Proteomic analysis of the *Anopheles gambiae* response to a *Plasmodium berghei* infection

Présentée à l'Université de Strasbourg

Pour obtenir le titre de:

Docteur de l'Université de Strasbourg

par

Martin Rono

Soutenue le 15 Janvier 2009 devant la commission d'examen:

Prof. Hendrik Stunnenberg
Department of Molecular Biology, Radboud University Nijmegen

Dr. Suzanne Eaton
Max Planck Institute of Molecular Cell Biology and Genetics, Dresden

Prof. Flaminia Catteruccia
Division of Cell and Molecular Biology, Imperial College, London

Prof. Jean-Luc Imler
Institut de Biologie Moléculaire et Cellulaire, Strasbourg

Prof. Jules Hoffmann
Institut de Biologie Moléculaire et Cellulaire, Strasbourg

Dr. Elena Levashina
Institut de Biologie Moléculaire et Cellulaire, Strasbourg

DEDICATION

I dedicate my work to my mother Mrs Betty Rono and my late father David Rono for the support to their Children.

ACKNOWLEDGEMENTS

First of I would like to convey my sincere gratitude to my supervisor Elena Levashina for her support, encouragement and guidance, and to my co-director Henk Stunnenberg from whose laboratory we performed the mass spectrometry work. I would like to extend my appreciation to Professor Jules Hoffmann for accepting me to undertake my research work at UPR9022 and for his contribution as a member of my Thesis Advisory Committee (TAC) in the BioMalPar programme.

I also want to thank the members of my thesis Jury for accepting to participate in my defense.

I am greatly indebted to all the members of the “mosquito group” both past and present for their various support both in and out of work. I would particularly want to mention Christine Kappler and Marie-Eve Moritz for their technical support and Julien Soichot who constantly supplied us with the dear mosquitoes we needed for our experiments. I also mention my special thanks to Hidehiro Fukuyama and his group.

I would like to acknowledge the special contribution to my work from Miranda Whitten with whom we established the lipid transport molecule project dubbed “M&M” project later renamed “MM&E” having worked closely with Eric Marois from whose experience in lipophorin we investigated the interesting aspects of lipid transport system in Mosquitoes and its implications in reproduction and antiparasitic responses.

To my colleagues the PhD students (Malou, Martine, Steffi, Marina, Sandrine and lastly Annika who doubles up as a fellow student in the BioMalPar programm. I thank you all for understanding me and sharing your experiences and knowledge as students. I also want to remember the BioMalPar colleagues “CSP” group.

Finally, I thank BioMalPar international PhD programme for the opportunity and funding.

Abstract

Malaria is a mosquito-borne infectious disease yearly affecting an estimated 500 million humans, of which 1 to 2 million (mostly children in Sub-Saharan Africa) succumb to the disease. Malaria transmission is initiated when a female mosquito ingests gametocytes during a blood meal, required for ovary development. Thus, feeding on a malaria-infected host will simultaneously activate oogenesis and allow malaria parasites to invade mosquito tissues. However, the parasites undergo massive losses during their development in the vector, due to the powerful immune response that mosquitoes mount against the invading parasites. The basis of this antiparasitic response has been investigated previously using reverse genetic approaches and has identified several antiparasitic molecules including TEP1, a homologue of vertebrate complement factor C3, which mediates parasite killing in a complement-like manner. However, additional mosquito factors involved in this killing mechanism including effector molecules are yet to be identified. To this aim, transgenic mosquitoes with *TEP1 gain-of-function* (*GOF*) and *loss-of-function* (*LOF*) were established, and transcriptional analysis of their immune response during parasite development performed as a basis for examining the pathway. Because transcript levels do not always correlate with protein abundance, we complemented the microarray analysis with a proteomic analysis of the mosquito response towards a *Plasmodium berghei* infection in the midgut tissues.

We observed that mosquitoes respond to parasite infection by inducing the expression of putative antiparasitic molecules including thioester containing proteins (TEPs); leucine rich repeat molecules (LRRs); galectins; and serine protease inhibitors (SRPNs). In addition, the proteomics data confirmed the transcriptional profiles of *P. berghei* infected mosquitoes. We showed that *GOF* mosquitoes induced more putative antiparasitic molecules compared to *LOF* mosquitoes. Furthermore, we have provided the first global proteomic analysis of the mosquito midgut during parasite infection.

Next, we extended our analysis to the nutrient transport system in mosquitoes comprising lipophorin (Lp) and the phospholipoglycoprotein vitellogenin (Vg), a precursor of the yolk storage protein vitellin. Lp has been shown to be important for oogenesis and parasite survival. We find that Lp promotes parasite survival by reducing the parasite-killing activity of TEP1. Furthermore, antiparasitic factors such as TEP1 are secreted into the hemolymph by the mosquito blood cells and may

associate with lipophorin, since such an association has been reported between human complement factor C3 and lipoproteins. In order to examine this we purified and analyzed mosquito lipophorins using immunobloting and mass spectrometry approaches. We found that Lp associated with prophenoloxidase (PPO), an enzyme that catalyzes melanization reactions in insects. This association was specific to PPO as no other immune factor (including TEP1) could be detected in the lipid complexes.

Next, we functionally characterized Vg and established that it impinged on TEP1 activity in a manner similar to Lp. Further analysis by gene silencing and IFA revealed a surprising network of genetic interactions between lipophorin, vitellogenin, NF-kB/Rel transcription factors and the capacity of TEP1 to bind and kill ookinetes. In addition, preliminary results indicate that besides their role in regulating immunity, NF-κB factors are also implicated in the regulation of the TOR pathway, which controls Vg expression, through the TSC1/TSC2 complex. These results provide a molecular basis to explain the trade-off between reproduction and immunity.

Résumé

Le paludisme est une maladie infectieuse transmise par un moustique, affectant chaque année environ 500 millions d'humains dont 1 à 2 millions y succombent; principalement des enfants d'Afrique sub-saharienne. La transmission du paludisme commence lorsqu'un moustique femelle ingère des gamétocytes lors d'un repas sanguin requis pour le développement de ses ovaires. Ainsi, le fait de se nourrir sur un hôte infecté va simultanément activer l'oogénèse et permettre aux parasites responsables du paludisme d'envahir les tissus du moustique. Toutefois, les parasites subissent des pertes massives au cours de leur développement dans le vecteur, dues à une puissante réponse immunitaire que les moustiques développent vis-à-vis des parasites envahisseurs. Les bases de cette réponse antiparasitaire ont été précédemment étudiées par l'utilisation d'approches de génétique inverse qui ont permis l'identification de plusieurs molécules antiparasitaires incluant TEP1, une protéine homologue au facteur C3 du complément des Vertébrés, responsable de l'élimination du parasite d'une manière similaire au complément. Cependant, d'autres facteurs du moustique sont impliqués dans ce mécanisme d'élimination du parasite, y compris des molécules qu'il reste encore à identifier. Dans ce but, nous avons utilisé des moustiques transgéniques présentant un *gain de fonction* (*gain-of-function, GOF*) ou une *perte de fonction* (*loss-of function, LOF*) de TEP1 pour effectuer l'analyse transcriptionnelle de leurs réponses immunitaires durant le développement du parasite. Parce que l'abondance des ARNm messagers ne reflète pas forcément l'abondance des protéines, nous avons complémenté l'analyse des puces à ADN par une analyse protéomique de la réponse du moustique à l'infection de ses tissus intestinaux par *Plasmodium berghei*.

Nous avons observé que les moustiques répondent à l'infection par *Plasmodium* en induisant l'expression de molécules antiparasitaires présomptives, dont des protéines contenant un groupement thioester (thioester containing proteins, TEPs) ; des protéines à répétition riches en leucine (leucine-rich repeat, LRR) ; des galectines ; ainsi que des inhibiteurs de protéases à sérine (serine protease inhibitors, SRPNs). Nous avons obtenu une bonne corrélation entre nos données protéomiques et nos profils transcriptionnels. Nous avons montré que les moustiques *GOF* induisent plus de molécules antiparasitaires présomptives que les moustiques *LOF*. Ce travail représente la première analyse protéomique globale de l'intestin de moustique lors de l'infection par le parasite.

Nous avons ensuite étendu nos analyses aux systèmes de transport de nutriments dans les moustiques, comprenant la lipophorine (Lp) ainsi que la vitellogenine (Vg), une phospholipoglycoprotéine précurseur de la protéine du vitellus : la vitelline. Il a été prouvé que la Lp est requise pour l'oogénèse du moustique ainsi que pour la survie du parasite. Nos résultats montrent que l'effet protecteur de la Lp pour le parasite s'explique par une diminution de l'activité antiparasitaire de TEP1. Par ailleurs des facteurs antiparasitaires tels que TEP1 sont sécrétés dans l'hémolymphe par les cellules sanguines du moustique et pourraient s'associer avec la lipophorine, car une telle association a été rapportée entre le facteur du complément humain C3 et les lipoprotéines. Pour tester cette hypothèse, nous avons purifié et analysé les lipophorines de moustique par immunoblotting et spectrométrie de masse. Nous avons trouvé que la Lp est associée à une prophénoloxidase (PPO), enzyme catalysant les réactions de mélanisation chez les insectes. Cette association est spécifique de PPO, puisqu'aucun autre facteur immunitaire (y compris TEP1) n'a pu être détecté dans les complexes lipidiques.

Ensuite nous avons caractérisé fonctionnellement la vitellogénine et établi qu'elle affecte l'activité de TEP1 de manière semblable à la lipophorine. Les analyses suivantes d'invalidation de gènes et d'immunomarquages ont révélé un réseau d'interactions génétiques inattendu entre la lipophorine, la vitellogénine, les facteurs de transcription NF-kB/Rel et la capacité de TEP1 à opsoniser et éliminer les oocinètes. Enfin, des résultats préliminaires indiquent que les facteurs NF-kB, outre leur rôle de régulateurs de l'immunité, sont impliqués dans la voie TOR (qui contrôle l'expression de la vitellogénine) *via* le complexe TSC1/TSC2. Ces résultats apportent un début d'explication moléculaire au phénomène d'exclusion mutuelle entre reproduction et immunité observée chez le moustique.

TABLE OF CONTENTS

ABBREVIATIONS

2D	Bidimensional
2D-DIGE	2D-Difference Gel Electrophoresis
ACT	Artemisinin Combination Therapy
APOII/I	Apolipophorin II and I
CBB	Coomassie Brilliant Blue
DD	Death Domain
DDT	Dichloro-diphenyl-trichloroethane
DIF	Dorsal-related Immune Factor
DREDD	Death-Related ced-3/Nedd2-like protein
dsRNA	Double stranded RNA
DTT	Dithiothreitol
EANMAT	East Africa Network of Monitoring Antimalarial Treatment
EDTA	Ethylenediaminetetraacetic acid
ESI	Electrospray Ionization
FADD	Fas Associated Death Domain protein
GFP	Green fluorescence protein
GNBP	Gram-Negative Bacteria Binding Proteins
IκB	Inhibitor of NF-κB
IMD	Immune Deficiency
IPT	Intermittent Prophylaxis Treatment
IRD5	Immune Response Deficient 5
JNK	Jun Kinase
KD	Knock Down
LC-MS/MS	Liquid Chromatography coupled to tandem Mass Spectrometry
Lp	Lipophorin
LPS	Lipopolysaccharide
LRR	Leucine Rich Repeat
MALDI-TOF	Matrix Assisted Laser Desorption Ionisation-Time Of Flight
NF-κB	Nuclear factor kappa B
pI	Isoelectric Point
PO	Phenoloxydase
PPAE	Prophenoloxydase Activating Enzyme
PPO	Prophenoloxydase
PRR	Pattern Recognition Receptors
PSH	Persephone

RBC	Red Blood Cells
RFABP	Retinoic and Fatty Acid Binding Protein
RNAi	RNA interference
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
SCP	Sarcoplasmic Calcium-binding Protein
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	SDS-Polyacrylamide Gel Electrophoresis
SPZ	Spaetzle
TEMED	N,N,N',N'-tetramethylethylenediamine
TEP	Thio Ester containing Proteins
TOR	Target of Rapamycin
TSC1/2	Tuberous sclerosis one and two
Vg	Vitellogenin
WB	Western blotting
WHO	World Health Organization

CHAPTER 1

GENERAL INTRODUCTION

1.1 Malaria

Malaria, a term derived from Italian “bad air” (mal’aria), has been known for more than 4000 years. The symptoms of malaria were described in ancient Chinese medical writings dating back 2700 BC (http://www.cdc.gov/malaria/basics.htm). The causative agents of malaria were unknown until 1880 when Charles Leveran, a French military doctor working in Algeria, discovered protozoan parasites of the genus *Plasmodium* in the blood of infected patients. There are four species of *Plasmodium* parasites that cause human malaria. One of these, *P. falciparum,* is responsible for most of the infections and if left untreated can be fatal. Malaria is exclusively transmitted by mosquitoes of the genus *Anopheles*.

Malaria remains the most important tropical parasitic disease. Every year approximately 300 to 500 million cases of malaria infections are reported, resulting in over 1 million deaths due to malaria complications. The majority of the people afflicted by malaria originate from Sub-Saharan Africa (Figure 1). Malaria is a complex disease affecting multiple organs and tissues and takes several clinical presentations. Severe malaria in children or non-immune adults may result in severe anemia (extremely low red blood cells count) or cerebral malaria (deep coma). Pregnant women present a special kind of severe malaria characterized by sequestration of parasitized erythrocytes in the placenta, causing harm to both the mother and the unborn child.

Malaria thrives in tropical regions due to favourable climatic conditions that permit vector breeding. *Anopheles* mosquitoes select small sunlit pools of water to lay their eggs. Clearing tropical forest for agricultural purposes provides optimum conditions and proximity to human hosts that mosquito require to thrive and transmit malaria. Because of the dependence on human/vector contact, malaria is termed “the disease of the poor”. Poor people are often physically marginalised, living closer to degraded land prone to mosquito invasions.

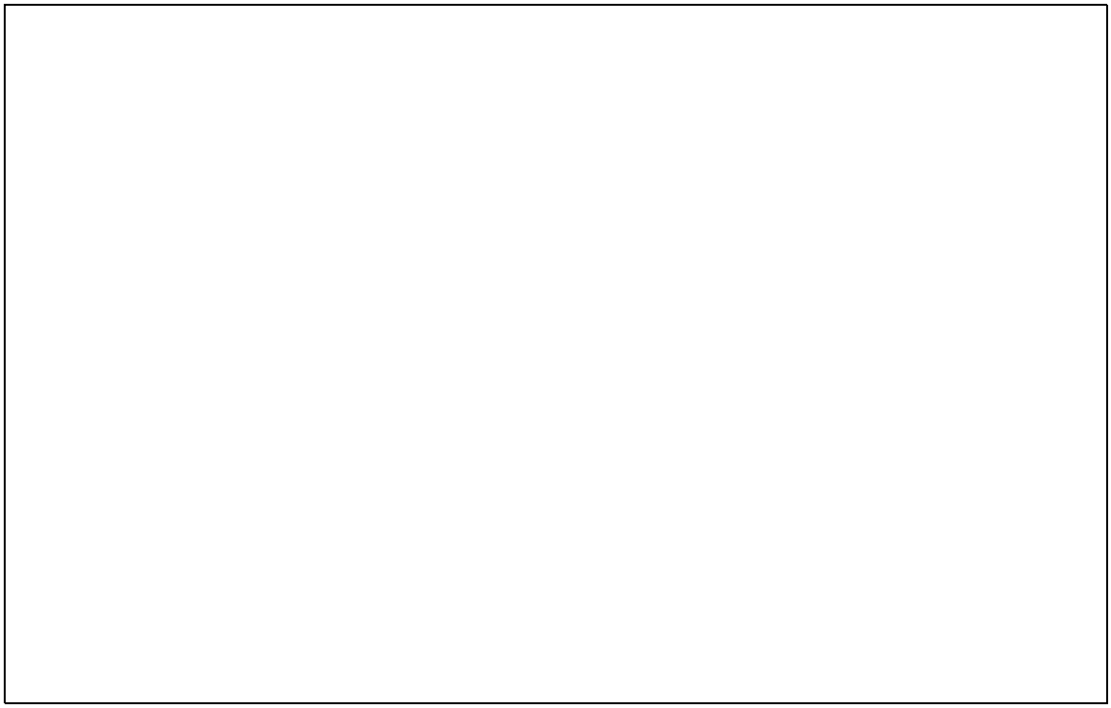


Figure 1. *P. falciparum* Malaria Risk Defined by Annual Parasite Incidence, Temperature, and Aridity Populations at risk in areas defined as having very high (dark green) and low endemicity (light green) (WHO, 2005; Snow et al., 2008)

1.2 Malaria control strategies

The control of malaria has been undertaken through the use of antimalarial agents for the treatment and prevention of the disease and the deployment of insecticides and mosquito nets to kill or prevent mosquitoes from biting humans. For many years chloroquine (CQ), a derivative of quinine, was successfully used for treating uncomplicated malaria while quinine was reserved for complicated malaria. CQ was used with considerable amount of success in terms of treatment, availability and keeping down the costs of treatment. However this is about to change with the ever-emergence and widespread of drug resistant parasites, resulting in malaria treatment failure, not to mention the ability of malaria parasites to exhibit cross-resistance among drugs of the same family. This led to the urgent need for reviewing malaria treatment procedures in many endemic countries (2003). New drugs that target different facets of the parasite life cycle or metabolic processes were introduced where CQ had failed. Most of these drugs were based on a combination of sulphadoxine and pyremithamine (SP), two drugs that interrupt the parasite's folate pathway. The combination of these drugs was expected to achieve better efficacy and prolong the therapeutic life of the drugs. This treatment approach has already

been exploited for the control of highly drug-resistant infectious diseases such as tuberculosis, infection with human immunodeficiency virus, or acquired immunodeficiency syndrome (AIDS) with considerable success. However, mutations in the *P. falciparum dihydrofolate reductase* (dhfr) and in *dihydropteroate synthase* (dhps) genes reduced parasite sensitivity to the antifolates (Plowe et al., 1997).

Currently, the World Health Organization (WHO) recommends the use of artemisinin combination therapies (ACT) for the treatment of uncomplicated malaria in endemic countries (Snow et al., 2008). It is important to note that the increase in drug resistance has severely shortened the useful life of most antimalarial drugs. At this rate we may rapidly exhaust the resources for malaria treatment. This calls for an urgent evaluation of the underlying cause of the observed increase in the rate of *Plasmodium* mutations and resistance to antimalarials. Some of the factors leading to drug resistance could be traced to the widespread use and misuse of antiinfective agents in developing countries. The sale of antimalarial drugs or other therapeutic agents in poorly controlled health care systems, the dumping of obsolete products, intensive marketing, lack of diagnostic facilities and the receptive cultural attitudes to “wonder drugs” such as antibiotics have resulted in unnecessary use of chemotherapeutic agents. These have negatively impacted the control and prevention of malaria. An ideal approach for malaria treatment should be guided by microbiological tests to confirm the type of infection and a good knowledge of the existing pattern of resistance before treatment. Thus there is need for general information concerning malaria treatment, stricter legislation curbing the dumping of obsolete drugs, essential drug lists, national drug policies, better diagnostic facilities, better knowledge about drug beliefs and communication with local healthcare providers (Gundersen,1992). Several “umbrella bodies” serving malaria endemic countries such as the East Africa Network for Monitoring Antimalarial Treatment (EANMAT) have been formed specifically to formulate policies of malaria treatment. Their role is to monitor the prevalence patterns of drug resistance, formulate new treatment regimens and advice member states about the intervention strategies (2003).

The second aspect of malaria management deals with prevention at the vector level. A combination of environmental management and the use of insecticides have greatly reduced the burden of malaria by interrupting disease transmission. In Europe and America malaria was eradicated in a mass campaign involving the use of

residual sprays formulated from dichloro-diphenyl-trichloroethane (DDT). DDT proved very effective against the malaria transmitting mosquitoes, but the benefits of this agent were not extended in Africa due to sustainability and environmental concerns among other reasons. The use of DDT was therefore abandoned until recently, when the fight against malaria faced the resurgence of malaria infections and an increase in both resistant parasites and vectors. In 2006, WHO recommended the use of DDT for indoor residual spraying (IRS), to control the malaria vector in epidemics and in endemic regions. However, this goes against the 2001 Stockholm convention, which targets DDT as one of the persistent organic pollutants to be banned (Sadasivaiah et al., 2007). The use of DDT therefore faces an uncertain future. Other malarial vector control strategies such as personal protection using repellants, insecticide treated materials and fumigants have greatly reduced the disease burden, renewing interest in vector control approaches for the control of malaria. The sequencing of both the *Anopheles* and *Plasmodium* genomes provides a unique opportunity for developing better intervention strategies for disease control. At the moment new perspectives in vector control are emerging from molecular studies on mosquito immunity. Genetically modified mosquitoes have been developed. Their evaluation to replace or suppress existing wild vector populations and reduce transmission hence delivering public health gains are imminent prospects and may offer novel approaches for malaria control (Toure et al., 2004; Blandin et al., 2008; Knols et al., 2007).

An efficient way to control mosquitoes is to find and destroy their breeding sites, thus referred to “source reduction”. This approach is ideal for mosquito control especially when mosquito species targeted concentrate in a few discrete habitats. Therefore source reduction eliminates immature mosquitoes before they reach the stage that is responsible for disease transmission. Larval breeding sites may be destroyed in several ways; by filling depressions that collect water, draining swamps or ditching marshy areas to remove stagnant water. Educating people to remove standing water in used containers, cups and covering water reservoirs, can eliminate container-breeding mosquitoes. However some mosquitoes habitats such as land under irrigation schemes may not be destroyed, thus insecticides may be applied to reduce mosquito breeding. However, due to environmental concerns alternative methods that are less destructive have been preferred. A thin layer of biodegradable oil may be applied on the surface of water, thus suffocating larvae and pupae. Biolarvicides such as bacterial toxins from *Bacillus thurigiensis var. israeliensis* (BTi) and *Bacillus sphaericus* can be applied just as other chemical insecticides to aquatic

developmental stages of mosquitoes (Das and Amalraj, 1997). Larvivorous fishes such as *Gambusia* and *Poecilia* (Tilak et al., 2007) or predacious copepods of the genus *Mesocyclops* (Kay and Vu, 2005) may also be used to deter mosquito breeding. Other potential biological agents for vector control include fungi and nematodes (Mohanty et al., 2008; de Valdez, 2006)

1.3 The malaria parasite life cycle

Plasmodium parasites require two hosts, usually a vertebrate and an insect vector - to complete their development cycle. In humans the predominant stages involve the asexual forms, while the sexual stages develop in the mosquito. A good knowledge of the parasite biology and developmental cycle, would be essential in formulating new control measures such as novel chemotherapeutic agents for treatment and prophylaxis of human malaria as well as new strategies for transmission blocking.

Asexual life cycle of parasite (human host)

Malaria infection starts when female mosquito injects sporozoites into the dermis of a host. Majority of the sporozoites migrate to the liver via the blood stream and invade hepatocytes (Yamauchi et al., 2007; Amino et al., 2006). However, some sporozoites do not invade liver cells but enter lymph nodes draining the site of infection, where they are internalized by dendritic cells, with some initiating development (Amino et al., 2006, 2008). Sporozoites have been observed to traverse several liver cells before settling into a final hepatocyte, where they form a parasitophorus vacuole (pv) (Amino et al., 2008). Here the parasites multiply and differentiates into thousands of liver stage merozoites that are released into the blood stream upon maturity. Merozoites will then invade red blood cells (RBC's) and form a parasitophoruos vacuole within which the parasite hijacks the proteins of a host cell and sets up its own system for nutrient transport and protection. The parasite replicates and matures into schizonts, rupture and release thousands of merozoites that can infect new RBC's. The re-invasion of blood cells by newly emerged merozoites can occur repeatedly over several times leading to massive destruction of RBCs, marked by the periodic malaria fevers every 48 h.

In order to invade RBC's, the merozoites use surface proteins such as erythrocyte-binding antigen (EBA175) or proteins with Duffy binding-like (DBL) domains that facilitate the interaction with host cells. These interactions are currently being explored for vaccine development. During each amplification cycle, a small proportion of merozoites commit to differentiate into male and female gametocytes.

These sexual stages precede the next phase of the parasite cycle in the mosquito (Figure 2).

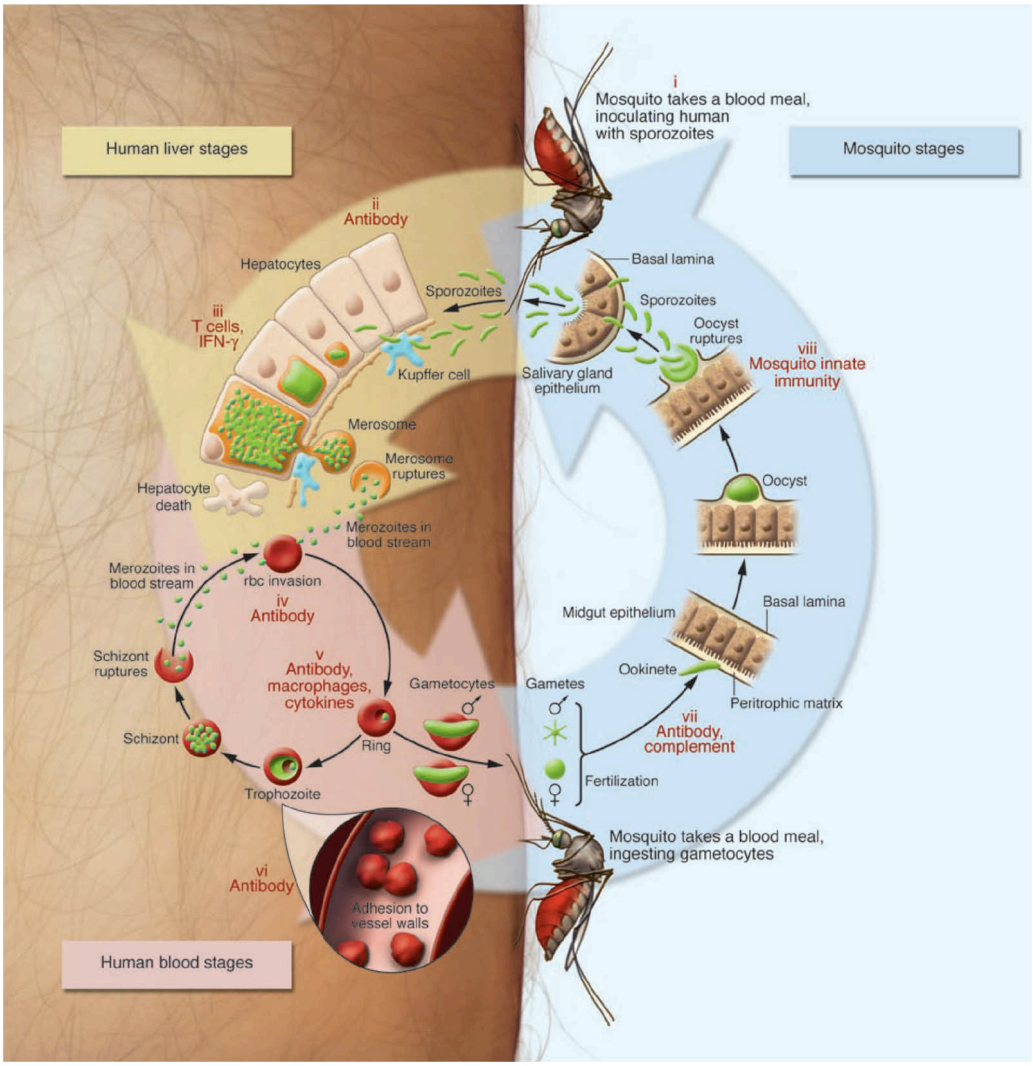


Figure 2. The life cycle of malaria-causing *Plasmodium* parasites.

During a blood meal female mosquitoes ingests male and female gametocytes that transform within minutes into gametes. Gametes fuse to form a zygote, the zygote undergoes transformations forming motile ookinetes that invade and traverse the midgut to settle in the basal side. Here the ookinetes transform into an oocyst, which develops by undergoing several rounds of mitotic divisions, 1-2 weeks later mature oocysts rupture, releasing several thousands of sporozoites. Sporozoites invade salivary glands where they mature and are ready to infect the next vertebrate host. Once injected into the vertebrate dermis, sporozoites invade hepatocytes where they multiply and undergo differentiation leading to the release of thousands of liver stage merozoites, which infect red blood cells initiating erythrocytic cycle. Some of the merozoites commit to forming gametocytes and when taken up by a mosquito during a blood meal complete the development cycle. Thus, the *Plasmodium* development cycle is marked by various transitions and stages, which could potentially be targeted for antimalarial drugs or vaccine development (Greenwood et al., 2008)

Sexual cycle of parasite development (vector)

After a mosquito has ingested gametocytes during a blood meal, a drop in temperature, the presence of xanthurenic acid and other factors in the mosquito

midgut triggers the exflagellation of male gametocytes. This is followed by fusion of male and female gametocyte to form a non-motile zygote. The zygote transforms into a motile ookinete, which penetrates and traverses the midgut epithelium and settles on the basal side. Upon reaching the basal side of the midgut, ookinetes change into oocysts and undergo several rounds of mitotic divisions. The mature oocyst ruptures releasing thousands of sporozoites, which invade salivary glands, mature and become ready to be inoculated into a new human host during a subsequent blood meal, therefore perpetuating the malaria life cycle (Mies et al., 1983, reviewed in Sinden, 1999).

1.4 Mosquito regulates parasite infection

Parasite development in mosquitoes experiences major hurdles, marked with massive losses of parasites in numbers during three transition stages: between gametocytes and ookinetes, ookinetes and mature oocysts, and between midgut and salivary gland sporozoites (Alavi et al., 2003, reviewed in Blandin and Levashina, 2004, reviewed in Sinden, 1999). Naturally the vector may attempt to block competition for resources with the parasite as well as avoid suffering from tissue damage caused by the parasite's journey through various tissues. Thus, the mosquito is implicated in the reduction of parasites numbers during ookinete transition to mature oocyst and between midgut and salivary gland sporozoites. These stages are marked by host cell invasion by the parasites and may be detrimental to the host survival. Indeed, mosquito responds to *Plasmodium* infection by an elaborate immune response to oppose parasite infection. To understand the mosquito immune response much can be learned from comparison with *Drosophila*, the fruit fly whose immune response towards pathogens has been characterized extensively and used as a model system for studying other organisms.

Drosophila immune response

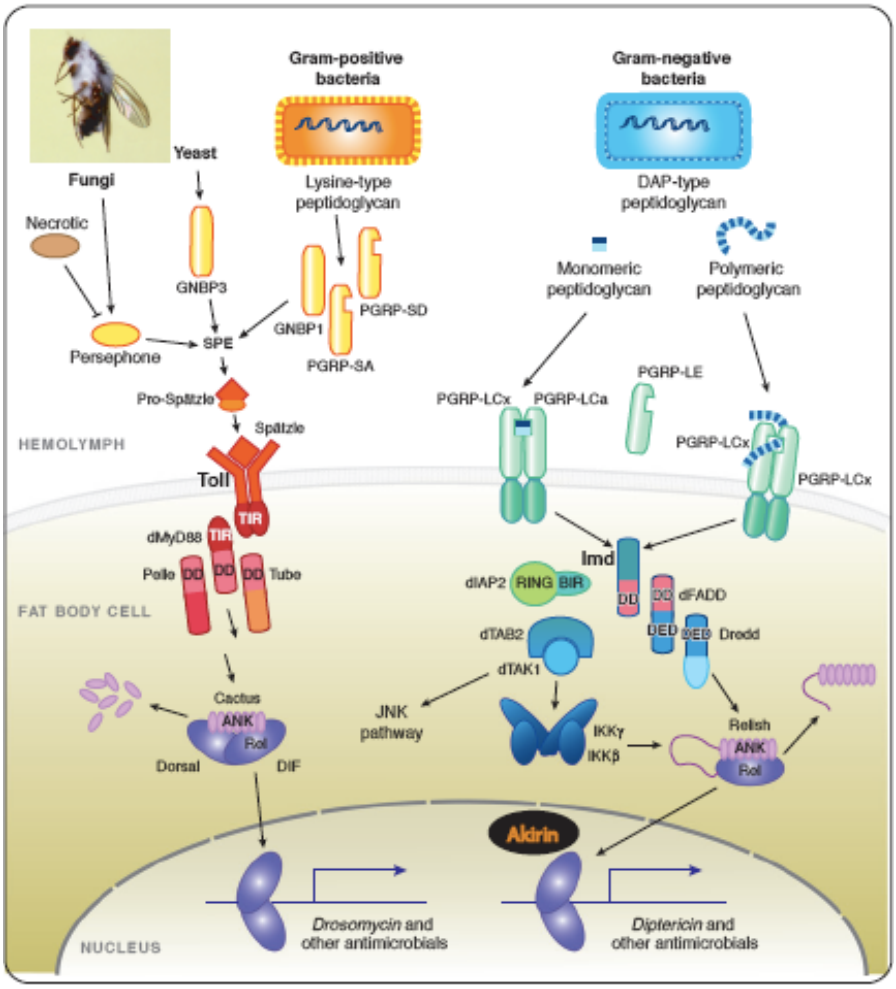
Drosophila, like other insects, is able to mount efficient responses to inhibit microbial infections. The basis of these antimicrobial responses resembles the vertebrate innate immunity, and can be divided into cellular and humoral reactions (reviewed in Hoffmann, 2003).

The cellular response in adults involves specialized blood cells called plasmatocytes, lamellocytes and crystal cells, and in larvae plasmatocytes (reviewed in Crozatier and Meister, 2007). The primary role of plasmatocytes is to phagocytose foreign particles such as invading bacteria and fungi (Ramet *et al*., 2001; Kocks *et al*., 2005);

on the other hand lamellocytes (only found in larval stages) form a multilayered capsule around pathogens too big to be phagocytosed (such as parasitoid wasp eggs). The capsule is melanized through the prophenoloxidase activity associated with crystals cells (Braun et al., 1998, Ashida and Brey, 1997).

The basis of humoral reactions is the induction of antimicrobial peptides in the fat body after pathogen challenge. Seven types of antimicrobial peptides (AMPs) have been identified. Infection by Gram-positive bacteria elicits the synthesis production of defensin, fungi drosomycins and metchnikowin; whereas Gram-negative bacteria induce the synthesis of attacins, drosocin, diptericins and cecropins (reviewed in Hoffmann, 2003, and in Ferrandon et al., 2007). The expression of AMPs depends on two *Drosophila* members of nuclear factor kB (NF-kB) family: Dif (dorsal-related immunity factor) and Relish. The activation of Dorsal and Relish by bacteria and fungi occurs through two distinct signaling cascades, namely the Toll and immune deficiency (Imd) pathways. Both Gram-positive bacteria and/or fungi preferentially induce Toll pathway, Imd by Gram-negative bacteria (reviewed in Hoffman 2003 and in Ferrandon et al., 2007).

Toll signaling involves several factors that were initially discovered to control dorsal ventral patterning in embryos (Anderson and Nusslein-Volhard, 1984) and later shown to be involved with immune response in adult flies (Lemaitre et al., 1996). Toll signaling shows similarities to mammalian signaling downstream the interleukin receptor (IL-1R) and the toll-like receptors (TLRs) pathways. The activation of Toll is dependant on the proteolytic cleavage and binding of spaetzle to the ectodomain of Toll. This initiates intracytoplasmic signaling through three adaptor proteins (MyD88,Tube and Pelle*)* that culminates in the phosphorylation and degradation of IkB (Cactus). In the absence of Cactus, Dif translocates to the nucleus and initiates the synthesis of antimicrobial peptides such as Drosomycin (Figure 3).



modified from Lemaitre and Hoffmann, 2007

Figure 3. Drosophila Toll and IMD pathways

Toll pathway: Gram (+) bacteria and fungi induce the cleavage of Spaetzle, mature spaetzle binds to and activates Toll, This leads to the recruitment of the adaptor proteins dMyD88, Tube and Pelle, initiating a cascade of activities that culminates in the phosphorylation and degradation of Cactus (IkB), releasing NF-kB factor DIF. DIF translocates to the nucleus to initiate the synthesis of antimicrobial peptides such as Drosomycin. IMD pathway: The binding of peptidoglycan from Gram (-) bacteria to PGRP-LC, leads to the recruitment of adaptor protein Imd. Imd interacts with dFADD which binds to Dredd through the death domains, initiating a series of enzymatic activities that leads to the release of Relish from its inhibitory ankyrin repeats sequence. Relish translocates to the nucleus initiating the synthesis of antimicrobial peptides such as Diptericin. Alternatively, downstream of dTAK1, Imd pathway may branch to the JNK pathway leading to the synthesis of cystoskeletal genes.

Imd is primarily involved in the defense against Gram-negative bacterial and is similar to tumor necrosis factor receptor (TNFR) in mammals. Imd is activated via PGRP-LC, which in addition to IMD interacts with adaptor proteins, FADD and DREDD. This activation leads to the degradation of ankyrin repeats of Relish and to the translocation of Relish into the nucleus and initiate the transcription of antimicrobial peptides. The mechanism resulting in the release of Relish from its

inhibitory sequences is not fully understood. Imd pathway downstream of TAK1 branches into two pathways: (i) Relish mediated synthesis of AMPs and (ii) the JNK (c-Jun N-terminal kinase) pathway expression of cytoskeleton genes. Thus, It has been suggested that Imd pathway may coordinate host defense response by the Relish arm (antimicrobial synthesis) and JNK arm (wound healing) (reviewed in Hoffmann, 2003).

Immune response in mosquitoes

Comparative bio-informatics analysis of the *Drosophila, Anopheles* and *Aedes* mosquito genomes has revealed significant divergences as well as conserved features in the repertoire of recognition and effector molecules, probably reflecting adaptation to specific environmental requirements imposed by the distinct modes of life of each insect species (Waterhouse et al., 2007).

In *Drosophila*, two major signaling pathways, namely Toll and Imd, which employ NF-kB transcription factors, control antimicrobial responses. Some members of these pathways have been identified in the mosquito genome. The activation of Toll pathway in *Drosophila* is mediated by the binding of a cleaved ligand, Spaetzle, to the extracellular domain of Toll. It is yet, to be established how this pathway is activated in *A. gambiae* since Spaetzle homologs have not been identified even though three paralogs have been characterized in the *Aedes* genome (Waterhouse et al., 2007, Levashina, 2004). However, several known *Drosophila* immunity gene families including thioester containing proteins (TEPs), antimicrobial peptides, prophenoloxidase (PPOs), clip domain serine protease (CLIPs) and serine protease inhibitors (SRPNs) have been identified in the mosquito genome (Waterhouse et al., 2007, Christophides et al., 2002). Therefore mosquitoes may use similar strategies observed in the fruit flies to respond to microbial infections.

Antimicrobial peptides in mosquitoes

Seven antimicrobial peptide families exist in *Drosophila* but only three have been identified in the mosquito genome: Cecropins, Defensins and Gambicins. Therefore Drosomycin, Diptericin, Metchnikowin, Dosocin and attacins seem to be specific to *Drosophila*, where as Gambicins are only found in the mosquitoes. In *A. gambiae* Gambicin codes for 81 amino acid (aa) protein, which is processed to a 61-aa mature peptide containing eight cysteines forming four disulfide bridges. Gambicins (mature peptide) have been shown to contain bacterialcidal and morphogenic effect against filamentous fungus (Vizioli et al., 2001).

Cecropins have been identified in both insects and mammals (reviewed in Boman et al., 1991), and show broad spectrum of activity against Gram-negative and Gram-positive bacteria as well as some fungi (Vizioli et al., 2000). In the *Anopheles* mosquito, four cecropins have been identified, with several homologs found in *Aedes* (Waterhouse et al., 2007).

Defensins are small cationic peptides, four members of this family have been identified in *A. gambiae*. *In vitro* activity of Defensin 1 has been established to be directed against Gram-positive bacteria, ookinete, although it does not seem to affect *Plasmodium* development in mosquitoes (Richman et al., 1997). Interestingly the expression of this gene is up regulated by parasite infection (Richman et al., 1997).

