
PB000182.01.0	conserved hypothetical protein	PFC0720w				
PB000240.03.0/ PB000689.01.0	conserved hypothetical protein		Ubiquitin-like protein, (PY)			Ubiquitin domain
PB000409.03.0	conserved hypothetical protein	PFF0505c				PWI domain
PB000599.02.0/ PB000634.01.0	hypothetical protein		WD repeat protein orthologs			wd repeat
PB000626.02.0	ubiquinol-cytochrome c reductase hinge protein	PF14_0248				ubiquinol-cytochrome c reductase
PB000789.01.0/ PB000308.00.0/ PB000740.00.0	hypothetical protein		protein kinase all orthologs			kinase-like
PB000829.01.0	conserved hypothetical protein	MAL13P1.435				
PB000943.03.0	conserved hypothetical protein	MAL13P1.260	Pfemp3 (PY)			
PB000947.00.0/ PB000158.03.0/ PB000341.03.0/ PB000600.00.0	calcium-dependent protein kinase				√	kinase-like
PB000956.03.0/ PB404215.00.0	hypothetical protein					ATPase
PB000962.00.0	conserved hypothetical protein	PF10_0151	omeobox-containing protein (PY)			DNAJ
PB001016.01.0	conserved hypothetical protein	PF10_0310				GYF domain
PB001095.00.0	conserved hypothetical protein	MAL7P1.98				
PB300953.00.0	conserved hypothetical protein	MAL8P1.121			√	Coatomer subunit
PB001129.00.0/ PB000823.03.0	conserved hypothetical protein		DnaJ protein all orthologs			
PB401857.00.0/ PB400440.00.0	hypothetical protein					Transposase
PB000712.00.0	conserved hypothetical protein	PF14_0274				Diphthamide synthesis
PB000418.03.0	conserved hypothetical protein	PFI0945w	PDI-like protein (Py, Pk)			PDI- thioredoxin domain manual annota
PB000627.01.0	conserved hypothetical protein	MAL13P1.251			√	RNA Binding Domain
PB000653.00.0	conserved hypothetical protein	PF08_0116	c-13 antigen (Py)	√	√	PP2Cc
PB000317.00.0	conserved hypothetical protein	PF08_0008	Pb000129.01.0 sharing ORF	√	√	
PB000773.02.0	conserved hypothetical protein	PF11_0115				
PB108456.00.0/ PB000759.02.0/ PB000760.02.0/ PB300784.00.0	hypothetical protein	MAL8P1.154				
PB300785.00.0/ PB000674.01.0/ PB001555.02.0	conserved hypothetical protein	PF13_0058	RNA binding protein (Pf,Pk)			RNA recognition motif#
PB000254.00.0	conserved hypothetical protein	PFB0765w				AAA-Filament manual annotation
PB000508.03.0	conserved hypothetical protein	PF08_0119				
PB000808.02.0	conserved hypothetical protein	MAL13P1.103			√	
PB001180.00.0	conserved hypothetical protein	PFL0260c				
PB001289	conserved hypothetical protein	PFE0360c			√	
PB001343.02.0	conserved hypothetical protein	PFF0975c			√	
PB001363.02.0	conserved hypothetical protein	PFA0270c				
Oocyst						
PB000318.01.0	conserved hypothetical protein	MAL13P1.328				Histidine-kinase like ATPase
PB001002.00.0	conserved hypothetical protein	PFD0330w				
PB106944.00.0	hypothetical protein		protein kinase all orthologs		√	
PB000538.00.0/ PB108843.00.0	hypothetical protein		Pfemp3 (Py,Pf,Pk)			
PB000890.02.0	conserved hypothetical protein	PFE1330c			√	
PB105834.00.0/ PB000964.01.0	conserved hypothetical protein		PB000964.01.0 Sharing ORF			Serine threonine phosphatase
PB000111.00.0/ PB000180.00.0	conserved hypothetical protein	PF07_0116			√	Ribonuclease, domain#
PB000679.03.0	conserved hypothetical protein	PF14_0162	PB108027.00.0 sharing ORF			
PB000692.01.0/ PB000112.00.0	hypothetical protein	PF13_0221				
PB000882.03.0	conserved hypothetical protein	PF14_0410				
PB000918.03.0/ PB3007005.00.0/ PB108513.00.0	conserved hypothetical protein	MAL13P1.228			√	
PB000712.03.0/ PB300324.00.0	conserved hypothetical protein	PF11_0176			√	
PB001632.02.0	cell differentiation protein rcd1	PFE0375w				Rcd1
PB000467.02.0	conserved hypothetical protein	PFF1230c			√	
PB301371.00.0	conserved hypothetical protein	PF07_0114				Prenyltransferase+
PB000747.03.0	conserved hypothetical protein	PF10_0076			√	
Early blood bolus stages, ookinete & Sporozoite						
PB000504.00.0	cyclin 4, putative	PF13_0022				cyclin like
PB000767.01.0/ PB300506.00.0	conserved hypothetical protein					
PB001296.02.0	conserved hypothetical protein	PFE0950c				Duf
PB001507.02.0	conserved hypothetical protein	PFB0620w				
PB103620.00.0	hypothetical protein					
PB104754.00.0	hypothetical protein					
PB402742.00.0	hypothetical protein					IBN-N
PB000251.01.0	conserved hypothetical protein	PFD0425w			√	
PB300759.00.0/ PB000103.01.0	hypothetical protein	PF14_0060			√	GDPD+

Appendix Table 1: List of candidate genes Genes are grouped according to their expression profile as predicted by in vivo transcriptome analysis in the mosquito host. Four groups of genes are presented including Pbs21-like expressed, ookinete specific, oocyst expressed and a group of genes expressed both during early blood bolus stages or ookinete, as well as sporozoite development. PlasmoDB description, manual annotation, Transmembrane domains (TM), Signal Peptides (SP) and gene functional features are shown. In colour (blue and green) candidate genes for QRT-PCR validation of transcriptional profile while in green candidates for further functional characterization. Pb; *Plasmodium berghei*, Pf ; *Plasmodium falciparum*, Py; *Plasmodium yoelii*, Pk; *Plasmodium knowlesi*, Pv; *Plasmodium vivax*, ORF ; open reading frame ;‡ indicates domains of selected candidates that were revealed after PlasmoDB's reannotation, and were later excluded.

