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A dissertation submmited to obtain a Doctor of Philosophy degreee in Biomedical Sciences, Parasitology speciality

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Sumário

Este trabalho pretende contribuir para o conhecimento geral da resposta imunológica do mosquito ao parasita da malária, uma vez que a elucidação das interacções entre vector e parasita poderão facilitar o desenvolvimento de medidas eficientes para bloquear a transmissão. As experiências realizadas neste trabalho incluíram o uso de *Drosophila melanogaster* como modelo de estudo das respostas imunológicas do mosquito e a avaliação do impacto da presença de esporozoítos de *Plasmodium* na hemolinfa do mosquito através da determinação de alterações no número de hemócitos, activação da reacção de melanização e do padrão de expressão de proteínas na hemolinfa do mosquito.

O fármaco antimalárico cloroquina promove a transmissão no mosquito e tem sido relacionado com a expressão diferencial de péptidos antimicrobianos no mosquito. Para avaliar o efeito da cloroquina na sua produção usámos o modelo *Drosophila*, uma vez que a expressão e síntese de péptidos antimicrobianos na mosca está bem caracterizada, assim como as vias de sinalização da resposta imunológica. Os resultados deste trabalho não conseguiram provar algum efeito do fármaco na expressão e/ou síntese de péptidos antimicrobianos de *Drosophila*. O tratamento com cloroquina *in vivo* não afectou as vias de sinalização Toll e Imd, avaliado pela expressão de *drosomicina* e *diptericina* em moscas infectadas. Experiências *in vitro* em que se utilizaram linhas celulares derivadas de hemócitos de moscas produziram os mesmos resultados para a síntese de Drosomicina e Atacina. Experiências de sobrevivência de moscas infectadas e tratadas com cloroquina também não evidenciaram qualquer efeito do fármaco na resposta imunitária de *Drosophila*. Como este fármaco antimalárico tem um efeito conhecido na resposta imune do mosquito, propomos que a cloroquina tenha uma acção sobre moléculas específicas dos mosquito ou sobre diferentes vias de activação da sinalização que possam estar presentes apenas no mosquito. Por outro lado, a informação conhecida acerca do efeito da cloroquina na imunidade foi obtida após tratamento de humanos, ratinhos ou linhas celulares de mamíferos, implicando a metabolização do fármaco. Como tal, não é claro se o efeito observado resulta da acção do própria fármaco ou de um metabolito específico.

Neste trabalho pretendeu-se também determinar as respostas do mosquito aos esporozoítos de *Plasmodium* na hemolinfa, uma vez que nesta fase da infecção no mosquito o parasita sofre uma grande redução no seu número. No hemocélio do

mosquito podem ser activadas respostas celulares e humorais. Durante o seu desenvolvimento no oocysto, os esporozoítos são cobertos por uma camada de proteína circumsporozoítica, que constitui o seu maior antigénio de superfície. Quando o oocisto rompe e os esporozoítos são libertados, esta proteína pode ser reconhecida pelas moléculas de reconhecimento presentes na hemolinfa levando à activação de respostas imunes. A activação de respostas imunitárias celulares contra os esporozoítos foi testada com base na determinação de variação do número de hemócitos quando estimulados com a proteína circumsporozoítica de *P. falciparum*. Apenas uma das doses (5ng) de proteína utilizadas para estimular linhas celulares de hemóctios causou uma redução significativa no números de hemócitos. Isto pode ser um reflexo de uma cinética de divisão celular mais lenta ou de destruição celular, apoptose, que poderia ser despoletada pela fagocitose de parasitas, por exemplo. Não foi possível obter uma resposta correlacionada com a dose usada para estimulação. No entanto, os hemócitos do mosquito parecem reconhcer a proteína do parasita e responder à sua presença.

A activação da reacção de melanização durante a invasão da hemolinfa por esporozoítos foi testada, através da determinação da activação da enzima profenoloxidase e da actividade da fenoloxidase. Verificou-se que a actividade enzimática da fenoloxidase varia com o tempo em mosquitos submetidos a uma refeição sanguínea não infectante. A infecção por *P. berhgei* não pareceu impor variações na actividade da fenoloxidase. Diferenças subtis foram observadas aos dias 9, 12 e 15 pós-infecção, sendo a actividade enzimática mais elevada em mosquitos infectados. Os esporozoítos foram detectados na hemolinfa de mosquitos a partir do dia 9 pós-infecção, indicando que o parasita pode induzir um aumento subtil na activação da melanização. A actividade da fenoloxidase parece ser mantida constitutivamente num nível baixo, mesmo em mosquitos não infectados, o que pode explicar que apenas pequenas diferenças sejam observadas em mosquitos infectados. Injecções da proteína circumsporozoítica de *P. falciparum* em mosquitos não revelaram indução da actividade da enzima fenoloxidase. Apesar de não ter sido possível demonstrar conclusivamente a melanização de esporozoítos na hemolinfa, experiências de inibição da fenoloxidase mostraram que a actividade desta enzima é necessária para controlar o número de esporozoítos na hemolinfa e nas glândulas salivares.

A hemolinfa é extremamente rica em proteínas, e conhecida por albergar a maior parte das moléculas do sistema imunológico necessárias ao reconhecimento, sinalização e à resposta efectora. Como tal, de modo a caracterizar o proteoma da hemolinfa durante a infecção por *P. berhgei* ao dia 13 pós-infecção, usámos uma

abordagem que incluiu electroforese bidimensional e espectrometria de massa *MALDI-TOF*, visando identificar proteínas diferencialmente reguladas em mosquitos infectados. As proteínas com níveis alterados na hemolinfa de mosquitos infectados poderão estar envolvidas em processos fisiológicos como metabolismo de ácidos gordos, glicólise e transporte de iões. Estes resultados indicam que o parasita impõe alterações no metabolismo do mosquito, quer directamente quer levando o mosquito a alterar o seu próprio metabolismo como forma de conter a infecção. De facto, não há evidência se as alterações observadas são danosas ou necessárias para o desenvolvimento do parasita. No entanto, os nossos resultados sugerem que mecanismos fisiológicos do mosquito podem ter um papel na resposta imune. Um dado interessante obtido neste trabalho foi a inexistência de correlação entre a regulação a nível proteico e a nível do RNA na hemolinfa. Isto pode derivar de uma janela de tempo diferente entre expressão génica e síntese proteica, uma vez que as amostras foram recolhidas ao mesmo tempo, ou pode reflectir um fonte diferente de RNA e proteína. O RNA amplificado para avaliar a expressão génica era originário dos hemócitos presentes na hemolinfa, enquanto que as proteínas podem ter sido produzidas quer pelos hemócitos quer pelo corpo gordo, que sintetiza a maior parte das moléculas imunes que são secretadas para a hemolinfa. No entanto, é importante tem em atenção que a informação resultante da análise de expressão génica ter de ser avaliada cuidadosamente, pois pode não ter uma regulação correspondente ao nível da proteína. A biosíntese de eicosanóides teve dois impactos distintos e opostos no desenvolvimento do parasita, promovendo e bloqueando a transmissão. Os eicosanóides parecem ser importantes para o desenvolvimento do parasita numa fase da infecção em que os esporozoítos se desenvolvem nos oocistos, enquanto que numa fasemais tardia, estas moléculas parecem ser importantes para controlar o número de parasitas na hemolinfa. Os nossos resultados sugerem que o parasita possa imunosuprimir o mosquito.

A resposta do mosquito ao *Plasmodium* parece ser muito complexa, envolvendo acções de ambos os organismos. Para responderà invasão da hemolinfa pelos esporozoítos, o mosquito parece depender de diferentes mecanismos, como a fagocitose e a melanização. Para além destes, moléculas envolvidas em processos fisiológicos do mosquito são também afectadas pela infecção. Os nossos resultados sugerem que a resposta imunológica do mosquito possa envolver mecanismos para além daqueles que são tradicionalmente relacionados com a imunidade, como a biosíntese de eicosanóides. Verificámos também que pode não existir correlação entre a expressão génica e a síntese de proteínas, e como tal, a resposta imune deveria ser analisada em primeiro lugar por uma abordagem proteómica.

Abstract

This work aimed at contributing to the general knowledge of the mosquito immune responses to the malaria parasite, in hope that the elucidation of vector/parasite interactions will facilitate the development of effective transmission blocking measures. Experiments performed here include the use of *Drosophila melanogaster* as a model for immunity studies in the mosquito and the evaluation of *Plasmodium* sporozoites presence in mosquito hemolymph impact on hemocyte numbers, melanization reaction responses and protein expression pattern.

Chloroquine promotes malaria transmission in mosquito and it has been linked to differential AMP gene expression in mosquitoes. As *Drosophila* AMP expression and synthesis is well understood and as we have a good knowledge about immune signaling pathways, we chose this model to evaluate chloroquine effect on AMP production upon infection. Our results failed to show any drug effect on *Drosophila* AMP expression and/or synthesis. Drug treatment *in vivo* did not affect either the Toll or Imd immune signaling pathways, as shown when accessing *drosomycin* and *diptericin* expression in infected flies, and *in vitro* experiments using hemocyte-like cell lines produced the same results for Drosomycin and Attacin synthesis. Survival experiments were also performed in drug treated flies and failed to indicate any effect. For all the mechanisms tested, chloroquine did not seem to have any effect on *Drosophila* immunity. As this antimalarial drug has a known effect on mosquito immunity we propose that chloroquine may act on particular mosquito immune molecules or on different routes for pathway activation operating in the mosquito. Also, data obtained for chloroquine action on immunity were collected from treatment of humans, mice or mammalian cell-lines, implying that the drug is metabolized. Thus it is not clear if the observed effect results from an action of the drug itself, or from a specific metabolite. This would explain why direct drug feeding to flies would fail to produce an effect on *Drosophila* immunity.

Another purpose of this work was to determine the mosquito responses to *Plasmodium* sporozoites in the hemolymph, as parasite development in the mosquito suffers a major bottleneck at this stage of infection. Both cellular and humoral responses may be triggered in the mosquito hemocel. Upon development inside the oocysts, sporozoites are covered with a layer of circumsporozoite protein, its major

surface antigen. Recognition molecules present in the hemolymph may recognize sporozoites upon oocyst burst, possibly through its surface protein and activate immune responses towards it. Cellular responses towards sporozoites were tested, based on the evaluation of hemocyte number variation upon stimulation with the circumsporozoite protein of *P. falciparum*. Only one dose (5ng) stimulated hemocyte-like cell lines and led to a significant reduction in cell numbers. This may reflect a slower cell-division kinetics or cell destruction, by apoptosis, following phagocytosis. We failed to show any dose-dependent response. Nevertheless, it seems that the CS protein is recognized by mosquito hemocytes that respond to its presence only in specific conditions.

We also tested for activation of melanization reaction upon sporozoite invasion of the hemolymph by accessing PPO activation and PO activity. PO activity was found to vary over time in blood fed mosquitoes. *P. berghei* infection did not seem to impose variations in PO activity. Subtle differences were observed at D9, 12 and 15pi, when PO activity was higher in infected mosquitoes. Sporozoites were first detected in the hemolymph at D9pi indicating that parasite recognition may induce subtle increases in melanization activation. PO activity seems to be maintained at a low level even in non-infected mosquitoes. This may explain the fact that no great variations were observed upon infection. Pf-CS protein injections in mosquitoes failed to show PO activity induction. Although we could not conclusively determine sporozoite melanization, PO inhibition experiments showed that its activity is necessary for control of sporozoite load in the hemolymph and salivary glands.

Hemolymph is an extremely protein rich environment, and known to harbor most of the immune molecules necessary for recognition, signaling and effector mechanisms. As such, we used a two dimensional electrophoresis approach coupled with MALDI-TOF mass spectrometry to compare the hemolymph proteome of *P. berghei* infected and non-infected *An.* gambiae mosquitoes at D13pi, aiming at the identification of differentially regulated protein in infected mosquitoes. Proteins found to have altered levels in the hemolymph of infected mosquitoes are predicted to be involved in physiological processes such as fatty acid metabolism, aminoacid synthesis, glycolysis and ion transport. This indicated that the parasite imposes alterations in the overall mosquito metabolism, either directly, or secondarly to combat infection. Actually we have no evidence if the alterations observed are harmful or necessary for parasite development. Yet, the results suggest that mechanisms operating in mosquito physiology may have a role on immune responses. An interesting fact is that protein regulation in the hemolymph did not correlate at any level with gene transcription. This may reflect a different time frame between transcription and protein synthesis, as

samples were collected at the same time, or a different source for the RNAs and the tested proteins. RNA amplified to evaluate transcription was hemolymph, *ie*, hemocyte-derived, while hemolymph proteins may have been produced not only by hemocytes, but also by fat body cells, that synthesize the majority of immune-related molecules secreted into the hemolymph. Nevertheless, it is important to bear in mind that data resulting from gene expression analysis have to be carefully analyzed as it may not indicate direct protein synthesis. Eicosanoid biosynthesis was found to have two distinctive and opposite impacts in parasite development: both promoting and blocking transmission. Eicosanoids seem to be important for parasite biology and development, at a time when sporozoites are developing inside oocysts. At a later stage, these molecules seem to restrain sporozoite infection in the hemolymph. Evidence also point to mosquito immunosuppression by the parasite.

Mosquito responses to *Plasmodium* seem to be highly complex, involving actions from both organisms. To respond to hemolymph invasion by sporozoites, the mosquito seems to rely on different mechanisms, such as phagocytosis and melanization. Additionally, molecules involved in physiological processes are affected by hemolymph infection. Data obtained by this work suggests that immune responses may include mechanisms other than those traditionally related to immunity, as in the case of eicosanoid biosynthesis. Also, our results indicate that correlation between transcription and protein synthesis is not sure to exist and thus, immune responses should be analyzed by proteomics in a first approach.

List of abbreviations

2D – Two dimensional

2DE - Two dimensional Electrophoresis

AA – Arachidonic Acid

ACN - Acetonitrile

Ae. - Aedes

AMP – Antimicrobial Peptide

An. – Anopheles

APO II/I – Apolipophorin II/I

Att – Attacin

B. – Beauveria

BSA – Bovine Serum Albumin

CBB – Colloidal Brilliant Blue

cDNA - complementary Deoxyribonucleic Acid

CEC – Cecropin

CEC1 – Cecropin 1

CEC2 – Cecropin 2

CEC3 – Cecropin 3

CECA – Cecropin A

Chl – Chloroquine

COX - Ciclooxygenase

CSP – Circumsporozoite Protein

CTL4 – C-Type Lectin 4

CTLGA – C-type Lectin Galactose binding

CTLMA – C-Type Lectin Mannose binding

CTLMA2 – C-Type Lectin Mannose binding 2

CTLSE – C-type Lectin Selectin

CTRP – Circumsporozoite and TRAP Related Protein

Cys - Cysteine

D. – Drosophila

dCTP – Deoxycytidine Triphosphate

DEF1 – Defensin 1

DEF2 – Defensin 2

DEFA – Defensin A

DEPC - Diethylpyrocarbinate

DEX - Dexamethasone

Dipt –Diptericin

DNA - Deoxyribonucleic Acid

dNTP - Deoxyribonucleotide Triphosphate

Dome - Domeless

Drs – Drosomycin

DTT - Dithiothreitol

E. - Escherichia

EM – Electron Microscopy

En. - Enterococcus

EST – Expressed Sequence Tag

FA – Fatty Acid

FCS – Fetal Calf Serum

FBN9 – Fibronectin 9

FBN23 – Fibronectin 23

FBN39 – Fibronectin 39

GALE – Galectin

GALE5 – Galectin 5

GALE8 – Galectin 8

GAM – Gambicin

GFP – Green Fluorescent Protein

GNBP – Gram Negative Binding Protein

GNBP1 - Gram Negative Binding Protein 1

GNBP3 – Gram Negative Binding Protein 3

GNBPA1 – Gram Negative Binding Protein A1

GNBPB1 – Gram Negative Binding Protein B1

GNBPB4 - Gram Negative Binding Protein B4

GPI - Glycosylphosphatidylinositol

h - hours

HIV/AIDS – Human Immunodeficiency Virus / Acquired immune deficiency Syndrome

HK – Heat-Killed

hpi – hours post-infection

HRP – Horseradish Peroxidase

IEF – Isoelectric Focusing

IFN-γ – Interferon-γ

IGALE20 – Infection-responsive Galactose Lectin 20

IMCR14 - Immune-responsive alpha-macroglobulin and complement C3- related protein 14

IMD – Immunodeficiency

IN – Indomethacin

IPG – Immobilized pH gradient

IRSP5 – Immune Responsive Short Secreted Peptide 5

Key - Kenny

JNK – Janus Kinase

KD – Knock-Down

L-DOPA – 3,4-dihydroxy-L-phenylalanine

LOX - Lipooxygenase

LPS – Lipopolysaccharide

LRIM – Leucine-rich Repeat Immune Protein

LRIM1 – Leucine-rich Repeat Immune Protein1

LRR – Leucine Rich Repeats

M. – Micrococcus

Ma. - Manduca

MALDI-TOF MS – Matrix Assisted Laser Desorption Ionization – Time Of Flight Mass Spectrometry.

MAPK – MAP Kinase

MDL1 – MD2-like receptor 1

min – minutes

Mix bact – Mixed bacteria

MMLV-RT – Moloney Murine Leukemia Virus Reverse Transcriptase

mRNA – messenger Ribonucleic Acid

MS – Mass Spectrometry

NDGA - Nordihydroguaiaretic acid

NF-KB – Nuclear Factor kB

ni – non-infected

NL – Non-Linear

NO – Nitric Oxide

NOI – Nitric Oxide Intermediates

NOS – Nitric Oxide Synthetase

ON - Overnight

ORF – Open Reading Frame

P. – Plasmodium

PAE – Prophenoloxidase Activating Enzyme

PAMP – Pathogen Associated Molecular Pattern

PBS - Phosphate-buffered saline

PbS21 – *Plasmodium berghei* Surface Protein 21

PbS28 – *Plasmodium berghei* Surface Protein 28

PCR – Polymerase Chain Reaction

Pf-CS – *Plasmodium falciparum* Circumsporozoite Protein

PG – Prostaglandin

PGE2 – Prostaglandin E2

PGN – Peptidoglycan

PGRP – Peptidoglycan Recognition Protein

PGRP-L – Peptidoglycan Recognition Protein Long

PGRP-LB - Peptidoglycan Recognition Protein Long B

PGRPL-C – Peptidoglycan Recognition Protein Long C

PGRPL-Ca – Peptidoglycan Recognition Protein Long Ca

PGRPL-Cx – Peptidoglycan Recognition Protein Long Cx

PGRP-LE - Peptidoglycan Recognition Protein Long E

PGRP-S – Peptidoglycan Recognition Protein Short

PGRP-SA - Peptidoglycan Recognition Protein Short A

PRGP-SD - Peptidoglycan Recognition Protein Short D

pI – Isoelectric point

PLA2 – Phospholipase A2

PO – Phenoloxidase

PO2 – Phenoloxidase 2

PPO – Prophenoloxidase

PPO1 – Prophenoloxidase 1

PPO2 – Prophenoloxidase 2

PPO3 – Prophenoloxidase 3

PPO4 – Prophenoloxidase 4

PPO6 – Prophenoloxidase 6

PPO9 – Prophenoloxidase 9

PRR – Pattern Recognition Receptor

Psh – Persephone

PTU – Phenylthiourea

PUFA – Polyunsaturated Fatty Acid

QTL – Quantitative Trait *Loci*

R. – Rhodnius

RNA – Ribonucleic Acid

RNAi – Ribonucleic Acid interference

ROI – Reactive Oxygen Intermediates

RP49 – Ribosomal Protein 49

rt – room temperature

RT-PCR – Reverse-Transcriptase PCR

S. - Serratia

SAGE – Serial Analysis of Gene Expression

SDS-PAGE – Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

sec - second

SM1 – Short Secreted Peptide 1

SOAP – Secreted Ookinete Adhesive Protein

SOD – Superoxide Dismutase

SP – Serine Protease

SP14D – Serine Protease 14D

SP24D – Serine Protease 24D

SPH – Serine Protease Homologue

SPZ – Spaetzle

SRPN1 – Serpin 1

SRPN2 – Serpin 2

SRPN3 – Serpin 3

SRPN6 – Serpin 6

SRPN10 – Serpin 10

SRPN27A – Serpin 27A

SRPN43AC – Serpin 43Ac

SSC - Saline Sodium Citrate

TEP1 – Thioester-like Protein 1

TEP3 - Thioester-like Protein 3

TEP4 - Thioester-like Protein 4

TLR – Toll-like Receptor

Tm – melting Temperature

TNFα – Tumor Necrosis Factor α

TRAP – Thrombospondin-Related Adhesive Protein

TYR – Tyrosine

UPD3 – Unpaired 3

WARP – Willebrand Factor A Related Protein

WHO – World Health Organization

X. - Xenopus

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I – Introduction

I.1 – Malaria

Malaria is one of the most life-threatening diseases affecting the human population. Together with HIV/AIDS and tuberculosis, it has become a calamity with worldwide proportions, mainly in sub-tropical countries. The WHO reports around 500 million cases of malaria each year, resulting in one million deaths, particularly of children under 5 years old.