Cellular Responses

Cellular defense involves melanization and phagocytosis of pathogens. Two types of melanization responses have been described in insects: formation of a hemocytic capsule around pathogens that is subsequently melanized by PPOs (Gotz, 1986) and cell-free humoral encapsulation, involving the deposition of a proteinatious capsule around the invading microorganism (reviewed in Dimopoulos, 2003). In mosquitoes, the formation of a melanotic capsule around parasites involves cell-free humoral reactions mediated by the PPO activity (reviewed in Dimopoulos, 2003). Phagocytosis involves the uptake and degradation of microorganism by hemocytes. Phagocytosis is mediated by pattern recognition receptors (PRR), that can bind microorganism surfaces and trigger intracellular cascades that lead to their internalization (Aderem and Underhill, 1999). Phagocytosis has been shown to be mediated by TEP1, acting as an opsonin. TEP1 binds to the surface of bacteria and parasites and activates two distinct types of immune responses: phagocytosis of bacteria and parasite killing via lysis, followed by actin polymerization and melanization in the refractory strain (reviewed in Blandin et al., 2008)

Mosquito immune responses to *Plasmodium* infection

The life cycle of the malaria parasites in mosquito involves several developmental transformations and translocations through mosquito tissues (reviewed in Dimopoulos, 2003). During these transitions parasites undergo several bottlenecks marked by massive reduction in numbers of parasites. In some refractory strains of *A. gambiae*, parasite development is completely blocked (Vernick et al., 1995, Collins et al., 1986), therefore, showing mosquitoes are able to oppose parasite infection.

The basis of the antiparasitic response has been under intense investigations. At the moment, several genes that affect the outcome of parasite development especially at the ookinete stage have been identified and can be grouped according to their knock down phenotypes on parasite survival. The first group of proteins include the Thioester containing Protein1 (TEP1) homologous to vertebrate complement factor C3 (Baxter et al., 2007, Levashina et al., 2001) and two Leucine Rich Repeat genes (LRR) LRIM1 and APL1, which are the key antiparasitic molecules that mediate parasite killing in a complement-like manner, involving lysis and melanization of dead parasites (Blandin et al., 2004, 2008, Osta et al., 2004. Riehle et al., 2006). Furthermore, the depletion of TEP1 in a refractory strain of *A. gambiae* is sufficient to convert refractory mosquitoes to be permissive to *P. berghei* infections (Frolet et al., 2006). Interestingly, TEP1 activity is not limited to malaria vector strains of *Anopheles gambiae* but equally observed in a non-vector species such as *Anopheles quadriannulatus*. The silencing of TEP1 and or the LRR proteins (LRIM1 and APL1) converts *A quadriannulatus* to a competent vector of *P. berghei* (Habtewold, 2008), therefore, showing the importance of TEP1 in mosquito antiparasitic responses including refractoriness to parasite infection.

TEP1 is constitutively present in the mosquito hemolymph but its expression is further induced by *Plasmodium* infection. TEP1 is cleaved, binds to the parasite surface in a thioester-dependent manner initiating a series of events that lead to parasite lysis in susceptible mosquitoes. TEP1 acts as an opsonin facilitating the phagocytosis of some Gram-negative bacteria (Levashina et al., 2001) a conserved function of most thioester-containing proteins (reviewed in Dodds and Law, 1998). Recently the crystal structure of the refractory allele TEP1r was resolved. This revealed the similarities between TEP1 and the vertebrate complement component C3 (Baxter et al., 2007). TEP1 shares 31% sequence similarity with C3 within the thioester-containing domain (TED), but lacks the anaphylatoxin and C345C domains present in C3. The crystal structure also suggests that the TEP1r protease-sensitive region is more accessible to proteases than in C3. This implies that TEP1r cleavage may not require specific convertases as for C3, an idea consistent with the presence of multiple cleavage sites for diverse proteases including trypsin, chymotrypsin and thermolysin (Baxter et al., 2007, Blandin et al., 2004), Thus TEP1 may be cleaved by an endogenous protease that may be set free by injury of by protease of pathogen origin (Levashina et al., 2001).

The second class of proteins includes those whose knock down phenotypes lead to reduced parasite survival, a phenotype opposite to that of TEP1, which implies that they may be involved in the negative regulation of mosquito antiparasitic responses. Osta et al., (2004) showed that the depletion of two C-type lectins: CTL4 and CTLMA2 leads to a reduction in parasite numbers in the midgut. Likewise, the silencing of lipophorin (Lp), the insect lipid transport molecule by dsRNA compromises parasite development as well as blocking oogenesis (Vlachou et al., 2005). However, it is not known whether parasites die due to starvation in the absence of the nutrient transporter or enhanced TEP1 activity. It is possible that lipophorin may impinge on TEP1 activity through the formation of immune complexes that may negatively regulate TEP1 activity. Regulatory factors that control prophenoloxidase activity and coagulation reactions that inactivate bacteria toxins in *Lepidoptera* have been associated to Lp (Rahman et al., 2007), in mosquitoes the knock down of Lp leads to reduced melanization of parasites in refractory mosquitoes (Mendes et al., 2008). On the other hand the depletion SRPN 2 or SRPN 6 increases the deposition of melanin in the mosquito tissues and negatively imparts on parasite development in the midgut (Michel et al., 2005; Abraham et al., 2005). All put together, mosquitoes are able to sense and respond to *Plasmodium* infection by inducing the expression of antiparasitic molecules such as TEP1 to limit parasite infection. However, the complete pathway of parasite killing mediated by TEP1 is yet to be elucidated with a view of identifying the missing links such as the pattern recognition event and parasite-killing effecter molecules.

1.5 Molecular-Genetic tools for studying *Plasmodium-mosquito interactions*

The malaria parasite has a complex life cycle revolving between a vertebrate host and the mosquito. The knowledge of parasite-host interactions is an essential step towards developing new control strategies for malaria treatment, vaccine development or transmission blocking (Alavi et al., 2003). Vector control has currently gained new interest with the aim of establishing novel control strategies such as establishing mosquitoes refractory to human malaria (Alphey, 2002). In order to establish refractory mosquitoes, first we need to understand interactions between the vector and parasites that lead to refractoriness or susceptibility to *Plasmodium* infection, similarly it is important to locate the site and time of parasite killing. However, quantitative measurements of parasite development *in vivo* were previously limited by imaging techniques until recently, when molecular tools that

permit the modification, disruption and introduction of transgenes were made available.

Green fluorescent protein (GFP)-expressing *P. berghei* (Franke-Fayard et al., 2004; Amino et al., 2008) have been developed and are now widely used to study host parasite-interactions both *in vivo* (Amino et al., 2008) and *in vitro* (Prudencio et al., 2008). Furthermore, parasite motility can now be investigated using GFP parasites on a Matrigel-based *in vitro* system. Therefore, giving insight into how mosquito tissues such as salivary glands are invaded by parasites, with a potential to develop strategies for blocking invasion, hence limiting the success of malaria transmission (Akaki and Dvorak, 2005).

TEP1 plays a central role in mosquito antiparasitic responses. In order to understand further the TEP1-mediated parasite killing mechanism, it is essential to identify molecules that may be involved in the pathway. Therefore, it may be envisaged that artificially inducing or repressing TEP1 expression will affect molecules whose expression are regulated by TEP1 and may be involved in the antiparasitc reponses. Changes in gene expression or protein content following these perturbations can then be analyzed using standard techniques such as microarray or proteomic analysis to identify potential candidates for further analysis. Thus, two transgenic mosquitoes lines were developed using the *piggyBac* transposon–mediated gene transfer. In the first mosquito line the construct contained *TEP1* under the control of *Drosophila* heat shock protein 70 (HSP70) promoter and *dsRED* as the selection marker under the control of Pax3 promoter. This led to elevated levels of *TEP1* expression compared to wild type mosquitoes hence referred to *TEP1 gain-of-function*. In the second line a similar construct was used but, the transgene lead to diminished expression of TEP1 thus referred hereafter as *TEP1 loss-of-function*. However, the exact silencing mechanism affecting TEP1 in this line is not fully understood. The diminished expression of TEP1 may be through RNA interference mediated by production of *TEP1*-antisense mRNA due to the reverse orientation of *TEP1* with respect to the ornithine promoter at the insertion site (Blandin S., E. Levashina et al., unpublished work). Microarray analysis on the *TEP1* transgenic mosquito lines is currently being exploited in order to investigate the mosquito antiparasitic response. In this work we intended to complement the microarray data by proteomic analysis in order to gain more insight into the parasite killing mechanisms with a keen interest of establishing the effector molecules involved.

Proteomics complements transcriptional analysis to investigate vector-parasite interactions

The expression of genes is tightly regulated depending on the cell state at a particular time. Thus, careful analysis of the changes in gene expression that manifest in the message (messenger RNA) or product (protein), can reflect the differences between cellular states (Cox and Mann, 2007). Such changes in gene expression could be due to infection, stress, growth or development. Historically, molecular biologists performed on a "one gene one experiment" basis to determine changes in gene expression, which resulted in time consuming, low throughput experiments in which the "whole picture" of gene function was hard to obtain despite the immense information about individual genes. The advent of DNA microarrays and proteomic analysis has paved the way to whole-genome analysis also called "the factory approach" giving more information on biological processes.

The first genome-wide expression analysis involved the hybridization of mRNA on complementary sequences immobilized on a chip. However despite the possibility to cover the whole transcription repertoire, microarray analysis falls short of precisely predicting the level of proteins which are in essence the effectors of most metabolic and regulatory processes for a cell's survival (Gygi et al., 1999, Cox et al., 2005). Protein levels not only depend on the mRNA levels but on a host of translational controls and regulated degradation (Li et al., 2007), direct measurements of which cannot be performed by transcriptional analysis. Thus, proteomic analyses should complement microarray analyses.

Furthermore, the existence of open reading frames (ORF) in genomic sequences does not mean the existence of a functional gene. Although the sequencing of other related organisms can be used in comparative analyses to predict genes, the accuracy of such methods is still low (Pandey and Mann, 2000). Moreover, fast evolving genes, or genes without a model for comparison may be missed by such methods. In addition, post-translational modifications such as oxidation glycosylation and phosphorylation of proteins, which determine protein activity, cannot be assessed by genomic analysis, justifying that proteomics should complement genomics.

Proteomics analysis

The term "proteome" refers to the entire protein complement of a genome, cell, tissue or organism (Wasinger et al., 1995). The study of this protein complement is thus

referred to Proteomics (Ong and Mann, 2005). Proteins serve diverse functions in the cells ranging from transport, transcription regulation, signal transduction, defense, cell-to-cell communication, growth and development, and driving metabolic processes. Perturbations in such activities may lead to toxicological or disease events. Proteins involved in these events can be identified by comparing two different cell states such as diseased and healthy individuals or treated versus untreated. The identified proteins can then be explored as novel targets for vaccine and drug development (Fried et al., 1998, Pandey and Mann, 2000), as well as biological markers for diagnostic kits.

Mass spectrometry based proteomics

Mass spectrometry (MS) is a venerable technique (whose use dates to the early days of the last century). MS measures an intrinsic property of a molecule (mass) with high precision and sensitivity hence gaining a wide range of applications. However, MS gained popularity in biological sciences recently mainly because mass spectrometers require ionized gaseous molecules for analysis. Most bio-molecules (such as proteins) are large and polar, however they are difficult to convert into charged gaseous state, therefore limiting the application of MS. Electron-spray-ionization (ESI) and matrix-assisted-laser-desorption-ionization (MALDI) are two techniques developed in the late 1980's that revolutionalized MS analysis in life sciences. Several algorithms were also developed at the same time that permitted the correlation between mass spectrum of a protein with protein databases.

MALDI and peptide-mass fingerprinting

Peptide mass fingerprinting is one of the two main techniques used in mass spectrometry for protein identification initially suggested by Henzel et al., (1993). In this technique the mass spectrum of eluted peptides is acquired and used for protein identification. Trypsin is used to digest peptides, because it cleaves the protein backbone at arginine and lysine residues. This generates peptide fragments whose masses can be predicted theoretically for any entry in a protein database. The predicted peptide masses are compared to those experimentally determined by MALDI analysis. A protein can then be identified if there are sufficient corresponding matches between those in the database, giving a high statistical score using special algorithms, such as mascot search, designed for the comparisons. High scores are required for unambiguous protein identification avoiding false positive. Thus the success rate of this approach is enhanced with the presence of all the predicted protein sequences in the database (Pandey and Mann, 2000). However, it is difficult to estimate the specificity of gene prediction in a less mature genome annotation

such as *A. gambiae* which lacks well annotated reference genome regions. By using full-length cDNA, the Ensembl *A. gambiae* genome was predicted to be 99% specific but with low coverage 37% due to under prediction associated with comparative gene prediction model used (Li et al., 2006).

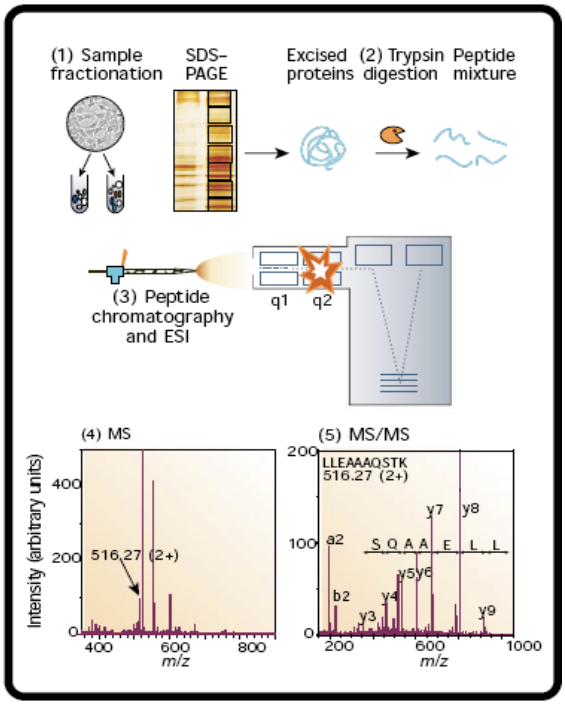
The matrix used in MALDI is normally derived from small organic molecules that absorb at the wavelength of the laser; two types of matrices are constantly used in proteomics namely: alpha-4-cyanohydroxycinnamic acid (alpha-cyano) and dihydrobenzoic acid (DHB). The choice of matrix depends on the desired amount of fragmentation, for example alpha-cyano achieves the highest amount of fragmentation (high sensitivity) but only lasting for a shorter time (microseconds) therefore, used in MALDI-based mass spectrometers, while mass spectrometers based on time-of-flight (TOF) require long lasting fragmented ions therefore, use DHB matrices, produce ions with long half-lives (milliseconds) (Mann et al., 2001). The ionization mechanism involved in MALDI MS steal remains unclear, but the signal intensity depends on a number of factors including, incorporation of peptide into crystal and the possibility of the analyte to capture a proton and retaining it during desorption. Therefore, it is difficult to correlate sample quantity to signal intensity, moreover mass range below 500 daltons (DA) is usually obscured by matrix related ions thus MALDI analysis is limited to peptide identification.

Electrospray ionization (ESI) and tandem mass spectrometry

There are two major mass spectrometric strategies that apply ESI approach. In the one method, the liquid carrying the analyte is applied to a low-flow device called a nano-electrospray (Wilm and Mann, 1996), which has a small aperture, just big enough to disperse the mixture as aerosol into the mass spectrometer. The liquid evaporates rapidly imparting its charge on the analyte molecules without fragmentation occurring. Individual peptides from the mixture are isolated in the first step and fragmented in the second step to obtain the sequence information of the peptides (thus tandem mass spectrometry). The second strategy utilizes a liquid chromatography to separate peptides followed by sequencing as they elute into the electrospray ion source. It is possible to bypass protein separation by gel electrophoresis since the protein mixture can be digested in solution and analysed together. Theoretically each protein in the sample is identified by several peptide hits generated from the sequencing event.

Searching protein database using tandem mass spectrometry

Peptide fragmentation is achieved through collision with gas molecules in the mass spectrometer. The derived fragments are spaced by a difference of the mass of one amino acid, which reveals the identity and location of that amino acid in the peptide. In principle only two such amino acids of known location within a peptide (sequence tag) are required for sufficient peptide identification in a large sequence database (Mann and Wilm, 1994). Thus complex protein mixtures can be analyzed with high sensitivity and specificity (at picomole range) and the corresponding data searched against expressed sequence tag (ESTs) or genomic database. Advances in tandem mass spectrometry have improved the sensitivity and dynamic range of protein identification in fairly complex mixtures. To date, some of the largest (high-throughput) proteomic studies ever undertaken, such as the yeast and *Plasmodium* proteomes, owe their credits to tandem mass spectrometry (Washburn et al., 2001)



Modified from Aebersold and Mann, 2003

Figure 4. Mass spectrometry based proteomics

Complex protein mixtures are fractionated according to solubility and molecular size using SDS PAGE. Protein bands of interest are excised, trypsin digested and analyzed by a tandem mass spectrometer coupled to liquid chromatography. Ionization of peptides is achieved by ESI.

Quantitative proteomics

Apart from protein identification, quantitative analysis of protein expression can be achieved from relatively small amounts of sample or without performing the gel-based protein separation before analyzing the samples by a mass spectrometer. Proteins from one or two states are labeled chemically or metabolically with either heavy or light isotopes, followed by mixing of samples before mass spectrometry analysis. Two versions of a given peptide with a mass difference corresponding to

the isotope used are compared by peak ratios of the light and heavy isotopes (reviewed in Steen and Pandey, 2002, Henzel et al., 1993)

The signal generated by MS for any given peptide is determined by many factors, but most importantly the ease to form ions in an electrospray. Therefore, direct quantification of proteins using standard non-gel based mass spectrometry approaches is difficult. However, there is a general correlation between the number of peptides sequenced per protein and the amount of protein present in the sample (Rappsilber et al., 2002). Thus, a protein abundance index (PAI) was developed relating the number of peptides sequenced to the number of observable peptides predicted *in silico*. Proteomic analysis on the human spliceosome showed that PAI values obtained at different concentrations of serum albumin exhibited a linear relationship with the logarithm of protein concentration in tandem mass spectrometry experiments. In order to perform absolute quantification, PAI was thus converted to exponentially modified PAI (emPAI), which is proportional to protein content in a protein mixture (Ishihama et al., 2005). Therefore, differential protein expression analysis can be performed within different states by using PAI or emPAI values.

Protein interactions

In addtion to accurately determining when and where a protein is expressed, a key question is with which other proteins it interacts. Protein-protein interactions mediate cellular functions and responses such as signal transduction, immune and stress responses. These protein interactions could be transient as observed in protein kinases or form long-lived complexes such as the lipid transport system (Harjes et al., 2006, Link et al., 2005, Pandey and Mann, 2000)

The best way to study protein-protein interaction is to purify proteins when they are interacting in their native form. There are several methods available including pulldowns using glutathione-S transferase (GST) fusion proteins, antibodies, DNA, RNA or small proteins with affinity to cellular targets with varying degrees in sensitivity and specificity. But the gold standard assay remains co-immunoprecipitation performed on endogenous proteins (Bonifacino et al., 1995). The protein of interest is isolated together with its interacting partners using specific antibodies. The co-precipitated proteins are then detected using immunoblotting analysis or mass spectrometry based proteomic approaches. Interacting partners can also be purified by unbiased approaches such as biochemical fractionations of protein complexes by density gradients (Link et al., 2005). Schal et al (2001) used

both KBr density gradient and co-immunoprecipitation to show that lipophorins were implicated in the transport of hydrocarbons and sex pheromone in the house fly, *Musca domestica*.

1.6 Proteomic studies in insects

The sequencing of the *A. gambiae* genome, together with published data from other related insects genomes, have generated sequence databases which can be exploited for large scale protein expression analysis. Such studies can be designed to investigate the interaction between the malaria vector and *Plasmodium* parasites, potentially inspiring new approaches for malaria intervention.

A wealth of information has been made available concerning the fruit fly immune response using genetic studies (reviewed in Hoffmann, 2003 and in Ferrandon et al., 2007), however several aspects still remained to be established, for instance the identity of immune molecules recruited in the hemolymph to fight microbial infections. In order to complement the data obtained from the genetic studies and gain more insight into the fruit fly immune response to pathogens, Levy et al., (2004a) investigated changes in the fruit fly hemolymph proteome upon different microbial infections. In one approach a differential proteomic analysis was performed using 2D-gel in order to detect changes in proteins of molecular weight (>15KDa). Infection with filamentous fungi elicited more changes in the hemolymph compared to either Gram (+) or Gram (-) bacterial infections. In addition there was only a small overlap between the proteins identified from various types of immune challenges. Some of the proteins identified belonged to known immune factors such as proteases, pattern recognition molecules, prophenoloxidases, serpins and thioester containing proteins (TEPs); in addition several other proteins including odorant binding proteins, proteins involved in iron metabolism were differentially regulated by infection. In the second approach the analysis of *Drosophila* immune-induced molecules (DIMs) was investigated by a non-gel protein analysis, in which samples are directly loaded to a mass spectrometry via a liquid chromatography referred to peptidomic approach. Several infection-induced molecules were identified including known antimicrobial peptides such as drosocin, defensin and cecropin as well as novel DIMs that may be involved in Drosophila immune responses to microbial infections (Levy et al., 2004b).

Proteomic analysis of mosquito response to microbial infections

Mosquito immune response to microbial infections including human malaria (Gorman et al., 2000, Rodriguez et al., 2007, Mendes et al., 2008) has been extensively

characterized using transcriptional data and explored by reverse genetic approaches involving gene disruption to identify mosquito factors that affect parasite development. It has been shown that TEP1 is the key antiparasitic molecule that kills parasites in a complement-like manner (Blandin et al., 2004), however some aspects of this pathway such as effector molecules involved downstream TEP1 are yet to be identified. Furthermore, it has been shown that most of the antiparasitic molecules are secreted by hemocytes but, the knowledge of how molecules are recruited on the basal labyrinth of the midgut, where parasite killing occurs is yet to be established. Such questions may be difficult to address using transcriptional approaches. In addition, it is widely accepted that transcriptional analysis has poor correlation with proteomics (Gygi, 1999), since it does not depict posttranslational changes occurring in proteins that may determine their activity, localization and half-life, which will greatly influence their turnover. Shi and Paskewitz (2004) performed a peptidomic analysis on mosquito hemolymph and established that two chitinase-like proteins AgBR1 and AgBR2 were induced shortly after exposure to bacteria or peptidoglycan. Proteins from a closely related family such as *Drosophila* Ds47 have been shown to promote cell proliferation and regulate migration of immune cells (Recklies et al., 2002), thus suggesting that AgBR1 and BR2 may be involved with mosquito immune response and need to be investigated further.

The mosquito midgut has been shown to be a crucial organ that plays a major role in determining parasite development and vectorial capacity. Transcriptional data has shown that both male and female midguts show similar gene expression profile in the absence of a blood meal, however upon feeding the female midgut undergoes changes in gene expression in part attributed to its hematophagous nature, but most importantly distinct changes were observed in different compartments. For example, the cardia was indicated as a major site for the synthesis of antimicrobial peptides. Following these observations, peptidomic analysis was performed on the cardia and identified 10 secreted peptides, among them three known antimicrobial peptides cecropin, defensin1 and gambicin as well as lysozymes (Warr et al., 2007). Defensin 1 was shown to be induced in the hemolymph after bacterial challenge and 24h after parasite infection (Richman et al.,1997), however the depletion of this gene was shown not to affect parasite development. These results suggest that defensin 1 may not be one of the determinants of vectorial competence (reviewed in Levashina, 2004). *Plasmodium* infection has equally been shown to induce changes in the mosquito head proteome, which may potentially regulate mosquito behavior such as

host seeking for blood meal (Lefevre et al., 2007) and possibly ensure the success of malaria transmission.

Hemolymph, which is equivalent to blood in higher organisms, is the fluid responsible for the supply of nutrients to tissues and organs in arthropods. It is a complex mixture of whole cells (hemocytes), proteins such as lipophorin (the lipid transport protein) and vitellogenin, the major yolk protein, lipids, nucleic acids, as well as degradation products. Most importantly, it has been shown to contain some factors of the immune system such as such as hemocytes, antimicrobial peptides and prophenoloxidases. However, there is lack of data concerning proteomic analysis of mosquito hemolymph especially during pathogen infection, which may be essential for identifying novel secreted molecules that influence parasite survival. In addition, there is a need to improve genome annotation in the current protein databases such as Ensembl that rely on comparative models for genome prediction. Two studies that covered mosquito salivary gland proteins (Kalume et al., 2005) and mosquito hemolymph (Li et al., 2006) have shown the limitations in protein coverage and predictions encountered when searching protein databases using mass spectrometry data. Improvements in genome annotation were proposed through combining two gene prediction tools based on *ab initio* gene prediction model (GENESCAN) and comparative model (GENEWISE) (Li et al., 2006). Therefore, a new data set of coding sequences (CDS) referred to as ReAnoCDS05 was generated and shown to improve protein identification using mass spectral data (Figure 4A) (Li et al., 2006). By extending the genome coverage, proteins that were previously left out due to lack of existing comparative models, can now be identified. Some of these proteins may have been specific due to the evolutionary adaptations between the malaria parasite and its vector, and may be good targets to interrupting malaria transmission.

Figure 4

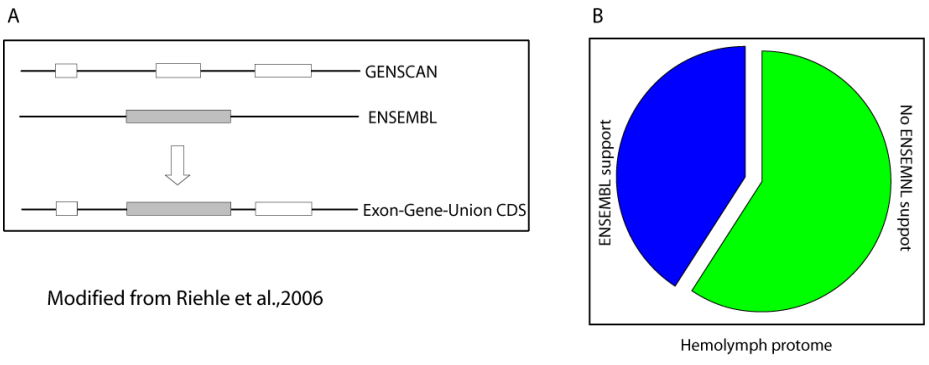


Figure 4. Diagram of Exon gene union (EGU) algorithm

A: The algorithm considers all exons predicted by GENSCAN and Ensembl as potential exons of a final CDS, and examines exon boundaries to assemble a new gene model. If exons from GENESCAN

and Ensembl have different boundaries, the algorithm extends the exon boundary to include all nucleotides of the *ab initio* and comparative predictions next, the open reading frame selection tool chooses the best translatable frame to yield the final ReAnoCDS05. B; Hemolymph proteome prediction by Ensembl fails to account to over half of the proteins (Adapted from Li et al., 2006)

With improved genome annotation, proteomic analysis may be used to address other aspects of parasite killing such as antiparasitic responses of the entire midgut tissues, since previous analysis was limited to the cardia section and only focused on short secreted molecules such as antimicrobial peptides (Warr et al., 2007). Similarly, it would be interesting to investigate the lipid transport system, which may shed light on how molecules are recruited in the hemolymph, and transported to the basal labyrinth where parasite killing occurs.

How are antiplasmodial factors recruited in the hemolymph and the midgut?
Published data has shown that TEP1 among other immune factors is produced by mosquito blood cells (hemocytes), but it is not clear how they are recruited on the basal side of the midgut where ookinete killing occurs. It may be envisaged that immune factors form complexes with the lipid transfer protein lipophorin hence transported and delivered on the basal side of the midgut epithelium. Lipoproteins have been shown to associate with immune factors such as human C3 (Vaisar, et al., 2007) and in mosquitoes, lipophorins have been implicated in antiparasitic responses (Sinnis et al., 1996), thus analysis of mosquito lipophorins would be essential to establish if it associates with immune factors.

1.7 Lipid transport in insects

"Oil and water do not mix", an easily observed phenomenon that all living things need to manage. In insects lipids are produced in the fat body, an organ functionally equivalent to the mammalian liver, but are required or deposited in different organs such as the ovaries, where they support egg development. An elaborate transport system is required to transport cholesterol and fatty acids through aqueous environments. Therefore, insects just like other animals have developed a vehicle for lipid transport composed of apolipoprotein (also known as lipophorin particles).

Apolipoproteins are proteins that bind to lipids by forming a biochemical assembly of lipid and proteins. In mammals apolipoprotein belongs to a multigene family of proteins with six structural variants (apoA, apoB, apoC, apoD, apoE, apoH) with several subclasses. Lipoprotein particles consists of two parts, the inner core (hydrophobic in nature) composed of cholesterol esters and triacylglycerols,

surrounded by a monolayer of polar phospholipids and cholesterol. Lipids may be transported as cargo by docking to the inner part of the complex and shuttled through aqueous environment. Upon reaching the target organ, the lipidic cargo is released and internalized through receptor-mediated endocytosis, the free lipoprotein can then be recycled (Kawooya and Law, 1988). It's not clear if lipoprotein itself needs to be endocytosed in order to release cargo.

Most classifications of lipoproteins are operational, depending upon physical properties such as charge, density or particle size. These properties are used to distinguish four classes of lipoproteins namely: high-density lipoprotein (HDL), low~ (LDL) and very low~ (VLDL) and chylomicrons (reviewed in Lewis, 1973). However, it has been suggested that these lipoproteins form a dynamic system within which mass transfer of lipids and proteins occurs (Sigurdsson et al., 1975). The injection of heparin in rabbits and human was shown to lead to decreased levels of VLDL and an increase of LDL in plasma (Yang et al., 1999). Similarly the injection of radio labeled VLDL into humans lead to some proportion of labeled LDL (Marzetta et al., 1990). It has also been shown that ApoIII binding to HDLp in locusts converts it into LDL (van der Horst et al., 1991). This facilitates the binding and transportation of lipids to energy consuming flight muscles, while the dissociation of ApoIII reverses the process and leads to the release of lipids from the complex (Adamo et al., 2008, Weers et al., 1999). In this way lipids can be shuttled between the sources of production to the storage organs. Thus ApoIII acts as a molecular switch controlling the loading and release of lipid cargo by either lowering or raising the density of the lipoprotein complexes (Weers et al., 1999).

Functions of the lipid transport system in insects

The major role of the lipophorins is the shuttling of lipids from the site of synthesis to the site of storage or utilization which include energy-consuming tissues, including muscles; rapidly developing imaginal organs in larvae; and the ovaries in adult females. In addition to lipids, lipophorin serves as a vehicle for morphogen proteins in the imaginal discs of *Drosophila* larvae (Panakova et al., 2005). Interestingly, vertebrate lipoproteins have been shown to be involved in host defense responses against pathogens. Published data implicated human HDL to contain lytic factors on *Trypanosoma* (Raper, 1996), and established that apolipoprotein L-1 (apoL-1) is the key antiparasitic molecule (Vanhamme et al., 2003). Similarly, lipophorin forms a detergent-insoluble aggregate with LPS thereby protects the silkworm and Galleria from toxic microbial secretions (Taniai et al., 1997, Kato et al., 1994, Ma et al., 2006).

Lipophorin has been shown to harbor some fraction of pattern recognition molecules and regulatory proteins that control prophenoloxidase (PPO) activity (Rahman et al., 2006). Furthermore, lipophorin and PPO play a crucial role in clotting and are the main coagulating factors in mosquito plasma. Clotting protects the host from excess bleeding and prevents microbial invasion (Agianian et al., 2007). Apolipoproteins have been also shown to interact with vertebrate complement factors such as human and fish C3 (Lange et al., 2005; Vaisar et al., 2007) however the role of this association has not been clearly defined. Interestingly, TEP1 shares structural and functional similarities to vertebrate C3 (Baxter et al., 2007, Blandin et al., 2004) therefore, it may be envisaged that TEP1 may associate with lipophorin, just like vertebrate C3 and lipoproteins (Vaisar et al., 2007). Such an association may negatively regulate TEP1 activity, since it has been shown that lipophorin negatively regulates parasite killing (Vlachou et al., 2005), which is largely shown to be TEP1-dependent (Blandin et al., 2004, Frolet et al., 2006). However, it is not known if the two molecules (TEP1 and lipophorin) do interact as previously observed for C3 and Apolipoprotein in vertebrates (Lange et al., 2005). We attempted to investigate if such an association existed and report our findings (Chapter 3)

Lipid transport in mosquitoes

The *A. gambiae* lipophorin gene consists of 8 exons, encoding 10,516 nucleotide-long transcript. Lipophorin is translated into a proapolipophorin, which is processed by proteolysis to generate two mature apolipophorins: apolipophorin-I (Mr = 280 KDa) and apolipophorin-II (Mr = 81 kDa) (Marinotti et al., 2006, Atella et al., 2006). Unlike other insects that form reusable lipid transport system incorporating an exchangeable lipoprotein (ApoIII), mosquito lipophorin relies only on the non-exchangeable system consisting of ApoI and ApoII and equal amounts of proteins and lipids with a small percentage of carbohydrates (2%) (Atella et al., 2006, Marinotti et al., 2006). Mosquito lipophorin has been associated with diverse functions including hemolymph clot in insect larvae by coagulation reactions involving lipophorin-prophenoloxidase complexes (Karlsson et al., 2001) and melanization of dead parasites (Atella et al., 2009, Mendes et al., 2008).

Vlachou et al., (2005) demonstrated, through RNAi approaches, that lipophorin is involved in parasite survival and oogenesis in a mosquito. Lipophorin-depleted mosquitoes showed reduced parasite development, while ovary development was totally abolished (Vlachou et al., 2005, Mendes et al., 2008). However, how this is

achieved has not been established. Furthermore, *Plasmodium* infection induces lipophorin expression in mosquitoes (Vlachou et al., 2005, Cheon et al., 2006). It may be envisaged that immunity factors such as TEP1, LRIM1 or PPO may be scaffolded in complexes with lipophorin. Therefore, parasites may negatively regulate host immunity (reviewed in Hurd, 2001) by inducing lipophorin expression. However this needs to be established. We undertook to investigate if lipophorin transports or inactivates TEP1, presented in Chapter 3.

Vitellogenesis

The synthesis of yolk protein precursors (YPP) such as vitellogenin (Vg) is a key event in the reproductive cycle of anautogenous insects referred to vitellogenesis and is strictly dependant on a blood meal (Raikhel et al., 2002, Roy et al., 2007, Attardo et al., 2003).

The expression of Vg has been shown to be controlled by the nutrient sensitive target of rapamycin (TOR) pathway (Hansen et al., 2004). Vg is produced in the fat body alongside other yolk proteins, secreted into the hemolymph and transported to the ovaries where it is stored and proteolytically cleaved by vitellogenin cathepsin B (VCB) (Cho et al, 1999). Vg is a large protein of 2051 amino acids with a putative lipid transport and von Willebrand factor domains (vWF). Interesting, similar domain structure has been observed in lipophorin the major lipid transport molecule, therefore potentiating the role of Vg as lipid transporter and most importantly involved in the mosquito antiparasitic responses just as observed for lipophorin (Vlachou et al., 2005, Mendes et al., 2008). Thus, we investigated the role of Vg during parasite development in mosquitoes using RNAi approaches, we extended our analysis to include on VCB and report our findings in Chapter 3 and 4.

1.8 General objective of the thesis project

The current understanding of the TEP1-dependent parasite killing has been based on transcriptional analysis that led to the identification of several molecules that participate in the parasite killing. However the complete picture, including from signaling to effecter molecules, is yet to be established. We intended to complement the microarray analysis with a proteomic approach in order to identify the unknown factors involved in the killing mechanism by:

- Performing global proteomic analysis of mosquito midgut tissues, to establish which molecules are induced by *Plasmodium* infection

- Investigate why the nutrient transport system in mosquitoes using proteomic analysis. We were keen to establish if nutrient transport system was involved in the transport of immunity factors such as TEP1

CHAPTER 2

Global proteomic analysis of the *Anopheles gambiae* midgut during a *Plasmodium berghei* infection

Introduction

Malaria transmission occurs when a mosquito, particularly the major malaria vector *Anopheles gambiae*, ingests gametocytes of the *Plasmodium* parasite during a blood meal derived from an infected host. Within the mosquito, parasites undergo differentiation and replication during which they experience three severe population bottlenecks. These occur in the transitions between gametocytes and ookinetes, between ookinetes and mature oocysts, and between midgut sporozoites and salivary gland sporozoites (reviewed in Blandin et al., 2008), and are considered vulnerable steps in the parasite life cycle during which the parasite is easily destroyed by the vector's immune response. Therefore, the knowledge of vector-parasite interactions may be critical to identify potential targets for disrupting the parasite cycle and blocking malaria transmission from the vector. Studies have been undertaken to investigate the mosquito antiparasitic responses and have identified a number of molecules that affect the outcome of parasite development in mosquitoes. Thioester-containing protein 1 (TEP1) and two leucine rich repeat proteins encoded by the *LRIM1* and *APL1* genes have been shown play a central role in the mosquito antiparasitic responses (Blandin et al., 2004, Osta et al., 2004, Riehle et al., 2006 and 2008). Several other molecules have been identified whose knock down leads to either increased or reduced parasite development (reviewed in Blandin et al., 2008) and may be molecularly connected to the TEP1 killing mechanism. However, the complete undertanding of the parasite killing mechanism is yet to be established. Furthermore, most of the studies previously done to investigate the molecular aspects involved in vector-parasite interactions were based on transcriptional profiles of mosquito immune responses (Vlachou et al., 2005, Dong et al., 2006, Mendes et al., 2008), which are based on the assumption that most changes in the expression of mosquito genes that affect parasite survival occur at the transcriptional level. However, proteins are the ultimate effectors of biological functions including the immune response, and protein abundance and transcript levels are not always correlated (Gygi et al., 1999). Therefore, a proteomic analysis of *A. gambiae* infected with the malaria parasite may be envisaged as a complement to microarray data and can be used to identify other factors involved in the parasite killing.

The mosquito midgut plays a central role in the development and subsequent transmission of malaria. Exflagellation of the male gametocyte is facilitated by a drop in temperature and by xanthurenic acid among other host factors (Arai et al., 2001). Furthermore, interactions of the parasite with the host factors (for instance laminin and annexin is critical for a successful invasion. Interestingly, parasites are the most

vulnerable to mosquito immune responses mediated by TEP1 during the ookinete stage (Blandin et al., 2004) early oocysts. In addition, it has been shown that antibodies directed against mosquito midgut epitopes can minimize *Plasmodium* transmission (Suneja et al., 2003). Put together, these observations underline the importance of the midgut as a focal point for novel malaria control strategies. We undertook to investigate the immune response of *A. gambiae* during *P. berghei* infection by a proteomic approach. We were keen on identifying mosquito factors that were induced by ookinete invasion of midgut tissues and most importantly those that associated with strong (*TEP1 GOF*) or impaired (*TEP1 LOF*) immune responses. We identified over 700 mosquito proteins expressed 24 h after infectious feeding in the midgut. Several known or putative immune factors were identified such as thioester-containing proteins (TEPs), peptidoglycan recognition proteins (PGRPs) and serine protease inhibitors (SRPNs). As expected we detected more antiparasitic molecules in *GOF* compared to *LOF* mosquitoes, which is consistent with the higher efficiency of *TEP1 GOF* to oppose parasite development.

Experimental procedures

Sample preparations and analysis by tandem mass spectrometry

Transgenic mosquitoes: *TEP1GOF* and *TEP1LOF*, developed in the laboratory were reared under standard mosquito care previously described. Newly emerged females were maintained with 10% sucrose for six days before infection with *P. berghei* parasites Midguts were dissected on ice 24 hpi and homogenized in TRIS buffer supplemented with a cocktail of protease inhibitors (Complete, Roche). Soluble protein extracts were used for further analysis after quantification by Bradford assay (Bradford, 1976)

SDS gel electrophoresis was used to resolve 1 mg of mosquito protein. The gel was cut into 15 slices and subjected to a standard in-gel digestion protocol (Shevchenko et al., 1996). The digested peptides were purified using Stop and go tips (STAGTIPS) then eluted with trifluoroacetic acid (TFA). The organic solvent was evaporated in a vacuum centrifuge and TFA added to a final concentration (2% TFA) before analyzing samples by tandem mass spectrometry (Rappsilber et al., 2003).

Peptides were sequenced with a nano-high-pressure liquid chromatography Agilent 1100 nano-flow system connected to a 7-Tesla linear quadruple ion-trap Fourier transform (LTQ-FT) mass spectrometer (Thermo Electron) as described previously (Olsen et al., 2004). The MS equipment was operated in a data-dependent mode to

automatically switch between MS, MS2 and MS3 acquisition as described (Pilch and Mann, 2006).

The acquired data was searched against the International protein index human protein sequence database, the *A. gambiae* proteome (Ensembl) and the *P. berghei* proteome database (Sanger/TIGR) downloaded from the Ensembl and NCBI database respectively, using the automated data search program Mascot (Matrix science, London UK). Spectra were searched with the following parameters: mass tolerance of 5 ppm for MS data and 0.5 Da for MS/MS data with up to 3 missed trypsin cleavage sites allowed. Carbamidomethyl cysteine was set as fixed modification, and oxidation of methionine and deamidation set as variable modifications. MS^2 spectra were automatically scored with MSQuant spectra (MSQuant at Sourceforge).