A brief description of a number of featured predicted domains illustrated in Appendix Table 1 follows

Skp1 : Family of Skp1 (kinetochore protein required for cell cycle progression) and elongin C (subunit of RNA polymerase II transcription factor SIII) homologues. (Connelly & Hieter, 1996).

DNAJ domain : The folding of proteins and the assembly of protein complexes within subcompartments of the eukaryotic cell is catalysed by different members of the Hsp70 protein family. The chaperone function of Hsp70 proteins in these events is regulated by members of the DnaJ-like protein family, which occurs through direct interaction of different Hsp70 and DnaJ-like protein pairs that appear to be specifically adapted to each other (Bork et al., 1992; Cyr et al., 1994).

V-ATP synthase subunit D: Vacuolar-ATPases are primarily found in eukaryotic vacuoles, catalysing ATP hydrolysis to transport solutes and lower pH in organelles.(Wilkins et al, 2005)

Prefoldin: Prefoldin is a hexameric molecular chaperone complex built from two related classes of subunits and present in all eukaryotes and archaea. Prefoldin interacts with nascent polypeptide chains and, *in vitro*, can functionally substitute for the Hsp70 chaperone system in stabilizing non-native proteins for subsequent folding in the central cavity of a chaperonin.(Siegert et al., 2000)

Histone H3-lysine 4-specific methyltransferase: Catalysis of the addition of a methyl group onto lysine at position 4 of the histone H3 protein; one of the key mechanisms of post translational modification. It is suggested to be playing role in transcriptional regulation (Wang et al., 2001).

Semaphorin domain: Semaphorins are a class of secreted and membrane proteins that act as axonal growth cone guidance molecules. They primarily act as short-range inhibitory signals and signal through multimeric receptor complexes.(Cohen et al., 2005) (See Table 3.1.2 for reasons of non selection)

Alpha-haemoglobin stabilising protein: α -Hemoglobin stabilizing protein (AHSP) is a and abundant erythroid-specific protein that binds specifically to free α -(hemo)globin and prevents its precipitation (Gell et al.,

2002). *Plasmodium* erythrocyte invasion and intraerythrocytic development is dependent on intact haemoglobin and RBC structure (Senok et al., 1997).

Fibronectin: Fibronectin is a high-molecular weight (~440kDa) extracellular matrix glycoprotein that binds to membrane-spanning receptor proteins (Pankov & Yamada, 2002). It is involved in several functions including cell adhesion, growth, migration and differentiation. (See Table 3.1.2. for reasons of non selection)

ELM2: The ELM2 domain is a small domain of unknown function. It is usually found to the N terminus of a myb-like DNA binding domain and a GATA binding domain. ELM2, in some instances, is also found associated with the ARID (AT-rich interaction domain) DNA binding domain. This suggests that ELM2 may also be involved in DNA binding, or perhaps is a protein-protein interaction domain. (Solari et al., 1999)

ALBA DNA/RNA binding domain: The DNA/RNA-binding protein Alba binds double-stranded DNA tightly but without sequence specificity. It binds rRNA and mRNA in vivo, and may play a role in maintaining the structural and functional stability of RNA, and, perhaps, ribosomes. It is distributed uniformly and abundantly on the chromosome. (Wardleworth et al. 2002)

TPR-HAT: The HAT (Half A tetratricopeptide repeat) repeat is structurally and sequentially similar to TPRs (tetratricopeptide repeats), though they lack the highly conserved alanine and glycine residues found in TPRs. HAT-repeat-containing proteins appear to be components of macromolecular complexes that are required for RNA processing while the repeats may be involved in protein-protein interactions. (Preker & Keller, 1998)

Papain family cysteine protease: Mammalian cysteine proteases of the papain family have been implicated in general protein degradation and turnover within the endosomal/lysosomal system (Buhling et al., 2000). In *Plasmodium*, members of this family have been implicated in *Plasmodium* schizogony (Miller et al., 2002) and late sporogony (Aly & Matuschewski, 2005).

WD40 domain: WD-repeat proteins are a large family found in all eukaryotes and are implicated in a variety of functions ranging from signal transduction and transcription regulation to cell cycle control and apoptosis. The underlying common function of all WD-repeat proteins is coordinating multi-protein complex assemblies, where the repeating units serve as a rigid scaffold for protein interactions. In this case, the respective domain is found in the context of a chromatin assembly factor 1 protein, molecule that is essential for chromatin assembly and remodelling during replication, transcription, recombination, and repair of DNA in eukaryotic cells (Hoek & Stillman, 2003), including *Plasmodium* (Navadgi et al, 2006).