In old days, malaria was attributed to the fetid marshes around Rome, hence the name of "bad-air" (*mal-aria*, in Italian), or Roman fever. In the late 19th century, scientists realized that a single-cell organism is responsible for the illness of malaria.

I.1.1 – The Parasite

The single–cell organism is a protozoan parasite from the genus *Plasmodium*. Taxonomically, it belongs to the Alveolata phylum, the Apicomplexa order, the Haemosporida family, and the *Plasmodium* genus, Levine, 1988. Biologically, the parasite has an exceedingly complex life cycle in which it alternates between a vertebrate host and an anopheline mosquito vector. In the host, the parasite is responsible for the illness observed in malaria-infected people, while in the vector it multiplies and develops compromising the mosquito's fitness. Figure I.1.1 shows the detailed life cycle of the *Plasmodium* parasite.

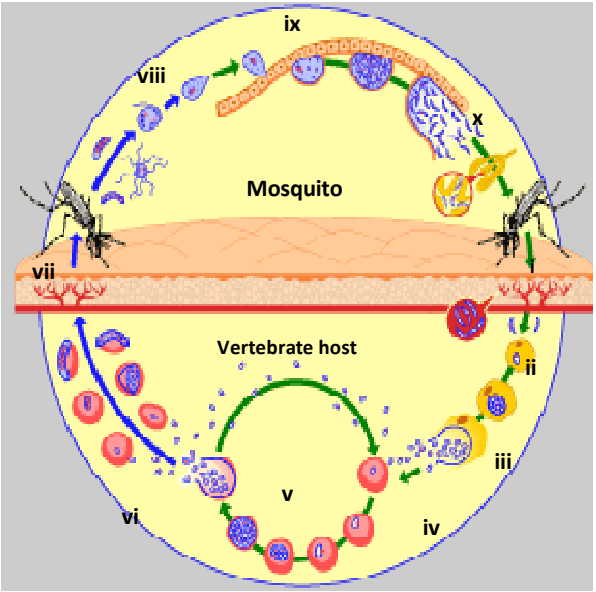


Figure I.1.1 – *Plasmodium* life cycle. (Adapted from www.who.int/tdr/diseases/malaria/lifecycle) Parasites are passed on to the vertebrate host through the mosquito bite (i). The parasite rapidly infects the liver (ii) where it multiplies forming new invasive forms (merozoites) – hepatic schizogony. These are released into the blood stream (iii) and invade red blood cells (iv). Merozoites mature into schizonts and undergo a series of divisions forming new invasive merozoites (v), initiating cycles of erythrocyte invasion, maturation and rupture, causing the disease – erythrocytic schizogony. Some merozoites differentiate into gametocytes (vi) that are taken up by the mosquito in a blood meal (vii). In the mosquito midgut the parasite activates gametocytes maturation into gametes. Fertilization occurs (viii) and a motile zygote is formed (ookinete). It traverses the midgut epithelia and settles down underneath the basal lamina, forming a cyst (oocyst) (ix). Inside, thousands of new invasive forms develop (sporozoites). Upon oocyst burst, these are released into the mosquito hemolymph (x) and flow to the salivary glands, where they accumulate until the next bite – sporogonic development, initiating a new cycle (i).

I.1.2 – The disease

Malaria illness occurs during erythrocytic schizogony. Successive cycles of parasite invasion result in elevated numbers of red blood cells destroyed, causing acute anemia. At the same time, erythrocyte rupture releases pyrogenic substances into the blood causing high fevers. Thus, anemia, high fivers chills and renal insufficiency are the general symptoms of malaria, and are caused by infection with any of the four species of *Plasmodium* that infect humans: *Plasmodium malariae*, *Plasmodium ovale*, *Plasmodium vivax* and *Plasmodium falciparum* (Tuteja, 2007). The later has an additional property: it induces cytoadherence in the infected red blood cells that easily adhere to the blood capillaries wall, hindering oxygen delivery to the major organs. If this occurs in the brain, it leads to coma, and inescapably to death. Cerebral malaria is the worst form of malaria known, and *P. falciparum* is the deadliest of the malaria parasites.

In the last few decades, the number of malaria-ill people has been rising, and the situation is becoming even more serious, since it is estimated that the numbers will (at least) double by 2020 (Breman, 2001). Several factors are contributing for this increase: i) the increasing development of parasites resistant to antimalarial drugs; ii) the existence of different *Plasmodium* species and their inherent antigenic variation that amplifies parasite variability; iii) the geographical and socio-economical conditions of endemic countries, and the development of other so called poverty diseases, like HIV/AIDS and Tuberculosis; iv) the hot and humid weather, the poor health and sanitary system, and insecticide resistance that contribute for the large scale development and reproduction of the mosquito vector, and v) the breakdown of control programs.

Therefore, to efficiently combat malaria, an integrated action will have to be created, so that all these factors are restrained and controlled. This will include: i) the development of new reliable drugs (or a combination of drugs to reduce resistance emergence), ii) a new efficient and highly protective vaccine, iii) the improvement of sanitary and health conditions, iv) vector control to decrease transmission (removal of mosquitoes breeding places, use of insecticide impregnated bed nets and the control of parasite development inside the mosquito), and vii) sustainability of malaria control programs.

I.1.3 – The mosquito vector

The parasite depends on its ability to develop in the mosquito in order to infect a human host. Thus, controlling transmission through the mosquito greatly reduces malaria outspread.

Transmission can be impaired by several strategies, as reducing the number of mosquitoes (using insecticides and destroying potential breeding sites), avoiding the contact between mosquitoes and humans (using insecticide-impregnated bednets) or blocking the development of the parasite in its vector. For this, it is vital to understand

the biology of parasite and mosquito, their interactions, mosquito permissiveness to parasite, and factors in parasite and mosquito necessary for an efficient transmission.

The mosquito responsible for malaria transmission belongs to the genus *Anopheles*, and is widespread through the major temperate and tropical areas. Even though the *Anopheles* genus is composed by 400 species only about 60 are able to efficiently transmit malaria. This reveals a high degree of specificity for the parasite/mosquito combination. In fact, each mosquito species has a specific permissiveness to a particular species of *Plasmodium*. Mosquito and parasite factors and interactions that may account for this specificity are not yet known. It is clear, however, that both organisms engage in a series of interactions in which they are able to recognize and respond to each other (Sinden, 2002).

For instance, the blood meal alone triggers the transcriptional regulation of a set of mosquito genes, required for functions such as digestion and immunity against bacteria infected blood. A *Plasmodium* infected blood meal however, triggers a different set of genes, suggesting that the mosquito recognizes the parasite and mounts a response to its presence through the activation of specific genes. Moreover, the parasite itself has to undergo several morphological changes that are also regulated at the genomic level and activated with precision both temporally and spatially in the mosquito (Dimopoulos *et al.*, 1998, 2002; Richman *et al.*, 1997; Sinden, 2002).

Additionally, there are factors external to both organisms able to influence the outcome of infection, such as temperature, humidity and blood meal constituents. The impact of these factors on parasite development is generally characterized by an increase/decrease of parasite load in the midgut.

Thus, to be able to control transmission it is essential to fully understand which and how factors (from parasite, mosquito or external) are determinant for a successive infection.

I.2 – The parasite in the mosquito

There is considerable amount of information regarding the sporogonic development of the parasite. Figure I.2.1 summarizes mosquito infection by malaria parasites and the successive bottlenecks that the parasite encounters during its development.

All interactions between parasite and mosquito, and the fact that mosquito directed antibodies are able to prevent infection by *Plasmodium* (Dinglasan *et al.*, 2003), come as hope for new targets for transmission control. As malaria infection is able to compromise mosquito's fitness, it is imperative that the mosquito controls parasite numbers for survival. Actually, the mosquito is capable of mounting efficient bottlenecks to the development of the parasite, illustrated by the dramatic losses in number suffered by the parasite along the sporogonic cycle (Figure I.2.1; Alavi *et al.*, 2003, Sinden & Billingsley, 2001; Sinden, 2002). What mechanism(s) are responsible for the constraint in parasite number, and how they are activated remains to be fully elucidated. Nevertheless, it has become clearer that the mosquito immune system plays an important role.

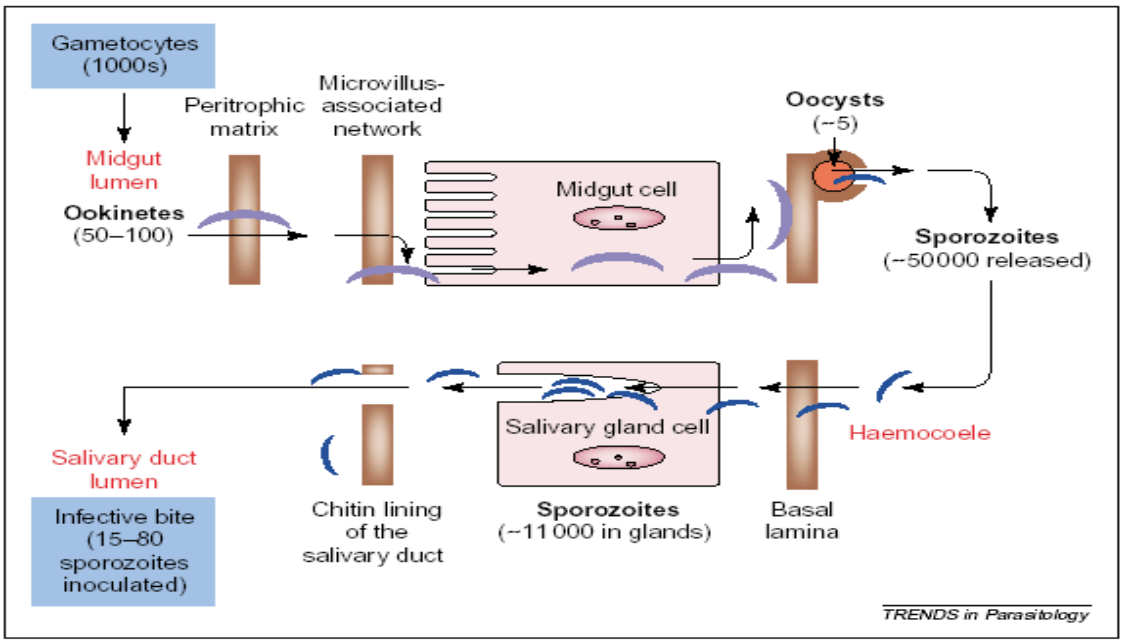


Figure I.2.1 – The sporogonic cycle of malaria parasites development in the mosquito. Source: Sinden & Billingsley, 2001.

Upon a blood meal the parasite enters the mosquito midgut, where it senses changes in environment, as temperature drop, pH increase, and the presence of a mosquito factor, xanturenic acid. These cause an increase of cytoplasmic calcium release in *Plasmodium* gametocytes (Billker *et al.*, 2004), triggering the differentiation into gametes. In a few minutes fertilization occurs, and a motile ookinete develops. Of the tens of thousands of gametocytes ingested by the mosquito only 50-100 ookinetes are formed. These have to evade from the midgut, traversing the peritrophic matrix, and the midgut epithelium, until settle on the basal lamina. This process involves midgut epithelium recognition and some parasite proteins have been implicated, as the secreted ookinete adhesive protein (SOAP), and the circumsporozoite- and TRAP-related protein (CTRP). Without these, the parasite's ability to invade the midgut epithelium and progress in infection is reduced (Dessens *et al.*, 1999, 2003). PbS21 has also been shown to be necessary for its binding to the basal lamina itself, and maturation into oocyst (Arrighi & Hurd, 2002). How the parasite recognizes the midgut cells, if it actually targets a specific type of cells, and how it reaches the basal lamina is in centre of debate and seems to differ with parasite/vector combinations. In some combinations ookinetes take an intracellular route of invasion, passing through several cells in the midgut epithelium that undergo apoptosis, and are released into the midgut lumen. This calls for a tissue repair system, directed from the surrounding cells and involving the actin cytoskeleton and microtubule remodelling that build a cover for the ookinete, in the hemocel side of the basal lamina (Han *et al.*, 2000, Vlachou *et al.*, 2004, 2005). Of the 50-100 ookinetes only 5 successfully develop into an oocyst that matures, producing within sporozoites that are released into the hemocel in numbers that reach tens of thousands. It is not known if the oocyst burst is a mechanical result of the growth and development of the cells within, or a result from a specific action of sporozoites. In the hemocel, sporozoites encounter a new environment, with different cells and organs of the mosquito, ultimately reaching the salivary glands. Again, only a small proportion of sporozoites (15-80) invade the salivary glands with success. How sporozoites recognize salivary glands and differentiate them from the other organs is still unknown. But it is sure that there is a specific recognition of the salivary glands, invasion of the distal lateral and medial lobes (Ghosh *et al.*, 2001, 2002), and that gliding is essential for cell invasion, parasite locomotion in host tissues, and possibly, a target to control transmission (Frischknecht *et al.*, 2004; Matuschewski *et al.*, 2002). Some studies also refer to chemotatic attraction that could help to bring the parasite closer to the binding site for invasion (Akaki & Dvorak, 2005). Several proteins have been identified as essential for sporozoite invasion of the salivary glands, like the parasite proteins CSP and TRAP (Myung *et al.*, 2004; Sultan *et al.*, 1997) and the mosquito SGS1. The later is a salivary gland specific protein, which localizes at the region that is preferentially invaded by the sporozoites. Antibodies anti-SGS1 inhibit sporozoites invasion in *Aedes aegypti*, making it a good candidate for the sporozoites receptor in the salivary gland epithelium (Korochkina *et al.*, 2006).

I.3 – The insect immune response

Numerous studies have shown that insects have the ability to recognize pathogens and trigger the activation of effective mechanisms to control or clear an infection. The current knowledge about insect response to pathogens is summarized in Figure I.3.1.

Insects show several lines of defense against pathogens: i) the external cuticle avoids massive infections hindering pathogen entry into the hemocel, ii) chitinous membranes, acids and enzymes in the digestive tract help restrain infection by ingested microorganisms, iii) if pathogens manage to enter the hemocel the insect is able to mount a complex immune response to clear the infection.

Insects do not possess an adaptative immune system as vertebrates, relying solely upon an innate immune response. Nevertheless, this can be highly complex, presenting outstanding specificity and effectiveness.

Insects have several tissues able to engage in immunity: hemocytes, fat body and epithelium. Upon an infection, one or more tissues may be stimulated to participate in the response. How they signal to each other is not very clear, but it may require cytokine-like molecules.

Hemocytes (or blood cells) are the immune-responsive cells by excellence. They move freely through the hemolymph and possess the arsenal to combat infections, and are probably the first to be activated upon an infection in the hemocel.

Fat body is the equivalent to the mammalian liver. This organ is responsible for the production in large scale of pathogen killing molecules such as antimicrobial peptides, when a systemic response is in order.

Epithelium encloses the local immune responses. Epithelium responses are critical, as localized immunity avoids massive spread of infection, facilitating pathogen clearance.

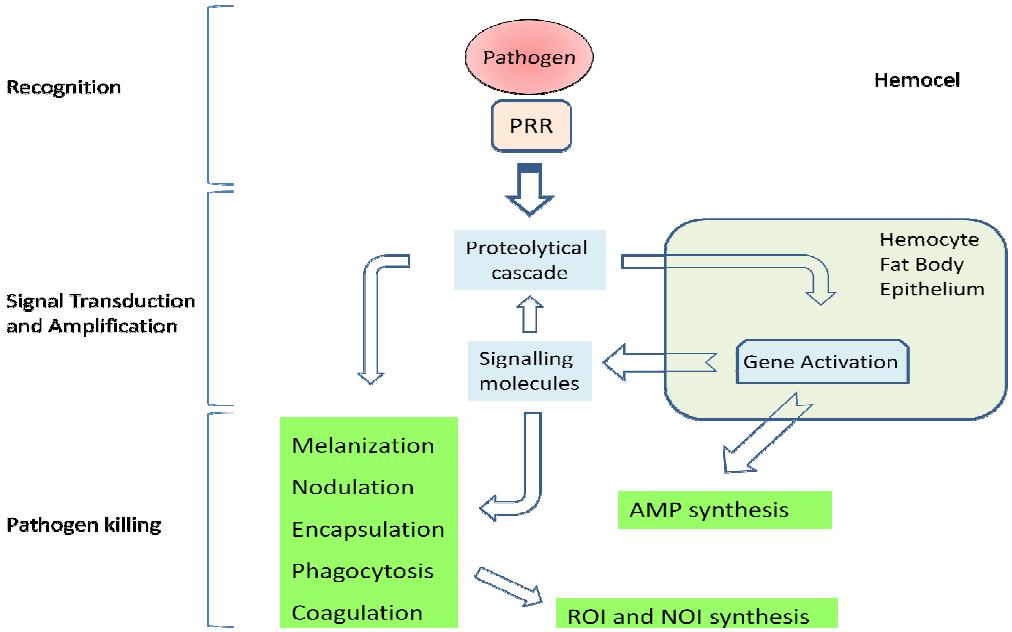


Figure I.3.1 – Insect immune responses to pathogen infection. Pathogens are recognized in the hemocel by specific receptors that activate proteolytical cascades. These may directly trigger pathogen killing mechanisms or activate intracellular signal transduction pathways. These result in the transcription of immune related genes whose products are released into the hemocel. These molecules can be either effector molecules with antimicrobial activity (AMPs, NOIs and ROIs), or secondary signaling molecules that contribute to the activation of other effector mechanisms for pathogen killing. As such, insect immune responses can be roughly divided into three phases: 1) Recognition of non-self molecules by highly conserved pattern recognition receptors (**PRR**); 2) Signal transduction and amplification through extracellular proteolytical cascades (comprised serine proteases and their negative regulators, serpins) and/or intracellular immune signaling pathways; and 3) Activation of effective pathogen killing mechanisms. **AMP:** Antimicrobial Peptide; **NOI**: Nitric Oxide Intermediate; **PRR:** Pattern Recognition Receptor; **ROI**: Reactive Oxygen Intermediate.

As for the way how different effector mechanisms are activated, the response can be classified as humoral or cellular. A humoral response is characterized by the use of effector and signaling molecules already present in the hemocel, and subsequent release (by signal amplification) of the same molecules by either of these tissues into the hemocel where they exert their action. This includes the production of small peptides with antimicrobial activity (antimicrobial peptides - AMP), generation of reactive intermediaries of oxygen and nitrogen (ROI and NOI, respectively), and activation of complex cascades that lead to responses such as melanization and coagulation. The presence of hemocytes at the infection site is mandatory for cellular

immune responses. These are able to engage in responses such as phagocytosis, nodulation, aggregation, encapsulation and cytotoxic reactions. As humoral factors affect the hemocytes, and these are necessary for the production of the same factors, both responses are overlapping and operating when combating an infection.

Pathogen killing mechanisms in insects include phagocytosis, nodulation, encapsulation, coagulation, melanization and AMP synthesis. One or more of these mechanisms may be activated upon an infection.

Phagocytosis involves the engulfment and intracellular digestion of non-self particles by the hemocytes. It starts with binding of non-self molecules to specific receptors on hemocytes, which triggers the target engulfment via an actin polymerization-dependent mechanism. The target is destroyed within phagosomes by lysosomal enzymes. The digestion of pathogens may lead to the production of secondary signals sent to the fat body to stimulate other immune responses. Insects are able to recognize and phagocyse bacteria, yeast, parasites, virus, and also synthetic particles such as negatively charged Sephadex beads (da Silva *et al*., 2000; Hernandez *et al*., 1999; Hillyer *et al.*, 2003; Kocks *et al*., 2005; Lamprou *et al.,* 2007; Mizutani *et al.*, 2003; Moita *et al.*, 2005).