The Mascot search engine (Matrix science) was used to search mass spectrum data against the human (NCBI), mosquito (Ensembl version 43) and *Plasmodium* (GeneDB) protein database to generate a list of peptides. Only peptides with i) at least six amino acids, and ii) a mascot score above 20 were considered for further analysis. Three or more unique peptides were used for protein identification. Proteins identified by either one- or two-peptide hits were manually verified as previously described (Pilch and Mann, 2006). Relative protein abundance between different samples was based on the total number of unique peptides identified for each protein.

A. gambiae midgut proteins induced by *P. berghei* infection

To investigate changes in the protein expression in the mosquito midguts and to focus on those associated with ookinete invasion, we chose to infect mosquitoes with two different strains of *P. berghei* parasites: *Pb*GFP (midgut invasion) and the ANKA 2.33 strain (also referred as *PbMut*, a strain unable to form gametocytes, used as a control triggering non-invasive responses). Using these parasites we compared mosquito protein sets that were characteristic of *TEP1 GOF* and *LOF* transgenic mosquitoes. We chose to prepare the midgut proteins for mass proteomic analysis at 24 hours post infection (hpi). We selected this time point with reference to *TEP1* expression (Figure 2-1 A and B), and envisaged that antiparasitic molecules involved in the TEP1 pathway would be co-regulated with *TEP1*, while those that inhibit parasite killing would be down counter-regulated in the presence of excess TEP1.

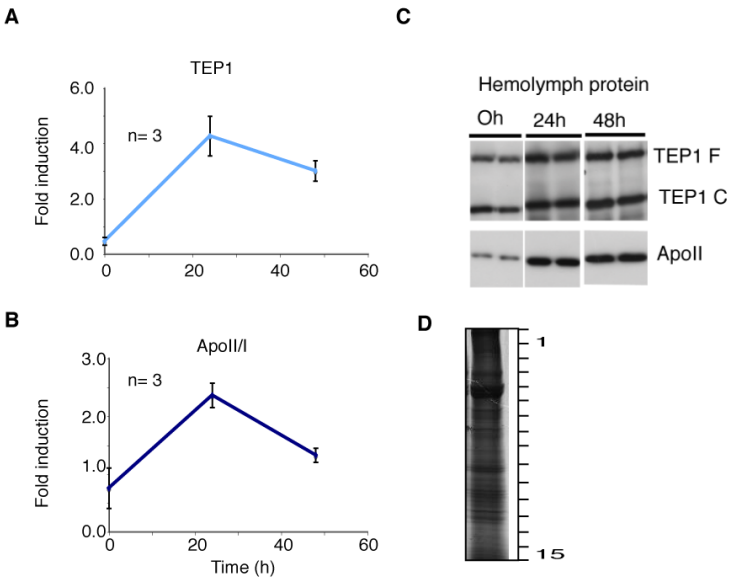


Figure 2-1. *TEP1* and *ApoII/I* expression in *P.berghei* -infected *A. gambiae*

Susceptible mosquitoes (G3) were infected with *P. berghei*. A and B expression of *TEP1* and *ApoII/I* was analyzed by qRTPCR at 0, 24 and 48 hpi. C: Imunnobloting analysis of TEP1 and ApoII/I proteins in mosquito hemolymph at 0, 24 and 48 hpi, D: soluble midgut proteins were resolved by SDS-gel, that was reduced to 15 equivalent gel slices, trypsinized and analyzed by tandem mass spectrometry.

Midgut proteins from *P. berghei* infected mosquitoes were extracted in TRIS buffer (pH 6.8) containing a cocktail of protease inhibitors. The soluble proteins sample was resolved by SDS gel, trypsinized and sequenced by tandem mass spectroscopy as previously described (Lasonder et al., 2002). We identified 724 unique mosquito proteins expressed in the midgut 24 hpi, corresponding to approximately 7 % of the proteins predicted in the mosquito genome (Ensembl version 43), however only relatively few parasite proteins were identified due to the huge predominance of mosquito proteins in the sample (see figure 2-2).

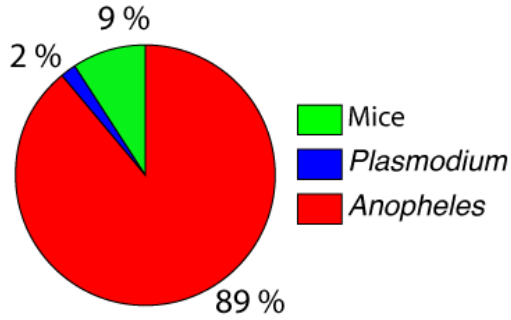


Figure 2-2. Proteins identified from the mosquito midguts after *P. berghei* infection.
TEP1 GOF and *TEP1 LOF* transgenic mosquitoes were infected by *P. berghei* parasites. Soluble proteins prepared from dissected midguts 24 hpi, resolved by SDS PAGE and analyzed by tandem mass spectrometry. The pie chart represents the total number of proteins identified according the respective proportions.

Large scale proteomic analysis potential generates a large a mount of data that may be difficult to understand. In order to gain a general overview of the biological changes during *Plasmodium* infection, we organized our proteomic data into functional categories. We observed an over representation of proteins associated with catalytic activity, binding and lipid transport. These proteins may be involved in the digestion of blood, lipid biosynthesis and transportation of nutrients to storage organs such as ovaries. Proteins associated with cell adhesion, molecular chaperones and apoptosis were also induced by parasite infection (figure 2-3).

Figure 2-3. Proteins identified from *P. berghei* infected mosquito midguts classified according to functional categories. Proteins identified by mass spectrometry were organized according to their biological functions using gene ontology (GO) terms, a summary of the main functional categories is given in the pie char

Blood feeding induces the expression of putative immune genes

In order to investigate the specific interactions between the vector and parasite during *Plasmodium* invasion of midgut cells. We first attempted to eliminate changes in proteins expression associated with blood feeding. To this end we analyzed proteins expressed after infection with a mutant parasite that does not produce gametocyte (*Pb Mut*) hence fails to invade midgut cells. Figure 2-4 summarises the proteins identified under this condition. We show that most proteins identified were constituively expressed in the *TEP1 GOF* and *TEP1 LOF* mosquitoes (226 proteins), but surprisingly we observed less strain specific proteins (34) in *TEP1 GOF* compared to *TEP1 LOF*.

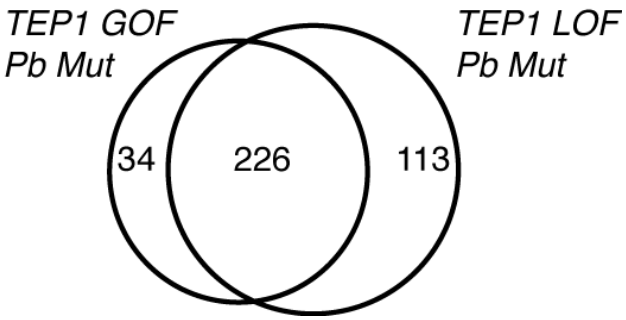


Figure 2-4. Mosquito proteins expressed constitutively after blood feeding. *TEP1 GOF* and *LOF* transgenic mosquitoes were infected with a mutant *P. berghei* parasite (*PbMut*) that does not produce gametocytes to identify proteins that are expressed constitutively during blood feeding. The venn diagram gives a summary of the proteins identified in the two strains. Most of proteins were equally distributed between the two strains.

Interestingly, some of the proteins constituitively expressed after blood feeding included putative immune proteins such as scavenger receptor with sushi domain (Agap000550), Catalase and leucine rich repeat proteins. The digestion of blood products generates free radicles as by products including reactive oxygen species (ROS) which may be detrimentous to the cells, Inorder to maintain a low level of ROS, catalase and superoxide dismutase expression is induced. Scavenger receptors (SRs) a large family of membrane receptors that bind oxidized low density lipoprotein and a wide variety of other ligands many of which are derived from apoptotic cells and pathogens. However, the ability of some SRs to function as PRRs through their binding of a wide variety of pathogens, potentiate their role in host defence. The expression of Agap000550 could be in response to apoptotic cells or probably sensing the presence of parasites without midgut invasion. A summary of other putative immune molecules is found in table 2-1 and appendix 1

A. gambiae* responds to midgut invasion by *P. berghei

Next, we compared proteins differentially identified in the *TEP1 GOF* and *LOF* mosquitoes during ookinete invasion. Over 70% (415 proteins) were equally distributed in both transgenic mosquitoes. We observed more proteins specific to *TEP1 GOF* (133 proteins) compared to *TEP1 LOF* (101 proteins) figure 2-5. This difference in the number of unique proteins may be attributed to the strong immune response observed in *TEP1 GOF*.

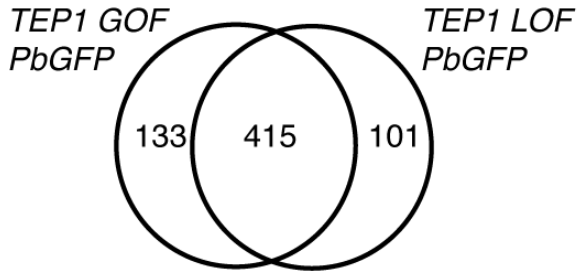


Figure 2-5. Comparison of proteins expressed in *TEP1 GOF* and *LOF* mosquito during parasite infection. *TEP1 GOF* and *TEP1 LOF* transgenic mosquitoes were infected with GFP expressing *P. berghei* parasites. Midgut proteins were identified by tandem mass spectrometry analysis. Proteins identified in the two transgenic mosquito lines were compared and displayed by the venn diagram according to their enrichment i.e train specific and those common in both mosquito lines

However, we also observed some variations in the peptide abundance for some proteins under the two conditions (*GOF* versus *LOF*). For instance, we identified (71) unique peptides for lipophorin (AGAP001826-PA) in *TEP1 LOF* while only (3) in *TEP1 GOF*, on the contrary there were fewer peptides matching an LRR protein AGAP005744-PA in the *TEP1 LOF* (4) compared to *TEP1 GOF* (12) see appendix 1 for more details. Therefore, suggesting a differentially expressions of these proteins in the two transgenic mosquitoes during midgut invasion by ookinetes. Previously, Ishihama et al., (2006) developed a model referred to the exponential protein abundance index (emPAI). Therefore, further analysis on the proteomic data will be

essential to provide more insights into the changes caused by parasite infection. Table 2-2 gives a summary of some of the interesting molecules identified by the proteomic analysis during midgut invasion (for more details see appendix 2 and 3). Some of the proteins identified include known immune factors such as inhibitors of serine proteases (SRPNs), proteins involved in the negative regulation of prophenoloxidase activity (Michel et al., 2006). For example SRPN 6 is induced in the salivary glands upon sporozoite invasion (Pinto et al., 2008) and may facilitate the invasion of salivary glands. We identified several proteins from the thioester containing proteins (TEPs) family, petidoglycan recognition proteins (PGRPs) as well as proteins containing leucine rich repeats (LRR) domains including LRIM1, which has been shown to antagonize *P. berghei* development in mosquitoes (Osta et al., 2004). Some of the immune factors we identified in the midgut (for instance LRIM1 and TEPs) are produced by hemocytes but are required in the basal labyrinth of the midgut where parasite killing occurs. Induction of SRPN 10 has been shown to occur specifically in the parasite-invaded midgut epithelial cells and its expression requires parasite surface proteins P25/28 (Danielli et al., 2005).

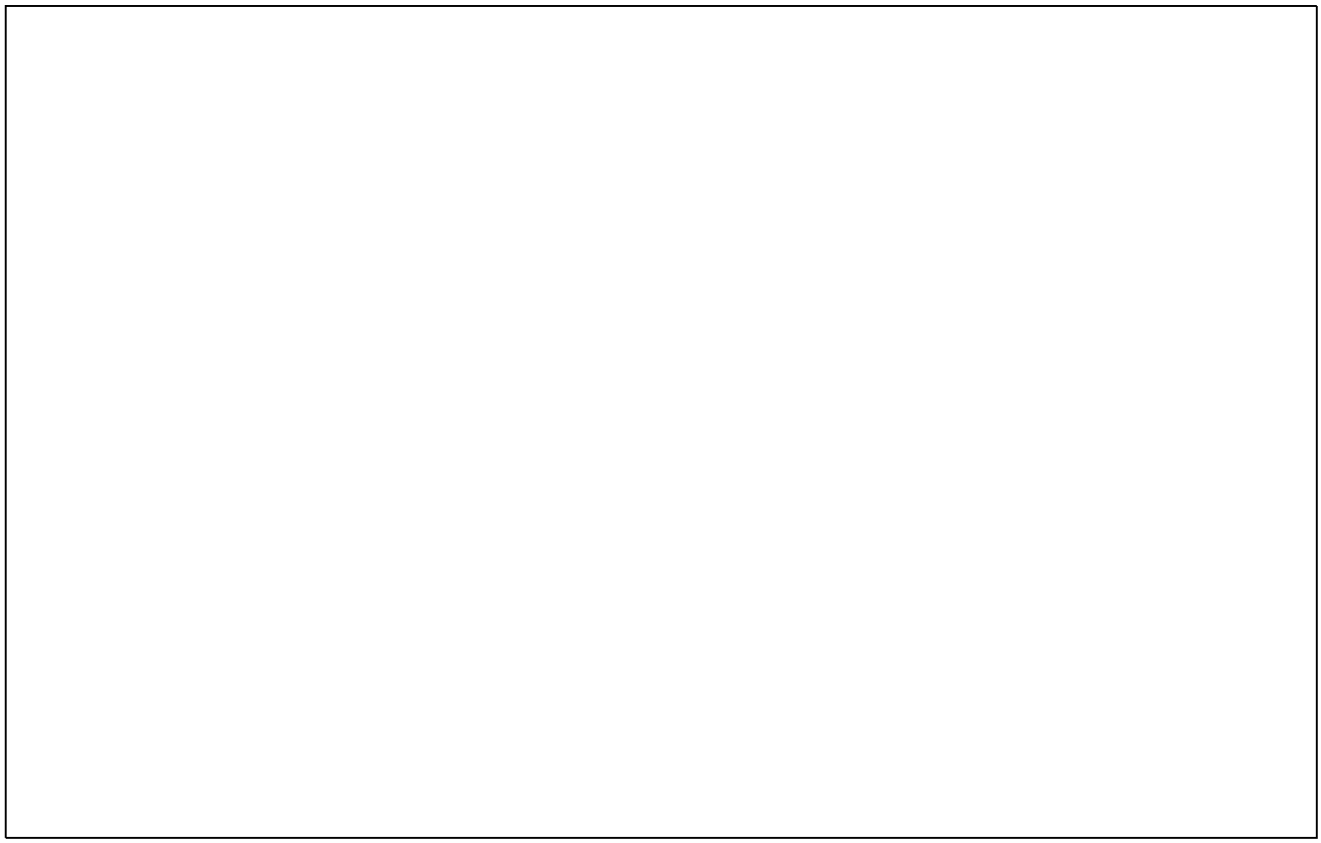


Table 2-2. Proteins identified in mosquitoes during midgut invasion by *P. berghei*

Proteins identified during midgut invasion (24 hpi). Proteins in red were only present in *TEP1 GOF* mosquitoes e.g. LRRD10, 11 which may be involved in antiparasitic response, while those in blue only present in *LOF* strain which may be important for parasite survival. Proteins found in both strains are displayed in black

Furthermore, we show that the *TEP1 GOF* and *LOF* strains of mosquitoes to parasite infection by expressing more putative antiparasitic molecules which was determined by the absence or presence of a given molecule in the respective strain such as LRRD10, SOD2 were only present in *GOF* and SRPN 14 and SRPN 4 only in *LOF* (table 2-3).

Table 2-3. Putative immune proteins differentially enriched between *TEP1 GOF* and *TEP1 LOF* after parasite infection.

Proteins that were uniquely identified in *TEP1 GOF* or *LOF* mosquitoes during parasite infection are displayed above. Some of the proteins include LRRD10, FBN 24 and GALE 8

Therefore, we suggest further analysis of some of these molecules to establish their role in mosquito antiparasitic responses. We discuss in detail some of the molecules identified during parasite infection below.

LRR proteins

Proteins with LRR domain facilitate protein-protein interactions and have been attributed diverse biological functions including disease resistance in plants (Huang et al., 2008), inflammatory response (Wilmanski et al., 2007) and, most importantly, antiparasitic responses in mosquitoes (Osta et al., 2004; Riehle et al., 2008). We identified several proteins from this family including: LRRD10, LRRD11 and LRIM1 in our proteomic analysis, in addition, these proteins were only identified in the parasite infected samples. Previously it was shown that the depletion of LRIM1 and that of another LRR protein APL1 leads to increased parasite survival in a manner similar to the phenotype observed after silencing of *TEP1* (Osta et al., 2004, Riehle et al., 2006, Blandin et al., 2004). Recently, Fraiture et al., (2009) showed that binding of TEP1 to parasites requires LRIM1 and APL1. The knock down of either LRIM1 or/and APL1 leads to precocious deposition of mature TEP1 on the mosquito self-tissues and therefore abolishes TEP1 binding to and killing of ookinetes. In addition, LRIM1 and APL1 might form a heterodimer, to persist in the circulation since the knock down of either of the two genes leads to the disappearance of both from the hemolymph (Fraiture et al., 2009). Furthermore, the two LRR proteins co-precipitate with TEP1 in pull down experiments, therefore suggesting that these three proteins

form a complex in the mosquito hemolymph. Future RNAi experiments will determine if the additional LRR-containing proteins identified here also assist TEP1 function.

LRR proteins have been associated with the sensing and activation of immune response such as Toll pathway in insects, and mammalian Toll-like receptors (TLRs) (reviewed in Hoffmann, 2003). LRRD 10 and 11 were reported to be induced by parasite infection and have been suggested to play a role in the mosquito antiparasitic response (Dong et al., 2003). Our data demonstrated that LRR proteins were enriched in the *TEP1 GOF* mosquitoes compared to *TEP1 LOF* thus suggesting that many more LRR proteins may be involved in the *TEP1*- dependent antiparasitic responses.

We performed functional analysis on the LRR genes (LRRD 10, LRR through gene disruption mediated by dsRNA injection in mosquitoes and gauged parasite development 7 dpi (figure 2-6)

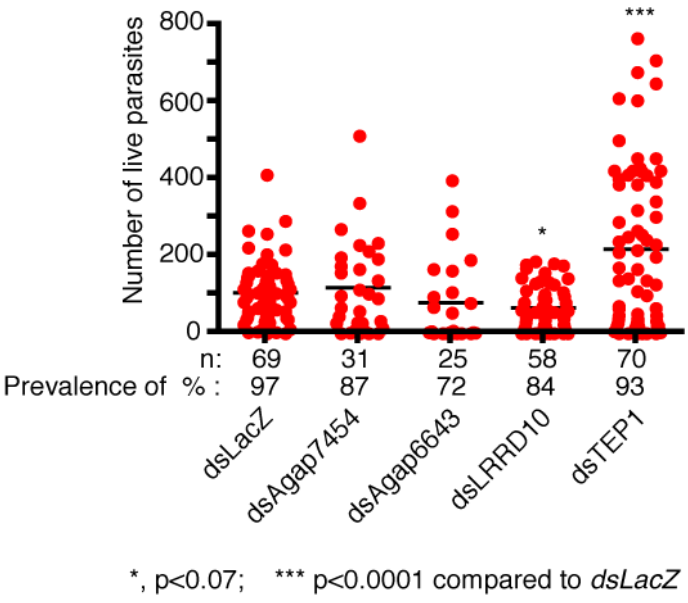


Figure 2-6 Analysis of *Plasmodium* development in mosquitoes after depletion of of LRR protein coding genes. Mosquitoes were injected with dsRNA directed against respective LRR genes. 4 days later infected mosquitoes were infected with *P. berghei* and parasite developed gauged 7dpi.

Here we show that apart LRRD10 whose depletion results in significant reduction in parasite numbers, the depletion of other LRR proteins did not affect parasite development. Similarly we did not observe change in ovary development in the injected mosquitoes.

Lectins

Insect galectins are important players in embryonic development and in immunity against pathogens, in addition some members of this family for instance a sand fly protein PpGalec, are essential for parasite development in the vector (Kamhawi, 2004). In our proteomic screen of midgut proteins Gale 7 and Gale 8, were identified after parasite infection. Further studies are required to establish the role of these proteins in the mosquito antiparasitic responses.

Lipid transport molecules

Female mosquitoes require a blood meal for ovary development and reproduction. After a blood meal several events occur simultaneously, including the synthesis and transportation of yolk proteins and lipids to developing ovaries as well as immune responses towards invading parasites. We identified several molecules implicated in synthesis and transportation of lipids including lipophorin (Lp) and low-density lipoprotein receptor (LDLr), induced by *P. berghei* infection in the midgut. Endocytic receptors (LDLr) are cell-surface proteins that transport large molecules into cells through a process known as receptor-mediated endocytosis (Defesche et al., 2004), In insects LDLr are expressed in various tissues including fat body, midgut, brain and oocytes (Dantuma et al., 1999). AGAP010896 is latently induced by blood feeding in ovaries, midguts and fat body (Marinotti et al., 2006), suggesting it may be involved in the internalization of Lp and vitellogenin in oocytes. In chicken, LR8, a protein from LDLr family was shown to facilitate transport and deposition of vitellogenin and C3 in oocytes (Recheis et al., 2005). Interestingly, based on the number of unique tryptic peptides identified, Lp was more abundant in *TEP1 LOF* mosquitoes compared to *TEP1 GOF* (See table1 appendix). This observation was consistent with the previous report showing that the depletion of Lp leads to significant decrease in parasites survival (Vlachou et al., 2005). Therefore suggesting that there might be an interaction between immune factors and molecules involved in reproduction. We investigated this in detail in the manuscript presented with this work (chapter 3).

Reactive oxygen species detoxifying enzymes.

Reactive oxygen species (ROS) are produced as by-products of mitochondrial respiration or immune response towards pathogens (reviewed in Molina-Cruz et al., 2008). High levels of ROS in the mosquito hemolymph have been shown to limit *Plasmodium* development (Kumar et al., 2003), however it is potentially harmful to host cells and has been implicated in the aging process (Harman, 2003). Therefore, the concentration of ROS is kept low by detoxifying enzymes such as catalase, superoxide dismutase (SOD), thioredoxin peroxidase and glutathione peroxidase

(Gpx). We identified catalase (AGAP004904), SOD2 (AGAP005234) and two thioredoxin peroxidases (AGAP007543, AGAP011054) as being more abundant during parasite infection. Previously it was shown that catalase is induced in a tissue-specific manner and its knock down leads to increased parasite lysis and reduction in fecundity (Molina-Cruz et al., 2008, Dejong et al., 2007). The increased parasite lysis may be attributed in part to enhanced TEP1 activity, since TEP1 facilitates parasite killing through lysis. To address this question, it would be essential to investigate if catalase may be involved in the negative regulation of TEP1 expression, cleavage or binding on parasite surface. Similarly, the role SOD2 and peroxidase during parasite invasion needs to be established.

Actin dynamics and cytoskeleton reorganization

Microarray data suggested that a large set of genes associated with cytoskeleton dynamics is induced during ookinete midgut crossing (Vlachou et al., 2005). We identified several candidate molecules that may be involved with actin dynamics including F actin cross linkers (AGAP010895, AGAP006686) that may be involved in the polymerization of actin and possibly the formation of an actin zone (AZ), which has been proposed to be a parasite disposal mechanism or a form of wound healing (Shiao et al., 2006). The formation of AZ has been shown to depend on TEP1, Frizzled and Cdc 42 (Shiao et al., 2006).

TEPs and Alpha 2 macroglobulins

Parasite killing in *A. gambiae* mainly occurs through a complement-like killing mechanism involving TEP1 (Blandin et al., 2004). We identified TEP15 and two alpha 2 macroglobulins (AGAP008366-PA and AGAP008367-PA) proteins from infected mosquitoes ookinete. These genes were previously shown to be induced at the transcript level by *P. berghei* infection (Wyder et al., unpublished data). We speculate that these proteins they may be involved in the TEP1 mediated parasite killing through the formation of a membrane attack complex reminiscent of the vertebrate complement system. Therefore, further analysis of these proteins by RNAi approaches will be essential to establish their role of during parasite invasion.

SRPNs

Serine protease inhibitors (SRPNs) have been implicated with the regulation of the melanization response and parasite killing (Michel et al., 2005, 2006). The knockdown of SRPN2 provokes spontaneous melanization of ookinetes in susceptible mosquitoes. We identified five SRPNs (SRPN 4, 6, 10 and 14), Moreover, SRPN 4 and 14 were abundantly expressed in *TEP1 LOF* mosquitoes

compared to *TEP1 GOF*, whereas SRPN 6 was only present in *TEP1 GOF*. Through an unknown mechanism. Therefore, *TEP1* may repress the expression of these negative regulators to enhance efficient parasite killing. Therefore, future RNAi experiments should explore whether these SRPNs might modulate the efficiency of parasite killing.

Other immune related proteins

Down syndrome cell adhesion molecule (*Dscam*), a transmembrane receptor composed of immunoglobulin and fibronectin domains (Graveley et al., 2004). Dscam was induced in *TEP1 GOF* mosquitoes after *P. berghei* infection. The depletion of Dscam was shown to increased parasite numbers and proposed that this protein may be involved with parasite sensing (Dong et al., 2006) hence leading to efficient parasite elimination in mosquito midguts.

Mosquito response to *bacteria* and *Plasmodium* has been shown to induce similar immune factors including complement factors and bacterial binding proteins (Blandin et al., 2004, Dimoupulos et al., 2002) which suggests a general mechanism against invading parsites. We identified bacteria responsive protein 2 (AGAP008060-PA) induced after *P. berghei* infection. AGAP008060-PA was only found in *TEP1 GOF* mosquitoes, suggesting it may be involved in mosquito antiparasitic response.

Put together, our data confirms at the protein level that mosquitoes to respond to parasite infection and that the *TEP1 GOF* mosquito line induces more known and putative immune factors in response to invading parasites compared to *TEP1 LOF* transgenic mosquitoes (see appendix 2). Our findings are consistent with the enhanced parasite surveillance and killing observed in the *TEP1 GOF* transgenic mosquitoes (Levashina, Blandin et al., unpublished data).

Proteomics confirms transcriptional analysis of *A. gambiae* midgut responses to *Plasmodium* infection.

Transcriptional profile of a particular gene may not necessary correlate with the protein level (Gygi et al., 1999). However, there is added power of discovery if two or more methods can confirm the presence or absence of the altered transcript and its corresponding protein (Sigdel and Sarwal, 2008). Thus, we overlaid the proteomic and transcriptional profile data of *P. berghei*-infected midgut tissues from susceptible G3 and *GOF* and *LOF* transgenic mosquitoes.15 % of the proteins identified were

either induced or repressed at the transcriptional level with 3 fold change in the *GOF* versus G3, or in the *LOF* versus G3 at 24hpi (Figure 2-3 and 2-4).

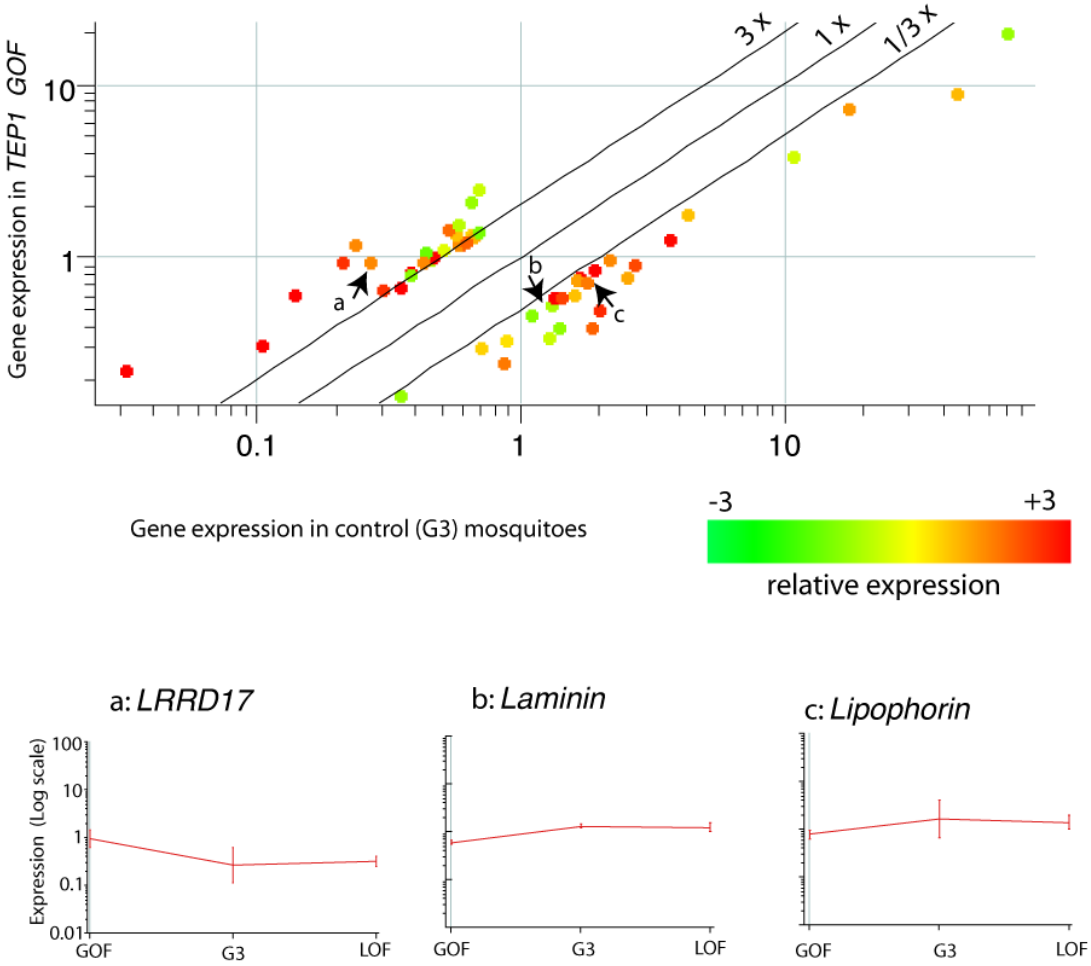


Figure 2-7. Transcriptional profiles of molecules identified in the proteomic analysis

Proteomic data was imported into Genespring microarray platform using corresponding Ensembl transcript identities. The data was filtered for fold change difference in gene expression 24hpi. Genes with at least a three-fold (3X) difference in *TEP1 GOF* versus control G3 are displayed by individual spots on the scatter plot. Some of the *genes* differentially expressed indicated on the scatter plot include: a (*LRRD17)*, b (*Laminin)* and c (*Lp*)

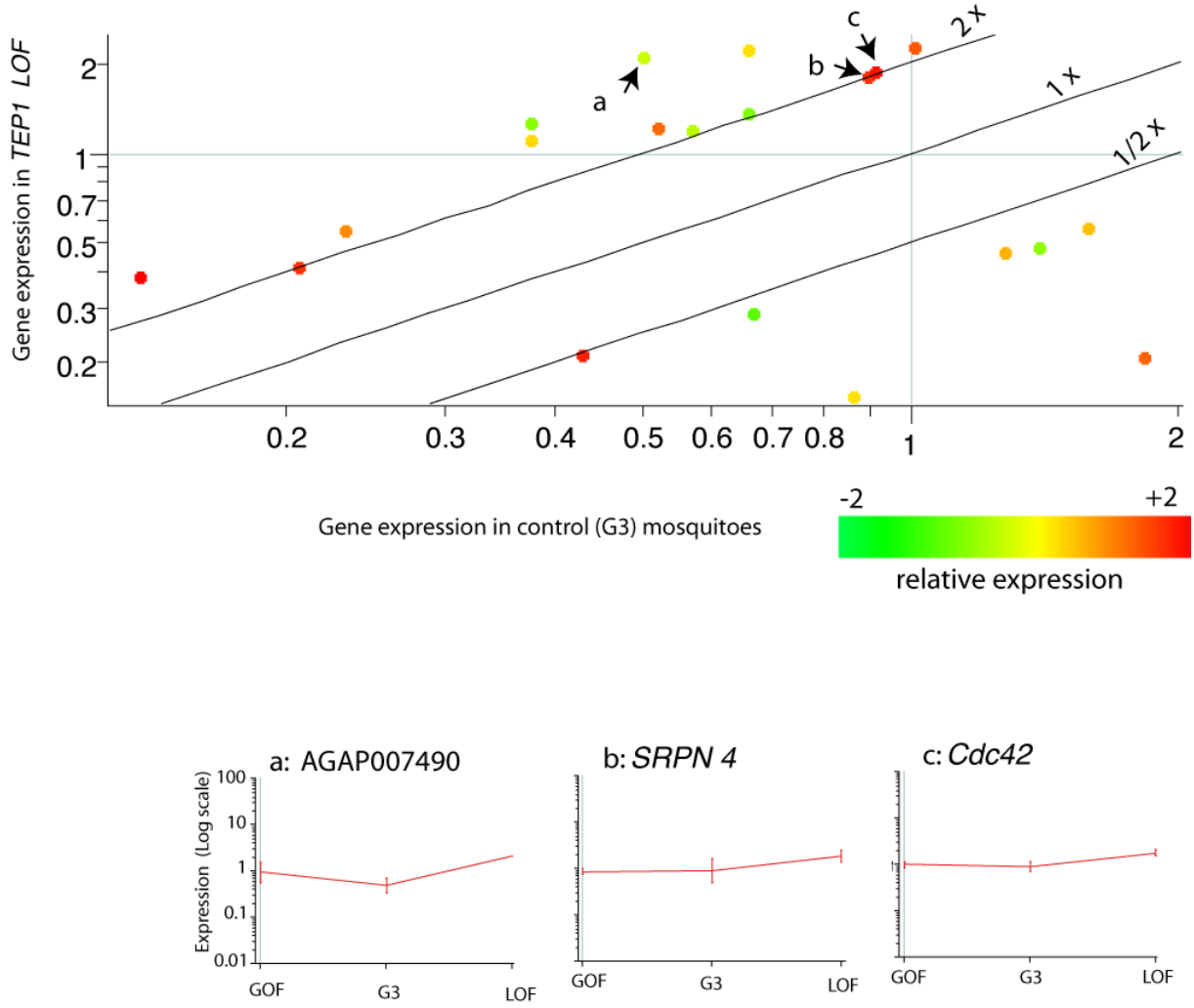


Figure 2-8. Transcriptional profiles of molecules identified in proteomic analysis after parasite infection.

Proteomic data was imported into Genespring microarray platform using their corresponding Ensembl transcript identities. The data was filtered for fold change difference in gene expression 24 hpi. Genes with at least (2X) fold difference in *TEP1 LOF* versus control G3 are displayed by individual spots on the scatter plot. Some of the *genes* differential expressed on the scatter plot include:, a(ENSANGT19056), b(*SRPN 4*) and c(*Cdc 42*)

With this approach we could show that some of the genes encoding proteins we identified in our proteomic analysis (e.g. SRPN 4, laminin and Cdc42) were also differentially expressed at the transcript level. Interestingly, these genes were up regulated in the *LOF* mosquitoes compared to *GOF* or G3 mosquitoes. Shiao et al. (2006) showed that Cdc42, a guanoside triphosphate (GTP) binding protein, is involved together with the Frizzled receptor in the formation of an actin zone (AZ) around dead parasites and in the melanization response in refractory mosquitoes. In addition the melanization response and AZ formation were shown to be TEP1-dependent. Therefore, suggesting an upregulation of Cdc42 in the *TEP1 LOF* mosquitoes could be compensatory expression of antiparasitic factors to minimize

the effects of TEP1 loss. Our data confirms that combining transcriptional and proteomic data improves the power of discovery to identify new factors that may be implicated in mosquito antiparasitic responses.

Functional analysis of candidate molecules identified from the proteomic analysis on mosquito midgut tissues during *P. berghei* infection

To further understand the interactions between the malaria parasite and mosquitoes , we selected some candidate molecules that were differentially expressed during parasite development and belonged to family of proteins such as LRR proteins that have previously been implicated with antiparasitic responses. The selected molecules were cloned into an expression vector for preparaing dsRNA. Mosqutioes were injected with the dsRNA specific for each candidate gene,

Functional analysis of LRR proteins

Improved genome annotation using proteomic

The completion of the *A. gambiae* genome sequencing provided architectural scaffolding for mapping, identifying and selecting genes for functional studies (reviewed in Kalume et al., 2005). Approximately 85 % of the genome has been assembled with over 15000 genes, of which a majority has been automatically predicted. However, there are only about 700 known proteins in the Ensembl database; moreover protein identification by mass spectrometry relies on searching databases of known proteins or predicted transcripts. Therefore, novel proteins may be missed with this approach (figure 2-5).

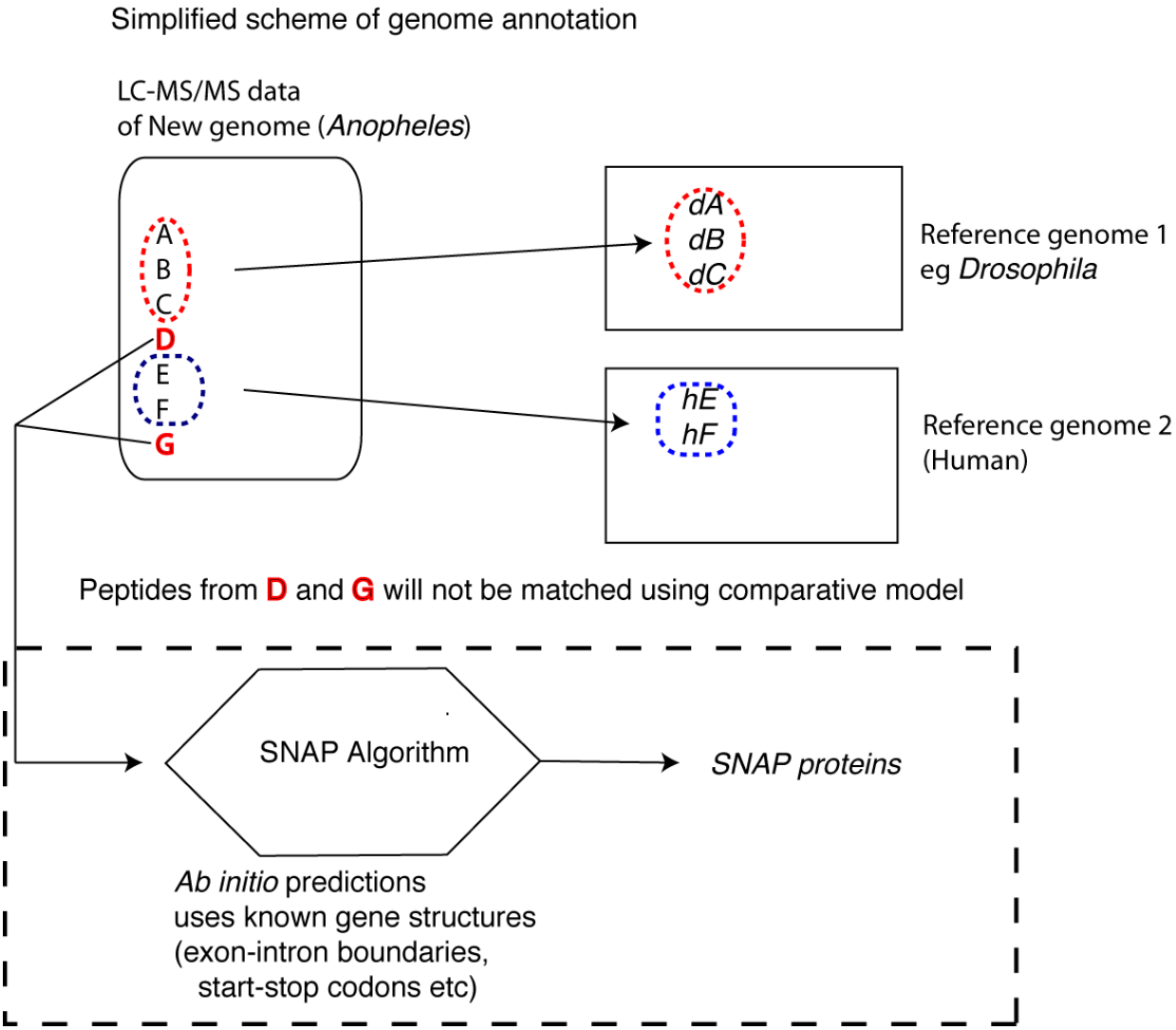


Figure 2-5. Geneome annotation using mass spectrometry

Tandem mass spectrometry of *Anopheles*, a new genome, is searched against reference genomes (e.g *Drosophila* and Human), peptides that find matches in the reference genomes are identified. Peptides that lack gene structures in the reference genomes are analyzed using special algorithm that gives *ab initio* protein predictions based on gene structures present (e.g exon–intron boundaries).

