Epithelial and Cellular Innate Immune Responses of *Anopheline* Malaria Vectors to *Plasmodium* Parasites

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Oral examination

Epithelial and Cellular Innate Immune Responses of *Anopheline* Malaria Vectors to *Plasmodium* Parasites

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Thesis Abstract

Anopheles gambiae is the most prominent vector of human malaria in Africa. The causative agents of this disastrous disease are unicellular eukaryotic parasites of the genus *Plasmodium* which are transmitted to humans by infected female mosquitoes when they take a blood meal. During its development in the mosquito, *Plasmodium* undergoes massive losses suggesting that mosquitoes are able to mount an immune response that limits parasite infection. However, the molecular mechanisms underlying parasite invasion, immune evasion, its recognition and killing via the vector are not well understood.

We have functionally analysed the orthologous *SRPN6* genes from *Anopheles stephensi* and *Anopheles gambiae* and showed that they are specifically expressed in midgut cells invaded by *Plasmodium* ookinetes. Phenotypic analysis via RNAi knock down indicates that *AsSRPN6* is involved in the parasite killing process, whereas *AgSRPN6* acts on parasite clearance, by promoting parasite lysis. Furthermore, SRPN6 is also a parasite induced salivary gland epithelial marker located at the basal side of epithelial cells in proximity to invading sporozoites. Knockdown of SRPN6 had no effect on oocyst rupture but significantly increased the number of sporozoites present in salivary glands.

Midgut invasion is vital for the parasite life cycle progression. We show that *Δpplp5* ookinetes cannot invade midgut epithelial cells and are retained attached to the midgut, possibly, due to the fact that, these mutant parasites can not form pores in the plasma membrane.

Haemocytes secrete immune factors such as opsonins, proteases and their negative regulators as well as antimicrobial peptides, all crucial for immune responses. We report the first genome wide molecular characterisation of *Anopheles gambiae* circulating haemocytes, presenting a list of 1587 genes we strongly suggest are expressed by these cells.

Zusammenfassung

Anopheles gambiae ist der wichtigste Überträger menschlicher Malaria in Afrika. Die Erreger dieser Krankheit sind einzellige eukaryotische Parasiten des Genus *Plasmodium*. Parasiten werden auf den Menschen übertragen, wenn infizierte weibliche Mücken eine Blutmahlzeit nehmen. *Plasmodium* Parasiten durchlaufen mehrere Lebensstadien in der Mücke, die erhebliche Verluste erleiden. Diese Verluste verdeutlichen, dass die *Anopheles* Mücken durchaus im Stande sind, Immunreaktionen gegen den Parasiten zu bilden, die deren Infektivität beschränkt. Die molekularen Mechanismen, die dieser Immunantwort sowie die der Parasiteninvasion und Immunevasion unterliegen, sind jedoch weitgehend ungeklärt.

Diese Dissertation beschreibt die funktionelle Analyse der orthologen serpin (SRPN)-6 Gene von *An. gambiae* und einer nahe verwandten Species, *An. stephensi. SRPN6* wird spezifisch in denjenigen epithelialen Zellen des Mitteldarms exprimiert, die vom *Plasmodium* Ookineten-Stadium invadiert werden. Phänotypische Analyse von funktionellen Knockdowns, erzielt mittels RNA-Interferenz, zeigt, dass AsSRPN6 an der Abtötung von *Plasmodium* Parasiten im Mitteldarm der Mücke maßgeblich beteiligt ist. AgSRPN6 hingegen, beeinflusst die spätere Lyse abgetöteter Parasiten. AgSRPN6 wirkt zusätzlich als Marker für die Parasiteninvasion des Speicheldrüsen-Epitheliums, und ist in diesen Zellen basal nahe der Sporozoiten lokalisiert. Der Knockdown von SRPN6 in der späten Phase der Parasitenentwicklung in der Mücke (13-21 Tage nach Infektion) hatte keinen Einfluss auf das Freisetzen von Sporozoiten aus den Oozysten im Mitteldarm, erhöhte jedoch signifikant die Anzahl dieser Parasiten in the Speicheldrüsen.

Die Invasion des Mitteldarm-Epitheliums ist essentiell für die Entwicklung des Parasiten. Ookineten, denen das Gen *pplp5* fehlt, können den Mitteldarms von *Anopheles* Mücken nicht infizieren. Die Ursache ist möglicherweise, dass diese Parasiten nicht fähig sind Poren in die Membranen der epithelialen Zellen zu bilden.

Haemozyten sekretieren wichtige Immunefaktoren, wie zum Beispiel Opsonine, Proteasen und deren Inhibitoren, sowie antimikrobielle Peptide. Das letzte Kapitel dieser Dissertation beschreibt die erste vollständige genomische molekulare Charakterisierung der zirkulierenden Haemozyten von *An. gambiae*. Die Transkripte von 1587 Genen wurden in diesen Zellen detektiert. Das Kapitel beschreibt weiterhin, in welchem Maß die Transkriptome von Haemozyten verschiedener Diptera konserviert sind.

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To all my friends and family, you know who you are!….. thank you!

Ao meu pai e ao meu irmao, embora longe eu sei que estao sempre comigo…. Muito obrigada!

To my beloved mother…

… a quem eu devo tudo o que sou!

"There is no malaria without Anopheles...
... [but] there is certainly a biological race of Anopheles
mosquitoes that does not bite man."
G.B.Grassi

Table of Contents:

Contribution:

The work presented by SB Pinto in her thesis dissertation has been part of several different collaborative research efforts. Bellow is stated the contribution of SB Pinto in each study:

Chapter 2 was previously published *in:*
Abraham, EG *, **Pinto SB*** Gosh A, Vanlandingham D, Budd A, Higgs S, Kafatos FC, Jacobs-Lorena M and K Michel, (2005) An immune-responsive serpin (SRPN6) mediates mosquito defense against the malaria parasite, *PNAS,* Nov 8;102(45):16327-32
* joint first authorship

SB Pinto contributed with experimental design, performed all research related to SRPN6 analysis in An gambiae, analysed results and was involved in the writing to produce the final manuscript.

.
Chapter 4 was previously published *in*:
Ecker A, **Pinto SB**, Baker KW, Kafatos FC and RE Sinden
Plasmodium berghei: *Plasmodium* Perforin- like Protein 5 is required for mosquito midgut invasion in *Anopheles stephensi (Exp. Parasitology*)
(in press)

***SB Pinto** contributed to the experimental design, execution and data analysis of the results presented in Figure 4.3 A-C. As also assisted in writing and revising the manuscript.*

Chapters 3 and 5:

SB Pinto contributed to the experimental design, performed all research, analysed the results and wrote the manuscripts. Both chapters are currently manuscripts in preparation.

Pinto SB, Kafatos FC and K Michel
The parasite invasion marker, SPRN6 reduces the number of sporozoites in salivary glands (in preparation)

Pinto SB, Koutsos AC, Waterhouse RM, Kafatos FC and K Michel
Genome wide analysis of the molecular repertoire of *An. gambiae* haemocytes (in preparation)

Prof. Fotis C Kafatos

List of Abbreviations:

#, number
***Ae**., Aedes*
***AgSRPN**, Anopheles gambiae serpin*
AMPs, Antimicrobial peptides
***An.,** Anopheles*
APL, *Anopheles Plasmodium*-responsive leucine-rich repeat
***Ar.,** Armigeres*
AsSRPN, *Anopheles stephensi serpin*
cDNA, complementary deoxyribonucleic acid
CelTOS, cell traversal protein for ookinete and sporozoites
CSP, circumsporozoite protein
CTL, C-type lectins
CTRP. Circumsporozoite and thrombospondin-related anonymous protein-related protein
***D.,** Drosophila*
dhfr/ts, dihydrofolate reductase/thymidylate synthase
DNA, deoxyribonucleic acid
dpi, days post infection
dsRNA, double stranded RNA
DTT, dichlorodiphenyltrichloroethane
ECP, Egress cysteine protease
EE, exo-erythrocytic
EEF, exo-erythrocytic form
EST, expressed sequence tag
GFP, green fluorescent protein
GO, gene ontology
hpi, hours post infection
IFA, immunofluorescence analysis
IPR, InterPro domain
IPT intermittent preventive treatment
IRS indoor residual spraying
ITN insecticide treated nets
IVT, in vitro transcription
kb, kilo base
KD, knock down
kDa, kilo dalton
KO, knock out
L, instar larvae
LRIM, Leucine rich repeat immune protein
MACPF, membrane attack complex pore forming
MAEBL, merozoite apical erythrocyte binding like
MAOP, membrane-attack ookinete protein
mRNA, messenger ribonucleic acid
MS, mass spectrometry
MSP, merozoite surface proteins
NOS, nitric oxide synthase
ONN, O'nyong-nyong virus
***P.,** Plasmodium*
PAMP, pathogen-associated molecular pattern
PBS, phosphate buffered saline
***Pen**, Plasmodium* encapsulation
Pf/v, *Plasmodium falciparum/vivax* protein
PGRP, peptidoglycan recognition protein
PPAE, prophenoloxidase activating enzymes
PPLP, *Plasmodium* perforin-like protein
PPO, prophenoloxidase
PRR, pattern recognition receptor
PV, parasitophorous vacuole
qRT-PCR, quantitative real time reverse transcription PCR
QTL, quantitative trait loci
RBP, reticulocyte-binding protein homologue
RCL, reactive center loop
RIFIN, repetitive interspersed
RNA, ribonucleic acid
RNAi, RNA interference
SD, standard deviation
SE, standard error
SPECT, sporozoite micronemal protein essential for cell traversal
SR, scavenger receptor
SRPN, serine protease inhibitor
STEVOR, sub-telomeric variable open reading frame
TEP, Thioester containing protein
TRAP, thrombospondin related adhesive protein
UIS, upregulated in infective sporozoites
WHO, World Health Organisation
wt, wild type

List of Figures and Tables

CHAPTER 1:

General Introduction

1.1 The discovery of malaria and initial control strategies

Malaria is an old and wide spread disease. The first documented observations of the etiology and pathology of this disease remote back to Hippocrates who determined its prevalence in the Mediterranean world and compiled a detailed clinical description, suggesting that “tertian” malarial fevers were distinct and more severe than “quartan” fevers. Solely in 1885, these fevers were shown by Camillo Golgi to be due to different infections by either *Plasmodium vivax* or *Plasmodium malariae,* respectively.

In the seventeenth century, Jesuit missionaries learned from South American natives that the bark of the cinchona tree can be used to treat the fevers associated to malaria. This observation led to the discovery of quinine, which was later developed into the safer and cheaper chloroquine, a potent and widely used antimalarial drug.

The disease was named malaria from the Latin word “mal aria” (bad air) as it was believed to be caused by putrid and poisonous swamp vapors. As early as 95 BC, Lucretious suggested that swamp fever might result from living organisms nevertheless, only in 1880, Charles L. A. Laveran saw for the first time under a microscope a blood stage malaria parasite and put forward the hypothesis that these organisms could be responsible for the relapsing fevers. Nearly 20 year later, Manson and Ross identified a mosquito stage parasite, an oocyst attached to the mosquito midgut wall. This discovery led them to determine the entire life cycle of the parasite. At the same time, the pioneering work of Battista Grassi and colleagues showed that the only human transmitters are the *Anopheline* species of mosquitoes and importantly not all species, just few can act as vectors (reviewed in *Essential Malariology* (2002)) .

Malaria is not restricted to humans, it can infect other mammals, birds and even reptiles. However any given species has a limited host range, as it is only able to infect a single or few closely related vertebrates. Furthermore, it is usually transmitted by a small number of *Anopheline* species.

In the 1950s-1960s following the discovery of the protozoan parasite and its transmission by the mosquito vector, global eradication strategies were put

forward: on one hand, there was widely available human treatment with chloroquine and sulphadoxine/ pyrimethamine and on the other hand, there was also systematic control of the insect vector. This was conducted on two fronts: reclamation of marshy environments, and direct biological and chemical control of the mosquito. Although these eradication measurements were successful in temperate regions, such as Mediterranean Europe and North America, in the Tropics (where malaria still today remains a pestilence) parasite resistance to the cheap most effective drugs and mosquito resistance to the best insecticides quickly emerged. Throughout the 1970s up to early 1990s, interest in the malaria problem declined with very little investment in research, drug discovery or control programs. It is agreed that during this period the malaria situation deteriorated and mortality increased (and many believe is still increasing) especially in Sub-Saharan Africa. Only in 1992 malaria control was re-established as a global health priority by both the international scientific community and governmental agencies such as the World Health Organisation (WHO).

1.2. The malaria burden

Endemic in 107 countries, malaria remains a global problem putting at risk of infection 3.2 billion people. It is estimated that more than 300 to 500 million clinical episodes occur per year leading to 1.5 to 2.7 million deaths worldwide (WHO, world malaria report 2005, www.rbm.who.int/wmr2005/). After tuberculosis, malaria is the second most frequent infectious disease in the world. Eighty percent of fatal malaria cases are in sub-Saharan Africa, where malaria accounts for 20% of all childhood deaths. Outside Africa, two-thirds of the cases occur in just three countries: Brazil, India and Sri Lanka (WHO, wmr 2005).

The association of malaria presence and poverty in the world is striking (Fig1.1). It has been calculated that the economic burden associated with this disease originates in a reduction of 1.3% in the annual economic growth rate leading to a reduction in the gross national product of more that 50% (Sachs and Malaney, 2002). Malaria perpetuates poverty: it affects workers productivity and augments absenteeism, increases medical costs and premature mortality, decreases saving, foreign investment and tourism. Reciprocally, poverty

augments malaria transmission as it reduces the expenditures on prevention, treatment and on government control programmes.

The malaria situation has worsened in Africa during the past decades (Fig1.1A)(reviewed in (Greenwood and Mutabingwa, 2002)). A series of environmental and climatic changes – global warming, floods associated with rains, construction of small dams - have elevated the number of local mosquito populations leading to an increase in infective bites. Political instability such as civil wars and refugee migrations allowed the transfer of malaria from country to country. In addition, the collapse of health services and the increase in populations is expected to double the number of people at risk.

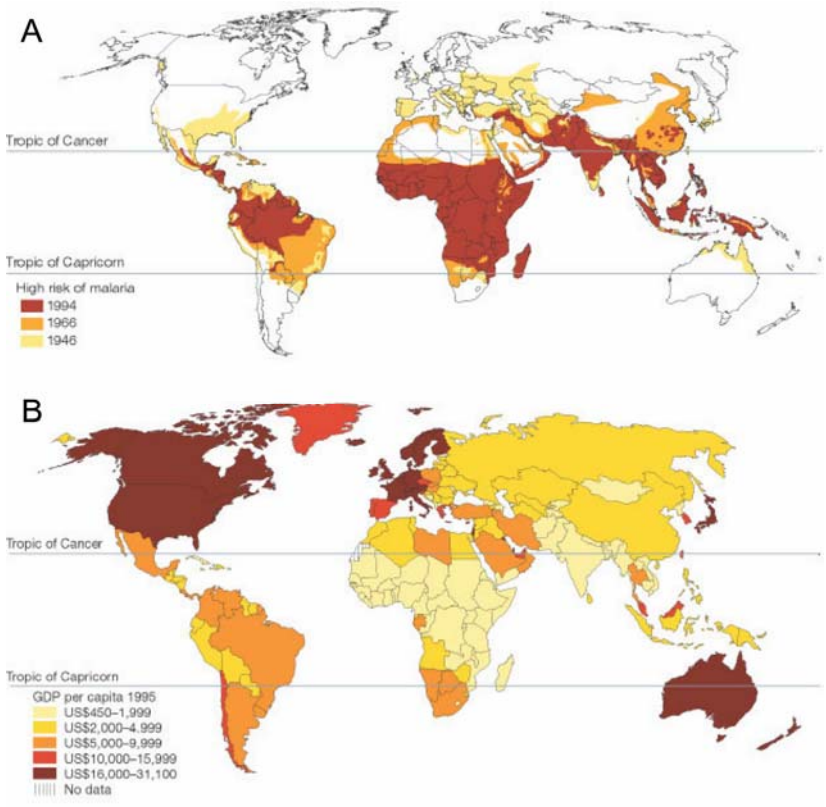

Figure 1.1 Correlation of malaria with poverty.
(A) Global distribution of malaria risk from 1946 to 1994. **(B)** Global distribution of per capita gross domestic product 1995. (Picture adapted from (Sachs and Malaney, 2002)).

Undeniably, two factors have led to the dramatic increase in malaria: resistance of mosquito vectors to insecticides and resistance of *Plasmodium*

parasites to drug treatments. The majority of insecticides that have been used for vector control are common chemicals developed primarily for agricultural pests. Resistance to commonly used compounds such as DTT, carbamates, organophosphates and pyrethroids has been observed (reviewed in (Hemingway and Ranson, 2000)). The molecular basis of insecticide resistance has been attributed to the existence of mutations in target site genes or metabolic alterations at the level of the activity of the detoxification proteins (Hemingway et al., 2004).

Several drugs have been developed for the treatment of malaria symptoms (reviewed in (Ridley, 2002)). However, compounds such as quinoline, antifolates and atovaquone/proguanil have been limited in their use due to high costs, rapid resistance and poor results. Chloroquine and sulphadoxine/ pyrimethamine have been the most successful drugs to alleviate malaria symptoms, but the emergence of resistance makes them, by themselves, ineffective to control this disastrous disease. Artemisinin and its derivatives are the only malarial drugs for which no resistance has been observed so far. Therefore, treatment policies for malaria are now encouraged to contain an artimisinin derivative in a combinational therapy (WHO, wmr 2005).

Malaria also propagates a genetic burden by maintaining in the affected population hereditary illnesses confering resistance to malaria infection. The phenomenon of 'heterozygote advantage' perpetuated by these ilnesses, can maintain in high frequencies the diseased traits in African populations. Among them, sickle cell anaemia is a well known example as reviewed in (Williams, 2006). Other abnormalities include hemoglobin C, α and β thalassemias, glucose-6-phosphate dehydrogenase deficiency and hereditary ovalocytosis (Williams, 2006). Several other polymorphisms have been implicated with increased susceptibility to malaria infection. Examples of these are the polimorphisms in the ICAM-1 putative receptor (Fernandez-Reyes et al., 1997) and in the promoter region of tumor necrosis factor alpha, TNF-α (McGuire et al., 1994).

The clinical features of the disease can vary from person to person depending on their immunological history and the type of malaria. Generally, the incubation period until the first fever, lasts one to four weeks. The classic symptoms include a persistent fever, shivering, joint pains, headaches and vomiting. Severe and complicated malaria can cause anemia, renal failure, hypoglycemia, pulmonary edema, shock, coma and death (Haldar et al., 2007). The 3 primary pediatric causes of death are: severe malaria anemia, cerebral malaria and acidosis (Haldar et al., 2007). If diagnosed on time and treated adequately malaria can be cured. However, only 60% of all malaria sufferers have prompt access to appropriate treatment within 24 hours of the onset of symptoms (WHO, wmr 2005). Due to partially effective immunity *Plasmodium* may persist in the blood at low non-clinical levels. In western travellers and migrant workers without partial immunity mortality can occur within 24 hours from the first appearance of symptoms.

1.2.1 Malaria Control in the 21st century: New efforts to fight and old disease

In 1998, the WHO launched the “Roll back malaria” partnership, which has committed itself to reduce by half the burden of malaria by the year 2010. Priority malaria control strategies have been attributed according to epidemiological settings (WHO, wmr 2005). Treatment stratergies consists of early and effective case management including combinational therapy for suspected cases. In stable endemic transmission areas where the availability of health support is reduced, home management is also implemented. Prevention strategies include insecticide treated nets (ITN) for children under 5 and pregnant women, indoor residual spraying (IRS) and intermittent preventive treatment (IPT) during pregnancy. In unstable transmission areas, prevention also includes larvae control (larviciding) and environmental and agricultural management strategies to minimize mosquito breading and concomitant human bites (WHO, wmr 2005).

In addition support from applied scientific research aims to develop new tools such as new drugs, vaccines, transmission blocking vaccines, and also new strategies to eradicate the vector or control its vectorial capacity. The remaining

of the chapter will review this novel approaches together with the current knowledge of the parasite life cycle and its interactions with the vertebrate and mosquito hosts.

1.3 Overview of the malaria infection cycle:

Malaria is a successful epidemic disease in part because of its transmission via mosquito vectors. There are more than 2500 known mosquito species worldwide. However, only 50-60 *Anopheline* species distributed throughout the globe are important malaria transmitters (Fig 1.2). The *Anopheles gambiae sensu lato (s.l.)* complex consists of six sibling species (*An. gambiae, An. arabiensis, An. quandriannulatus, An. bwambae,* East African salt-water breeder *An. merus* and West African salt-water breeder *An. melas*) with all taxa of this group being native to sub-Saharan Africa (Powell et al., 1999).

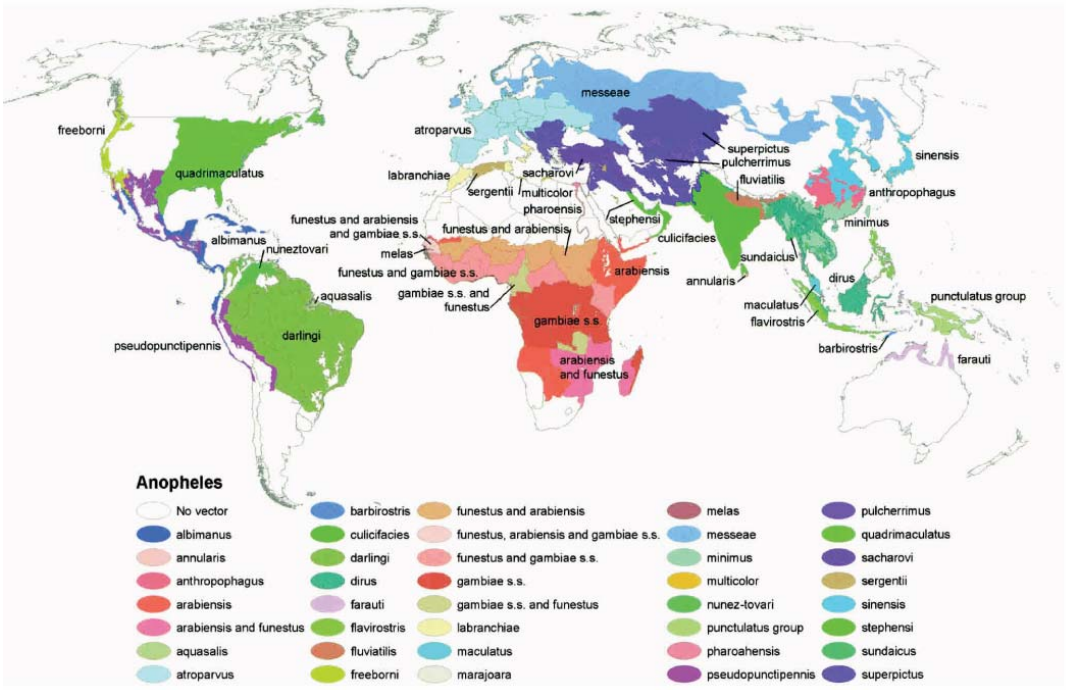

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Figure 1.2 Global distribution of dominant malaria vectors

Adapted from (Kiszewski et al., 2004)

Anopheles gambiae is the most prominent human malaria transmitter due to its highly anthropophilic behavior; populations are commonly found around

human habitations and blood feed almost exclusively on humans. In addition, it is long lived and has the highest rate of *P. falciparum* sporozoite development.

Of the 120 *Plasmodium* species known, only 4 are able to sustain a human infection: *Plasmodium ovale, Plasmodium malariae, Plasmodium vivax and Plasmodium falciparum.* The later two species account for more than 80 percent of all deaths worldwide (WHO, wmr 2005).

Throughout its life cycle, the parasite undergoes a complex development in both the vertebrate and the vector host. In brief, when an infected female mosquito feds on a vertebrate host it will inject with its saliva sporozoites that once taken to the liver invade hepatocytes (Fig1.3). Maturation time depends on the malaria species and the parasite differentiates into an exo-erythrocytic (EE) trophozoite. When the next parasite stage (the exo-erythrocytic schizont,) has grown to a large size, eventually differentiating into EE merozoites, the infected hepatocyte will bust and release parasites into the blood (reviewed in (Frevert, 2004; Yuda and Ishino, 2004)). This single cycle is called the exo-erythrocytic schizogony. In *P. vivax and P. ovale* dormancy in the liver in the form of a hipnozoite can be observed at this stage with relapses occurring up to several years after the initial illness.

The erythrocytic schizogony phase (Fig 1.4) starts with the invasion of the red blood cell by the merozoite. Similarly to the liver, the invaded merozoite will give rise to an early trophozoite or ring stage, followed by a late trophozoite, an immature and a mature schizont. Once merozoites are fully formed the red blood cell will burst and release the parasites into the circulation where they can re-invade un-infected erythrocytes. Gametocytogenesis or sexual development occurs during the erythrocytic cycle when some parasites will give rise to female and male gametocytes (Fig 1.4).

When a mosquito female feeds on an infected vertebrate gametocyte carrier it will ingest a few sexual staged parasites. In the mosquito midgut, gametocytes escape from red blood cells within minutes to undergo differentiation and form female and male gametes (Fig 1.5). After fertilisation the zygote, the only diploid parasite form, undergoes meiosis and differentiates into a

motile haploid ookinete. To continue the cycle, the ookinete will invade the midgut epithelial until it reaches the basal epithelial cell surface, where it will round up and form an oocyst. During the next 10 days, the oocyst will undergo synchronized endomitotic divisions to yield thousands of haploid nuclei which can then undergo cytokinesis and form sporozoites. These will be released into the mosquito haemocoel and will invade the salivary glands (Fig 1.6). The cycle is completed when the infectious mosquito bites and inoculates a new individual (reviewed in (Ghosh et al., 2000)).

1.4 Molecular interactions between parasite, host and vector:

1.4.1 Exo-erythrocytic stage

An infectious mosquito can hold thousands of sporozoites in its salivary glands. In spite of this, in the initial seconds of the fed, deposits a substantially small number of parasites in the vertebrate's skin (Amino et al., 2006; Vanderberg and Frevert, 2004). Nevertheless, as little as two to ten sporozoites, have been shown to be sufficient to initiate an infection (Khusmith et al., 1994). By active "crawling" a few of the skin deposited sporozoites find a capillary and successfully invade it (Amino et al., 2006; Vanderberg and Frevert, 2004). It is not known if chemotactic signals are used to find a blood vessel or if the invasion of the vessel requires specific receptors, however acquired immunity has been suggested to delay this invasion process (Vanderberg and Frevert, 2004).

Once in the liver sinusoids, the sporozoite glides along the endothelia until it finds a Kupffer cell to invade (Fig 1.3). It then rests shortly in the space of Disse and promptly invades a series of hepatocytes via cell traversal motility, before it chooses a final cell where it will form a parasitophorous vacuole (PV) and differentiate into an exo-erythrocytic form (EEF) (Mota et al., 2002).

Several parasite molecules have been shown to be crucial for liver stage invasion. These include circumsporozoite protein (CSP), thrombospondin related adhesive protein (TRAP), sporozoite micronemal protein essential for cell traversal (SPECT) (Ishino et al., 2004), *Plasmodium* perforin-like protein 1 (PPLP1 or SPECT2) (Kaiser et al., 2004b)and cell traversal protein for ookinete

and sporozoites (CelTOS) (Kariu et al., 2006). While CSP and TRAP have been implicated in motility and attachment/recognition of hepatocytes by sporozoites, the latter three seem to have a role in either Kupffer cell invasion (PPL1) or cell traversal ability.

The liver is often characterised as an “immune compromised” organ due to its particular inflammatory responses under the phenomenon portal vein tolerance (Cantor and Dumont, 1967). Several studies have been performed to understand specific immune responses against *Plasmodium* liver stages, as infection might be completely averted by a vaccine against this pre-expansion stage (reviewed in (Doolan and Martinez-Alier, 2006)). A vaccine has been shown to generate complete liver stage-specific protective immunity (Chatterjee et al., 1996). It comprises irradiated sporozoites which are able to infect the liver but cannot differentiate. Although the mechanisms underling this protective immunity are not fully understood, the infection mediated by these irradiated sporozoites has been suggested to induce different inflammatory responses to those triggered by infectious sporozoites (reviewed in (Krzych et al., 2000)). A second EE vaccine (RTS,S) based on CSP antigen has been shown to reduce risk of clinical malaria, delay time to new infection, and reduce episodes of severe malaria in African children (Alonso et al., 2004). It also confers partial protection in African children aged 1-4 years living in rural endemic areas against a range of clinical disease caused by *P. falciparum* for at least 18 months (Alonso et al., 2005).

Alternatively, immunization could be achieved via genetically attenuated sporozoites. Three genes UIS3, UIS4 (upregulated in infective sporozoites) (Mueller et al., 2005a; Mueller et al., 2005b) and P36p (van Dijk et al., 2005) when disrupted do not allow *P berghei* parasites to sustain an infection. Similarly to attenuated sporozoites, these knock out (KO) parasites are motile, invade Kupffer cells and hepatocytes, but no EEF development is seen. Furthermore protective immunogenicity was found for UIS3 and P36p when used as immunogens. All three genes have homologues in *P. falciparum*, thus increasing

our knowledge about the protein-protein interactions between host and parasite offers new avenues for anti-parasitic strategies.