Nodulation and Encapsulation are observed exclusively in invertebrates. They are activated in response to microorganisms too large to be phagocised. Nodules refer to multicellular aggregates of hemocytes surrounding a high number of bacteria or large pathogens in an extracellular material. Encapsulation refers to binding of hemocytes to larger targets, or even nodules. Nodules and capsules can be subsequently melanised, *ie,* covered by a melanin layer, that hinders the delivery of oxygen to pathogens. The production of cytotoxic quinones and free radicals from ROI and NOI (by-products of melanin synthesis), and of AMP's by hemocytes may also contribute to pathogen killing (Jiravanichpaisal *et al.*, 2006).

Melanization is a mechanism present in various groups of invertebrates including insects. Melanin is produced for several purposes, such as hardening of the egg chorion, wound healing, cuticle tanning and immunity. Upon an infection, a melanin layer can be deposited extracellularly over the invading microorganism, or intracellularly over phagocised microorganisms. The melanin layer keeps the pathogen immobilized, avoiding the infection to spread while hindering nutrient and oxygen exchanges between the microorganism and the surrounding media. In addition, several toxic molecules, such as cytotoxic quinones and ROI and NOI species produced in the process of melanin synthesis, may assist in pathogen clearance. Melanization can be itself a pathogen killing mechanism or it can be activated as a complement to phagocytosis, encapsulation or nodulation. The machinery to produce melanin is synthesized in hemocytes and fat body, and delivered to the hemocel. Upon infection or wounding melanin is rapidly synthesized to avoid massive infection or loss of hemolymph (reviewed by Barillas-Mury, 2007; Soderhall & Cerenius, 1998).

Coagulation, as melanization, is vital for wound healing and immunity. It is essential to avoid hemolymph losses when a wound opens in the cuticle and to clear pathogen infections. In this process, microorganisms are entrapped inside a clot comprised of extracellular protein aggregates and hemocytes (Theopold *et al.*, 2002). It is not known whether the clot itself participates in pathogen killing (by the production of toxic side products or asphyxiation) or if it functions only to entrap pathogens to be cleared by other mechanisms.

Antimicrobial peptides are small cationic molecules with antimicrobial properties. These molecules are produced by the fat body and the hemocytes and are released into the hemocel (Bulet *et al.*, 1999) and have the ability to kill microorganisms generally through the destruction of the cell membrane, by permeabilzation. Increasing evidence shows some degree of specificity in the pathogen-AMP relation. For instance, in *Drosophila melanogaster* the antimicrobial peptide Drosomycin was

shown to be synthesized upon infection with fungi or Gram-positive bacteria, while Diptericin is produced in response to Gram-negative bacteria.

I.3.1 – The *Drosophila* immune response

Nowadays, one of the most studied insect immune systems is that of the fruitfly *Drosophila melanogaster*, whose powerful genetic tools allow an accelerated study at many molecular levels. Although hematophagous insects such as mosquitoes have a higher diversity in pathogen microorganism to which they are exposed (due to the nature of the blood meal), the mosquito complexity of breading, the lack of suitable genetics and technology of genetic manipulation permit only a limited view of the immune responses, making *Drosophila* a useful model to study immunity even in hematophagous insects (Meister *et al.*, 2004).

The *Drosophila'*s immune response to infection as been recently reviewed by several authors: Hoffmann (2003), Agaisse & Perrimon (2004), Leclerc & Reichhart (2004), Naitza & Ligoxygakis (2004), and Lemaitre & Hoffmann (2007).

The best studied pathogen killing mechanism in *Drosophila* is the synthesis of antimicrobial peptides (AMP). AMP synthesis is activated by bacteria and fungi and is regulated by two intracellular signal transduction pathways (TOLL and IMD). A third pathway (JAK/STAT) is triggered in response to viruses. The recognition of pathogen associated molecular patterns in microorganisms (PAMPs) by pattern recognition receptors (PRRs) (Medzihtov & Janeway, 2002) activates a specific signal transduction pathway that results in the translocation of a cytoplasmatic NF-kB transcription factor to the nucleus, where it starts synthesis of AMPs and other immune molecules. These pathways are represented in Figure I.3.2.

Apart from AMP production, other effector mechanisms are known to be essential for immune responses in *Drosophila*, as phagocytosis, encapsulation, coagulation and melanization of microorganism, which have been observed upon immune challenge. However, their activation and regulation was not yet disclosed.

Additionally, molecules like the iron up-taking molecule transferrin (Yoshiga *et al.*, 1999) and the inducible nitric oxide synthase NOS (Foley & Farrell, 2003; Nappi *et al.*, 2004) have been implicated in microorganism killing, although a link to regulation by one of the signal transduction pathways is still missing.

The fly's immune response is a complex and intricate net of signalling pathways that lead to the successive activation of several reactions that work together in order to restrain the microbial infection, and to heal the wounded tissues and damaged cells of the fly.

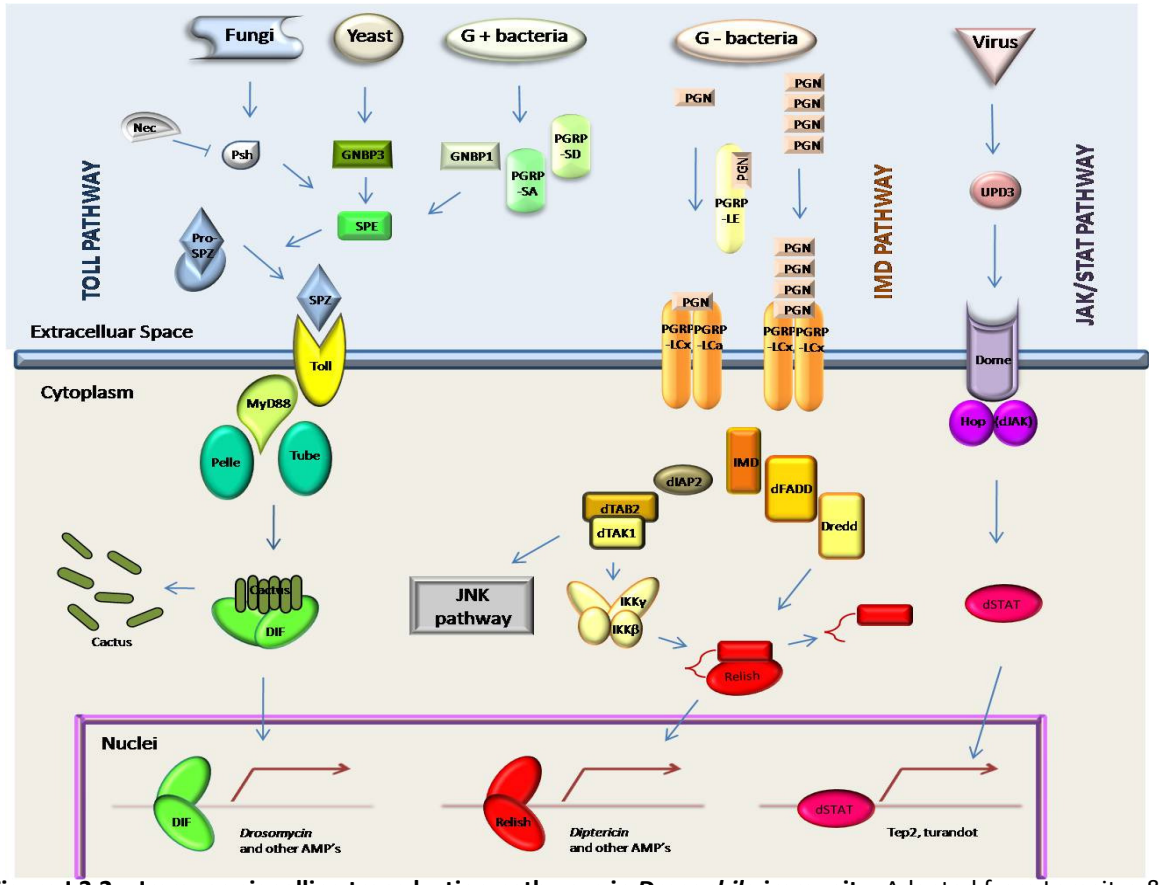


Figure I.3.2 – Immune signalling transduction pathways in *Drosophila* immunity. Adapted from Lemaitre & Hoffmann, 2007.

Toll pathway (left) is activated by fungi, yeast and gram-positive bacteria. Recognition involves PRR's like Persephone (**Psh**), **GNBP3** and **GNBP1/PGRP-SA/PGRP-SD**. Mutants for this pathway have a compromised survival for fungal and Gram-positive bacterial infections (Hoffmann & Reichhart, 2002). Recognition activates a Spaetzle activating enzyme (SPE) that converts pro-Spaetzle into Spaetzle (**SPZ**). This binds to the receptor **Toll** (Lemaitre *et al.*, 1996; Imler & Hoffmann, 2001), triggering an intracellular cascade, involving **Myd88**, **Tube** and **Pelle**. This results in degradation of the inhibitor Cactus and release of **DIF** that translocates into the nucleus, starting the transcription of AMP's like Drosomycin. Toll pathway is also associated with melanization (Ligoxygakis *et al.*, 2002), along with hemolymph components, like serine proteases and their negative regulators, serpins. Loss-of-function mutants for serpin 43Ac have constitutive cleavage of Spz and *drosomycin* expression (Levashina *et al.*, 1999; Ligoxygakis *et al.,* 2002). Serpins seem to protect against microbial proteinases, and to regulate endogenous proteinases, preventing over activation of hemolymph coagulation, proteolytic cytokine and prophenoloxidase (Kanost, 1999).

IMD pathway is activated by Gram-negative bacteria. Bacterial peptidoglycan (PGN) is recognized by the peptidoglycan recognition protein LE (**PGRP-LE)**. Monomeric PGN's activate a dimeric receptor composed by **PGRP-LCx** and **PGRP-LCa**, while polymeric PGN's activate a dimeric receptor composed by **PGRP-LCx**. Mutants for this pathway have a compromised survival for bacterial infection (Hoffmann & Reichhart, 2002). Recognition activates the **IMD** protein that in turn activates **dFADD** and **Dredd**, which cleaves the inhibitory domain of the **Relish**, causing it to translocate into the nucleus and start the transcription of AMP's such as *diptericin* and *attacin*. **IMD** may also activate **dIAP2**, **dTAB2** and **dTAK1**. The later can activate **IIKγ** and **IKKβ** leading to **Relish** activation, or trigger the **JNK pathway**. IMD is also involved in apoptosis, through the activation of the caspase-like protein DREDD (Georgel *et al.*, 2001).

JAK/STAT pathway is triggered by virus, as mutants for this pathway are resistant to bacterial and fungal expression, but susceptible to *Drosophila* Virus C (Dostert *et al.*, 2005). Recognition leads Unpaired-3 (**UPD3**) to bind to the receptor Domeless (**Dome**), activating **JAK**, and leading **STAT** to translocate into the nucleus and trigger the transcription of genes such as *tep2* and *turandot*. Overexpression of this pathway leads to the formation of melanotic pseudotumors (Hanratty & Dearolf, 1993; Harrison *et al.*, 1995; Luo *et al.*, 1997). Agaisse *et al.*(2003) also suggested this pathway to be involved in the response to tissue damage.

I.4 – The mosquito immune response to malaria

The mosquito immune system is believed to be responsible, at least in part for the parasite losses occurring along its sporogonic development. Thus, its complete understanding will provide a powerful tool to block malaria transmission.

The completion sequencing of *Anopheles gambiae* genome (Holt *et al.*, 2002) allowed a comparative analysis between the genomes of *An. gambiae* and *D. melanogaster* and the description of correspondent protein families in the mosquito. This comparison revealed that about half of the genes are orthologs 1:1 and identified 242 genes that potentially code for components of the immune system (Zdobnov *et al.*, 2002).

The comparative analysis of 18 gene families from the fly and the mosquito revealed a 2 fold deficit in 1:1 orthologs in immune-related genes, when compared to the whole genome. In contrast these genes present specific gene expansions in the mosquito, when compared to the fly. Components of the immune system or gene families that include these components have evolved faster than the rest of the genome. Gene families coding for recognition, signal modulation and effector mechanisms are poor in ortholog genes reflecting species specific expansions and possibly a strong selective pressure (Christophides *et al.*, 2002). On the contrary, genes belonging to intracellular immune signaling pathways are highly conserved, even more than the genome as a whole, probably due to the high range of functions in which they are involved, such as development. Although comparative genetics is of vital importance to highlight immune-related genes, it is insufficient to point out which of these genes are indeed involved in the response to *Plasmodium*.

Several approaches have been pursued in order to understand parasite-vector interactions and to attempt to block malaria transmission in the mosquito. These included transcription profile studies of infected and non-infected mosquitoes, and the establishment of mosquito strains refractory to malaria infection.

I.4.1 – Immune gene expression studies

Work in mosquito immune responses has focused mainly in differential expression of immune-related genes upon infection. Different transcriptional profiling approaches included:

- Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) for specific immune-related genes. Dimopoulos *et al.*, 1997 found some immune related genes to be up-regulated, both locally (midgut) and systemically, upon midgut invasion by ookinetes. These genes were also found to be up-regulated in later steps of parasite development (Dimopoulos *et al.*, 1998).
- Subtractive libraries, enriched for genes up-regulated after bacterial challenge (Oduol *et al.*, 2000), genes expressed in midguts after ookinete invasion and early stages of oocyst development (Abraham *et al.*, 2004), and genes expressed in midguts during late stages of oocyst development (Srinivasan *et al.*, 2004).
- EST libraries constructed of immunocompetent cell lines (Dimopoulos *et al.*, 2000), blood fed and non-blood fed mosquitoes (Ribeiro, 2003).
- Microarrays (derived from the immunocompetent cell line EST library), used to analyze genome responses to injury, bacterial challenge and malaria infection (Christophides *et al.*, 2002; Dimopoulos *et al.*, 2002). Microarray studies contributed to the identification of several genes with altered expression during midgut invasion (Christophides *et al.*, 2002; Dimopoulos *et al.*, 2002; Kumar *et al.*, 2003; Vlachou *et al.*, 2005). Vlachou *et al.* (2005) found that 7% of the assessed mosquito genes are differentially regulated at this point in infection, including genes belonging to functional classes such as cytoskeleton remodelling, apoptosis, immunity, redox metabolism, cell adhesion and extracellular matrix maintenance. Microarrays are now a widely used technique and a powerful tool to study mosquito responses to the parasite, and have been used to target differential expressed genes in the whole genome or in specific subsets of genes (immunity, stress, and others).

The massive data obtained by transcription profiling has not yet been functionally validated by reverse genetics, available through the use of techniques such as RNA interference (RNAi) (Blandin & Levashina, 2008). As far as proteomic studies, very few have been conducted, thus not much information is available regarding post-transcriptional protein regulation.

I.4.2 – Laboratory models for refractoriness to malaria

Another approach to attempt to underline the mechanisms used by the mosquito to restrain malaria infection was the genetic selection of refractory mosquito strains under laboratory conditions. These strains are able to totally (or nearly) eliminate *Plasmodium* ookinetes, either by lysis in the midgut epithelium cells, or melanization in the basal lamina of the epithelium (Carton *et al.*, 2005).

In the lab, strains of *Anopheles* mosquitoes were selected for refractoriness to malaria:

- The *An. gambiae* L3-5 strain restrains infection by melanization of ookinetes from *P. berghei* and allopatric strains of *P. falciparum* (Collins *et al.*, 1986).
- The *An. gambiae* SUAF2 strain lysis *Plasmodium gallinaceum* ookinetes while traversing the midgut epithelium (Vernick *et al.*, 1995).
- A selected line from *An. dirus* melanizes ookinetes from *P. yoelii* (Somboon *et al.*, 1999).

Malaria infection in these mosquitoes, in particular in *An. gambiae* L3-5, has been exhaustively studied to understand how the mosquito is able to control parasite infection. A multidisciplinary approach, comprising morphological, biochemical and genetic studies have revealed large physiological differences between refractory and

sensitive strains (Blandin *et al.*, 2004; Kumar *et al.*, 2003; Paskewitz *et al.*, 1998; Volz *et al.*, 2006; Zheng *et al.*, 2003).

I.4.3 – The mosquito response to the parasite

Even though the mosquito response to *Plasmodium* is far from disclosed, data obtained so far by transcription profiling, genetic selection of refractory mosquitoes, or RNAi-based reversed genetics functional studies, have highlighted several molecules that might be implicated in this process. These molecules have been grouped here by their function in immune responses.

I.4.3.1 – Recognition

I.4.3.1.1 – Peptidoglycan Recognition Proteins

PGRPs bind to peptidoglycan with high affinity. These molecules have a domain similar to bacterial amidase, which enables PGN cleavage for bacteria clearance.

Mosquito PGRP's are grouped in two classes: the long and short forms of PGRP. The long form (PGRP-L) may be intracellular or transmembrane, while the short form (PGRP-S) is extracellular. PGRPs are processed by alternative splicing that is influenced by several immune factors (Michel & Kafatos, 2005). PRGP-LC seems to be involved in Gram-negative bacteria (*E. coli*) phagocytosis and influence *Plasmodium* development in the mosquito (Moita *et al.*, 2005). PGRP-LB is up-regulated after *Plasmodium* infection in adult mosquitoes (Dimopoulos *et al.*, 2002) and sustains a high level of expression all through the parasite life cycle (Christophides *et al.*, 2002).

I.4.3.1.2 – Gram Negative Binding Proteins

GNBPs share significant similarities with the catalytic region of bacterial β,1-3 and β,1-3-1,4 glucanases. GNBPB1 and GNBPA1 were found to be up-regulated after *Plasmodium* infection. GNBPB1 is also induced in salivary glands and responds to bacteria (Christophides *et al.*, 2002; Dimopoulos *et al.*, 2002). GNBP4 weakly co-localized with *Plasmodium berghei* ookinetes in the mosquito midgut epithelium (Warr *et al.*, 2008).

I.4.3.1.3 – Thioester Proteins

TEPs are thioester-containing complement-like protein. The *Anopheles* TEP family shares significant similarities with the vertebrate complement factors C3, C4 and C5 and with the universal protease inhibitors, α2-macroglobulins.

The protein is activated by cleavage that exposes the thioester bond promoting covalent binding to the pathogen that is then marked for phagocytosis or lysis.

Several TEP proteins (TEP1, 3 and 4) are up-regulated by bacterial and/or *Plasmodium* infection (Blandin *et al.*, 2004; Chistophides *et al.*, 2002; Levashina *et al.*, 2001; Oduol *et al.*, 2000), although TEP1 is the most studied.

The thioester promotes binding of TEP1 to Gram-positive and Gram-negative bacteria that are opsonized and targeted for phagocytosis by hemocytes *in vitro* (Levashina *et al.*, 2001).

TEP1 is secreted by hemocytes into the hemolymph and enters the basal labyrinth of the midgut epithelium where it binds to ookinetes promoting lysis (Blandin *et al.*, 2004).

In the susceptible *An. gambiae* mosquito strain (G3), TEP1 binds to ookinetes from 24h reaching a maximum of TEP1 marked ookinetes at 48hpi. In the refractory mosquito

L3-5 strain TEP1 also binds to ookinetes, but much faster: at 24hpi nearly all ookinetes are marked by TEP1 and become later on melanized (Blandin *et al.*, 2004).

Knock-Down (KD) of TEP1 in adult mosquitoes completely abolishes melanotic encapsulation of ookinetes in L3-5 mosquitoes allowing the correct development of *P. berghei*. Additionally, mosquitoes KD for TEP1 are unable to melanize Sephadex beads (Warr *et al.*, 2006), revealing an essential role in melanization. In the susceptible strain, however, it causes hyperinfection – 5 fold increase in oocyst number and ookinete survival (Blandin *et al.*, 2004). TEP1 binds to ookinete surface after crossing the midgut epithelium in both sensitive and refractory strains but with different timing leading to degeneration. TEP1 associated ookinetes are lysed in midgut cells by a process that includes degeneration, represented by parasite blebbing, loss of the vital marker GFP, nuclear abnormalities, fragmentation, disturbances in the distribution of PbS28 (ookinete surface protein). TEP1 is responsible for the death of 100% of *P. berghei* ookinetes in L3-5 and 80% in G3 (Blandin *et al.*, 2004).