From our proteomic data approximately 8000 peptides sequenced by tandem mass spectrometry could not be matched to any entries in the protein databases we used (mouse-*Anopheles*-*Plasmodium*), and may reflect poor coverage in protein prediction. Li et al., (2006) proposed running two different gene prediction algorithms for synthesizing new coding sequences for *A. gambiae* (ReAnoCDS05) to improve genome annotation. Therefore, we performed new protein searches using tandem mass data from midgut proteins against ReAnoCDS05 and compared this data to the previous analysis using Ensembl predictions (Figure 2-6A). We increased our protein coverage by 300 new proteins by using approximately 70% of the 8000 orphan peptides. Among newly identified proteins was a maltase-like protein encoded by

SNAP-ANOPELES0000011847, a gene located on Chr. 3L (AgamP3: 3L: 41725998:41732061) flanked by two predicted Ensembl protein coding genes *AGAP012400* and *AGAP012401*. We confirmed the protein prediction by 39 high scoring peptides with an average mass tolerance within the acceptable range (<10ppm) (Figure 2-6B and 2-6C).

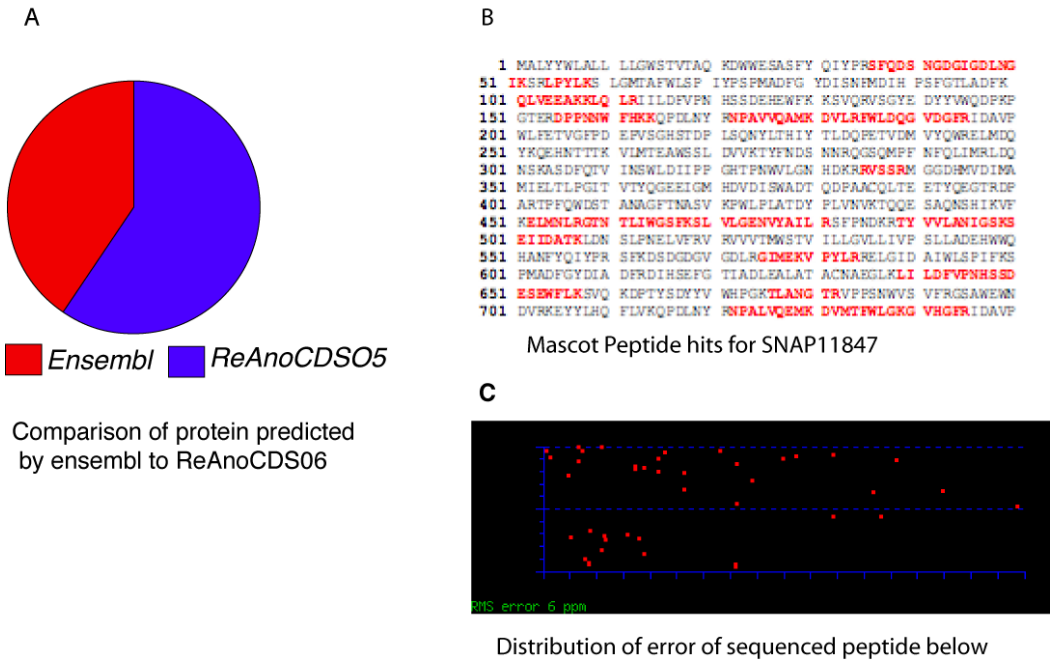


Figure 2-6. Improved genome annotation using tandem mass spectrometry data

A: Pie chart comparing the protein coverage by searching tandem mass against Ensembl or ReAnoCDS05, B: Protein coverage by trypsin digested peptide identified by tandem mass spectrum of SNAP-ANOPELES0000011847, C: Corresponding distribution of error of the sequenced peptides

Plasmodium proteins identified in the mosquito midguts

Plasmodium parasites have a complex life cycle alternating between a vertebrate host and mosquitoes. It has been shown that parasites are most vulnerable to host defense mechanisms during the vector stages. This provides an opportunity for developing malaria control strategies, that may be focused on disrupting the parasite life cycle and reduce malaria transmission. Identifying parasite proteins expressed during this stage may provide targets for such interventions. We identified several parasite proteins present 24 hpi in the midgut tissues; most of these were conserved between *Plasmodium* species or expressed in several parasite stages. However, we also identified an aspartyl protease (PB000864.03.0) that is highly produced by ookinetes: Interestingly the knock out of this gene blocks sporozoites egress but does not affect ookinete survival (Ecker et al., 2008). Table 2-4 summarizes some of the parasite proteins identified. Out of the 15 proteins, 10 are unknown.

Accession num	Mass (da)	Protein family	References
PB000556.01.0	35231	Phosphoglycerate mutase	Hall et al., 2005
PB000589.00.0	141137	Conserved hypothetical protein	Hall et al., 2005
PB000666.01.0	112552	Conserved hypothetical protein	
PB000698.01.0	78345	Hypothetical protein	Hall et al., 2005
PB000754.02.0	211826	POM1, putative	Hall et al., 2005
PB000958.01.0	68068	Conserved hypothetical protein	Hall et al., 2005
PB000997.00.0	63134	Putative uncharacterized protein	Hall et al., 2005
PB001053.03.0	71728	Conserved hypothetical protein	Hall et al., 2005
PB103303.00.0	2872	Hypothetical protein	
PB404206.00.0	6999	Hypothetical protein	Hall et al., 2005
PB405795.00.0	13619	Conserved hypothetical protein	Hall et al., 2005
PB000896.02.0	32988	ATP synthase beta chain	Hall et al., 2005
PB000997.00.0	63134	Conserved hypothetical protein	Hall et al., 2005
PB000864.03.0	52230	Aspartyl protease	Ecker et al., 2008, Hall et al., 2005
PB001096.02.0	16483	Histone H2A variant	Hall et al., 2005

Table 2-4. *P. berghei* proteins identified from mosquito midguts during infection

A. *gambiae* mosquitoes were infected with *P. berghei* , midgut proteins prepared 24 hpi, resolved by SDS gel and analyzed by tandem mass spectrometry. Parasite proteins are displayed with corresponding accession numbers and predicted mass.

2.4 Discussion

Plasmodium development in mosquitoes is completed in approximately two- three weeks. During this time parasites must undergo several developmental stages and transitions. These transitions are considered weak links in the parasite cycle since parasites are most vulnerable to host defenses and suffer severe losses in numbers (Alavi et al., 2003), therefore providing a unique opportunity for disrupting the malaria transmission cycle. Several studies have been undertaken focusing on either cellular responses or transcriptional profiles of host immune responses towards malaria parasites, combined with reverse genetic screen of selected genes (Han et al., 2000; Dong et al., 2006; Vlachou et al., 2005; Mendes et al., 2008). Candidate molecules have been identified based on the assumption that the mosquito immune response is largely regulated at the mRNA levels (Dong et al., 2006, Osta et al., 2004, Michel et al., 2005). We performed proteomic analysis of midgut responses to parasite infection in order to complement the transcriptional analysis and improve our understanding of the changes induced by ookinete invasion in the mosquito midgut. We were keen on understanding the TEP1-mediated parasite killing mechanism and used *TEP1 GOF* and *TEP1 LOF* transgenic mosquitoes infected with *P. berghei*. Previously it had been shown that mosquitoes could sense the presence of parasite without breaching the midgut barrier (Dong et al., 2006). Therefore we used two different parasites *Pb*GFP and a mutant parasite *PbMut* (ANKA 2.33, non gametocyte producers) to discriminate between midgut invasion and general response towards infected blood. We were able to confirm that some putative immune factors such as gale 8, catalase and gelsolin were constitutively expressed after blood feeding in the absence of midgut invasion. Lectins often paly an immunity role as agglutinins and as opsonins in PPO activation process, linked to melanization of pathogens (). Published data has shown that catalase is necessary for ovary development and has been proposed that catalase may protect ovaries from ROS. Over all we identified over 700 mosquito proteins from midgut tissues prepared at 24 hpi, 15% of which were differentially regulated between *TEP1 GOF* and *TEP1 LOF* transgenic mosquitoes with at least a 3-fold difference. We identified four more antiparasitic molecules in *GOF* compared to *LOF.* Conversally, molecules that negatively regulate parasite killing such as SRPNs and lipohorin were more abundantly found in *TEP1 LOF* mosquitoes infected with malaria parasites.

Using a direct comparison between transcriptional and proteomics data, we confirmed the microarray data perfomed on the transgenic mosquitoes during midgut

invasion. Several molecules were differentially induced at the transcript as well as the protein levels, for instance: *Cdc 42, SRPN 4* and *Lp* were upregulated in *TEP1 LOF* mosquitoes compared to *TEP1 GOF* mosquitoes while *LRRD10 and LRRD17* were induced in *TEP1 GOF* mosquitoes (Wyder et al., unpublished data, Vlachou et al., 2005). The knock down of *Lp* has been shown to reduce parasite development and block reproduction (Vlachou et al., 2005). Furthermore, developing oocysts endocytose Lp (Agianian et al., 2007). Therefore, in order to meet these nutritional requirements and to ensure successful egg production mosquitoes might induce the expression of genes involved in lipid and fatty acid biosynthesis. We identified Lp in the midgut protein samples. Previously, the Lp transcript was shown to be highly induced in the midgut after parasite infection (Vlachou et al., 2005), suggesting that it may be synthesized in the midgut apart from its main source, the fat body (Marinotti et al., 2006).

We equally observed a large set of proteins involved in actin dynamics and cytoskeleton genes. Some of these have been shown to facilitate actin polymerization and formation of an actin zone around dead parasites (Shiao et al., 2006) and may represent host response to wound repair of damaged tissues and disposal of dead parasites. The digestion of blood leads to the release of ROS, we observed several proteins involved with the detoxification of ROS including catalase and SODs. Catalase is induced by parasite infection and its depletion lead to increased parasite lysis and reduced fecundity (Molina-Cruz et al., 2008, Dejong et al., 2006). Therefore, it would benefit the mosquito to have elevated levels of catalase and probably other ROS detoxifying enzymes for its own survival and reproduction. However, it remains to be established how parasite lysis is enhanced in the absence of catalase (Molina-Cruz et al., 2008). Since TEP1 parasite killing involves parasite lysis, it is tempting to speculate that Catalase may negatively regulate TEP1 activity.

We also show that mass spectrometry data can be used to improve genome annotations by confirming putative transcripts with protein evidence and by mapping sequenced peptides on genomic locations not covered by annotation algorithms based on comparative analysis. Indeed, we identified more proteins using such *ab initio* predictions, for example SNAP-ANOPELES0000011847. Therefore, the proteomic data we have generated may be explored to improve Ensembl protein

annotation and provided a tool for understanding mosquito-*Plasmodium* interactions with a view towards developing new malaria control strategies.

CHAPTER 3

Analysis of lipid transport system in mosquitoes

Introduction

Lipid transportation in arthropods is achieved by specialized lipoproteins referred to as lipophorin (Lp). However, there is a wealth of data that suggests that Lp is also involved in other functions such as regulation of coagulation reaction in mosquiotoes (Agianian et al., 2007), morphogen signaling in Drosophila (Panakova et al., 2005) and most importantly, insects defense response including detoxification of bacterial toxins and antiparasitic responses in mosquitoes (Vlachou et al., 2005). Interestingly, Lp has been shown to associate with vertebrate immune factors such as the human C3 (Vaisar et al., 2007). We were keen to investigate if mosquito immune factors, such as TEP1 wee associated with Lp through the formation of immunocomplexes. To this end LP was characterized during Plasmodium invasion by immunobloting and tandem mass spectrometry. We demonstrated that Lp associates with PPO but not other immune factors. In addition we showed that Vitellogenin (Vg) co-fractionates with Lp in the low density fraction of a potassium bromide gradient. The knock down of Vg reduced parasite survival by 2-fold and negatively impacted on ovary development. Unexpectedly, Vg negatively regulated TEP1 activity and its expression was unde the control of NF-κB/Rel factors.

Vitellogenin inhibits TEP1-dependent parasite killing in *Anopheles gambiae* mosquitoes

Martin Rono[1], Miranda M. A. Whitten[1,2], Pascal Jansen[3], Adrian Cohen[3], Hendrik Stunnenberg[3], Mustapha Oulad-Abdelghani[4], Elena A. Levashina[1], and Eric Marois[1 #]

[1]UPR9022, CNRS, 15 rue Descartes, F-67084 Strasbourg, France

[2]Department of Environmental and Molecular Biosciences, School of the Environment and Society, Swansea University, Singleton Park, Swansea SA2 8PP, U.K.

[3]Department of Molecular Biology, Radboud University Nijmegen, P.O. Box 9101 6500 HB Nijmegen, The Netherlands

[4]IGBMC, 1 rue Laurent Fries, BP10142, 67404 Illkirch CEDEX

Correspondence should be addressed to: E.Marois@ibmc.u-strasbg.fr

Summary

When feeding on a malaria infected vertebrate, female *Anopheles gambiae* mosquitoes, the major vector of human malaria, simultaneously ingest a blood meal that will activate mosquito reproduction and infect themselves with the malaria parasite. Mosquitoes mount a potent immune response against the invading parasite. Here, we show that molecular processes involved in delivering nutrients to maturing mosquito ovaries also modulate the antiparasitic response. Lipophorin and vitellogenin, two nutrient transport proteins, reduce the parasite-killing efficiency of the antiparasitic factor TEP1. This phenomenon does not seem to involve a physical association between lipidic particles and known proteins of the TEP1 pathway, as a proteomic analysis of mosquito lipophorin particles did not reveal an association with known immune factors other than the prophenoloxidase PPO. We further show that the Cactus/Rel1/Rel2 NF-kB transcription factors, known to control the expression of immune factors, also regulate vitellogenin expression. Our results reveal links at several levels connecting reproduction and immunity, providing a molecular basis for a long-suspected trade-off between these two processes.

Introduction

Malaria is a mosquito-borne infectious disease yearly affecting an estimated 500 million people, of which 1 to 2 million (mostly children in sub-Saharan Africa) succumb to the disease (WHO, 2005). Several parasite species in the *Plasmodium* genus cause malaria, the most dangerous being *P. falciparum. Anopheles gambiae*, the African malaria mosquito, is an obligate vector of human malaria. To initiate their ovary development and production of eggs, mosquito females absolutely require a blood meal. Thus, feeding on a malaria-infected host will simultaneously activate oogenesis and allow malaria parasites to invade mosquito tissues. In the mosquito midgut, ingested *Plasmodium* gametocytes differentiate within minutes into gametes. After fertilization, zygotes rapidly turn into ookinetes, motile cells that traverse the midgut epithelium between 16 and 48 h post infection (hpi). Once they reach the hemolymph-bathed basal side of the midgut epithelium, those ookinetes that escaped destruction turn into oocysts, protected capsules within which the parasite will multiply asexually. Previous studies have established that the ookinete is the parasite stage most vulnerable to the mosquito's powerful immune response. As a consequence of this response, most mosquito species eliminate 100% of invading ookinetes and the parasite cycle is aborted. In a few parasite/mosquito combinations, up to 20% of the ookinetes survive and the disease can be further transmitted. A number of mosquito humoral antiparasitic proteins have been characterized (reviewed in (Blandin et al., 2008). The molecularly best characterized and phenotypically most prominent defense pathway mediating *Plasmodium* killing in *A. gambiae* involves a thioester-containing protein (TEP1) homologous to vertebrate complement factor C3 (Blandin et al., 2004; Baxter et al., 2007). Depletion of TEP1 by RNA interference renders mosquitoes hypersusceptible to *Plasmodium* infection, resulting in abnormally high infection levels.

Simultaneously to the midgut crossing by ookinetes, the physiology of the mosquito is profoundly modified to prepare for the laying of a clutch of eggs. Within 2 to 3 days, the ovary grows massively while 100-150 oocytes are maturing. Amino acids acquired during the blood meal signal via the TOR pathway to initiate massive synthesis of nutrient transport proteins in the mosquito fat body. These transport proteins include the lipid transporter lipophorin (Lp, Agap001826) (also called apolipoprotein II/I or retinoic and fatty acid binding protein, RFABG/P) and the phospholipoglycoprotein vitellogenin (Vg, Agap004203), a precursor of the yolk storage protein vitellin (Hagedorn and Judson, 1972; Valle et al., 1993). Both proteins are secreted into the hemolymph

(mosquito blood) 24 h post feeding (hpf) and transported to the ovaries. Vg, a large protein of 2051 residues with putative lipid binding and von Willebrand Factor (vWF) domains, is internalized by developing oocytes where it is proteolytically cleaved to generate vitellin (Cho et al., 1999). Lp, encoded by a single transcript, is composed of two subunits (of X and Y kDa) that together scaffold a lipidic particle —analogous to vertebrate LDL and HDL— containing a core of fatty acids and sterols, surrounded by an outer leaflet of phospholipids. These lipidic particles deliver lipids and fatty acids to energy-consuming tissues, including muscles, rapidly developing imaginal organs in larvae, and the ovaries in adult females. In addition to lipids, Lp particles serve as a vehicle for morphogen proteins in the imaginal discs of *Drosophila* larvae (Panakova et al., 2005). Interestingly, human HDL has been shown to host a fraction of complement factor C3 (Vaisar et al., 2007) as well as a trypanosome-killing activity of controversial molecular nature (Pays et al., 2006). In mosquitoes, recent studies (Mendes et al., 2008; Vlachou et al., 2005) have revealed that Lp is important both for reproduction and *Plasmodium* survival in the mosquito. Vlachou et al (2005) demonstrated that experimental depletion of Lp by RNA interference inhibited ovary development, but also reduced the number of developing *Plasmodium* oocysts in the mosquito midgut, which is the opposite phenotype to that observed upon depletion of the immune factor TEP1. This could be explained by a nutritional requirement for Lp in the early stages of parasite development. Indeed, Lp has recently been shown to be a source of lipid for developing *Plasmodium* oocysts (Atella et al., 2008). An alternative explanation is that the increasing levels of Lp following a blood meal may negatively impact mosquito immunity against parasites. Artificially blocking the physiological elevation of lipophorin levels would then allow the immune system to exert its full strength against the parasite. Here, we investigated the role of Lp in mosquito antiparasitic responses using the popular laboratory model of malaria transmission: *A. gambiae* mosquitoes infected with the rodent parasite *P. berghei* (Warr et al., 2008, Mendes et al., 2008, Blandin et al., 2004). We show that the reported effect of Lp on parasite survival depends on the immune protein TEP1. Therefore, we were particularly keen to know whether TEP1 and/or its partners are carried in the hemolymph by lipidic particles and whether this association negatively regulates TEP1 activity. We found that Lp associates with prophenoloxidase (PPO), an enzyme that catalyzes melanization reactions in insects. This association is specific to PPO as no other immune factor (including TEP1) could be detected in the lipid complexes. Still, we observed that depleting Lp, but also Vg, strongly stimulates

TEP1 binding to the parasite surface (and therefore, parasite killing). Further, while we observed that Lp (but not Vg) is absolutely required for oogenesis, the negative impact of Lp depletion on *Plasmodium* survival appeared to be predominantly mediated by Vg. We find that Vg expression is controlled both by Lp and by the Cactus/REL1/REL2 NF-kB transcription factors. Our results reveal an unexpected network of interactions between the expression of two nutrient transport proteins, NF-kB transcription factors known for their involvement in regulating immunity, and the capacity of TEP1 to bind and kill ookinetes.

Results

TEP1 knockdown rescues the *Lp* knockdown phenotype

We first examined whether the decrease in parasite loads and arrest in ovary development obtained after *Lp* knockdown (Vlachou et al., 2005) required TEP1 function. To this end, we compared the numbers of developing parasites in single *TEP1; Lp* and in double *TEP1/Lp* RNAi knockdowns by injecting double-stranded RNA (*dsRNA*). Four days after dsRNA injections, mosquitoes were fed on an infected mouse carrying GFP-expressing parasites (Franke-Fayard et al., 2004). 8 to 10 days later, mosquitoes were dissected to gauge prevalence of infection and mean oocyst numbers per midgut (supplementary Figure 1A). To our surprise, depleting TEP1 at the same time as Lp completely rescued the Lp phenotype, i.e. restored the high oocyst counts typically observed upon *TEP1* silencing. Therefore, the low oocyst counts seen in Lp-depleted mosquitoes are not due to a nutritional dependence of ookinetes on Lp-derived lipids, although lipophorin is required later for optimal oocyst growth (supplementary Fig 3 and Atella et al., 2008); instead, the absence of Lp renders ookinetes highly vulnerable to TEP1-dependent killing. In contrast, concomitant TEP1 silencing did not rescue the inhibition of the ovary development induced by Lp silencing, suggesting that the function of Lp in reproduction is independent of TEP1.

PPO2 and Vg, but not TEP1, associate with lipidic particles

The above results prompted us to examine whether TEP1 activity is modulated by a physical association of TEP1 with Lp. Lipidic particles from mosquito adults were purified using potassium bromide (KBr) gradient fractionation (Sun et al., 2000), and 10 fractions of 2 ml were recovered from the gradients. Coomassie staining of SDS-PAGE gels showed that the two subunits of lipophorin accounted for virtually all the protein

detectable in the top fraction (supplementary Fig 2 A), which is expected to contain the low-density lipoprotein. To determine whether some TEP1 co-fractionates with Lp, we performed western blot analysis with anti-TEP1 antibodies to probe these purified lipoproteins. We could not detect TEP1 in the Lp-containing gradient fractions at any time points before or after infection, suggesting that the major *Plasmodium*-killing factor does not molecularly interact with the lipidic particles under the conditions of this experiment (data not shown). Interestingly, a prophenoloxidase (PPO) recognized by the PPO2 antibody was present in the Lp-containing fractions (supplementary Fig 2B), indicating that some facets of insect immunity may involve an interaction between Lp and PPO. Indeed, such an association has been reported in studies of bacterial lipopolysaccharide aggregation in Lepidoptera (Rahman et al., 2006) and of hemolymph clotting in mosquito larvae (Agianian et al., 2007).

The absence of TEP1 in lipophorin-containing fractions might be due to disruption of some molecular interactions at the high salt (KBr) concentrations used for gradient fractionation. Therefore, we sought to purify Lp particles using the following independent method with more physiological buffers. Lp purified by KBr fractionation was used to immunize mice to generate monoclonal antibodies. We recovered eight antibodies, all of which recognized either the large or the small subunit of lipophorin. We selected one directed against the small subunit that proved efficient for immunoprecipitation. This allowed us to purify Lp without gradient fractionation. Western blot analysis of the immunoprecipitated Lp particles confirmed the absence of TEP1, although a fraction of the C-terminal TEP1 cleavage product was pulled down non-specifically by Sepharose beads (supplementary Figure 2C). Similarly, while control Sepharose beads in combination with non-specific antibodies did not pull down any Lp, they non-specifically pulled down high amounts of PPO and vitellogenin. This prevented us from obtaining an independent confirmation for the co-purification of PPO (or Vg, see below) with Lp observed in low-density KBr fractions.

We extended this analysis to two known components of the TEP1 pathway, APL1 and LRIM1, and asked whether Lp could modulate TEP1 function by sequestering these proteins. To this end, we probed the blots using anti-LRIM1 and anti-APL1 antibodies. No signal was detected with either antibody, suggesting that these proteins do not

associate with Lp purified either by KBr fractionation or by immunoprecipitation (data not shown).

To establish the protein composition of lipidic particles in an unbiased manner and detect other potential immune proteins in association with Lp, we submitted the Lp-enriched KBr fractions to gel electrophoresis and tandem mass spectrometry (MS) (Bilch and Mann, 2006). To maximize the sensitivity of protein identification, even the least abundant proteins in the complexes were included in the analysis using the "target approach", which involves the preparation of an exclusion list of highly abundant peptides followed by a second analysis of precursor ions to identify the less abundant proteins (Picotti et al., 2007).

The MS analysis of the top fraction of KBr gradients (which predominantly contains Lp according to Coomassie staining) revealed the presence of peptides corresponding to PPO2 and vitellogenin, but not to TEP1 or any other known immune factor (supplementary Table 1). Mass spectrometry analysis of immunoprecipitated Lp did not reveal additional mosquito proteins compared to the KBr gradient-purified Lp fraction (Vinh, Marois, Rono, Levashina, unpublished data).

Thus, although Lp co-purifies with a fraction of PPO2 and perhaps with other, yet unknown, immune factors, its adverse effect on TEP1 activity is apparently not explained by a physical interaction between Lp and the known components of the TEP1 pathway.

Vg is necessary for parasite and egg development

To investigate whether the adverse effects on immunity are a specific property of Lp or may be generalized to other nutrient transport factors, we tested if vitellogenin modulates TEP1 activity. Double stranded RNA against *Vg* (*dsVg*) targeting its lipid-binding and vWF domains was prepared as previously described (Blandin et al., 2002). We injected mosquitoes with *dsVg* and compared its effect on parasite development with *dsLacZ* and *dsTEP1* controls (Figure 1A). A significant, 4-fold reduction in mean parasite numbers was observed in the *dsVg* group compared with *dsLacZ* controls ($p<0.0001$). This effect was more profound than that of *dsLp* (Figure 2A). We then examined whether depletion of the major yolk protein would compromise ovary development. In contrast to *Lp*, knockdown of *Vg* did not completely block ovary

development but caused resorption of approximately 50% of ovaries compared to *dsLacZ* control mosquitoes (Figure 1B). Surprisingly, the efficiency of RNAi-mediated depletion of Lp and Vg could be verified directly by Coomassie staining of PVDF membranes (supplementary Figure 2D). To investigate whether Vg and Lp cooperate to sustain both oogenesis and parasite development, or are involved in independent processes, we performed double-knockdown experiments by simultaneously injecting *dsVg-dsLp* to compare with single injections of *dsVg* and *dsLp* controls prior to infectious blood feeding. As expected, *dsLp* completely blocked ovary development and the same was observed in concomitant *dsLp-dsVg* knockdowns. We then focused our attention on parasite development. Single *dsVg* ($p=0.0001$) and double *dsLp-dsVg* ($p<0.0001$) knockdowns caused similar reductions in oocyst counts; the reduction in oocyst numbers was stronger than in the single *dsLp* knockdown ($p=0.024$) (Figure 2A and 2B). These results suggest that Lp and Vg have distinct roles in reproduction and immunity. Lp appears to be more crucial than Vg for ovary development, whereas Vg influences the mosquito / *Plasmodium* interactions more strongly than does Lp. Furthermore, we note that the phenotypes of *Vg* and *Lp* knockdowns on parasite counts do not appear to be additive, suggesting that the two proteins are involved in a single process benefiting parasite development.

Vg, like Lp, inhibits TEP1-dependent parasite killing

We next determined whether the effect of Vg on parasite development was mediated by TEP1. To address this question, we performed triple knockdown experiments by injecting combinations of *dsTEP1, dsVg, dsLp* or control *dsLacZ*. The efficiency of *TEP1* and *Lp* silencing was confirmed by immunoblotting (Figure 4D). Again, total inhibition of ovary development was observed in all dsRNA combinations that included *dsLp*, suggesting that the ovary development is independent of TEP1 but absolutely requires Lp. In striking contrast, high parasites loads similar to that detected in *dsTEP1* single knockdowns were obtained when TEP1 was depleted simultaneously to Vg and/or Lp (Figure 3A and 3B). These findings imply that blocking the transport of lipids and vitellogenin-derived nutrients does not deter parasite survival. We conclude that the biggest impediment for parasite development around the ookinete stage is the TEP1-mediated immune pressure exerted by the vector. If this constraint is removed via TEP1 depletion, *Plasmodium* parasites can effectively exploit even reduced vector resources and proceed with the formation of oocysts. We note that oocysts may still exploit lipophorin for their later growth. Indeed, oocyst size at day 9 post infection was markedly

reduced when lipophorin (but not vitellogenin) was depleted (supplementary Fig 3), indicating that Lp but not Vg contributes nutrients to oocyst development. Secondly, the silencing phenotype of *Vg*, and to a lesser extend of *Lp*, on parasite development indicates that physiological levels of Vg and to a lesser extent of Lp both oppose TEP1-dependent parasite killing.

Vg and Lp do not affect *TEP1* expression or cleavage, but Lp is necessary for proper *Vg* expression

Previous work by Frolet et al. (2006) demonstrated that boosting mosquito basal immunity via inactivation of the inhibitory IkB protein Cactus up-regulates *TEP1* expression and completely blocks parasite development. Therefore, we asked whether the knockdown of *Vg* and *Lp* could mimick the effect of Cactus depletion and increase TEP1 expression levels, providing an explanation to the above observations. We inactivated *Lp* and/or *Vg* and examined the transcript levels of *TEP1, Lp* and *Vg* after infective blood feeding using quantitative real time polymerase chain reaction (qRT-PCR). Knockdown of these two nutrient transport genes did not alter *TEP1* expression (Figure 4A). Surprisingly, silencing of *Lp* delayed the expression of *Vg,* resulting in a clear shift of the *Vg* peak of expression from 24 to 48 h after blood feeding. In contrast, the depletion of *Vg* had no effect on *Lp* expression (Figure 4B and 4C). This result suggests that Lp transiently fine-tunes the expression of *Vg* after blood feeding.

We then evaluated the effect of *Lp* and *Vg* silencing on TEP1 protein amounts and cleavage in the hemolymph by western blot using anti-TEP1 polyclonal antibodies. This analysis did not reveal significant changes in the amounts of full length or cleaved TEP1 proteins (Figure 4D). Similarly, Lp protein levels were unaffected by *dsVg* injection. Monitoring the depletion of Vg and Lp by Coomassie staining of PVDF membranes (supplementary figure 2D) allowed us to detect a difference in Vg levels at 24 hpf in *dsLp*-treated mosquitoes compared with the controls, confirming that Lp is indeed facilitating Vg expression at early time points post blood-feeding. Therefore, we propose that the observed effect of *Lp* knockdown on parasite counts may be an indirect consequence of delayed Vg expression. In this scenario, Vg may be the main negative regulator of TEP1 activity, consistent with its stronger KD phenotype compared with Lp as far as parasite development is concerned.

Vg and Lp inhibit TEP1 binding to the parasite surface

How do Vg and Lp reduce the antiparasitic activity of TEP1? Binding of cleaved TEP1 to the parasite surface is one of the first steps leading to parasite killing; either increasing or reducing this event greatly influences the outcome of the killing efficiency (Frolet at al., 2006). We did not observe any change in the expression or cleavage of TEP1 in mosquitoes lacking Vg and/or Lp; yet parasite killing efficiency was increased. We suspected that physiological levels of Vg and Lp might reduce the binding of TEP1 to parasites, thereby inhibiting parasite killing. To test this hypothesis, we performed time course analyses of TEP1 binding to ookinetes and their subsequent killing in dsRNA-injected mosquitoes. At early time points (18 and 24 hpi) TEP1 binding to ookinetes did not differ in the Vg or Lp depleted versus control mosquitoes; but at 48 hpi more than 75 % of ookinetes were TEP1 positive (either dead or moribund) in *dsVg* or in ds*Vg-Lp* injected mosquitoes versus only approximately 40% in *dsLacZ* controls (Figure 4E), similarly we observed increased TEP1 activity in *dsLp* (supplementary figure 1B). This strongly suggests that physiological levels of Vg and Lp inhibit TEP1 binding to parasites once the invasion phase is completed.

Depletion of Cactus represses *Vg* expression

Previously, we established that expression of several components of the TEP1 pathway is controlled by NF-kB factors (Frolet et al., 2006). Boosting the basal immunity by inactivating the repressor Cactus (IkB) upregulates the activity of NF-kB factors REL1 and REL2, elevating the expression of TEP1 and other immune factors, leading to a complete block in parasite development. Cactus depletion not only promotes parasite killing but also arrests ovary development (supplementary figure 2E, to be included). These observations prompted us to investigate whether NF-kB factors Rel1, Rel2 and Cactus control expression of *Vg* and *Lp*. To this end, mosquitoes were injected with either: *dsRel1, dsRel2, dsCactus* or co-injected with *dsRel1-dsRel2*, *dsRel1-dsCactus*, *dsRel2-dsCactus* and *dsLacZ* control. Silenced mosquitoes were fed on an infected mouse and expression of *Vg* and *Lp* was monitored by qRT-PCR. Strikingly, *Vg* expression was drastically reduced in *dsCactus* at 24 hpi; conversely, the depletion of NF-kB Rel1 or Rel2 at this time point induced expression of *Vg* above the *LacZ* control. Thus, while *TEP1* expression is upregulated, *Vg* expression is directly or indirectly repressed by Rel1/2. Therefore, Cactus regulates TEP1 and Vg in opposing directions. We extended our analysis to Lp, but its expression was unaffected by the knockdown of

NF-kB factors (Figure 5A and 5B). Interestingly, concomitant silencing of *Cactus/Rel1* restored *Vg* expression to physiological levels (Figure 5C)

Taken together, our findings reveal that depletion of *Cactus* lowers the levels of an inhibitor of TEP1 activity *(Vg)* while simultaneously inducing TEP1 expression, thereby stimulating TEP1 activity at two different levels.

Discussion

The first indication that nutrient transport after a blood meal influences mosquito immunity against *Plasmodium berghei* was provided by Vlachou et al. (2005), who demonstrated that experimental depletion of the lipid transporter protein Lp by RNA interference reduces the number of developing oocysts in the mosquito midgut. Recently, the same was observed in the interaction of *A. gambiae* with *P. falciparum* (Mendes et al., 2008). Without invoking immunity-related interpretations, this could suggest that developing *Plasmodium* consume high levels of lipids and must hijack some of their host's lipidic particles for their growth, which was indeed recently demonstrated at the oocyst stage (Atella et al., 2008). However, parasite starvation through lipid deprivation does not necessarily imply the death of the parasite at the ookinete stage. We report here that high numbers of parasites do survive and turn into oocysts even in the absence of the nutrient transport proteins Lp and Vg, as long as the immune factor TEP1 is also experimentally depleted. These oocysts will not reach as large a size as those found in control mosquitoes (supplementary Figure 3), in agreement with the fact that oocysts tap some of the host's lipophorin for their growth (Atella et al., 2008). Therefore, physiological levels of both nutrient transport proteins following a blood meal normally dampen the strength of the immune defense and allow a larger number of ookinetes to escape destruction by the TEP1 pathway. The effects of LP and Vg depletion on parasite survival are not additive, suggesting that the two proteins act in the same process. We observed that the Vg effect on parasite survival is stronger than that of Lp, and that Lp is required for the full induction of Vg expression (both at the mRNA and protein level) following a blood meal. Thus, Lp may only indirectly inhibit TEP1 activity via its influence on Vg levels, whereas Vg would impinge directly –or more closely- on the actual molecular process that inhibits parasite killing.

Our quantitative PCR studies of Vg expression refined our knowledge of the transcriptional events influencing parasite success after a blood meal. *Vitellogenin* expression in the fat body is thought to be induced by the blood meal, through activation

of the TOR signaling pathway by released amino acids (Hansen et al., 2004). In addition, we show here that *Vg* expression is influenced by the levels of lipophorin (itself also induced by the blood meal) and by NF-kB factors Cactus, Rel1 and Rel2, known to control innate immunity (Frolet et al., 2006). Additional signals arising from the parasite's invasion render this picture still more complex. Indeed, ookinete invasion of the midgut induces lipophorin mRNA expression further than does an uninfected blood meal (Vlachou et al., 2005, Cheon et al., 2006). At the protein level, we did not observe a corresponding increase in Lp amounts using specific antibodies (data not shown), which may reflect consumption of the extra lipophorin by parasites and suggest that Lp homeostasis is under tight physiological regulation. Conversely, Ahmed et al. (2001) reported that parasite invasion reduces the abundance of the *Vg* transcript but that Vg protein is only transiently reduced before accumulating in the hemolymph. Therefore, the production of both proteins is subjected to multiple physiological switches. To our knowledge, Vg and the Cactus NF-kB transcription factor are the first molecules reported to occupy a central position between reproduction and immunity, providing a handle to explore the long-suspected trade-off between these two processes (Reviewed in Hurd, 2001).

What is the molecular basis of the negative effect of the two nutrient transport proteins on the TEP1 pathway? We initially hypothesized that lipophorin-scaffolded lipidic particles could sequester components of the TEP1 pathway in an inactive state. This idea was supported by a body of literature linking (1) vertebrate HDL and some effectors of innate immunity in vertebrates, including complement factor C3 (Raper, 1996, Whitten et al., 2004, Lange et al., 2005, Vaisar et al., 2007), which belongs to the same family as TEP1; (2) lipophorin and immune reaction in insects (Taniai et al., 1997, Kato et al.,1994); and (3) the known role of Lp particles as vehicles for morphogen and gpi-linked protein transport in *Drosophila* imaginal discs (Panakova et al., 2005). This prompted us to examine Lp-associated proteins in mosquito adults. We purified lipophorin particles using two independent methods (KBr gradient fractionation and immuno-precipitation with a mouse monoclonal anti-Lp antibody) and searched for associated proteins either by western blot with antibodies directed against known immune factors, or in a more unbiased manner by mass spectrometry analysis of the purified Lp particles. Indeed, we observed that a fraction of prophenoloxidase (PPO), an enzyme mediating melanotic encapsulation during insect defense, co-purifies with Lp in

potassium bromide gradients. PPO may interact directly with Lp, or co-fractionate in the low-density part of the gradient via its association with uncharacterized lipid compounds. PPO mediates melanization of dead *Plasmodium* ookinetes (Volz et al., 2006, Blandin et al., 2004). However, previous work in our laboratory (Shiao et al., 2006) established that it probably does not play a part in parasite killing in *A. gambiae*. TEP1 and its interacting partners LRIM1 and APL1 were not detectable in the Lp extracts, suggesting that the *Plasmodium*-killing machinery is probably not transported on lipophorin particles. This left intact the question of the mechanism by which Lp/Vg impinge on TEP1 activity. Further examination of the *Plasmodium* infection process in Lp/Vg-depleted mosquitoes allowed us to demonstrate that the negative effect of the transport proteins on *Plasmodium* killing results from a slower kinetics of parasite opsonization by TEP1. Indeed, RNAi-mediated depletion of Lp and especially of Vg accelerated TEP1 binding to the surface of ookinetes, promoting their killing. We can speculate that Vg (and perhaps Lp, to a lesser extent) may be recruited to the parasite surface, where they could mask a binding site for TEP1. Alternatively, there may be a physical interaction between TEP1 and Vg that would inhibit TEP1 activity. The tools to test these hypotheses are under development in our laboratory.

Experimental procedures

Lipophorin purification

100 mosquitoes were severed by opening the thorax and abdomen cuticles with fine forceps and bled on ice in a 2-ml Eppendorf tube containing1 ml TNE buffer with protease inhibitors (Complete, Roche). The sample was cleared by multiple centrifugations (twice 2000 g, three times16,000 g) to remove mosquito debris, fat and cells. Resulting extracts were gently rocked for 1h at 4°C with 1.5 µl of mouse ascites containing an irrelevant IgG2ak monoclonal antibody, 35 µl of protein A-sepharose slurry were added and samples were again rocked for 1h at 4°C. Sepharose beads were removed by centrifugation, 1.2 µl of mouse ascites with anti-lipophorin IgG2ak monoclonal antibody was added to the supernatant, incubated 1h as above, then 1h with protein A sepharose beads. Beads were collected by centrifugation and washed 5 times 5 minutes in TE buffer with or without 500 KCl, alternatively. Lipophorin and associated proteins were eluted from the beads using protein sample buffer and submitted to mass spectrometry.

Polyacrylamide gel electrophoresis

Approximately 1 mg of protein sample was resolved on 10% SDS gel and cut into 15 gel slices of coomassie stained protein bands and subjected to standard in-gel trypsin digestion protocol (Shevchenko et al., 1996, Ishihama., 2006). Digested peptides were cleaned, concentrated and selectively enriched using simple, self-made stop-and-go-extraction tips (StageTips) (Rappsilber et al., 2003).

Nano-LCMS^3

Tandem mass spectrometry analysis (LC-MS3) was performed on an Agilent 1100 nanoflow system coupled to a LTQ-FT mass spectrometer (Thermo Electron, Bremen, Germany) equipped with a nanoelectrospray source (Proxeon Biosystems, Odense, Denmark). The mass spectrometer was operated in data-dependent mode to automatically switch between MS, MS2 and MS3 acquisition as previously described (Pilch and Mann, 2006).