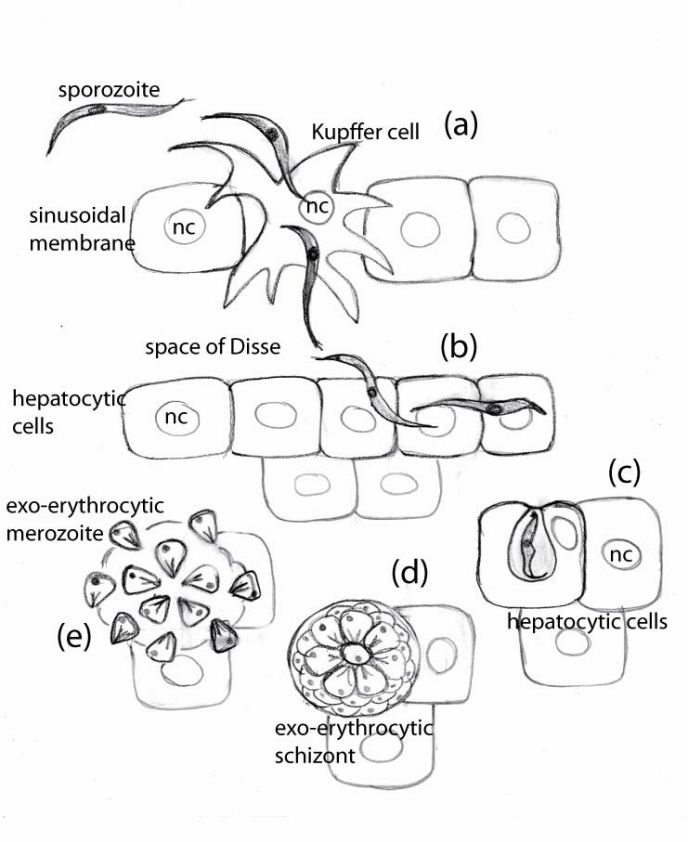

Figure 1.3 Exo-erythrocytic schizogony.

Injected sporozoites migrate to the liver sinusoids and invade a Kupffer cell to gain access to the space of Disse **(a)**. The parasite reaches the liver parenchyma where it invades several hepatocytes in a process called cell traversal **(b)**, until it reaches a final hepatocyte where it forms a parasitophorous vacuole required for subsequent development **(c). I**n the infected hepatocyte the parasite differentiates into an exo-erythrocytic schizont **(d)** and when maturation is complete the infected cell bursts releasing the exo-erythrocytic merozoites **(e)** which migrate to the blood stream. nc, nuclei

1.4.2 Erythrocytic stage

Once in the blood, the merozoite must recognize, attach and enter an erythrocyte for infection to continue (Fig 1.4) (reviewed in (Cowman and Crabb, 2006)). This process occurs very rapidly has the surface antigens of this extracellular form of the parasite are highly susceptible to immune attack. Attachment occurs at any point of the parasite surface and a reorientation event follows in order to juxtapose the apical end of the merozoite with the blood cell membrane. In this position the parasite initiates invasion of the erythrocyte in part mediated via the action of its apical organelles: the micronemes, rhoptries and dense granules. Initially a tight junction is formed and moves from the apical to the posterior end of the merozoite mediated by the parasite actin-myosin motor

(Keeley and Soldati, 2004). As the parasite moves into its host cell it creates a PV isolating himself away from the host cytoplasm (Fig 1.4). The molecule or molecules involved in the initial attachment are not known but this recognition is of long distance, of low affinity and reversible (Bannister and Dluzewski, 1990). Several families have been suggested to potentially play a role in this process: 6-cys family members, the abundant merozoite surface proteins (MSPs) and the peripheral proteins (reviewed in (Cowman and Crabb, 2006)). There is some evidence that MSP1, the most abundance surface protein and dominant antigen, may mediate the initial contact (Goel et al., 2003). For invasion, several parasite proteins have been implicated: reticulocyte-binding protein homologue 1 (RBP1) (Rayner et al., 2001; Triglia et al., 2005), RBP2b (Duraisingh et al., 2003b) and RBP4 (Stubbs et al., 2005) as well as erythrocyte binding A 175 (EBA-175) (Duraisingh et al., 2003a), EBA-140 (Orlandi et al., 1992) and EBA-181 (Gilberger et al., 2003). Also, some erythrocyte receptors for these ligands have been identified. These include glycophorin A (Orlandi et al., 1992) and C (Maier et al., 2003).

Thought to be essential for parasite survival although its molecular function remains to be elucidated, MSP1 is a leading malaria vaccine candidate (Holder et al., 1999). Another vaccine candidate is apical membrane antigen 1 (AMA1) a micronemal associated protein, dispensable for initial attachment but required for reorientation and invasion (Mitchell et al., 2004). Although its function is not perfectly delineated it appears to be required to establish the apical interaction by parasite adhesins located initially at the neck of the rhoptries and in the micronemes.

Once invasion has been established, parasite mediated erythrocyte remodeling is undertaken to facilitate the export of virulence and pathogenic factors to the host membrane. The variant antigen (VAR) family, the sub-telomeric variable open reading frame (STEVOR) family and the repetitive interspersed (RIFIN) family, all know for their antigenic variation, are amongst the highly secreted proteins (Rasti et al., 2004). The best described exported protein is PfEMP1.

Multiple parasite ligands, invasion pathways and antigenic variation have important implications for the infection. The molecules on the erythrocyte are highly polymorphic in the human population (Zimmerman et al., 2003) and vary according to the lifespan of the erythrocyte. Also host immune responses could block invasion pathways and recognize and eliminate infected erythrocytes. Therefore, multiple ligands/receptors and variant erythrocyte infected antigen presentation could improve parasite fitness within the host and should be kept in mind in any protective strategy.

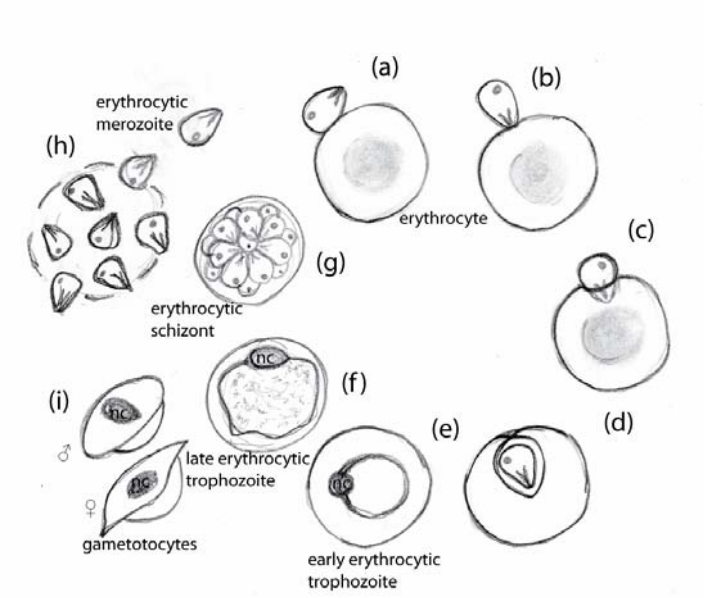

Figure 1.4 Erythrocytic schizogony
Attachment to the erythrocyte occurs at any point of the parasite surface **(a)** and is followed by a reorientation event leading to the formation of a tight junction between the apical end of the merozoite and the erythrocyte membrane **(b)**. In an actin-myosin motor dependent movement the parasite initiates invasion by moving the tight junction from its apical to its posterior end **(c)** creating around it a parasitophorous vacuole **(d)**. Early- followed by late-erythrocytic development and subsequent schizont formation **(e-g)** will ensue and the erythrocytic schizont will develop into merozoites that with the burst if the infected blood cell **(h)** can infect new erythrocytes **(a).** Sexual development occurs when a few parasites escape the erythrocytic schizogony cycle and differentiate into female and male gametocytes **(i).** nc, nuclei

1.4.3 Sexual development

Sexual development of the malaria parasite starts when gametocytes arise from asexual schizonts (Fig 1.4). What stimulates gametocytogenesis has been the target of much investigation, but remains a black box (review in (Talman et al., 2004). Although clinical symptoms appearance (Miller, 1958) and immune stress (Smalley and Brown, 1981) have been suggested to play a role they still remain to be validated. Recently, gametocyte specific mRNA has been

detected as early has the first erythrocytic schizogony (Schneider et al., 2004), suggesting that a regular proportion of gametocytes would develop during each growth cycle. Knowledge of gametocyte carriage rates could also be used to monitor intervention strategies as any decrease in the transmission intensity can shift the disease burden as well as the pattern of infectiousness (reviewed in (Drakeley et al., 2006)).

The presence of gametocytes is clearly vital for transmission however the correlation between gametocyte densities and transmission is not well understood. Nevertheless, the theory of reproductive restrain has been put forward, suggesting that gametocytemia is kept low in order to avoid transmission blocking immunity to be generated (Taylor and Read, 1997). Specific immune responses towards the sexual stages of the parasite are not well established. Antibodies against proteins found on the surface of the gametocytes (Pfs/vs230) or gametes (Pfs/vs 48/45) were identified to naturally arise in infected populations (Bousema et al., 2006). These may reduce the infectivity of humans to mosquitoes and could therefore be used in a potential transmission blocking vaccine. Transmission-blocking vaccines are one strategy for controlling malaria, whereby sexual-stage parasites are inhibited from infecting mosquitoes, and common targets for this approach would be surface or essential secreted molecules. Other known gametocyte surface antigens are members of the VAR and STEVOR family (Sharp et al., 2006), which could contribute to immune evasion mechanisms.

1.4.4 Mosquito stages: Gamete-to-ookinete transition

The mosquito ingests a substantial blood meal containing a small amount of *Plasmodium* G_0-arrested gametocytes (Fig 1.5). The process of gametogenesis has been studied in detail and some factors are thought to be essential: a sudden drop of temperature and possibly pH due to the presence of xanthurenic acid in the mosquito midgut (Billker et al., 1998). The male gamete undergoes further differentiation resulting in the sudden release of 8 flagella in a process termed ‘exflagellation’. Each flagellum produces a mature male microgamete, which swims freely until it encounters a female macrogamete.

Fertilisation is achieved by nuclear fusion and produces a zygote, which then transforms into a motile, 'banana-shaped' structure called ookinete (9-36 hours post infection (hpi), depending on the parasite specie). A major switch in surface protein repertoire seems to occur at this transitional stage where most known surface molecules are shed and replaced by ookinete molecules required for defense and host interaction (Kaushal et al., 1983).

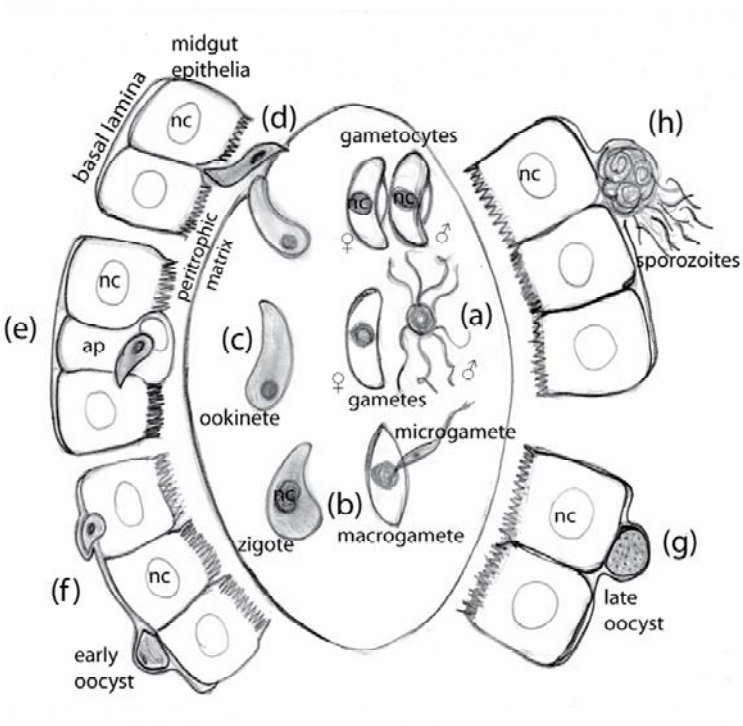

Figure 1.5 Sporogony
Inside the mosquito, the ingested gametocytes differentiate into gametes **(a).** Once gametogenesis is complete the male microgamete swims freely in the blood bolus until it encounters a female macrogamete in order for fertilization to occur culminating in the formation of a zygote **(b)**. The zygote further develops into an ookinete **(c)**, which migrates to the periphery of the blood bolus breaks through the peritrophic matrix and invades a midgut cell **(d).** The ookinete will traverse several cells which will undergo apoptosis and extrusion from the epithelium **(e).** It will eventually reach the basal lamina where it will round up and form an oocyst **(f).** Oocyst growth and maturation **(g)** will lead to the formation of thousands of sporozoites that once fully differentiates will egress from the oocyst and be released into the haemolymph **(h).**

1.4.5 Mosquito stages: Ookinete-to-oocyst transition

The ookinete is an invasive stage. It moves via gliding motility, migrating through the blood meal until it crosses two barriers in the mosquito: the peritrophic matrix (an acellular chitinous mesh approximately 2-10 μm in thickness, synthesized shortly after ingestion of the blood meal, which coalesces around the entire bolus of remaining erythrocytes (Shao et al., 2001)) and the midgut epithelium (Fig 1.5).

Several parasite and vector molecules are essential for this ookinete-to-oocyst transition. Parasite-derived chitinase secretion mediates peritrophic matrix dissolution (Langer and Vinetz, 2001), allowing the parasite to pass to the ectoperitrophic space. Circumsporozoite and thrombospondin-related anonymous protein-related protein (CTRP), has been shown to be required for ookinete migration and thus essential for invasion (Dessens et al., 1999). PPLP3 (or membrane-attack ookinete protein, MAOP) KO analysis (Kadota et al., 2004) suggested that midgut invasion is mediated via an initial receptor-ligand attachment (independent of *pplp3* function) followed by membrane breaching potentially mediated by a pore forming activity of PPLP3 (and PPLP5, see chapter 4). CelTOS, which has been shown to be necessary for cell traversal motility in hepatocyte invasion (see 1.4.1), is also required for ookinete midgut traversal migration (Kariu et al., 2006). The two most abundant ookinete surface proteins, P25 and P28 have partially redundant functions during parasite infection of the mosquito vector (Tomas et al., 2001). Ookinetes lacking both P25 and P28 are still able to cross the midgut epithelium and establish oocyst development albeit at a low efficiency when compared with either single knock downs or wild type parasites. A similar phenotype has been seen for a cystein-rich secreted ookinete protein (secreted ookinete adhesive protein, SOAP (Dessens et al., 2003). Nevertheless, antibodies against P25 and P28 block oocyst formation and are currently on trials as a transmission blocking vaccine (Hisaeda et al., 2000).

There is currently no significant knowledge on the direct molecular interactions of the ookinete with the mosquito tissue, other than that sialic acid moieties are involved in ookinete binding to the epithelia wall and that this binding is inhibited by the expression of SM1 peptide (Ito et al., 2002) or the pre-treatment of guts with the snake venom PLA2 (Moreira et al., 2002). However, following invasion of the midgut tissue significant parasite loses are commonly seen and have been suggested to result from a variety of defence mechanisms mounted by the vector (Huff, 1927; Sinden, 1999). This hypothesis is supported by laboratory selection of mosquito strains that are refractory to the parasite. In

these strains the parasites are killed while traversing the midgut epithelium, by melanisation in the L3-5 strain (Blandin et al., 2004; Collins et al., 1986) or by lysis in the SUAF2 strain (Vernick et al., 1995). Refractoriness appears to have a genetic basis. It is thought to be conveyed by natural resistance alleles (Niare et al., 2002; Riehle et al., 2006b) that limit parasite development in the vector to small oocysts numbers, typically fewer than ten (Billingsley, 1994; Pringle, 1966). Furthermore, the genetic control of refractoriness is complex involving several quantitative trait loci (QTL) each with variable relative contributions dependent on the parasite species (Zheng et al., 1997; Zheng et al., 2003).

Several mosquito immune factors have been identified. Thioester containing protein 1 (TEP1) (Blandin et al., 2004), which is produced by haemocytes and secreted in the haemolymph, binds to the surface of the parasites mediating their killing. Silencing of *TEP1* in the L3-5 strain inhibits melanisation and increases the number of developing oocysts. The absence of two other factors, Leucine rich repeat immune protein 1 (LRIM1) (Osta et al., 2004) or Anopheles *Plasmodium*-responsive leucine-rich repeat 1 (APL1) (Riehle et al., 2006b) leads to substantially increased numbers of oocysts, similarly to the *TEP1* KD, and thus are thought to act in the same or parallel pathways of parasite killing. On the contrary, two C-type lectins (CTLs) – *CTL4* and *CTLMA2* – seem to be exploited by *P. berghei* to protect it from *LRIM1*-mediated killing. Silencing of either of these CTLs leads to killing and melanisation of almost all *P. berghei* ookinetes invading the mosquito midgut (Osta et al., 2004).

Other genes implicated in the mosquito responses to parasite invasion are serine proteases that have been shown to be transcriptionally activated and involved in ookinete killing and melanisation (Dimopoulos et al., 2002; Volz et al., 2006; Volz et al., 2005). A serine protease inhibitor (SRPN) with homology to prophenoloxidase activating enzymes (PPAE), SRPN2 (Michel et al., 2005) has been shown to be a negative regulator of ookinete killing and melanisation.

1.4.6 Mosquito stages: Oocyst-to-salivary gland sporozoite transition

Once the ookinete reaches the basal side of the midgut epithelium, basal lamina components such as laminin and collagen IV interact with parasite

surface proteins and seem to trigger parasite differentiation (Adini and Warburg, 1999; Arrighi et al., 2005). This 10-20 day development (the longest in the malaria life cycle) is accompanied by a series of complex changes (Terzakis et al., 1967) and is intrinsically programmed to "run to completion" (Fig 1.5). But efficiency in sporozoite differentiation can be compromised due to environmental pressures such as nutritional deprivation (the oocyst scavenges from the vector important nutrients for its growth and maturation) or immunological attack (Sinden et al., 2004).

Two genes have been shown to play a crucial role in sporozoite formation. CSP (Menard et al., 1997) and scavenger receptor like protein (SR) (Claudianos et al., 2002)) are essential for sporozoite formation as KO mutants for each protein do not form sporozoites. A third gene, inner membrane complex (IMC) 1a (a family member of the subpellicular network resident IMC1 protein (Khater et al., 2004) is required for invasion as *⊿imc1a* parasites have lost their capacity to invade salivary glands.

After sporogony is complete, sporozoites must be release into the mosquito haemocoel to continue the cycle (Fig 1.5). Sporozoite egress seems to be an active process involving parasite proteases which breach the oocysts capsule to release the parasites. Egress cysteine protease 1 (ECP1) a member of the SERA family of proteases, is involved in this active egress as mutant parasites form viable sporozoites but are incapable of exiting oocysts (Aly and Matuschewski, 2005).

Once in the haemocoel, sporozoites appear to be at the mercy of the heamolymph flow beeing passively taken to all parts of the mosquito body (Hillyer et al., 2006). Only a small proportion of parasites seems to reach the salivary glands (Hillyer et al., 2006; Sinden, 1999) were invasion is mediated via receptor-ligand interactions (Fig 1.6). Sporozoite proteins implicated in this stage of the life cycle are: CSP an important protein not only for sporozoite formation, but also for salivary gland invasion (Myung et al., 2004), and as mention above, liver stage development. MAEBL (Kariu et al., 2002) is required for salivary gland

invasion but not gliding motility and TRAP although it affects gliding motility it is also involved in salivary gland attachment/recognition (Sultan et al., 1997).

The mosquito receptors remain unknown, but a potential candidate (SGS1) has been proposed (Korochkina et al., 2006). Interestingly, the synthetic peptide SM1 which can block ookinete midgut invasion (as mentioned in 1.4.5) can also block sporozoite salivary gland invasion (Ito et al., 2002), suggesting some conservation in receptor-ligand interactions in the two mosquito invaded epithelia.

The successful sporozoites that invade de salivary glands, quickly migrate through the secretory cavities, towards the duct where they can reside for a long time unharmed. With the next bite of the female mosquito they can be deposited into the skin of the vertebrate host, perpetuating the infection cycle.

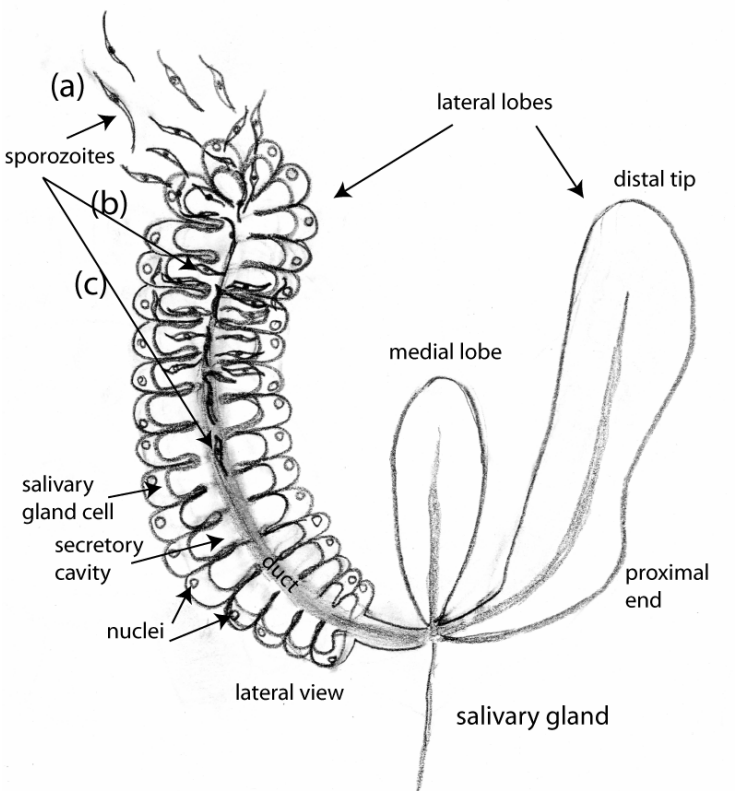

Figure 1.6 Salivary gland invasion
Sporozoites commonly invade the salivary glands through its distal tip **(a)**. They then migrate throught the cytoplasm of the salivary gland cells into the secretory cavities **(b)**. While most parasites reside in the gland cell cytoplasm or secretory cavities, a few sporozoites rest in the duct **(c)** waiting for the female to blood fed.

1.5 Mosquito vector control

Two major concepts arise when thinking of vector based control strategies to reduce malaria transmission. These include the reduction or irradication of the mosquito populations or the abolishment of vectorial capacity.

1.5.1 Reduction/elimination of mosquito populations:

The irradication or reduction of mosquito populations can be achieved via chemical, biological or genetic approaches towards adult or larval stages.

Insecticides such as DTT or pyrethroids have long been used in population control, but increased resistance requires new insecticides to be developed.

Biological agents can be used against either adults or larval stages. For the biological control of larval stages larvivorous fish (Mohamed, 2003; Singh et al., 2006), several arthropods (Aditya et al., 2006; Rey et al., 2004) and *Bacillus thuringiensis* (Bt) are commonly used, with Bt being a highly promising control agent (Mittal, 2003).

Recent studies have provided evidence that entomopathogenic fungi, could yield up to 80% reduction of mosquito populations which come in contact with the treatment (Blanford et al., 2005; Scholte et al., 2005). Furthermore, fungal-infected mosquitoes showed reduced propensity to blood feed which could led to a reduction of human bites (Blanford et al., 2005; Scholte et al., 2006).

Sterile insect technique (SIT), consist in the sterilization of males (via irradiation or genetic means) prior to their release so that they mate with wild females causing them to lay sterile eggs. The success of the technique is dependent on the efficiency of the sex separation, the sterilization procedure and the fitness and mating competitiveness of the lab reared sterile males and there are strategies currently under development (Benedict and Robinson, 2003; Handler, 2002).

Mosquito midguts are full of bacteria, which could be exploited into para-transgenic approaches that could either kill the mosquito or block the

transmission of important pathogens through mosquito populations (Riehle et al., 2006a). Also, *Wolbachia* a common maternally inherited bacterial endosymbiont, confers reproductive advantage to infected females, spreads quickly into uninfected populations thus could be exploited as a drive system to introduce fatal or vectorial blocking transgenes (Sinkins and Godfray, 2004).

1.5.2 Targeting vectorial capacity: mosquito tissue responses modulating malaria development:

Parasite losses in the mosquito are documented at three decisive developmental transitional stages: the gamete-to-ookinete, the ookinete-to-oocyst and the midgut sporozoite-to-salivary gland sporozoite transitions (Alavi et al., 2003; Sinden, 1999; Sinden, 2002). The development of the parasite inside the vector depends on essential parasite genes and positive and negative mosquito factors (review above in *3.4-3.6)*. This is consistent with the notion of parasite killing and melanisation in a refractory strain being complex and depending on multiple quantitative trait loci, (Zheng et al., 1997), allelic variations and polymorphisms. The *TEP1* gene sequence, which is in the *Pen2* region, displays allelic variation between refractory and susceptible mosquitoes (Blandin et al., 2004); and the *Pen1* region shows clusters of extensive sequence polymorphisms that may relate to the refractory phenotype (Thomasova et al., 2002). Also, strong physiological differences have been shown to exist in refractory and susceptible strains (Kumar et al., 2003).

Haemocyte and fat body (systemic immune) responses:

In any insect, systemic immune reactions are mediated by molecules either induced or constitutively secreted into the haemolymph by the fat body cells or haemocytes. Upon binding to pathogens, the recognition proteins (or opsonins) immediately trigger mainly two types of innate immune responses: cellular responses and humoral responses.

In humoral responses, the binding of the opsonins to pathogen-associated molecular patterns activates (commonly through post-translational activation)

proteolytic cascades, which lead to the activation of signaling pathways which themselves cause the transcriptional activation of hundreds of immune-inducible molecules (Dimopoulos et al., 2002).

Antimicrobial peptides (AMPs) represent a well characterised insect humoral reaction and although mainly produced by the fat body and the haemocytes, various other epithelia have been shown to express them (Lehane et al., 1997; Richman et al., 1997; Tzou et al., 2000). Several AMPs are upregulated in *An gambiae* upon *Plasmodium* infection (Dimopoulos et al., 2002; Rosinski-Chupin et al., 2006; Vizioli et al., 2001). Two of then have been implicated in parasite development: Gambicin, an antimicrobial peptide found only in the mosquito lineage, is marginally lethal to *Plasmodium berghei* ookinetes (Vizioli et al., 2001), while the overexpression of Cecropin A results in a 60% reduction in the number of oocysts (Kim et al., 2004).

In Anopheles gambiae, the activation of the prophenoloxidase (PPO) cascade leading to melanotic encapsulation of ookinetes is an example of a refractory mechanism. The prophenoloxidase activating system is composed of an enzyme cascade consisting of pattern recognition proteins, several serine proteases and their negative regulators culminating in the local activation of PPO (Christensen et al., 2005). PPO is believed to be synthesised in oenocytoids (see below (Castillo et al., 2006)) and is activated by PPO-activating enzymes (PPAEs).

Haemocytes are the main mediators of cellular immunity (Lemaitre and Hoffmann, 2007; Meister, 2004). These cells are involved in cellular processes such as phagocytosis and encapsulation, as well as, in part, being involved in the humoral responses, through the production of antimicrobial peptides, opsonizing factors and products of the melanisation cascade (reviewed in (Lavine and Strand, 2002)).

Three haemocyte types have been described in mosquitoes: a) granulocytes perform most of the phagocytosis and represent the main population of haemocytes; b) prohaemocytes may act has undifferentiated stem-cell like precursor cells and finally, c) PPO-expressing oenocytoids are thought to

express the components of the melanisation cascade (Castillo et al., 2006). These cells are mainly found attached to body tissues with a small proportion circulating freely in the haemolymph

Phagocytosis, although important for clearing bacterial infection in mosquitoes (Hernandez-Martinez et al., 2002; Hillyer et al., 2003a; Hillyer et al., 2003b; Moita et al., 2005), in *An gambiae* and *An albimanus* seems not to have a role in eliminating the sporozoite (Hernandez-Martinez et al., 2002; Hillyer et al., 2006), the only parasite stage with which the haemocytes come in direct contact with. However, many genes which have been shown to be involved in immune responses against the ookinete stage during midgut invasion are expressed by haemocytes. These include phenoloxidases (PPOs), thio-ester containing proteins (TEPs), CLIPB serine proteases and serpins. It would be of great interest to enrich the knowledge on the role of these very important cells in the immune response of the malaria vector.