I.4.3.1.4 – C-type Lectins

C-type lectins (CTLs) are either membrane bound or secreted proteins that recognize and bind to carbohydrates, in a CA^{2+} binding dependent fashion. In the mosquito CTLs have been grouped according to sugar binding specificity: mannose binding (CTLMA), galactose binding (CTLGA), selectins (CTLSE) or other CTLs. These molecules mediate processes such as cell adhesion, cell-cell interactions, glycoprotein turnover and pathogen recognition leading to immune responses, usually activating melanization (Christophides *et al.*, 2004).

CTL4 and CTLMA2 are predominantly expressed in carcasses during midgut invasion and protect the parasite. KD of either these molecules leads to massive ookinete melanization (97% of ookinetes melanized in CTL4 KD and 48% in CTLMA2 KD) in G3 susceptible mosquito strain (Osta *et al.*, 2004), reflecting a melanization inhibitory role

and transmission permissiveness. However, KD of either CTL4 or CTLMA2 does not affect the melanization of negatively charged Sephadex beads (Warr *et al.*, 2006).

I.4.3.1.5 – Leucine rich immune genes

LRIM (Leucine rich immune genes) proteins can be secreted, membrane-bound or cytoplasmic. These PRRs contain numerous leucine-rich repeats (LRR) that are similar to motifs found in several immune-related molecules and may mediate protein-protein interactions.

LRIM1 is up-regulated in infections by bacteria and *Plasmodium* (Dimopoulos *et al.*, 2002). It is predominantly expressed in carcasses, when compared to midguts and is specifically up-regulated in carcasses of infected mosquitoes. The expression in the midgut of infected mosquitoes is strong and transient, between 24 and 48hpi. So far no homologous genes to LRIM1 have been found in other species (Osta *et al.*, 2004, Moita *et al.*, 2005).

LRIM1 limits parasite development in the mosquito (Osta *et al.*, 2004), as its KD leads to a 4 fold increase in oocyst number in the sensitive strain. Also, mosquitoes KD for this genes show inability to melanize Sephadex beads (Warr *et al.*, 2006), suggesting a role in melanization.

Double KD of *CTL4* and *LRIM1* results in an increased number of live oocysts (4 fold) without any melanization, suggesting that LRIM1 acts upstream of CTL4 and CTLMA2 and that parasite death occurs before melanization (Osta *et al.*, 2004).

I.4.3.1.6 – Galectins

Galectins are galactoside binding lectins (GALE) that are thiol dependent.

GALE8 was found to be up-regulated by bacteria and *Plasmodium* (Dimopoulos *et al.*, 1998), while GALE5 is only marginally up-regulated by bacteria infection and midgut invasion by the parasite (Christophides *et al.*, 2002).

I.4.3.1.7 – Fibronectins

FBN have a domain similar to the carboxy-terminus of the fibrinogen γ chain. These molecules are involved in microorganism recognition and agglutination.

Some FBNs are up-regulated upon infection with bacteria, as FBN9, and/or malaria infection, like FBN9 and FBN23 (Christopides *et al.*, 2002, Dimopoulos *et al.*, 2002).

I.4.3.2 – Signal modulation by extracellular protease cascades

I.4.3.2.1 – CLIP domain serine proteases (CLIPs)

CLIPs are proteases characterized by an N- terminal disulphide-knotted domain (CLIP domain), and a C-terminal trypsin-like domain. CLIP proteins modulate the extracellular signal that activates several intracellular pathways involved in development, AMP synthesis, melanization and hemolymph clotting and are negatively regulated by serpins.

In *An. gambiae*, the 41 CLIP genes were grouped into 4 classes. CLIPs belonging to the B group are probable candidates for PPO cascade activation (Volz *et al.*, 2005).

Some CLIPs are regulated by *Plasmodium* infection: CLIPB1, B4 (Gorman *et al.*, 2000), B14 and B15 (Christophides *et al.*, 2004) are up-regulated, while ClipA6 is down-regulated (Christophides *et al.*, 2004). CLIPB14 and B15 share significant features with proteases known to be involved in the PPO cascade activation. These proteins have a

role in melanization, affecting the vectorial capacity of *An. gambiae* to *P. berghei* (Volz *et al.*, 2005).

I.4.3.2.2 – Serpins

Serpins (SRPNs) are usually inhibitory proteins that act as suicidal substrates for serine proteases. SRPN proteolytical activation results in a conformational change of the protein that binds irreversibly to the target protease. Other proteins of this group that are not inhibitory can have roles in hormone transport, storage and blood pressure regulation (Silverman *et al.*, 2001). The mosquito has 14 genes coding for serpins, 10 of which are inhibitory.

SRPN1, 2 and 3 are orthologs of *Drosophila* SRPN27A, involved in melanization activation, and SRPN2 regulates the PPO cascade in the mosquito (Michel *et al.*, 2005). SRPN2 is a negative factor for ookinete death and melanization. KD of SRPN2 results in the formation of melanotic masses, death and melanization of parasites. It does not affect the *P. berghei* ookinete formation, but strongly reduces the oocyst numbers through an increase in ookinete lysis and melanization (Michel *et al.*, 2005). Nonetheless, it has no effect on the development of *P. falciparum* strains (Michel *et al.*, 2006).

SRPN6 is expressed in ookinete invaded midgut cells in both *An. gambiae* and *An. stephensi*. KD of SRPN6 in *An. stephensi* increases parasite load, while in *An. gambiae* it does not alter the oocyst number, but the ratio of melanized vs. lysed oocysts. L3-5 refractory mosquitoes KD for SRPN6 have and increased number of melanized ookinetes, while in G3 mosquitoes SRPN6 KD delays parasite lysis progression (Abraham *et al.*, 2005).

SRPN10 has 4 different intracellular isoforms that inhibit different and specific proteases. Two of these isoforms (KRAL and RCM) are induced in midgut cells invaded by the parasite where they translocated from the nucleus into the cytoplasm (Danielli *et al.*, 2003, 2005), causing the cell to detach from the epithelium into the midgut

lumen and undergo apoptosis. Hence this protein is a good marker for invasion and apoptosis of epithelial cells (Danielli *et al.*, 2005).

I.4.3.3 – Intracellular signal transduction pathways

I.4.3.3.1 – Toll pathway

All intracellular components of the Toll pathway are highly conserved between *An. gambiae* and *D. melanogaster* (Figure I.3.1). As for the extracellular ligand of the Toll receptor, the mosquito has at least 6 similar to *Drosophila* Spaetzle.

I.4.3.3.2 – IMD pathway

Like the Toll pathway, all the intracellular components of the IMD pathway are well conserved between *An. gambiae* and *D. melanogaster* (Figure I.3.1; Christophides *et al.*, 2002).

In *An. gambiae,* REL2 (ortholog of *D. melanogaster* Relish) regulates the intensity of infection by *P. berghei* and has a role in melanization. Double KD of REL2/IMD leads to a 2 fold increase of oocyst number, as does the single KD of REL2-F. The single KD of REL2 results in a limited occurrence of melanized ookinetes (~5%) (Meister *et al.*, 2005).

In *Drosophila*, each pathway seems to be activated by specific microorganisms, while in the mosquito the response to *Plasmodium* seems to require both Toll and IMD pathways. In fact, expression of AMPs such as CEC1, DEF1 and GAM was found to be regulated by both Toll and IMD pathways (Luna *et al.*, 2006). Also, in a pre-invasion stage of the midgut, the response seems to be regulated by both REL1 (ortholog of *D. melanogaster* Dorsal) and REL2, *ie*, by both TOLL and IMD pathways. Double KD for REL1 and REL2 results in a decreased expression of *tep1* and *lrim1*, compromising mosquito resistance to *P. berghei*. Additionally, KD of *cactus* (REL1 inhibitor) up-regulates TEP1 and other immune factors and completely blocks parasite

development. In this stage, in non-KD mosquitoes, there is a basal expression of *tep1* and *lrim1* that is regulated by REL1 and REL2. However, in a post-invasion stage, there is an up-regulation of TEP1, LRIM1 and CTL4 that is independent of REL1/REL2 regulation. KD of CACTUS at this stage has no effect on oocyst numbers (Frolet *et al.*, 2006).

I.4.3.4 – Immune effector mechanisms for parasite clearance

Evidence shows that some immune effector mechanisms are implicated in the mosquito response to the parasite, as AMP synthesis, melanization, lysis and phagocytosis.

I.4.3.4.1 – AMP synthesis

The mosquito has genes coding for AMPs, such as defensin, cecropin, attacin and gambicin. The efficiency of the antimicrobial activity of the different AMP's depends on the vector-parasite combination (reviewed by Carton *et al.*, 2005).

Defensin1 (DEF1) corresponds to DEFA characterized by Richman *et al.* (1996). It has antimicrobial activity against several Gram-positive bacteria, *E. coli*, yeasts and filamentous fungi (Vizioli *et al.*, 2001b). Administration of exogenous defensin from *Aeschna cyanea* and *Phormia terranovae* has a profound toxic effect in isolated sporozoites and in the development of *P. gallinaceum* oocysts in *Ae. aegypti* (Shahabuddin & Pimenta, 1998). Defensin lead to a reduction in oocyst number when injected (Shahabudin & Pimenta, 1998), and is transcriptionally induced in *An. gambiae* mosquitoes in response to midgut epithelium invasion by *Plasmodium* ookinetes (Richman *et al.*, 1997). Nevertheless, KD of defensin has no impact on *P. berghei* infection rate (Blandin *et al.*, 2002).

Cecropin 1 (CEC1 - CECA as described by Richman *et al.*, 1996) is active against several strains of Gram-positive and Gram-negative bacteria and fungi (Vizioli *et al.*, 2000). In mosquitoes CEC1 is up-regulated upon *Plasmodium* infection from D2pi (Christophides *et al.*, 2002; Dimopoulos *et al.*, 2002, Vizioli *et al.*2000). Overexpression of this AMP results in a significant reduction of oocyst number (Kim *et al.*, 2004).

Gambicin is an AMP unique and exclusive to *Anopheles* and *Aedes* mosquitoes. It has 8 Cys residues that form 4 dissulfide-bridges. *Plasmodium* parasite induces gambicin expression both locally (midgut) and systemically (fat body) in early and late stages of infection. Gambicin has a weak inhibitory activity against *Plasmodium* ookinetes (Vizioli *et al.*, 2001a).

I.4.3.4.2 – Melanization

Melanization was one of the first mechanisms to be proposed as a parasite killing mechanism in the mosquito. In 1986, Collins *et al.* were able to select, under laboratory conditions, a strain of *An. gambiae* mosquitoes (L3-5) that clears *P. cynomolgi* B parasites by melanizing ookinetes, as soon as they emerge from the midgut epithelium. The L3-5 strain is refractory by melanization to many but not all *Plasmodium* isolates (Collins *et al.*, 1986; Zheng *et al.*, 2003). Melanin polymerization prevents gas diffusion, leading the parasite to suffocate, and its synthesis leads to the production of reactive species of oxygen (ROI). The L3-5 strain has higher levels of ROI than the susceptible G3 strain, and these levels increased even further with the blood meal suggesting that refractoriness may result (at least in part) from a deficient detoxification of ROI (Kumar *et al.*, 2003).

Multiple Quantitative Trait *Loci* (QTL's) in the mosquito genome were found to be associated with this phenomenon (Gorman *et al.*, 1997; Zheng *et al.*, 1997, 2003). In the model *An. gambiae* – *P. cynomolgi* B there are 3 QTL's involved: one major *locus* (*Pen1*) and two minor (*Pen2, 3*). In the model *An. gambiae* – *P. cynomolgi Ceylon*, a

different but closely related parasite species, refractoriness depends on 3 different *loci*: *Pen2R, Pen3R, Pen3L*. The genomic sequence of *Pen1* showed that 38 of the 46 ORF's have orthologs in *Drosophila* (Thomasova *et al.*, 2002).

Some mosquito molecules have been implicated in *P. berghei* ookinete melanization. They act both for and against the parasite: KD of *TEP1* and *LRIM1* blocks ookinete killing and melanization, while KD of *CTL4* and *SRPN2* increases the number of melanized ookinetes. These molecules are up-regulated upon ookinete invasion of the midgut (Whitten *et al.*, 2006). TEP1 however, is not synthesized *de novo* (as there is only a modest up-regulation at 24h and 4d pi) but is already present in the basal labyrinth and hemolymph when the ookinete invades the midgut epithelium.

Melanization is activated by a serine protease cascade that culminates with the proteolytical activation of the prophenoloxidase (PPO) enzyme into Phenoloxidase (PO). This enzyme is responsible for melanin production, catalyzing the oxidation of phenols to quinones that polymerize nonenzymatically into melanin (Soderhall & Cerenius, 1998). The *An. gambiae* genome comprises nine genes coding for PPOs (Christophides *et al.*, 2002). PPO1 to PPO4 are expressed in immature stages of the mosquito, while PPO6 and PPO9 are expressed in adults. PPO2 and PPO3 are up-regulated upon blood feeding (Muller *et al.*, 1999). In this work the authors selected a mosquito hemocyte-like cell line (4A-3B) that constitutively expressed 6 PPO genes. This cell line responds to bacterial infection through the induction of DEF and GNBP, but not by inducing any PPO gene. Immune challenge does not induce PPO gene expression instead it is believed to activate the enzyme at a post-transcriptional level (conversion of PPO into PO), through the conjugated regulation of serine proteases and serpins.

Phenoloxidase enzyme is activated by a cascade of CLIP serine protease. Some CLIPs need an inactive CLIP serine protease (serine protease homologue – SPH) as a co-factor to activate PPO. In *An. gambiae*, two classes of CLIP proteases exist: CLIPA proteins are SPHs and CLIPB proteins comprise the functional serine proteases (Barillas-Mury, 2007).

Silencing of some CLIPs (*CLIPA2*, *CLIPA5* and *CLIPA7)* in L3-5 refractory mosquitoes leads to an increase of melanization suggesting an inhibitory role. In the same mosquitoes, CLIPA8 was found to have the opposite action, *ie*, to be essential for PPO activation cascade. However CLIPA8 KD did not increase the number of live ookinetes, suggesting that ookinete death occurs prior to melanization. On the other hand *CLIPA8* KD in the mosquito sensitive strain represses the *CTL4* KD melanization phenotype, and increases the number of live parasites in contrast to *CTL4* KD mosquitoes, suggesting that in this strain melanization is a parasite killing mechanism. In the sensitive mosquitoes, melanization induced by KD of CTL4 or CLIPA2/A5 is an efficiently ookinete killing mechanism (Volz *et al.*, 2006).

CLIPB14 and CLIPB15 act synergistically promoting ookinete lysis, in both refractory and susceptible mosquito strains. KD of CLIPB14 revealed a role in the activation of PO in a susceptible strain on *An. gambiae*. Double KD of CTL4 and CLIPB14 in a G3 background lead to a significant decrease in melanized ookinetes reversing the CTL4-KD phenotype, and a corresponding increase in live developing ookinetes. The KD of either CLIPB14 or CLIPB15 independently or jointly, results in a significant increase of oocyst number in the G3 strain, while it leads to an increase in the number of melanized ookinetes in L3-5. Neither one of these CLIP's blocks melanization in L3-5 suggesting that these proteins do not function in the regulation of melanization in the refractory strain (Volz *et al.*, 2005).

In fact, in refractory strains, melanization seems to be a post-mortem event, as melanin has never been observed associated with live parasites, only over dead ones. Melanization intermediaries may account for some parasite killing, but around 80% of the parasites are killed in a sensitive mosquito strain without melanization (Whitten *et al.*, 2006).

I.4.3.4.3 – Lysis

Ookinete lysis is characterized by organelle disintegration, cytoplasmic vacuolization and membrane blebbing, and has been observed for *P. berghei* ookinetes.

As melanization seems now to be a mechanism to clear parasites in L3-5 refractory mosquitoes and not a killing mechanism *per se*, a two step model has been proposed for the response of *An. gambiae* to *P. berghei*. Firstly, the ookinetes pass through the midgut epithelium and reach the basal labyrinth where they come in contact with soluble elements from the hemolymph, as TEP1 that binds to live ookinetes targeting them to death, both in refractory and sensitive strains. Secondly dead parasites are degraded either by melanotic encapsulation or lysis in the refractory strain, or by lysis in the sensitive strain (Whitten *et al.*, 2006).

In mosquitoes KD for TEP1 some ookinetes survive and some die without TEP1 labeling, suggesting that other products control parasite development (Whitten *et al.*, 2006).

I.4.3.4.4 – Phagocytosis

TEP1 was found to be implicated in phagocytosis responses to bacterial infection in mosquito (Levashina *et al.*, 2001). However, it does not seem to be a mechanism involved in ookinete clearance, as TEP1 marked ookinetes are targeted for lysis and not phagocytosis.

Nonetheless, phagocytosis of sporozoites in *An. gambiae* mosquitoes has been observed in the hemolymph (Hillyer *et al.*, 2007) and in the salivary glands (Korochkina *et al.*, 2006). *Ae. aegypti* were also found to be able to phagocyse *P. gallinaceum* sporozoites in the hemolymph (Hillyer *et al.,* 2003).

I.4.3.4.5 – Other killing Mechanisms

Some other mechanisms, or in some cases specific molecules, have been linked to the mosquito response to the parasite.

Oxidative stress appears to be a helpful tool in parasite killing, as it is present during both melanization and lysis phenomena. In agreement, *in vivo* inhibition of NOS increases parasite survival (Luckhart *et al.*, 1998). Midgut epithelium invasion induces NOS in the midgut and in the carcass (Han *et al.*, 2000; Luckhart *et al.*, 1998). NOS expression and activity are also induced upon sporozoite egress from the oocyst, which is reflected by the increased levels of nitrite and nitrate in the hemolymph of infected mosquitoes (Luckhart *et al.*, 1998). NO can rapidly be converted into nitrite that, in the presence of hydrogen peroxide (H_2O_2), mediates tyrosine (Tyr) nitration causing extensive degeneration of the invaded cell (Kumar *et al.*, 2004).

Different systemic levels of ROS were proposed to affect melanotic responses to *Plasmodium* (Kumar *et al.*, 2003), as H_2O_2 levels in the hemolymph of L3-5 refractory mosquitoes are twice as high as those of the susceptible G3 strain.

Although NOS and peroxidase activity and subsequent Tyr nitration are induced in *An. stephensi* mosquitoes upon *P. berghei* infection, the same is not observed in the *Ae. aegypti – P. gallinaceum* and *An. stephensi-P. gallinaceum* combinations, suggesting that epithelial responses are not universal to every parasite-vector combinations (Gupta *et al.*, 2005).

Vlachou *et al.* (2005) found that midgut epithelium remodeling, involving actin cytoskeleton and microtubules, is a major response to ookinete invasion. Other responses include innate immunity, extracellular matrix remodeling and apoptosis. RNAi studies have shown that among actin dynamics regulators there are agonists and

antagonists of parasite development and that actin polymerization is inhibitory to the parasite.

Also, there is a dual role in hemolymph lipid transporters for the parasite and egg production (Vlachou *et al.*, 2005). RFABG (retinoid and fatty acid binding glycoprotein) encodes an apolypophorin precursor that is involved in lipid transport. It is strongly induced upon ookinete invasion and has a role in egg development and ookinete survival. Silencing this gene leads to a 3.9 fold reduction in oocyst number and to an inhibition of egg development in the ovaries (Vlachou *et al.*, 2005).

I.4.3.5 – Immune responses after the midgut stage

The fact that the major part of parasites is lost during midgut invasion (Vlachou & Kafatos, 2005) led to numerous works regarding the midgut epithelium response to invading ookinetes.

Not so much is known about the response to emerging sporozoites and salivary gland invasion, as only few studies have paid attention to these stages that also reflect major bottlenecks in parasite development in the mosquito. Among others we find the works of Dimopoulos *et al.*(1996, 1998), Hillyer *et al.*(2003, 2007) and Rosinski-Chupin *et al.*(2007).