Data analysis

A peak list was generated containing the precursor masses and the corresponding MS/MS fragment masses were generated from the original data file using and searched in the annotated A. gambiae database (Ensembl version 43) combine with P. berghei database (GeneDB) and mouse IPI database (European Bioinformatics Institute) using the Mascot program (Matrix Science). MS3 spectra were automatically scored with MSQuant, open-source software, a validation tool which parses Mascot peptide identifications and enabling their manual and automated validation. Proteins were considered positively identified if they had at least two fully tryptic peptides of more than six amino acids and a Mascot score of at least 26 (95% significance level) for one of the peptides and at least 33 (99% significance level), proteins identified by single peptide hit required a combined score from MS3 above 43 (Pilch and Mann.,2006). Relative protein abundance between the different samples was based on the total number of unique peptides identified for each protein (Lasonder et al., 2002).

Cloning and dsRNA production

A Vg1 HincII-HincII 630-bp-long fragment was cloned from cDNA library clone into pLL10 vector. DsRNA was synthesized as previoulsy described (Blandin et al 2004). Other genes were cloned in the same manner from corresponding cDNA library clones or PCR products (see table 1)

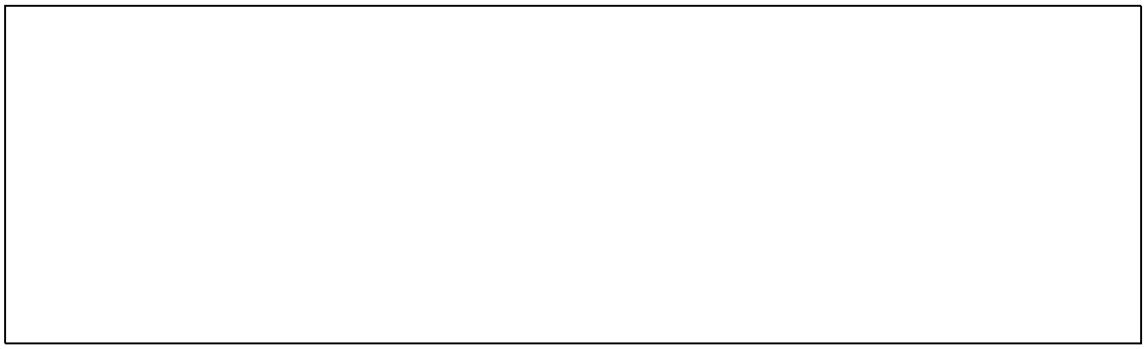


Table 1. Cloning and transcriptional analysis of selected genes

The table displays primer pairs (forward:frw, reverse:rev) used to examine transcriptional profiles of selected genes using quantitative real-time polymerase chain reaction(qRTPCR) and the respective conditions used to clone them into expression vector for dsRNA sythesis.

dsRNA injection in Mosquito and infection

A. gambiae–susceptible G3 strain were maintained at 28 °C, 75%–80% humidity, and a 12/12 h light/dark cycle. 2 days-emerged adult female mosquitoes from the same cohort were injected with 0.2 µg of dsRNA using a Nanoject II injector (Drummond, http://www.drummondsci.com). Co-injection experiments were performed by injecting a double volume of 1:1 mixtures of 3-µg/µl solutions of dsRNAs. Four days after dsRNA injection mosquitoes were fed on an a mouse carrying with *P. berghei* GFP-con 259cl2 as previously described (Blandin et al., 2004, Shiao et al., 2006).

Quantitative Real-Time PCR

Total RNA from 10 mosquitoes was extracted with Trizol reagent (Invitrogen) before and after dsRNA injection or after feeding of. Approximately 2µg of RNA was reverse transcribed using M-MLV enzyme and random primers (Invitrogen). Specific primers were designed using PrimerSelect (DNA Star).

To asses the expression of Vg, Lp, TEP1 among other selected genes, the following primer pairs were selected (see table 1), and an internal control Ribosomal protein L19 (RPL19), forward 5- CCAACTCGCGACAAAACATTC-3′, reverse 5′-ACCGGCTTCTTGATGATCAGA-3′. The reactions were run on an Applied Biosystems 7500 Fast Real-Time PCR System using Power SYBR Green Mastermix (http://www.appliedbiosystems.com).

Fluorescence microscopy.

In order to guage the number of surviving GFP expressing parasites, mosquito midguts were dissected between between 7 and 10 dpi and prepared as previously described (Blandin et al., 2004, Shiao et al., 2006) and observed under a fluorescence microscope. To asses TEP1-binding to ookinete, mosquito midguts were dissected at 18, 24 and 48 hpi, washed on ice, fixed in 4% paraformaldehyde at room temperature for 45 min, then washed with phosphate buffered saline and stained with anti TEP1 antibodies as previously described (Blandin et al., 2004, Frolet et al., 2006). Parasite numbers were scored using a Zeiss fluorescence microscope (Axiovert 200M) equipped with a Zeiss Apotome module (http://www.zeiss.com). Live parasites were identified by GFP expression while dead parasites were GFP negative. Differential TEP1 staining on ookinete were gauged at 18, 24 and 48 hpi. Atleast three independent experiments were conducted for per treatment group with a minimum of five mosquito midguts

Manuscript Figures

Figure 1

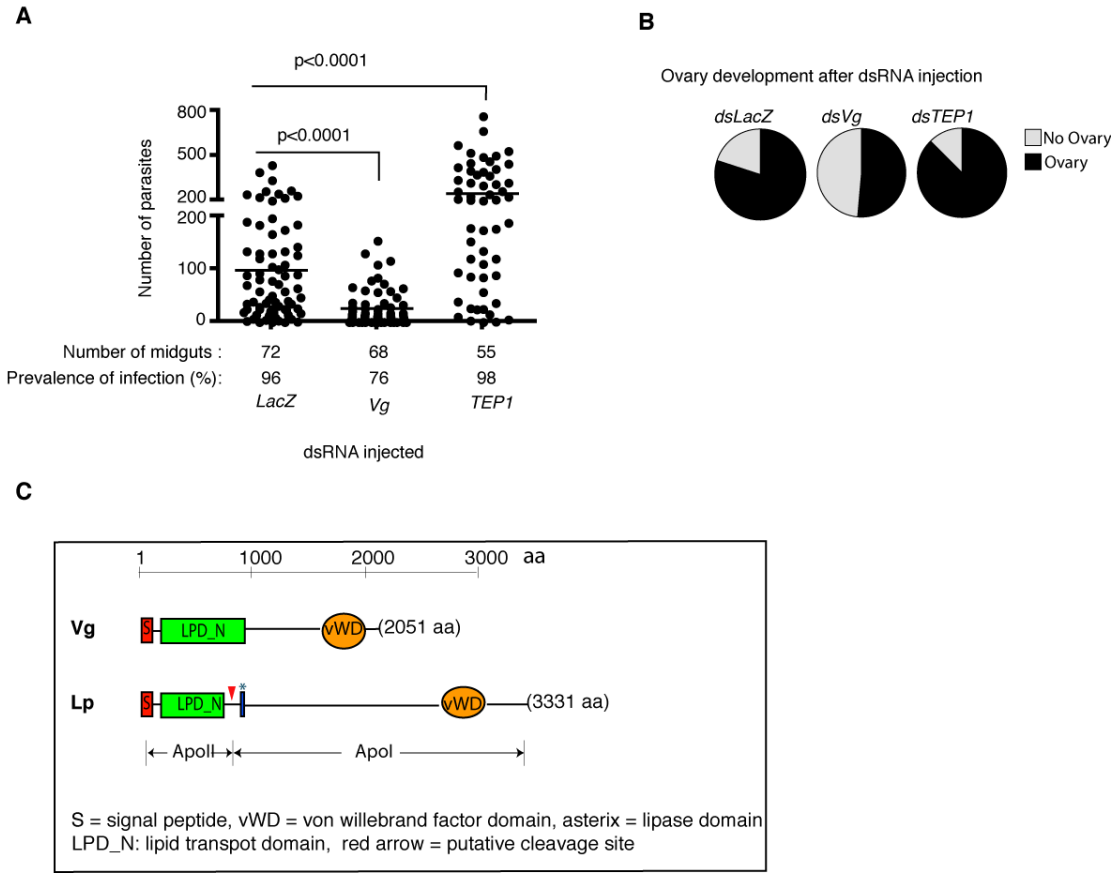


Figure 1. Depletion of *Vg*, a putative lipid transport molecule reduces parasite survival and negatively impacts on ovary development**.**
A: Mosquitoes were injected with *dsLacZ, dsVg* and *dsTEP1*, infected with *P. berghei*, parasite development gauged 7-9 days post infection. Each dot represents the number of oocyst developing per midgut. The depletion of *Vg* statistically reduced parasite development, B: KD *Vg* negatively impacted on ovary development 7dpi, C: Vg and Lp protein sequences display similar structural features (lipid transport and von willebrand factor domains).

Figure 2

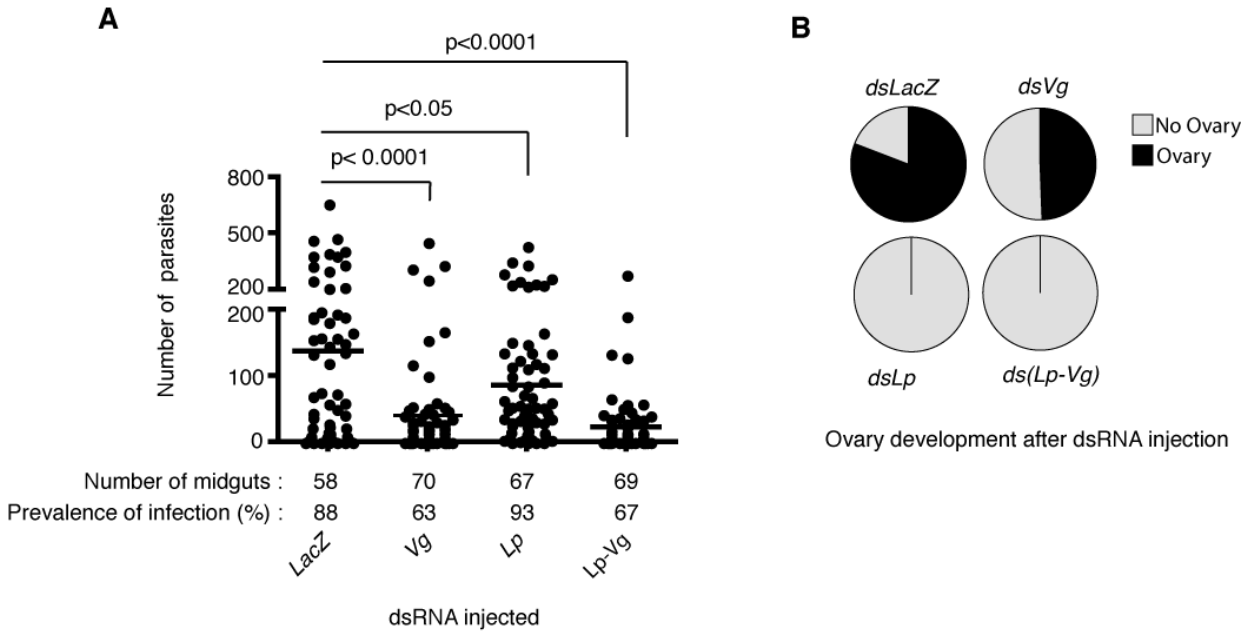


Figure 2. Vg and Lp are involved in the same mechanism involving parasite and ovary development.
Mosquitoes were injected with *dsVg, dsVg-Lp or dsLp*, infected with *P. berghei*. A: parasite and B: ovary development development was gauged 7-9 days post infection and compared to controls *dsLacZ/ dsTEP1*. The depletion *Vg* in single or double KD with *Lp* drastically reduced parasite development, while the KD of *Lp* completely blocked ovary development.

Figure 3

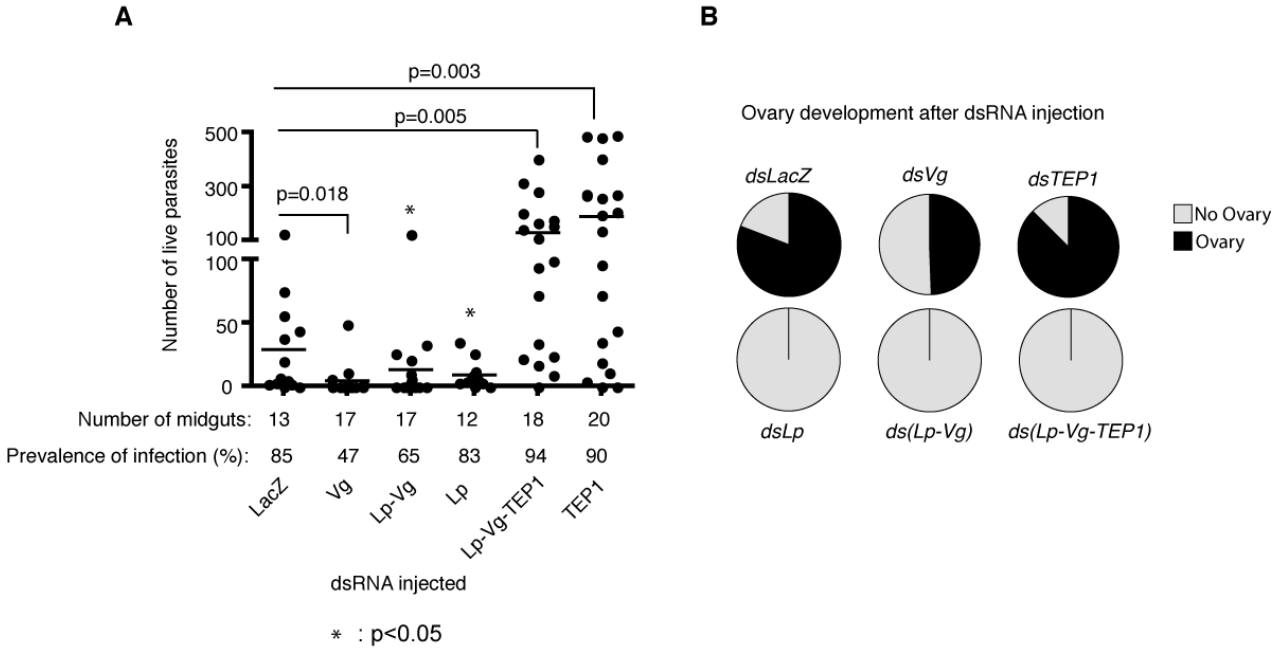


Figure 3. Vg and Lp are involved in TEP1-depent parasite killing mechanism.
Concomitant silencing of *TEP1-Vg-Lp* A: reversed parasite killing observed in the depletion of *Lp* or *Vg* in single or double KD and B: the triple KD *TEP1-Lp-Vg* did not rescue ovary development

Figure 4

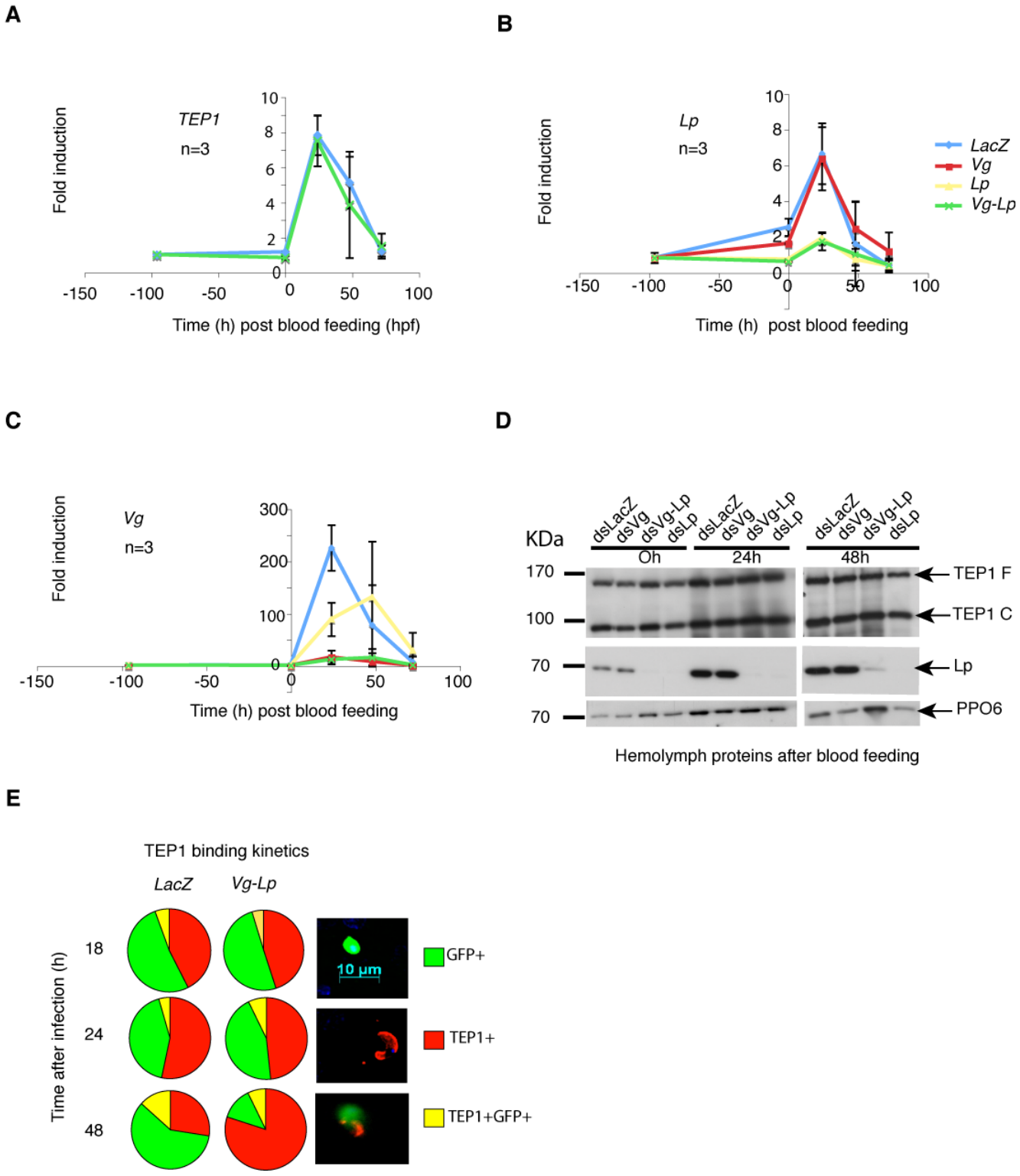


Figure 4. Vg and Lp do not affect TEP1 expression and processing but *Vg* inhibits TEP1 binding to Parasites.

Mosquitoes were injected with *dsLp, dsVg* or *dsLp-Vg*. In A, B and C: *TEP1, Vg* and *Lp* expression were gauged at several time points after *P. berghei* infection using quantitative real time PCR (qRTPCR) and in D: The amounts of TEP1 (full length and processed) in mosquito

hemolymph was gauged by immunoblotting. E: TEP1 binding to ookinetes was gauged by immunoflouresence assay (IFA) after the depletion of *Vg* and *Lp*

Figure 5

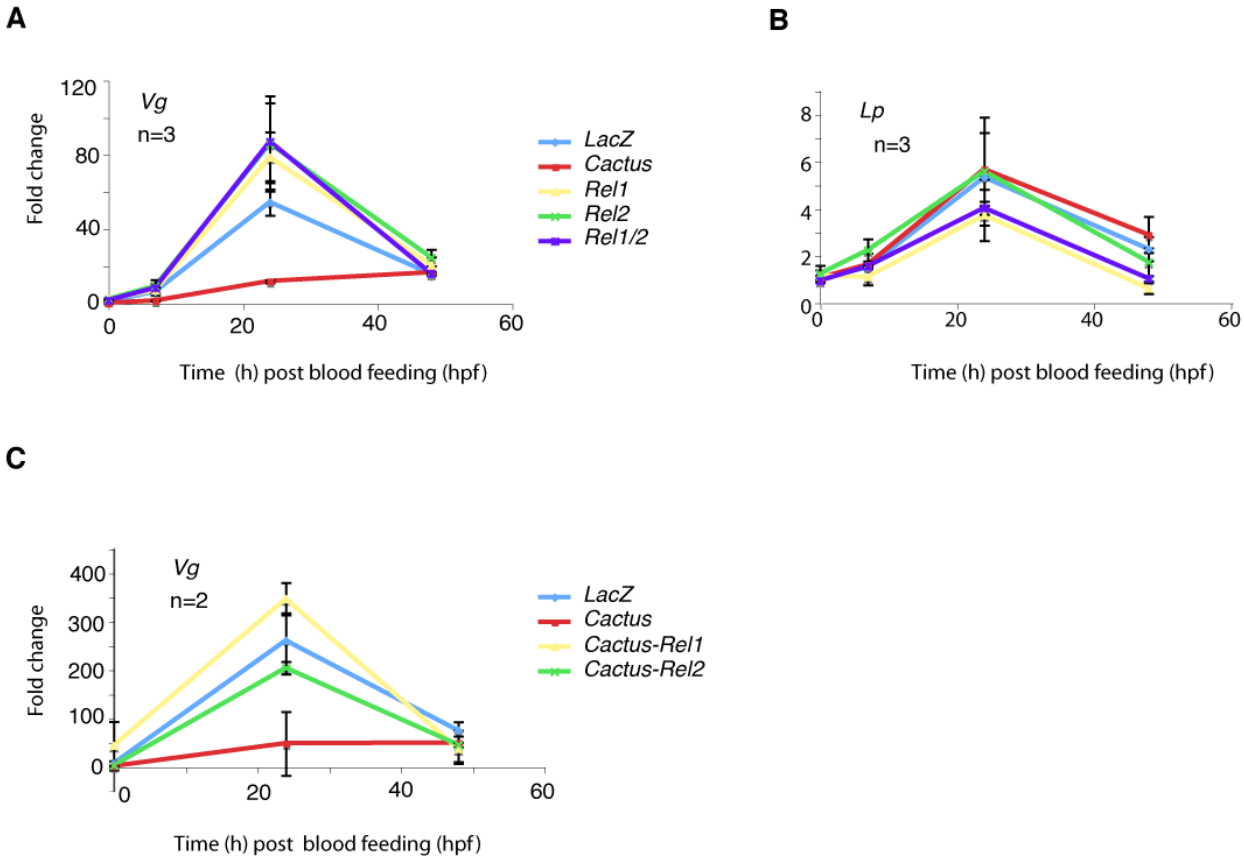
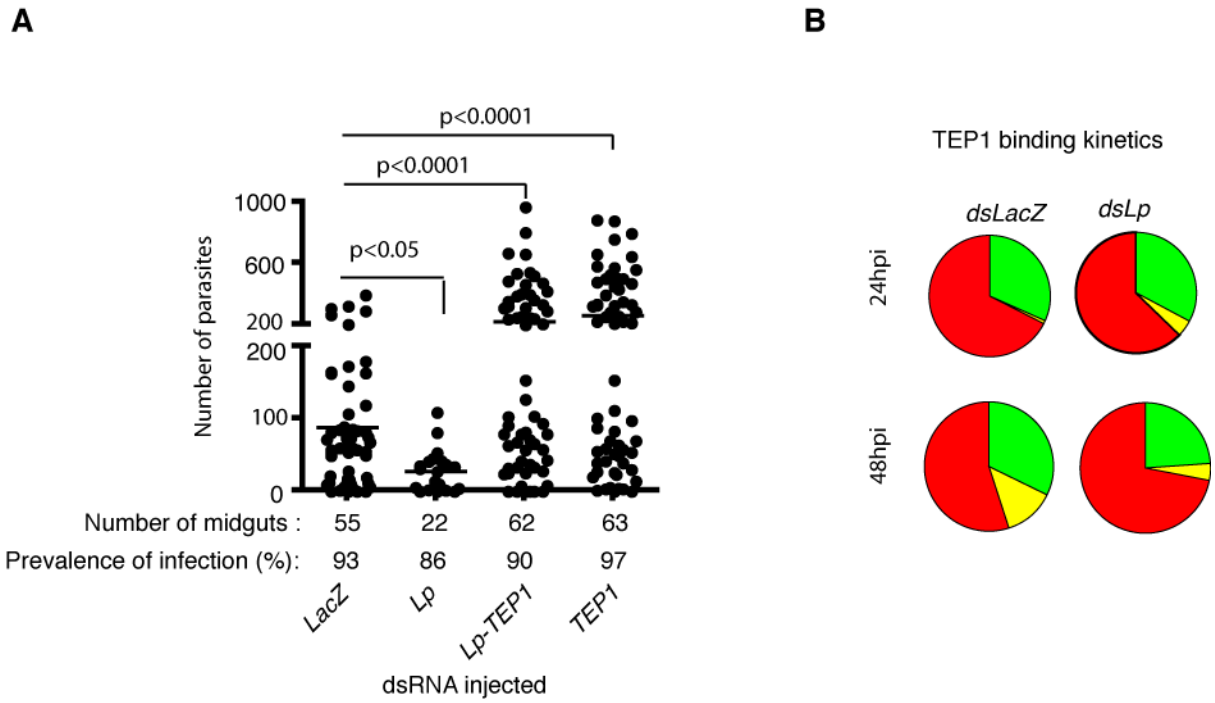


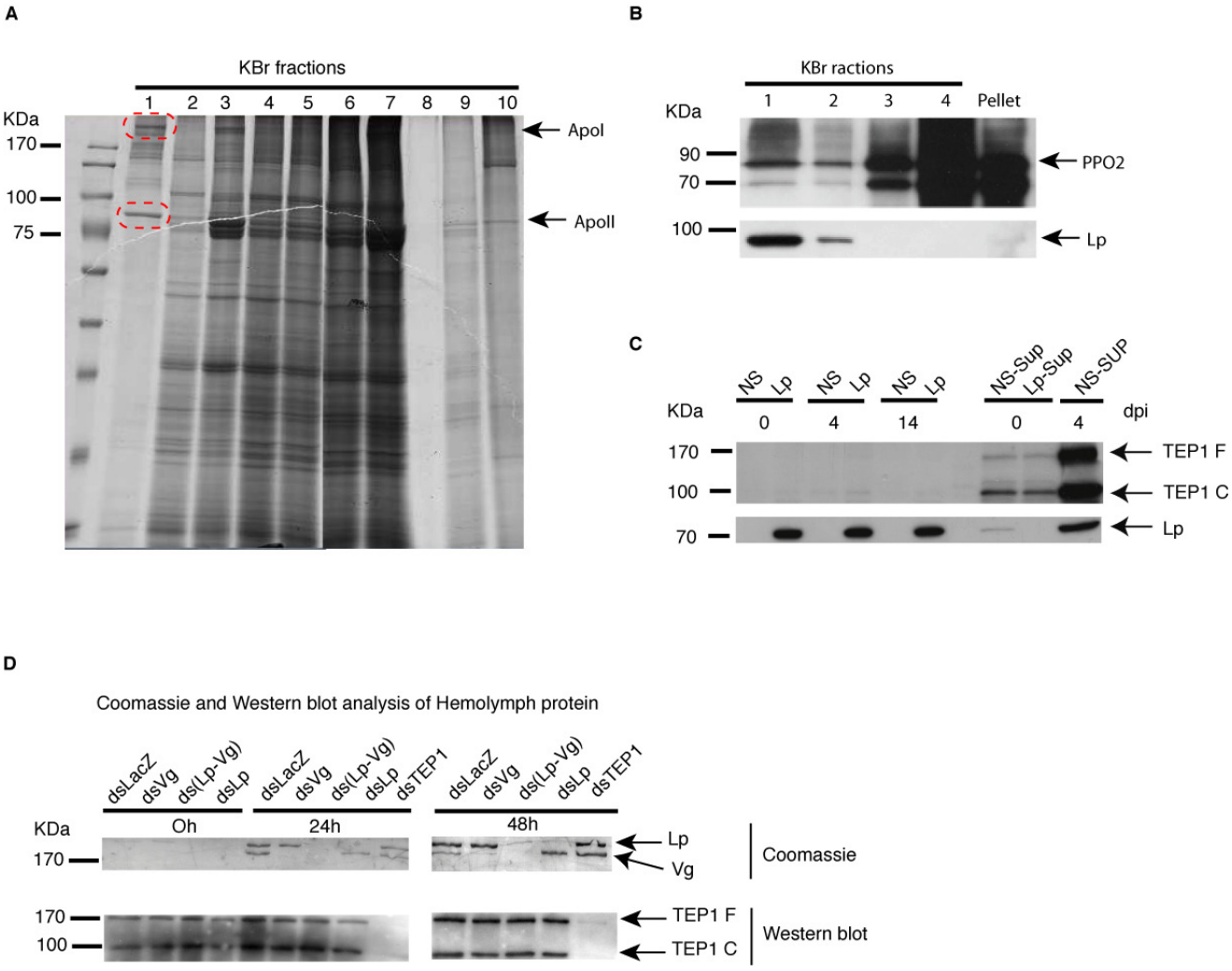
Figure 5. *Vg* expression is negatively controlled by NF-κB factors Rel1/Rel2
In A and B: Mosquitoes were injected with either *dsCactus, dsRel1 or dsRel2*, blood fed and the expression of *Vg* and *Lp* examined by qRTPCR at various time points after infectious feeding and compared to *dsLacZ* control. *Vg* expression was inhibited in *dsCactus* while elevated in *dsRel1/Rel2* treatment groups, C: concomitant depletion of *Cactus* and *Rel1* restored *Vg* expression.

Supplementary Figure 1



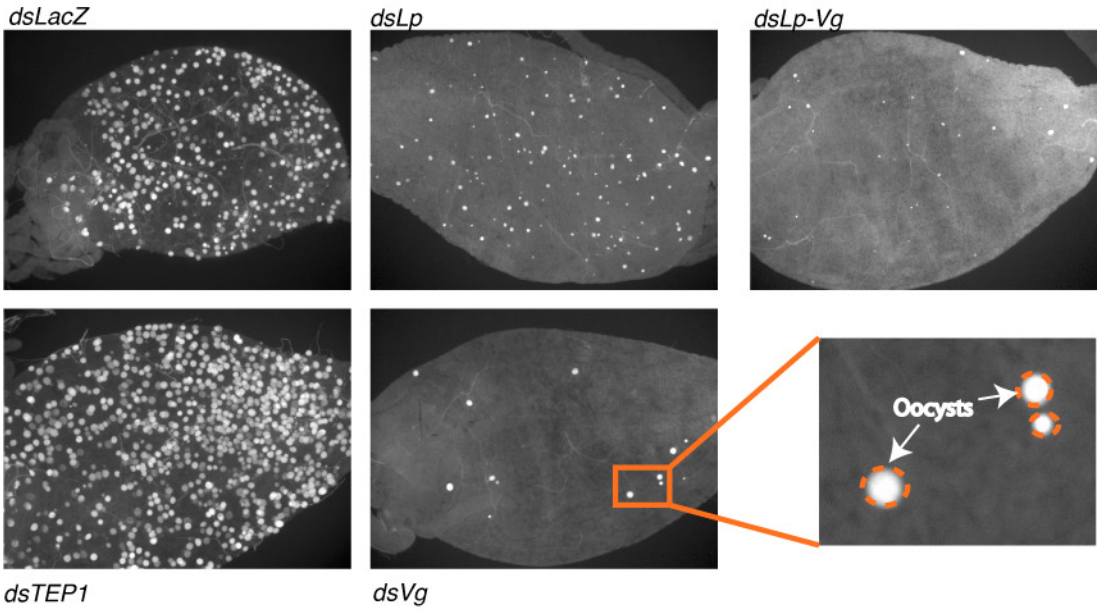
Supplementary figure 1. Parasite killing in *dsLp* rescues TEP1 activity
Mosquitoes were injected with either *Lp, LacZ*, *TEP1* or *dsLp-TEP1* and infected with *P. berghei*. Parasite development was gauged 7dpi by counting the number of GFP expressing oocysts. Concomitant silencing of *TEP1* and *Lp* significantly reversed parasite killing observed in *dsLP*

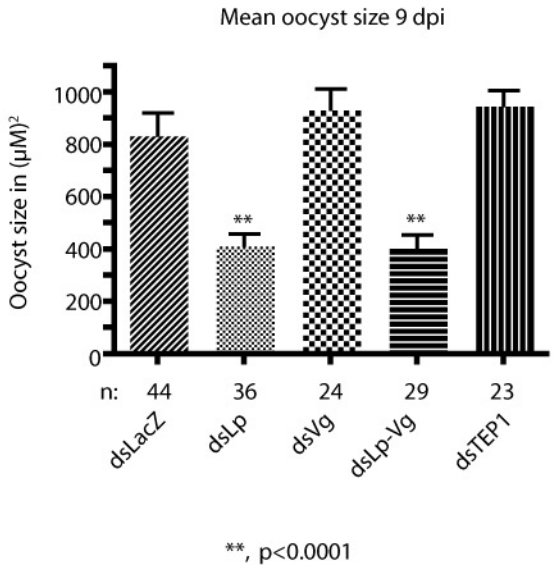
Supplementary Figure 2



Supplementary figure 2. PPO2 associates with Lp but not TEP1
A: SDS PAGE of Potassium bromide gradient from mosquito tissues, lane 1 mosquito lipophorins, B: Immunobloting analysis of KBr fractions (1-4 and pellet) with anti PPO2 antibodies. PPO2 associates with lipophorins and in C: Immunobloting analysis of lipophorins purified by immunoprecipitation with antibodies directed against purified lipophorin. TEP1 does not associate with lipophorins at different times after *P. berghei* infection

Supplementary Figure 3





Supplementary figure 3. Lp is required for oocyst development
Parasite development was gauged 8 dpi by estimating the size of oocysts in mosquitoes after the depletion of *Lp, Vg* or double KD *Lp-Vg* compared to controls KD *TEP1* and *LacZ.* Pictures of dissected midguts were analyzed by axiovision software and parasite size estimated by the area covered by each individual oocyst and averaged as a mean size for each gene. The depletion of *Lp* in single or double statistically reduced the size of parasite development compared to controls KD *LacZ*

Vitellogenin Cathepsin B inhibits parasite killing in *A. gambiae*

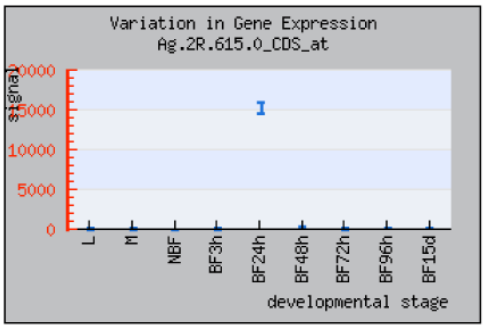
Introduction

After a female mosquito has ingested a blood meal, accumulation of amino acids in the mosquito hemolymph activates signaling by the nutrient-sensitive target of rapamycin (TOR) pathway to initiate the production of *Vg* in the fat body (Hansen et al., 2004). Vg is internalized and accumulates in developing follicles. Vg is cleaved by vitellogenin cathepsin B (VCB), a cystein (proapoptotic) protease that is similarly produced 24h after blood feeding by the fat body and accumulates in the ovaries (Marinotti et al., 2006, Cho et al., 1999). Previously, we established that Vg, the major yolk protein in mosquitoes negatively regulates parasite killing by inhibiting TEP1 binding to ookinetes. We also demonstrated that NF-κB factors Rel1 and Rel2 negatively regulate the expression as *Vg*. Having made these observations, we were keen to identify other genes that function with *Vg* and interfere with parasite killing. To this end, we interrogated a combined expressional database (Marinotti et al., 2006; Wyder et al., unpublished data) to find genes whose expression was similar to Vg in various knock downs. Of particular interest to us, were genes only induced 24h after blood feeding as well as being under the control of NF-κB factors Cactus/Rel1.

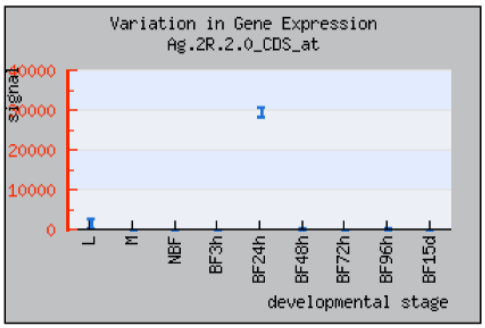
We selected three genes according to their expression profiles that matched *Vg*: Two members of the cathepsin B family (AGAP004531), (AGAP004534, also referred to VCB) and MD-2 like protein with a lipid recognition domain (AGAP002851). We extended our analysis to include a third cathepsin B which had a distinct expression profile (AGAP004533) as a negative control (Figure 3-1 and Marinotti et al., 2006). Interestingly, Vg and VCB were shown to be induced in female mosquitoes lower reproductive tracts after mating (Rogers et al., 2008). Therefore, suggesting that these genes may be under similar regulatory mechanism, potentiating for their involvement in similar activities such as antiparasitic responses. Thus we functionally analyzed these genes by dsRNA injections to gauge their role in parasite and ovary development. Here we show that VCB negatively regulates TEP1 dependent parasite killing and that the NF-κB factors Cactus/Rel are implicated in the expression of VCB, which reminiscent of our previous observation with Vg.

Candidate genes selected by expression profile after blood feeding

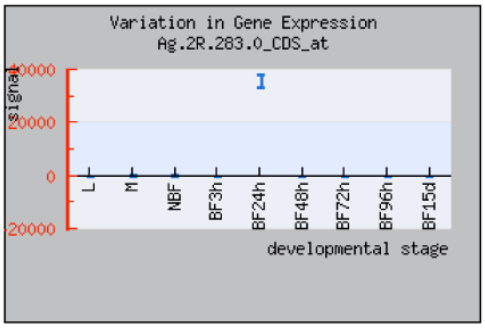
Agap004534 (VCB)



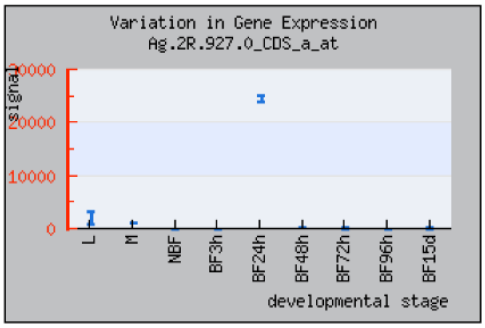
Agap004203 (Vg)



Agap004531(CathB)



Agap002851(MD-2 like)



Agap004533(CathB)

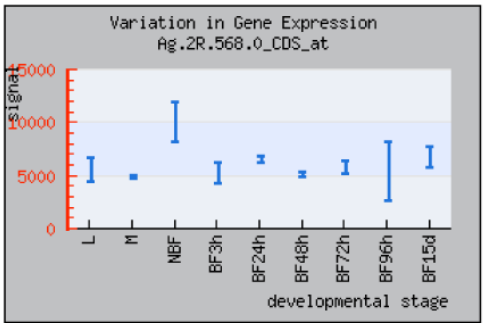


Figure 3-1. Transcriptional profiles of candidate molecules in susceptible mosquitoes after at blood feeding

The expression of candidate molecules in mosquitoes at different time points compared to non blood fed (NBF) larvae (L) and male (M) (Marinotti et al., 2006).

VCB is negatively regulated by NF-κB factors Rel1/2 and repressed in *TEP1 GOF* transgenic mosquitoes

In order to examine the expression of VCB (among other candidate genes) in various genetic backgrounds modified by the deletion of NF-KB factors. We designed qRTPCR primers specific to *VCB, MD-2*, *AGAP004531(VCB-2)* and *AGAP004533 (VCB-3)*, and examined the expression of these genes at various time points after *P. berghei* infection in mosquitoes treated with *dsCactus, dsRel1* or *dsRel2*. We did not observe any significant difference in the expression of *AGAP004531* and *AGAP004531* after depletion of the NF-κB factors compared to *dsLacZ* (data not shown). Strikingly similar to *Vg*, the depletion of *Cactus* inhibited *VCB*, while *dsRel1* or *dsRel2* elevated the expression of *VCB.* Moreover, concomitant injection of *dsCactus-Rel1* rescued the *dsCactus* effect on VCB expression to physiological levels, whereas only partial rescue was observed in *dsCactus-Rel2* double KD (Figure 3-2 A & B). These results suggest that Rel1/Cactus signaling cassette negatively controls the expression of VCB.