Midgut epithelial responses

It remains controversial whether ookinetes migrate inter- or intracellularly, as different routes have been reported for various mosquito-parasite combinations (Han et al., 2000; Meis et al., 1992; Shahabuddin and Pimenta, 1998; Vlachou et al., 2004; Zieler and Dvorak, 2000). Then again, a general consensus is starting to arise (Baton and Ranford-Cartwright, 2005), which argues that ookinete entry may always be intracellular, but the parasite route can either be intracellular or extracellular. Agreed is the fact that, upon parasite penetration there is extensive ookinete cell traversal, the invaded cells up-regulate several markers such as nitric oxide synthase (NOS) (Han et al., 2000) , peroxidases (Kumar et al., 2004) and SRPN10 (Danielli et al., 2003) and commit to apoptosis. They also undergo extensive cytoskeletal remodeling, culminating in an actin–based extrusion of the invaded cell from the midgut epithelia into the lumen (Gupta et al., 2005). The molecular interactions inducing cell death are not fully understood but P25/28 and subtilisin 2 (SUB2), a parasite secreted protease are shed into the invaded cells (Danielli et al., 2003; Han et al., 2000). The baso-lateral movements of the

ookinete have been suggested to represent an immune evasion strategy to escape high levels of reactive oxygen species induced in the invasion process.

Parasites suffer significant losses during midgut invasion, in refractory mosquitoes and in fully susceptible mosquitoes (Blandin et al., 2004). The kinetics of parasite killing and the observation of bubble-like projections in close proximity to dying ookinetes suggest that ookinetes are lysed in the midgut epithelium and that this accounts for major parasite losses associated with midgut invasion in both strains (Blandin et al., 2004). In the L3-5 refractory strain, melanisation does not kill parasites, but occurs after parasite death, possibly to isolate dead parasites from surrounding mosquito tissues (Blandin et al., 2004). However different genetic backgrounds can kill parasites directly via melanisation. This is the case, when mosquitoes are depleted of CTL4 (and partially CTLMA2) (Osta et al., 2004) or of CLIPA2 and CLIPA5 comcomitantly (Volz et al., 2006).

Recently, it has been shown that ookinete killing occurs in the extracellular space of the midgut cell, between the basal lamina and the plasma membrane (Shiao et al., 2006). All negative and positive regulators identified so far have been shown (Blandin et al., 2004; Michel et al., 2005) or have been suggested to be constitutive haemolymph proteins (Frolet et al., 2006; Osta et al., 2004; Riehle et al., 2006b), but their function in modulating killing or survival of the parasite occurs in the midgut epithelium. This would suggest that their activation is dependent on some midgut factor which is itself activated due to ookinete invasion.

Furthermore, transcriptomic microarray analysis of midgut epithelial responses as revealed that as much as 7% of the surveyed mosquito transcriptome may be regulated upon parasite invasion – this includes genes involved in cytoskeletal remodeling, apoptosis, immune responses, the redox state, cell adhesion and the extracellular matrix (Vlachou et al., 2005). Discovered in this study, *RFABG* encodes the precursor of lipophorin a lipid transport vehicle that is a positive factor of parasite development and mosquito egg development.

The mosquito midgut local epithelial responses and their interplay with (at least) the haemolymph derived systemic responses are central for the survival and development of *Plasmodium*, and is thus also one of the most attractive sites for novel targeted malaria control strategies.

Salivary gland responses:

In contrast to the midgut epithelium, the salivary gland cells seem not to undergo major damage upon invasion and the sole route of migration seems to be intracellular (Pimenta et al., 1994; Sterling et al., 1973).

Only recently, 57 *An. gambiae* genes (Rosinski-Chupin et al., 2006) have been identified to be differentially expressed in infected salivary glands, but no local salivary gland epithelial response involved in the development of the malaria parasite has been described to date.

Unfortunately the current knowledge of mosquito immune responses after the completion of the sporogonic cycle is extremely limited. The importance of the sporozoite has the only parasite stage capable of naturally infecting humans justify a need to increase the understanding of the mosquito innate immune system, together with a much detailed description of mosquito-parasite molecular interactions at this stage.

1.6 Aims of the thesis:

Vector-parasite interactions are central for the development and transmission of malaria to humans. The genomic revolution and the development of functional tools in both the vector and the parasite such as transgenesis and RNAi allow a detailed analysis of the interactions between the malaria parasite and the mosquito vector at the molecular level.

To develop novel vector based intervention strategies, it is central to increase the knowledge of the molecular interactions and immune responses of the vector mosquito with the *Plasmodium* parasite. To address that, the general objectives of the project presented here were to characterise mosquito tissues responses in response to parasite invasion by:

1) The functional characterisation of SRPN6, a serine protease inhibitor gene strongly upregulated in midguts after ookinete invasion and in salivary glands after sporozoite invasion, in both invaded epithelia and its putative role in vectorial capacity (Chapters 2 & 3).

2) The further understanding of the mechanism of ookinete invasion through the functional characterisation of a parasite protein (PPL5) essential for midgut traversal (Chapter 4).

3) Determination of the molecular make-up of *An gambiae* haemocytes by characterising their gene expression profile leading to the discovery of novel immunity genes and molecular markers for these cells (Chapter 5).

MOSQUITO EPITHELIAL IMMUNE RESPONSES AGAINST *PLASMODIUM*

The effect of SRPN6 on *Plasmodium* development

CHAPTER 2:

An immune-responsive serpin, SRPN6, mediates mosquito defense against malaria parasites.

This study has been published in:
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** joint first authorship*

***SB Pinto** contributed with experimental design, performed all research related to SRPN6 analysis in An. gambiae, analysed results and was involved in the writing to produce the final manuscript.*

Abstract

We have functionally analyzed the orthologous *SRPN6* genes from *Anopheles stephensi* and *Anopheles gambiae* using phylogenetic, molecular, reverse genetic and cell biological tools. The results strongly implicate *SRPN6* in the innate immune response against *Plasmodium*: This gene belongs to a mosquito-specific expansion cluster that includes three additional *Anopheles* serpins. Its expression is induced by *E. coli* and both rodent and human malaria parasites. It is specifically expressed in midgut cells invaded by *Plasmodium* ookinetes, as well as in circulating and attached haemocytes. Knockdown of *SRPN6* expression by RNAi in susceptible *An. stephensi* leads to substantially increased parasite numbers, whilst depletion in susceptible *An. gambiae* delays progression of parasite lysis without affecting the number of developing parasites. However, the *An. gambiae SRPN6* knockdown increases the number of melanized parasites in the L3-5 refractory strain and in susceptible G3 mosquitoes depleted of *CTL4*. These results indicate that *AsSRPN6* is involved in the parasite killing process, whereas *AgSRPN6* acts on parasite clearance, by inhibiting melanisation and/or promoting parasite lysis. We propose that these observed phenotypic differences are due to changed roles of the respective target serine proteases in the two mosquito species.

2.1 Introduction

Malaria is one of the most devastating infectious diseases with 550 million cases every year (Snow et al., 2005). The causative agents of malaria are protozoan *Plasmodium* parasites, which are transmitted to humans exclusively by anopheline mosquito vectors. In the mosquito midgut lumen, *Plasmodium* gametocytes differentiate into gametes. After fertilization the zygotes differentiate into motile ookinetes that invade the midgut epithelial cells approximately 20-36 h post infection (hpi). Upon emerging from the basal epithelial cell surface, ookinetes differentiate into oocysts each producing thousands of sporozoites within 10d. After release into the mosquito hemocoel, sporozoites invade the salivary glands and the cycle is completed when the mosquito bites and inoculates a new individual with stored sporozoites mixed with the saliva (Ghosh et al., 2000)

Only a limited number of mosquito species are able to transmit a *Plasmodium* parasite. Moreover, individual mosquitoes differ considerably in their permissiveness; large losses of parasites are observed in any mosquito as the parasites develop, predominantly during ookinete midgut invasion (Niare et al., 2002) (Sinden, 2002). Melanotic encapsulation and parasite lysis are two mechanisms responsible for attrition of parasites during midgut invasion (Collins et al., 1986; Vernick et al., 1995). Lysis takes place as the ookinetes traverse the epithelial cells, and encapsulation begins when the ookinetes emerge from the midgut. The genetic basis for variation in mosquito permissiveness to parasite development is still not fully understood. Several mosquito immune genes respond transcriptionally to midgut invasion by malaria parasites (Dimopoulos et al., 2002; Richman et al., 1997; Tahar et al., 2002), but their mechanisms of action remain mostly unclear. Recent studies have demonstrated that ookinete coating with a complement-like mosquito protein (TEP-1) is one mechanism by which ookinetes are lysed in mosquito midguts (Blandin et al., 2004). Using gene silencing Osta *et al*. (Osta et al., 2004) have shown that a leucine rich-repeat

protein (LRIM1) also acts as an antagonist of ookinete development whereas two C-type lectin immune proteins (CTL4 and CTLM2) act in the same pathway to protect the parasite. These proteins are expressed in naïve mosquitoes and are induced in the midgut (including attached haemocytes) during ookinete invasion (Osta et al., 2004). Furthermore, knockdown (KD) of *SRPN2*, the *An. gambiae* ortholog of the *Drosophila* gene Spn27A, accelerates parasite lysis and causes melanisation of the remaining ookinetes (Michel et al., 2005). The specific roles that these molecules play in mosquito-parasite interactions, as well as the mechanisms of their regulation, remain to be elucidated.

We are interested in understanding interactions between parasites and vector mosquitoes at the molecular level. Recently we identified numerous genes up-regulated in the midgut in response to *P. berghei* (Abraham et al., 2004; Srinivasan et al., 2004; Vlachou et al., 2005). One of these, *SRPN6*, a member of the serine protease inhibitor (serpin) family, is the subject of this report. Its expression is strongly induced in both *An. stephensi* and *An. gambiae* midguts during ookinete invasion. *AgSRPN6* is also induced by the presence of *Escherichia coli* in the midgut lumen. *SRPN6* KD leads to substantially increased parasite numbers in *An. stephensi*, while in *An. gambiae*, *SRPN6* KD increases the number of melanized parasites, both in the L3-5 refractory strain and in susceptible G3 mosquitoes depleted of CTL4. We propose that *SRPN6* acts in synergy with CTL4 as a component of the midgut epithelial immune response system.

2.2 Results

Isolation and phylogenetic analysis of SRPN6 genes

The *SRPN6* gene of *An. gambiae* (*AgSRPN6*), encoding a predicted protein of 494 amino acids, was identified in the annotated genome sequence (Christophides et al., 2002). The corresponding *An. stephensi* gene (*AsSRPN6*) was identified in a library enriched for *An. stephensi* midgut cDNAs expressed during *P. berghei* invasion and development (Abraham et al., 2004; Srinivasan et al., 2004) and encodes a predicted protein of 497 amino acids.

Using BLAST (Altschul et al., 1997) we identified and then incorporated into a protein alignment of arthropod inhibitory serpins (Michel et al., 2005) additional *AgSRPN6*-like sequences: from GENBANK (Benson et al., 2005) the *An. aegypti* gene index (http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=a_aegypti, *Ae. aegypti* gene index), and the honey bee genome via ENSEMBL (Hubbard et al., 2005). Improved annotation of the honeybee serpin using GENEWISE (Birney et al., 2004) resulted in an almost full length coding sequence. Both Baysian Inference (BI) and Maximum Likelihood (ML) analyses of the combined alignment identified the same set of ten sequences as clustering stably with AgSRPN6 (results not shown). Further phylogenetic analysis of this set using both BI and ML found all the mosquito sequences clustering together to the exclusion of the *Drosophila* and honeybee sequences, strongly suggesting an expansion of this serpin clade limited to the mosquito lineage (Fig. 2.1A). Additionally, AgSRPN6 clusters stably with AsSRPN6 (posterior probability 1.0, bootstrap 100%), clearly indicating orthology. Equally stable clustering identified two *An. gambiae*/ *Ae. aegypti* pairs (SRPN5/TC47107; SRPN16/TC39371) as orthologous.

Serpins play a wide variety of roles in the physiology of many organisms (Gettins, 2002; Silverman et al., 2001) Each serpin interacts with its target protease via an exposed C-terminal reactive center loop (RCL), which places the so called P1-P1' scissile bond of the reactive center in an accessible position for cleavage by the protease. The serpin then undergoes a drastic conformational change, trapping the protease in a stable complex with the inhibitor. Thus, serpins act as suicide substrates whose target specificity is determined primarily by the scissile bond sequence. Fig. 1B compares the 28-residue RCL loop regions of SRPN6 and related serpins (cf. Fig. 2.1A). All ten are indeed similar, but only in AsSRPN6 and AgSRPN6 are they identical and have the same scissile bond, strongly indicating similar target specificities.

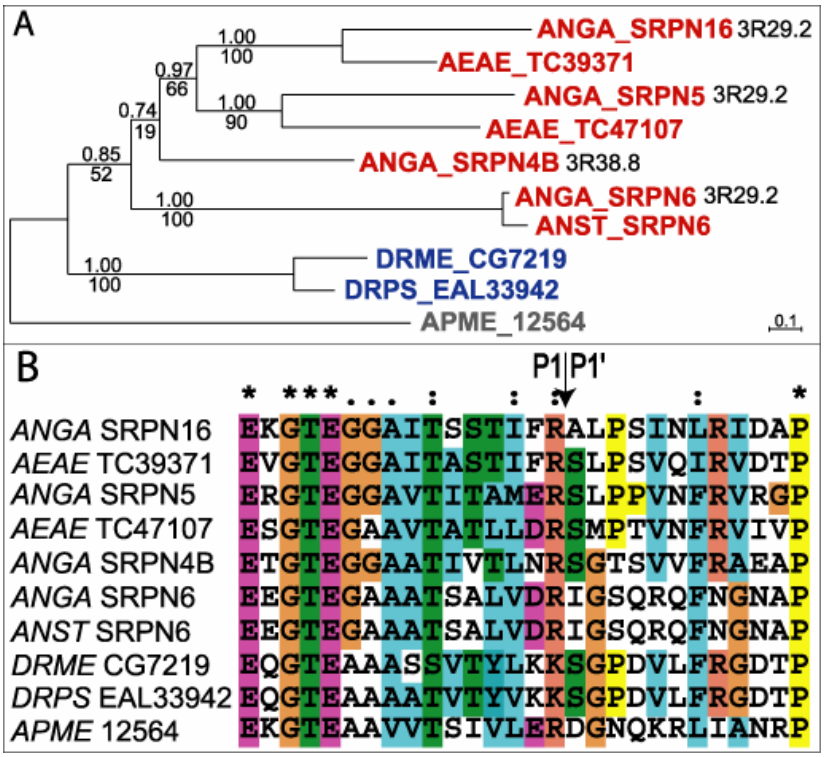

Figure 2.1. Phylogenetic analysis of the SRPN6 mosquito-specific expansion cluster.
A. Baysian inference of phylogenetic relationships of arthropod serpins, related to SRPN6. *An. gambiae* SRPN4, 5, 6 and 16 form a mosquito-specific expansion cluster together with *An. stephensi* SRPN6 and *Ae. aegypti* TC47107 and TC39371. Red labels correspond to mosquitoes, blue to drosophilids, and grey to honeybee. ANGA, *An. gambiae*; ANST, *An. stephensi*; DRME, *D. melanogaster*; DRPS, *D. pseudobscura*; APME, *Apis mellifera*. Chromosomal arm (3R) are specified in Mb. Numbers on each branch indicate posterior probability (top) and bootstrap values (bottom). **B.** Sequence alignment of the RCL loops. P1-P1' sites flank the arrow.

Ookinete invasion induces SRPN6

Since *AsSRPN6* is enriched in infected midgut tissues (Abraham et al., 2004; Vlachou et al., 2005), we analysed the expression of both *SRPN6* genes in more detail. Using RNA blot (Northern) analysis we were unable to detect significant *AsSRPN6* expression at any stage during development or in adult females fed with uninfected blood (Fig. 2.2A), but detected strong expression in midguts after feeding with blood containing gametocyte-competent but not incompetent (Paton et al., 1993) *P. berghei*. Furthermore, strong *AsSRPN6* induction was observed in infected midguts during the 18 to 48 hpi period (Fig. 2.2B; maximum at 30 hpi), coinciding with the period when ookinetes invade the midgut epithelium. Using quantitative real time RT-PCR (qRT-PCR), *SRPN6* expression was detected at low levels at all life stages of *An. gambiae*, and was highest in adults (Fig. 2.2F). The observed differences between the two species probably reflect the higher

sensitivity of qRT-PCR. *AgSRPN6* expression is unaltered after a non-infective bloodmeal, as in *An. stephensi*, but is induced dramatically in the adult midgut after an infective *P. berghei* bloodmeal, maximally during the ookinete invasion period (20-24 hpi in *An. gambiae*; Fig. 2.2D). Interestingly, a small 4-fold increase in *AgSRPN6* expression was also detected in carcasses of mosquitoes 48 hpi (Fig. 2.2E).

AsSRPN6 expression was also induced after infection with the human malaria parasite, *P. falciparum* (Fig. 2.2F) at a level significantly lower than after *P. berghei* infection (30 as compared to 25 cycles of RT-PCR). Similarly, both prevalence (30-40% vs. 90%) and intensity (0.25-0.4 vs. 121 oocysts/gut) were considerably lower for *P. falciparum* as compared to *P. berghei* infection. It is possible that *SRPN6* upregulation increases with the number of invading ookinetes.

E. coli but not o'nyong-nyong virus induces* AgSRPN6 *expression

We investigated whether other foreign organisms induce *SRPN6* expression. *E. coli* (3 x 10^6/gut) fed to mosquitoes (Fig. 2.2G) led to a considerable increase in *AgSRPN6* expression, unlike feeding with buffer alone. No significant difference in induction was observed among mosquitoes fed on 10, 100 and 1000-fold fewer bacteria; as few as $3x10^3$ *E. coli* are sufficient to fully induce SRPN6 expression (data not shown).

O'nyong-nyong (ONN) is an alphavirus transmitted by *An. gambiae* and is usually acquired via infection of midgut epithelial cells (Vanlandingham et al., 2005). To determine whether the virus activates *SRPN6* gene expression, ONN-infected mosquitoes were examined by Northern blot analysis. No expression of *AgSRPN6* mRNA could be detected in either gut or carcass even though hybridization of the blot with an ONN probe confirmed that the midguts were infected with virus (Fig. 2.2H; (Sim et al., 2005)).

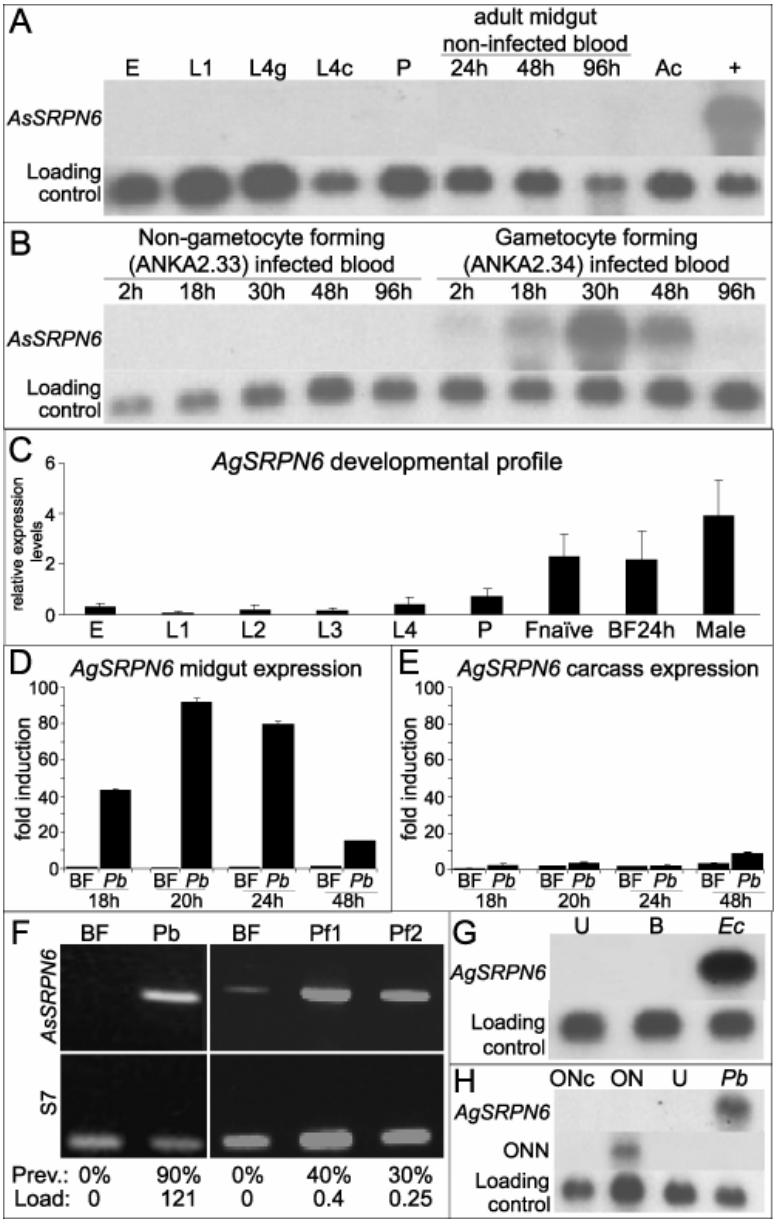

Figure 2.2. *AsSRPN6* and *AgSRPN6* are strongly up-regulated by immune challenge. **A.** *AsSRPN6* expression at different developmental stages, tissues and after non-infectious bloodmeal was not notable by Northern analysis. E, 14 h-old embryos; L1, first instar larvae; L4g, fourth instar guts; L4c, fourth instar carcasses; P, 16 h-old pupae; Ac, adult carcasses. RNA from mosquitoes dissected at 24h after ingesting *P. berghei*-infected blood was used as a positive control (+). Loading control in Fig. **2A**, **B**, **G** and **H** is *An. gambiae* mitochondrial rRNA probe. **B**. Northern analysis revealed strong upregulation of *AsSRPN6* expression in midgut tissues 18-48 h after infection with gametocyte-forming *P. berghei*. **C**, **D** and **E**. Q-RT-PCR analysis of *AgSRPN6* expression during development (**C**), and after *P. berghei* infection (**D, E**). Expression of *AgSRPN6* was mainly detected in adult stages, and was strongly upregulated in midgut tissues 20-24 h post infection (weakly so in 48 hpi carcasses). Data were normalized to S7 expression and calibrated to the average of all developmental stages or 18 h BF. Samples were E, embryos; L, larval stages; P, pupae; Fnaive, sugar-fed females; BF, bloodfed; *Pb*: *P. berghei*-infected. **F.** RT-PCR analysis of *AsSRPN6* induction by *P. falciparum*. PCR amplifications were performed using 25 cycles for *P. berghei* and 30 cycles for *P. falciparum* experiments. Prevalence and parasite load for each experiment are indicated. BF, bloodfed; Pb, *P. berghei*-infected; Pf1, Pf2, *P. falciparum*-infected (two independent experiments). The lower panels show amplification of the ribosomal protein S7 loading control. **G.** Northern analysis of *An. gambiae* midgut tissue total RNA 6 h after ingestion of $3x10^5$ *E. coli* or O'nyong-nyong (ONN) virus (**H)**. ONN virus infection was verified using a virus-specific probe (ONN). B, buffer fed; ON, o'nyong-nyong-infected; ONc, O'nyong-nyong-infected carcass; U, uninfected; Pb, *P. berghei*-infected gut.

SRPN6 protein is expressed in infected midgut cells and in haemocytes

We raised polyclonal antibodies against AsSRPN6 and AgSRPN6 to investigate protein accumulation and localisation. Immunoblot analysis of *P. berghei*-infected *An. stephensi* midgut sheet extracts detected a protein of ~55 kDa at 24 and 48 hpi but not at 8 hpi, indicating that AsSRPN6 appears at later stages of ookinete development (Fig. 2.3C, right lanes). Consistent with the RNA analysis, no protein was detected after a non-infected blood meal (Fig. 2.3C, left lanes). The affinity-purified antibody also recognized the slightly larger recombinant thioredoxin-AgSRPN6 fusion protein and three putative degradation products around 36, 23 and 16 kDa. Consistent with this interpretation, similar size bands were detected by Western analysis of overloaded purified recombinant SRPN6 samples (1 µg/lane; data not shown). The SignalP (www.cbs.dtu.dk/services/SignalP/) algorithm predicted that both *As* and *AgSRPN6* genes encode secreted proteins and abundant AgSRPN6 protein was detected (Fig. 2.3D) in Westerns of media conditioned by the immune responsive *An. gambiae* cell line, 4A3B (Muller et al., 1999). However, no specific bands were detected on Western analysis of hemocoel or gut luminal contents from mosquitoes dissected at 24 or 48 hpi (data not shown). The mode and extent of extracellular SRPN6 release from cells remains to be determined.

The SRPN6 protein distribution in the midgut epithelium was investigated by immunofluorescence microscopy using antibodies to SRPN6 and P28, a major ookinete surface protein of *P. berghei* (Paton et al., 1993). As expected, neither antibody reacted with non-infected midgut sheets. In *An. stephensi* (Fig. 2.3A), AsSRPN6-positive cells were detected at 24 hpi, 85% of which co-localised with invading parasites; the frequency dropped to 48% at 40 hpi and 2% at 72 hpi. The prevalence of AsSRPN6-positive cells and invading ookinetes decreased in parallel over time. This suggests that positive cells are those invaded by parasites, as observed in the case of nitric oxid synthase (NOS) and SRPN10 (Danielli et al., 2005; Han et al., 2000). At 24 hpi, ca. 5% of single parasites were surrounded by several SRPN6-positive cells (Fig. 2.3A, Pb24h - i). Similar clusters have been observed previously for SRPN10 (Danielli et al., 2005). In *An.*

gambiae, confocal microscopy revealed a similar correlation: the number of AgSRPN6-positive cells increased from 24h to 32h and dropped sharply thereafter. The frequency of parasites in the direct vicinity of AgSRPN6-positive cells was 57% at 24 hpi, increased to 79% at 32h and dropped to only 6% at 40 hpi (Table 2.1; see also Fig. 2.3B). The decrease in association between parasites and SRPN6-positive cells at later stages of infection may be due to exfoliation of SRPN6-positive cells, movement of ookinetes in the basal extracellular space after exiting the invaded cells, or degradation of SRPN6 once the parasite exits.

Table 2.1 Co-localisation of SRPN6 protein with *P. berghei* parasites in mosquito midguts

Time after infection [h]	No. of SRPN6-positive events[a]		Total # of parasites		SRPN6/parasite co-localisation [%][b]	
	As	*Ag*	*As*	*Ag*	*As*	*Ag*
24	166	229	196	402	**85**	**57**
32	nd	412	nd	524	nd	**79**
40	91	30	190	570	**48**	**6**
48	nd	19	nd	454	nd	**4**
72	3	nd	185	nd	**2**	nd

[a] an ookinete within or contacted/surrounded by one or more SRPN6-positive cell is counted as one SRPN6-positive (invasion) event. If more than one ookinete was detected in the vicinity, the number of SRPN6-positive events was considered to equal the number of ookinetes. Ookinetes were detected in *An. stephensi* with an anti-P28 antibody, and in *An. gambiae* by endogenous GFP expression. [b] Number of SRPN6-positive events divided by the total number of parasites multiplied by 100; nd, not determined.

High resolution confocal analysis also detected AgSRPN6 in circulating haemocytes (Fig. 2.3E) and in small-nucleated cells, most likely haemocytes, attached to the basal side of the midgut facing the hemocoel (Fig. 2.3F and G). SRPN6 also accumulated in pericardial cells (Fig. 2.3H), the scavenging nephrocytes of insects. It showed a granular cytoplasmic distribution, which has been described previously for other secreted proteins such as Sp22D and TEP1

(Danielli et al., 2000; Levashina et al., 2001). Confocal sections through the center of invaded midgut epithelial cells showed wide-spread cytoplasmic distribution of SRPN6, especially in granules (Fig. 2.3A, Pb24h-ii, Fig. 2.3B, Pb27-i).