Hillyer *et al.* (2003) observed both phagocytosis and melanization of *P. gallinaceum* sporozoites in the hemocel of *Ae. aegypti*, phagocytosis being the major response. These authors also found that when sporozoites egress from the midgut the hemolymph flux through the dorsal vessel helps parasites to find salivary glands to invade. When purified sporozoites (*P. berghei/P. gallinaceum*) are injected into the mosquito hemocel (*An. gambiae/Ae. aegypti*) only a small proportion (19%) successfully invades the salivary glands. The invasion occurs shortly after oocyst burst (~8h) and the sporozoites that do not invade the salivary glands are rapidly killed and

degraded. The mechanisms behind this parasite clearance are not known, but 2% of the mosquito hemocytes contain sporozoites trapped due to phagocytosis (Hillyer *et al.*, 2007).

Rosinski-Chupin *et al.*(2007) provided the first *An. gambiae* salivary gland transcriptome analysis upon *P. berghei* infection. Through a Serial Analysis of Gene Expression (SAGE) 57 genes were found to be differentially regulated at the transcript level in infected mosquitoes. These included genes belonging to different functional classes such as transport, lipid and energy metabolism, stress response and immunity. Immunity-related gene class alone was represented by 37 genes. Of particular interest was the upregulation of *Defensin 1 (DEF1), GNBP, Serpin6 (SRPN6)* and *Cecropin2 (Cec2)*. The first two were also shown to respond to *P.berghei* infection in *An. gambiae* by Dimoupolos *et al.* (1998), and to be expressed in the salivary gland. *SRPN6* is also involved in the response to *P. berghei* upon midgut infection (Abraham *et al.*, 2005).

Figure I.4.1 summarizes the knowledge of the mosquito response to the parasite:

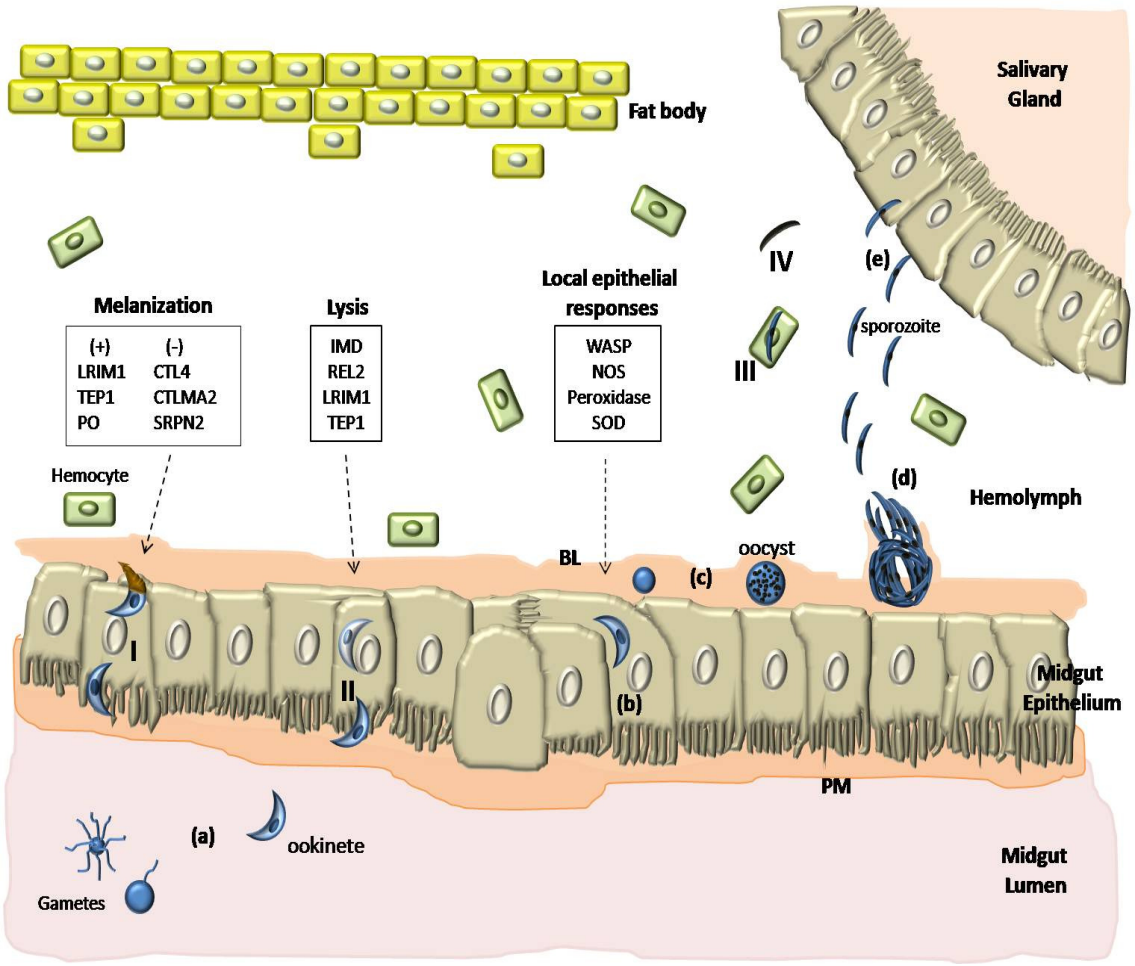


Figure I.4.1 – Interactions between *Plasmodium* and *An. gambiae*. Adapted from: Vlachou & Kafatos (2005).
Mosquito molecules putatively involved in positive (+), negative (-) and unclassified reactions to the parasite are indicated in boxes. Responses include melanization, lysis and local epithelium responses. Following gamete development and fertilization (**a**) ookinetes (**ook**) traverse the cytoplasm of several midgut cells (**1**, **2** and **3**) before reaching the basal lamina of the epithelium emerging basolaterally into the extracellular space (**b**). During midgut invasion, parasite induces local responses such as the induction of directional lamelliopodia protrusions (**lam**) beneath the invaded apoptotic cells, and the formation of cytoplasmatic lamellar protrusions (**hood**) of the invaded cell, which tightly embrace the parasite as it exits from the midgut (**c**).In the space between the epithelium and the basal lamina (**BL**) the parasite develops into an oocyst (**ooc**) in which thousands of sporozoites (**spz**) are produced (**d**). Some days later the oocyst busts and releases the sporozoites into the hemolymph where they journey to the salivary glands that are then invaded (**e**). During this journey, parasites are exposed to the immune response of the fat body and hemocytes. **CP**: Capping Protein; **IMD**: immune deficiency gene; **PM**: Peritrophic Matrix; **PO**: Phenoloxidase; **SOD**: Superoxide Dismutase.

I.4.4 – Studying rodent vs. human malaria

The majority of work that has done so far in mosquito immune response to malaria relies on the use of rodent (*P. berghei, P. yoelii*), and in some cases avian (*P. gallinaceum*) malaria parasites. These models are easy to handle and the work can be performed in safe conditions, as none of these parasites is infective to humans. *P. berghei* has been the parasite used most often as it is amenable to manipulation, and several transgenes now exist including GFP parasites that exhibit green fluorescence. However, it is extremely hard to predict if the mosquito responses to *P. berghei* determined under lab conditions correlate to some extent to the ones displayed to *P. falciparum* in the field, but data suggests that the responses to these parasites are in fact different. Yet, some molecules and mechanisms seem to be induced by both parasites but it is not sure if the similar responses observed are universal for all *P. falciparum* strains.

For instance, *P. berghei* and *P. falciparum* have different optimal temperatures for sporogonic development (21ºC and 25ºC, respectively) that may account *per se* for the induction of different molecules and/or effector mechanisms.

SRPN2 is able to bind to and inhibit a heterologous PAE, from *Manduca sexta*, and its subsequent melanization activation *in vitro*. KD of SRPN2 increases melanin deposition in negatively charged Sephadex beads, and affects negatively *P. berghei* development in the mosquito. However, this effect is not seen in the development of field isolates of *P. falciparum* in *An. gambiae* isolated from the same region (Michel *et al.*, 2006). Silencing of *SRPN2, LRIM1* or *CTL4* markedly affects *P. berghei* infection but does not influence the development of *P. falciparum* in *An. gambiae*, suggesting that the PPO cascade is under different regulation against the two *Plamsodium* species (Cohuet *et al.*, 2006).

A microarray study that aimed at the comparison of the *An. gambiae* response to *P. berghei* and *P. falciparum* showed that *P. berghei* has a more profound effect on the mosquito transcriptome, leading to an alteration in expression of several genes, belonging to different functional classes, while *P. falciparum* elicits a broader immune response, at the transcription level. Seven out of 12 immune-related genes chosen for KD affect mosquito resistance to both parasite species. However, some immune genes are involved in species specific resistance: MDL1 and FBN39 are specific for the resistance to *P. falciparum*, while Gambicin and IRSP5 are specific for the resistance to *P. berghei*. Another interesting fact is that all genes affecting *Plasmodium* development also affect bacterial infection. These results show that the mosquito response to malaria at the transcription level is diverse, depending on universal and species specific factors (Dong *et al.*, 2006).

Bonnet *et al.* (2001) found that 16 genes of *An. gambiae* are regulated by the presence of *P. falciparum* in the blood meal, including a profilin gene. Of these, only 4 are up-regulated by invasive forms. Profilin, an actin cytoskeleton regulator is also induced in midguts infected with *P. berghei* (Vlachou *et al.*, 2005). These results suggest that response to invasion of epithelial cell might be similar for both parasites that cross epithelia in a similar way.

In a study from Tahar *et al.* (2002) 9 genes were found to be up-regulated in *An. gambiae* infected with *P. falciparum* gametocyte carriers, including NOS, defensin and GNBP. The later is also induced in *P. berghei* infection, but with a different expression pattern (Christophides *et al.*, 2002). As for DEF, GNBP, ICHIT, IGALE20, IMCR14 and ISPL5, they are also up-regulated by *P. berghei* infection in *An. gambiae* (Dimopoulos *et al.*, 1997, 1998; Oduol *et al.*, 2000).

Although mosquitoes collected in the field are highly efficient in melanizing Sephadex beads, it is extremely rare to find melanized *P. falciparum* ookinetes (Niare *et al.*, 2002; Schwartz & Koella, 2002). The L3-5 strain is able to melanized ookinetes from *P. berghei, P. gallinaceum* and *P. cynomolgi* B, and allopatric but not from sympatric strains of *P. falciparum*. Nevertheless, one African strain of *An. gambiae* was found to

melanize/encapsulate *P. falciparum*, although at a lower level than L3-5 (Brey, 1999). Mosquitoes in the field have a higher frequency of resistant alleles that attenuate infection intensity (Niare *et al.*, 2002).

Mendes *et al.* (2008) used microarrays and reverse genetics to study 5 genes known to regulate *P. berghei* development and field isolates of *P. falciparum* in geographically related mosquitoes. The transcriptional response seems to be similar in general, but not identical. Two genes were found to affect both parasite species in the same fashion: WASP and APOII/I. Both act at the midgut invasion level and have opposite effects. WASP is an activator of the local actin cytoskeleton dynamics that limits parasite development. APOII/I is a lipid transporter that affects the first stages of oocyst formation and promotes parasite development.

Lim *et al.* (2005) found that *An. stephensi* infection by *P. falciparum* leads to the induction of NOS in midguts and the production of inflammatory levels of NO, by the recognition of parasite glycosylphosphatidylinositol (GPI) anchors. Although GPI anchors were not referred in either work of Luckhart *et al.*(1998) or Han *et al.*(2000), NOS induction and subsequent NO production was also found to occur in *P. berghei* ookinetes invaded cells of the midgut.

These studies indicate that, even tough *P. berghei* is a useful and necessary model to use in laboratory work, the mosquito response so this parasite may not correlate entirely to the response to *P. falciparum*.

I.4.5 – Vector control by mosquito manipulation

Parasite development in the mosquito depends on differences in genetics, ecology and behaviour of different mosquito species. The presence of different vector species in one area in the field may vary with season and ecology, making transmission control difficult. The relative importance of each mosquito species in the field also has a great impact on the parasite transmission pattern (Catteruccia, 2007).

Several attempts have been made in order to restrain transmission in the lab, including dietary provision of transmission blocking compounds, and the construction of transgenic mosquitoes that express foreign proteins making them resistant to malaria infection.

Examples include *Aedes aegypti* (Jansinskiene *et al.*, 1998), *Anopheles stephensi* (Catteruccia *et al.*, 2000), *An. gambiae* (Grossman *et al.*, 2001) and *Anopheles albimanus* mosquitoes (Perera *et al.*, 2002). Since stable germline integration was established, a lot of progress has been made in order to construct mosquitoes able to restrain parasite development (Riehle *et al.*, 2003). Examples of stable gene expression (either by inoculation of virus as gene carriers or by transgenesis) with an impact on parasite development in mosquitoes are present in Table I.4.1.

Table I.4.1

Stable gene expression in mosquitoes

	Model		Gene	Expression	Phenotype	Reference
	Parasite	Mosquito				
Viral expression	*P. gallinaceum*	*Ae. aegypti*	Anti-CS MAB	Sindbis virus	Reduction in salivary gland invasion	De Lara Capurro *et al.*, 2000
	P. berghei	*An. stephensi*	SM1	f88.4 Phage	Reduction in oocyst number and salivary gland invasion	Ghosh *et al.*, 2001
Transgenesis	*P. berghei*	*An. stephensi*	SM1	Pcarboxypep	Reduction in oocyst number	Ito *et al.*, 2002
	P. berghei	*An. stephensi*	Bee PLA2	Pcarboxypep	Reduction in oocyst number	Moreira *et al.*, 2002
	P. berghei	*An. gambiae*	CEC	Pcarboxypep	Reduction in oocyst number	Kim *et al.*, 2004
	P. gallinaceum	*Ae. aegypti*	DEFA	Pvitellog	Inhibition of parasite development	Kokoza *et al.*, 2000
	P. berghei *P. falciparum*	*An. stephensi*	Sea cucumber CEL III	Pcarboxypep	Inhibition of oocyst formation	Yoshida *et al.*, 2007

In viral expression the correspondent genes were expressed in viruses that were introduced in the mosquitoes either by injection or feeding. **MAB**: Monoclonal antibody. **SM1**: Salivary gland peptide 1. **Pvitellog**: Vitellogenin promoter. **Pcarboxypep**: Carboxypeptidase promoter.

In transgenesis mosquitoes were constructed to express the desired gene under the influence of a promoter that is specifically induced in the midgut upon a blood meal (**Pcarboxypep** or **P vitellog**). **PLA_2**: Phospholipase A_2. **CEC**: Cecropin. **DEFA**: Defensin A. **CELIII**: C-type lectin CEL III.

Although successful mosquito transformations impairing transmission have been achieved, it is atill hard to predict their efficiency in the field (Scott *et al.*, 2002). Catteruccia *et al.* (2003) have observed that in laboratory conditions, transgenes input a great burden in *An. stephensi* fitness. Yet, Moreira *et al.* (2004) found that transgenic

expression of PLA_2 in *An. stephensi* mosquitoes had no impact on mosquito fitness, in terms of mortality and egg production.

Another attempt to block transmission has been made by introducing of foreign agents into the mosquitoes, either by feeding or injection. Examples follow in Table I.4.2.

Table I.4.2

Introduction of foreign agents in mosquitoes to block transmission

	Model		*Agent*	*Phenotype*	*Reference*
	Parasite	Mosquito			
Feeding	*P. gallinaceum* *P. falciparum*	*Ae. aegypti* *An. gambiae*	Snake PLA_2	Inhibition of midgut invasion	Zieler *et al.*, 2001
Feeding	*P. falciparum* *P. berghei*	*An. stephensi*	Gomesin	Inhibition of exflagellation	Moreira *et al.*, 2007
Feeding	*P. berghei* *P. falciparum*	*An. stephensi*	NOS	Reduction in oocyst numbers	Luckhart *et al.*, 1998
Feeding	*P. gallinaceum*	*Ae. aegypti*	Anti-CTRP AB	Inhibition of oocyst formation	Li *et al.*, 2004
Feeding	*P. gallinaceum* *P. falciparum*	*Ae. aegypti* *An. stephensi* *An. gambiae*	Anti-WARP AB	Inhibition of oocyst formation	Li *et al.*, 2004
Feeding	*P. berghei*	*An. stephensi*	Anti-PbS21 AB	Inhibition of oocyst formation	Ranawaka *et al.*, 1994
Feeding	*P. gallinaceum* *P. falciparum*	*Ae. aegypti* *An. freeborni*	Allosamidin	Inhibition of oocyst development	Shahabuddin *et al.*, 1993
Injection	*P. gallinaceum* *P. berghei*	*Ae. aegypti* *An. gambiae*	Bacteria	Reduction in oocyst numbers	Lowenberg *et al.*, 1999

Agents were introduced in mosquitoes either by feeding (blood meal or diet) or injection. **PLA_2**: Phospholipase$_2$. **NOS**: Nitric oxide synthase. **Anti-CTRP AB**: Anti circumsporozoite and TRAP relate protein antibody. **Anti-WARP AB**: Anti Willebrand factor A protein antibody. **Anti-PbS21 AB**: Anti *P. berghei* surface protein 21 antibody. **Gomesin**: APM from spider. **Allosamidin**: chitinase inhibitor.

I.5 – External factors influence on mosquito malaria infection

Although much attention has been paid to the mosquito immune responses, other factors may affect the sporogonic development of the malaria parasite.

In the blood meal, along with the red blood cells and the parasites, are introduced a series of factors that can affect the outcome of infection. For instance, components of the mammalian immune system may have a parasite inhibitory effect: the presence of mammalian growth factors in the blood meal seems to be associated with an increased expression of NOS and a reduction in the parasite number (Luckhart *et al.*, 2003) and the presence of leukocytes seems to be related to a reduction in gamete differentiation (Lensen *et al.*, 1997).

Other blood meal components, like antibodies and drugs also have an influence. An immunized blood meal against *Plasmodium* sporozoites interferes with parasite development in the mosquito and affects gene expression of both parasite and mosquito (Lopes *et al.*, 2007). Many studies have been conducted in order to evaluate the effect of antimalarial drugs in malaria transmission.

Several antimalarial drugs were found to have a transmission blocking activity, damaging ookinetes and/or reducing oocyst numbers in the mosquito midgut. For instance, Atovaquone decreases infectivity of *P. berghei* to mosquitoes and Primaquine has both gametocytocidal and sporotoncidal effects on *P. falciparum*. Drugs such as Proguanil, Pyrimethamine and the combination Sulfadoxine/Pyrimethamine were found to have an inhibitory effect on the parasites development in the mosquito without affecting gametocytes. On the other hand, Sulfadoxine by itself, Artemisinin and Mefloquine show no effect on *P. falciparum* development in the mosquito. The latter, however, is able to reduce oocyst numbers of *P. berghei* (Butcher, 1997).

On the contrary to other drugs, Chloroquine has a transmission promoting effect, increasing oocyst numbers on *P. falciparum, P. berghei* and *P. yoelii nigeriensis* infections, even though it has a gametocytocidal action against immature gametocytes

(Butcher, 1997, Buckling *et al.*, 1997; Buckling & Read, 1999; Hogh *et al.*, 1998; Ichimori *et al.*, 1990).

The mechanisms by which chloroquine may affect increased parasite transmission in the mosquito have not been unveiled. One hypothesis is that it affects the mosquito immune response, promoting parasite infectivity. This was recently reported for another antimalarial drug, Nitorquine that increases the melanotic encapsulation of *P. yoelii* in *An. stephensi*, which is accompanied by an induction of PPO activity and mRNA. Interestingly, it has no effect on PPO expression or activity in non-fed mosquitoes or mosquitoes fed on non-infected blood, suggesting that it specifically aids response of the mosquito to the parasite (Zhang *et al.*, 2008).

I.5.1 – Chloroquine

Chloroquine was, until a decade ago, the major antimalarial drug used around the world. However the constant and widespread development of resistance by the parasite has made its use as a safe prophylactic drug nearly impossible.

Chloroquine is a diprotic weak base that shows a remarkable ability to enter acidic compartments in cells and raise its pH (Krogstad *et al.*, 1985), exerting a lysosomotrophic action and compromising its function. The exact mechanism through which chloroquine exerts its antimalarial action is still unclear. Yet, it is sure that it interferes with the hemoglobin digestion by the parasite, upon blood cell invasion. Hemoglobin digestion occurs in an acidic compartment of the parasite, the digestive vacuole, resulting in the production of a toxic waste. It is thought that chloroquine affects several detoxification mechanisms, leading to parasite killing (Ginsburg *et al.*, 1999).