Cyt-b6: In the mitochondrion of eukaryotes and in aerobic prokaryotes, cytochrome b is a component of respiratory chain complex III - also known as the bc1 complex or ubiquinol-cytochrome c reductase. Although erythrocytic stages of malarial parasites derive their energy through glycolysis with little or no contribution by mitochondria to the cellular ATP pool functional mitochondria and an electron transport system are critical for parasite survival and growth (Vaidya et al., 1993)

PWI domain: The PWI motif is a highly conserved nucleic acid-binding domain that facilitates pre-mRNA processing (Szymczyna et al., 2003)

Bromodomain: A bromodomain is a protein domain that recognizes acetylated lysine residues such as those on the N-terminal tails of histones. The precise function of the domain is unclear, but it may be involved in protein-protein interactions and may play a role in assembly or activity of multi-component complexes involved in transcriptional activation.(Jeanmougin et al., 1997)

Ran BP1 protein: Ran is a conserved member of the Ras superfamily, which regulates all receptor-mediated transport between the nucleus and the cytoplasm. Ran Binding Protein 1 (RanBP1) has guanine nucleotide dissociation inhibitory activity, specific for the GTP form of Ran and also functions to stimulate Ran GTPase activating protein (GAP)-mediated GTP hydrolysis by Ran. RanBP1 contributes to maintaining the gradient of RanGTP across the nuclear envelope high (GDI activity) or the cytoplasmic levels of RanGTP low (GAP cofactor)(Steggerda & Paschal, 2002)

HSA domain: This domain of unknown function is found in helicases and other DNA-binding proteins of eukaryotes (Doerks et al., 2002)

Fibrillarin: Fibrillarin is a component of a nucleolar small nuclear ribonucleoprotein (SnRNP), functioning *in vivo* in ribosomal RNA processing (Jansen et al., 1991)

SPOIII: The exact function of this family is unknown. SpoIII is the an uncharacterised protein that appears to act as part of a cascade of events leading to endospore formation (Mizuno et al., 1996)

GYF domain: GYF domains are small, versatile adaptor domains that recognize proline-rich sequences (PRS). So far, only a few physiological binding partners for GYF domains have been identified, and the exact role of GYF domain-containing proteins is poorly understood. Involvement in splicing or splicing-associated processes is a recurrent functional theme for proteins containing GYF domains (Kofler & Freund, 2006)

Coatomer subunit: The coatomer is a cytosolic protein complex that binds to dilysine motifs and reversibly associates with Golgi non-clathrin-coated vesicles, which further mediate biosynthetic protein transport from the ER, via the Golgi up to the trans Golgi network. Coatomer complex is required for budding from Golgi membranes, and is essential for the retrograde Golgi-to-ER transport of dilysine-tagged proteins (Watson et al., 2004)

PDI-thioredoxin: Protein disulfide isomerase (PDI) is a member of a large family of dithiol/disulfide oxidoreductases, the thioredoxin superfamily. They are mainly implicated in catalyzing thiol/disulfide exchange reactions, including disulfide formation, reduction and isomerization.(Wilkinson & Gilbert, 2004)

PP2C: Protein phosphatase 2C (PP2C) is one of the r major classes of serine/threonine specific protein phosphatases. They can be implicated in a variety of processes, through mediation of signal conduction regulation (Bork et al., 1996; Mamoun et al., 1998)

Prenyltransferase: Prenylated proteins are involved in protein prenylation, and among other cellular regulatory processes have been shown to function in signal transduction (Chakrabarti et al., 1998)

Cyclin : Cyclins are a family of proteins which control the progression of cells through the cell cycle by activating cyclin-dependent kinase (Cdk) enzymes. *Plasmodium*'s complex life cycle has been found to be tightly regulated and controlled by the above proteins (Merckx et al., 2003)

DUF domain: domains of unknown function (Jaroszewski et al., 2009)

IBN-N: Members of this family (Importin β – IBN) represent a large number of soluble transport receptors mediating macromolecular import processes across the nuclear envelope. (Strom & Weis, 2001)

GDPD : Members of the Glycerophosphoryl diester phosphodiesterase (GDPD) family are implicated in glycerol metabolic process, hydrolysing a broad spectrum of phosphodiesterases including glycerophosphocholine, glycerophosphoethanolamine, glycerophosphoglycerol, and bis (glycerophosphoglycerol) (Tomassen et al., 1991)