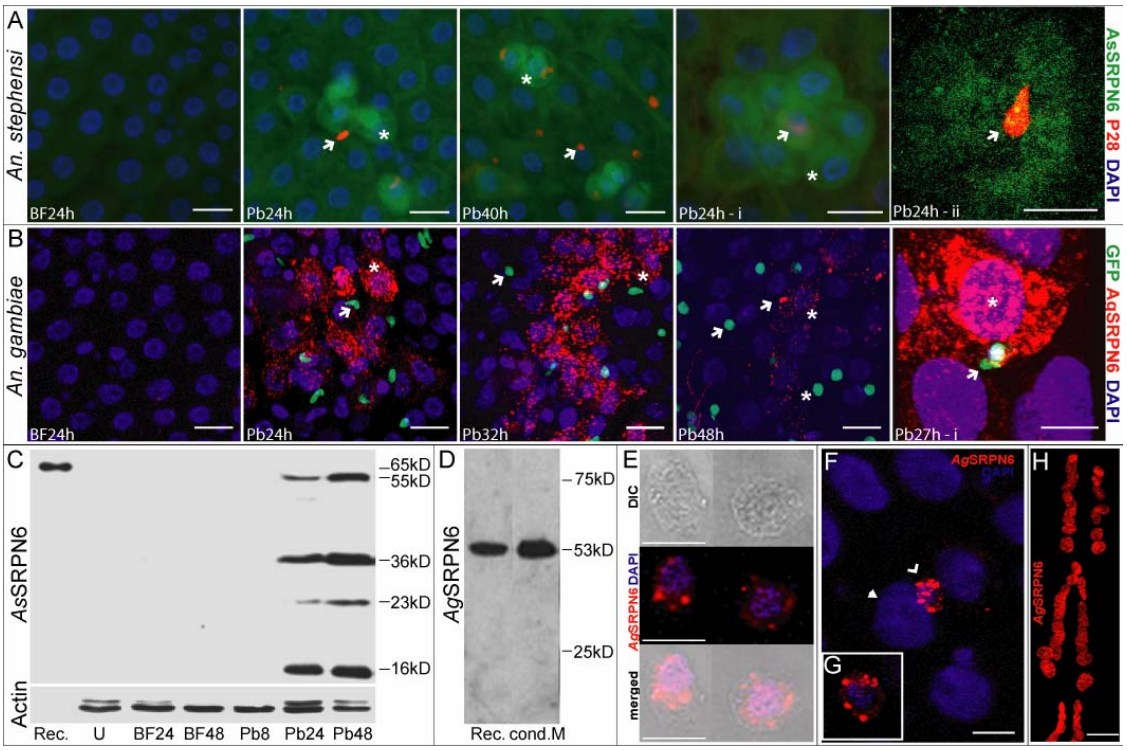

Figure 2.3 Immunolocalisation and immunoblotting of *As* and *Ag*SRPN6 protein. Localisation of AsSRPN6 by Immunofluorescence (**A**) and AgSRPN6 by confocal microscopy (**B**) in midgut sheets at indicated time points after *P. berghei* infection (Pb). The majority of invading parasites was either in close proximity or within SRPN6-positive cells (**A**, Pb24h-i, Pb24h-ii). Number of AgSRPN6-positive cells dropped strongly by 48 hpi. Parasites were either visualized with monoclonal anti-P28 antibody (**A**) or by endogenous GFP expression (**B**). No SRPN6 protein was detected in midgut epithelia after non-infected blood feeding (BF). Arrows indicate invading parasites. Examples of SRPN6-positive midgut epithelial cells are marked by asterisks. Scale bars = 10 µm for **A**, Pb24h–ii and **B**, Pb27h-i. All other scale bars in **A** and **B** = 20 µm. Note the granular distribution of AgSRPN6 and the frequent association with parasites revealed by confocal microscopy. In **B**, Pb27h-i, a parasite is seen emerging from a previously invaded cell, which stains intensely for AgSRPN6 (asterisk). **C**. Immunoblot of *An. stephensi* midgut sheets before (U) and after either a non-infected (BF) or *P. berghei*-infected blood meal (Pb; 90% infection prevalence, mean oocysts number 200/gut). Numbers after BF and Pb indicate hours after blood ingestion. Blots were incubated with rabbit affinity-purified AsSRPN6 antibody, washed and detected using anti-rabbit HRP-conjugated antibody. The equivalent of two gut sheets was loaded per lane. Rec, 25ng of bacterially-expressed thioredoxin-AgSRPN6 fusion protein. A ~55 kDa protein band

was detected only in infected midgut sheets. The membrane was stripped and reprobed with anti-actin antibody as a loading control (lower panel). **D.** AgSRPN6 protein was detected in immunoblots of conditioned medium of an *An. gambiae* cell line probed with anti-AgSRPN6 rabbit antibody (1:1000), indicating that the protein is secreted. Rec, 10 ng of bacterially expressed full-length AgSRPN6. **E**. AgSRPN6 is expressed in circulating haemocytes. Scale bar = 10 µm. **F.** 30° y-plane projection of a confocal stack reveals expression of AgSRPN6 in some small nucleated cells attached to the haemolymph-facing basal side of the midgut epithelium. Arrowhead, nucleus of midgut epithelial cells; open arrowhead, small nucleated cells. Scale bar = 10 µm. **G**. 2 µm confocal slice of the same cell. **H.** Constitutive presence of AgSRPN6 in pericardial cells. Scale bar = 50 µm.

SRPN6 limits malaria parasite infectivity to mosquitoes

We used dsRNA-based gene silencing to investigate whether *SRPN6* influences *P. berghei* development in the mosquito. Experimental or control mosquitoes were injected with *SPRN6* or *GFP* dsRNA, respectively, followed one day (*An. stephensi*) or four days (*An. gambiae*) later by infective blood meals. RT-PCR or qRT-PCR analysis of RNA extracted 1 dpi showed efficient *SRPN6* RNA depletion in experimental as compared to control midguts (Fig. 2.4A and B).

KD of *AsSRPN6* significantly increased the number of developing oocysts, 2.5 fold (Mann Whitney U-test, $p<0.001$) in *An. stephensi* (Fig. 2.5A, Table 2.2), suggesting that SRPN6 may act in a parasite-killing pathway, of the type revealed by the antagonistic effects of TEP1 or LRIM on *P. berghei* in *An. gambiae* (Blandin et al., 2004; Osta et al., 2004). Surprisingly, KD of *AgSRPN6* did not affect oocyst numbers in three *An. gambiae* strains susceptible to *P. berghei* infection (G3, Yaounde, A69; Fig. 2.5B, Table 2.2). In contrast, it increased the numbers of melanized ookinetes 2.9 fold in the refractory L3-5 strain, which melanizes ookinetes as they exit the midgut epithelium (Fig. 2.5C, Table 2.2). This increase was highly significant (Mann Whitney U-test, $p<0.001$).

To investigate further whether this effect is dependent on melanisation, we altered genetically the susceptible G3 mosquitoes to a melanizing refractory phenotype, by KD of *CTL4*. Consistent with previous results (Osta et al., 2004), this KD caused melanisation of 81% of invading parasites, but did not significantly increase the total number of parasites. Interestingly, the double-KD of *CTL4* and *AgSRPN6* increased the number of melanized parasites significantly

(1.8 fold) and caused melanisation of 95% of parasites (Fig. 2.5D & 2.6, Table 2.3).

These results indicate genetic interaction, with *SPRN6* functioning as an enhancing modifier of the *CTL4*-KD melanisation phenotype. Evidently, when active these two genes cooperate in down-regulating ookinete melanisation.

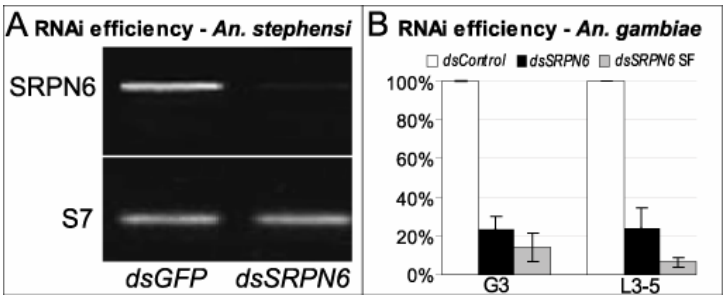

Figure 2.4 RNAi efficiency. A. RT-PCR analysis revealed that dsRNA treatment one day before infection effectively abolished *AsSRPN6* induction by *P. berghei* 24 hpi. DsGFP treated mosquitoes served as control. Similar results were obtained in four independent experiments **B.** Midgut *AgSRPN6* expression levels were measured by qRT-PCR 5 d after dsRNA injections from sugar-fed (SF) or *P. berghei* infected mosquitoes 24 hpi. Data were normalized to S7 expression and calibrated to SRPN6 expression in infected midguts from *dsGFP*-injected mosquitoes. Reduction of *AgSRPN6* expression was comparable between different strains of *An. gambiae* (G3 and L3-5). Results present means ±1SE of three independent experiments.

Table 2.2 Effect of *SRPN6* KD on the development of *P. berghei* parasites

Species	Strain	Gene KD	Repeats [#]	Midguts [#]	Prev. [%]	Parasite load†	Fold diff	P value‡
An. stephensi		*SRPN6* *Control*	4	57 57	96 88	289±21 115±21	+2.5	**<0.001**
An. gambiae	G3	*SRPN6* *Control*	8	115 115	83 85	66 ±7 77±8	-1.2	0.568
	Yaounde	*SRPN6* *Control*	3	53 53	81 87	26±7 43±9	-1.7	0.445
	A69	*SRPN6* *Control*	3	42 42	81 83	36±9 43±9	-1.2	0.799
	L3-5	*SRPN6* *Control*	6	96 96	0 (98)* 0(94)*	0 (176±15)* 0 (60±7)*	(+2.9) *	**<0.001**

Control: dsGFP was used as the control treatment. †mean number of developing parasites per midgut, 8 -15 dpi. ‡Mann Whitney U-test. *No developing parasites were observed in L3-5. Numbers in parentheses reflect the presence of melanized ookinetes.

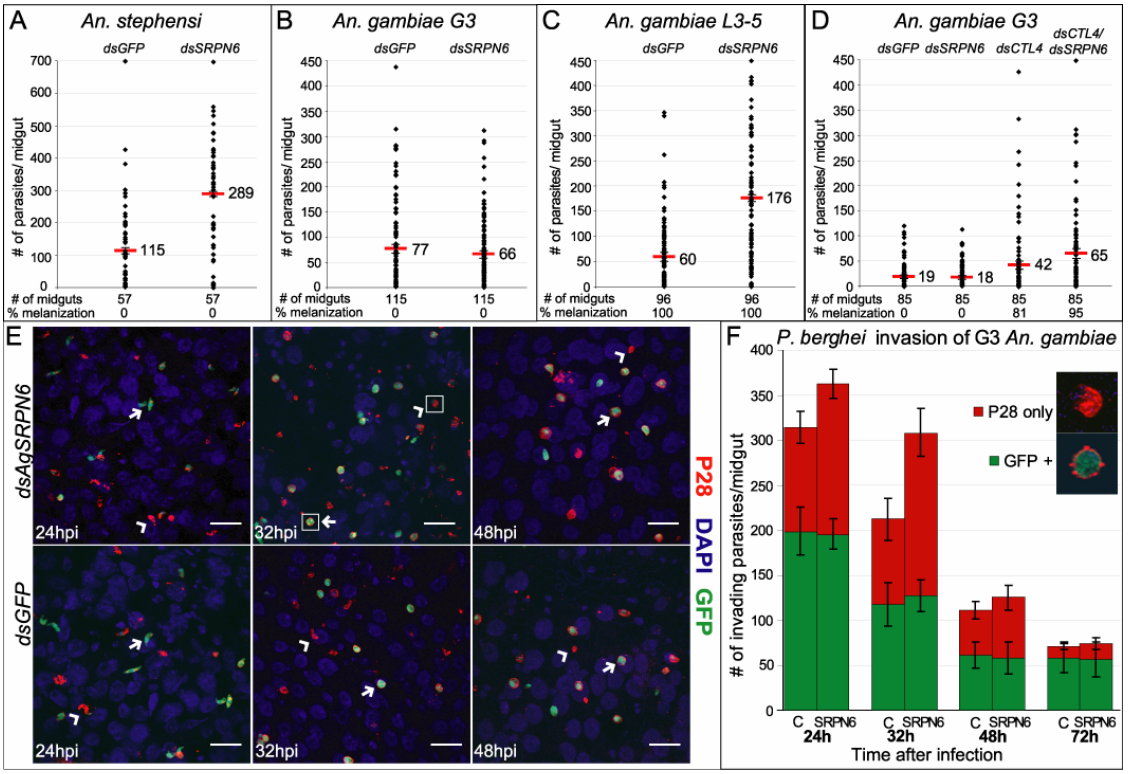

Figure 2.5 Effects of *SRPN6* KD on *P. berghei* development in the mosquito.
A–D. Graphs show the distribution of overall numbers of parasites per midgut 8 to 15 dpi in *dsSRPN6* or control *dsGFP*-treated mosquitoes. Geometric means ±1SE in the pooled datasets from at least three independent experiments, number of midguts examined, and the percentage of melanized parasites per midgut are indicated. See also Supplementary materials, 2 and Fig. S1. *SRPN6* KD significantly increases the number of developing parasites in susceptible *An. stephensi* (**A**), but not in susceptible *An. gambiae* (**B**, **D**); the numbers of melanized parasites increase significantly after *dsSRPN6*-treated refractory L3-5 (**C**) and *dsCTL4* KD *An. gambiae* (**D**). **E.** 3D projections of midguts dissected and stained with anti-P28 antibody and DAPI at indicated times after infectious blood meal. Mosquitoes had been treated with either *dsAgSRPN6* or *dsGFP* 4 d prior to infection. Living parasites (arrows) appear green and yellow, as they are double-labeled with anti-P28 antibody (red) and endogenous GFP (green). Open arrowheads indicate P28-only parasites that are in the process of lysis. White squares, close-ups are shown in insets in **F**. Scale bar = 20 m. **F.** Graphical representation of temporally changing parasite phenotypes detected in *dsGFP* and *dsSRPN6*-treated mosquito midguts at indicated times after infection. Bar graphs, coded as indicated in the insets, represent GFP-fluorescent live parasites (green) or lysing P28-only parasites (red). Insets show confocal micrographs of these two parasite classes. Error bars represent standard error in the pooled data set of three independent experiments.

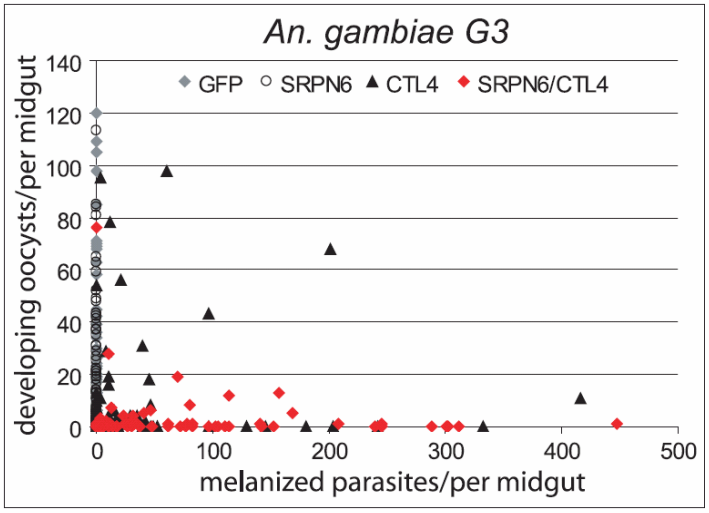

Figure 2.6 Melanized parasite and developing oocyst distribution per midgut in G3 *An. gambiae* 8dpi after double-KD of *CTL4* and *AgSRPN6*. Graph shows the distribution of melanized parasites and developing oocysts 8 dpi in each midgut after dsRNA treatment. Double-KD of *AgSRPN6* and *CTL4* increases significantly the percentage of melanized parasites and the overall number of parasites per midgut as compared to single KDs and the control treatment (GFP).

Table 2.3 Effect of double-KD of *CTL4* and *SRPN6* on *P. berghei* development in G3 *An. gambiae*

Gene KD	Repeats [#]	Midguts [#]	Parasite classes	Prev. [%]	Parasite load	Fold Diff.*
Control	4	85	oocysts	81	19	
			melanized	0	0	
			total	81	19	
SRPN6	4	85	oocysts	79	18	
			melanized	0	0	
			total	79	18	-1.1*
CTL4	4	85	oocysts	56	9	
			melanized	66	34	
			total	78	42	+2.2*
SRPN6/CTL4	4	85	oocysts	38	2	
			melanized	82	62	***+3.3*****
			total	85	65	***+3.4****

* Fold difference in parasite load as compared to number of oocysts in the *dsGFP* control treatment. ** Fold difference in melanized parasites as compared to the number of melanized oocysts in the *dsCTL4* treatment. Numbers in bold and italics indicate statistically significant differences (Mann-Whitney U-test, $p \leq 0.002$) as compared to control.

To investigate whether *AgSRPN6* KD has an effect on parasite lysis, we performed a separate, detailed time-course analysis of ookinete midgut invasion in G3 mosquitoes, using GFP as a marker for live parasites and P28 as a surface

marker for live (P28+GFP positive) parasites and dead (P28-only) ookinetes in the process of lysis (Blandin et al., 2004; Michel et al., 2005). In accordance with previous data (10, 12), we found that even in these susceptible mosquitoes about 80 % of invading ookinetes are killed and subsequently eliminated by parasite lysis during the first 72h of infection. As expected from the above results (Fig. 2.5B and Table 2.4) we did not find significant differences between control and *AgSRPN6*-KD G3 mosquitoes in the number of live (GFP+) parasites during the first 72 hpi (Fig. 2.5E, F). However, P28-only dead parasites in the process of lysis were initially more numerous after *SRPN6*-KD. This difference was maximal at 32 hpi and completely disappeared by 72 hpi indicating that in susceptible *An. gambiae*, KD of *AgSRPN6* transiently slows down parasite lysis.

Table 2.4. Effect of SRPN6-KD on early stages of *P. berghei* during midgut invasion of *An. gambiae* G3 mosquitoes

Time post infection [h]	Repeats [#]	Midguts [#]	Parasite classes	Gene KD *dsGFP*	Gene KD *dsSRPN6*
24	3	28	GFP+	199±27	196±17
			P28-only	116±18	168±16
32	3	31	GFP+	117±24	127±17
			P28-only	95±23	***182±26****
48	3	29	GFP+	61±14	58±17
			P28-only	50±10	67±14
72	3	25	GFP+	58±16	57±20
			P28-only	14±4	18±6

* P28-only parasites, in the process of lysis are statistically significantly more numerous in the midguts of *SRPN6*-KD mosquitoes 32hpi, indicating that SRPN6 KD slows down lysis.

2.3 Discussion

The *An. gambiae* serpin genes *SRPN5*, *6*, and *16* are clustered within 20 kb on chromosome 3. Our phylogenetic analysis indicated that this genomic proximity is the result of tandem gene duplications, which occurred after the divergence of mosquitoes from drosophilids. The phylogenetic analysis also identified *An. gambiae* and *An. stephensi SRPN6* genes as an orthologous pair; similarly, the

An. gambiae SRPN5 and *SRPN16* genes are orthologs of *Ae. aegypti* TC39371 and TC47107 sequences, respectively. This mosquito-specific expansion led to gene diversification, possibly reflecting selection pressures to adapt to differential ecological and physiological challenges, among them haematophagy and exposure to blood-borne pathogens and parasites (Christophides et al., 2002). Interestingly, *D. melanogaster* CG7219 has been shown to be up-regulated after bacterial challenge (De Gregorio et al., 2001), suggesting that an early function in innate immunity might have been elaborated after gene reduplication.

SRPN6 sequences were identified as overrepresented in an *An. stephensi* subtraction library enriched for sequences expressed in the midgut of *P. berghei*-infected mosquitoes (Abraham et al., 2004), and in microarray midgut invasion-specific signals from *An. gambiae* (Vlachou et al., 2005). Our detailed expression analysis of SRPN6 verified these findings, firmly establishing that *SRPN6* is strongly induced during the parasites' passage through the midgut epithelium. Importantly, *AgSRPN6* is also upregulated after infection with the human malaria parasite, *P. falciparum*. Therefore, induction seems to be independent of parasite species and host blood factors, but depends on ookinete invasion: non-invasive parasites in the blood meal do not induce *SRPN6* expression. These findings suggest that *SRPN6* expression in the mosquito midgut epithelium is dependent on direct interaction of microorganisms with the midgut cell. The failure of ONN virus to induce *SRPN6* expression suggests some pathogen specificity of this response, although a quantitative explanation cannot be excluded.

Immunolocalisation experiments indicated that SRPN6 transiently co-localizes with the parasites in both mosquito species. The co-localisation coincides with the parasite's passage through the midgut epithelium and then subsides, suggesting transient expression combined with a short SRPN6 half life. Alternatively, loss of co-localisation may reflect the known propensity of post-invasion ookinetes to move laterally within the subepithelial space, and of invaded cells to be extruded from the epithelium (Danielli et al., 2005; Vlachou et al., 2004). It has been shown that a single ookinete can invade multiple midgut cells before emerging on the basal side of the epithelium (Danielli et al., 2005;

Vlachou et al., 2004), and this fact may explain the observed SRPN6-positive cell clusters. While no SRPN6 protein was detected in the midgut lumen or the haemolymph at various time points after infection, the prediction of a strong signal peptide, its granular cellular distribution and its detection in cell line-conditioned medium strongly suggest that SRPN6 can be secreted. Further work is needed to distinguish between these hypotheses and to determine the extent of co-localisation with additional markers within the invaded and apoptotic cells.

As SRPN6 expression is closely linked to invasion of *P. berghei* within its mosquito vector, we examined if this serpin indeed has an influence on the outcome of infection. RNAi induced by dsRNA injection led to significant *SRPN6* reduction in *An. stephensi* and *An. gambiae* midgut tissues, and, importantly, affected strongly the vectorial capacity of *An. stephensi*. Similar to the KD phenotype of TEP1 and LRIM1 (Blandin et al., 2004; Osta et al., 2004), *AsSRPN6*-KD significantly increased the number of developing oocysts. Future work will address whether all three molecules act within the same pathway of parasite killing in *An. stephensi*. In contrast, KD of *AgSRPN6* had no effect on prevalence or oocyst load and therefore is not involved in parasite killing in *An. gambiae*. However, detailed cell biological analysis of the *P. berghei* midgut invasion process in *AgSRPN6*-KD mosquitoes showed an effect on lysis of dead parasites, by slowing down its progression. This delay in lysis would make more parasites available for melanisation and could thus explain the observed increase numbers of melanized parasites in the CTL4/SRPN6 double-KD as well as in the refractory L3-5 mosquitoes.

Blandin *et al.* (Blandin et al., 2004) hypothesized that parasite clearance is a two-step process, where parasites are killed and subsequently, dead parasites are eliminated by lysis or become melanized, in the case of the refractory L3-5 strain. Our results suggest that *AsSRPN6* is either directly or indirectly involved in the parasite killing process, whereas *AgSRPN6* apparently acts further downstream on parasite elimination, by promoting parasite lysis. The sequence similarity between these two orthologs and their identical hinge region strongly indicate similar target specificities. It is therefore likely that their observed

phenotypic differences are due to changed modes of action of the respective putative target serine proteases in the two mosquito species. Thus far, all insect serpins known to play a role in insect immunity, including *AgSRPN2*, do so by downregulating immune pathways (De Gregorio et al., 2002; Levashina et al., 1999; Ligoxygakis et al., 2002; Michel et al., 2005; Park et al., 2000; Wang and Jiang, 2004). Interestingly, results presented here indicate that the role of *SRPN6* differs between mosquito species and are subject to interactions with other genes implicated in antiparasitic responses. Future work is needed to elucidate how SRPN6 mediates specific mosquito defenses against foreign organisms in the context of different genetic backgrounds.

2.4 Materials and Methods

Mosquito cultures, parasite infection, O'nyong-nyong infection and bacterial treatment

An. stephensi mosquitoes and *An. gambiae* Keele, G3 and L3-5 strains were maintained under standard conditions (Richman et al., 1996). The *P. berghei* ANKA strain clone 2.34, or the non-gametocyte-forming strain 2.33 (Paton et al., 1993) were fed to mosquitoes as described (Srinivasan et al., 2004) and infection was assessed by counting oocysts at 15 days post infection (dpi). The *P. berghei* GFP-CON transgenic 259cl2 strain (Franke-Fayard et al., 2004) was passaged in Balb-C female mice and infections were performed as described in (Sinden et al., 1996). Parasite prevalence and load was determined in dissected midguts, 5-7 dpi, by fluorescent light microscopy. *Plasmodium falciparum* strain NF54 was maintained and gametocyte cultures were fed to mosquitoes as described before (Ifediba and Vanderberg, 1981) *P. falciparum* oocysts were counted eight days post feeding by mercurochrome staining of midguts. *E. coli* was washed in phosphate buffered saline (PBS) and suspended in latex feeding buffer (Moskalyk et al., 1996). Four to five day-old adult female mosquitoes were starved overnight and provided the bacterial meal. *An. gambiae* adult mosquitoes

were infected with o'nyong-nyong virus as described (Vanlandingham et al., 2005).

Isolation of AsSRPN6 cDNA

To construct a midgut cDNA library, *An. stephensi* mosquitoes were fed on *P. berghei*-infected mice and RNA was prepared from midguts dissected 24-72 h after feeding. cDNA was prepared from this RNA and inserted into the Lambda TripEX2 vector using a SMART library construction kit (Clontech). This library was screened using EST 3108 (Abraham et al., 2004) as a probe. The remaining of the 5' coding sequence was isolated by reverse transcription polymerase chain reaction (RT-PCR) of *P. berghei*-infected *An. stephensi* midgut total RNA using a degenerate primer designed from the translation initiation region of the *AgSRPN6* gene (5'-TGYTCNACNATGAAACAYYTNCARATG-3') and primer AsR-RT (5-CCGCACTGTCAATCATGTTCG-3'). The full coding sequence was isolated using an overlapping PCR of the library-isolated insert and PCR amplified 5' region.

Expression analysis

For Northern and semi-quantitative RT-PCR, *Anopheles* total RNA was extracted with TRI reagent (Molecular Research Center Inc.) from at least 20 engorged female mosquito midguts, at selected time points after an infected or a non-infected blood meal. For the developmental profile, total RNA was extracted from 100 eggs, 50 first instar larvae (L1), 20 fourth instar larval (L4) guts or carcasses and 20 pupae or adults. Northern analysis was performed as previously described using a mosquito mitochondrial rRNA gene was used as a loading control (Edwards et al., 1997). For semiquantitative RT-PCR, 1 µg of total RNA was reverse transcribed using oligo $d(T)_{12-15}$. SRPN6 transcript abundance in *An. stephensi* was determined using AsF-RT (5-CCAGCTCCAGCCTGGTAATC-3') and AsR-RT. *Anopheles* ribosomal protein (S7) primers were used for normalization (forward 5'-TGCGGCTTCAGATCCGAGTTC-3' and reverse 5'-TTCGTTGTGAACCCAAATAAAAATC-3').

Real-time quantitative reverse transcription PCR (qRT-PCR) were performed as described in Michel *et al*. (Michel et al., 2005). Primers qRT-AgSRPN6f (5'-CGGTCAGTGGAATCCGGTACTACA-3') and qRT-AgSRPN6r (5'-GCCGTACGCACCATTGGT-3') were designed using Primer ExpressTM software (Applied Biosystems, USA).

Antibody production

Polyclonal rabbit antibody against AsSRPN6: Proteins comprising the 205 amino acids from the C-terminus of AsSRPN6 or the full-length AgSRPN6 were expressed using pET-15b or pET32b expression system (Novagen, DE) in *E. coli,* BL21 (DE3) pLysS. The *An. stephensi* recombinant protein was purified on a nickel column (Qiagen, DE) and used to raise antibodies. The AgSRPN6 protein was immobilized using the AminoLink® Plus Immobilization Kit (Pierce, USA) and then used to affinity purify the SRPN6 antibody.

Polyclonal rabbit antibody against *Ag*SRPN6: The *AgSRPN6* coding region was amplified from cDNA of three day old *P. berghei* infected females and cloned into pGEM T-easy using the following primer pair: Exp-SRPN6f (5'-CCATGGCTTCCAATCGGTACTACACACAAGCC-3'), Exp-SRPN6r (5'-TGAAGCTTGGAGCAAGCAATTAGCG-3'). Subsequently, a Nco I and HindIII-fragment, encoding AgSRPN6 amino acids 17-489 was sub-cloned into pETM-11. Recombinant N-terminally His-tagged AgSRPN6 fusion protein was expressed in *E. coli*, BL21 (DE3) strain, purified using standard procedures, and used for antibody production. Rabbit antibody against *Ag*SRPN6 was affinity-purified using FPLC-purified antigen coupled to activated CNBr Sepharose beads as described (Harlow and Lane, 1988).

Immunofluorescence and immunoblotting

An. stephensi midgut sheets were prepared at selected time points after a *P. berghei*-infected or non-infected blood meal as described in (Abraham et al., 2004). Sheets were incubated with purified *As*SRPN6 antibody (1:1000) and P28 monoclonal antibody (1:1000). SRPN6 was detected with Alexa Fluor® 488-

labeled goat anti-rabbit IgG (1:1000, green, Molecular Probes, USA) and P28 was detected with Rhodamine red™-X-labeled goat anti-mouse IgG (1:1000, red, Molecular Probes, USA). Cell nuclei were stained with DAPI (Roche Applied Science, CH). Midgut sheets were viewed with a Leica DMLB fluorescent microscope. For confocal analysis, sheets were mounted using Slowfade (Molecular Probes, USA, USA) and examined using a Zeiss LSM 410 confocal microscope. For immunoblotting, ten mosquito gut sheets from mosquitoes fed on infected or non-infected blood were suspended in 70 µl of 1 X Laemmli buffer, boiled for 5 min, and the equivalent of two midgut sheets was separated on 12% SDS-PAGE followed by electro-transfer to a polyvinylidene fluoride membrane. The membrane was incubated with purified anti-*As*SRPN6 antibody (1:10,000) and bound antibody was detected with a horseradish peroxidase-linked anti-rabbit IgG (Pierce, USA, 1:25,000 dilution) by exposing the blots to X-ray films. Membranes were stripped by two 30 min washes in 100 mM 2-mercaptoethanol, 2% (w/v) SDS, 62.5 mM Tris-HCl, and pH 6.7 at 50 ^{o}C. Stripped membranes were incubated with Actin antibody (1:500; A2066, Sigma, USA) as loading control. For westerns on cell line supernatant, 3 d old conditioned medium of the immune responsive haemocyte-like cell line, 4A3B (Muller et al., 1999) was harvested and 15µl were loaded per lane. Blots were incubated with 1:1000 rabbit polyclonal anti-AgSRPN6 antibody, and 1:20000 goat anti-rabbit-HRP conjugated secondary antibody (Promega, USA).