At the same time this drug seems to have a series of effects in the human organism. Some of these have special influence in the immune system, and are sometimes

controversial. Evidence exists that it promotes nitric oxide synthesis from endothelial cells (Ghigo *et al.*, 1998), but also that it strongly inhibits IFN-γ induced NO synthesis in macrophages (Hrabák *et al.*, 1998). It inhibits proinflammatory cytokine release (Hong *et al.*, 2004; Karres *et al.*, 1998), but induces NF-kB activation and expression of proinflammatory cytokines in human astroglial cells (Park *et al.*, 2003a). In addition, it inhibits platelet aggregation *in vitro* (Nosál *et al.*, 2000), and pH dependent steps of virus replication (Savarino *et al.*, 2003).

In the mosquito it was shown to promote *Plasmodium* infection (Enosse *et al.*, 2000; Gautret *et al.*, 2001; Hogh *et al.*, 1998; Ramkaran & Peters, 1969). The exact mechanism behind it is still unclear, but accumulating data on chloroquine action on mammalian immunity indicate that it might also affect the mosquito immune responses to the parasite, thereby increasing its permissiveness to the parasite.

Abrantes *et al.* (2005) characterized chloroquine effect in the mosquito immune response at the serine protease and AMP synthesis level. After a blood meal, the expression of serine protease (*Sp14D, Sp24D, ISPL5* and the serine protease *1644280*) and AMPs (*gam, def* and *cec*) are induced. Remarkably, this induction is suppressed in mosquitoes fed on mice treated with chloroquine and AMP expression seems to be more affected by chloroquine than serine proteases expression. Following a *P. berghei* infected blood meal chloroquine has the same suppressive action at the exception of *Sp14D, def* and *1644280*. However, in infected mosquitoes chloroquine action is less pronounced, suggesting that the response to the parasite can overcome the drug effect.

A microarray-based study performed to obtain more detailed information on chloroquine effect on the mosquito responses, revealed that this drug affects the expression of mosquito genes involved in several physiological processes, such as immunity, apoptosis, cytoskeleton remodeling and oxidative stress. As all these phenomena have been in some way linked to the response to the parasite and it is understandable that this drug is able to modulate malaria development in the mosquito (Abrantes *et al.*, 2008).

Understanding how chloroquine and other external factor can impair the mosquito immune response to the parasite and manipulate infection may provide an insight on the complex array of interactions between parasite and vector. This is a requisite to finally manipulate mosquitoes and block transmission.

To successfully eradicate malaria an integrated action will have to be created, covering areas such as vaccination, treatment, prevention, sanitary and health conditions improvement and transmission blocking. Each of these areas invokes an enormous amount of effort and labor. For transmission blocking alone it is imperative to understand the biology of parasite and mosquito, their interactions throughout the infection step (including mosquito immune responses and parasite evasion), and the factors extrinsic to the parasite and the mosquito that may influence the outcome of the infection. Ultimately, this will allow the construction of transgenic mosquitoes resistant to malaria infection and fit for field survival and spreading.

II – Objectives

II.1 – General objectives

Efficient control of malaria requires integrated actions, covering areas such as sanitary and health conditions improvement, sustainable control programs, vaccination, reliable drug therapy and transmission control. For this it is vital to understand the biology and interactions of both parasite and vector. In this context, the mosquito immune responses to the parasite are of critical importance. As such, this work aims at contributing to the knowledge about mosquito immunity towards the malaria parasite. This will be accomplished by using the insect model for immunity studies *Drosophila melanogaster* and by testing several insect immune responses that the mosquito might use to control malaria infection at a specific life cycle stage: the hemolymph invasion by *Plasmodium* sporozoites. This will involve *in vivo* systems of *D. melanogaster* and *An. gambiae* infections and *in vitro* systems of both organisms. Molecular techniques will involve gene expression studies, *Plasmodium* detection PCR techniques and proteomics.

II.2 – Specific objectives

II.2.1 – Objective 1 – Evaluation of Chloroquine effect on mosquito immune responses using the *Drosophila melanogaster* model.

Chloroquine is known to affect malaria transmission in the mosquito vector. When present in a blood meal it has a *Plasmodium* development promoting action, enhancing oocyst numbers in the mosquito midgut. Accumulating evidence suggests that chloroquine has an impact on mosquito immunity. Knowledge about mosquito immunity is still limited and scattered. Oppositely, current understanding about *Drosophila* immune responses is straight forward, particularly in respect to

intracellular immune pathway activation and AMP synthesis. Thus, it will be a useful model to study the effect of Chloroquine on AMP induction in mosquito immunity.

Chloroquine effect on fly immunity will be evaluated on AMP (Drosomycin, Attacin and Diptericin) gene expression and protein synthesis, using both *in vivo* and *in vitro* systems, upon infection or immune stimulation, and on survival, upon infection.

II.2.2 – Objective 2 – Determination of mosquito immune effector mechanisms involved in the hemolymph response to *Plasmodium* sporozoites.

Mosquito vector immune responses to malaria parasites have been widely studied. The massive data accumulated so far refers mainly to the responses triggered by ookinete invasion of the mosquito midgut. Knowledge about mosquito responses to hemolymph invading sporozoites is scarce. We aim at understanding which immune effector mechanisms may be triggered on the mosquito hemolymph allowing the mosquito to restrain sporozoite numbers and avoid massive salivary gland infection.

a) Determination of hemocyte number variation upon stimulation with a *Plasmodium* sporozoite surface protein.

Hemocyte numbers in insect hemolymph may vary according to the specific immune response triggered: microaggregation reactions reduce the number of circulating hemocytes, while other responses may require the induction of hemocyte proliferation to amplify the response itself. This will be tested *in vitro* using hemocyte-like cells that will be stimulated with the major surface antigen from *P. falciparum* sporozoites.

b) Evaluation of melanization activation and role upon sporozoite invasion of the mosquito hemolymph.

Massive ookinete melanization has been observed in midguts of malaria refractory mosquitoes and on sporozoites in the hemolymph of *Ae. aegypti* mosquitoes. We aim at evaluating melanization induction in mosquito hemolymph in response to the presence of *Plasmodium* sporozoites and determining the potential role of this response in the control of sporozoite numbers in the mosquito hemolymph.

Melanization in the hemolymph will be followed *in vivo* through *P. berghei* infection in the mosquito and correlated with the presence of sporozoites. It will also be determined *in vivo* upon stimulation with the major surface antigen from *P. falciparum* sporozoites. Its role on parasite control in the hemolymph will be tested by inhibition of melanization in infected mosquitoes and evaluation of sporozoite load on salivary glands.

c) Proteomic analysis of mosquito hemolymph upon sporozoite invasion.

The insect hemolymph is a protein rich environment to which several immune proteins are secreted. Soluble proteins include PRRs, protease cascades and their regulators for signalization and activation of immune effector mechanisms, and AMPs. Some of these proteins exist at a basal level in the hemolymph to assure a quick response or are produced and secreted upon infection. Thus the hemolymph proteome at a specific stage of a particular infection is expected to be unique for the pathogen. We will use a proteomic analysis to determine differential regulation of hemolymph proteins upon sporozoite invasion of the hemolymph. Gene expression of genes coding for the identified proteins will add further knowledge about the response itself. Immune effector mechanisms will be evaluated according to the predicted functions of the identified proteins.

III – Chloroquine and the *Drosophila* immune response

III.1 – Introduction

Drosophila melanogaster flies represent a powerful model to study physiological processes taking place in several organisms, from insects to humans (Alarco *et al.*, 2004; Bilen & Bonini, 2005; Schneider, 2000). These flies are easy to handle and to maintain in the lab. Additionally, their powerful genetic tools allow an accelerated study at many molecular levels, also through the establishment of mutant lines, and the possibility to knock-down virtually any gene. This is of particular importance in the understanding of how the components of a given pathway are ordered. These properties allowed identifying components of the intracellular transduction pathways (namely TOLL and IMD, Figure I.3.2) and of the extracellular protease cascades acting during the immune response. Conversely, mosquitoes are hard to handle and the establishment of mutant lines is complex and difficult. Nevertheless, the completion of the *An. gambiae* genome sequencing allowed a comparative analysis of the genomes of the fly and the mosquito, including immunity genes. This revealed a great conservation of the components of intracellular transduction pathways, as opposed to extracellular immune-related molecules. Thus, the fly came as a good model for mosquito immunity in particular for the activation of intracellular transduction pathways.

In flies, TOLL and IMD pathway activation is marked mainly by AMP synthesis, the best studied effector mechanism in *Drosophila*. TOLL pathway is known to be responsible for the production of AMPs like Drosomycin (Drs) in response to fungi or Gram + bacterial infection, while the IMD pathway leads to apoptosis and to the synthesis of AMPs such as Diptericin (Dipt) and Attacin (Att) in response to Gram – bacterial infection. Experiments in which mutant flies were challenged with fungi or bacteria revealed that survival to fungal, but not to bacterial infection, was severely compromised in TOLL-deficient mutants, and, by contrast, IMD mutants were markedly affected by bacterial infections but resisted fungi with a survival pattern similar to that of wild-type flies (Hoffmann & Reichhart, 2002).

The antimalarial drug chloroquine was shown to have a transmission boosting action in the mosquito of both rodent and human malaria species (Enosse *et al.*, 2000; Gautret *et al.*, 2001; Hogh *et al.*, 1998; Ramkaran & Peters, 1969), and it was proposed to facilitate transmission through the mosquito by affecting mosquito-parasite interactions, especially by interfering with the mosquito immune response.

Chloroquine can be expected to act at the extra or intracellular level. It has been documented to affect proteolytical activity (Staszczak *et al.*, 2000), thus it may interfere with extracellular signalling between the microbial recognition and the activation of surface receptors and intracellular signalling.

Chloroquine is a diprotic base with a remarkable ability to enter acidic compartments in cells, such as phagosomes, lysosomes and transport vesicles. These are of particular importance in immune processes such as phagocytosis, secretion of proteins to the extracellular space and the delivery of recognition receptors to the cell wall. The accumulation of chloroquine in these compartments results in a pH increase, exerting a lysosomotrophic action and affecting processes depending on endo/exocytic pathways (Krogstad *et al.*, 1985; Weber *et al.*, 2000). One of the mechanisms that could be compromised is the recycling of immune related receptors to the cell surface. In this case, no recognition of the extracellular immune signal would occur, no intracellular signalling would be produced, and no immune response would be observed. It could also happen that the few receptors already present at the cell surface by the time of administration of the drug, are enough to receive, transmit and amplify the immune signal. But impairment of the exocytic pathway would prevent the correct extracellular localization of several immune related molecules, which depends on it to be secreted. Additionally, chloroquine was shown to interfere with the function of transcription factors (Park *et al.*, 2003a). Thus chloroquine might be expected to interfere with processes necessary to fight several types of infections.

In *Drosophila* the response to different pathogens is quite well understood, particularly at the intracellular level and the AMP synthesis, both *in vivo* and *in vitro*, making it a good model to study the effect of chloroquine on immunity.

III.2 – Methodology

III.2.1 – Fly stocks

Wild type *Drosophila melanogaster* Oregon R flies were kept at 25ºC, on standard medium. Three to five days old female flies were utilized for toxicity tests, antimicrobial peptide expression and survival experiments.

DD1/DD1; Droplet/TM6c flies were used to qualitatively evaluate the expression of the antimicrobial peptides Drosomycin and Diptericin on a gut-localized immune response. These flies carry two reporter transgenes, a *drosomycin-GFP* gene (*P[w$^+$mC Drom::GFP = pDrs-GFP S65T*), in which the *GFP* gene is under control of the *drosomycin* promoter, and a *diptericin-β-galactosidase* gene *(P[w$^+$mC Dipt:: LacZ = pDipt-LacZ]*) that has a *β-galactosidase* gene under the control of the promoter of *diptericin*.

III.2.2 – Chloroquine solutions and administration

Chloroquine diphosphate salt (Sigma) was diluted in water, in order to make a stock solution at 10mM , and aliquots were stored at -80ºC.

For *in vivo* experiments, dilutions of 2µM and 10µM were prepared in a 1% glucose solution, and this was used as a control. All solutions were administered to flies by feeding. A filter paper was soaked with a few millilitres of each solution, and placed in an empty vial, along with the flies. Toxicity tests were performed by feeding flies on solutions ranging from 0mg/l to 500mg/l of chloroquine for 9 days, and checking mortality.

Solutions for i*n vitro* assays were simply diluted in water (0.1, 0.5, 1, 5, 10µM). Cells were checked morphologically for drug toxicity.

III.2.3 – Chloroquine impact on AMP production

Chloroquine effect on the *Drosophila* immune response was primarily checked on AMP synthesis, using both *in vivo* and *in vitro* systems. In adult flies, AMP expression was determined at a systemic level, while *in vitro* AMP *de novo* synthesis was evaluated on hemocyte-like cell lines.

Figure III.2.1 represents the experimental design for AMP expression and synthesis.

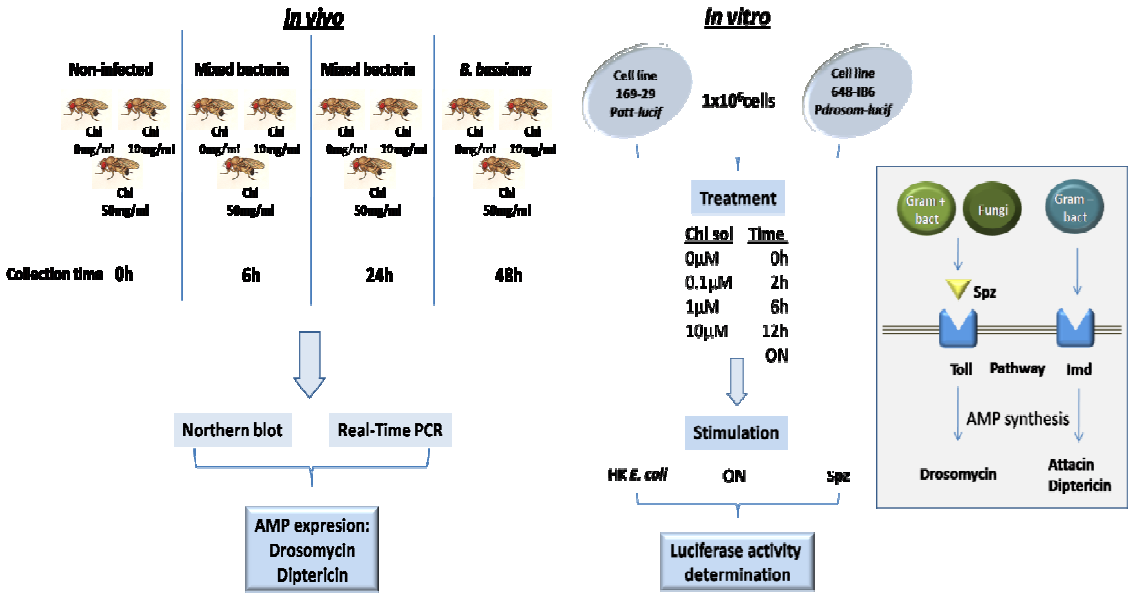


Figure III.2.1 – Experimental design for chloroquine effect on AMP synthesis response of *Drosophila* to infection.
In vivo assay: wild type flies Oregon R were fed on different amounts of chloroquine (**Chl** – 0, 10 and 50 mg/l) and infected with mixed bacteria or *B. bassiana*. Non-infected flies were collected at 0h post infection, mixed bacteria at 6 and 24h post-infection, and *B. bassiana* infected flies at 48h post-infection. RNA from collected flies was used for Northern blot and Real-time PCR to determine drosomycin and diptericin expression levels.
In vitro assay: cells from hemocyte-like cell lines were plated on a 24-well plate ($1x10^6$cells/well) and conditioned to different chloroquine solutions (**Chl sol** – 0, 0.1, 1 and 10µM) for different times (0, 2, 6, 12h and ON). Cells were stimulated ON with heat killed (**HK**) E. coli bacteria (129-69 cell line, transfected with a *attacin-luciferase* (***Patt-lucif***) gene) or the Spaetzel protein (**Spz** – 648-IB6 cell line, transfected with a *drosomycin-luciferase* (***Pdrosom-lucif***) gene). After stimulation, luciferase activity was determined for each sample.
Boxed figure: Toll pathway is activated by Gram + bacteria and fungi. Pathogen recognition leads to binding of Spaetzel to the receptor Toll, resulting in the expression and synthesis of AMPs such as Drosomycin. Imd pathway is activated by Gram – bacteria (like E. coli) and leads to the expression and synthesis of AMPs such as Diptericin and Attacin.

III.2.3.1 – *In vivo* AMP expression

III.2.3.1.1 – *Drosophila* infections

Wild type Oregon R flies were treated by oral feeding with different doses of chloroquine: glucose 1%; 10mg/l chloroquine/ 1% glucose; 50mg/l chloroquine/ 1% glucose for two days.

Flies were either pricked with a thin needle dipped into a concentrated mixture of overnight grown bacterial cultures of *Escherichia coli* 1106 and *Micrococcus luteus* CIP A270 or shaken in a Petri dish containing a sporulating culture of the entomopathogenic fungus *Bauveria bassiana*.

Non-infected flies were collected at time 0 (infection time). Flies infected with mixed bacteria were collected at 6h and 24h post-infection (pi). *B. bassiana* naturally infected flies were collected at 48h pi. Twenty flies of each group were frozen for RNA collection, for Northern blot and Real Time semi-quantitative PCR.

Chloroquine treatments were maintained during the course of all infections.

III.2.3.1.2 – Northern blot analysis

RNA was extracted from flies frozen at -80ºC with Trizol Reagent (Invitrogen). Briefly, flies were crushed and ressuspended in 1ml of Trizol® reagent and incubated at room temperature (rt) for 5min. To this was added 200µl of chloroform (Sigma) and solution was vigorously mixed and incubated at rt for 3min. samples were centrifuged at 12 000x*g* for 15min at 4ºC. The RNA containing aqueous phase was transferred to a new tube and precipitated with 500µl of isopropanol (Sigma) by incubating at rt for 10min. Samples were then centrifuged at 12 000x*g* for 10min at 4ºC. The supernatant was discarded and RNA precipitates were washed with 75% ethanol (Sigma), vortexed and centrifuged at 7 500x*g* for 5min at 4ºC. The supernatant was discarded and pellets were left to dry until all ethanol had evaporated. RNA was ressuspended in 20µl of

diethyl pyrocarbonate (DEPC – Sigma) treated water by incubating at 60ºC for 10min and vortexing. RNA samples were kept at -80ºC.

Twenty µg of RNA was ran on a 1% agarose, 6.7% formaldehyde denaturing gel, and transferred ON to a positively charged nylon membrane by capillarity on a 10X saline sodium citrate (SSC) buffer solution.

Radioactive (^{32}P-labeled) probes were synthesized using the Rediprime VI Random Prime Labeling System (dCTP – GE Hralthcare). Probes were prepared by a reverse transcription reaction from *rp49, drs* and *dipt* cDNA's. Primer sets are described in table III.2.1. Hybridizations were performed at 42ªC, ON, sequentially for *rp49, drosomycin* and *diptericin*.

Signal was visualized in a Bioimager film (Bio-Rad), and on an X-ray film, and quantified using the Bioimager software (Bio-Rad). The *rp49* signal was used as an internal loading control. Five independent experiments were performed, and data was analyzed for statistical differences with a Mann-Whitney test, using the GraphPad software (Prism).

Table III.2.1

Primers used for RT-PCR

Gene	***Primer sequence***
rp49	Fwd: ATACAGGCCCAAGATCGTGA
	Rev: GTGTATTCCGACCACGTTACA
drosomycin	Fwd: AGCTCCGTGAGAACCTTTTCC
	Rev: CATCCTTCGCACCAGCACTTC
diptericin	Fwd: AGTTCACCATTGCCGTCGCC
	Rev: GTAGGTGTAGGTGCTTCCCA

III.2.3.1.3 – Real-Time analysis

Samples of 5 flies were frozen at -80ºC and crushed with a Mixer Mill 300 (Retch). Total RNA was prepared using the Nucleospin 96 kit (Macherey Nagel) with the vacuum technique and RNAs eluted in 100 µl of RNase free water. Two µl were used in a Reverse Transcription reaction using Superscript II reverse transcriptase (Invitrogen) and random primers (Invitrogen) according to the supplier's instructions.