Appendix Table 2 Transcriptome and Proteome expression

Plasmodium Transcription Programmes in the mosquito vector (unpublished)	Proteome					Transcriptome				
	ABS	Gc/Gm	Ook	Ooc	Spz	ABS	Gc/Gm	Ook	Ooc	Spz
Pbs21 - like										
PB000001.03.0	+ ²	Gc ^{1,3,4} Gm ³	+ ²			R ⁹ , T ⁹ , S ⁹ , M ⁹	Gc ⁹			+ ⁹
PB000017.03.0										
PB000019.02.0	+ ^{2,4}					R ⁹ , T ⁹ , S ⁹ , M ⁹	Gc ⁹			+ ⁹
PB000089.00.0	T ¹	Gc ^{1,3,2}			+ ¹					
PB000142.00.0	R ^{5,4} T ^{3,5} S ^{3,5} M ^{1,5}	Gc ^{1,3} Gm ³			+ ¹	R ⁹ , T ⁹ , S ⁹ , M ⁹	Gc ⁹			+ ⁹
PB000417.00.0										
PB000149.01.0	M ¹	Gc ¹	+ ¹	+ ¹		R ⁹ , T ⁹ , S ⁹	Gc ⁹			
PB000250.03.0	T ³ S ³	Gc ⁴ Gm ³				S ⁹	Gc ²			
PB000270.03.0										
PB000053.00.0	+ ² T ^{1,3} S ³ M ¹	Gc ^{1,2,3,6} Gm ³	+ ²	+ ^{2,10}	SGS ¹⁰	R ⁹ , T ⁹ , S ⁹ , M ⁹	Gc ⁹			
PB001532.02.0										
PB300823.00.0										+ ⁹
PB000311.01.0	+ ²					R ⁹ , T ⁹ , S ⁹	Gc ⁹			
PB000314.03.0	+ ⁴					R ⁹ , T ⁹ , S ⁹	Gc ⁹			+ ⁹
PB000319.03.0										
PB000325.03.0	+ ² T ³ S ³ M ¹	Gc ¹ Gm ³			+ ¹	R ⁹ , T ⁹ , S ⁹ , M ⁹	Gc ⁹			+ ⁹
PB000332.02.0	M ¹				+ ¹	S ⁹	Gc ^{7,9}			+ ⁹
PB000371.00.0	+ ²									
PB000370.00.0										
PB000434.03.0	+ ² T ^{1,3} S ³	Gc ^{1,4}	+ ²			R ⁹ , T ⁹ , S ⁹ , M ⁹	Gc ⁹			+ ⁹
PB000465.02.0										
PB300869.00.0										
PB000473.03.0	+ ^{2,4}	Gc ^{3,2}								
PB000536.02.0	+ ² M ¹									
PB000752.01.0	T ¹					R ⁹ , T ⁹ , S ² , M ⁹	Gc ^{2,9}			+ ⁹
PB000779.00.0	+ ^{2,4} R ⁵ T ^{1,3,5} S ^{3,5} M ^{1,5}	Gc ^{1,2,4} Gm ³			+ ¹	R ⁹ , T ⁹ , S ² , M ⁹	Gc ^{2,9}			
PB000812.02.0	+ ^{2,4} R ⁵ T ^{1,3,5} S ^{3,5} M ^{1,5}	Gc ^{3,4} Gm ³	+ ²		ODS/SGS ¹⁰	R ⁹ , T ⁹ , S ⁹ , M ⁹	Gc ⁹			+ ⁹
PB000819.00.0						R ⁹ , T ⁹ , S ⁹				
PB000840.00.0	+ ^{2,4} R ⁵ T ^{3,5} S ^{3,5} M ^{1,5}	Gc ^{2,3,4} Gm ³	+ ²	+ ²	+ ¹	R ⁹ , T ⁹ , S ⁹ , M ⁹	Gc ⁹			+ ⁹
PB000856.03.0										
PB000590.03.0	+ ^{2,4} T ¹ M ¹	Gc ^{1,2,4}	+ ²			R ⁹ , T ⁹ , S ⁹ , M ⁹	Gc ⁹			+ ⁹
PB000866.03.0					+ ¹⁰	+ ¹ ODS/SGS ¹⁰	R ⁹ , T ⁹ , S ⁹ , M ⁹	Gc ⁹		+ ⁹
PB000892.02.0	+ ^{2,4} T ³ S ³ M ¹	Gc ^{2,3,4} Gm ³	+ ²			R ⁹ , T ⁹ , S ⁹	Gc ^{7,9}			+ ⁹
PB000909.03.0	+ ⁴ R ⁵ T ^{3,5} S ^{3,5} M ^{1,5}	Gc ^{1,3,4} Gm ³			+ ¹ SGS ¹⁰	R ^{2,9} , T ^{2,9} , S ⁹	Gc ^{2,9}			
PB000922.00.0						R ⁹ , T ⁹ , S ⁹	Gc ^{7,9}			+ ⁹
PB001081.02.0				+ ²		R ⁹ , T ⁹ , S ⁹	Gc ⁹			
PB001198.00.0						R ⁹ , T ⁹ , S ⁹	Gc ⁹			+ ⁹
PB001232.02.0	+ ² T ^{1,3} S ³	Gc ^{1,2}	+ ²			R ⁹ , T ⁹ , S ⁹ , M ⁹	Gc ⁹			+ ⁹
PB001306.00.0				+ ²		R ⁹ , T ⁹ , S ⁹ , M ⁹				+ ⁹
PB100823.00.0										
PB405718.00.0										
PB101572.00.0				+ ²						
PB101678.00.0										
PB102173.00.0	+ ²	Gc ²	+ ²							
PB105908.00.0										
PB106158.00.0	+ ² T ^{3,5} S ^{3,5} M ¹	Gc ^{1,3}								
PB107035.00.0				+ ²						
PB000704.03.0	+ ²			+ ²						
PB108856.00.0/ PB108056.00.0	+ ² R ⁵ T ¹ S ⁵ M ⁵	Gc ^{1,3} Gm ³				T ⁹ , S ⁹ , M ⁹	Gc ⁹			+ ⁹
PB401579.00.0										
PB405164.00.0										
PB300953.00.0	T ³ S ³					T ⁹ , S ⁹	Gc ⁹			
PB300508.00.0				+ ²						
PB103734.00.0										
PB000801.00.0	T ³ S ³	Gc ³ Gm ³		+ ²		R ⁹ , T ⁹ , S ⁹ , M ⁹	Gc ⁹			
PB000867.00.0										
PB103082.00.0										
PB105866.00.0										
PB102403.00.0	+ ²	Gc ²	+ ²							
PB000551.00.0										
PB000893.01.0	+ ^{2,4} T ^{1,5} M ^{1,5}	Gc ^{1,3,4} Gm ³	+ ²							
PB001039.00.0	+ ^{2,4} T ^{3,5} S ^{3,5} M ^{1,5}	Gc ^{1,4}	+ ²			R ⁹ , T ⁹ , S ⁹ , M ⁹	Gc ⁹			+ ⁹
PB104218.00.0										
PB301555.00.0		Gm ³					Gc ⁹			
PB107098.00.0										
PB000489.03.0	+ ^{2,4}	Gc ^{3,2}	+ ²		SGS ¹⁰					
PB000846.01.0										
PB000874.00.0						R ⁹ , T ⁹ , S ⁹ , M ⁹	Gc ⁹			+ ⁹
PB001229.02.0	+ ⁴ T ¹	Gc ^{4,2}					Gc ⁹			
PB001312.02.0	+ ^{2,4} R ⁵ T ^{1,3,5} S ^{3,5} M ^{1,5}	Gc ^{1,2,3,4} Gm ³	+ ²	+ ²	+ ¹ ODS/SGS ¹⁰	R ⁹ , T ⁹ , S ⁹ , M ⁹	Gc ⁹			+ ⁹