An. gambiae midgut and abdominal wall tissues were dissected in PBS, fixed in 4% paraformaldehyde, blocked in 1% BSA, 0.1% Triton X-100, 1x PBS, and incubated with primary antibodies against *Ag*SRPN6 (1:800) and P28 (1:500). Binding was detected using goat α-rabbit and α-mouse Alexa 546 secondary antibody (Molecular Probes, USA, 1:1500), respectively. Circulating haemocytes were collected by proboscis clipping into 2 μl of Schneider medium (Gibco, USA). Cells were left to settle (5-10 min), fixed (4% paraformaldehyde, 10 min), permeabilized (0.2% Triton X-100 in PBS, 2 min), blocked (2% BSA in PBS, 1 h) and incubated with anti-AgSRPN6 antibody as described above. Nuclei were counter-stained with DAPI (Roche Applied Science, CH). Samples were

mounted using the ProLong Antifade Kit (Molecular Probes, USA) and analyzed using a Zeiss LSM 510 META confocal microscope.

Double stranded RNA KD

Plasmid pBluescript II KS was modified by inserting a T7 promoter between the *Xho* I and *Eco* RI sites (pBSIIΔT7). An *An. stephensi* SRPN6 cDNA (900-1780 bp region) was cloned into the *Eco* RI site between two T7 promoters of pBSIIΔT7. A 400 bp of GFP sequence was also cloned into pBSIIΔT7 to use as control. DsRNAs were produced using MEGAScript™ RNA kit (Ambion, USA, USA) according to manufacturer's instruction. *An. stephensi* female mosquitoes were injected with ~500ng of *dsAsSRPN6* or *dsGFP*. *P. berghei* infections were performed 24h post injection and the effect on *AsSRPN6* expression was monitored 24 hpi.

DsRNAs were produced from plasmids pll6ds (GFP control; (Levashina et al., 2001), pll6.1, pll6.3 (AgSRPN6) and injected according to (Blandin et al., 2002). To construct pll6.1 and pll6.3, primer pairs 6.1f (5'-TTCTCACCGCTCAGCATCATTACC-3'), 6.1r (5'-GGCAGCCGCACCATCGTC-3'), 6.3f (5'-GGCAACGCTCACCGGCAAGATG-3') and 6.3r (5'-GGAGCGGCGCACTAAATAAATAACG-3') were used to amplify the respective *AgSRPN6* cDNA fragments. Fragments were cloned into pGEM T-easy vector (Promega, USA) and subcloned into the *Eco*RI site of pll10. *P. berghei* infections were performed 4d post injection and the effect on *AgSRPN6* expression was monitored 24 hpi.

Phylogenetic analysis

Sequence databases were searched by BLAST (Altschul et al., 1997) available on their respective webservers. Coding sequences were predicted from the honey bee and *An. gambiae* genomes using GENEWISE (Birney et al., 2004) with default parameters, aligning a local-alignment model HMM built using HMMER (http://hmmer.wustl.edu/, HMMER Software Package) with default parameters from an alignment of full-length serpin sequences most similar to

SRPN6 against the genomic sequence. Multiple alignments were constructed as in Michel *et al* (Michel et al., 2005). Both Bayesian inference (BI) and maximum likelihood (ML) phylogenetic analyses accounted for between-site rate heterogeneity using a gamma distribution estimated by eight discrete categories plus an additional category of invariant sites. BI was carried out using MRBAYESv3.1 (Ronquist and Huelsenbeck, 2003) using model jumping between fixed amino acid models run with two chains for 250000 generations. Chain convergence was assessed by comparing standard deviations of split frequencies between runs. ML was carried out using PHYML (Guindon and Gascuel, 2003) using the WAG substitution matrix with 100 non-parametric bootstraps.

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CHAPTER 3:

The parasite invasion marker, SPRN6 reduces the number of sporozoites in salivary glands.

Abstract

Plasmodium sporozoites must infect the salivary glands of their mosquito vectors in order for malaria transmission to occur. This study reports that an immune responsive *Anopheles* serpin, SRPN6, participates in a local salivary gland epithelial response against the rodent malaria parasite, *Plasmodium berghei*. We have shown previously that SRPN6, an immune inducible midgut invasion marker, influences ookinete development. Here we report that, unlike other midgut invasion markers such as SRPN10 and NOS, SRPN6 is also specifically induced in salivary glands upon sporozoite invasion. Detailed time course analysis using immunofluorescence revealed that SRPN6 protein is first observed in salivary gland epithelial cells with the onset of sporozoite invasion. Thereafter, SRPN6 remains detectable throughout infection and the protein is always found located in the basal region of epithelial cells in proximity to invading sporozoites. Knockdown of SRPN6 by RNAi has no effect on oocyst rupture but significantly increases the number of sporozoites present in salivary glands. Taken together, the data presented here implicate SRPN6 in limiting parasite development. To our knowledge this is the first report of a local salivary gland epithelial response that limits the number of invading parasites. Despite many cell biological differences between the *Plasmodium* parasite's passage through the midgut and the salivary gland, this study identifies a striking overlap between the molecular responses and functional consequences of SRPN6 in these two epithelia in response to parasite invasion.

3.1 Introduction:

The causative agents of malaria are apicomplexan protozoa of the genus *Plasmodium*. Of the 120 known *Plasmodium* species (Sinden, 2002), only four cause human disease and are transmitted exclusively via anopheline mosquitoes. *Plasmodium falciparum*, the most deadly human malaria parasite, is estimated to cause around 500 million clinical malaria cases and kills up to three million people each year (Snow et al., 2005), mainly in sub-Saharan Africa. Furthermore, malaria has devastating economic consequences in many of the world's poorest countries (Sachs and Malaney, 2002).

Malaria transmission occurs because a small proportion of invading parasites are able to develop unharmed within the vector. Individual mosquitoes differ considerably in their permissiveness to parasite infection and large parasite losses have been observed in different mosquito vector/parasite combinations (Collins et al., 1986; Sinden, 2002). The parasite undergoes complex developmental changes during its passage through the mosquito where it must cross two epithelial barriers: The ookinete must traverse the midgut and the sporozoite must cross the salivary gland. Our knowledge of vector responses to midgut invasion has grown rapidly during the last five years as reviewed in (Whitten et al., 2006). Surprisingly little is known about mosquito responses to salivary gland invasion.

Salivary gland invasion by *Plasmodium* parasites is thought to be mediated via receptor-ligand interactions as summarized in (Beerntsen and Christensen, 1990). Five sporozoite proteins, CS (Sidjanski et al., 1997), TRAP (Sultan et al., 1997), MAEBL (Kariu et al., 2002), and PCRMP 1 and 2 (Thompson et al., 2007) have been implicated in this process. However, only one mosquito candidate receptor SGS1 has been proposed so far (Korochkina et al., 2006). Interestingly, a synthetic peptide, SM1, can block both ookinete midgut and sporozoite salivary gland invasion suggesting that there may be similarities in the invasion process across these distinct epithelia (Ito et al., 2002). Recently,

transcriptional analysis of *An. gambiae* salivary glands found 57 genes to be differentially expressed after infection with *P. berghei* (Rosinski-Chupin et al., 2006). One of these genes encodes a serine protease inhibitor, serpin 6 (*SRPN6)*, which we had previously shown to be a midgut invasion marker for both rodent and also human malaria parasites (Abraham et al., 2005).

Serpins constitute a large protein family of mainly serine protease inhibitors represented in most eukaryotes (Silverman et al., 2001). In insects, serpins have been shown to inhibit various immune reactions, including activation of the Toll pathway (Levashina et al., 1999) and the melanisation response (Ligoxygakis et al., 2002; Michel et al., 2005; Zhu et al., 2003).

While the precise molecular function of SRPN6 remains to be elucidated, this molecule has been shown to affect survival of *P. berghei* during the ookinete to oocyst transition (Abraham et al., 2005). The apparent upregulation in infected salivary gland tissues prompted us to explore in more detail its potential role in salivary gland epithelia by immunofluorescence analysis (IFA) using confocal microscopy and reverse genetic methods. Here we report the first study that functionally characterised a local salivary gland epithelial response to *Plasmodium* invasion and revealed its role in the parasite development.

3.2 Results

SRPN6 is an invasion marker in salivary glands

To examine how extensively the epithelial immune responses of the midgut and salivary glands overlap, we investigated in infected salivary glands the protein expression of three known midgut invasion markers: SRPN6 (Abraham et al., 2005), SRPN10 (Danielli et al., 2005) and nitric oxide synthase, NOS (Luckhart et al., 1998).

The immunofluorescence analysis detects, SRPN6 (Fig 3.1A, B) protein but not SRPN10 (Fig 3.1C) or NOS (Fig 3.1D), in salivary glands 17 days post infection (dpi) with *P. berghei.* No SRPN6 staining was observed in salivary

glands dissected from blood-fed uninfected mosquitoes (Fig. 3.1A). Furthermore, SRPN6 was only detected in the lateral distal lobes of the salivary glands.

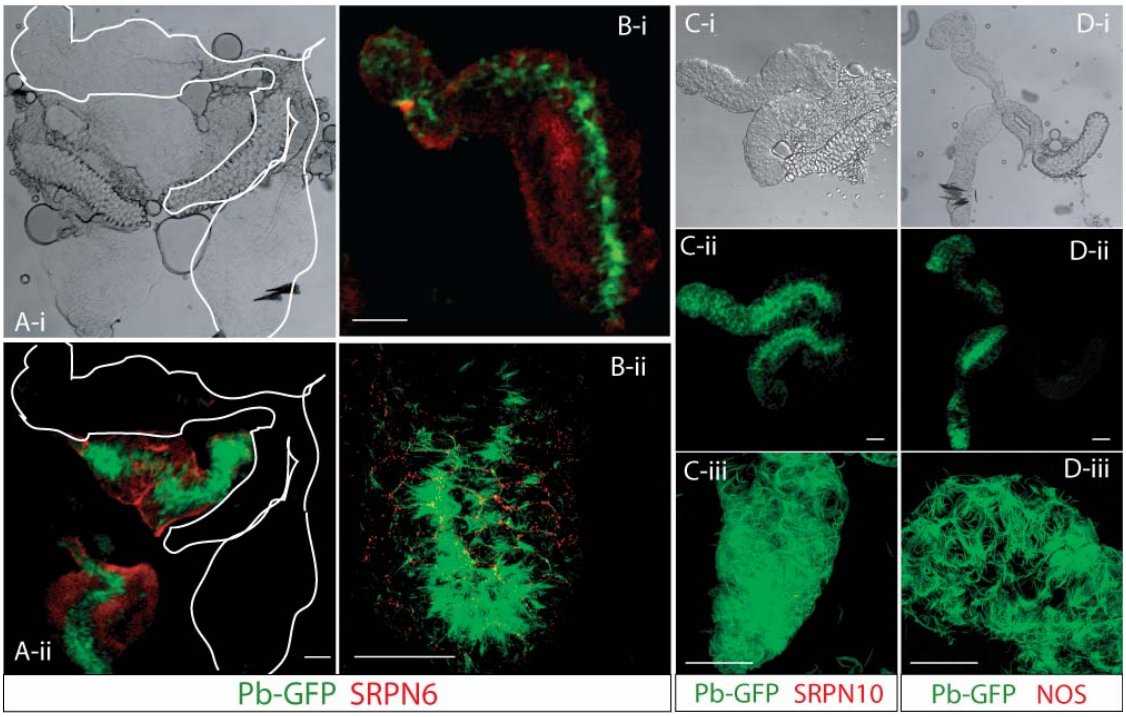

Figure 3.1 SRPN6 expression in *P. berghei* infected salivary glands. Salivary glands from infected 17 dpi adult female mosquitoes were dissected and immunostained for known midgut invasion markers. **(A)** SRPN6 was strongly detected in infected glands. Note that in **A-i** and **A-ii** there are two salivary glands, one from infected and the other from blood fed but not infected mosquitoes (white line delimited) as indicated by GFP expressing *P.berghei* absence. Detail of a single lateral lobe **(B-i)** and the tip of a lateral lobe **(B-ii)**. SRPN6 expression is granular and found throughout the lateral lobe in both distal and proximal regions. SRPN10 **(C)** and NOS **(D)** showed no signal. Scale bars 50 μm.

We next explored how far SRPN6 expression correlated with the course of salivary gland infection and sporozoite relative quantities. Oocyst decay was monitored in infected mosquitoes between 11-23dpi, by immunofluorescence. Reproducibly, under our rearing conditions, very few oocysts burst before 13dpi. After that, oocyst numbers significantly decline with the most prominent decrease usually seen between 15 to 17dpi (Fig 3.2A).

Circumsporozoite protein 1 (CSP1), an abundant sporozoite surface protein, was used as a marker for parasite presence and relative abundance in

Western Blot analysis of dissected salivary glands. The stronger the CSP signal detected, the higher the number of parasites present. In concordance with the oocyst decay data presented above, CSP was detected at very low levels at 13 dpi. It subsequently increased overtime until 17dpi and appeared to remain more or less constant until 23 dpi (Fig3.2B). This could imply that a high number of sporozoites invade between 13-17dpi and subsequently fewer sporozoites invade thereafter. Monitoring the same blot for SRPN6 expression revealed low levels of SRPN6 protein detectable at 13 dpi.The expression subsequently increased overtime until 17dpi, where a clear peak of expression was seen. Thereafter the detection decreased in intensity, but remained present at low levels until 23 dpi (Fig3.2B). Importantly, in individual experiments, SRPN6 presence was detected as early as 13dpi, but only if CS protein was also detected.

To understand the location of the parasites inside the salivary gland cells during the course of infection and how that correlates to SRPN6 expression, parallel immunofluoresce assays where performed. At 13dpi, parasites could already be seen in dissected glands although they where mainly resting at the basal side of the cells, possibly just attached to the epithelial surface (Fig 3.2C, 13dpi, 90y, yellow arrowhead). Low amounts of SRPN6 were detected at this time point (Fig 3.2C). At 15 dpi and 17dpi the quantity of parasites clearly increased in the glands and duct colonization was evident, as determined by the presence of parasites in the middle plane of the lobe (Fig 3.2C, 15dpi or 17dpi, white arrowheads). At these time points, SRPN6 protein levels were considerably elevated (Fig3.2C, 15dpi or 17dpi, 90y, asterix) with the strongest expression detected at 17dpi. Interestingly, SRPN6 expression was mainly detected at the basal side of the epithelial cells (Fig3.2C, 15dpi or 17dpi, asterixs). At 19 and 20 dpi SRPN6 presence strongly decreased as shown by the weak fluorescent staining detected (Fig3.2C, 19dpi and 21dpi).

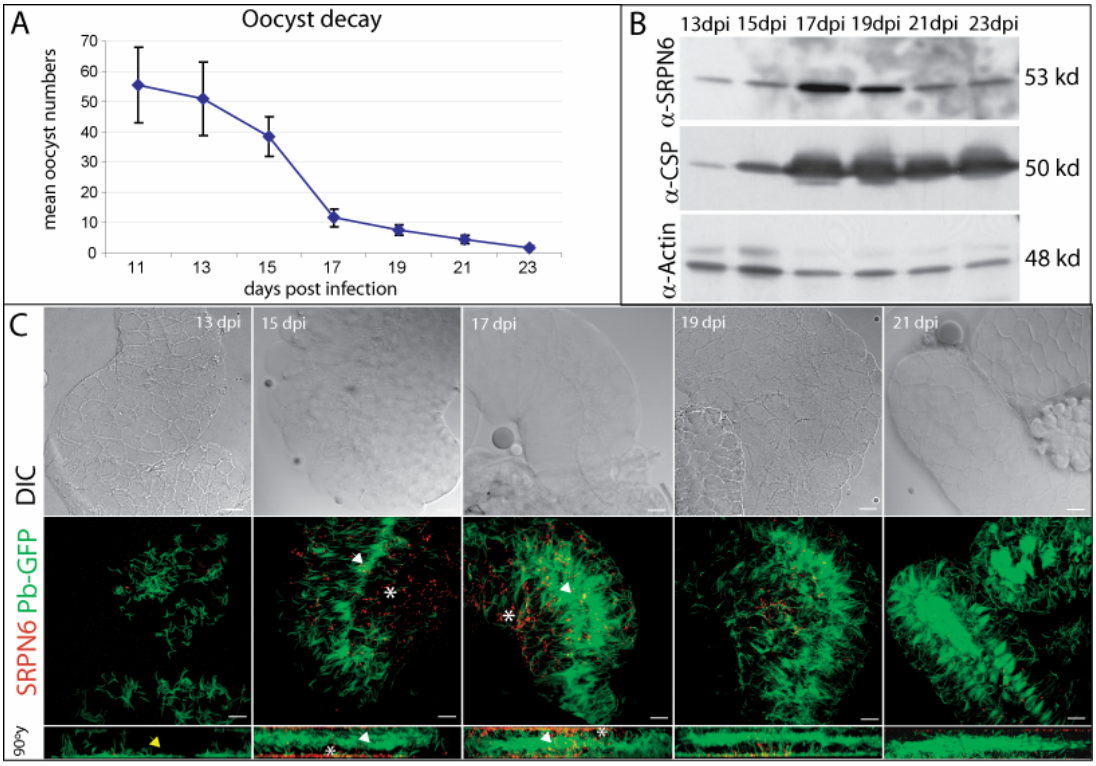

Figure 3.2 Immunolocalisation and immunobloting of SRPN6 in salivary glands. (A) Chart shows the decrease of oocysts in a typical *P. berghei* infection between 11 to 23 dpi. Very few oocysts burst between 11-13 dpi, while a significantly higher amount has sporulated between 13-15 dpi. The most marked decrease in oocyst numbers is seen between 15-17 dpi. Error bars represent standard error (SE) of average between two biological repeats. **(B)** Western blot analysis of a time course of infection for SRPN6 and the *P. berghei* CS protein, used as a marker of infection and parasite quantity. A peak of SRPN6 expression is clearly seen at 17dpi together with a strong increase and high amount of CS protein. Actin was used as a loading control. **(C)** Confocal projection of a z-stack analysis through the total width of the infected distal lobe immunostained for SRPN6 and concomitant dynamics of parasite presence at given time points. Lower panel represents 90º y axis projection of the stack showing. SRPN6 was mainly found at the basal side of the epithelial cells. Scale bars: 10μm.

SRPN6 is found basally in gland cells near sites of invasion

Salivary glands of *An. gambiae* are composed of three lobes (one medial and two lateral), with each lobe duct coalescing into a main salivary duct. The lobes are commonly divided into distal tip and proximal end, with the sporozoites presenting a preference for distal invasion. The salivary epithelial cell is highly polarized with its apical side surrounding a large extracellular space, a reservoir for secretory products, found connected to the duct (Stark and James, 1996), , see Fig. 3.3B). Using confocal microscopy, we further examined the localisation

of SRPN6 in these cells. The protein was found in both proximal and distal lateral lobes when parasites were present (Fig. 3.1A,B). Interestingly, SRPN6 was never found in the medial lobes even when these were infected (data not shown).

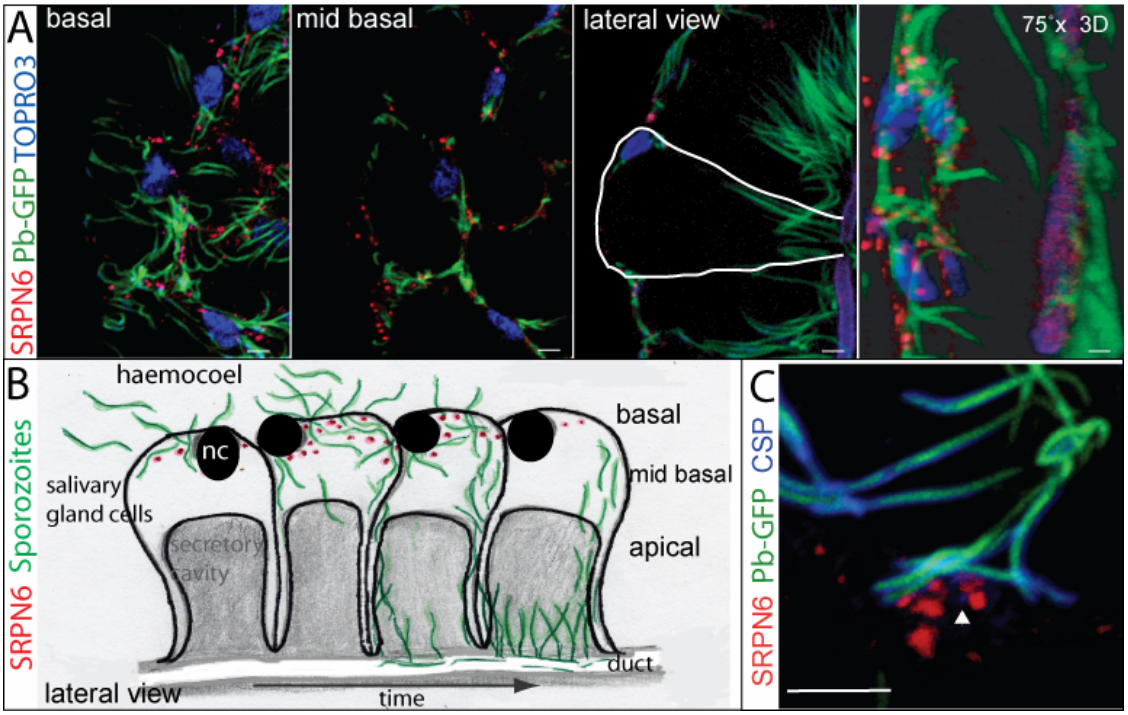

Figure 3.3 Sub-cellular localisation of SRPN6 in invaded salivary glands. (A) First 3 panels represent single slices of a confocal z-stack analysis of SRPN6 localisation in gland cells. Rightmost panel shows the 3D projection, tilted 75 degrees on the x axis of the respective z slices. Anti-SRPN6 antibody staining was granular and was found predominantly in the same focal plane as the nuclei. The staining adjoined but did not overlap with sporozoites at the basal side of the epithelial cells. White line in lateral view highlights the putative cell bounderies **(B)** Graphic interpretation of SRPN6 expression throughout an invasion event. First cell on left represents first invaded cell. SRPN6 (red granules) is restricted to basal site and is most prominent during sporozoite (green) cell invasion. **(C)** Detail of a site of infection showing SRPN6 in close association with putative shedded CS protein (white arrowhead). No co-localisation between SRPN6 and sporozoites was observed. Scale bars: 5 μm. nc, nuclei

Anti-SRPN6 antibody staining in lateral lobes of infected salivary glands was not diffusely localised in the cytoplasm but present in granular structures, resembling small vesicles (Fig. 3.3A-C). Furthermore, SRPN6 was found in the same focal plane as the epithelial cell nuclei, at the basal side of the cells near invading sporozoites (Fig. 3.3A-basal). At a mid-basal plane, where the

cytoplasm of the cell may be already surrounding the large central secretory cavity, SRPN6 granules were seen near the periphery of the cells suggesting their presence to be limited to the cytoplasm of the gland cells (Fig. 3.3A-mid basal). No SRPN6 staining was found in distal regions of the gland cells or apical cavities. SRPN6 granules did not co-localize with sporozoites (Fig. 3.3-lateral view and 3D projection). However, in some cases SRPN6 expression was found in close proximity to cytoplasmic, non-sporozoite (absence of green fluorescence) CSP signal, possibly arising from the shedding of CSP during invasion (Fig. 3.3C, arrowhead). Using filter fedding assays (Billingsley, 1994), we were unable to detect SRPN6 in the saliva of infected mosquitoes (data not shown) and would suggest that SRPN6 predominantly remains intracellularly.

SRPN6 limits the number of invading sporozoites

To assess the potential role of SRPN6 in either parasite development or maturation the protein was depleted in salivary glands overtime, after SRPN6-specific dsRNA injection. A recent study suggested that dsRNA-mediated silencing in *An. gambiae* salivary glands required larger quantities of dsRNA to be injected compared to other mosquito tissues (Boisson et al., 2006). To determine the required amount of dsRNA to obtain efficient SRPN6 knockdown dsRNA was injected into 14 days-old female mosquitos (10dpi) with either 612ng - a comparable dose used to silence SRPN6 in midgut tissue (Abraham et al., 2005)- or 310 ng per mosquito - a similar amount used to silence genes in fat body or haemocytes ~207ng, (Blandin et al., 2002; Volz et al., 2005). SRPN6 protein levels were assessed 5, 7 and 9 days after injection (15, 17, 19 dpi) via Western blot analysis. The protein was silenced similarly using either of these two concentrations (Fig. 3.4A) and therefore no increased dosage of dsRNA treatment was necessary. It may be that in salivary glands as in other tissues, silencing efficiency by dsRNA injection is gene-dependent.

DsRNA injection into adult mosquitoes is an invasive treatment and so far has only been performed in 1-3 day old adults. The survival of dsRNA-injected females was monitored and 20% death of mosquitoes was observed on the initial

48hrs after injection. This could suggest a slight negative effect of the injection procedure in the treated mosquitoes however, not significant to impair a quantitive assay to be performed, as 60% of injected mosquitoes survive for at least 10 days post injection (Fig. 3.4B). The specific knockdown of SRPN6 did not significantly affect negatively or positively the lifespan of treated mosquitoes, when compared to control KDs (Fig. 3.4B).

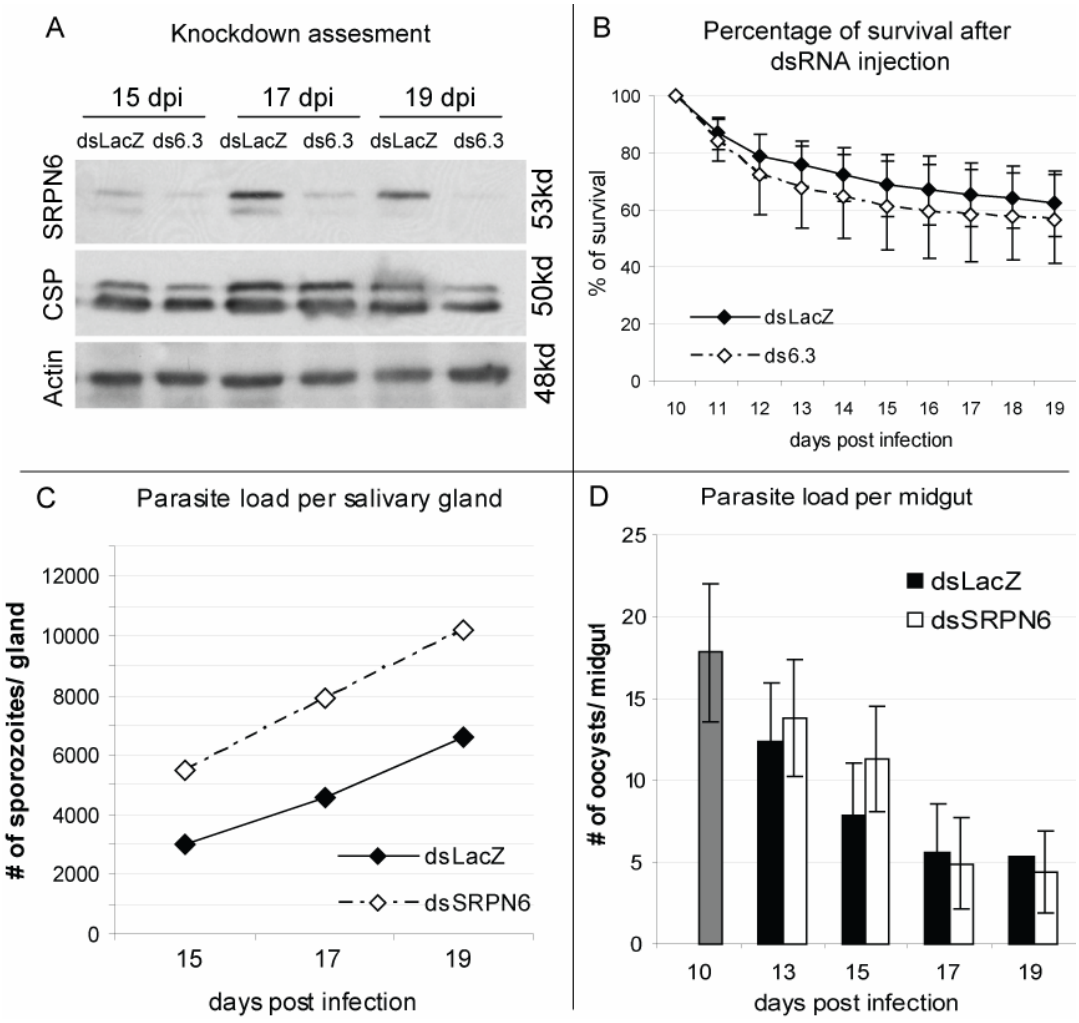

Figure 3.4 Effect of *dsSRPN6* on *P berghei* development after sporulation. (A) Efficiency of knockdown after dsRNA treatment was examined by Western blot (representative blot of 4 repeats). Salivary glands of 10 females were dissected at each time point. The equivalent of 5-7 salivary glands was loaded per lane. Note that although a KD is evident, it is not complete and low levels of SRPN6 are always present. CSP is used as an infection control and α-actin as a loading control. **(B)** Chart represents the percentage of survival of dsRNA-treated mosquitoes throughout the time course of infection. DsRNA treatment of infected mosquitoes at 10dpi does not influence their

survival. Error bars represent standard error (SE) of the mean percentage of survival in four independent biological repeats. **(C)** Plot shows the average number of parasites found in salivary glands at indicated times after infection in *dsSRPN6* and *dsLacZ* mosquitoes of four independent biological repeats (For SDs see table 3.1). SRPN6-KD significantly increases the number of invading sporozoites in *An. gambiae* at all time points tested. **(D)** Graph indicates average number of oocysts (+/-SD) present in the midgut of females scored for sporozoite infection in *dsSRPN6* and *dsControl-KDs* at the indicated time points. Grey bar at 10dpi shows mean number of oocysts found in the infected mosquito population before differential dsRNA treatment. As expected, oocyst numbers decline over time. SRPN6 knockdown has no effect on oocyst numbers at any time point. Data on oocyst and sporozoite numbers are listed in Table 3.1.