Real-Time PCR reactions were set up using the qPCR kit (Eurogentec – SYBRGreen final concentration of 1/50000 to 1/75,000) and the synthesized cDNAs. Real-time PCR was performed on an i-cycler iQ (Biorad). PCR conditions comprised preincubation at 95°C, 40 cycles of 15sec at 95 °C and 1min at 60 °C. Reactions were performed in duplicates. To check for specificity of the PCR reaction, melting curves were analyzed for each data point. The levels of expression of the gene of interest were normalized against the level of the RNA coding rp49 determined in each sample. Primers used in Real-Time PCR are detailed in Table III.2.2.

Three independent experiments were performed, and data was analyzed for statistical differences with a Mann-Whitney test, using the GraphPad software (Prism).

Table III.2.2

Primers used for Real-Time PCR

Gene	***Primer sequence***
rp49	Fwd:GACGCTTCAAGGGACAGTATCTG
	Rev: 5'AAACGCGGTTCTGCATGAG
drosomycin	Fwd:CGTGAGAACCTTTTCCAATATGATG
	Rev: TCCCAGGACCACCAGCAT
diptericin	Fwd: GCTGCGCAATCGCTTCTACT
	Rev: TGGTGGAGTGGGCTTCATG

III.2.3.2 – *In vitro* AMP synthesis

Two hemocyte-like cell cultures originally derived from *Drosophila* embryos were used to test antimicrobial peptide synthesis. Both cell lines derive from *Drosophila* S2 from transfection: 648-ib6 cells which contain a stable insertion for a *drosomycin-luciferase* gene, and 169-29, with an *attacin-luciferase* gene insertion. Cells were grown at 25 °C in Schneider's medium (Biowest) supplemented with 10% fetal calf serum, 10^5 units/liter penicillin, 100 mg/liter streptomycin, and 1 µg/ml puromycin.

Both cell cultures were plated in a 24 well plate ($1x10^6$ cell/well), and let to rest overnight. The next day cells were treated with 0, 0.1, 1 and 10µM of Chloroquine, for 0, 2, 6, 12h and ON.

After treatment, cells were washed and let for 30 minutes with new medium (no chloroquine). Cells were then stimulated either with 5nM SPZ protein (648 – IB6 cell line) or $4x10^7$ heat killed *E. coli* bacteria (169-29 cell line).

After ON stimulation, cells were collected and disrupted using a lysis buffer (Promega). Luciferase substrate (luciferin; Promega) was added and luciferase activity was immediately read on a luminometer (BCL Book, Promega).

III.2.4 – Chloroquine impact on local immune responses

For localized immune response study, DD1 flies were fed on different doses of chloroquine (0, 10, 50 mg/l in 1% glucose) for 48h. Then, solutions were removed and flies were fed with the Gram-negative bacteria *Serratia marcescens* for 2-3 days.

Serratia marcescens infected flies were dissected in order to allow the visualization of the gut, which was fixed in a glutaraldehyde 1% solution for 10–15 min, and stained in a 30 µl/ml X-gal stock solution (5% in DMF). The colorimetric reaction was observed in a light microscope.

III.2.5 – Chloroquine impact on fly's survival to infection

To test chloroquine effect on the survival of flies upon an infection, wild type Oregon R flies were treated by oral feeding with different doses of chloroquine: glucose 1%; 10mg/l chloroquine/ 1% glucose; 50mg/l chloroquine/ 1% glucose for two days. Flies were then either challenged with bacteria solutions (mixed bacteria, *E. coli, M. luteus, Enterococcus fecalis* or *Staphylococcus aureus*) or shaken in a Petri dish containing a sporulating culture of *B. bassiana*. Control groups included non-infected flies.

Fly mortality was followed for 7 days, by counting the number of dead flies. Chloroquine treatments were maintained during the course of experiment.

III.3 – Results

III.3.1 – Chloroquine toxicity to flies

During 9 days, no lethality occurred with any of the chloroquine treatments, suggesting that chloroquine can be used, even at high concentrations, without compromising the fly's survival.

Solutions of 10 and 50 mg Chl/l were used in all experiments, since they correspond to the sub-therapeutical and therapeutical dosages of the drug used to treat mice in the mosquito's experiments.

III.3.2 – AMP expression and synthesis

III.3.2.1 – *In vivo* AMP expression

The effect of chloroquine in the activation of specific immune pathways as Toll or Imd, was accessed *in vivo* by determining AMP gene expression in wild type flies Oregon R and the DD1 (*drosomycin-GFP)* flies. The *drs* (*drosomycin*) expression was taken as readout for the Toll pathway activation. The *dipt* (*diptericin*) and *att* (*attacin*) expressions were used as readout for the Imd pathway activation. The housekeeping gene for the ribosomal protein Rp49 was used as an internal control.

III.3.2.1.1 – Northern blot analysis

Drosophila drs and *dipt* gene expression was determined by Northern blot by determining signal intensity in the films. Figure III.3.1 shows a representative Northern blot signal for *rp49, drs* and *dipt* genes of Oregon R flies, as determined in experimentI.

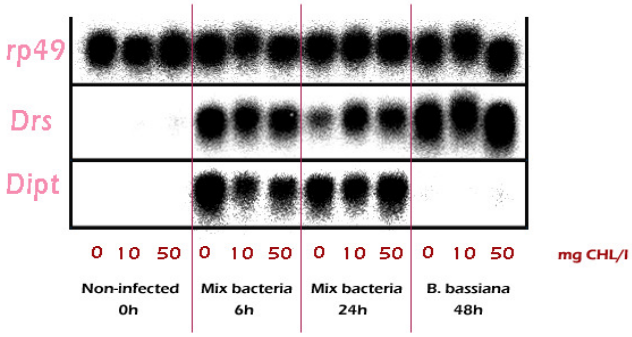


Figure III.3.1 - Northern blot of *rp49*, *drs* and *dipt* gene expression in Oregon R flies. Flies were treated with different doses of Chloroquine (0, 10, 50 mg/l). Non-infected flies were collected at 0h, mixed bacteria infected at 6h and 24h, and flies infected with *B. bassiana* at 48h. Radioactive gene-specific probes were incubated with fly RNA.

Providing a qualitative analysis, Northern blots observation allow to have a rough idea of AMP induction. All groups of flies show similar levels of rp49, indicating that approximate amounts of RNA were loaded into the gel. *Drs* gene expression was induced in flies infected with mixed bacteria (*M. luteus* + *E. Coli*, in a 1:1 ratio) at 6 and 24h pi and in flies infected with *B. bassiana* at 48h pi, in accordance with the expected

activation of the Toll pathway by Gram-positive bacteria and fungi. *Dipt* gene expression was induced in flies infected with mixed bacteria, at 6 and 24h pi, which confirms the IMD pathway activation by Gram-negative bacteria.

The same pattern of AMP induction was seen in all experiments, indicating that the readouts are functional and reproducible.

Chloroquine treatments did not seem to cause noteworthy alterations in AMP expression. Only small alterations were observed in mixed bacterial infection. Chloroquine treated flies seemed to have an increased *drs* expression at 24h after mixed bacterial infection, and a reduction in *dipt* expression at 6h after mixed-bacterial infection.

Nevertheless, the band pattern allows only a rough view of alterations in gene expression. It has a low sensitivity, and the relation between the gene of interest and the control *rp49* can only be accessed qualitatively. To quantify gene expression, the intensity of the bands in Northern blots was determined with the bioimager software. All values obtained for gene expression of *drs* and *dipt* were normalized with the ones for *rp49*.

Relative expression of *drs* in flies submitted to different chl treatments and infections is shown in Figure III.3.2.

Chloroquine treated flies showed the same pattern of *drs* induction upon infection as control flies (0mg Chl/l). No significant differences in gene expression were observed.

Nevertheless, a slight reduction (20%) in *drs* expression was detected in non-infected flies treated with the lower dose of Chl (10mg/l) when compared to non-treated flies, which was not observed for the higher dose (50mg/l).

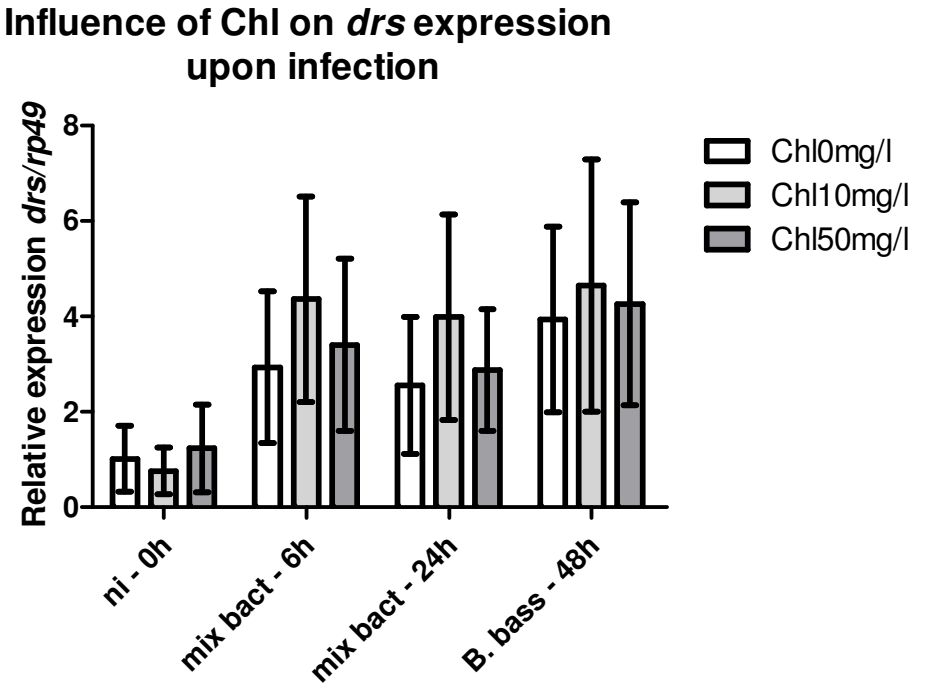


Figure III.3.2 – Northern blot evaluation of the influence of Chl on *drs* expression upon infection. Oregon R flies were submitted to different chloroquine (**Chl**) treatments – 0, 10 and 50mg/l – and infections – Non-infected (**ni**), Mixed bacteria (**mix bact**) and *B. bassiana* (**B. bass**). RNA was collected from flies at 0, 6, 24 and 48h, respectively to infection. Gene expression of *drosomycin* (***drs***) in flies was determined by Northern blot and normalized with the expression of *rp49* gene.

Flies infected with mixed bacteria and treated with the lower dose of chloroquine (10mg Chl/l) showed a 1.2fold increase in *drs* expression at 6hpi and a 1.5fold increase in *drs* expression at 24h when compared with non-treated controls. This trend was observed in all experiments performed. The same pattern of induction was not seen with the higher dose of Chl. Although flies infected with mixed bacteria treated with the higher dose of chloroquine (50mg Chl/l) showed a 1.3fold increase at 24hpi relative to controls, this trend was not consistent in all experiments performed.

AS for *dipt*, relative gene expression in flies submitted to different chl treatments and infections is shown in Figure III.3.3.

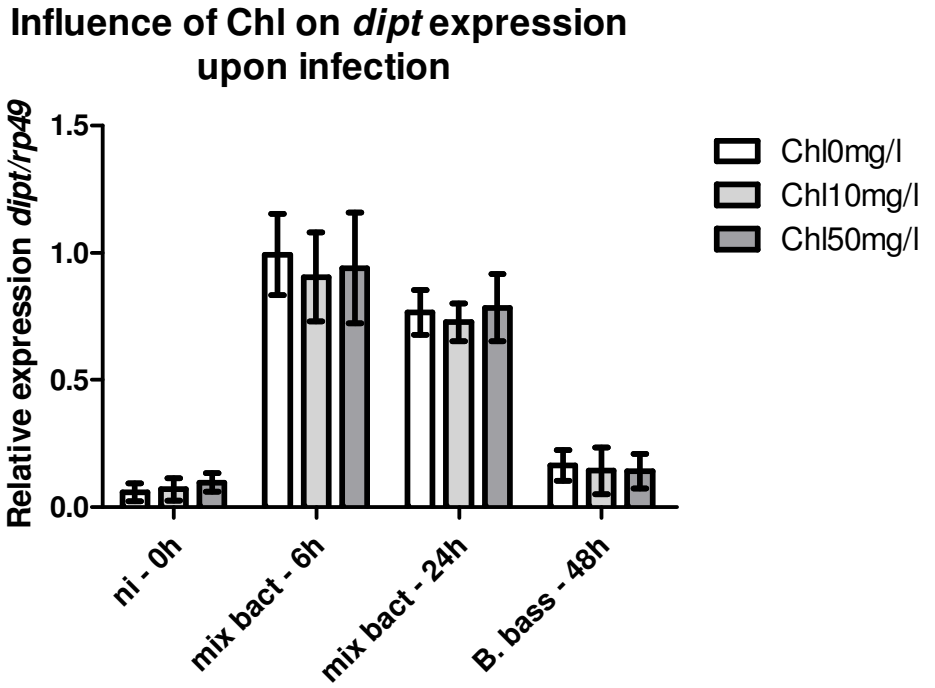


Figure III.3.3 –Northern blot evaluation of the influence of Chl on *dipt* expression upon infection. Oregon R flies were submitted to different chloroquine (**Chl**) treatments – 0, 10 and 50mg/l – and infections – Non-infected (**ni**), Mixed bacteria (**mix bact**) and *B. bassiana* (**B. bass**). RNA was collected from flies at 0, 6, 24 and 48h, respectively to infection. Gene expression of *diptericin* (***dipt***) in flies was determined by Northern blot and normalized with the expression of *rp49* gene.

No significant differences were observed in *dipt* induction pattern for any of the chl treatments. However, a slight induction of *dipt* was observed in non-infected flies treated with the higher dose of Chl (50mg/l).

III.3.2.1.2 – Real-Time analysis

Expression of *drs* and *dipt* was further analyzed by Real-Time PCR.

Relative expression of *drs* in flies submitted to different chl treatments and infections is shown in Figure III.3.4.

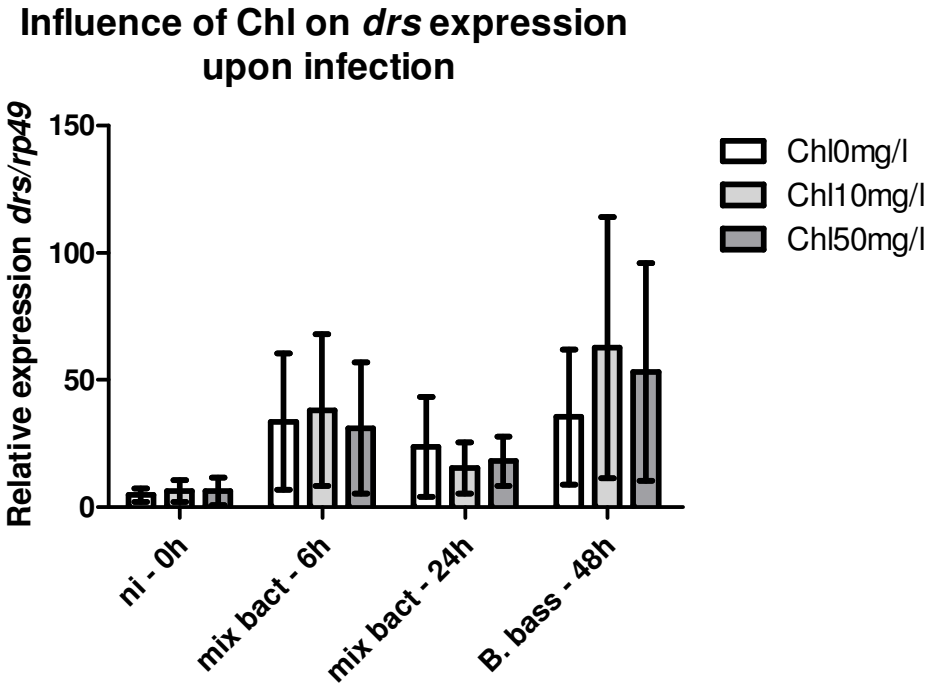


Figure III.3.4 – Real-Time evaluation of the influence of Chl on *drs* expression upon infection. Oregon R flies were submitted to different chloroquine (**Chl**) treatments – 0, 10 and 50mg/l – and infections – Non-infected (**ni**), Mixed bacteria (**mix bact**) and *B. bassiana* (**B. bass**). RNA was collected from flies at 0, 6, 24 and 48h, respectively to infection. Gene expression of *drosomycin* (***drs***) in flies was determined by Real-Time PCR and normalized with the expression of *rp49* gene.

As for Northern blot results, results obtained by Real-Time PCR show that *drs expression* seems to be induced by mixed bacterial infection, both at 6 and 24hpi, and strongly induced by *B. bassiana* infection at 48hpi, emphasizing the strength of this assay.

Chloroquine did not seem to have any effect on *drs* induction in any of the treated fly groups tested, as no significant differences were observed between Chl treatments for any of the infected or the control flies.

A faint induction of *drs* expression was seen at 6hpi in flies infected with mixed-bacteria and treated with the lower dose of Chl (1.2fold) when compared to non-treated flies, and was in accord with the results from Northern blot. Although small, this induction was reproducible between experiments. On the contrary, a minor

reduction (20%) was observed in the same flies, treated with the higher dose of Chl. This reduction pattern was observed in all experiments performed.

Flies infected with *B. bassiana* showed a slight *drs* induction when treated with both doses of Chl, contrasting with Northern blot results, where no differences were observed. While flies treated with 10mg/l showed increased *drs* induction (1.5fold) in all experiments, flies treated with 50mg/l (1.2fold *drs* induction) showed inconsistency between experiments.

Additionally, a slight reduction was observed for both chloroquine treatments at 24h in flies infected with mixed bacteria, contrasting with the Northern blot results. However, this trend was not consistent in all experiments performed and thus is probably due to experimental variation.

Real-Time determined *dipt* relative gene expression in flies submitted to different chl treatments and infections is shown in Figure III.3.5.

Real-Time determined *dipt* gene expression was strongly induced in flies infected with mixed bacteria at 6 and 24hpi, confirming the robustness of the AMP induction readouts.

Yet, no significant differences were observed in *dipt* expression between Chl treatments.

Only a slight reduction in *dipt* expression was observed in flies infected with mixed bacteria at 6h and treated with the highest dose of Chl (45% reduction relative to non-treated control flies), contrasting with results obtained by Northern blot, where no differences were observed. This trend was seen in all experiments performed.

Another result that contrasts to Northern blot analysis is the non-observation of *dipt* expression reduction in flies infected with *B. Bassiana* and treated with Chl. Although a slight reduction was observed with the lower dose treatment (30%) it was not consistent through experiments.