Next, we compared VCB expression in GOF (strong immune response and a slight elevation of TEP1) and LOF (hyper susceptible to parasite infection, diminished TEP1 expression) transgenic mosquitoes. We used microarray data of *A. gambiae* midgut tissues infected with *P. berghei* (S. Wyder, P. Irving, S.H Shiao, L. Troxler et al., unpublished data). Surprisingly *VCB* was negatively regulated in the *TEP1 GOF* mosquito strains compared to the laboratory susceptible model G3 mosquitoes (Figure 3-2C & D). Our data suggests that VCB is repressed with increased TEP1.

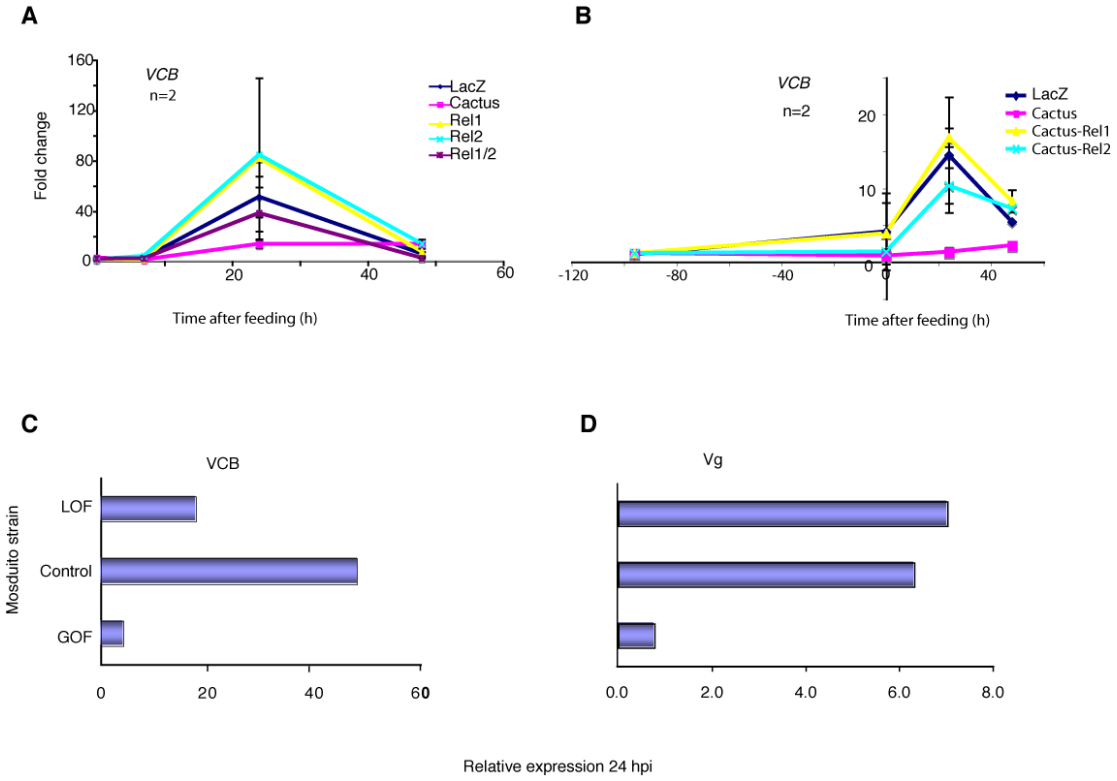


Figure 3-2. *VCB* expression is negatively regulated by Cactus signaling cassette

A: The expression of *VCB* was monitored by qRTPCR at different time points after blood feeding in mosquitoes silenced for *Cactus, Rel1* or *Rel2*, **B**: *VCB* expression was analyzed by qRTPCR after concomitant knockdown (kd) of *Cactus-Rel1* or *Cactus-Rel2* compared to single kd *Cactus* at various time points after feeding, **C & D:** Microarray analysis of Vg and VCB 24 h after infection with *P. berghei* in midgut tissues of the *TEP1 GOF* and *LOF* mosquitoes compared to susceptible strain (Wyder et al unpublished data).

VCB inhibits TEP1-mediated parasite killing

Next we examined the function of VCB in mosquitoes during parasite development by gene silencing. Indeed similarly to *Vg*, *VCB* depletion resulted in a 2-fold reduction in the number of developing oocysts (Figure 3-3A). We did not detect any significant effect on ovary development (Figure 3-3B). However, there were no significant changes in ovary or parasite development in mosquitoes injected with dsRNA directed against VCB-2, VCB-3 or *MD-2* (Figure 3-3D).

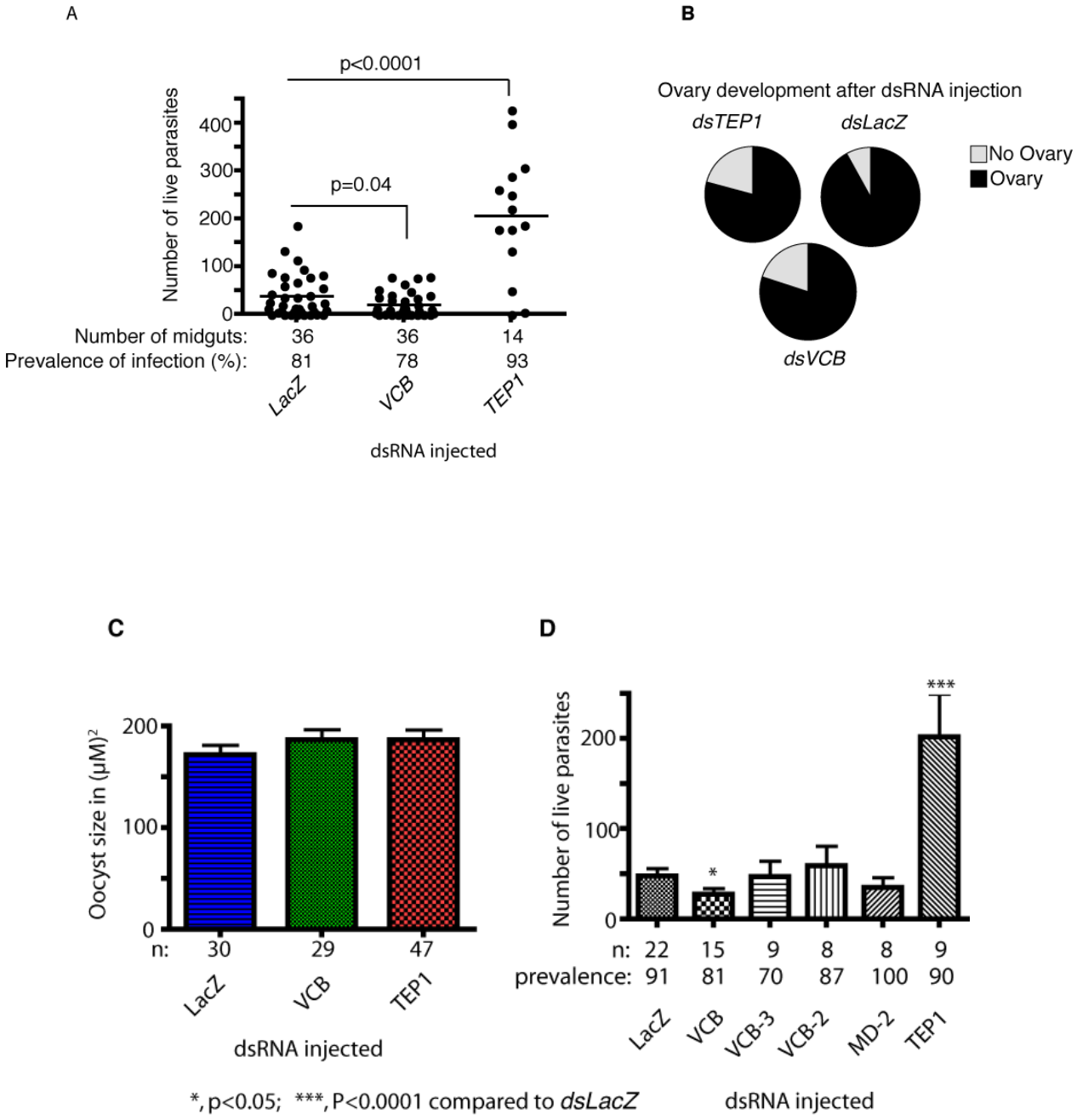


Figure 3-3. The effect of silencing *VCB* to parasite and ovary development in mosquitoes

A: Female G3 mosquitoes were injected with *dsVCB, dsTEP1* and *dsLacZ*, and then infected with *P. berghei*. Parasite development was monitored in the midguts 7 days after infection B: ovary development in *VCB* depleted mosquitoes compared to *LacZ* and *TEP1* controls, C: mean parasite size (area) determined 7 dpi after in dsRNA injected mosquitoes **D**: Parasite development is gauged 7 dpi in mosquitoes injected with VCB, CathB4533, CathB4531 and MD-2; ***, p<0.001; *, p<0.05

Conclusion and Discussion

Previously we showed that Vg was involved in negative regulation of TEP1 activity, we then investigated the role of VCB, a proapototic cystein protease that cleaves Vg and show that just like Vg, it inhibits parasite killing in female *A. gambiae* susceptible mosquitoes. Furthermore, we showed that NF-κB/Rel transcription factors negatively regulate VCB expression in manner similar to Vg but, opposite that of the major antiparasitic molecule TEP1. We did not observe any significant changes in ovary or parasite development in knockdown of cathepsin B isoforms (Agap004531 and Agap004533) or MD-2. Similarly these genes were not under the control of NF-κB factors Cactus/Rel. Put together, we have demonstrated that NF-κB/Rel may be implicated in the regulation of genes that respond to nutrient supply and involved in mosquito reproduction. Therefore, we propose to investigate the mechanism by which the Cactus cassette regulates the nutrient sensitive TOR pathway.

Chapter 4

Perspectives

Introduction

Previously we showed that Vg, the major yolk protein, and VCB, a cystein protease that processes Vg, were involved in parasite development in mosquitoes by inhibiting TEP1-mediated parasite killing. Vg inhibited the binding of TEP1 to parasite surface, therefore reducing the killing efficiency. Published data has shown that expression of Vg is induced by blood feeding under the control of the nutrient-sensitive TOR pathway (Hansen et al., 2004), reaching its peak of expression 24 h post blood meal. Interestingly, we showed that lipophorin was required for the normal expression of *Vg* and that depleting Cactus (IκB) inhibited *Vg* synthesis. Furthermore, silencing of *Cactus* negatively regulated the expression of *VCB*, a yolk protein involved in the cleavage of Vg. Frolet et al., (2006) and showed that inactivating Cactus boosts the basal expression of antiparasitic molecules such as TEP1, leading to complete elimination of invading parasites and negatively impacted on fecundity. This suggests a connection between immunity and reproduction. Indeed, our data has shown that *Vg* is involved in both processes (immunity and reproduction); moreover it is regulated by NF-κB factors. In addition, the depletion of Cactus has been shown to upregulate the expression of lipophorin in *Aedes* mosquitoes (Cheon et al., 2006), however this does not rescue ovary development despite the fact Lp was shown to be absolutely essential for eggs production in mosquitoes (Vlachou et al., 2005). These observations strongly suggest that there are other mosquito factors, yet to be identified, that play a crucial role in reproduction.

We attempted to establish the link between NF-κB factors and *Vg* expression by investigating the TOR pathway and targeted upstream events that regulate Vg expression. Hansen et al., (2004) showed that in the absence of amino acids TOR activity is kept under nutritional arrest which is brought about by TSC1/2 complex (see Figure 4-1).

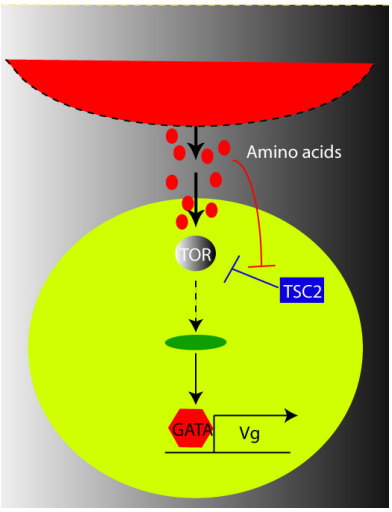


Fig. 4-1. A model for *Vg* gene activation in the mosquito fat body after blood feeding.

After blood feeding, the amino acid concentration in the hemolymph rises and induces TOR signaling which activates a GATA transcription factor to the synthesis of Vg. Similarly, increased amino acid concentration inactivates TSC2 the inhibitor of TOR

Modified from Hansen et al., 2004

To investigate this we analyzed the expression of *TOR* and *TSC1/2* in Cactus knock down and used microarray data (Frolet et al., unpublished data). Strikingly, the depletion of Cactus lead to an elevation of *TSC1* and down regulated *TOR* expression in midgut tissues (Figure 4-2), suggesting that in the absence of Cactus, the inhibitor of the TOR pathway is induced which may contribute to the down regulation of TOR and subsequent reduction on the expression of *Vg*.

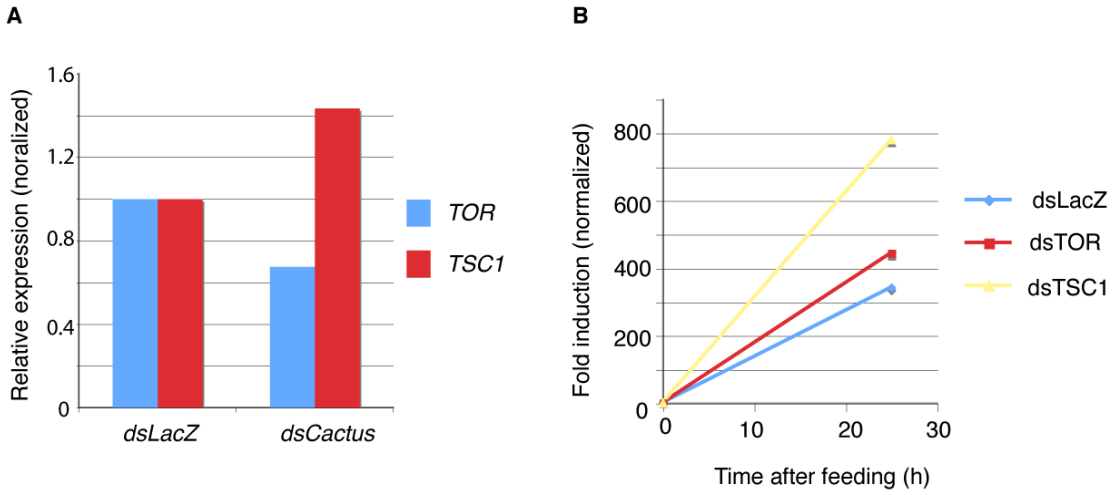


Figure 4-2. Cactus regulates expression of *Vg* via *TSC1*

A: Microarray analysis of Cactus depleted mosquitoes was conducted using the affymetrix platform and analyzed by dCHIP software. The expression of *TSC1* and *TOR* in *dsCactus* treated mosquitoes is compared to *dsLacZ* control. B: The expression of *Vg* was examined by qRT-PCR in mosquitoes after injection of *dsTSC1, dsTOR* and *dsLacZ*

Depletion of TSC1 upregulates *Vg* expression in mosquitoes

Next, we prepared *dsRNA* directed against *TOR* and *TSC1*, injected mosquitoes and examined the expression of *Vg* using qRT-PCR at different times after blood feeding. In our preliminary experiments we did not observe any change in *Vg* expression in *TOR* depleted mosquitoes which was probably due to incomplete inactivation of *TOR.* Interestingly, depletion of TSC1, the inhibitor of TOR, leads to elevated levels of *Vg* expression to almost 2-fold compared to control mosquitoes. These results suggest that TSC1 via the TSC1/TSC2 complex may negatively regulate *Vg* expression as compared to *Cactus* knock down. We speculated that TSC1/TSC2 complex may be implicated in the loss of reproductive capacity in Cactus depleted mosquitoes. To investigate this, we performed concomitant injection of *dsTSC1-Cactus* and single injections *dsTSC1*, *dsCactus* and *dsLacZ* and examined ovary and parasite development 7 dpi. Just as expected, silencing of *Cactus* completely blocked parasite and ovary development while single knockdown of *TSC1* lead to increased parasite survival (Figure 4-3 A and B). Surprisingly, nearly all mosquitoes injected with *dsTSC1-Cactus* had fully developed ovaries; in addition we observed some midguts with GFP expressing parasites (Figure 4-3C). This rescue of the Cactus knockdown phenotype by TSC1 knockdown suggests that TSC1 inhibition by Cactus is required for optimal oogenesis, but only partially rescues killing of parasites.

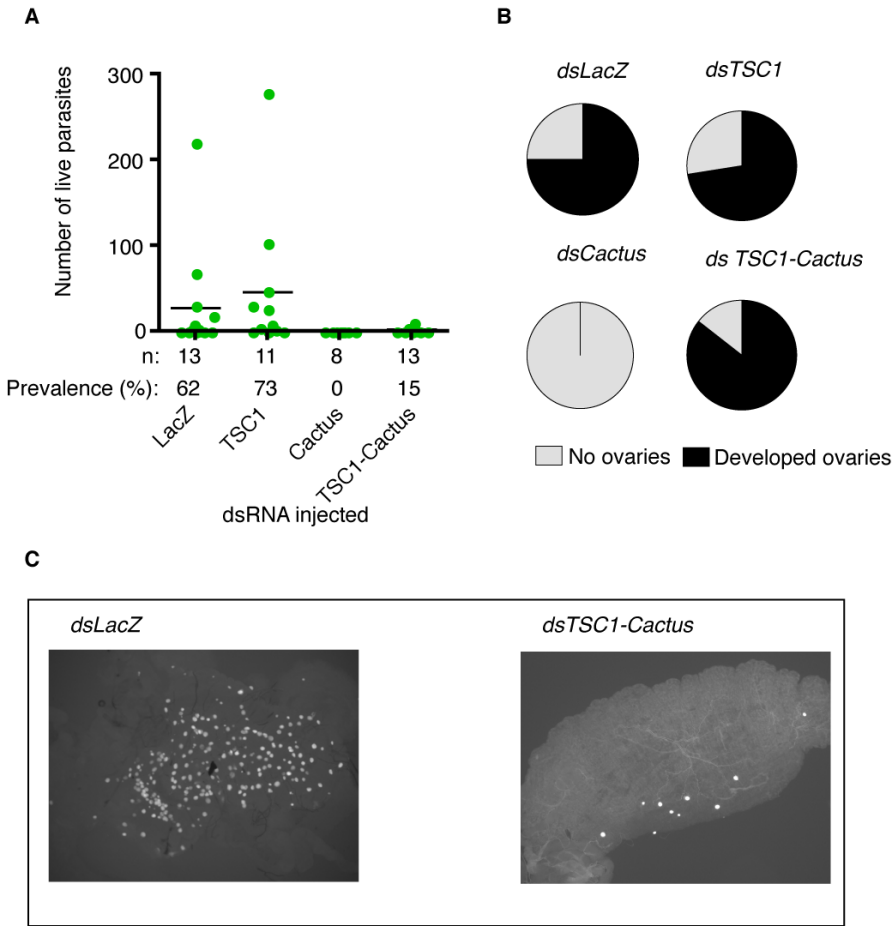


Figure 4-4. The effect of double knock down of *TSC1-Cactus* on ovary and parasite development in susceptible mosquitoes.

A: Parasite development and B: ovary development was gauged 7 dpi after concomitant silencing of *TSC1-Cactus* compared to single knock downs C: Example of dissected midguts (7dpi) shows GFP parasites in the injected mosquitoes

Our data has shown that concomitant silencing of *TSC1-Cactus* rescues ovary development, which is blocked in *Cactus* knockdown, as well as allowing some parasite to escape the immune pressure. From these observations we are tempted to speculate that concomitant silencing of *Cactus-TSC1-TEP1* may restore parasite survival similar to those observed in single knock down of *TEP1*. In addition we propose that Cactus is located upstream of Akt, where it may facilitate the activation of Akt or block its inhibitor. Akt kinase phosphorylates and inactivates TSC2 (Inoki et al., 2002). Alternatively Cactus may activate a nutrient sensitive repressor of TSC2/TSC1 pathway and subsequent expression of Vg (Figure 4-5).

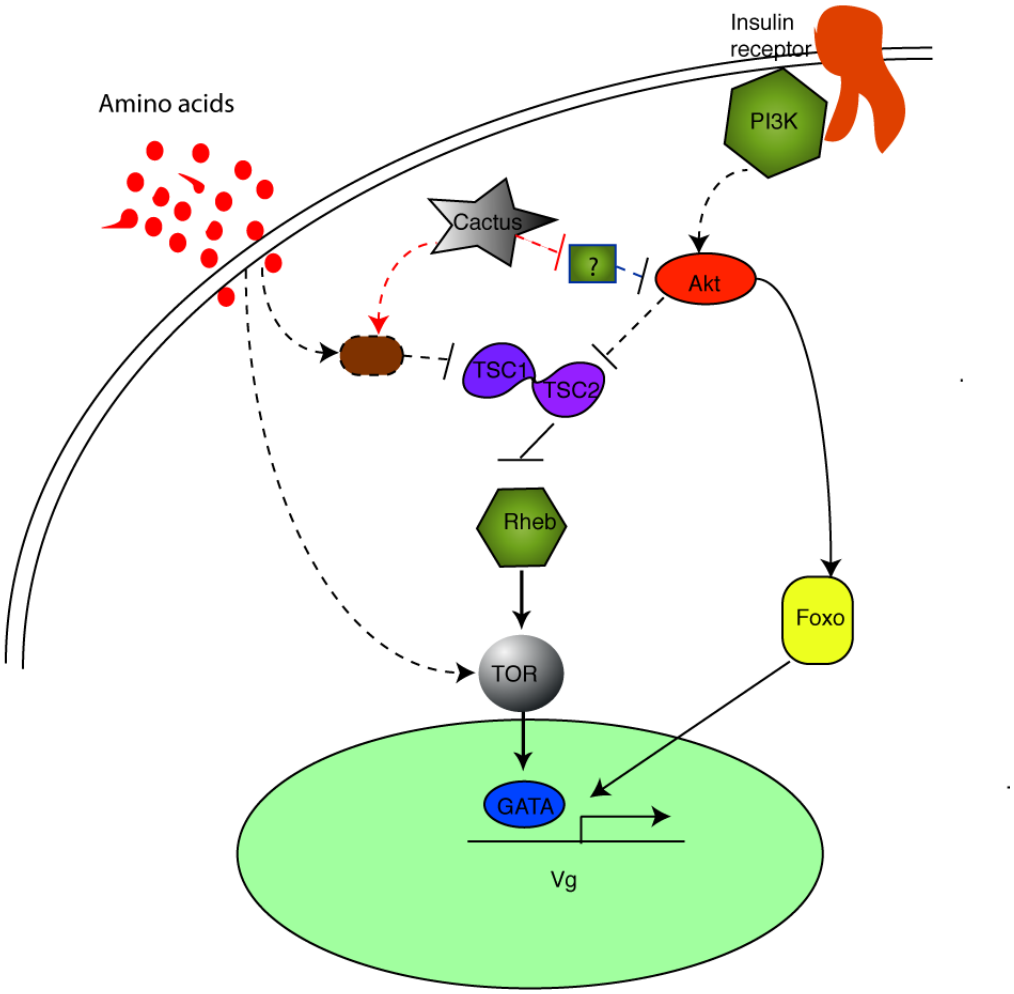


Figure 4-5. A model for Vg activation in the fat body under the regulation of Cactus
Amino acids derived from a blood meal activate TOR kinase and suppresses its inhibitor TSC1/TSC2 complex. Rheb, a GDP binding protein initiates TOR's activity leading to the synthesis of Vg via a GATA factor. Rheb is kept inactive by interacting TSC1/TSC2, while TSC2 is phosphorylated and inactivated by the protein kinase Akt, which itself is activated by PI3K. In addition Akt activates a transcription factor FOXO, leading to the transcription of FOXO sensitive genes. We propose two possible mechanisms in which Cactus could suppress TSC1/TSC2; 1) by suppressing an inhibitor of Akt or 2) by activating a nutrient sensitive inhibitor of TSC1/TSC2.

In order to gain more understanding of the model we propose epistatic analysis on some target molecules downstream of Cactus such as Rheb and Akt, to establish if Cactus controls their expression and activity in order to confirm the position of Cactus in the model. It would also be worth establishing if events upstream of Akt affect the TOR/TSC pathway and subsequent expression of *Vg*.

Résumé de thèse en français

Analyse protéomique de la réponse d'*Anopheles gambiae* à l'infection par *Plasmodium berghei*

1.0 Introduction

Le paludisme reste un problème de santé important dans certaines régions d'Asie et d'Amérique du Sud, mais son impact est majeur en Afrique sub-saharienne (Greenwood and Mutabingwa, 2002). Les parasites du genre *Plasmodium* sont les agents responsables de cette maladie et sont transmis aux humains par une piqûre infectieuse du moustique *Anopheles* (WHO 2005 ; Lind *et al*., 2005). La lutte contre le paludisme est grandement dépendante de l'utilisation d'agents thérapeutiques pour son traitement ainsi que sa prophylaxie. Cependant cette stratégie présente plusieurs inconvénients en raison de l'émergence de parasites toujours plus résistants aux médicaments, du coût considérable du traitement et de son inaccessibilité notamment pour les personnes ayant peu ou pas de ressources dans les régions endémiques du paludisme (Greenwood et al., 2008). Par ailleurs, aucun vaccin contre le paludisme n'est pour le moment disponible à la commercialisation en dépit d'un bon potentiel (Gupta et al., 1999 ; Carter, 2001). Le contrôle du moustique vecteur a combiné l'utilisation d'insecticides, la gestion de l'environnement et la mise en place d'un dispositif de protection des personnes par l'utilisation de matériels traités aux répulsifs ou aux insecticides. Cela a grandement réduit le fardeau qu'est le paludisme et a conduit à rechercher de nouvelles stratégies ciblant la transmission du paludisme du vecteur à l'humain.

Une combinaison appropriée vecteur-parasite est nécessaire au *Plasmodium* pour permettre son futur développement dans le moustique. Néanmoins, un faible nombre de parasites complète leur cycle dans le moustique en raison d'une forte réponse antiparasitaire mise en évidence par une réduction drastique du nombre d'oocinètes envahissant l'intestin (Sinden 1999). Parfois, dans certaines souches de moustique, le développement du parasite est totalement avorté (Collins et al., 1991). Les mécanismes sous-jacents d'élimination du parasite n'étaient pas totalement compris jusqu'à récemment lorsque des outils de biologie moléculaire ont été développés suite à la publication du génome d'*Anopheles*. Cela a conduit à l'identification de plusieurs molécules antiparasitaires. La « Thio-Ester containing Protein 1 » (TEP1) ainsi que deux molécules riches en répétitions de leucine LRM1 et APL1, jouent un rôle central dans les réponses antiparasitaires du moustique (Blandin et al., 2004 ; Osta et al., 2004 ; Riehle et al., 2006) qui se traduisent par la lyse du parasite ou la mélanisation des parasites morts dans les moustiques réfractaires. Plusieurs autres molécules, supportant ou s'opposant à la survie du parasite, ont été identifiées (Blandin et al., 2008) et semblent

être interconnectées au mécanisme d'élimination par TEP1. Cependant, une vue complète de ce mécanisme d'élimination (du signal aux molécules effectrices) n'a pas encore été établie.

Nous essayerons de répondre aux questions formulées ci-dessus en utilisant l'analyse protéomique pour étudier les réponses induites par le parasite dans *A. gambiae* durant une infection *à P. berghei*. Nous étendrons ensuite notre analyse au rôle du système de transport des lipides dans le moustique. Parmi d'autres molécules non caractérisées, plusieurs gènes connus de l'immunité sont différentiellement régulés lors d'une infection à *Plasmodium*. L'interaction entre le transport lipidique, la reproduction et la réponse immunitaire du moustique a été établie. Par ailleurs, nous avons identifié deux molécules sous le contrôle de facteurs NF-κB qui inhibent le mécanisme d'élimination TEP1-dépendant.

1.1 Protéomique globale de l'intestin *d'A. gambiae* durant une infection à *P.berghei*.

L'efficacité de fixation de TEP1 aux parasites détermine l'ampleur de l'élimination de *Plasmodium*. La stimulation de l'expression basale de TEP1 augmente l'efficacité de fixation et avorte le développement du parasite dans le moustique, tandis que la disparition de TEP1 transforme des moustiques réfractaires en susceptibles à l'infection par *Plasmodium* (Frolet et al., 2006 ; Blandin et al. 2004). Afin de comprendre la fonction de TEP1, des moustiques transgéniques avec TEP1 gain-de-fonction (GOF) (réfractaire à l'infection par *P. berghei*) et perte-de-fonction (LOF) (hypersusceptible à l'infection par *P. berghei*) ont été établis. Nous avons exploré la réponse immunitaire de ces deux lignées transgéniques suite à une infection par *P. berghei* en utilisant une analyse protéomique. Nous souhaitions établir quelles molécules étaient associées avec une forte réponse immunitaire (GOF) et celles présentant une faible réponse immunitaire (LOF). Nous avons identifié plus de 1000 protéines uniques dans l'intestin de moustique 24h après l'infection à *P. berghei*. Elles représentent approximativement 7% du protéome du moustique selon les prédictions de ENSEMBL (version 43 ENSEMBL). Plusieurs protéines provenant de gènes connus de l'immunité tels que les protéines contenant des groupements thioester (TEPs), celles riches en répétition de leucine (LRRs), les galectines et les serpines ont été identifiées. Nous avons également identifié des enzymes impliquées dans la détoxification d'espèces à oxygène réactif incluant la superoxide dismutase (SOD) et la catalase ; cette dernière étant réprimée lors d'une

infection à *P. berghei* et influençant positivement le développement du parasite (Molina-Cruz et al., 2008). L'infection à *Plasmodium* induit des changements dans l'actine et la dynamique du cytosquelette au niveau protéique. L'invalidation de WASP, un régulateur positif des dynamiques du cytosquelette, augmente la survie du parasite (Mendes et al., 2008).

Nous avons observé des molécules plus antiparasitaires dans les moustiques *GOF*, incluant plusieurs protéines LRRs lesquelles sont peut être influencées par l'augmentation de l'expression de TEP1 ; de même, des molécules plus proparasitaires ont été associées avec les souches de moustique transgéniques *LOF*. Cependant, nous n'avons pas pu détecté par analyse protéomique de peptide antimicrobien connu induit par l'infection à *Plasmodium*, au moins dans l'intestin. Nos données présentaient également de nouvelles protéines autant que des peptides orphelins, pouvant contribuer à l'amélioration de l'annotation du génome. Certaines protéines parasitaires ont été identifiées incluant une aspartyl-protéase spécifique de l'oocinète. Si l'on considère tout cela, *A. gambiae* répond à l'infection en induisant des molécules antiparasitaires afin de limiter le développement de *Plasmodium* et subi des changements dans le cytosquelette, probablement dans le but de protéger l'hôte de dommages aux tissus dus aux substances toxiques relarguées par les parasites (Shiao et al., 2006).

1.2 Le système de transport lipidique régule l'activité de TEP1 dans *Anopheles*

Après qu'un moustique ait ingéré un repas sanguin infecté, plusieurs évènement ont lieu simultanément : la transformation d'acides aminés dérivés du sang, le transport de nutriments extra-ovariens et les réponses immunitaires à l'encontre des parasites envahisseurs. De plus, ces événements se déroulent dans un même organe tel que l'intestin ou le corps gras (Marinotti et al., 2006). Il est possible qu'ils puissent s'influencer l'un l'autre ou qu'ils soient coordonnés par un mécanisme général. La Lipophorine (le transporteur majeur de molécules lipidiques) interagit avec des molécules ayant un motif de reconnaissance, des protéines impliquées dans la régulation de l'activité de la phénoloxidase et inactive LPS (Schmidt et al., 2005). Nous voulions établir si TEP1 et/ou ses partenaires forme un complexe avec la Lipophorine. Une telle association peut réguler négativement l'activité de TEP1 puisque l'invalidation de la Lipophorine a conduit a une survie réduite du parasite et bloque la reproduction dans les moustiques (Vlachou et al., 2005). Nous avons essayé de répondre à cette question en réalisant des analyses

protéomiques sur des lipophorines purifiées de moustique. Nos résultats n'ont pas permis d'établir une interaction physique entre TEP1 et la Lipophorine, même si la Lipophorine régule le processus d'élimination du parasite TEP1-dépendant. Cependant, PPO2, qui est impliqué dans la mélanisation des parasites, était partiellement associé à la Lipophorine. Nos données suggèrent que certains aspects de l'immunité du moustique sont associés avec le transport de nutriments. De façon intéressante, nous avons identifié la Vitellogenine (Vg), une protéine majeure du jaune d'œuf et un transporteur lipidique. Vg diminue l'activité de TEP1 d'une manière rappelant la Lipophorine. De analyses complémentaires de ce processus par invalidations multiples et immunofluorescence ont révélées de manière intéressante un réseau d'interactions entre la Lipophorine, Vg, la Vitellogenine Cathepsine B (VCB), les facteurs NF-KB/Rel et la capacité de TEP1 à se lier et détruire les oocinètes.

Appendix

Appendix 1		Proteins constitutively present after blood feeding		Sequenced peptides			
AGAP Ids	OLD Protein ID	Interpro Annotation	Mass (Da)	*GOF GFP*	*LOF GFP*	*GOF PbMut*	*LOF PbMut*
AGAP012407-PA	ENSANGP00000026077	Endoplasmic reticulum targeting sequence	55667	40	35	25	24
AGAP004192-PA	ENSANGP00000012893	Endoplasmic reticulum targeting sequence	72813	39	33	13	14
AGAP004362-PA	ENSANGP00000025315	Glu/Leu/Phe/Val dehydrogenase	63003	39	37	36	19
#N/A	ENSANGP00000019887	ATP/GTP-binding site motif A (P-loop)	71206	37	43	18	6
AGAP003656-PA	ENSANGP00000022422	EGF-like domain	471125	36	33	27	20
AGAP004904-PA	ENSANGP00000021298	Catalase	55257	32	25	29	17
AGAP000881-PA	ENSANGP00000017723	Aldehyde dehydrogenase	56323	31	28	28	21
AGAP007827-PA	ENSANGP00000018531	ATP/GTP-binding site motif A (P-loop)	46883	31	28	26	27
AGAP007393-PB	ENSANGP00000027211	Thioredoxin type domain	54791	31	21	14	14
AGAP012401-PA	ENSANGP00000011672	Alpha amylase, catalytic domain	57316	29	22	24	20
AGAP004563-PA	ENSANGP00000011721	Neutral zinc metallopeptidases, zinc-binding region	143738	29	32	41	30
AGAP010025-PA	ENSANGP00000011972	Rab GTPase activator	50071	27	21	10	7
AGAP012167-PA	ENSANGP00000014040	Phosphoglucose isomerase (PGI)	62332	27	17	21	20
AGAP003153-PA	ENSANGP00000024697	ATP/GTP-binding site motif A (P-loop)	68507	26	27	16	8
AGAP000862-PA	ENSANGP00000010269	Glycoside hydrolase, family 31	107573	25	24	18	13
AGAP000565-PB	ENSANGP00000017329	Pyridine nucleotide-disulphide oxidoreductase, class I	55176	25	20	24	20
AGAP004212-PA	ENSANGP00000012895	Endoplasmic reticulum targeting sequence	46614	24	27	4	11
AGAP004596-PA	ENSANGP00000021580	Pyruvate kinase	56842	24	22	6	17
AGAP002564-PA	ENSANGP00000024159	Fructose-bisphosphate aldolase, class-I	39621	24	23	14	6
AGAP011476-PA	ENSANGP00000014382	Macrophage scavenger receptor	94851	23	21	10	8
#N/A	ENSANGP00000025086	Neutral zinc metallopeptidases, zinc-binding region	86939	23	8	23	14
AGAP004598-PA	ENSANGP00000027966	ATP/GTP-binding site motif A (P-loop)	40184	23	17	20	15
AGAP003869-PA	ENSANGP00000005638	Cytosol aminopeptidase	52042	22	16	4	7
AGAP009907-PA	ENSANGP00000013312	Neutral zinc metallopeptidases, zinc-binding region	72634	22	24	20	16
AGAP005246-PA	ENSANGP00000015833	SRPN 10	44022	22	19	17	13
AGAP004993-PA	ENSANGP00000010787	EGF-like domain	423593	21	17	7	3
AGAP009944-PA	ENSANGP00000013314	Aldehyde dehydrogenase	52352	21	21	22	14
AGAP001884-PA	ENSANGP00000020828	Fumarate lyase	50532	21	19	10	5
AGAP003995-PA	ENSANGP00000024978	Glycoside hydrolase, family 31	61668	21	12	15	16
AGAP004860-PA	ENSANGP00000001203	Membrane alanine aminopeptidase	85263	20	20	10	11
AGAP006225-PA AGAP006220-PA	ENSANGP00000020618	2Fe-2S ferredoxin	145199	20	24	12	13
AGAP002509-PA	ENSANGP00000021922	G-protein beta WD-40 repeat	66321	20	19	13	17
AGAP002858-PA	ENSANGP00000022526	ATPase, E1-E2 type	111759	20	22	22	26
#N/A	ENSANGP00000007943	Neutral zinc metallopeptidases, zinc-binding region	97965	19	12	25	16
AGAP012400-PA	ENSANGP00000008952	Alpha amylase, catalytic domain	68568	19	17	15	10
AGAP004394-PA	ENSANGP00000013056	Peptidase family M49	81444	19	10	6	5
AGAP003696-PA	ENSANGP00000025222	Neutral zinc metallopeptidases, zinc-binding region	106704	19	26	25	27

Appendix 1	Continued…	Proteins constitutively present after blood feeding		Sequenced peptides			
AGAP Ids	OLD Protein ID	Interpro Annotation	Mass (Da)	*GOF GFP*	*LOF GFP*	*GOF PbMut*	*LOF PbMut*
AGAP001037-PA	ENSANGP00000028747	Metallopeptidase family M24	66016	19	17	14	14
AGAP007793-PA	ENSANGP00000010332	Senescence marker protein-30 (SMP-30)	34786	18	19	5	4
AGAP006616-PA	ENSANGP00000011486	ATP/GTP-binding site motif A (P-loop)	112844	18	21	2	5
AGAP004809-PA	ENSANGP00000003366	Neutral zinc metallopeptidases, zinc-binding region	76522	17	13	21	12
AGAP007809-PA	ENSANGP00000010351	ATP/GTP-binding site motif A (P-loop)	52976	17	17	7	12
AGAP003398-PA	ENSANGP00000026746	Inorganic pyrophosphatase	33827	17	9	2	5
AGAP005558-PA	ENSANGP00000017821	Beta and gamma crystalline	52294	16	5	8	6
AGAP000184-PA	ENSANGP00000019421	Malic oxidoreductase	63550	16	14	4	5
AGAP003077-PA	ENSANGP00000020286	Neutral zinc metallopeptidases, zinc-binding region	118646	16	15	6	4
AGAP003785-PA	ENSANGP00000015052	Glucose-methanol-choline oxidoreductase	69474	15	4	7	5
AGAP000679-PA	ENSANGP00000019075	Peptidase M20/M25/M40	47030	15	9	3	2
AGAP000367-PA	ENSANGP00000022030	Glyoxalase/Bleomycin resistance protein/dioxygenase domain	32607	15	15	12	8
AGAP011939-PA	ENSANGP00000025684	Alpha amylase, catalytic domain	32654	15	14	15	10
AGAP009610-PA	ENSANGP00000021023	D-isomer specific 2-hydroxyacid dehydrogenase, NAD binding domain	36609	14	13	9	10
AGAP004142-PA	ENSANGP00000011707	Aminotransferase, class-I	45083	13	15	13	15
AGAP012757-PA	ENSANGP00000012865	Neutral zinc metallopeptidases, zinc-binding region	75479	13	13	10	9
AGAP000154-PA	ENSANGP00000019433	AMP-dependent synthetase and ligase	61860	13	10	7	5
AGAP006099-PA	ENSANGP00000020419	Insulinase-like peptidase, family M16	42009	13	10	7	8
AGAP012053-PA	ENSANGP00000008628	Glycoside hydrolase, family 37	65066	12	10	3	6
AGAP010404-PA	ENSANGP00000010247	Glutathione S-transferase, N-terminal	23384	12	11	12	10
AGAP007088-PA	ENSANGP00000011257	Peptidyl-prolyl cis-trans isomerase, cyclophilin type	21802	12	9	8	4
AGAP000883-PA	ENSANGP00000013107	Elongation factor 1, gamma chain	48795	12	14	6	2
AGAP003475-PA	ENSANGP00000014285	Eukaryotic thiol (cysteine) protease	53351	12	4	16	14
AGAP001420-PA	ENSANGP00000015800	Phosphoglycerate/bisphosphoglycerate mutase	28749	12	4	13	12
AGAP000935-PA	ENSANGP00000024967	Insulinase-like peptidase, family M16	49481	12	5	5	8
AGAP012056-PA	ENSANGP00000026391	Actin-binding, cofilin/tropomyosin type	17151	12	14	8	7
AGAP005744-PA	ENSANGP00000027859	Leucine-rich repeat	45150	12	4	4	4
AGAP002464-PA	ENSANGP00000030559	FERRITIN	26317	12	12	14	9
AGAP004775-PB	ENSANGP00000010783	Xaa-Pro dipeptidase/Xaa-Pro aminopeptidase	52423	11	12	16	8
#N/A	ENSANGP00000012480	Acyl-CoA dehydrogenase	64764	11	11	2	2
AGAP000550-PA	ENSANGP00000017306	Protein prenyltransferase, alpha subunit	144389	11	7	20	17
AGAP011053-PA	ENSANGP00000019779	Aldo/keto reductase	36262	11	6	6	7
AGAP007745-PA	ENSANGP00000022245	Cell surface glycoprotein 1	34305	11	11	11	8
AGAP010429-PA	ENSANGP00000010243	Fumarate reductase/succinate dehydrogenase, FAD-binding site	73047	10	6	10	9
AGAP010479-PA	ENSANGP00000013769	Serine/threonine dehydratase, pyridoxal-phosphate attachment site	32527	10	10	8	8
AGAP011453-PA	ENSANGP00000014482	Transferrin	27585	10	6	7	2
AGAP011584-PA	ENSANGP00000015082	Protein kinase	87909	10	5	21	17