Table 3.1 Effect of *dsSRPN6* on *P. berghei* development after sporulation.

			Midgut infection				Salivary gland infection		
Time [dpi]	**Gene KD**	**n**	**Oocyst load***	***P*******	**Prev. [%]**	**$\chi^{2‡}$**	**Sporozoite Load/gland***	**Fold diff.**	***P*$^{|}$**
10	na	90	18 ± 4		90		nd	na	
13	*ds6.3*	70	14 ± 4	0.29	87	0.28	nd	na	
	dsLacZ	70	12 ± 3		90		nd	na	
15	*ds6.3*	93	11 ± 3	0.24	84	0.17	5498 ± 0.52	1.8	Time: 0.029
	dsLacZ	93	8 ± 4		82		3025 ± 0.62		
17	*ds6.3*	75	5 ± 3	0.38	79	0.04	7910 ± 0.76	1.7	dsRNA: 0.015
	dsLacZ	75	6 ± 3		80		4563 ± 0.68		
19	*ds6.3*	74	4 ± 3	0.23	74	0.04	10199 ± 0.54	1.5	
	dsLacZ	74	5 ± 3		74		6630 ± 0.54		

*****Oocyst and sporozoite load represent the geometric mean (+/- one Standard Deviation) of parasite numbers in four independent biological repeats. n, number of mosquitoes tested (from each mosquito both salivary glands were analysed), Prev, prevalence, percentage of infected mosquitoes per experiment; *na*, not applicable; *nd*, not determined,**Mann-Whitney U-Test on oocyst load. ‡Chi-Square Test on oocyst prevalence. §Paired t-test on sporozoite load. |Two-way Anova analysis.

Oocysts and salivary gland sporozoites present in mosquitoes during sporogony and sporozoite salivary gland invasion were counted after *dsSRPN6* or control (*dsLacZ*) treatment. The *dsSRPN6* knockdown did not affect oocyst prevalence or load. Furthermore, oocyst numbers declined similarly during sporulation, indicating no effect of *dsSRPN6* treatment on sporozoite egress (Fig. 3.4D, Table 3.1). However**,** *SRPN6* depletion resulted in higher numbers of sporozoites at 15dpi (early invasion stage), 17dpi (bulk invasion stage) and 19dpi (maturation stage) (Fig. 3.4C, Table 3.1). Interestingly, at all time points *dsSRPN6* treated glands presented statistically significant higher sporozoite

numbers than control injections (Fig. 3.4C, Table1; Two-way Anova ,Df=1, F value=7.28, *P*=0.015). Within treatments, sporozoite numbers increased continuously between 15-19dpi (Two-way Anova, Df=2, F value=4.33, *P*=0.029). This increase indicated continuous salivary gland invasion over several days likely due to the asynchronous egress of sporozoites from the oocysts. No interaction between treatment and time was observed (Df=2, F value=0.06, *P*=0.938).

To test whether *SRPN6* knockdown affected not only sporozoite numbers but also their infectivity, transmission assays were performed. The same number of *dsSRPN6* and control-treated female mosquitoes were allowed to feed on naïve mice at 18dpi. All mice, which received mosquito bites, developed parasitaemia at similar levels within the same pre-patent period (data not shown). This was irrelevant of differential dsRNA treatment, supporting no role for SRPN6 in sporozoite maturation and infectivity. Prepatency periods correlate with the number of sporozoites injected into the vertebrate host (Nussenzweig et al., 1966; Winger and Sinden, 1992) and there seems to be no corrolation between the number of sporozoites present in the salivary glands and the number of host-injected parasites. Therefore a two-fold increase of sporozoite numbers due to SRPN6 knockdown was not expected to increase *P. berghei* transmission.

3.3 Discussion

Plasmodium parasites have to invade two epithelia of their vector to be transmitted to their vertebrate host. Ookinetes invade the midgut epithelium within 24 hours after the mosquito has taken an infectious blood meal. This invasion process causes massive changes in the midgut cells, which ultimately leads to apoptosis and expel of the invaded cells from the epithelium -Time bomb theory, (Han et al., 2000)-. The process is accompanied by changes in cytoskeleton of the invaded and neighbouring cells (Vlachou et al., 2004) as well as extracellular matrix remodelling (Vlachou et al., 2005). Additionally, midgut

and haemolymph-derived factors lead to the killing of nearly 80% of the invading ookinete population during midgut traversal (Blandin et al., 2004; Michel et al., 2005). Three genes have been identified as midgut invasion markers for *P. berghei* ookinetes: NOS (Han et al., 2000), SRPN10 (Danielli et al., 2005) and SRPN6 (Abraham et al., 2005). The upregulation of both, NOS and SRPN10, are restricted to *P. berghei*-induced, luminal-protruding apoptotic cells (Danielli et al., 2005; Han et al., 2000). The precise molecular function of SRPN10 remains to be elucidated however the tight link between its expression and apoptosis suggests a role in epithelial parasite-induced damage. In contrast, SRPN6 upregulation is not restricted to these cells, but can also be detected in neighboring cells with normal appearance. Moreover, in parallel IFAs of SRPN6 and SRPN10 in infected midguts, higher number of cells are consistently found to stain for SRPN6 than for SRPN10 (SBP, unpublished results). These differences in expression suggest that SRPN6 may act by a mechanism different than that of the “time bomb” process of invasion.

The invasion of salivary gland epithelia by sporozoites is accompanied by surprisingly little effects on the invaded tissue. Previous studies examining this invasion process showed, via electron microscopy, that *P. berghei* invasion of *An. stephensi* epithelial cells leads to an increase in microtubules in the distal area of the invaded cells (Sterling et al., 1973), potentially providing a supporting system for damaged cells. It was also shown that *Plasmodium gallinaceum* is transiently surrounded by mitochondria during its passage through the salivary gland epithelial cells of *Ae. aegypti*, potentially exploiting the host cells energy source (Pimenta et al., 1994). Invasion does not seem to be detrimental to the epithelium and no apoptosis occurs. Some differences between the expressional responses to invasion in these tissues suggest differences between these two parasite invasion stages (Rosinski-Chupin et al., 2006; Vlachou et al., 2005).

Surprisingly, the midgut invasion marker, *SRPN6* was identified as a highly abundant EST in a library derived from *P. berghei* infected salivary glands (Rosinski-Chupin et al., 2006). The present study further analysed the potential function of SRPN6 in invaded salivary glands. The results presented here confirm

the findings by Rosinski-Chupin *et al*. (2006) and establish that *SRPN6* expression directly correlates with sporozoite invasion of the salivary gland epithelium.

Oocyst sporulation is asynchronous, and under our rearing conditions oocysts start bursting at low numbers from 13dpi onwards with the most pronounced decrease seen between 15 to 17 dpi. Low numbers of intact oocysts were found as late as 24dpi. Western and immunofluorescence analysis showed that from 13 to 17dpi the majority of salivary gland invasion occurs corresponding to a strong increase in parasite numbers present in the salivary glands. Thereafter few invasion events were observed. SRPN6 expression correlated with the course of invasion: Protein levels increased gradually from 13 to 17 dpi, where they typically peaked, and declined subsequently to low levels which remained thereafter. It is therefore likely that SRPN6 salivary gland expression is dependent on and directly responding to sporozoite invasion.

SRPN6 knockdown in invaded salivary glands increased the numbers of sporozoites during early invasion (15dpi), peak of invasion (17dpi) and sporozoite maturation phase (19dpi). Furthermore, the difference between sporozoite numbers in SRPN6- versus control-treated salivary glands is constant through out the time course. At what point between sporulation and reaching the salivary gland extracellular space does SRPN6 limit sporozoite progression? The comparison of oocyst numbers from 10-19dpi in *dsSRPN6* and control-treated mosquitoes, clearly shows that SRPN6 does not alter the timing or rate of sporozoite egress from oocysts. This strongly suggests that the effect of SRPN6 on sporozoite numbers in salivary glands in not attributable to increased numbers of sporozoites released into the haemolymph. SRPN6 could limit parasite numbers at a pre or early invasion stage, and as no or little killing of sporozoites can be observed after invasion, the initial increase in parasite numbers would be kept constant over time.

We have previously shown that SRPN6 in *An. stephensi* controls the number of invading ookinetes via directly or indirectly eliminating the parasite during its passage through the midgut epithelium. In *An. gambiae*, SRPN6 acts in

promoting lysis of previously killed ookinetes (Abraham et al., 2005). Similarily, SRPN6 may act directly or indirectly in eliminating sporozoites either in the haemolymph or during invasion and that this killing is dependent on its upregulation in the salivary glands. Alternatively, SRPN6 may block sporozoite invasion, by e.g. inhibiting a parasite-derived serine protease required for invasion. Parasite serine proteases, such as subtilisin-1 have been identified to play a crucial role in erythrocyte invasion of the asexual merozoite stage (Blackman et al., 1998). However, the analogous protease required for sporozoite salivary gland invasion has yet to be identified.

Taken together this study characterises the first salivary gland epithelial response implying that the salivary gland epithelium is capable of sustaining a local immune response against the malaria parasite. In addition, the data suggest an important role for SRPN6 in reducing the number of invading sporozoites, highlighting striking parallels between midgut and salivary gland epithelial immune responses.

3.4 Materials and Methods:

Insect and parasite cultures

The *A. gambiae* G3 strain was reared as previously described (Richman *et al.,* 1996). The *P. berghei* GFP-CON transgenic 259cl2 strain (Franke-Fayard *et al*., 2004) was passaged in TO female mice, and infections were performed as described by Sinden (1996). Parasite prevalence and load were determined in dissected midguts (10-19 dpi) by fluorescent light microscopy and in salivary glands (13-19 dpi), by light microscopy.

DsRNA production and injection

DsRNAs were produced from plasmids, pLL100 (LacZ control: (Blandin et al., 2004)), pLL6.3 (SRPN6;(Abraham et al., 2005)). Fourteen day old females were injected 10 dpi with dsRNAs (4.5μg/μl) as described by Blandin *et al* (Blandin et al., 2002).

Western Blotting

Salivary glands were dissected in PBS, placed in 20mM Hepes buffer, pH 7.5, 30mM NaCl, and EDTA-free Protease Inhibitor Cocktail (Roche), and homogenized by repetitive freeze-thawing in liquid nitrogen after addition of SDS loading buffer. Samples were separated on 12% SDS-PAGE followed by electro-transfer to a polyvinylidene fluoride membrane. The blot was incubated with the following antibodies: AgSRPN6 rabbit antiserum at 1:1,000 dilution (Abraham et al., 2005), mouse monoclonal antibody 3D11 against *P. berghei* CS protein at 1:2,000 dilution (Yoshida et al., 1980), and the antibody against α-actin (20-33) at 1:1,000 dilution (Sigma, USA). Primary antibody binding was detected using anti-mouse and anti-rabbit goat IgG horseradish peroxidase conjugated secondary antibodies at a 1:20,000 dilution each (Promega, USA). The western blots were developed using Western Lightning Chemiluminescence Reagent Plus kit (Perkin Elmer, USA). For complete removal of primary and secondary antibodies blots were incubated at 60°C for 1 hour in 62.5mM Tris-HCl, pH6.7, 2% SDS, and 100mM β-Mercaptoethanol.

Immunostainings for confocal microscopy

Immunostainings were performed essentially as previously described (Danielli et al., 2000). Briefly, salivary glands and midguts were dissected in PBS, fixed in 4% paraformaldehyde and blocked in PBT (1% bovine serum albumin, 0.1% Triton X-100, 1x PBS). Dependent on the experiment, fixed tissues were incubated with one or several of the following primary antibodies: rabbit affinity-purified polyclonal antibody against SRPN6 (1:800 (Abraham et al., 2005)), rabbit affinity-purified polyclonal antibody against SRPN10 (1:300,(Danielli et al., 2003)), mouse monoclonal antibody 3D11 against *P. berghei* CS protein (1:200,(Yoshida et al., 1980)), and the rabbit Anti-Nitric Oxide Synthase universal antibody (1:300, Oncogene, USA). This was followed by incubation with secondary antibody α-rabbit or α-mouse Alexa 568 and 647 (Invitrogen, USA, 1:1,500). Cell nuclei were stained with TOPRO 3 (1:2,000, Invitrogen, USA) or

DAPI (1ng/ml, Roche Applied Science, CH). Samples were mounted using the Vectashield (Vector Laboratories, USA) and analysed using a Leica TCS SP2 confocal microscope.

Oocyst and Sporozoite quantification

Midguts were dissected in PBS, and oocysts were counted 10-19dpi in individual midguts using fluorescence microscopy. At 13 to 19 dpi, the salivary glands of 20 to 25 females were dissected, pooled and homogenised using a 0.1ml tissue homogeniser to release sporozoites from the cells and duct. Sporozoites were subsequently counted by light microscopy using a haemocytometer.

For statistical analysis numbers of oocysts and sporozoites were log-transformed, and analysed using Mann-Whitney U-Test and Two-way Anova (GenStat program, VSN International, UK). Data are presented as back-transformed arithmetic means of log-transformed data $\pm$ one standard deviation. Prevalences were analysed using Chi-square test.

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MOSQUITO-*PLASMODIUM* INTERACTIONS:

The effect of *P. berghei* PPL5 in midgut invasion

CHAPTER 4:

Plasmodium Perforin- like Protein 5 is required for mosquito midgut invasion in *Anopheles stephensi*

This study has been published in:
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(in press)

***SB Pinto** contributed to the experimental design, execution and data analysis of the results presented in Figure 4.3 A-C. Has also assisted in writing and revising the manuscript.*

Abstract

During its life cycle the malarial parasite *Plasmodium* forms three invasive stages which have to invade different and specific cells for replication to ensue. Invasion is vital to parasite survival and consequently proteins responsible for invasion are considered to be candidate vaccine/drug targets. *Plasmodium* Perforin-like proteins (PPLPs) have been implicated in invasion because they contain a predicted pore forming domain. Ookinetes express three PPLPs, and one of them (PPLP3) has previously been shown to be essential for mosquito midgut invasion. In this study we show through phenotypic analysis of loss-of-function mutants that PPLP5 is equally essential for mosquito infection. *Δpplp5* ookinetes cannot invade midgut epithelial cells, but subsequent parasite development is rescued if the midgut is bypassed by injection of ookinetes into the haemocoel. The indistinguishable phenotypes of *Δpplp5* and *Δpplp3* ookinetes strongly suggest that these two proteins contribute to a common process.

During the malaria life cycle, three invasive stages are formed, which selectively invade their respective host cells. Invasion is an active process driven by the parasite's acto-myosin motor and requires secretion from specialized apical organelles, including micronemes and rhoptries (Opitz and Soldati, 2002). In contrast to the merozoite, which invades red blood cells exclusively via the formation of a PV, an alternative mode of cell invasion, termed "cell traversal" or "cell breaching", is used by the ookinete to cross the mosquito midgut epithelium. Cell traversal does not involve the formation of a PV, probably reflected by the absence of rhoptries in the ookinete (Sinden, 2004), and ultimately results in death of the invaded midgut cell by apoptosis (Han et al., 2000). Sporozoite invasion of hepatocytes occurs first by cell traversal, while entry into the final host cell, in which further development takes place, involves the formation of a PV (Mota et al., 2001).

A protein family implicated in cell traversal are the *Plasmodium* Perforin-Like Proteins (PPLPs), a family of five putative secreted proteins conserved across the *Plasmodium* species (Kaiser et al., 2004a). PPLPs are characterised by a MACPF-like domain, which in other proteins has been shown to play a role in the formation of transmembrane channels in lipid bilayers. While direct biochemical proof of a poreforming activity of the PPLP MACPF domain is still lacking, it has been suggested that this pore formation may either weaken the target cell membrane or allow injection of micronemal proteins into the target cell (Ishino et al., 2005). Accordingly, during the malaria life cycle PPLPs have been detected by MS mainly in the invasive stages (Florens et al., 2002; Hall et al., 2005; Lasonder et al., 2002). As further evidence for a role in cell invasion, at least two family members, PPLP1 and PPLP3, have been shown to localise to the micronemes (Kadota et al., 2004; Kaiser et al., 2004a) and the *pplp1/spect2* and *pplp3/maop* gene disruptions abolished cell traversal in the sporozoite and ookinete, respectively (Ishino et al., 2005; Kadota et al., 2004).

However the detection of PPLP2 in *P. falciparum* merozoites and of PPLP5 in *P. falciparum* gametocytes (Florens et al., 2002) argues for additional roles of PPLPs other than in cell traversal, such as in exit from the host cell.

Ookinete midgut invasion is a major population bottleneck in the malaria life cycle and proteins essential for invasion, such as PPLP3, may be prime targets for transmission blocking vaccines. Besides PPLP3, *P. berghei* ookinetes reportedly express PPLP4 (Hall et al., 2005; Raibaud et al., 2006), and we report here, for the first time, evidence for expression of PPLP5 in the ookinete. PPLP5 was detected by MudPIT in a surface enriched ookinete proteome (R. R. Stanway, unpublished data) and expression was confirmed by RT-PCR (Fig. 4.1) on cDNA prepared from *P. berghei* gametocytes and purified ookinetes. Interestingly *pplp5* was also amplified from day 5 and day 10 oocyst cDNA, indicating that the gene may be expressed throughout parasite development in the mosquito. This is consistent with data from *P. falciparum*, where PPLP5 was detected by MS in gametocytes and sporozoites (Florens et al., 2002).

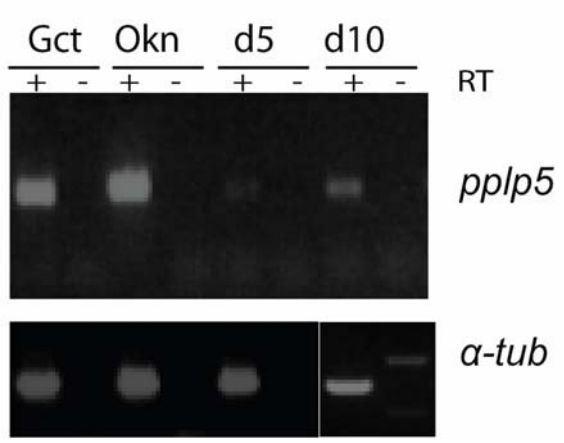

Figure 4.1 RT-PCR analysis of *pplp5* expression during mosquito development.
Routine parasite maintenance in and mosquito infections from Theiler's Original mice were carried out as previously described (in Sinden RE et al (Doolan, 2002)) . *Plasmodium berghei* ANKA 2.34 gametocytes (Gct) were harvested from mice treated for 2 days with sulfadiazine in the drinking water to decrease asexual parasitaemia, and purified by ammonium chloride lysis at 4°C. Ookinetes (Okn) were cultured *in vitro* and purified using α-Pbs28 antibody (13.1) coupled to magnetic beads (Dynabead) as previously described (Siden-Kiamos et al., 2000). Infected *An. stephensi* midguts were dissected on day 5 (d5) or d10 (d10) of infection. Total RNA was isolated using TRIzol (Invitrogen, USA), contaminant genomic DNA was removed by treatment with TURBO DNA-*free*™ (Ambion, USA, USA) and RNA was cleaned up using the RNeasy Mini Kit (Qiagen, DE). Reverse transcription was performed on 1 µg of RNA using the TaqMan ® Reverse Transcription Reagents with a mixture of Oligo-dT primers and Random Hexamers (Applied Biosystems, USA) and the resulting cDNA was used in diagnostic PCR reactions. Primers N-ter F (5'-TGAATTCATGGGTGATCCACTATTTACT-3') and N-ter R (5'-TTCTCGAGTTAAAACTTATAACTCTTATATTCATCATC- 3') amplify a 318 bp fragment of *pplp5*, and primers TubF (5'- CCAGATGGTCAAATGCCC -3') and TubR (5' - CTGTGGTGATGGCCATGAAC -3') a 432 bp fragment of the *α-tubulin* gene. + and – denote presence or absence of RT.

In an attempt to understand why the ookinete expresses more than one PPLP protein and to investigate their respective functions, we removed the entire coding region of *pbpplp5* (PB000511.01.0) by double cross-over homologous recombination and integration of a modified *Toxoplasma gondii dihydrofolate reductase/thymidylate synthase* (*dhfr/ts*) gene cassette which confers resistance to the antimalarial drug pyrimethamine.

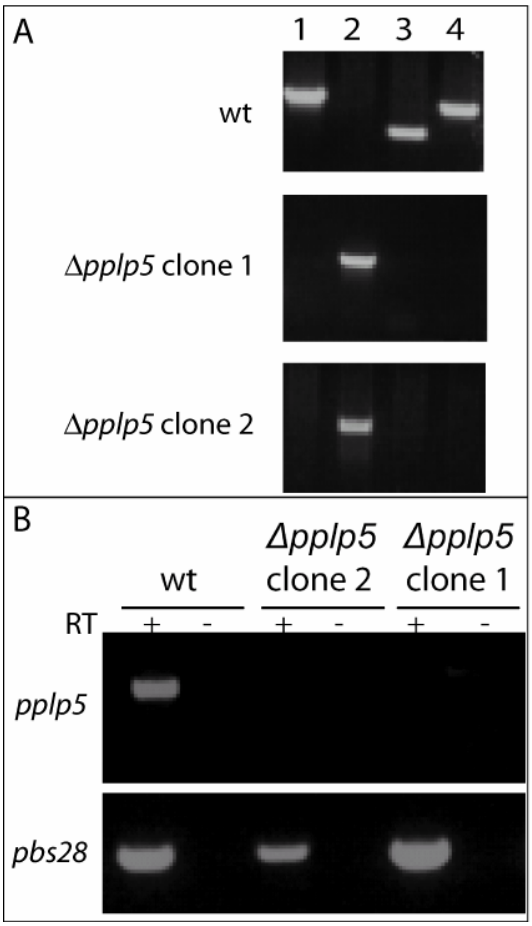

Figure 4.2 Generation of *Δpplp5* parasites.

Generation of constructs for targeted disruption of *pplp5* by double homologous recombination were carried out as previously described (Dessens et al., 1999). Briefly, an upstream homology region of 469 bp was PCR amplified from *Plasmodium berghei* ANKA clone 2.34 genomic DNA using primers AE27A (5'-TT**GGGCCC**GTTGAATATGCATAGACAACATC-3') and AE27B (5'-CC**AAGCTT**TCACAAATATAGGCTACTCTTGC -3') and cloned into pBS-DHFR via ApaI and HindIII (restriction sites in bold). A downstream homology region of 570 bp was PCR amplified using primers AE27C (5'-T**GAATTC**TCATATTGAATAGGCCTTATATC-3) and AE27D (5′-GG**GGATCC**TTTATCACTTCATATCCCAATAC-3') and cloned into the plasmid with the upstream homology region via EcoRI and BamHI. The targeting cassette was released by ApaI and BamHI digestion. Parasite transfection using the Human T Cell Nucleofector Kit (amaxa), selection by pyrimethamine and dilution cloning were carried out as previously described (Janse et al., 2006; Waters et al., 1997). Diagnostic PCR **(A)** on genomic DNA from two independent *Δpplp* clones and control *wt* parasites. PCR reactions in lanes 1 (27KO 5'-TTAGAATATTTTAAGCATTGGCTATC-3' and 27WT 5'- CAAATGCCAACCAAATGCAC- 3'), 3 (N-ter F and N-ter R) and 4 (MACPF-F 5'- TGAATTCGACCCATTTTTTATAAATATGTTGAA-3' and MACPF-R 5'- TTCTCGAGTTAGCTAGAATAATATTCTAGAGCT-3') are specific for the *wt* allele. The PCR reaction in lane 2 is specific for integration of the gene targeting cassette (primers 27KO and 248 5'-GATGTGTTATGTGATTAATTCATACAC-3'). RT-PCR analysis **(B)** of *pplp* expression on total RNA isolated from purified *in vitro* cultivated ookinetes demonstrates absence of transcript in the *Δpplp5* clones. *pplp5* primer as in Figure 1, p28F (5'- GCGAGATCTATGAATTTTAAATACAGTTTTATTTTTTTA-3') and p28R (5'- GCGCCTAGCATTACTATCACGTAAATAACAAGTA-3') amplify the *pbs28* gene (642 bp).

Two independent transfections were carried out to generate two independent Δ*pplp5* clones, clone 1 and 2, which were characterised by diagnostic PCR (Fig. 4.2A). Successful gene disruption was further confirmed by our failure to amplify *pplp5* mRNA from Δ*pplp5* ookinete cDNA (Fig. 4.2B).

Δ*pplp5* parasites showed normal asexual and sexual blood stage development and were able to form ookinetes *in vitro* and *in vivo* in numbers comparable to *wt* (data not shown). However when mosquitoes were allowed to feed on mice infected with Δ*pplp5*, no oocysts were observed in midguts dissected on day 10 of infection (Table 4.1A). Δ*pplp5* parasites also failed to infect when ookinetes were cultured *in vitro* and fed to mosquitoes via membrane feeding (Table 4.1B). Accordingly, no Δ*pplp5* sporozoites were observed in salivary glands of these mosquitoes on day 21 of infection. However, the block in infection was not absolute, as we observed a single oocyst each in two of 50 dissected mosquitoes in one experiment. Moreover, in another experiment a single sporozoite was observed in salivary gland dissections (under identical conditions more than 500 were observed in the respective *wt* control).

Strikingly, these mosquitoes were able to transmit Δ*pplp5* parasites to a C57BL/6 mouse, a mouse strain which is highly susceptible to infection by sporozoites (Jaffe et al., 1990). Diagnostic PCR on genomic DNA prepared from the resulting blood stage infection confirmed that these parasites were indeed Δ*pplp5*, indicating that while midgut invasion is almost entirely blocked, the parasites seem to be able to complete the rest of their life cycle.

To test whether bypassing the midgut would thus completely rescue the mutant phenotype, Δ*pplp5* ookinetes were cultured *in vitro* and either fed to mosquitoes by membrane feeding or injected into the mosquito haemocoel. Since ectopic oocysts can develop virtually anywhere in the mosquito haemocoel (Paskewitz and Shi, 2005), their quantification is unreliable and we therefore determined salivary gland sporozoite numbers on day 20 – 22 of infection (Table 4.2).

Table 4.1 Development of *Plasmodium berghei* ∆*pplp5* parasites in *Anopheles stephensi*

A		Oocysts					Salivary gland sporozoites	
Exp	Parasite	#	Prevalence	Mean	SEM	p-value	#	Mean
1	wt	52	88%	221	22	-	20	4836
	∆ppl5 cl1	50	0%	0	0	p<0.001	30	0
2	*wt*	50	96%	111	20	-	30	3104
	∆ppl5 cl1	50	0%	0	0	p<0.001	30	0
	∆ppl5 cl2	50	0%	0	0	p<0.001	30	0 §
3	*wt*	50	96%	218	31	-	30	5166
	∆ppl5 cl1	50	0%	0	0	p<0.001	30	0

B		Oocysts					Salivary gland sporozoites	
Exp	Parasite	#	Prevalence	Mean	SEM	p-value	#	Mean
1	*wt*	50	100%	249	17	-	15	9324
	∆ppl5 cl1	25	0%	0	0	p<0.001	22	0
	∆ppl5 cl2	50	4%	0.04	0	p<0.001	13	0
2	*wt*	50	98%	31	3	-	nd	nd
	∆ppl5 cl1	50	0%	0	0	p<0.001	nd	nd

(A) direct (gametocyte) feed on infected mice; **(B)** membrane feeding of *in vitro* cultivated ookinetes; Exp, experiment number; n, number of mosquitoes; Prevalence, percentage of mosquitoes with oocysts; Mean, mean number of oocysts or salivary gland sporozoites per mosquito, respectively; SEM, standard error of the mean; P-value as determined by z-test; n.d. not done; § 1 single sporozoites observed

Haemocoel injection completely restored mosquito infectivity of Δ*pplp5* ookinetes, indicating that the block in infection is specifically due to the inability of Δ*pplp5* ookinetes to cross the midgut epithelium. Ectopic Δ*pplp5* oocysts appeared morphologically normal (data not shown) and, importantly, Δ*pplp5* sporozoites were able to infect C57BL/6 mice by tail vein injection and by direct bite-back with prepatent periods similar to *wt*. Both *wt* and Δ*pplp5* parasites were first detected in Giemsa stained blood smears 4- 5 days post bite/injection,

indicating that sporozoites were fully infectious and both hepatocyte infection and liver stage development were not affected.