Appendix Table 2 Transcriptome and Proteome expression *continued*

Plasmodium Transcription Programmes in the mosquito vector	Proteome					Transcriptome				
Ookinete	ABS	Gc/Gm	Ook	Ooc	Spz	ABS	Gc/Gm	Ook	Ooc	Spz
PB000096.01.0	+ ⁴ T ³ S ^{3,5} M ^{1,5}	Gm ³	+ ²		+ ¹ SGS ¹⁰	R ⁹ ,T ⁹ ,S ⁹ ,M ⁹	Gc ^{7,9}			
PB000182.01.0	T ^{1,3} S ³	Gc ⁴ Gm ³	+ ²		+ ¹	S ⁹				
PB000240.03.0/ PB000689.01.0	+ ^{2,4} R ⁵ T ^{1,3,5} S ^{3,5} M ^{1,5}	Gc ^{3,4} Gm ³	+ ²			R ⁹ ,T ⁹ ,S ⁹ ,M ⁹	Gc ⁹			
PB000409.03.0/ PB000599.02.0/ PB000634.01.0	M ¹					R ⁹ ,T ⁹	Gc ⁹			+ ⁹
PB000626.02.0/ PB000789.01.0/ PB000308.00.0/ PB000740.00.0/ PB000829.01.0						R ⁹ ,S ⁹	Gc ⁹			+ ⁹
PB000943.03.0/ PB000947.00.0/ PB000158.03.0/ PB000341.03.0/ PB000600.00.0/ PB000956.03.0/ PB404215.00.0			+ ²							
PB000962.00.0/ PB001016.01.0										
PB001095.00.0/ PB300953.00.0	+ ² T ^{1,3} S ³ M ¹				+ ¹					
PB001129.00.0/ PB000823.03.0	+ ^{2,4} M ¹	Gc ⁴ Gm ³			+ ¹	R ⁹ ,T ⁹ ,S ⁹ ,M ⁹	Gc ⁹			+ ⁹
PB401857.00.0/ PB400440.00.0										
PB000712.00.0/ PB000418.03.0		Gc ²		+ ²	+ ¹ ODS/SGS ¹⁰	R ⁹ ,T ⁹ R ⁹ ,S ⁹	Gc ⁹ Gc ⁹	+ ⁸		
PB000627.01.0/ PB000653.00.0										+ ⁹
PB000317.00.0/ PB000773.02.0	M ¹	Gc ³ Gm ³			+ ¹ SGS ¹⁰	R ⁹ ,T ⁹ ,S ⁹ R ⁹ ,S ⁹	Gc ⁹ Gc ⁹			+ ⁹ + ⁹
PB108456.00.0/ PB000759.02.0/ PB000760.02.0/ PB300784.00.0				+ ²		T ⁹ ,S ⁹ ,M ⁹	Gc ⁹			+ ⁹
PB300795.00.0/ PB000674.01.0/ PB001555.02.0			+ ²		ODS/SGS ¹⁰		Gc ⁷			
PB000254.00.0/ PB000508.03.0		Gc [*] 6		+ ²	+ ² SGS ¹⁰	R ⁹ ,T ⁹ ,S ⁹ ,M ⁹ T ⁹ ,S ⁹ ,M ⁹	Gc ⁹ Gc ⁹			+ ⁹ + ⁹
PB000808.02.0/ PB001180.00.0	+ ²	Gc ¹ Gm ³				T ⁹ ,S ⁹	Gc ⁹			+ ⁹
PB001289	M ¹	Gm ³				R ⁹ ,T ⁹ ,S ⁹ ,M ⁹	Gc ⁹	+ ⁸		+ ⁹
PB001343.02.0/ PB001363.02.0		Gc [*] 6		+ ¹⁰	ODS ¹⁰			+ ⁸		
Oocyst										
PB000318.01.0/ PB001002.00.0/ PB106944.00.0		Gc ⁴ Gm ³			+ ¹	R ⁹ ,T ⁹ ,S ⁹ ,M ⁹	Gc ⁹			+ ⁹
PB000538.00.0/ PB108843.00.0										
PB000890.02.0/ PB105834.00.0/ PB000964.01.0						T ⁹ ,S ⁹	Gc ⁹			
PB000111.00.0/ PB000180.00.0					+ ²	R ⁹ ,T ⁹				
PB000679.03.0/ PB000918.03.0/ PB3007005.00.0/ PB108513.00.0		Gc ⁴ Gm ³				R ⁹ ,T ⁹ ,S ⁹ ,M ⁹				
PB000692.01.0/ PB000112.00.0										
PB000882.03.0/ PB000712.03.0/ PB300324.00.0				+ ²		R ⁹ ,T ⁹ ,S ⁹ ,M ⁹				
PB001632.02.0	+ ⁴	Gc ⁴ Gm ³			+ ¹	R ⁹ ,T ⁹ ,S ⁹ ,M ⁹	Gc ⁹			+ ⁹
PB000467.02.0/ PB301371.00.0	+ ⁴					S ²				
PB000747.03.0		Gc [*] 6								
Early blood bolus stages, ookinete & Sporozoite										
PB000504.00.0/ PB000767.01.0/ PB300506.00.0										
PB001296.02.0/ PB001507.02.0	T ^{1,3} S ³	Gc ^{1,3} Gm ³				R ⁹ ,T ⁹	Gc ⁹			+ ⁹
PB103620.00.0/ PB104754.00.0		Gc [*] 6								
PB402742.00.0/ PB000251.01.0	+ ²		+ ²							
PB300759.00.0/ PB000103.01.0		Gc [*] 6		+ ¹⁰	+ ^{1,2} ODS/SGS ¹⁰			+ ⁸		+ ⁹