Appendix 1	**Continued…**	**Proteins constitutively present after blood feeding**		**Sequenced peptides**			
AGAP Ids	**OLD Protein ID**	**Interpro Annotation**	**Mass (Da)**	***GOF GFP***	***LOF GFP***	***GOF PbMut***	***LOF PbMut***
AGAP011302-PA	ENSANGP00000015648	Alkaline phosphatase	56426	10	10	10	4
AGAP008988-PA	ENSANGP00000019490	Glutamine synthetase, beta-Grasp domain	45055	10	4	5	3
AGAP000462-PA	ENSANGP00000020778	Peptidyl-prolyl cis-trans isomerase, cyclophilin type	18507	10	9	13	9
AGAP011050-PA	ENSANGP00000023237	Aldo/keto reductase	35847	10	12	3	4
AGAP007120-PA	ENSANGP00000011253	Nucleoside diphosphate kinase	19055	9	12	9	9
AGAP004382-PA	ENSANGP00000013058	Glutathione S-transferase, N-terminal	21508	9	10	8	4
AGAP005929-PA	ENSANGP00000013603	Carbohydrate kinase, PfkB	34316	9	7	7	9
AGAP000414-PA	ENSANGP00000020750	ATP/GTP-binding site motif A (P-loop)	38772	9	10	6	4
AGAP011895-PA	ENSANGP00000021292	Uroporphyrinogen decarboxylase (URO-D)	39184	9	9	3	3
AGAP006729-PA	ENSANGP00000021628	Ester hydrolase C11orf54	35409	9	6	4	2
AGAP010718-PA	ENSANGP00000027573	Proteasome B-type subunit	31498	9	5	7	3
AGAP011208-PA	ENSANGP00000028361	Hexokinase	51443	9	14	11	7
AGAP007505-PA	ENSANGP00000009426	Serine carboxypeptidase (S10)	53038	8	5	3	4
AGAP003578-PA	ENSANGP00000011393	Aldehyde dehydrogenase	52641	8	5	14	7
AGAP009172-PA	ENSANGP00000016749	Prolyl endopeptidase, serine active site	81364	8	2	4	6
AGAP006414-PA	ENSANGP00000020485	Glycoside hydrolase, family 18/2	57703	8	11	8	6
AGAP005630-PA	ENSANGP00000022801	ATP/GTP-binding site motif A (P-loop)	89877	8	3	3	2
AGAP003337-PA	ENSANGP00000000940	Beta-lactamase-like	28044	7	4	3	4
AGAP001501-PA	ENSANGP00000011757	Senescence marker protein-30 (SMP-30)	33745	7	5	3	2
AGAP008909-PA	ENSANGP00000013993	Phosphatidylethanolamine-binding protein	24273	7	7	7	3
AGAP003987-PA	ENSANGP00000014377	Mitochondrial glycoprotein	22577	7	4	3	3
AGAP010253-PA	ENSANGP00000014428	Proteasome subunit, A-type	30873	7	10	8	6
AGAP011303-PA AGAP011305-PA	ENSANGP00000015627	Alkaline phosphatase	56312	7	4	7	3
AGAP001630-PA	ENSANGP00000018152	Triosephosphate isomerase	22851	7	12	14	14
AGAP010733-PA	ENSANGP00000018472	Nitrilase/cyanide hydratase and apolipoprotein N-acyltransferase	59493	7	2	6	6
AGAP005749-PA	ENSANGP00000018735	Glutathione S-transferase, N-terminal	28452	7	13	6	7
AGAP002465-PA	ENSANGP00000022116	Ferritin	22788	7	7	8	6
AGAP002499-PA	ENSANGP00000022164	Aldehyde dehydrogenase	55452	7	2	9	5
AGAP001341-PA	ENSANGP00000022498	Eukaryotic thiol (cysteine) protease	53170	7	5	8	2
AGAP010130-PA	ENSANGP00000028309	Enoyl-CoA hydratase/isomerase	30393	7	13	8	10
AGAP012894-PA	ENSANGP00000000003	Histone H2B	13751	6	7	5	4
AGAP006400-PA	ENSANGP00000020398	Alkaline phosphatase	48571	6	7	12	6
AGAP010517-PA	ENSANGP00000020588	SOD 1	25753	6	6	6	4
AGAP005662-PA	ENSANGP00000021837	Acyl-CoA dehydrogenase	45902	6	2	7	5
AGAP008965-PA	ENSANGP00000006488	Glycoside hydrolase family 13	66978	5	4	5	3
AGAP010156-PA	ENSANGP00000012364	Succinyl-CoA ligase, alpha subunit	119215	5	3	3	2
AGAP009833-PA	ENSANGP00000015798	Porin, eukaryotic type	30854	5	11	18	17

Appendix 1	**Continued…**	**Proteins constitutively present after blood feeding**		**Sequenced peptides**			
AGAP Ids	**OLD Protein ID**	**Interpro Annotation**	**Mass (Da)**	***GOF GFP***	***LOF GFP***	***GOF PbMut***	***LOF PbMut***
AGAP010347-PA	ENSANGP00000016164	SOD 3A	15894	5	5	6	5
AGAP008837-PA	ENSANGP00000016798	Proteasome B-type subunit	23488	5	8	9	6
AGAP008296-PA	ENSANGP00000018367	Serine protease, trypsin family	29438	5	4	10	6
AGAP000416-PA	ENSANGP00000020737	Beta-lactamase-like	30712	5	5	3	2
AGAP006907-PA	ENSANGP00000021767	Adenosine/AMP deaminase	36737	5	4	2	4
AGAP002477-PA	ENSANGP00000003047	Single-strand binding protein	15232	4	3	2	2
AGAP001697-PA	ENSANGP00000012182	Proteasome B-type subunit	23589	4	2	3	2
AGAP001240-PA	ENSANGP00000013861	Esterase/lipase/thioesterase, active site	55984	4	6	8	8
AGAP008292-PA	ENSANGP00000018384	Serine protease, trypsin family	30045	4	3	6	4
AGAP001973-PA	ENSANGP00000018478	Proteasome subunit, A-type	27823	4	2	8	4
AGAP000513-PA	ENSANGP00000019404	Peptidase family S51	27020	4	3	4	4
AGAP001321-PA	ENSANGP00000020911	FLAVIN REDUCTASE	22364	4	3	6	6
AGAP005327-PA	ENSANGP00000021077	Endoribonuclease L-PSP	14577	4	4	5	3
AGAP006385-PA	ENSANGP00000021092	Serine protease, trypsin family	28446	4	7	14	8
AGAP003696-PA	ENSANGP00000021245	Neutral zinc metallopeptidases, zinc-binding region	85727	4	10	6	3
AGAP011158-PA	ENSANGP00000022034	1-aminocyclopropane-1-carboxylate synthase	47858	4	4	2	4
AGAP007790-PA	ENSANGP00000031572	SODIUM/POTASSIUM TRANSPORTER	35913	4	6	6	9
AGAP007258-PA	ENSANGP00000032020	IRON RESPONSIVE ELEMENT BINDINGE	33255	4	2	12	12
AGAP007927-PA	ENSANGP00000010158	Ubiquitin domain	15005	3	6	2	2
AGAP007543-PA	ENSANGP00000010951	Alkyl hydroperoxide reductase/ Thiol specific antioxidant/ Mal allergen	28101	3	6	7	4
AGAP007121-PA	ENSANGP00000011266	Cytochrome b5	14875	3	3	3	3
AGAP006625-PA	ENSANGP00000011535	Coatomer epsilon subunit	35506	3	5	2	2
AGAP007975-PA	ENSANGP00000012307	2-oxo acid dehydrogenase, acyltransferase component, lipoyl-binding	48464	3	10	5	6
AGAP008769-PA	ENSANGP00000012475	Acyl-CoA dehydrogenase	37597	3	2	3	2
AGAP001199-PA	ENSANGP00000013929	Serine protease, trypsin family	29514	3	2	7	6
AGAP003935-PA	ENSANGP00000015960	Proteasome subunit, A-type	27899	3	4	3	4
AGAP001826-PA	ENSANGP00000018348	Apolipophorin	360572	3	71	12	33
AGAP004376-PA	ENSANGP00000028444	Aldose 1-epimerase	41323	3	7	3	2
AGAP011442-PA	ENSANGP00000004895	Serine carboxypeptidase (S10)	48092	2	5	7	5
AGAP006228-PA	ENSANGP00000005974	Carboxylesterase, type B	60701	2	3	3	6
AGAP001995-PA	ENSANGP00000011336	Proteasome subunit, A-type	25962	2	8	6	4
AGAP012529-PA	ENSANGP00000016074	GALE 8	16106	2	5	8	5

GOF: *TEP1 GOF* ; *LOF* : *TEP1 LOF*; *GFP*: *GFP* expressing *P. berghei* ; *PbMut* : non-gametocyte producing *P. berghei*
Proteins were organized according to the those identified in *TEP1 GOF* infected with *P. berghei* (*GOF-GFP*)

Appendix 2		**Proteins enriched in *TEP1 GOF***					
AGAP Ids	**OLD Protein ID**	**Interpro Annotation**	**Mass (Da)**	***GOF GFP***	***LOF GFP***	***GOF PbMut***	***LOF PbMut***
AGAP003405-PA	ENSANGP00000000753	Adenylosuccinate synthetase	49399	2	0	0	0
#N/A	ENSANGP00000000938	Cytosolic fatty-acid binding protein	8302	2	0	0	0
AGAP010278-PA	ENSANGP00000002909	Dihydrofolate reductase	21648	2	0	0	0
AGAP011125-PA	ENSANGP00000008370	Aldo/keto reductase	39325	2	1	0	0
AGAP001678-PA	ENSANGP00000008541	Tensin	165155	2	0	0	0
AGAP012399-PA	ENSANGP00000008953	Alpha amylase, catalytic domain	54044	2	1	0	0
AGAP005108-PA	ENSANGP00000009913	Proliferation Associated 2G4 P38 2G4	44177	2	0	0	0
AGAP009736-PA	ENSANGP00000010108	HCO3- transporter	123670	2	1	0	0
AGAP000399-PA	ENSANGP00000010117	RNA-binding region RNP-1 (RNA recognition motif)	31787	3	1	0	0
AGAP008092-PA	ENSANGP00000010445	GDP L fucose synthetase	36451	2	0	0	0
AGAP008099-PA	ENSANGP00000010523	UFM1 conjugating enzyme 1	19276	2	1	0	0
AGAP002627-PA	ENSANGP00000010587	Putative esterase	31819	2	1	0	0
AGAP002600-PA	ENSANGP00000010598	Polynucleotide adenylyltransferase	49282	2	1	0	0
AGAP003516-PA	ENSANGP00000010992	Nitrilase/cyanide hydratase and apolipoprotein N-acyltransferase	30942	6	0	0	0
AGAP007550-PB	ENSANGP00000011013	Carbonic anhydrase, eukaryotic	30476	2	1	0	0
AGAP007593-PB	ENSANGP00000011016	ATP/GTP-binding site motif A (P-loop)	38616	4	0	0	0
AGAP002016-PA	ENSANGP00000011321	Metallo-phosphoesterase	51584	3	0	0	0
AGAP001512-PA	ENSANGP00000011664	Glutamate-cysteine ligase catalytic subunit	74258	4	1	0	0
AGAP003629-PA	ENSANGP00000011828	5'-nucleotidase, N-terminal	44066	4	0	0	0
AGAP004504-PA	ENSANGP00000012197	Epsin N-terminal homology	44147	2	1	0	0
AGAP001701-PA	ENSANGP00000012221	Ubiquitin domain	11015	2	1	0	0
AGAP012657-PA	ENSANGP00000012468	Bipartite nuclear localization signal	33494	2	1	0	0
AGAP010964-PA	ENSANGP00000012540	Butyrophilin subfamily 1	25784	2	0	0	0
AGAP006343_PA	ENSANGP00000012978	PGRP-SA	20358	2	0	0	0
AGAP008593-PA	ENSANGP00000013036	LRRD	47803	3	0	0	0
AGAP009949-PA	ENSANGP00000013149	6-phosphogluconate dehydrogenase, NAD binding domain	63977	2	1	0	0
AGAP009937-PA	ENSANGP00000013300	ATP/GTP-binding site motif A (P-loop)	166582	2	1	0	0
AGAP001749-PA	ENSANGP00000013320	MIR domain	21761	2	0	0	0
AGAP006461-PA	ENSANGP00000013449	Serine/Threonine protein kinase	95312	2	0	0	0
AGAP00	ENSANGP00000014095	LRRD 10	62187	2	0	0	0
AGAP012148-PA	ENSANGP00000014018	Map kinase	41832	2	0	0	0
AGAP011447-PA	ENSANGP00000014192	Protein DEK	52769	2	1	0	0
AGAP002399-PA	ENSANGP00000014848	Bipartite nuclear localization signal	66186	4	0	0	0
AGAP002387-PA	ENSANGP00000014917	Histidine acid phosphatase	47339	3	0	0	0
AGAP005175-PB	ENSANGP00000015662	Biotin-requiring enzyme, attachment site	263715	3	0	0	0
AGAP005160-PE	ENSANGP00000015684	ATP/GTP-binding site motif A (P-loop)	21930	2	0	0	0
AGAP005234-PA	ENSANGP00000015824	SOD 2	22174	3	1	0	0
AGAP001021-PA	ENSANGP00000016011	4Fe-4S ferredoxin, iron-sulfur binding domain	114513	4	0	0	0

Appendix 2	**Continued….**	**Proteins enriched in *TEP1 GOF***					
AGAP Ids	**OLD Protein ID**	**Interpro Annotation**	**Mass (Da)**	***GOF GFP***	***LOF GFP***	***GOF PbMut***	***LOF PbMut***
AGAP009673-PA	ENSANGP00000016609	Glutaminyl cyclase	33904	4	1	0	0
AGAP010564-PA	ENSANGP00000016990	PUR-alpha/beta/gamma, DNA/RNA-binding	31640	5	1	0	0
AGAP000534-PA	ENSANGP00000017318	Eukaryotic glutathione synthase	58854	4	0	0	0
AGAP000551-PA	ENSANGP00000017325	Dehydrogenase, E1 component	102029	3	0	0	0
AGAP000044-PA	ENSANGP00000017364	Thioredoxin type domain	44945	2	1	0	0
AGAP002101-PA	ENSANGP00000017588	Aminoacyl-tRNA synthetase, class I	140020	2	0	0	0
AGAP010821-PA	ENSANGP00000018116	Ig-like	36236	3	0	0	0
AGAP007339-PA	ENSANGP00000018434	Calcium-binding EF-hand	17229	4	1	0	0
AGAP011337-PA	ENSANGP00000018463	Golgin subfamily a member 2 cis golgi matrix GM130	72741	2	0	0	0
AGAP011350-PA	ENSANGP00000018510	Haloacid dehalogenase-like hydrolase	35085	7	0	0	0
AGAP007457-PA	ENSANGP00000019036	LRRD11	31653	4	1	0	0
AGAP011644-PA	ENSANGP00000019166	Endonuclease/exonuclease/phosphatase family	54200	2	1	0	0
AGAP000199-PA	ENSANGP00000019390	Phospholipase D/Transphosphatidylase	49009	2	1	0	0
AGAP005305-PA	ENSANGP00000019515	DNA-binding SAP	21812	2	0	0	0
AGAP008364-PA	ENSANGP00000019522	TEP 15	158397	3	1	0	0
AGAP010769-PA	ENSANGP00000019649	GCN5-related N-acetyltransferase	23069	6	1	0	0
AGAP012343-PA	ENSANGP00000019992	Immunoglobulin-like	83771	2	1	0	0
AGAP006070-PA	ENSANGP00000020462	Prefoldin-related KE2 protein	17576	2	0	0	0
AGAP006195-PA	ENSANGP00000020647	Ambiguous	20293	2	0	0	0
AGAP004645-PA	ENSANGP00000020907	ATP/GTP-binding site motif A (P-loop)	21712	2	0	0	0
AGAP001319-PA	ENSANGP00000020913	5'-nucleotidase domain-containing protein 1	49036	3	0	0	0
AGAP006937-PC	ENSANGP00000021736	Ubiquitin-conjugating enzymes	16744	3	0	0	0
AGAP002530-PA	ENSANGP00000022032	ADP-ribosylglycohydrolase	38424	3	0	0	0
AGAP002752-PA	ENSANGP00000022059	TPR repeat	56984	4	0	0	0
AGAP000561-PA	ENSANGP00000022750	ATP/GTP-binding site motif A (P-loop)	111810	2	0	0	0
AGAP006179-PC	ENSANGP00000022769	Calcium-binding EF-hand	17239	2	0	0	0
AGAP005246-PD	ENSANGP00000022846	Serine protease inhibitor (SRPN6)	42668	4	0	0	0
AGAP010876-PA	ENSANGP00000022995	Calcium-binding EF-hand	59259	2	0	0	0
AGAP004902-PA	ENSANGP00000023815	DSCAM	205902	2	0	0	0
AGAP005246-PE	ENSANGP00000023448	Serine protease inhibitor	42248	7	0	0	0
AGAP003794-PA	ENSANGP00000023706	RNA-binding region RNP-1 (RNA recognition motif)	52286	2	0	0	0
AGAP009075-PA	ENSANGP00000023803	ATP/GTP-binding site motif A (P-loop)	41939	2	1	0	0
AGAP004247-PA	ENSANGP00000024750	Glutathione peroxidase	18580	2	0	0	0
AGAP001315-PC	ENSANGP00000025312	Bipartite nuclear localization signal	93585	3	1	0	0
AGAP008990-PA	ENSANGP00000028095	Lecithin:cholesterol acyltransferase	42245	2	0	0	0
AGAP011537-PA	ENSANGP00000028556	Argonaute and Dicer protein, PAZ	92739	3	1	0	0
AGAP007207-PA	ENSANGP00000029820	Precursor	33869	2	1	0	0

GOF: *TEP1 GOF* ; *LOF* : *TEP1 LOF*; *GFP*: *GFP* expressing *P. berghei* ; *PbMut* : non-gametocyte producing *P. berghei*

Appendix 3		**Proteins enriched in *TEP1 LOF* infected with *PbGFP***					
AGAP Ids	**OLD Protein ID**	**Interpro Annotation**	**Mass (Da)**	***GOF GFP***	***LOF GFP***	***GOF PbMut***	***LOF PbMut***
AGAP009670-PA	ENSANGP00000004662	SRPN 4	40482	0	3	0	1
AGAP006513-PA	ENSANGP00000009987	RNA-binding region RNP-1	183283	1	4	0	1
AGAP000852-PA	ENSANGP00000010395	Ubiquitin domain	9480	1	2	0	0
AGAP002982-PA	ENSANGP00000010912	Antifreeze protein, type I	277460	1	5	0	1
AGAP011190-PA	ENSANGP00000011259	RNA binding S1	38135	1	3	0	1
#N/A	ENSANGP00000012302	Zn-finger, C2H2 type	18200	1	5	0	0
AGAP006348-PA	ENSANGP00000013041	LRIM1	57325	0	2	0	0
AGAP006818-PA	ENSANGP00000013211	Ribonucleotide reductase	46605	1	2	0	0
AGAP001760-PA	ENSANGP00000013323	Calcium-binding EF-hand	22253	1	2	0	1
AGAP001212-PA	ENSANGP00000013948	PGRP-LB	31924	1	2	0	0
AGAP005402-PA	ENSANGP00000014276	Beta tubulin	14502	1	2	0	0
AGAP009852-PA	ENSANGP00000014824	TPR repeat	10853	1	2	0	0
AGAP002336-PA	ENSANGP00000015403	Subtilase serine protease	74701	1	2	0	0
AGAP012014-PA	ENSANGP00000015770	ATP/GTP-binding site motif A (P-loop)	20732	1	9	0	0
AGAP012008-PA	ENSANGP00000015778	PDZ/DHR/GLGF domain	28546	1	2	0	0
AGAP003021-PA	ENSANGP00000018215	7-Fold repeat in clathrin and VPS proteins	193086	1	3	0	0
AGAP002878-PA	ENSANGP00000018765	Ambiguous	11170	1	2	0	0
AGAP001942-PA	ENSANGP00000019097	Fumarylacetoacetate hydrolase	25396	1	4	0	1
AGAP000145-PA	ENSANGP00000019908	Ubiquitin-conjugating enzymes	16896	1	3	0	0
AGAP009554-PA	ENSANGP00000019967	Calponin-like actin-binding	544216	1	2	0	0
AGAP011762-PA	ENSANGP00000019996	Apoptosis regulator Bcl-2 protein, BAG	37934	1	2	0	0
AGAP011768-PA	ENSANGP00000020137	SH2 motif	21486	1	3	0	0
AGAP000927-PA	ENSANGP00000021084	Calcium-binding EF-hand	20976	1	2	0	0
AGAP002521-PA	ENSANGP00000021339	Short-chain dehydrogenase/reductase SDR	28059	1	2	0	0
AGAP0O7692-PA	ENSANGP00000021521	SRPN 14	45922	0	2	0	0
AGAP002340-PA	ENSANGP00000023614	Bipartite nuclear localization signal	126853	1	2	0	0
AGAP009305-PA	ENSANGP00000027824	Glucosamine-6-phosphate isomerase	29239	1	3	0	0
AGAP007698-PC	ENSANGP00000027989	Syntaxin/epimorphin family	33571	1	3	0	0

GOF: *TEP1 GOF* ; *LOF* : *TEP1 LOF*; *GFP*: *GFP* expressing *P. berghei* ; *PbMut* : non-gametocyte producing *P. berghei*

Appendix 4

Proteins enriched in both *TEP1 LOF* and *GOF* infected with *PbGFP*

AGAP Ids	OLD Protein ID	Interpro Annotation	Mass (Da)	*GOF GFP*	*LOF GFP*	*GOF PbMut*	*LOF PbMut*
AGAP002408-PA	ENSANGP00000001212	Aldo/keto reductase	41012	3	4	0	1
AGAP010251-PA	ENSANGP00000002872	G-protein beta WD-40 repeat	141133	4	4	0	0
AGAP002472-PA	ENSANGP00000003051	Ribosomal protein S14	127530	3	2	0	0
AGAP002463-PA	ENSANGP00000003062	Ubiquitin-associated domain	84795	7	5	0	0
AGAP004335-PA	ENSANGP00000003616	Actin-binding, actinin-type	262226	7	9	0	0
AGAP009031-PA	ENSANGP00000005182	Eukaryotic ribosomal protein L5	38083	8	7	0	0
AGAP008530-PA	ENSANGP00000006552	Ubiquitin thiolesterase, family 2	123227	2	3	0	0
AGAP008294-PA	ENSANGP00000006721	Serine protease, trypsin family	29018	2	2	0	0
AGAP008061-PA	ENSANGP00000006959	Glycoside hydrolase, family 18	49828	7	6	0	0
AGAP001791-PA	ENSANGP00000008439	Neutral zinc metallopeptidases, zinc-binding region	88255	3	2	0	0
AGAP001601-PA	ENSANGP00000008588	Protein kinase	63830	3	4	0	0
AGAP011806-PA	ENSANGP00000008816	Orn/DAP/Arg decarboxylase 2	45616	7	5	0	0
AGAP012072-PA	ENSANGP00000009226	Glyoxalase I	20631	5	2	0	1
AGAP007523-PB	ENSANGP00000009410	ATP/GTP-binding site motif A (P-loop)	158670	10	20	0	0
AGAP000396-PA	ENSANGP00000009997	Alkyl hydroperoxide reductase/ Thiol specific antioxidant/ Mal allergen	26193	5	5	0	0
AGAP000380-PA	ENSANGP00000010003	Bipartite nuclear localization signal	70550	6	4	0	0
AGAP007806-PA	ENSANGP00000010348	Calcium-binding EF-hand	16779	4	5	0	0
AGAP003560-PA	ENSANGP00000010474	UDP glucose:glycoprotein glucosyltransferase	171585	2	2	0	0
AGAP002608-PA	ENSANGP00000010594	Carbohydrate kinase, PfkB	32510	4	4	0	0
AGAP009119-PA	ENSANGP00000010637	RNA-binding region RNP-1 (RNA recognition motif)	35162	3	4	0	0
AGAP004749-PB	ENSANGP00000010734	Coproporphyrinogen III oxidase	38256	5	7	0	0
AGAP004793-PA	ENSANGP00000010754	Aminotransferase class-III	45970	6	5	0	0
AGAP007619-PA	ENSANGP00000010942	Peptidase M20/M25/M40	53153	8	4	0	0
AGAP009510-PA	ENSANGP00000011006	Malate dehydrogenase	35470	19	17	0	0
AGAP002969-PA	ENSANGP00000011058	Aspartyl-tRNA synthetase, class IIb	64276	3	2	0	0
AGAP004071-PA	ENSANGP00000011155	Calcium-binding EF-hand	68049	13	13	0	0
AGAP006643-PA	ENSANGP00000011337	Leucine-rich repeat	78268	2	2	0	1
AGAP011396-PA	ENSANGP00000011363	Calcium-binding EF-hand	959682	6	2	0	0
AGAP006570-PA	ENSANGP00000011437	Inositol monophosphatase	29918	7	6	0	0
AGAP006607-PA	ENSANGP00000011568	G-protein beta WD-40 repeat	36568	4	3	0	1
AGAP004164-PA	ENSANGP00000011661	Glutathione S-transferase, N-terminal	23939	13	9	0	0
#N/A	ENSANGP00000011670	D-isomer specific 2-hydroxyacid dehydrogenase, NAD binding domain	35841	5	3	0	0
AGAP004159-PA	ENSANGP00000011712	Malic oxidoreductase	62623	18	15	0	0
AGAP000719-PA	ENSANGP00000011950	S-adenosyl-L-homocysteine hydrolase	48276	2	2	0	0
AGAP009985-PA	ENSANGP00000011965	PYRIDOXAL PHOSPHATE PHOSPHATASE	32655	9	12	0	0
AGAP004559-PA	ENSANGP00000012226	ATP/GTP-binding site motif A (P-loop)	24446	6	3	0	0
AGAP010917-PA	ENSANGP00000012405	Carboxylesterase, type B	53836	3	3	0	0

Appendix 4 Continued… **Proteins enriched in both *TEP1 LOF* and *GOF* infected with *PbGFP***

AGAP Ids	OLD Protein ID	Interpro Annotation	Mass (Da)	*GOF GFP*	*LOF GFP*	*GOF PbMut*	*LOF PbMut*
AGAP010935-PA	ENSANGP00000012449	Delta-aminolevulinic acid dehydratase	35607	2	2	0	0
AGAP000255-PA	ENSANGP00000012597	Guanylate kinase	87709	6	4	0	0
AGAP010957-PA	ENSANGP00000012700	Calcium-binding EF-hand	17246	6	4	0	0
AGAP006353-PA	ENSANGP00000012999	Histidine triad (HIT) protein	14191	3	6	0	0
AGAP006360-PA	ENSANGP00000013025	ATP/GTP-binding site motif A (P-loop)	228725	13	4	0	0
AGAP004396-PA	ENSANGP00000013071	Acetyl-CoA hydrolase/transferase	52283	11	8	0	1
AGAP008632-PA	ENSANGP00000013147	Saccharopine dehydrogenase	100923	14	10	0	1
AGAP004236-PA	ENSANGP00000013329	Beta-lactamase-like	32951	4	2	0	1
AGAP008648-PA	ENSANGP00000013338	Galectin-12 RELATED	23211	3	3	0	1
AGAP002219-PA	ENSANGP00000013477	ATP/GTP-binding site motif A (P-loop)	22479	3	3	0	0
AGAP002227-PA	ENSANGP00000013516	Pyridoxamine 5'-phosphate oxidase	28542	5	4	0	0
AGAP003277-PA	ENSANGP00000013568	Eukaryotic/viral aspartic protease, active site	42148	14	12	0	0
AGAP012140-PA	ENSANGP00000013937	RNA-binding region RNP-1 (RNA recognition motif)	80761	4	11	0	0
AGAP001264-PA	ENSANGP00000013946	Peptidase family M28	53479	5	2	0	0
AGAP005404-PA	ENSANGP00000014225	Legume-like lectin	58614	2	3	0	0
AGAP010229-PA	ENSANGP00000014344	Nitrilase/cyanide hydratase and apolipoprotein N-acyltransferase	43778	9	9	0	0
AGAP010267-PA	ENSANGP00000014418	Aminoacyl-tRNA synthetase, class I	88543	5	5	0	0
AGAP003331-PA	ENSANGP00000014464	Haloacid dehalogenase-like hydrolase	30543	5	2	0	0
AGAP009379-PA	ENSANGP00000014554	Cellular retinaldehyde binding/alpha-tocopherol transport	33285	6	6	0	0
AGAP003238-PA	ENSANGP00000014705	Ndr family	38531	8	3	0	0
AGAP003236-PA	ENSANGP00000014721	Protein of unknown function DUF227	32105	2	2	0	0
AGAP004002-PA	ENSANGP00000014839	Chaperonin Cpn60	61025	13	10	0	0
AGAP011938-PA	ENSANGP00000015219	Intermediate filament, C-terminal	67247	7	5	0	0
AGAP010331-PA	ENSANGP00000015293	Heat shock protein Hsp70	89965	5	7	0	0
AGAP002337-PA	ENSANGP00000015368	Eukaryotic translation initiation factor 3, subunit 7	63655	9	8	0	0
AGAP000308-PA	ENSANGP00000015576	Proteasome activator pa28, alpha subunit	28476	3	3	0	0
AGAP005162-PB	ENSANGP00000015605	ATP/GTP-binding site motif A (P-loop)	84575	2	3	0	0
AGAP012048-PA	ENSANGP00000015768	Citrate synthase	51712	11	10	0	0
AGAP001424-PA	ENSANGP00000015826	Heat shock protein Hsp90	68416	13	7	0	0
AGAP004654-PA	ENSANGP00000016061	Inositol monophosphatase	33436	6	6	0	0
AGAP001026-PA	ENSANGP00000016112	Zinc carboxypeptidase A metalloprotease (M14)	47641	5	2	0	0
AGAP002802-PA	ENSANGP00000016208	Myb DNA-binding domain	96276	10	5	0	0
AGAP003165-PA	ENSANGP00000016555	Aldehyde dehydrogenase	57242	12	12	0	0
AGAP012247-PA	ENSANGP00000016587	Haloacid dehalogenase-like hydrolase	25746	3	7	0	0
AGAP009197-PA	ENSANGP00000016648	Glutathione S-transferase, N-terminal	25197	8	8	0	0
AGAP009176-PA	ENSANGP00000016695	Beta-ketoacyl synthase	263589	7	3	0	0
AGAP009173-PA	ENSANGP00000016841	Inositol phosphatase/fructose-1,6-bisphosphatase	37873	12	8	0	0

Appendix 4 Continued… **Proteins enriched in both *TEP1 LOF* and *GOF* infected with *PbGFP***

AGAP Ids	OLD Protein ID	Interpro Annotation	Mass (Da)	*GOF GFP*	*LOF GFP*	*GOF PbMut*	*LOF PbMut*
AGAP001381-PA	ENSANGP00000016875	EGF-like domain	202403	5	6	0	0
AGAP012126-PA	ENSANGP00000016910	Phospholipase/Carboxylesterase	23732	3	3	0	0
AGAP001384-PA	ENSANGP00000016916	Protein kinase	32366	3	4	0	0
AGAP010586-PA	ENSANGP00000016933	t-RNA-binding region	33133	3	2	0	0
AGAP004440-PA	ENSANGP00000017034	Actin-binding, actinin-type	474540	15	17	0	0
AGAP006141-PB	ENSANGP00000017140	F-actin capping protein, alpha subunit	33110	3	4	0	0
AGAP000562-PA	ENSANGP00000017331	Band 4.1 domain	73949	7	9	0	0
AGAP005845-PA	ENSANGP00000017401	V-ATPase subunit C	92682	8	12	0	0
AGAP005860-PA	ENSANGP00000017432	Phosphoglucomutase/phosphomannomutase	61354	3	2	0	0
AGAP000733-PA	ENSANGP00000017457	Ubiquitin-associated domain	31910	2	3	0	0
AGAP000749-PA	ENSANGP00000017613	SM22/calponin	18871	3	4	0	0
AGAP000901-PA	ENSANGP00000017843	1-aminocyclopropane-1-carboxylate synthase	53194	14	16	0	0
AGAP000889-PA	ENSANGP00000017848	Actin-binding, cofilin/tropomyosin type	16627	3	4	0	0
AGAP001621-PA	ENSANGP00000018146	Protein of unknown function UPF0001	29316	2	4	0	0
AGAP001622-PA	ENSANGP00000018155	Calcium-binding EF-hand	22879	3	2	0	0
AGAP003016-PA	ENSANGP00000018242	Bipartite nuclear localization signal	21337	2	2	0	0
AGAP007378-PB	ENSANGP00000018337	3-hydroxyacyl-CoA dehydrogenase, C-terminal	34995	9	5	0	0
AGAP001930-PA	ENSANGP00000018356	RNA-binding region RNP-1 (RNA recognition motif)	43180	2	2	0	0
AGAP009291-PA	ENSANGP00000018358	Adaptin, N-terminal	98945	2	2	0	0
AGAP007852-PA	ENSANGP00000018525	RNA-binding region RNP-1 (RNA recognition motif)	83341	20	2	0	0
AGAP007474-PA	ENSANGP00000019107	Band 4.1 domain	277884	21	20	0	0
AGAP007864-PA	ENSANGP00000019124	F-actin capping protein, beta subunit	31152	7	7	0	0
AGAP010052-PA	ENSANGP00000019212	Carbonic anhydrase, eukaryotic	17419	3	4	0	0
AGAP008502-PA	ENSANGP00000019292	Short-chain dehydrogenase/reductase SDR	25818	3	7	0	0
AGAP002171-PA	ENSANGP00000019413	Pre-mRNA processing ribonucleoprotein, binding region	57457	4	2	0	0
AGAP008952-PA	ENSANGP00000019415	Lupus La protein	44718	6	8	0	0
AGAP007532-PA	ENSANGP00000019826	Vinculin	107540	17	4	0	0
AGAP012304-PA	ENSANGP00000020070	Calponin repeat	20444	3	7	0	0
AGAP001919-PA	ENSANGP00000020140	Endoplasmic reticulum targeting sequence	49103	6	5	0	1
AGAP011777-PA	ENSANGP00000020171	Ribosomal protein S2	31578	3	2	0	0
AGAP011824-PA	ENSANGP00000020201	Alkyl hydroperoxide reductase/ Thiol specific antioxidant/ Mal allergen	25221	11	10	0	0
AGAP001827-PA	ENSANGP00000020237	Heat shock protein Hsp70	102271	2	4	0	0
AGAP001313-PA	ENSANGP00000020389	SM22/calponin	21027	2	3	0	0
AGAP004098-PA	ENSANGP00000020422	ATP/GTP-binding site motif A (P-loop)	22088	2	2	0	0
AGAP006226-PA	ENSANGP00000020620	CO dehydrogenase flavoprotein C-terminal domain	16828	2	2	0	1
AGAP006227-PA	ENSANGP00000020622	Carboxylesterase, type B	63373	3	2	0	0
AGAP009621-PA	ENSANGP00000020847	Glycoside hydrolase, family 27	49572	5	2	0	0

Appendix 4 Continued… **Proteins enriched in both *TEP1 LOF* and *GOF* infected with *PbGFP***

AGAP Ids	OLD Protein ID	Interpro Annotation	Mass (Da)	*GOF GFP*	*LOF GFP*	*GOF PbMut*	*LOF PbMut*
AGAP006660-PC	ENSANGP00000020939	Isocitrate/isopropylmalate dehydrogenase	47288	14	5	0	0
AGAP002667-PA	ENSANGP00000021085	Translationally controlled tumor protein	19649	8	2	0	0
AGAP002661-PA	ENSANGP00000021099	Enoyl-CoA hydratase/isomerase	32838	4	4	0	0
AGAP005531-PA	ENSANGP00000021175	BRO1	94388	18	20	0	0
AGAP003700-PA	ENSANGP00000021308	Methionine aminopeptidase, subfamily 1	42760	4	3	0	0
AGAP000792-PA	ENSANGP00000021319	S-adenosyl-L-homocysteine hydrolase	51700	2	5	0	0
AGAP009143-PA	ENSANGP00000021503	Speract/scavenger receptor	348598	7	3	0	0
AGAP009159-PA	ENSANGP00000021504	AMP-dependent synthetase and ligase	60757	5	4	0	0
AGAP007692-PA	ENSANGP00000021521	SRPN 14	45922	0	2	0	0
AGAP007629-PA	ENSANGP00000021549	EGF-like domain	185132	12	13	0	0
AGAP006724-PA	ENSANGP00000021598	Carboxylesterase, type B	69847	3	3	0	0
AGAP005672-PA	ENSANGP00000021797	Staphylococcus nuclease (SNase-like)	104600	15	11	0	0
AGAP008193-PA	ENSANGP00000021850	Aspartic acid and asparagine hydroxylation site	146607	13	5	0	1
AGAP011157-PA	ENSANGP00000022042	Prostaglandin E synthase 3	18598	2	2	0	0
AGAP011172-PA	ENSANGP00000022045	Cys/Met metabolism pyridoxal-phosphate-dependent enzymes	43510	4	4	0	0
AGAP011173-PA	ENSANGP00000022049	Ribosomal protein L5	21912	4	2	0	0
#N/A	ENSANGP00000022061	ATP/GTP-binding site motif A (P-loop)	307499	6	7	0	1
AGAP001676-PA	ENSANGP00000022175	Actin	42207	5	3	0	0
AGAP007740-PA	ENSANGP00000022228	60S Acidic ribosomal protein	12562	3	3	0	0
AGAP004940-PA	ENSANGP00000022232	Cyclic nucleotide-binding domain	43619	8	4	0	0
AGAP003742-PA	ENSANGP00000022492	Regulator of chromosome condensation, RCC1	43328	3	3	0	0
AGAP009210-PA	ENSANGP00000023159	CAP-Gly domain	179589	6	2	0	0
AGAP008367-PA	ENSANGP00000023583	Alpha-2-macroglobulin, N-terminal	19763	2	3	0	0
AGAP000577-PA	ENSANGP00000023647	Adenosine/AMP deaminase active site	82826	6	7	0	0
AGAP001257-PA	ENSANGP00000024060	UTP--glucose-1-phosphate uridylyltransferase	58498	27	15	0	0
AGAP002520-PA	ENSANGP00000024539	Short-chain dehydrogenase/reductase SDR	22494	4	2	0	0
AGAP004097-PA	ENSANGP00000024573	Enoyl-CoA hydratase/isomerase	39974	11	10	0	0
AGAP004164-PB	ENSANGP00000024808	Glutathione S-transferase, N-terminal	23601	5	3	0	0
AGAP000720-PA	ENSANGP00000024884	Fibronectin, type III	132704	10	12	0	0
AGAP008225-PA	ENSANGP00000024945	Trehalose-phosphatase	28385	2	3	0	0
AGAP006615-PA	ENSANGP00000025399	Peptidylprolyl isomerase, FKBP-type	44932	6	5	0	0
AGAP002654-PA	ENSANGP00000025436	RNA-binding region RNP-1 (RNA recognition motif)	17022	3	6	0	0
AGAP011872-PA	ENSANGP00000025877	Ubiquitin-activating enzyme	116447	11	5	0	0
AGAP009347-PA	ENSANGP00000025947	Peptidylprolyl isomerase, FKBP-type	44301	3	3	0	0
AGAP005728-PA	ENSANGP00000026472	ATP/GTP-binding site motif A (P-loop)	98978	12	7	0	0
AGAP011369-PA	ENSANGP00000027635	Gelsolin	82493	14	7	0	0
AGAP002481-PA	ENSANGP00000029144	Proteasome/cyclosome, regulatory subunit	97475	2	6	0	0

Appendix 4 Continued… **Proteins enriched in both *TEP1 LOF* and *GOF* infected with *PbGFP***

AGAP Ids	OLD Protein ID	Interpro Annotation	Mass (Da)	*GOF GFP*	*LOF GFP*	*GOF PbMut*	*LOF PbMut*
AGAP008366-PA	ENSANGP00000029324	Alpha-2-macroglobulin	26212	4	5	0	0
#N/A	ENSANGP00000031812	Immunoglobulin-like	15585	2	2	0	0
AGAP010578-PA	ENSANGP00000029889	TYROSYL DNA PHOSPHODIESTERASE	26839	2	2	0	0
AGAP011051-PA	ENSANGP00000031808	Aldose reductase	35444	3	2	0	0
AGAP000570-PA	ENSANGP00000031874	Serum inhibited-related	17416	5	3	0	1
AGAP008060-PA	ENSANGP00000032017	Bacteria responsive protein 2.	48395	7	3	0	0

GOF: *TEP1 GOF* ; *LOF* : *TEP1 LOF*; *GFP*: *GFP* expressing *P. berghei* ; *PbMut* : non-gametocyte producing *P. berghei*

References

Abraham, E.G., Pinto, S.B., Ghosh, A., Vanlandingham, D.L., Budd, A., Higgs, S., Kafatos, F.C., Jacobs-Lorena, M., and Michel, K. (2005). An immune-responsive serpin, SRPN6, mediates mosquito defense against malaria parasites. Proceedings of the National Academy of Sciences of the United States of America *102*, 16327-16332.