Table 4.2 Hemocoel injection of Δ*pplp5* ookinetes

	Salivary gland sporozoites					
	Ookinetes fed by membrane feeding			Ookinetes injected into haemoceol		
Parasite	**Mean**	**#**	**Infectivity**	**Mean**	**#**	**Infectivity**
wt	9324[§]	15	yes[b]	27519	15	yes[a]
Δppl5 cl1	0[§]	22	no[b]	6670	30	yes[a]
wt	18492	30	yes[b]	8794	30	yes[b]
Δppl5 cl2	0	30	no[b]	14221	29	yes[b]

mean, mean number of *Plasmodium berghei* salivary gland sporozoites per *Anopheles stephensi* mosquito; n, number of mosquitoes; Infectivity, ability to infect C57BL/6 mice by ailvein injection (a) or mosquito bite (b). § corresponds to Exp. 1 in table 1 B.

To determine more precisely at which point during midgut invasion Δ*pplp5* ookinetes were blocked, infected mosquito midguts were also analysed by immunofluorescence microscopy (Fig. 4.3A-C). Twenty-four hours after infection most *wt* ookinetes had already crossed the mosquito midgut epithelium, reached the basal lamina side and begun rounding up (Fig. 4.3B). Extruding midgut epithelial cells (Fig. 4.3A) and upregulation of *Anopheles stephensi* Serpin 6 (Fig. 4.3B) - both markers for midgut invasion (Abraham et al., 2005; Han et al., 2000) - were also observed in *wt* infected guts. In contrast Δ*pplp5* ookinetes were found attached in large numbers to the apical side of the midgut (Fig. 4.3A) where they persisted (in decreasing numbers) until 48 hours post infection (data not shown). No signs of cell invasion were observed in these guts. In confocal cross-sections of these midgut preparations *wt* parasites were detected within and on the basal side of the midgut epithelium, whereas *Δpplp5* ookinetes remained on the apical side (Fig. 4.3C). These observations were confirmed by the analysis of toluidine-stained semithin sections of midguts that were fixed 24 hours post blood feed (Fig. 4.3D). Invasion by *wt* ookinetes had induced massive damage to the midgut epithelium, while in contrast, no invasion of the midgut epithelium by *Δpplp5*

ookinetes was observed. Interestingly, *Δpplp5* ookinetes were stuck within the microvilli layer but had successfully crossed the peritrophic matrix.

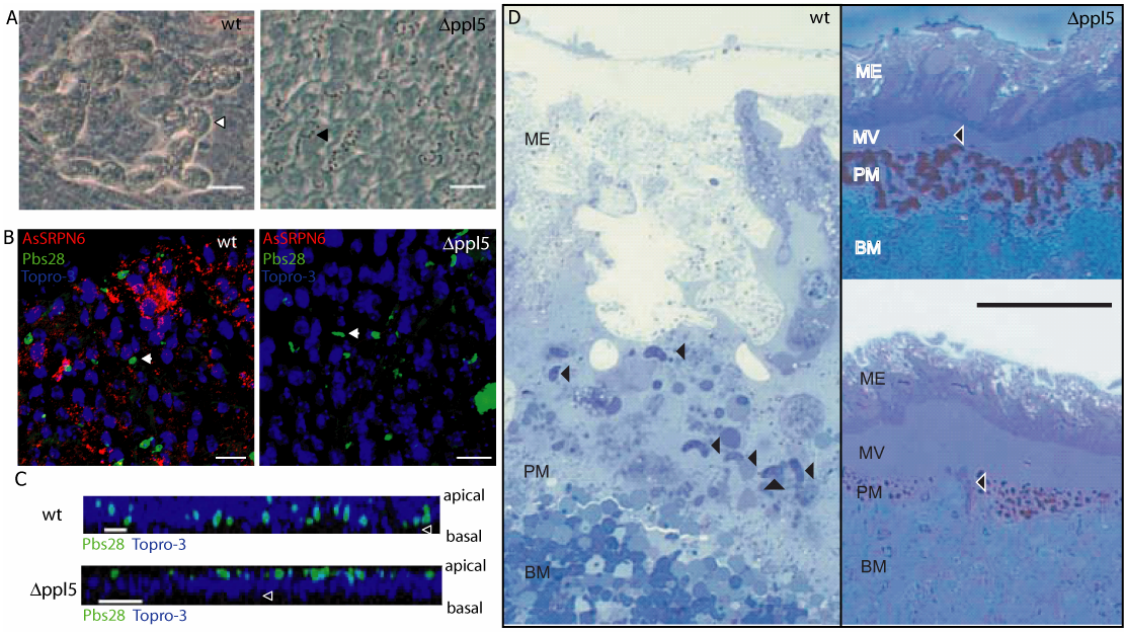

Figure 4.3 *Plasmodium berghei Δpplp5* ookinetes fail to invade and cross the *Anopheles stephensi* midgut

A – C: *An. stephensi* midguts were dissected 24 hours after feeding of *wt* or *Δpplp5* parasites and epithelia were prepared as previously described ((Danielli et al., 2000). Sheets were incubated with purified rabbit α-AgSRPN6 (1:1,000) and monoclonal α-Pbs28 (13.1; 1:1,000) antibody followed by secondary Alexa-Fluor-488-labelled-goat anti-mouse IgG and secondary Alexa-Fluor-568-labelled-goat anti-rabbit IgG (1:1,500, Molecular Probes, USA). Cell nuclei were stained with TO-PRO-3 (Molecular Probes, USA). Samples were analysed using a Leica SP2 confocal (B and C) or a Leica DMR fluorescence microscope and Leica DC500 digital camera (A). Scalebar = 20 µm. **A.** Differential interference contrast images show extruding midgut cells (white arrowhead) following invasion by *wt* ookinetes (left), and undamaged gut with large numbers of attached *Δpplp5* ookinetes (black arrowhead) (right). **B.** Confocal 3D projection of a z-stack shows that *wt* ookinetes have successfully invaded the midgut and started rounding up (green; white arrowhead; left) resulting in upregulation of *An. stephensi* Serpin 6 (red). No Serpin expression was detected in mosquitoes fed with *Δpplp5* parasites (white arrowhead; right). **C.** Z-stacks show that *wt* ookinetes (green) have crossed the midgut epithelium (top) while *Δpplp5* ookinetes (green) are still found on the apical side (bottom). Open arrowheads indicate nuclei of haemocytes, which are found attached to the basal side of the midgut epithelium. **D.** *An. stephensi* midguts were dissected 24 hours after feeding of *wt* or *Δpplp5* parasites, fixed as described in (Sinden et al., 1985) and semithin sections (500 nm) were prepared and stained with toluidine blue. Images were taken using a Leica DMR fluorescence microscope and Zeiss AxioCam digital camera. Scalebar = 50 µm. Invasion by *wt* ookinetes has caused massive damage to the midgut epithelium (ME) (left), while midguts of mosquitoes fed with *Δpplp5* remain unharmed (right, two examples shown). *Wt* ookinetes (arrowhead) are found within the midgut epithelium (left), whereas *Δpplp5* ookinetes (arrowhead)

have successfully crossed the peritrophic matrix (PM) but are stuck within the microvilli layer (MV) (right, two examples shown). BM, blood meal.

In summary we have shown that Δ*pplp5* ookinetes form normally *in vivo*, that they escape from the blood meal and move to the midgut epithelium, but are incapable of entering the midgut epithelial cells potentially due to a loss of cell-traversal activity. Importantly, if the midgut is bypassed by haemocoel injections of *in vitro* cultivated ookinetes, full infectivity to the mosquito is restored and the parasites are able to complete the rest of their life cycle. Thus, while expression of *pplp5* has also been detected in *P. falciparum* sporozoites in microarray (Le Roch et al., 2003) and proteomic studies (Florens et al., 2002), and in *P. yoelii* sporozoites by RT-PCR (Kaiser et al., 2004a), at least in *P. berghei* it is dispensable at this stage. While rescue of function by removal of a cellular barrier has not been shown for the *Δpplp3/maop* parasite and thus nothing can be concluded about the role of PPLP3/MAOP following midgut invasion (Kadota et al., 2004), full infectivity of *Δpplp1/spect2* parasites was restored by Kupffer cell depletion which allowed *Δplpp1/spect2* parasites direct access to hepatocytes (Ishino et al., 2005). Thus, both PPLP1 and PPLP5 play crucial roles only at single and different points in the parasite life cycle. Notably in both *Δpplp1/spect2* and *Δpplp5* parasites infectivity was not completely abolished. We observed natural transmission of *Δpplp5* parasites in one experiment, and Ishino *et al* report that *Δpplp1/spect2* parasites were capable of infecting rats (Ishino et al., 2005). We suspect that this low rate of transmission may occur should the cellular barrier be naturally compromised. Alternatively this low cell-traversal activity may be provided by other members of the PPLP family. The loss of infectivity of Δ*pplp5* ookinetes is striking, considering that the ookinete expresses three members of the PPLP family (PPLP3, 4 and 5) (Hall et al., 2005), and that *Δpplp3/maop* ookinetes were equally unable to cross the midgut epithelium (Kadota et al., 2004). The virtually identical phenotype of *Δpplp3/maop* and *pplp5* ookinetes strongly suggests that these two proteins may interact functionally.

Interestingly, the MACPF domain containing late complement components and perforin indeed function as polymers (Peitsch and Tschopp, 1991). If PPLP3

and PPLP5 formed a complex, this would obviously be lost in both individual knockouts. Alternatively these two proteins may function sequentially in the same pathway. We are currently raising antibodies to test these hypotheses and performing gene disruption experiments to determine the role of the remaining PPLP family members.

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MOSQUITO CELLULAR IMMUNITY

Molecular characterisation of haemocytes

CHAPTER 5:

Genome wide analysis of the molecular repertoire of *Anopheles gambiae* haemocytes

Abstract:

Besides mediating important cellular immune responses such as phagocytosis and encapsulation, haemocytes are important “factories” of secreted immune factors such as opsonins, proteases and their negative regulators as well as antimicrobial peptides. However, due to their tissue adhesiveness, relatively small abundance in the haemolymph and lack of primary culture methods, few studies have addressed the molecular composition of these important immune cells.

Here we report, the first genome-wide molecular characterisation of *Anopheles gambiae* circulating haemocytes. Haemocytes were collected from adult female mosquitoes, using a low contaminant yielding method. Their expression profiling identified a list of 1587 genes to be expressed by haemocytes. Many previously shown or speculated haemocyte-specific immune transcripts were detected. The study was extended to the comparison of available Dipteran haemocyte transcriptomes of *Drosophila melanogaster, Aedes aegypti* and *An. gambiae*. Interestingly, a reduced level of orthology compared to the genome as a whole was observed. The determination of a large number of haemocyte transcripts isolated in this study opens up the possibility of identifying pan- or subpopulation- specific haemocyte markers as well as novel immune genes to improve the functional characterisation of these cells in important immune responses.

5.1 Introduction

Innate immunity in insects consists of both humoral and cellular responses (reviewed in (Lavine and Strand, 2002; Lemaitre and Hoffmann, 2007)). Humoral immune responses are commonly defined as the result of a cascade of reactions, which are initially triggered through the recognition of non-self by the host. Recognition is thought to be mediated by pattern recognition receptors (PRRs) which can bind to specific pathogen-associated molecular patterns (PAMPs). This recognition is signaled to protease cascades, which subsequently lead to the activation of effector systems.

The main mediators of the cellular immune system in invertebrates are the haemocytes. Estimations proposed around 2000 haemocytes to be present in an adult mosquito (Christensen et al., 1989). Of these only a small fraction is circulating freely in the haemolymph while the rest is found attached to the body wall and organs such as midgut and tracheae. This small number of circulating cells that can be obtained from individual mosquitoes limits the work on mosquito haemocytes. It is therefore not surprising that the characterisation of cellular immunity lags much behind our knowledge of humoral immunity in mosquitoes.

Haemocytes are involved in important immune processes such as phagocytosis and encapsulation. They also contribute to humoral responses, through the production of antimicrobial peptides, opsonizing factors and molecules of the melanisation cascade (reviewed in (Lavine and Strand, 2002; Lemaitre and Hoffmann, 2007). In the model dipteran *Drosophila melanogaster,* haematopoiesis, distinct cell lineages, and functional characterisation of haemocyte responses have been described in some detail over the last years (reviewed in (Meister, 2004). Several studies have started the depiction of haemocyte-mediated immune effector mechanisms at the molecular level (Agaisse et al., 2003; Kurucz et al., 2003; Williams et al., 2005). Furthermore, a recent genome-wide transcriptional analysis of larval haemocytes (Irving et al., 2005) identified over 2500 genes present in at least one subpopulation of cells, giving for the first time insight into the molecular repertoire of these cells.

In mosquitoes, characterisation of haemocytes has been mainly based on morphological description (Castillo et al., 2006; Hillyer and Christensen, 2002; Hillyer et al., 2003a) and description of their ability to phagocytose and/or encapsulate foreign objects ((Hernandez-Martinez et al., 2002; Hillyer et al., 2003a; Hillyer et al., 2003b; Hillyer et al., 2004). Thus far, the molecular repertoire of mosquito haemocytes has only been addressed in *Aedes aegypti* and *Armigeres subalbatus*, where a single study identified over 2000 expressed sequence tags (ESTs) from bacterially challenged haemocytes in each specie (Bartholomay et al., 2004).

In *Anophelines*, haemocytes can phagocytize bacteria (cMoita et al., 2005; Levashina et al., 2001) and were reported to be associated with melanotic capsules (Hernandez et al, 1999, Hernandez-Martinez et al 2002). Interestingly, several agonist and antagonist of the development of the malaria parasite are expressed by haemocytes. These include phenoloxidases (PPOs) (Castillo et al., 2006), thio-ester containing proteins (TEPs) (Blandin et al., 2004; Frolet et al., 2006; Levashina et al., 2001), CLIPB serine proteases (Volz et al., 2005) and serpins (Abraham et al., 2005; Danielli et al., 2003). Three distinct subpopulations of circulating haemocytes in *An. gambiae* have been described (Castillo et al., 2006): Granulocytes, oenocytoids and prohaemocytes were distinguished based on morphological differences and with the aid of functional probes. However, none of the 17 markers used in that study unambiguously stained a single population in naïve and bacteria-challenged mosquitoes. Also, pan-specific haemocyte markers, which have been instrumental for detailed analysis of cellular responses in *Drosophila* (Goto et al., 2001; Kurucz et al., 2003; Tirouvanziam et al., 2004) still remain to be identified in *An. gambiae*.

The study presented here analyses the transcriptional profiles of circulating haemocytes in naïve female adult *An. gambiae* using Affymetrix, USA microarray technology. It aims to give insight into the molecular composition of these important cells in order to identify new markers and novel immune genes and to further characterise haemocyte-effector mediated responses. Additionally,

the results present a baseline for future analyses of haemocyte responses to different immune challenges.

5.2 Results

Haemocyte collection and RNA isolation

To find new genes as markers of *An. gambiae* haemocytes we decided to identify the expression profile of naïve adult haemocytes by Affymetrix Microarrays. Two established methods for collecting circulating mosquito blood cells were tested for yield and potential contamination from other tissues such as fat body. Proboscis-clipping (Chen and Laurence, 1987) yielded around 100 haemocytes per mosquito with virtually no contaminants as examined by microscopy. In contrast, displacement perfusion (Beerntsen and Christensen, 1990) yielded on average 300-400 cells but many contaminants such as fat body cells and cellular debris were also present in the perfusate. For any functional genomic study, which is aiming at identifying specific and exclusive haemocyte markers, contamination is a hazard, thus we opted to collect the heamocyte samples via proboscis clipping. Cells were collected from 1000 females and approximately 500ng of total RNA was consistently isolated from these samples.

To distinguish between transcripts specifically expressed in haemocytes as compared to other mosquito tissues, total RNA from carcasses (the remainder of the mosquito after removal of circulating haemocytes) and heads were isolated. Carcass samples were used to identify potential contamination of the haemocyte samples with fat body, which is abundantly and ubiquitously present in the abdomens. Mosquito heads contain a substantial amount of neuronal tissue and contain low numbers of haemocytes. Furthermore, previous transcriptomic analysis (AC Koutsos, FC Kafatos, and GC Chistophides unpublished), identified that important immunity transcripts had very low abundancy in head tissue as opposed to cascass or midguts making this tissue usefull for clustering analysis.

Microarray analysis

One vs. Two-cycle amplification

Due to the low amount of RNA isolated from the mosquito blood cells, a Two-Cycle Amplification protocol was chosen to label RNA prior to microarray hybridization. To address if potential bias could be introduced by the small sample amplification protocol, a comparison between one-cycle and two-cycle amplified carcass RNA was undertaken. Raw data were normalized as described in Materials and Methods, and only genes which were flagged as present in 3 out of 4 samples of each amplification protocol were considered. This left us with 3022 and 3089 genes for the one- and two- cycle amplification respectively. Of these roughly 84% (2558) of the genes are present in both lists and their intensities present an overall Pearson correlation coefficient of 0.908 (with 95% confidence intervals of 0.901 and 0.914 respectively, $P < 2.2E-16$, Fig 5.1).

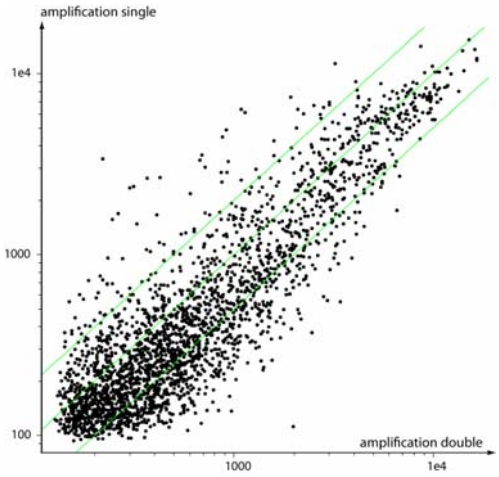

Figure 5.1 One vs Two-cycle comparison. Carcass sample were labeled via two different protocols: one and two cycle protocols. Of the 3022 and 3089 genes for the one and two cycle amplification respectively which were present in 3 out of 4 samples, 85% (2558) genes overlap in both lists. Their intensities are plotted presenting an overall Pearson correlation coefficient of 0.908 (with 95% confidence intervals of 0.901 and 0.914 respectively, and a p-value <2.2E-16).

Filtering Criteria

To identify haemocyte-specific transcripts a strict filtering criterion was applied to these samples. To detect above-background expression only transcripts with expression values two-fold higher than the standard deviation of global background intensities were considered. Of the 16050 initial elements, only 3505 passed this filter (Present or P List). Subsequently, to test for consistency in expression between the 4 replicates, solely transcripts that displayed a t-test p-value less than 0.05 in 3 out of 4 replicates were kept. This high stringency level was chosen to ensure high specificity for haemocyte expression and it should be

kept in mind that stringency probably decreases sensitivity in the detection of haemocyte-expressed genes. In total, 1587 transcripts were identified and considered consistently expressed in *An. gambiae* adult circulating haemocytes (Stringent or S List). By applying the same filtering criteria (two-fold higher intensity values than the standard deviation of global background intensities and t-test p-value less than 0.05) onto the carcass and the head samples 1391 and 1312 transcripts, for each tissue respectively, would not be flagged present.

A one-tailed Fisher's hypergeometric statistical analysis was performed to determine if the overlap between "absent in carcasses" and "present in haemocytes" would be expected to be detected more than by chance. A p-value of 0.0053 determined a significant difference characterised by an under-representation of absent carcass transcripts in the S List. This under-representation probably represents the attached haemocyte expression profile inherently present in the carcass samples.

In microarray analysis, presence is a more confident determinant of expression than is absence. Therefore, P land S lists were determined for carcass and head samples as previously done for the haemocytes, and the overlaps between the three tissue samples were investigated (Fig. 5.2A and B). For the P lists (Fig. 5.2A) over 2000 genes representing 64-73% of the genes in each list are shared between the 3 tissues, thus the percentage of tissue specific genes was very small ranging from 9 to 26%. However, when the S lists were analysed for gene overlap, 60-70% of the genes identified in each sample type are tissue specific with no overlap found between the 3 tested tissues.

Annotation of haemocyte-enriched transcripts

Using k-means clustering, transcripts in the S List were grouped into seven distinct co-expression clusters and a representation was chosen that focused mainly on the intensity of expression of genes in the haemocyte samples (Fig 5.2C). Clusters 1 to 4 represent high intensity value clusters (yellow), while clusters 5 to 7 represent genes with low expression in haemocytes (blue) as compared to ribosomal housekeeping expression. As many as 28% and 45% of

the genes in the high abundance (1-4) and low abundance (5-7) clusters, respectively have no assigned protein domain. For both cluster groups (high and low abundance) transmembrane domain predictions were found in equal proportions (12%). Signal peptide predictions were found twice as frequently in clusters 1-4 as compared to the low abundance clusters (Table 5.1). Cluster 4, consisting of the 82 most abundant genes, also presented by far the highest proportion of genes that bear a signal peptide (27/82) or a transmembrane domain (23/82).

To test for overrepresentation of Interpro or gene ontology (GO) terms a one-tail Fisher's exact test with Bonferroni multiple testing correction was used. Clusters 2, 4, 6, and 7 showed statistically significant enrichment for several different InterPro domains, and several GO terms were overrepresented in clusters 1, 2, 4, and 6 (Table 5.1). Specifically, cluster 2 and 4 showed an overrepresentation of peptidase domains; cluster 4 was enriched for transcripts encoding hemocyanin and Fibrinogen_C domains, which are components of PPO genes and domains implicated in coagulation responses. Cluster 6, the biggest cluster of the low abundance haemocyte transcripts was highly enriched for GO terms related to metabolic processes and energy transduction.

As haemocytes are considered an immunity-responsive tissue, we explored in how far the annotated immunity subgenome of *An. gambiae* (Christophides et al., 2002) is represented in the S list. Table 2 lists all identified immunity genes present in each of the seven clusters. Not surprisingly, given the overrepresentation of peptidase domains, almost all CLIPA and many CLIPB serine protease family members were found expressed in haemocytes. Additionally, a considerable number of SRPN and TEP genes were identified. TEP1, a previously identified haemocyte marker (Levashina et al., 2001) was not represented in the list, as its intensity value marginally did not pass the stringent first filter. Furthermore, a substantial number of thioredoxin dependent peroxidases (TPX) and glutathione transferase genes (GST) were also found. Many of the remaining genes identified in previous studies to be expressed in haemocytes, such as PP06 (Muller et al., 1999), SP22D (Danielli et al., 2000),

Lysozyme C (Castillo et al., 2006) and the low expressed PSMD3 (Castillo et al., 2006), were detected (Table 2).

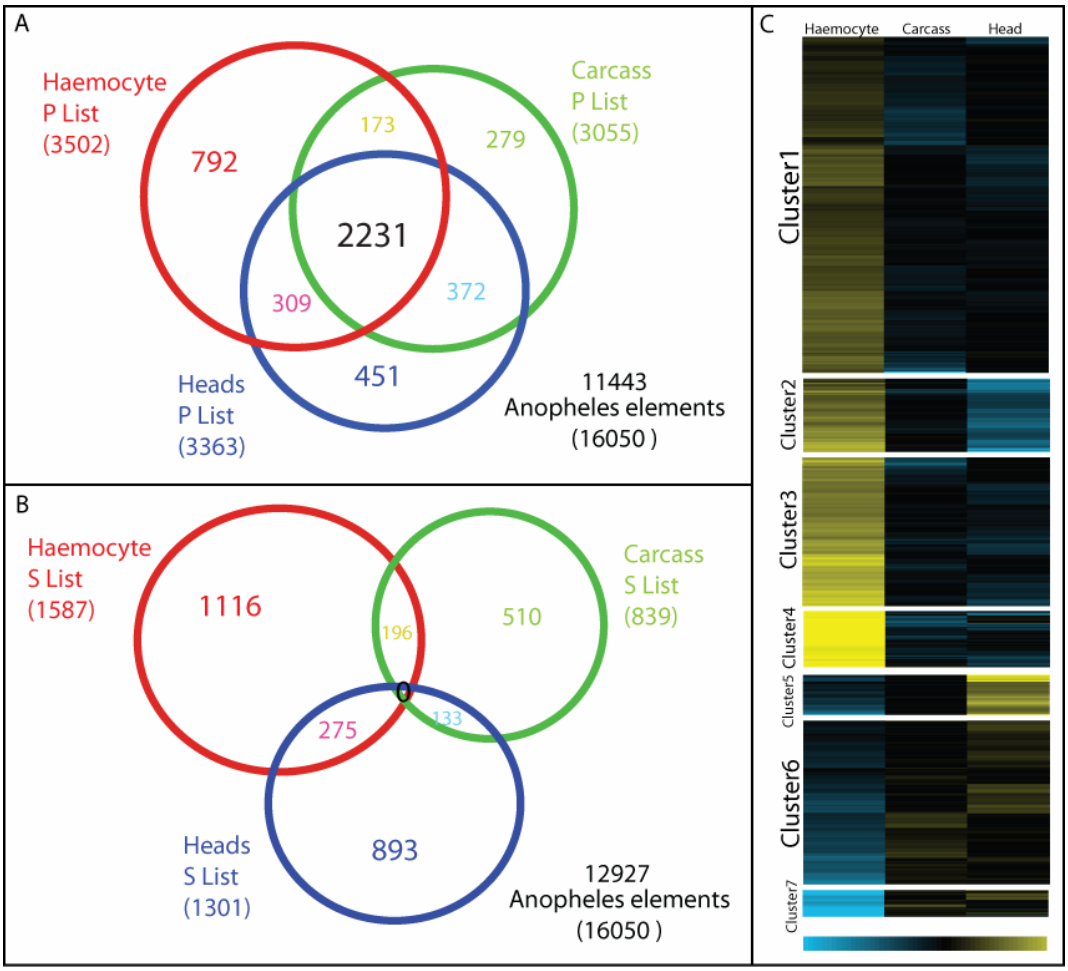

Figure 5.2 Characterisation of P and S lists.
Venn diagram representing the 3 way overlap between haemocyte (red), carcass (green) and head (dark blue) P Lists **(A)** and S Lists **(B).** Numbers in brackets refer to the total numbers of genes in each list. Different collours in the diagram represent different overlaps: yellow, carcass and haemocyte; purple, haemocyte and head; light blue, carcass and head and black represents the overlap between the 3 tissues. The same universe consisted of all *Anopheles* non-control elements present in the array. **(C)** Using a k-means algorithm, S List genes were clustered into seven distinct co-expression clusters. The representation chosen focused on distributing S List genes via their intensities of expression in the haemocyte samples. Note that yellow represents high intensity values while blue represents low intensity values. Black represents intensity values equal to the ribosomal genes used for normalisation (intensity value 1).