Appendix Table 2 Transcriptome and Proteome expression of 113 genes. Four groups of genes are presented according to their expression profile in *Plasmodium* transcription programmes (*Vlachou group, unpublished*) (under red headtitle). Cluster of genes showing similar expression patterns to already known genes critical for parasite development, i.e Pbs21-like expression, or appearing to be upregulated during ookinete or oocyst developmental stages were utilized. In addition a cluster of genes appearing to be expressed both during early blood bolus stages or ookinete, as well as sporozoite development was included. Available transcriptome and proteome data is shown, presence of significant hits for each developmental stage is indicated by “+” or M/R/T/S/Gc/Gm where specific information was available. In blue candidate genes for QRT validation of Transcriptional Profile, in Green candidates for further functional characterization . Asterisk (*) indicates post-translational regulation by DOZI (Meir et al 2007). Red and Blue Asterisk stand for increased and decreased suppression respectively. 1; Florens et al 2002, 2; Hall et al 2005, 3; Lasonder et al 2002, 4; Khan et al 2005, 5; Le Roch et al 2004, 6; Mair et al 2006, 7; Silvestrini et al 2005, 8; Rainabaud et al 2006, 9; Le Roch et al 2003, 10; Lasonder et al 2008 ABS; asexual blood stages, Gm; Gametes, Gc; ametocytes, Ook; ookinetes, Ooc; oocysts, Spz; sporozoites, R; ring forms, M; merozoites, T; trophozoite, S; Shizonts, ODS; oocyst derived sporozoites, SGS; salivary gland sporozoites, ♂; male, ♀; female

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                10      20      30      40      50      60
PB001289      MVLLNGKLYIAVVAIFYNLIILLVKEKFPYICTKKK-----FHAISNRILYEYLNNFVS
PC001277.02.0
PFE0360c     MIYLGKLLSCTLFVYFLYIHFFLLKQ--NNFCDVKVRERILEESINNDLSSKGENLHIY
PKH_102560   MLFTKERHRRVAVSTIFFHIIALLQFSGDATIHWCRRGSERVQRAMNGRLLEEGQIGKIP
PY04330      MVLLNGKFKYIAVVAIFYNLIILLVKEKFPYMCTQOK-----SHTISNRILYEYLNNFVS
Pv097925     MPFTKERQRHVVATIFCHLAALLLFGCDATVHRCRRGSEPLQRTVNGRVLLEGQVGSLA
Clustal Consensus