Adamo, S.A., Roberts, J.L., Easy, R.H., and Ross, N.W. (2008). Competition between immune function and lipid transport for the protein apolipophorin III leads to stress-induced immunosuppression in crickets. The Journal of experimental biology *211*, 531-538.

Aderem, A., and Underhill, D.M. (1999). Mechanisms of phagocytosis in macrophages. Annual review of immunology *17*, 593-623.

Agianian, B., Lesch, C., Loseva, O., and Dushay, M.S. (2007). Preliminary characterization of hemolymph coagulation in Anopheles gambiae larvae. Developmental and comparative immunology *31*, 879-888.

Ahmed, A.M., Maingon, R., Romans, P., and Hurd, H. (2001). Effects of malaria infection on vitellogenesis in Anopheles gambiae during two gonotrophic cycles. Insect molecular biology *10*, 347-356.

Akaki, M., and Dvorak, J.A. (2005). A chemotactic response facilitates mosquito salivary gland infection by malaria sporozoites. The Journal of experimental biology *208*, 3211-3218.

Alavi, Y., Arai, M., Mendoza, J., Tufet-Bayona, M., Sinha, R., Fowler, K., Billker, O., Franke-Fayard, B., Janse, C.J., Waters, A.*, et al.* (2003). The dynamics of interactions between Plasmodium and the mosquito: a study of the infectivity of Plasmodium berghei and Plasmodium gallinaceum, and their transmission by Anopheles stephensi, Anopheles gambiae and Aedes aegypti. International journal for parasitology *33*, 933-943.

Alphey, L., Beard, C.B., Billingsley, P., Coetzee, M., Crisanti, A., Curtis, C., Eggleston, P., Godfray, C., Hemingway, J., Jacobs-Lorena, M.*, et al.* (2002). Malaria control with genetically manipulated insect vectors. Science (New York, NY *298*, 119-121.

Amino, R., Giovannini, D., Thiberge, S., Gueirard, P., Boisson, B., Dubremetz, J.F., Prevost, M.C., Ishino, T., Yuda, M., and Menard, R. (2008). Host cell traversal is important for progression of the malaria parasite through the dermis to the liver. Cell host & microbe *3*, 88-96.

Amino, R., Thiberge, S., Martin, B., Celli, S., Shorte, S., Frischknecht, F., and Menard, R. (2006). Quantitative imaging of Plasmodium transmission from mosquito to mammal. Nature medicine *12*, 220-224.

Arai, M., Billker, O., Morris, H.R., Panico, M., Delcroix, M., Dixon, D., Ley, S.V., and Sinden, R.E. (2001). Both mosquito-derived xanthurenic acid and a host blood-derived factor regulate gametogenesis of Plasmodium in the midgut of the mosquito. Molecular and biochemical parasitology *116*, 17-24.

Atella, G.C., Bittencourt-Cunha, P.R., Nunes, R.D., Shahabuddin, M., and Silva-Neto, M.A. (2009). The major insect lipoprotein is a lipid source to mosquito stages of malaria parasite. Acta tropica *109*, 159-162.

Atella, G.C., Silva-Neto, M.A., Golodne, D.M., Arefin, S., and Shahabuddin, M. (2006).

Anopheles gambiae lipophorin: characterization and role in lipid transport to developing oocyte. Insect biochemistry and molecular biology *36*, 375-386.

Attardo, G.M., Higgs, S., Klingler, K.A., Vanlandingham, D.L., and Raikhel, A.S. (2003). RNA interference-mediated knockdown of a GATA factor reveals a link to anautogeny in the mosquito Aedes aegypti. Proceedings of the National Academy of Sciences of the United States of America *100*, 13374-13379.

Baxter, R.H., Chang, C.I., Chelliah, Y., Blandin, S., Levashina, E.A., and Deisenhofer, J. (2007). Structural basis for conserved complement factor-like function in the antimalarial protein TEP1. Proceedings of the National Academy of Sciences of the United States of America *104*, 11615-11620.

Blandin, S., Moita, L.F., Kocher, T., Wilm, M., Kafatos, F.C., and Levashina, E.A. (2002). Reverse genetics in the mosquito Anopheles gambiae: targeted disruption of the Defensin gene. EMBO reports *3*, 852-856.

Blandin, S., Shiao, S.H., Moita, L.F., Janse, C.J., Waters, A.P., Kafatos, F.C., and Levashina, E.A. (2004). Complement-like protein TEP1 is a determinant of vectorial capacity in the malaria vector Anopheles gambiae. Cell *116*, 661-670.

Blandin, S.A., Marois, E., and Levashina, E.A. (2008). Antimalarial responses in Anopheles gambiae: From a complement-like protein to a complement-like pathway. Cell Host & Microbe *3*, 364-374.

Boman, H.G., Faye, I., Gudmundsson, G.H., Lee, J.Y., and Lidholm, D.A. (1991). Cell-free immunity in Cecropia. A model system for antibacterial proteins. European journal of biochemistry / FEBS *201*, 23-31.

Bonifacio, E., Lampasona, V., Genovese, S., Ferrari, M., and Bosi, E. (1995). Identification of protein tyrosine phosphatase-like IA2 (islet cell antigen 512) as the insulin-dependent diabetes-related 37/40K autoantigen and a target of islet-cell antibodies. J Immunol *155*, 5419-5426.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical biochemistry *72*, 248-254.

Braun, A., Hoffmann, J.A., and Meister, M. (1998). Analysis of the Drosophila host defense in domino mutant larvae, which are devoid of hemocytes. Proceedings of the National Academy of Sciences of the United States of America *95*, 14337-14342.

Butler, D., Maurice, J., and O'Brien, C. (1997). Research 'essential' for control strategy. Nature *386*, 537.

Chen C, Rowley AF, Newton RP, Ratcliffe NA(1999). Identification, purification and properties of a beta-1,3-glucan-specific lectin from the serum of the cockroach, Blaberus discoidalis which is implicated in immune defence reactions. Comp Biochem Physiol B Biochem Mol Biol, *122*:309-319.

Cheon, H.M., Shin, S.W., Bian, G., Park, J.H., and Raikhel, A.S. (2006). Regulation of lipid metabolism genes, lipid carrier protein lipophorin, and its receptor during immune challenge in the mosquito Aedes aegypti. The Journal of biological chemistry *281*, 8426-8435.

Cho, W.L., Tsao, S.M., Hays, A.R., Walter, R., Chen, J.S., Snigirevskaya, E.S., and Raikhel, A.S. (1999). Mosquito cathepsin B-like protease involved in embryonic degradation of vitellin is produced as a latent extraovarian precursor. The Journal of

biological chemistry *274*, 13311-13321.

Christophides, G.K., Zdobnov, E., Barillas-Mury, C., Birney, E., Blandin, S., Blass, C., Brey, P.T., Collins, F.H., Danielli, A., Dimopoulos, G.*, et al.* (2002). Immunity-related genes and gene families in Anopheles gambiae. Science *298*, 159-165.

Collins, F.H., Sakai, R.K., Vernick, K.D., Paskewitz, S., Seeley, D.C., Miller, L.H., Collins, W.E., Campbell, C.C., and Gwadz, R.W. (1986). Genetic selection of a Plasmodium-refractory strain of the malaria vector Anopheles gambiae. Science (New York, NY *234*, 607-610.

Cox, B., Kislinger, T., and Emili, A. (2005). Integrating gene and protein expression data: pattern analysis and profile mining. Methods (San Diego, Calif *35*, 303-314.

Cox, J., and Mann, M. (2007). Is proteomics the new genomics? Cell *130*, 395-398.

Crozatier, M., and Meister, M. (2007). Drosophila haematopoiesis. Cellular microbiology *9*, 1117-1126.

Danielli, A., Barillas-Mury, C., Kumar, S., Kafatos, F.C., and Loukeris, T.G. (2005). Overexpression and altered nucleocytoplasmic distribution of Anopheles ovalbumin-like SRPN10 serpins in Plasmodium-infected midgut cells. Cellular microbiology *7*, 181-190.

Dantuma, N.P., Potters, M., De Winther, M.P., Tensen, C.P., Kooiman, F.P., Bogerd, J., and Van der Horst, D.J. (1999). An insect homolog of the vertebrate very low density lipoprotein receptor mediates endocytosis of lipophorins. Journal of lipid research *40*, 973-978.

Das, P.K., and Amalraj, D.D. (1997). Biological control of malaria vectors. Indian J Med Res *106*, 174-197.

de Valdez, M.R. (2006). Parasitoid-induced behavioral alterations of Aedes aegypti mosquito larvae infected with mermithid nematodes (Nematoda: Mermithidae). J Vector Ecol *31*, 344-354.

DeJong, R.J., Miller, L.M., Molina-Cruz, A., Gupta, L., Kumar, S., and Barillas-Mury, C. (2007). Reactive oxygen species detoxification by catalase is a major determinant of fecundity in the mosquito Anopheles gambiae. Proceedings of the National Academy of Sciences of the United States of America *104*, 2121-2126.

Dimopoulos, G. (2003). Insect immunity and its implication in mosquito-malaria interactions. Cellular microbiology *5*, 3-14.

Dodds, A.W., and Law, S.K. (1998). The phylogeny and evolution of the thioester bond-containing proteins C3, C4 and alpha 2-macroglobulin. Immunological reviews *166*, 15-26.

Dong, Y., Aguilar, R., Xi, Z., Warr, E., Mongin, E., and Dimopoulos, G. (2006). Anopheles gambiae immune responses to human and rodent Plasmodium parasite species. PLoS pathogens *2*, e52.

Dong, Y., Aguilar, R., Xi, Z., Warr, E., Mongin, E., and Dimopoulos, G. (2006). Anopheles gambiae immune responses to human and rodent Plasmodium parasite species. PLoS pathogens *2*, e52.

Ecker, A., Bushell, E.S., Tewari, R., and Sinden, R.E. (2008). Reverse genetics screen identifies six proteins important for malaria development in the mosquito. Molecular microbiology *70*, 209-220.

Ferrandon, D., Imler, J.L., Hetru, C., and Hoffmann, J.A. (2007). The Drosophila

systemic immune response: sensing and signalling during bacterial and fungal infections. Nat Rev Immunol *7*, 862-874.

Franke-Fayard, B., Trueman, H., Ramesar, J., Mendoza, J., van der Keur, M., van der Linden, R., Sinden, R.E., Waters, A.P., and Janse, C.J. (2004). A Plasmodium berghei reference line that constitutively expresses GFP at a high level throughout the complete life cycle. Mol Biochem Parasitol *137*, 23-33.

Fried, M., Muga, R.O., Misore, A.O., and Duffy, P.E. (1998). Malaria elicits type 1 cytokines in the human placenta: IFN-gamma and TNF-alpha associated with pregnancy outcomes. J Immunol *160*, 2523-2530.

Frolet, C., Thoma, M., Blandin, S., Hoffmann, J.A., and Levashina, E.A. (2006). Boosting NF-kappaB-dependent basal immunity of Anopheles gambiae aborts development of Plasmodium berghei. Immunity *25*, 677-685.

Gorman, M.J., Andreeva, O.V., and Paskewitz, S.M. (2000). Molecular characterization of five serine protease genes cloned from Anopheles gambiae hemolymph. Insect biochemistry and molecular biology *30*, 35-46.

Gotz, P. (1986) Encapsulation in arthropods. In Immunity in Invertebrates Brehelin, M. Berlin: Springer-Verlag, pp. 153–170.

Graveley, B.R., Kaur, A., Gunning, D., Zipursky, S.L., Rowen, L., and Clemens, J.C. (2004). The organization and evolution of the dipteran and hymenopteran Down syndrome cell adhesion molecule (Dscam) genes. RNA (New York, NY *10*, 1499-1506.

Gundersen, S.G. (1992). [Resistance problems in developing countries--use and misuse of antiinfective agents]. Tidsskr Nor Laegeforen *112*, 2741-2746.

Gygi, S.P., Rochon, Y., Franza, B.R., and Aebersold, R. (1999). Correlation between protein and mRNA abundance in yeast. Molecular and cellular biology *19*, 1720-1730.

Habtewold, T., Povelones, M., Blagborough, A.M., and Christophides, G.K. (2008). Transmission blocking immunity in the malaria non-vector mosquito Anopheles quadriannulatus species A. PLoS pathogens *4*, e1000070.

Hagedorn, H.H., and Judson, C.L. (1972). Purification and site of synthesis of Aedes aegypti yolk proteins. The Journal of experimental zoology *182*, 367-377.

Han, Y.S., Thompson, J., Kafatos, F.C., and Barillas-Mury, C. (2000). Molecular interactions between Anopheles stephensi midgut cells and Plasmodium berghei: the time bomb theory of ookinete invasion of mosquitoes. The EMBO journal *19*, 6030-6040.

Hansen, I.A., Attardo, G.M., Roy, S.G., and Raikhel, A.S. (2005). Target of rapamycin-dependent activation of S6 kinase is a central step in the transduction of nutritional signals during egg development in a mosquito. The Journal of biological chemistry *280*, 20565-20572.

Harjes, E., Harjes, S., Wohlgemuth, S., Muller, K.H., Krieger, E., Herrmann, C., and Bayer, P. (2006). GTP-Ras disrupts the intramolecular complex of C1 and RA domains of Nore1. Structure *14*, 881-888.

Harman, D. (2003). The free radical theory of aging. Antioxidants & redox signaling *5*, 557-561.

Henzel, W.J., Billeci, T.M., Stults, J.T., Wong, S.C., Grimley, C., and Watanabe, C. (1993). Identifying proteins from two-dimensional gels by molecular mass searching of

peptide fragments in protein sequence databases. Proceedings of the National Academy of Sciences of the United States of America *90*, 5011-5015.

Hoffmann, J.A. (2003). The immune response of Drosophila. Nature *426*, 33-38.

Huang, C.L., Hwang, S.Y., Chiang, Y.C., and Lin, T.P. (2008). Molecular evolution of the Pi-ta gene resistant to rice blast in wild rice (Oryza rufipogon). Genetics *179*, 1527-1538.

Hurd, H. (2001). Host fecundity reduction: a strategy for damage limitation? Trends in parasitology *17*, 363-368.

Inoki, K., Li, Y., Zhu, T., Wu, J., and Guan, K.L. (2002). TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. Nature cell biology *4*, 648-657.

Ishihama, Y., Oda, Y., Tabata, T., Sato, T., Nagasu, T., Rappsilber, J., and Mann, M. (2005). Exponentially modified protein abundance index (emPAI) for estimation of absolute protein amount in proteomics by the number of sequenced peptides per protein. Mol Cell Proteomics *4*, 1265-1272.

Ishihama, Y., Oda, Y., Tabata, T., Sato, T., Nagasu, T., Rappsilber, J., and Mann, M. (2005). Exponentially modified protein abundance index (emPAI) for estimation of absolute protein amount in proteomics by the number of sequenced peptides per protein. Mol Cell Proteomics *4*, 1265-1272.

Ishihama, Y., Rappsilber, J., and Mann, M. (2006). Modular stop and go extraction tips with stacked disks for parallel and multidimensional Peptide fractionation in proteomics. Journal of proteome research *5*, 988-994.

Kalume, D.E., Peri, S., Reddy, R., Zhong, J., Okulate, M., Kumar, N., and Pandey, A. (2005). Genome annotation of Anopheles gambiae using mass spectrometry-derived data. BMC genomics *6*, 128.

Kamhawi, S., Ramalho-Ortigao, M., Pham, V.M., Kumar, S., Lawyer, P.G., Turco, S.J., Barillas-Mury, C., Sacks, D.L., and Valenzuela, J.G. (2004). A role for insect galectins in parasite survival. Cell *119*, 329-341.

Kato, Y., Motoi, Y., Taniai, K., Kadono-Okuda, K., Yamamoto, M., Higashino, Y., Shimabukuro, M., Chowdhury, S., Xu, J., Sugiyama, M.*, et al.* (1994). Lipopolysaccharide-lipophorin complex formation in insect hemolymph: a common pathway of lipopolysaccharide detoxification both in insects and in mammals. Insect biochemistry and molecular biology *24*, 547-555.

Kawooya, J.K., and Law, J.H. (1988). Role of lipophorin in lipid transport to the insect egg. The Journal of biological chemistry *263*, 8748-8753.

Kay, B., and Vu, S.N. (2005). New strategy against Aedes aegypti in Vietnam. Lancet *365*, 613-617.

Knols, B.G., Bossin, H.C., Mukabana, W.R., and Robinson, A.S. (2007). Transgenic mosquitoes and the fight against malaria: managing technology push in a turbulent GMO world. Am J Trop Med Hyg *77*, 232-242.

Kocks, C., Cho, J.H., Nehme, N., Ulvila, J., Pearson, A.M., Meister, M., Strom, C., Conto, S.L., Hetru, C., Stuart, L.M.*, et al.* (2005). Eater, a transmembrane protein mediating phagocytosis of bacterial pathogens in Drosophila. Cell *123*, 335-346.

Kumar, S., Mishra, N., Raina, D., Saxena, S., and Kufe, D. (2003). Abrogation of the cell death response to oxidative stress by the c-Abl tyrosine kinase inhibitor STI571.

Molecular pharmacology *63*, 276-282.

Lange, S., Dodds, A.W., Gudmundsdottir, S., Bambir, S.H., and Magnadottir, B. (2005). The ontogenic transcription of complement component C3 and Apolipoprotein A-I tRNA in Atlantic cod (Gadus morhua L.)--a role in development and homeostasis? Developmental and comparative immunology *29*, 1065-1077.

Lasonder, E., Ishihama, Y., Andersen, J.S., Vermunt, A.M., Pain, A., Sauerwein, R.W., Eling, W.M., Hall, N., Waters, A.P., Stunnenberg, H.G.*, et al.* (2002). Analysis of the Plasmodium falciparum proteome by high-accuracy mass spectrometry. Nature *419*, 537-542.

Lefevre, T., Thomas, F., Schwartz, A., Levashina, E., Blandin, S., Brizard, J.P., Le Bourligu, L., Demettre, E., Renaud, F., and Biron, D.G. (2007). Malaria Plasmodium agent induces alteration in the head proteome of their Anopheles mosquito host. Paper presented at: 2nd International Workshop on 2-D DIGE Applications in Proteomics (Bochum, GERMANY).

Lemaitre, B., and Hoffmann, J. (2007). The Host Defense of Drosophila melanogaster. Annu Rev Immunol.

Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J.M., and Hoffmann, J.A. (1996). The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. Cell *86*, 973-983.

Levashina, E.A., Moita, L.F., Blandin, S., Vriend, G., Lagueux, M., and Kafatos, F.C. (2001). Conserved role of a complement-like protein in phagocytosis revealed by dsRNA knockout in cultured cells of the mosquito, Anopheles gambiae. Cell *104*, 709-718.

Levy, F., Bulet, P., and Ehret-Sabatier, L. (2004a). Proteomic analysis of the systemic immune response of Drosophila. Mol Cell Proteomics *3*, 156-166.

Levy, F., Rabel, D., Charlet, M., Bulet, P., Hoffmann, J.A., and Ehret-Sabatier, L. (2004b). Peptidomic and proteomic analyses of the systemic immune response of Drosophila. Biochimie *86*, 607-616.

Lewis, B. (1973). Classification of lipoproteins and lipoprotein disorders. Journal of clinical pathology *5*, 26-31.

Li, J., Riehle, M.M., Zhang, Y., Xu, J., Oduol, F., Gomez, S.M., Eiglmeier, K., Ueberheide, B.M., Shabanowitz, J., Hunt, D.F.*, et al.* (2006). Anopheles gambiae genome reannotation through synthesis of ab initio and comparative gene prediction algorithms. Genome biology *7*, R24.

Li, X., Gerber, S.A., Rudner, A.D., Beausoleil, S.A., Haas, W., Villen, J., Elias, J.E., and Gygi, S.P. (2007). Large-scale phosphorylation analysis of alpha-factor-arrested Saccharomyces cerevisiae. Journal of proteome research *6*, 1190-1197.

Link, J.M., Larson, J.E., and Schroeder, H.W. (2005). Despite extensive similarity in germline DH and JH sequence, the adult Rhesus macaque CDR-H3 repertoire differs from human. Molecular immunology *42*, 943-955.

Ma, G., Hay, D., Li, D., Asgari, S., and Schmidt, O. (2006). Recognition and inactivation of LPS by lipophorin particles. Developmental and comparative immunology *30*, 619-626.

Mann, M., and Wilm, M. (1994). Error-tolerant identification of peptides in sequence databases by peptide sequence tags. Analytical chemistry *66*, 4390-4399.

Mann, M., Hendrickson, R.C., and Pandey, A. (2001). Analysis of proteins and proteomes by mass spectrometry. Annual review of biochemistry *70*, 437-473.

Marinotti, O., Capurro Mde, L., Nirmala, X., Calvo, E., and James, A.A. (2006). Structure and expression of the lipophorin-encoding gene of the malaria vector, Anopheles gambiae. Comparative biochemistry and physiology *144*, 101-109.

Marzetta, C.A., Foster, D.M., and Brunzell, J.D. (1990). Conversion of plasma VLDL and IDL precursors into various LDL subpopulations using density gradient ultracentrifugation. Journal of lipid research *31*, 975-984.

Mendes, A.M., Schlegelmilch, T., Cohuet, A., Awono-Ambene, P., De Iorio, M., Fontenille, D., Morlais, I., Christophides, G.K., Kafatos, F.C., and Vlachou, D. (2008). Conserved mosquito/parasite interactions affect development of Plasmodium falciparum in Africa. PLoS pathogens *4*, e1000069.

Michel, K., Budd, A., Pinto, S., Gibson, T.J., and Kafatos, F.C. (2005). Anopheles gambiae SRPN2 facilitates midgut invasion by the malaria parasite Plasmodium berghei. EMBO reports *6*, 891-897.

Michel, K., Suwanchaichinda, C., Morlais, I., Lambrechts, L., Cohuet, A., Awono-Ambene, P.H., Simard, F., Fontenille, D., Kanost, M.R., and Kafatos, F.C. (2006). Increased melanizing activity in Anopheles gambiae does not affect development of Plasmodium falciparum. Proceedings of the National Academy of Sciences of the United States of America *103*, 16858-16863.

Mohanty, S.S., Raghavendra, K., Mittal, P.K., and Dash, A.P. (2008). Efficacy of culture filtrates of Metarhizium anisopliae against larvae of Anopheles stephensi and Culex quinquefasciatus. Journal of industrial microbiology & biotechnology *35*, 1199-1202.

Molina-Cruz, A., DeJong, R.J., Charles, B., Gupta, L., Kumar, S., Jaramillo-Gutierrez, G., and Barillas-Mury, C. (2008). Reactive oxygen species modulate Anopheles gambiae immunity against bacteria and Plasmodium. The Journal of biological chemistry *283*, 3217-3223.

Olsen, J.V., and Mann, M. (2004). Improved peptide identification in proteomics by two consecutive stages of mass spectrometric fragmentation. Proceedings of the National Academy of Sciences of the United States of America *101*, 13417-13422.

Olsen, J.V., Ong, S.E., and Mann, M. (2004). Trypsin cleaves exclusively C-terminal to arginine and lysine residues. Mol Cell Proteomics *3*, 608-614.

Ong, S.E., and Mann, M. (2005). Mass spectrometry-based proteomics turns quantitative. Nature chemical biology *1*, 252-262.

Osta, M.A., Christophides, G.K., and Kafatos, F.C. (2004). Effects of mosquito genes on Plasmodium development. Science (New York, NY *303*, 2030-2032.

Panakova, D., Sprong, H., Marois, E., Thiele, C., and Eaton, S. (2005). Lipoprotein particles are required for Hedgehog and Wingless signalling. Nature *435*, 58-65.

Pandey, A., and Mann, M. (2000). Proteomics to study genes and genomes. Nature *405*, 837-846.

Pays, E., Vanhollebeke, B., Vanhamme, L., Paturiaux-Hanocq, F., Nolan, D.P., and Perez-Morga, D. (2006). The trypanolytic factor of human serum. Nat Rev Microbiol *4*, 477-486.

Picotti, P., Aebersold, R., and Domon, B. (2007). The implications of proteolytic

background for shotgun proteomics. Mol Cell Proteomics *6*, 1589-1598.

Pilch, B., and Mann, M. (2006). Large-scale and high-confidence proteomic analysis of human seminal plasma. Genome biology *7*, R40.

Pinto, S.B., Kafatos, F.C., and Michel, K. (2008). The parasite invasion marker SRPN6 reduces sporozoite numbers in salivary glands of Anopheles gambiae. Cellular microbiology *10*, 891-898.

Plowe, C.V., Cortese, J.F., Djimde, A., Nwanyanwu, O.C., Watkins, W.M., Winstanley, P.A., Estrada-Franco, J.G., Mollinedo, R.E., Avila, J.C., Cespedes, J.L.*, et al.* (1997). Mutations in Plasmodium falciparum dihydrofolate reductase and dihydropteroate synthase and epidemiologic patterns of pyrimethamine-sulfadoxine use and resistance. The Journal of infectious diseases *176*, 1590-1596.

Prudencio, M., Rodrigues, C.D., Hannus, M., Martin, C., Real, E., Goncalves, L.A., Carret, C., Dorkin, R., Rohl, I., Jahn-Hoffmann, K.*, et al.* (2008). Kinome-wide RNAi screen implicates at least 5 host hepatocyte kinases in Plasmodium sporozoite infection. PLoS pathogens *4*, e1000201.

Rahman, M.M., Ma, G., Roberts, H.L., and Schmidt, O. (2006). Cell-free immune reactions in insects. Journal of insect physiology *52*, 754-762.

Rahman, M.M., Roberts, H.L., and Schmidt, O. (2007). Factors affecting growth in the koinobiont endoparasitoid Venturia canescens in the flour moth Ephestia kuehniella. Journal of insect physiology *53*, 463-467.

Raikhel, A.S., Kokoza, V.A., Zhu, J., Martin, D., Wang, S.F., Li, C., Sun, G., Ahmed, A., Dittmer, N., and Attardo, G. (2002). Molecular biology of mosquito vitellogenesis: from basic studies to genetic engineering of antipathogen immunity. Insect biochemistry and molecular biology *32*, 1275-1286.

Ramet, M., Pearson, A., Manfruelli, P., Li, X., Koziel, H., Gobel, V., Chung, E., Krieger, M., and Ezekowitz, R.A. (2001). Drosophila scavenger receptor CI is a pattern recognition receptor for bacteria. Immunity *15*, 1027-1038.

Raper, J., Nussenzweig, V., and Tomlinson, S. (1996). The main lytic factor of Trypanosoma brucei brucei in normal human serum is not high density lipoprotein. The Journal of experimental medicine *183*, 1023-1029.

Rappsilber, J., and Mann, M. (2002). Is mass spectrometry ready for proteome-wide protein expression analysis? Genome biology *3*, COMMENT2008.

Rappsilber, J., Ishihama, Y., and Mann, M. (2003). Stop and go extraction tips for matrix-assisted laser desorption/ionization, nanoelectrospray, and LC/MS sample pretreatment in proteomics. Analytical chemistry *75*, 663-670.

Recheis, B., Rumpler, H., Schneider, W.J., and Nimpf, J. (2005). Receptor-mediated transport and deposition of complement component C3 into developing chicken oocytes. Cell Mol Life Sci *62*, 1871-1880.

Recklies, A.D., White, C., and Ling, H. (2002). The chitinase 3-like protein human cartilage glycoprotein 39 (HC-gp39) stimulates proliferation of human connective-tissue cells and activates both extracellular signal-regulated kinase- and protein kinase B-mediated signalling pathways. The Biochemical journal *365*, 119-126.

Richman, A.M., Dimopoulos, G., Seeley, D., and Kafatos, F.C. (1997). Plasmodium activates the innate immune response of Anopheles gambiae mosquitoes. The EMBO journal *16*, 6114-6119.

Riehle, M.M., Markianos, K., Niare, O., Xu, J., Li, J., Toure, A.M., Podiougou, B., Oduol, F., Diawara, S., Diallo, M.*, et al.* (2006). Natural malaria infection in Anopheles gambiae is regulated by a single genomic control region. Science (New York, NY *312*, 577-579.

Riehle, M.M., Xu, J., Lazzaro, B.P., Rottschaefer, S.M., Coulibaly, B., Sacko, M., Niare, O., Morlais, I., Traore, S.F., and Vernick, K.D. (2008). Anopheles gambiae APL1 is a family of variable LRR proteins required for Rel1-mediated protection from the malaria parasite, Plasmodium berghei. PLoS ONE *3*, e3672.

Rodriguez, M., Tome, S., Vizcaino, L., Fernandez-Castroagudin, J., Otero-Anton, E., Molina, E., Martinez, J., De la Rosa, G., Lovo, J., and Varo, E. (2007). Malaria infection through multiorgan donation: an update from Spain. Liver Transpl *13*, 1302-1304.

Roy, S.G., Hansen, I.A., and Raikhel, A.S. (2007). Effect of insulin and 20-hydroxyecdysone in the fat body of the yellow fever mosquito, Aedes aegypti. Insect biochemistry and molecular biology *37*, 1317-1326.

Sadasivaiah, S., Tozan, Y., and Breman, J.G. (2007). Dichlorodiphenyltrichloroethane (DDT) for indoor residual spraying in Africa: how can it be used for malaria control? Am J Trop Med Hyg *77*, 249-263.

Schal, C., Sevala, V., Capurro, M.L., Snyder, T.E., Blomquist, G.J., and Bagneres, A.G. (2001). Tissue distribution and lipophorin transport of hydrocarbons and sex pheromones in the house fly, Musca domestica. Journal of insect science (Online) *1*, 12.

Sedlacek, Z., Shimeld, S.M., Munstermann, E., and Poustka, A. (1999). The amphioxus rab GDP-dissociation inhibitor (GDI) gene is neural-specific: implications for the evolution of chordate rab GDI genes. Molecular biology and evolution *16*, 1231-1237.

Shevchenko, A., Wilm, M., Vorm, O., Jensen, O.N., Podtelejnikov, A.V., Neubauer, G., Shevchenko, A., Mortensen, P., and Mann, M. (1996). A strategy for identifying gel-separated proteins in sequence databases by MS alone. Biochemical Society transactions *24*, 893-896.

Shi, L., and Paskewitz, S.M. (2004). Identification and molecular characterization of two immune-responsive chitinase-like proteins from Anopheles gambiae. Insect molecular biology *13*, 387-398.

Shiao, S.H., Whitten, M.M., Zachary, D., Hoffmann, J.A., and Levashina, E.A. (2006). Fz2 and cdc42 mediate melanization and actin polymerization but are dispensable for Plasmodium killing in the mosquito midgut. PLoS pathogens *2*, e133.

Sigdel, T.K., and Sarwal, M.M. (2008). The proteogenomic path towards biomarker discovery. Pediatric transplantation *12*, 737-747.

Sigurdsson, G., Nicoll, A., and Lewis, B. (1975). Conversion of very low density lipoprotein to low density lipoprotein. A metabolic study of apolipoprotein B kinetics in human subjects. The Journal of clinical investigation *56*, 1481-1490.

Sinnis, P., Willnow, T.E., Briones, M.R., Herz, J., and Nussenzweig, V. (1996). Remnant lipoproteins inhibit malaria sporozoite invasion of hepatocytes. The Journal of experimental medicine *184*, 945-954.

Snow, R.W., Guerra, C.A., Mutheu, J.J., and Hay, S.I. (2008). International funding for malaria control in relation to populations at risk of stable Plasmodium falciparum transmission. PLoS Med *5*, e142.

Steen, H., and Pandey, A. (2002). Proteomics goes quantitative: measuring protein

abundance. Trends in biotechnology *20*, 361-364.

Sun, J., Hiraoka, T., Dittmer, N.T., Cho, K.H., and Raikhel, A.S. (2000). Lipophorin as a yolk protein precursor in the mosquito, Aedes aegypti. Insect biochemistry and molecular biology *30*, 1161-1171.

Suneja, A., Gulia, M., and Gakhar, S.K. (2003). Blocking of malaria parasite development in mosquito and fecundity reduction by midgut antibodies in Anopheles stephensi (Diptera: Culicidae). Archives of insect biochemistry and physiology *52*, 63-70.

Taniai, K., Wago, H., and Yamakawa, M. (1997). In vitro phagocytosis of Escherichia coli and release of lipopolysaccharide by adhering hemocytes of the silkworm, Bombyx mori. Biochemical and biophysical research communications *231*, 623-627.

Tilak, R., Dutta, J., and Gupta, K.K. (2007). Prospects for the use of ornamental fishes for mosquito control: a laboratory investigation. Indian journal of public health *51*, 54-55.

Toure, Y.T., Oduola, A.M., and Morel, C.M. (2004). The Anopheles gambiae genome: next steps for malaria vector control. Trends Parasitol *20*, 142-149.

Vaisar, T., Pennathur, S., Green, P.S., Gharib, S.A., Hoofnagle, A.N., Cheung, M.C., Byun, J., Vuletic, S., Kassim, S., Singh, P*., et al.* (2007). Shotgun proteomics implicates protease inhibition and complement activation in the antiinflammatory properties of HDL. J Clin Invest *117*, 746-756.

Valle, D., Kun, J., Linss, J., Garcia Ede, S., and Goldenberg, S. (1993). cDNA cloning and expression of Rhodnius prolixus vitellogenin. Insect biochemistry and molecular biology *23*, 457-465.

Van der Horst, D.J., Van Doorn, J.M., Voshol, H., Kanost, M.R., Ziegler, R., and Beenakkers, A.M. (1991). Different isoforms of an apoprotein (apolipophorin III) associate with lipoproteins in Locusta migratoria. European journal of biochemistry / FEBS *196*, 509-517.

Vanhamme, L., Paturiaux-Hanocq, F., Poelvoorde, P., Nolan, D.P., Lins, L., Van Den Abbeele, J., Pays, A., Tebabi, P., Van Xong, H., Jacquet, A*., et al.* (2003). Apolipoprotein L-I is the trypanosome lytic factor of human serum. Nature *422*, 83-87.

Vernick, K.D., Fujioka, H., Seeley, D.C., Tandler, B., Aikawa, M., and Miller, L.H. (1995). Plasmodium gallinaceum: a refractory mechanism of ookinete killing in the mosquito, Anopheles gambiae. Experimental parasitology *80*, 583-595.

Vizioli, J., Bulet, P., Charlet, M., Lowenberger, C., Blass, C., Muller, H.M., Dimopoulos, G., Hoffmann, J., Kafatos, F.C., and Richman, A. (2000). Cloning and analysis of a cecropin gene from the malaria vector mosquito, Anopheles gambiae. Insect Mol Biol *9*, 75-84.

Vizioli, J., Bulet, P., Hoffmann, J.A., Kafatos, F.C., Muller, H.M., and Dimopoulos, G. (2001). Gambicin: a novel immune responsive antimicrobial peptide from the malaria vector Anopheles gambiae. Proc Natl Acad Sci U S A *98*, 12630-12635.

Vlachou, D., Schlegelmilch, T., Christophides, G.K., and Kafatos, F.C. (2005). Functional genomic analysis of midgut epithelial responses in Anopheles during Plasmodium invasion. Curr Biol *15*, 1185-1195.

Volz, J., Muller, H.M., Zdanowicz, A., Kafatos, F.C., and Osta, M.A. (2006). A genetic module regulates the melanization response of Anopheles to Plasmodium. Cellular microbiology *8*, 1392-1405.

Warburg, A., Shtern, A., Cohen, N., and Dahan, N. (2007). Laminin and a Plasmodium ookinete surface protein inhibit melanotic encapsulation of Sephadex beads in the hemocoel of mosquitoes. Microbes and infection / Institut Pasteur *9*, 192-199.

Warr, E., Aguilar, R., Dong, Y., Mahairaki, V., and Dimopoulos, G. (2007). Spatial and sex-specific dissection of the Anopheles gambiae midgut transcriptome. BMC genomics *8*, 37.

Warr, E., Das, S., Dong, Y., and Dimopoulos, G. (2008). The Gram-negative bacteria-binding protein gene family: its role in the innate immune system of anopheles gambiae and in anti-Plasmodium defence. Insect molecular biology *17*, 39-51.

Washburn, M.P., Wolters, D., and Yates, J.R., 3rd (2001). Large-scale analysis of the yeast proteome by multidimensional protein identification technology. Nature biotechnology *19*, 242-247.

Wasinger, V.C., Cordwell, S.J., Cerpa-Poljak, A., Yan, J.X., Gooley, A.A., Wilkins, M.R., Duncan, M.W., Harris, R., Williams, K.L., and Humphery-Smith, I. (1995). Progress with gene-product mapping of the Mollicutes: Mycoplasma genitalium. Electrophoresis *16*, 1090-1094.

Waterhouse, R.M., Kriventseva, E.V., Meister, S., Xi, Z., Alvarez, K.S., Bartholomay, L.C., Barillas-Mury, C., Bian, G., Blandin, S., Christensen, B.M.*, et al.* (2007). Evolutionary dynamics of immune-related genes and pathways in disease-vector mosquitoes. Science (New York, NY *316*, 1738-1743.

Weers, P.M., Narayanaswami, V., Kay, C.M., and Ryan, R.O. (1999). Interaction of an exchangeable apolipoprotein with phospholipid vesicles and lipoprotein particles. Role of leucines 32, 34, and 95 in Locusta migratoria apolipophorin III. The Journal of biological chemistry *274*, 21804-21810.

Whitten, M.M., Tew, I.F., Lee, B.L., and Ratcliffe, N.A. (2004). A novel role for an insect apolipoprotein (apolipophorin III) in beta-1,3-glucan pattern recognition and cellular encapsulation reactions. J Immunol *172*, 2177-2185.

WHO (2005) *International Travel and Health: Situation as on 1 January 2005*. World Health Organization (http://www.who.int)

Wilm, M., and Mann, M. (1996). Analytical properties of the nanoelectrospray ion source. Analytical chemistry *68*, 1-8.

Wilmanski, J.M., Petnicki-Ocwieja, T., and Kobayashi, K.S. (2008). NLR proteins: integral members of innate immunity and mediators of inflammatory diseases. Journal of leukocyte biology *83*, 13-30.

Yang, J.Y., Kim, T.K., Koo, B.S., Park, B.H., and Park, J.W. (1999). Change of plasma lipoproteins by heparin-released lipoprotein lipase. Experimental & molecular medicine *31*, 60-64.

Yu X-Q, Gan H., Kanost M.R. (1999). Immulectin, an inducible C-type lectin from an insect, Manduca sexta, stimulates activation of plasma prophenol oxidase. Insect Biochem Mol Biol *29*:585-597