Table 5.1 Cluster Annotation of S List

Cluster	Gene #	Signal Peptide [%]	Transmembrane Domain [%]	No IPR [%]	InterPro Domain overrepresentation	GO term overrepresentation
1	634	9	12	27	None	GO:0005515 protein binding, GO:0005488 binding, GO:0006397 mRNA processing, GO:0005732 small nucleolar ribonucleotide complex,GO:0005634 nucleus
2	142	17	8	30	IPR001254 Peptidase_S1_S6, IPR001314 Peptidase_S1A	GO:0004252 serine-type endopeptidase activity, GO:0006508 proteolysis, GO:0006012 galactose
3	267	18	9	29	None	None
4	82	27	23	28	IPR005204 hemocyanin_N, IPR002227Tyrosinase, IPR005203 hemocyanin_C, IPR000896Hemocyanin, IPR001314 Peptidase_S1A, IPR001254 Peptidase_S1_S6, signal peptide, IPR001827 Antennapedia, IPR002181 Fibrinogen_C, IPR002345 Lipocalin	GO:0005344 oxygen transporter activity, GO:0004252 serine-type endopeptidase activity,GO:0006508 proteolysis, GO:0008233 peptidase activity
5	69	4	13	59	None	None
6	339	6	13	41	None	GO:0046961hydrogen-transporting ATPase activity, rotational mechanism, GO:0015986 ATP synthesis coupled proton transport, GO:0046933 hydrogen-transporting ATP synthase activity, rotational mechanism, GO:0016469 proton-transporting two-sector ATPase complex, GO:0008137 NADH dehydrogenase (ubiquinone) activity GO:0016651oxidoreductase activity, acting on NADH or NADPH GO:0003735 structural constituent of ribosome GO:0005840 ribosome GO:0006118 electron transport GO:0006120 mitochondrial electron transport, NADH to ubiquinone GO:0004129 cytochrome-c oxidase activity
7	54	13	6	52	IPR000618 Insect_cuticle	None
Overall	1587	11	12	33		

IPR= InterPro domain, GO= Gene Ontology term, #= number

Table 5.2 Annotated and Immune genes present in S List

Clust	Gene name	Haem	Carc	Head
1	SRPN14	2.31	0.83	0.93
	CLIPD5	2.25	0.82	0.97
	PGRPS1	2.17	1.12	0.62
	TEP15	2.17	0.91	0.82
	SRPN4	2.13	0.83	1.02
	TPX3	1.98	0.77	0.92
	SRPN2	1.93	0.97	0.97
	CLIPA4	1.92	0.94	0.86
	TPX1	1.75	0.7	0.96
	GSTE5	1.67	0.88	0.84
	GSTT2	1.66	0.73	1.14
	REL1	1.58	0.84	0.97
	GSTO1	1.49	0.68	1.0
	TPX5	1.27	0.79	0.88
	PSMD3	1.2	0.97	0.66
	TRX1	1.18	0.84	1.05
	GSTT1	1.13	0.73	0.93
2	APL1	4.22	0.77	0.69
	CLIPB15	4.17	0.93	0.33
	CLIPA5	3.63	0.89	0.17
	CLIPB12	3.57	0.93	0.26
	TEP4	3.32	0.64	0.43
	CLIPA8	3.21	0.85	0.44
	CLIPA2	3.19	0.81	0.48
	CLIPA1	3.14	0.88	0.43
	CLIPC5	3	0.75	0.5
	CTLMA2	2.95	0.88	0.4
	TEP12	2.9	1.07	0.48
	CLIPB3	2.58	0.94	0.28
	CTL4	2.47	0.89	0.33
	PGRPLD	2.47	0.84	0.22
	PGRPLC2	2.33	0.93	0.54
	TEP14	2.31	0.93	0.48
	CLIPA7	2.19	0.89	0.51
	LRIM1	2.18	0.75	0.33
	LYSOZ-C	1.88	1.0	0.24
3	SCRB7	4.84	0.82	0.97
	GATA	4.65	0.48	0.93
	CTLMA4	3.95	0.77	0.76
	LRR	3.05	0.74	0.55
	SRPN1	3.04	0.6	0.92
	CLIPB13	2.99	0.74	0.98
	CLIPA9	2.94	0.93	0.6
	CLIPC7	2.93	0.78	0.81
	CLIPB1	2.9	0.86	0.7
	GPRMTHL4	2.89	0.73	0.93
	SRPN11	2.85	0.86	0.76
	SRPN16	2.56	0.68	0.9
	PGRPLC3	2.44	1.04	0.76
4	PPO2	44.25	0.4	0.89
	PPO9	14.68	0.34	1.14
	CLIPB8	13.98	1.21	0.62
	SCRC1	12.27	0.85	1.1
	PPO6	10.95	0.49	1.2
	PPO4	10.91	0.37	0.99
	GPRMTHL6	9.75	0.89	0.95
	SP22D	8.73	0.93	0.84
	CLIPD1	8.48	0.44	0.93
	CLIPB17	8.33	1.02	0.72
	TEP3	6.14	1.02	0.53
5	LRR	0.44	0.9	5
	TPX4	0.25	1.1	2.1
6	GSTE3	0.61	1.5	1
	IAP1	0.49	1.1	1.1
	GSTU1	0.3	1.2	0.8
7				

Clust, cluster; Haem, Haemocytes; Carc, Carcass: Values are normalized intensities of replicates. Genes in red have previously been shown to be expressed in haemocytes, while the expression of genes in blue were verified in the present study (see Fig. 5.4). , APL= Anopheles *Plasmodium*-responsive leucine-rich repeat, CLIP= clip domain serine proteases, IAP= inhibitor of apoptosis, CTLs = C-type Letins,, GPRMTHL=G-coupled receptor methuselah-like, GST= glutathione S transferase, LRIM= leucine rich repeat protein, LRR= leucine rich repeat, LYSOZ= lysozyme, PPO= prophenol oxidase, PGRP= peptidoglycan recognition protein, REL= relish, SCR= scavenger receptor, SP= serine protease, SRPN= serine protease inhibitor, TEP= thioester containing proteins, TPX= thioredoxin dependent peroxidase., TRX=thioredoxin

Comparison of Dipteran haemocyte transcriptomes

With the aim of identifing haemocyte homologous genes, the *An. gambiae* haemocyte-specific transcripts identified in this study were compared to those in similar studies performed in three other diptera. A microarray study in *D. melanogaster* (Irving et al., 2005) identified 2517 transcripts highly enriched in larval haemocytes, while an EST study in the mosquitoes *Ae. aegypti* and *Ar. subalbatus* (Bartholomay et al., 2004) established over 2000 putative bacterial responsive EST clusters per mosquito species. Of these, 1690 *Drosophila*, 604 *Aedes* and 763 *Armigeres* genes have putative *Anopheles* homologs (as defined by best reciprocal BLAST hit) present in the Affymetrix microarray (Table 5.3A). Surprisingly, the *Anopheles* haemocyte-specific gene list compiled in this study (S List) contains 367 (18%, of the homologues present in the Affymetrix array, see above), 191 (18%) and 247 (26%), *Drosophila, Aedes* and *Armigeres* putative homologs, respectively (Table 3A), with a remarkably low number of 37 genes overlapping in the 3 species.

This apparent lack of overlap prompted us to look into more detail at the orthology found between these dipteran species *D. melanogaster, An. gambiae,* and *Ae. aegypti*. As no genome sequence is available for *Armigeres* this mosquito species was excluded from the analysis. The *An. gambiae* transcripts present in the S and P lists, were mapped uniquely to 1180 and 2566 ENSANG genes, respectively (Table 3B). Of these, 83% (985) in the S and 86% (2219) in the P list fall into orthologous groups that also contains at least one *Drosophila* gene, while, 90% (1062) and 91% (2331) fall into an orthologous group containing at least one *Aedes* gene. These values represent the total number of orthologous genes present in the *An. gambiae* haemocyte-expressed gene lists that could potentially be found in the *Drosophila* and *Aedes* experiments. However, in the *Drosophila* blood cell transcriptome, of the possible 985 orthologs only 369 genes (38%) in the S and 865 genes (39%) in the P list are found. These numbers were even lower when comparing the *An. gambiae*/*Ae. aegypti* haemocyte transcriptomes: Out of the potential 1062 (S List) and 2331 (P

list) orthologs, merely 319 (30%) were present in the S and 661 (28%) in the P list (Table 5.3B).

Table 5.3 Comparative analysis of dipteran haemocyte transcriptomes

A. Homology	***D. melanogaster****	***Ae. aegypti****	***Ar. subalbatus****	
Gene #	2405	2687	2098	
An. gambiae homologues	1983 (1690[§])	1026 (604[§])	943 (763[§])	
Present in S list	***367***	***191***	***247***	
B. Orthology	***D. melanogaster***	***Ae. aegypti***	***An. gambiae***	
			S List	**P List**
Total Gene #	2756	1461	1180	2566
Genes in OG with Ag genes	2016	1138	1103	2219
Genes in OG with Dm genes	2214	1061	985	2423
Genes in OG with Aa genes	2086	1219	1062	2331
# of Ag genes with ortholog in Dm haemocyte* gene set			***369***	***865***
# of Ag genes with ortholog in Aa haemocyte** gene set			***213***	***432***

(A) Total gene numbers represent haemocyte transcriptomes identified in * (Irving et al., 2005) and ** (Bartholomay et al., 2004) [§]. *An. gambiae* homologous are determined by ENSEMBL predictions and the numbers in brackets represent the number of *An. gambiae* homologous genes present in the *Plasmodium*/Anopheles Affymetrix array. **(B)** Orthologous group is defined as a set of orthologous genes from at least two species.
#, number; Ag, *An. gambiae*; Aa, *Ae. aegypti*, Dm, *D. melanogaster*; OG, orthologous group

Determination of highly haemocyte-enriched transcripts

In order to identify likely haemocyte-specific transcripts the S list of haemocyte-expressed genes was further filtered removing all transcripts with less than 1.7 fold higher expression in the haemocyte vs. carcass samples. Of the 1587 S list transcripts 1023 genes, previously present in clusters 1-4, passed this filter (Haemocyte or H list). The H list was reclustered, into 6 cluster groups with a k-means algorithm, taking into consideration tissue specific patterns of expression (Fig. 5.3).

To test if the H list of haemocyte-expressed genes can be used to identify new haemocyte markers, all available antibodies against members of the H list

were tested in immunofluorescence analysis. Each of them, anti-PPO2, anti-CLIPA8, anti-CLIPB17 and anti-LRIM1 antibodies stained perfused haemocytes (Fig 5.4) validating the expression of these genes in the circulating haemocyte population. Moreover antibodies against two CLIPB family members (B4 and B8) and a PGRP protein (LC1) which were not detected in the microarray analysis but are closely relate to identified trancripts, were also tested. PGRP-LC1 and CLIPB4 were not detected thus acting has negative controls, CLIPB8 gave a convinsing signal suggesting that this protein was missed in the analysis.

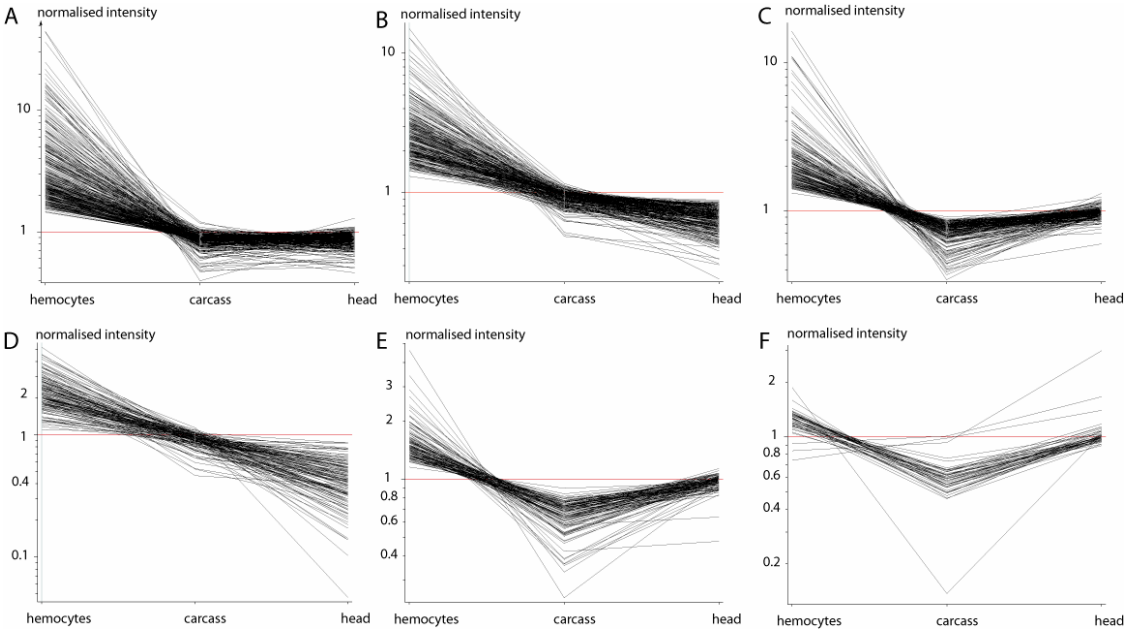

Figure 5.3 Clustering of the highly enriched haemocyte transcripts H List.
H list genes were clustered via a Pearson k-means algorithm. Six cluster groups were chosen as they identified singular tissue expression patterns. Each graph represents the expression pattern of a cluster **(A-E)** in the three different tissues. Top row shows the expression profiles of genes with high abundance in haemocytes and **(A)** low similar levels of carcass and head, **(B)** low expression in carcass but lower head and **(C)** low expression in head but lower carcass. Bottom row represents lower haemocyte expression clusters (note the scale). **(D)** Genes showed low expression in carcass but lower in head while genes in **(E)** showed low expression in heads and even lower expression in carcass samples. The last cluster contains a small group of transcripts with very low abundancy in haemocyte and head samples and even lower abundance in carcass.

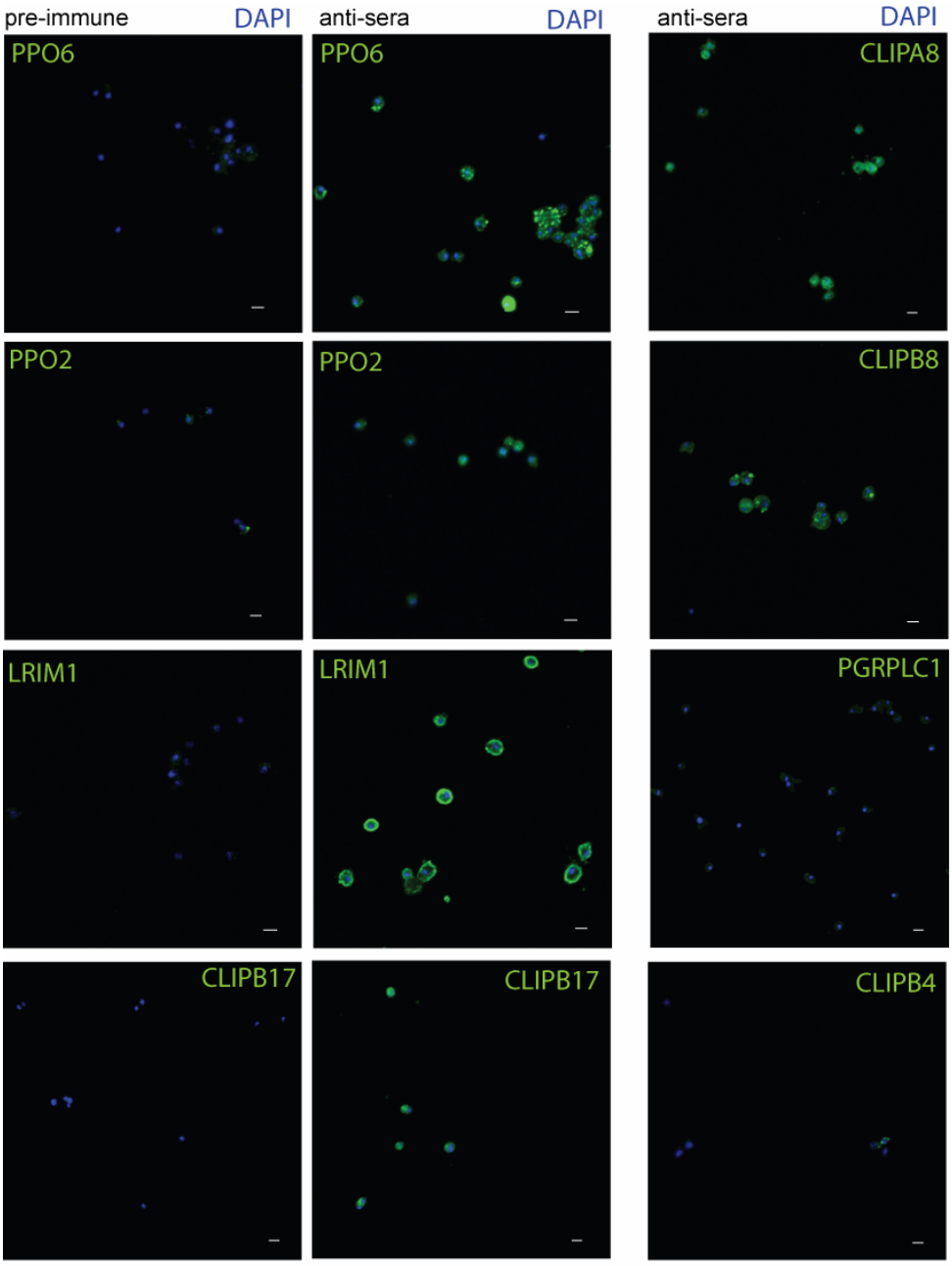

Figure 5.4. Immunolocalisation of identified markers in perfused haemocytes. Antibodies against four novel identified markers were used for validation of microarray results: LRIM1 (AC Koutsos, GK Christophides unpublished), CLIPA8 (M Osta, unpublished), CLIPB17 (J Volz, M Osta unpublished) and PPO2 (Leclerc et al., 2006) stained perfused haemocytes. PPO6 is used as a positive control. CLIPB8 (J Volz, M Osta unpublished), CLIPB4 (J Volz, M Osta unpublished), and PGRPLC1 (S Meister, GK Christophides unpublished) were not detected in the study. Note that while CLIPBA and PGRPLC1 serve as negative controls (no significant signal is detected), CLIPB8 seems to be expressed by haemocytes and was missed in the microarray analysis. Scale bar, 20μm

5.3 Discussion

Cellular immune responses are central in any invertebrate. Specialised blood cells are capable of recognizing foreign intruders and either eliminate them directly by engulfment or encapsulation, or may signal to other tissues to activate systemic or local responses (Lemaitre and Hoffmann, 2007). *D. melanogaster* has served as a model organism for the study of cellular immunity (Meister, 2004). However these studies have focused on the larval stage of the organism, which has been shown to have unique blood cell population types and responses. On the other hand, very little is know on the molecular mechanisms underlying cellular responses in mosquitoes, medically important insects, in spite of the fact that many of the molecules modulating malaria parasite development are expressed by these cells.

Adult mosquitoes possess an open circulatory system bathing every organ, in which haemocytes freely circulate throughout the body. Most haemocyte cells are sessile, attached to the body wall or other organs, making it very hard to isolate them. Castillo *et al* (2006) have recently tested different isolation techniques and assessed the samples for potential contamination. They concluded, as in this present study, that the isolation procedure is important to keep the samples contaminant-free. Therefore the cleanest isolation protocol (proboscis clipping) was used here to isolate circulating haemocytes from naïve female *An. gambiae* mosquitoes to determine their expression profile via Affymetrix microarrays.

A list of 1587 transcripts was compiled, which we consider to be consistently expressed in *Anopheles* haemocytes. The inexistent overlap between haemocyte, carcass and head S Lists and the concomitant high overlap of absent or aberrantly flagged S list haemocyte genes in the respective carcass and head samples suggests that the haemocyte S list identified in this study represents a pool of genes specifically expressed by circulating heamocytes.

More surprising is that neither by homology nor orthology we were able to identify a large proportion of orthologs to be expressed in the haemocytes of all four species. Decreasing the stringency of the filtering criteria expectedly

expanded the haemocyte gene P list and at the same time increased the number of orthologs found in the *Drosophila* and *Aedes* sets. However, this increase was proportional to the increase of potential orthologues in the initial unique gene list thus not specifically increasing the proportion of orthologous haemocyte-expressed genes. This discrepancy could be explained by the different experimental designs (larval vs adult and challenged vs unchallenged) and analysis criteria used in the three experiments. One could speculate that the distinct evolutionary adaptations of these dipteran species to respond to the environments they encounter could account for the differences seen in the molecular repertoire of these blood cells. However, to clarify this difference, systematic functional analysis between homologous and heterologous genes in these four species and their cellular responses should be performed.

Detailed annotation of the S list genes clustered them into seven co-expression clusters with distinct intensity patterns. Strikingly, a substantial number of annotated immunity genes (Christophides et al., 2002) were identified. A total of 17 members of the CLIPA, B, C and D serine protease families, five TEP genes, six SRPNs, four PPOs and three PGRP family members were detected. A complex module of CLIPA and B family members has been shown to mediate melanisation (Volz et al., 2006), and important regulators of melanisation such as CLIPA2, A5 and A8 were identified in the analysis. As in *Drosophila* (Irving et al., 2005), several PGRP genes had detectable expression in haemocytes which could potentially be involved in the activation of signaling cascades important for triggering key responses for pathogen clearing.

Several thioredoxin dependent peroxidases (TPX) were also identified and interestingly a mosquito TPX has been shown to be induced in the mosquito midgut upon parasite infection (Peterson and Luckhart, 2006). Moreover in a cell culture system this TPX protects cells against stresses that are relevant to malaria parasite infection (Peterson and Luckhart, 2006). Several members of another family of detoxifying enzymes was detected: glutathione transferases (GSTs) are a diverse family of enzymes which in the mosquito have been previously implicated in insecticide resistance and potentially in maintaining the

redox status of the mosquito cell upon parasite invasion (Ranson and Hemingway, 2005). Besides contributing to immune responses, haemocytes may be important players in maintaing the homeostatic redox balance of the mosquito.

The three clusters with low intensity values in haemocyte samples in comparison to either the carcasses or heads presented a high proportion of genes with InterPro or GO terms for metabolic and energy transducing processes, suggesting that these genes (339) are required for energy metabolism and are likely to be expressed in all mosquito tissues.

We extended the analysis to further enrich our list into putative specific haemocyte transcripts by only considering genes with an intensity value that was at least 1.7 times higher than the carcass tissue. Due to the high sensitivity in the Affymetrix array detection, this “low” value was chosen so that transcripts which would be expressed in other cells other than haemocytes would be eliminated, while the identification of some transcripts which are equally expressed in circulating and attached haemocytes (thus present in carcass samples) would remain in the analysis. A total number of 1023 genes were identified and an initial validation was performed for the identified proteins againts which there was available antibodies. The expression of CLIPA8, CLIPB17, LRIM1 and PPO2 in perfussed haemocytes was confirmed via immunoflourescence. In parallel, three proteins not identified by the analysis but closely related to detected proteins were used as negative controls. CLIPB8 was detected has a haemocyte protein and missed in the analysis, while PGRPLC1 and CLIB4 were not immuno-detected and further confirm the results presented.

Taken together, the data so far suggests that the analysis has determined a highly haemocyte specific list of genes. However, further and more extensive detailed validation and functional analysis is required. To address that, fifty candidates have currently been chosen for future validation via RNA in situ hybridisations and for functional characterisation in immune responses.

5.4 Materials and Methods

Mosquito rearing

An. gambiae G3 strain was maintained at 28ºC, 75% humidity, under a 12h day/night cycle according to (Richman et al., 1996). Larvae were fed on ground cat food and adults on a 10% sucrose solution.

RNA isolation

Haemocytes were harvested from 1000 females, 1-2 day old by clipping their proboscis and slightly squeezing their thorax with dissection forceps. The haemolymph drop was collected directly into RNeasy's buffer RLT (Qiagen, DE) and stored at -80ºC. RNA was extracted following the RNeasy Mini protocol for animal cells (Qiagen, DE). Twenty carcasses (the remainder of the mosquitoes after haemocyte collection) and 40 isolated heads were collected into Tryzol Reagent (Invitrogen, USA) and RNA extracted according to manufacturers protocol. All Tryzol isolated RNAs were run over a Mini-RNeasy column (Qiagen, DE) according to the RNA clean-up protocol prior to RNA labelling reactions.

RNA labeling and hybridization

For each sample type, 4 biological repeats were analysed. Total RNA was biotinylated according to the One-Cycle (only carcass control samples) or the Two-Cycle Eukaryotic Target Labeling protocol (Affymetrix, USA) using a start up amount of 1 µg and 80 ng, respectively. In brief, total RNA was reverse transcribed using a T7- Oligo(dT) promoter primer in a 1^{st}-strand cDNA synthesis. After RNase H mediated 2^{nd}-strand synthesis, the double stranded cDNA was purified. In case of the Two-cycle protocol an *in vitro* transcription (IVT) was performed, with unlabelled ribonucleotides and T7 RNA polymerase, to create antisense RNA (cRNA). Unlabelled cRNA was reversed transcribed using random primers, followed by a 2^{nd} strand cDNA synthesis with T7- Oligo(dT) promoter primers. One- and two-cycle double-stranded cDNA templates were amplified and labeled using biotinylated ribonucleotides in an IVT reaction. All labeled cRNAs were cleaned up, fragmented, hybridized to GeneChip®

Plasmodium/Anopheles Genome Arrays (Affymetrix, USA) and scanned following manufactures instructions.

Microarray data analysis

Data Manipulation

Signal and background intensities were determined with the GCOS v.1.3 softare (Affymetrix, USA) and the chp files were made with the default settings. The resulting data were introduced to GeneSpring v.7.2 programme (Agilent , USA) and each hybridisation was normalised according to the median of a set of 65 ribosomal housekeeping genes of *An. gambiae* (identified by (Marinotti et al., 2006) and according to the median of each gene in all hybridisations. If the Affymetrix probe corresponded to an ENSEMBL gene model, the expression profiles were averaged accordingly to ENSEMBL transcript ids. Annotation information was provided by Affymetrix and by querying the ENSEMBL v 28 database (Hubbard et al., 2007).

To determine above background expression, only spots with expression values above the corresponding background intensity plus 2 times the average standard deviation of the background intensity of each hybridisation were used. To determine haemocyte specific transcripts, only genes that displayed above background intensity in at least 3 of the 4 haemocyte sample experiments were considered further. These genes (3505) were named as 'present' in haemocytes (referred as P-list), whereas genes failing this criterion were considered 'absent' from haemocytes. Similar genes lists were made for other tissue samples. To determine consistent gene expression in the haemocyte samples, only genes with a t-test p-value less that 0.05 were further considered. This filtering criterion accounted for 1587 genes (referred to as "S-list').

Finally haemocyte H List genes were compiled, from the haemocyte S list by isolating the transcripts with intensity values 1.7 fold higher than the respective intensity value in the carcass set.

Clustering analysis

Gene expression clustering was performed with the Cluster v3.0 package (Eisen et al., 1998) using the k-means clustering algorithm and either the euclidean distance (S-lists) or the pearson correlation coefficient (H-list) as a similarity measurement. For each list, results yielding 3-10 k-means clusters were manually inspected and the result that yielded tight expression clusters was chosen. Results were visualised graphically either with the Java Treeview v1.0 (S-list.) or Genespring v7.2 programme (H-list).

GO and Interpro overrepresentation

Frequencies of GO terms and INTERPO domains of each cluster list were calculated and compared to the frequencies in all clusters combined. Statistically significant over- or under-representation of terms or domains was detected by applying a cut-off value of 0.05 in the hypergeometric test (one tail Fisher's exact test) with Bonferroni multiple testing correction, as implemented in the GeneMerge programme (Castillo-Davis and Hartl, 2003). All other calculations of the hypergeometric test were implemented in R statistical package (Team, 2006).

Homology comparison

A list of 2405 haemocyte enriched genes of a study in Drosphila melanogaster (Irving et al., 2005) was used to identify gene homologues in *An. gambiae*, based on homology information (best reciprocal hits) of ENSEMBL. As a result, 1983 homologues in *An. gambiae* were identified and their overlap to the S list was investigated. Similarly, 2686 *Aedes* and 2098 *Armigeres* haemocyte lists (Bartholomay et al., 2004), were used to identify homologues (best reciprocal hit) of *Anopheles gambiae*.

Orthology comparison

Orthologous groups were indentified in a four-way comparison between *D. melanogaster*, *Ae. aegypti*, *An. gambiae*, using *Apis mellifera* as an outgroup,

essentially as described in (Zdobnov and Bork, 2007). Required ENSANG ID numbers were retrieved as described below:

Drosophila FBgn IDs, which had two-fold higher values in the unchallenged haemocyte samples as opposed to whole larvae samples, were extracted from the supplementary data of Irving *et al*. (2005). Their respective Ensembl peptide IDs were obtained in BioMart (www.biomart.org), and a list of 2756 genes was assembled.

The 2686 *Ae. aegypti* haemocyte EST clusters (Bartholomay et al., 2004) were mapped to the Aedes genome assembly at Vectorbase (Hubbard et al., 2007) using BLAT (FastMap, (Kent, 2002) . Subsequently, only the longest matching *Aedes* transcripts with $\geq$ 50bp of match AND (>20% length of their probe or >20% of the Aedes transcript length). In total 1461 *Ae. aegypti* genes were identified.

Immunofluorescence analyses (IFA)

Circulating haemocytes were collected by proboscis clipping into 2 μl of Schneider medium (Gibco, USA). Cells were left to settle (5-10 min), fixed (4% paraformaldehyde in PBS, 10 min) and permeabilized (0.2% Triton X-100 in PBS, 2 min). Subsequently, cells were blocked (2% BSA in PBS, 1h), and incubated with anti-LRIM1 (1:300, AC Koutsos, GC Christophides unpublished), anti-CLIPA8 (1:10, M.Osta, unpublished), anti-PPO2 (1:500 (Leclerc et al., 2006)) anti-PPO6 (1:500, (Muller et al., 1999)), anti-CLIPB17 (1:100, J Volz, M.Osta, unpublished) anti-CLIPB4 (1:100, J Volz, M.Osta, unpublished)) anti-CLIPB8 (1:100, J Volz, M.Osta, unpublished) anti-PGRPLC1 (1:10, S Meister, GC Christophides unpublished) antibodies, respectively. When available, pre-immune sera was used at the same dilution as the respective anti-sera. Nuclei were counter-stained with DAPI (1ng/μl, Roche Applied Science, CH). Samples were mounted in Vecta Shield (Vector Laboratories, USA) and analysed using an Axiovert fluorescence microscope (Zeiss, DE).

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