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                70      80      90     100     110     120
PB001289      KDIFRRDITLK-----NLNFVQTNLKSDKDAEIKENRDTQSVDDNMFQRVYKFILNFFY
PC001277.02.0
PFE0360c     EKTNVSVQTFIKKERKNLSDNNINDKINNNNNNNNNNNNNNNNNNNIEDTTYYPTGNEKENIFL
PKH_102560   NVLHNYSKMNLS-----TKSFVQVKVDSDHDRNIRRGVTTSEGNYHIPNCDDTDESSNF
PY04330      KDIFRRDITLK-----NLNFVETNLKNDKDAEIKENSDTQSVDDNMFQRVYKFVLNFFY
Pv097925     DELHNYSKTNLS-----IPSFVQVKLKDSYDRKIRRGGATP-EGNYHIPNCDDTDGSANF
Clustal Consensus

```

REGION I

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                130     140     150     160     170     180
PB001289      GNKKNRINKSMN-----YGKYDMFNKINDIFEFMRNNGLPINITSVCLIDTGLNIK
PC001277.02.0
PFE0360c     ENKKDSTNKYMK-----YGKYEHFNKINDIFEFMRNNGLPINITSVCLIDTGLDIK
PKH_102560   KIFRYVKNWFPIKSSNNLKKTNINYEQVQENEFSKYILQNNMSIETTKVCLIGSGDSS
PY04330      LKKFCRSMKSFVGITPHDKDWCLKYEKFRRRKQHFVYMVQNNNLSLGKTRVCLIDTGLDLQ
Pv097925     GNKKNRINKSMN-----YGRYDMFNKINDIFEFMRNNGLPINITSVCLIDTGLNIK
Clustal Consensus

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                * : . . . . .      : : . . . . .      * * * * * : * : .

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                190     200     210     220     230     240
PB001289      DALINYFLNHDISTYNSYTYHSVNIN-----YKKPDSFNF
PC001277.02.0
PFE0360c     DEIINHFLNDDISTYNSDTFHSVNIN-----YKNADSFNF
PKH_102560   HDLIKQFLLHNVKYNRNYENDNSLN-----NKSGSISSYKYYEEIIQE
PY04330      DELINYFLNHDISTYNSYTYHSVNIN-----YKKPDSFNL
Pv097925     DEVLGHFVRMSRGGLNQGDNPGEQN-----ERRAD
Clustal Consensus

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REGION II

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                250     260     270     280     290     300
PB001289      GINSENCDEDNYSECESTFLENHNGHGKYEDKSTIQGDSLKLIEKYDKNVDLQRSGIDV
PC001277.02.0
PFE0360c     EINTENRDEDDYPEYESTFLENHNGDGSYEANSTSQGDPLKWGEKKYKGMDLQRSGIHV
PKH_102560   PYIIDRDICNKKKNCKESTLINYENNPKTLIGNIIIQSDILKNE-----KIFNMNRHF
PY04330      GINTERCNEENYARCQSSDIDDVDMHGTFIANTVIRRDLLMKR-----EMYKKNVEL
Pv097925     GVNSENCDEDSNSECESTFLENHNGYEKYEDRSKIQGDTLKLIEKKYEKNVDLQKSGINV
Clustal Consensus

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          310      320      330      340      350      360
...|...|...|...|...|...|...|...|...|...|...|...|
PB001289 EICKAFNNS-KEKKNLSN---IIPVIKLEYCKTKNVKIIHMDYNNINEQNEQLIQIMDDL
PC001277.02.0 ETCKAFDMS-KEKKNSSN---IIPVIKLEYCKTKNVKIIHIDYNNINEKNEELIEIIEDL
PFE0360c VVCKYFGSIPKTNIKINQSTLIQHLIKCLDYCKMEGVQYIYIGYNIYAANNKLIIEIMKKL
PKH_102560 IVCKAFGD---REETNSH---LMPLIKLEHCKGSGAKVIHVGYNVEGESEKLVEMQEL
PY04330 EICKTFDMS-KEKKNDSN---IIPVIKLEYCKTKNVKIIHIDYNNINEKNEQLMQIIEDL
Pv097925 IVCNAFAKSEFGNAVKNSH---LVPLIKLEMCKERGAKVIHVGYNVQGESEQLVKLMEEEL
Clustal Consensus *: * . : : :****: ** ...*:**: ...*:.....*

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          370      380      390      400      410      420
...|...|...|...|...|...|...|...|...|...|...|...|
PB001289 KNSEIFVILPSEKLFNEKPYEDN-----
PC001277.02.0 KNSEIFLLLPSKGLLNEKSYEDN-----
PFE0360c REHKIIIVTSSCKIYDDDNNDNNDNDFYNDNIYNNNIYNVHNDDEKIKKH IKKKKQNDY
PKH_102560 ERAQIVVVSPLRVYTCQSDSQ-----
PY04330 KKSEIFVVLPSKLFNEKSHGDN-----
Pv097925 QREEIIVVSPSLQVYHRNGGETN-----
Clustal Consensus ..*:..* : . :

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          430      440      450      460      470      480
...|...|...|...|...|...|...|...|...|...|...|...|
PB001289 -----SVIYPSSFFEKFEENVFFIGSLDYSMDSSDDADIASNFQIQKNEY-----L
PC001277.02.0 -----SVAYPSSFFENS-----
PFE0360c LYEYQRTQKDEEQKSNSSLYQNLLENVISISGLIYTDSSKKKNKNKNYIYDNEIKILDQKGN
PKH_102560 PRKEHGEEHSTEKLYPSSFADTFENVFSVGALRNSTQGGFVPI SGNGNPKGEKQKMKVLH
PY04330 -----SVIYPPFFFEDEFE-----HFEKFEK-----F
Pv097925 SKKEHLEEPSTQKMPASFADTFENVFSVGALRNSPQGGLVPI LGNANPRGEKPKGEQLH
Clustal Consensus . . : :

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          490      500      510      520      530      540
...|...|...|...|...|...|...|...|...|...|...|...|
PB001289 KYRKNNVFLLD SI--NSSLKKRDDHDILYEEIKYSSAFFINIITIIILNIYPMMSIKELRN
PC001277.02.0 -----
PFE0360c KKLNRNYISLFYFSYD TD IYEKIESDIIDDDHDLV SASFVNTLVLMHSINLKLSLGRLRK
PKH_102560 KRENTTLFSFSYG--KTFPFG RSPSSMVEDGEGYASADFVNILVMI LNVI PKLSIRRMRH
PY04330 EN----VFFLD SI--NSSLKKRDDHMLDYEIRYSSAFFINIITIIILNIYPNISIKELRN
Pv097925 KRENTTLFSFSYG--KTFPFG RSPSSMVEDAQAYASADFVNALVMI FNVNPKLSMKRVRL
Clustal Consensus

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          550      560      570      580      590      600
...|...|...|...|...|...|...|...|...|...|...|...|
PB001289 ILSYSIPSKETAQLKTENIFEGNFDINKFIHILLN-RGINSSGFVRKYKD VTPNESTNKI
PC001277.02.0 -----
PFE0360c I LNKSIIVKR--EELRHLSNRAYYHDMNTTFEDSLNQRKRSYKIFYLELKNKHKVLLSDA
PKH_102560 I LKRSIVKR--SEMKG LSKWGGYIDPLKVIDATLKERNE LCKTFFGELDL DLEAEGGSSS
PY04330 ILSYSILSKETPELKTENIFEGNFDINRFIHI LLN-RGI IYSD FVQKYEDVTPNEPTNKT
Pv097925 I LERSIGRR--SELKGLSKWGGYLDPEKLI AETLKERNE LCGRFFRELGGNLEEGGGG--
Clustal Consensus

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          610          620          630          640          650          660
.....|.....|.....|.....|.....|.....|.....|.....|.....|
PB001289  FLLEDQKQDADIEPQKQSSIDYICDE-----
PC001277.02.0
PFE0360c  NLKSMYQDNLPVNYNEEDHVKHNVQETSVERDIYKNNENSNNKNRKMDMDEGKG-----
PKH_102560 FRGDLKSGGFSGGSMTELDGWVEKPTTGTEEQIFDKTITGDVTPRLNDEQEESEKATGH
PY04330   SVLEGGQEDGEIEPQTDSSDIYYDEDGSGEGGSDEGGSGEGGSSEGGSGEGGSSEGGSGE
Pv097925  ----LLGGGLPRGEATEHLGDWVERPPNDEDQRSCGEATTGEATPRLD-----
Clustal Consensus

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          670          680          690          700          710          720
.....|.....|.....|.....|.....|.....|.....|.....|.....|
PB001289  -----GGSDEDIIVSTSNGLDVYSEY-----SHSNNKSDQLLNDEKGLKY-
PC001277.02.0
PFE0360c  ----TYIQNKESHKYNIHYPYNRKIQSLLN-----DNTLNHKPYVSFLNMSYYNED
PKH_102560 LEDQVIFPQORGEMEEMERHLVGETLNGLDPEERNDLGRYDMAHYDMDVISKWEEDFSDG
PY04330   GSGEGGSSEGGSGEDVLTSLNE LDDYSED-----SYSNNKSEQLLEDENGLLEDE
Pv097925  ----AAFPPQGGEELEEIERQFEEEAVRGLD-----HHNLGYHYHTDAISKWAEDFPDG
Clustal Consensus

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          730          740          750          760          770          780
.....|.....|.....|.....|.....|.....|.....|.....|.....|
PB001289  -----ETYKDLYSVKENDIYVFE SNNPTNSSFMQMNYYDDKIKSKYLDNLEEANY
PC001277.02.0
PFE0360c  IEKRNYNIYDDPSYTYDQGITYDDNYYIDDHDIHTRKKRKISYDGEDNNDYHMYDDRDNL
PKH_102560 VAALREDPASDTTTTFTSMYNYEENVVVFDEAKE SFYEDAGRVTVTGEMSSLPLGFSFLENH
PY04330   PLLEDENEFEGETS KDLYSVKENDIYVIESKNPTNSSFMQMNYYDDRIKSKYVDNLEDANY
Pv097925  GAAPSEGGPPSDTTYRSSYNDEEDVYTFDEANQSFYGNSSGGVAL-----
Clustal Consensus

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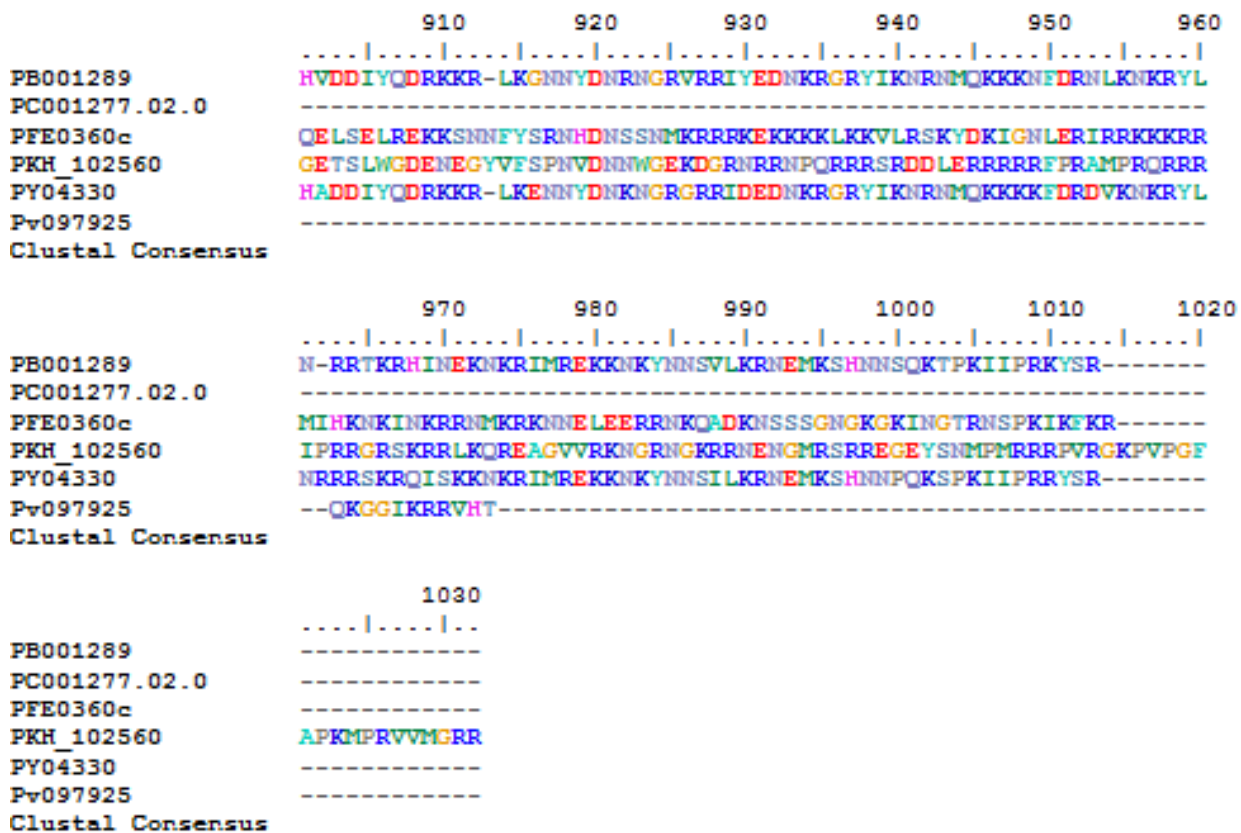
          790          800          810          820          830          840
.....|.....|.....|.....|.....|.....|.....|.....|.....|
PB001289  QNHR-TQFEINRNDNRYPIISEDN---LRDRQNPHNIQMLNDGINYSENANNIEELYDN
PC001277.02.0
PFE0360c  FHSN---LGNNKYEDDGNVHREKE-----KOLEPRFLYDPFANIENR-----
PKH_102560 TNDRGSDLPLYRTNERGQVYASGDGTPGLPLNRRSQDENGFPERWGCQGRQAMIEEDSGE
PY04330   QNHR-AQFEINRNGNRYPIFSEDN---LRDRQNTHNIQMVNDGINYSENGRNIEELYDN
Pv097925  -----EVVGEGE-----DLWVKVGS-----
Clustal Consensus

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          850          860          870          880          890          900
.....|.....|.....|.....|.....|.....|.....|.....|.....|
PB001289  DFEDYRQKDLNPEYNKIRDNNNNKINKEFGILNTRKKNDEGLYLSDSEESQPFKQMDM
PC001277.02.0
PFE0360c  -----DLETV
PKH_102560 GYHHGQDDWVTQMKGTKSVDDPEYDVGRMYRDSPEGIPAEELLRSWGRGTTPLSRWNRN
PY04330   DFEGYRQKDLNPEYNN---NNNNNNNNINREFGILNSWRKNDEGLYLDNNEEASQPFKQMDM
Pv097925
Clustal Consensus

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Appendix Figure 1. Multiple sequence alignment of Pb1289 orthologs as performed by ClustalW and visualised by Bioedit Sequence Alignment Editor. Regions I and II of increased conserved residues are indicated. Coloured blocks depict subtilisin active site as this predicted by Interpro Scan online prediction software

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