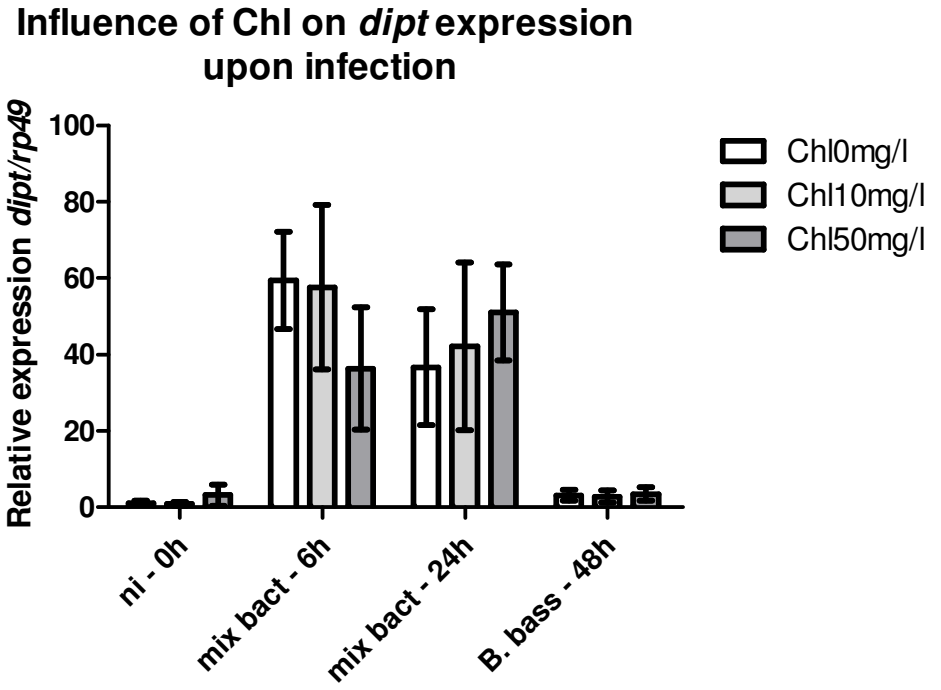


Figure III.3.5 – Real-Time evaluation of the influence of Chl on *dipt* expression upon infection. Oregon R flies were submitted to different chloroquine (**Chl**) treatments – 0, 10 and 50mg/l – and infections – Non-infected (**ni**), Mixed bacteria (**mix bact**) and *B. bassiana* (**B. bass**). RNA was collected from flies at 0, 6, 24 and 48h, respectively to infection. Gene expression of *diptericin* (***dipt***) in flies was determined by Real-Time PCR and normalized with the expression of *rp49* gene.

Single experiments performed to evaluate if Chl effect could be masked by the timepoints chosen to study the induction of each AMP and the mixture of Gram-positive and Gram-negative bacteria in one solution to administer. As such, AMP expression was evaluated in flies infected with mixed bacteria at 0, 3, 6 and 9hpi. Although an overall increasing in both genes expression through the first nine hours of infection was seen, no differences between Chl treated and non-treated flies were detected. Also, flies were treated with Chl and infected with *E. coli* and *M. luteus* separately, but once more no differences were observed between Chl treated and non-treated flies (data not shown).

Table III.3.1 resumes the *drs* and *dipt* expression induction and/or repression by Chl treatment determined by Northern blot and Real-Time PCR. Note that differences expressed are small and not significant, but consistent in all experiments performed.

Table III.3.1

***drs* and *dipt* expression regulation by chloroquine**

		drs		***dipt***	
infection	***treatment***	NB	RT	NB	RT
Ni 0h	Chl10mg/l	↓	=	=	=
	Chl50mg/l	=	=	=	=
Mix bact 6h	Chl10mg/l	↑	↑	=	=
	Chl50mg/l	=	↓	=	↓
Mix bact 24h	Chl10mg/l	↑	=	=	=
	Chl50mg/l	=	=	=	=
B. bass 48h	Chl10mg/l	=	↑	=	=
	Chl50mg/l	=	=	=	=

Ni: non-infected; **Mix bact**: Mixed bacteria; ***B. bass***: *B. bassiana*; **Chl**: Chloroquine; ***drs***: *drosomycin*; ***dipt***: *diptericin*; **NB**: Northern Blot; **RT**: Real-Time PCR; ↑; induction; ↓: reduction.

III.3.2.2 – *In vitro* AMP synthesis

To directly access the hemocytes immune response and *in vitro* assay was performed, using two stably transformed cell lines, derived from the *Drosophila* S2 hemocyte-like cell line – the 648 – IB6 cell line expresses a recombinant protein, which is codified by *luciferase* under the *drosomycin* promoter and the 169-29 cell line expresses an *attacin-luciferase* gene. AMP synthesis was evaluated by the induction of luciferase activity.

Drs synthesis was strongly induced in 648-IB6 cells by SPZ stimulation. In regard to Chl treatments, no differences were observed in Chl treated cells without SPZ stimulation, and only a modest reduction was observed after 6h incubation with 10µM of Chl and SPZ stimulation.

For Att synthesis, a high induction was observed upon 169-27 cells with heat-killed bacteria. Cells treated with Chl showed a slight induction of Att in both stimulated and non stimulated cells, after 6h of Chl treatment with the highest doses. These increases were very specialized in time, since it was only seen at 6h, and it was not dose dependent. Unfortunately, it was not consistent over time or with the different doses used.

Several attempts were performed to check if an altered AMP production could be seen after Chl treatment and different times of exposure to Chl. Nevertheless, none showed an altered response and thus, *in vitro* assays were not followed.

III.3.3 – Chloroquine impact on local immune responses

Another attempt to evaluate chloroquine effect in the fly immune response was made by using DD1 flies that contain a reporter gene in which the promoter region of *drs* is fused with the *gfp* gene, and a second reporter gene in which the promoter region of *dipt* is fused with the *β-galactosidase* gene.

The evaluation of local immune response may reveal localized differences that would be masked by the analysis of a systemic response, as such; flies were infected with a natural pathogen of the digestive track, the bacteria *Serratia marcescens*. No changes in coloration were detected between Chl treatments upon infection, reflecting no differences in *drs* and *dipt* expression pattern, suggesting that chl has no effect on the local immune response of the fly.

III.3.4 – Chloroquine impact on fly's survival to infection

As the fly's immune system is complex, other mechanism besides the activation of immune pathways and the production of antimicrobial peptides are implicated in the response to microorganisms. Nevertheless, no simple readouts are available to test these mechanisms. Thus, survival tests are used as a primary approach, to test if Chl treatment may compromise the fly ability to survive infection.

Survival experiments were conducted with pathogenic microorganisms such as *E. fecalis* and *E. cloacae*, and *B. bassiana* spores, all able to kill flies. In each infection, a mutant fly line was included as a control. For Gram-negative bacterial infection, the *key (kenny)* mutant was included. These flies are deficient for the Imd pathway, are not expressing *dipt* or *att*, and die faster than wild type flies after Gram-negative bacterial infection. For Gram-positive bacterial and fungal infection, the *dif* mutant was used. These flies are deficient for the Toll pathway, do not express *drs*, and die faster than wild type flies after Gram-positive bacterial and fungal infection.

Although Chl treatment seemed to affect survival of wild type flies to Gram – bacteria, as to they start to die somehow faster, at D5pi, they still showed, as non-treated flies, a 20% survival, while the mutant *key* show no survival at D1pi, suggesting that the drug does not seriously compromise the ability to survive to Gram-negative infection.

Survival to Gram + bacterial infection seemed to be slightly affected at the first days pi. Flies seem to die faster after treatment, in a dose dependent fashion, although they didn't show the mortality of *dif* mutants, which die 4 days after infection. Unfortunately, an opposite pattern was observed in second experiment, using the same microorganism. In this case, non-treated flies and flies treated with 10 mg Chl/l showed the same curve, reaching 20% survival at D5pi, but flies treated with 50 mg Chl/l had a survival curve comparable to non infected flies (around 80%), suggesting that this dose would protect them against infection.

Survival to fungal infection showed no differences between treatments in wild type flies or *dif* mutants. The few differences observed were modest, and non-reproducible, and thus, survival experiments were abandoned.

III.4 – Discussion

This work was conducted in order to understand if chloroquine was acting in the mosquito immune response. *Drosophila melanogaster* was the model used, as it provides a set of powerful tools and knowledge, particularly in immunity studies.

One of the most important and well known features of the fly immune response depends on the synthesis of antimicrobial peptides, and two immune pathways that lead to their production: the Toll and the Imd pathways. These are activated extracellularly, after the recognition of microorganisms, through a serine protease cascade or by direct binding of microorganisms to cell surface receptors. This results in an intracellular signal transmission that leads to AMP expression (synthesis of *drs* results from Toll pathway activation, and of *dit* and *att* upon Imd pathway activation).

Taking as a premise that the activation of any of the pathways results unconditionally in the expression of the respective peptides, we can state that the evaluation of their expression reflects the activation of the immune pathway. Thus AMP expression was a good candidate to evaluate chl influence on the *Drosophila* immune system.

Given that chloroquine seems to reduce this synthesis in the mosquito, and that there are evidences of similarities between the immune system backbone of both organisms (particularly at the intracellular signalling level), it is probable that the drug is affecting in any way either the Toll or the Imd signalling. Hence, it would be expected that after feeding chloroquine to the fly and infecting it with different classes of microorganisms, a reduction in the expression of either the *drs* or *dipt* or *att* would be observed, as a result of an impairment of either the Toll or the Imd pathways.

In this work, several attempts were made to test AMP gene expression both *in vivo* and *in vitro*, in the presence of chloroquine and infection.

The *in vivo* assays performed either by Northern blot or Real-Time PCR proved to be robust, as *drs* induction was detected by both techniques upon infection with mixed bacteria and the entomopathogenic fungi *B. bassiana,* as a result of Toll pathway

activation, which is known to happen in response to Gram-positive bacterial and fungal infections. As for *dipt*, it was induced upon mixed bacterial infection, resulting from Imd pathway signaling, which is known to be activated upon Gram-negative bacterial infection. Thus, AMP expression proved to be a good readout of pathway activation.

Neither technique used detected significant differences between Chl treated and non-treated flies, indicating that the antimalarial drug has no important effect on AMP expression in flies. Nevertheless, differences were found. These were too small to overcome experimental variation and to be considered significant. Nonetheless, they were reproducible between experiments, *ie*, all experiments performed revealed the same pattern of induction/repression (Table III.3.1). The only difference detected by both Northern blot and Real-Time PCR was found in *drs* expression at 6hpi in flies infected with mixed-bacteria. Flies treated with 10mg/l of Chl showed an induction of *drs* expression when compared with non-treated flies. The same pattern was not observed with the 50mg/l Chl dose, thus, the effect is not dose dependent. In fact, Real-Time analysis revealed *drs* repression with the higher dose of Chl, suggesting the opposite role for Chl. The fact that such patterns for *drs* expression were not detected for *B. bassiana* infection means that in case Chl is acting in the *Drosophila* immune response, it is doing so at an extracellular level, probably over the serine protease cascade that leads to Toll pathway activation. This is sustainable, as Chl is known to affect the activity of fungal proteases (Staszczak *et al*., 2000).

Overall, the antimicrobial peptide expression does not seem to be altered by chloroquine treatment *in vivo*.

In vitro assays also proved to be robust, as hemocyte-like cells efficiently induced the synthesis of Drs when stimulated with SPZ and Att when stimulated with heat-killed bacteria. The assays, however, did not reveal any alteration in AMP synthesis in immune stimulated hemocyte-like cells due to Chl treatment. This suggests that if the drug has any effect, even minor in the fly immune response, it is not noticeable in

hemocytes, thus it may affect other immune responses, such the one generated by the fat body or even local immune responses.

Gut localized immune responses, as assayed in DD1 flies infected with *S. marcescens*, also did not reveal any differences between Chl treated and non-treated flies.

Overall, AMP expression and synthesis induced by immune responses in *Drosophila* does not seem to be affected by Chl treatment. Only subtle variations were observed with the drug treatment, and their biological significance remains to be understood. However, the differences found do not seem to be enough either to compromise the survival neither to protect the fly upon infection with natural pathogens.

Contrary to what happens in the mosquito, Chl does not seem to affect fly immunity. The only differences observable relate to an induction of *drs* expression in flies infected with mixed bacteria as a result of Chl treatment, which is contrary to the AMP expression patterns observed in the mosquito, upon Chl treatment (Abrantes et al, 2005).

Several possibilities may account for the differences of chloroquine modulation of the immune responses of fly and the mosquito:

1) It can be acting over a specific molecule, responsible for the regulation of a response not tested by the readouts available. Phenomena like phagocytosis, encapsulation, melanization, hemocyte aggregation were not tested. But if it would seriously affect any of these, it would be observed by a decreased survival after infection.
2) It can be acting over a mosquito regulatory molecule that does not exist in the fly, and that is necessary to activate one of the major immune

pathways, like a serine protease, or a serpin. Flies and mosquitoes are distantly related and, although there are numerous similarities between the responses of the two organisms, they also show differences in immunity, especially through the presence of specific immune molecules, like gambicin in the mosquito.

3) For the same reason stated above, the response to the parasite can be dramatically different from the response to bacteria or fungi. A co-evolution of the mosquito and the parasite certainly has led to the appearance of highly specialized molecules of recognition and regulation of the immune response in the parasite. The antiparasitical response in the fly is still a choice of study.

4) It has to be kept in mind that there are no controls for the quantity of chloroquine that the mosquito ingests with the blood meal, meaning that the mosquito can take chloroquine, or a metabolite derived from its digestion. In mosquitoes experiments, mice were infected and treated with chloroquine 48h before the blood meal. As such, there is time for the mice to digest the drug and produce a series of metabolites. One of these could be the responsible for the raise observed in the number of parasite oocysts in the midgut (Hogh et al, 1998). These are conditions that cannot be reproduced in the fly model.

IV – Mosquito hemocyte proliferation

IV.1 – Introduction

Hemocytes are crucial players in the immune responses of insects, engaging in both humoral and cellular responses in the hemolymph. Contributions to humoral responses include AMP synthesis and production and secretion of extracellular proteolytic cascade components that activate processes such as melanization and coagulation. In cellular responses, hemocytes come in contact with the pathogen, either by phagocysing the microorganism or by forming a capsule or a nodule around it (Lavine & Strand, 2002).

Upon infection, if a cellular response is to be activated, hemocytes have to migrate to the site of infection, probably by stimulation and/or chemotatic attraction. A particular response may require high number of hemocytes (proliferation) and the differentiation into a specific cell type, *ie*, for phagocytosis hemocyte have to be able to phagocyse, for nodulation and encapsulation to attach, and for melanotic encapsulation to produce the machinery necessary for melanization. For instance *Drosophila* larvae infected with eggs of a parasitic wasp, *Leptopilina boulardi*, have an increased number of hemocytes that differentiate into lamellocytes which can restrain the parasite by melanotic encapsulation (Vass & Nappi, 2000; Russo *et al.*, 2001). Hemocyte proliferation and differentiation were found to be two independent phenomena. Proliferation itself seems to be under the regulation of several molecules/pathways, such as the Ras-MAPK pathway, the Toll pathway and PVF2, a growth factor that induces an increased hemocyte number when overexpressed (Asha *et al.*, 2003; Lemaitre *et al.*, 1995; Luo *et al.*, 1995; Matova & Anderson, 2006; Qiu *et al.*, 1998; Zettervall *et al.*, 2004).

Hemocyte proliferation also occurs in larvae of the fruitfly *Anostrepha oblique* infected with parasitoid wasps, in shrimps stimulated with LPS or *Fusarium* fungi, *Culex* mosquitoes infected with microfilaria, among others (Brayner *et al.*, 2007; Sequeira *et al.*, 1996; Silva *et al.*, 2002).

Hemocytes are produced and differentiated by a process called Hematopoiesis. When required, hemocyte proliferation is followed by differentiation into specialized cells.

Several classes of hemocytes have been described for a few insects, based on morphology and immune abilities. For instance, during *Drosophila* hematopoiesis, a precursor cell, the prohemocyte is able to differentiate into 3 types of hemocytes: plasmatocytes, the major class of hemocytes in the fly, that engage in phagocytosis; crystal cells, that represent less than 5% of the hemocytes and secrete enzymes necessary for humoral melanization; and lamellocytes that are produced only upon infection and engage in encapsulation of microorganisms too large to be phagocysed by plasmatocytes (Meister & Lagueux, 2003). As for mosquitoes, Hillyer & Christensen (2002) proposed a hemocyte classification for *Ae. aegypti* that included 4 types of cells, based on morphology, binding of selected lectins and enzymatic activity: granulocytes, the most abundant type that have attachment ability; oenocytoids that have phenoloxidase in the cytoplasm; adipohemocytes, the second most common type that have no attachment ability; and thrombocytoids that are rarely seen and are not circulating hemocytes, probably existing attached to fixed tissues. Castillo *et al.* (2006) found the same type of hemocytes in *An. gambiae* mosquitoes, with the exception of thrombocytoids. However, which type of hemocyte is induced upon a particular infection and engages in a specific mechanism is still unclear for both species.

Our knowledge on mosquito cellular immune responses to the malaria parasite is scarce. During ookinete invasion of the midgut and sporozoite invasion of the salivary glands the responses observed so far are humoral and epithelial. Conversely, the understanding of the mosquito response to sporozoites upon egress from the oocysts is still limited. At this stage, thousands of sporozoites are released into the hemolymph but less than 10% efficiently invade the salivary glands. Sporozoites are known to flow along with the hemolymph until reaching the salivary glands (Hillyer *et al.*, 2007). At this stage, sporozoites come in close contact with circulating hemocytes that are bound to recognize and combat the parasite. Sporozoites are known to be destroyed in the hemocel, in case they fail to reach the salivary glands (Hillyer *et al.*, 2007). Mosquito immune responses to hemolymph sporozoites detected so far include phagocytosis and melanization in *P. gallinaceum/Ae. agypti* and phagocytosis in *P. berghei/An. gambiae* models (Hillyer *et al.*, 2003, 2007). Other immune responses, such as nodulation or encapsulation, have not been disclosed yet.

Data available for hemocyte number variation in mosquitoes show that their number is reduced in the hemolymph of *Ae. aegypti,* correlated with aging and contributing to a higher mortality caused by *E. coli* infection (Hillyer *et al.*, 2005). In *Armigeres subalbatus*, hemocyte number decreases at 24h after a blood meal that might be associated with encapsulation, lowering levels of circulating hemocytes. Cell number returns to control levels at 48-72h post blood meal (Guo *et al.*, 1995). As for sporozoite invasion of the hemolymph there is are no data available for induction by the parasite of hemocyte proliferation and/or differentiation.

Data on hemocyte proliferation and differentiation (if possible) upon sporozoite egress from the hemolymph would disclose part of the mosquito response to the parasite at this stage.

IV.2 – Methodology

IV.2.1 – Cells, bacteria and recombinant Pf-CS

Sua 5.1* cells are hemocyte-like cells derived from larvae of the *An. gambiae* Suakoko strain. Cells were cultured in Schneider insect medium (Sigma) supplemented with penicillin (100 000U – Sigma), streptomycin (100mg – Sigma) and heat inactivated fetal calf serum (FCS – 10% - Sigma) and kept at 26ºC.

Bacteria solutions were prepared by mixing heat killed *Escherichia coli* and *Micrococcus luteus* in a 1:1 ratio, to a final concentration of $4x10^7$ bacteria/ml.

Recombinant Pf-Cs (sporozoite surface protein from *Plasmodium falciparum*) was obtained from a sporozoite detection kit (Sclavo Elisa kit – Sclavo Diagnostics). Protein was diluted in Schneider medium, aliquoted and kept at -80ºC at a concentration of 10ng/µl.

IV.2.2 – LPS contamination of recombinant Pf-CS

To evaluate if the Pf-CS solutions were contaminated with LPS (endotoxin), derived from cell walls of bacteria used for recombinant protein expression, Pf-CS protein solution was submitted to a LPS detection kit (E-Toxate Kit – Sigma), according to the manufacture instructions. LPS is detected in a sample by comparison with a standard curve of known Endotoxin solutions. Samples are incubated with a substrate that, in contact with LPS turns to a gel. Increasing endotoxin concentrations are reflected in a higher solidity of the gel formed.

Samples were prepared according to Table IV.2.1. Samples were gently agitated and incubated in a water bath for 1h at 37ºC., then inverted to verify gel formation.

Table IV.2.1

Sample preparation for LPS detection

Tube	*Sample*		*Endotoxin 4 EU/ml*	*E-toxate working solution*
A	Pf-CS 10ng/µl	100µl	-	100µl
A'	Pf-CS 1ng/µl	100µl	-	100µl
B	Pf-CS 10ng/µl	100µl	100µl	100µl
C	Negative control	100µl	-	100µl
D	Endotoxin 0.015EU/ml	100µl	-	100µl
E	Endotoxin 0.03EU/ml	100µl	-	100µl
F	Endotoxin 0.06EU/ml	100µl	-	100µl
G	Endotoxin 0.125EU/ml	100µl	-	100µl
H	Endotoxin 0.25EU/ml	100µl	-	100µl
I	Endotoxin 0.5EU/ml	100µl	-	100µl

Tube B has the purpose of detect any E-toxate reaction inhibitor present in the protein sample. Tubes D to I are the standard endotoxin solutions, prepared with endotoxin-free water.

IV.2.3 – Hemocyte proliferation assay

An *in vitro* approach was used to evaluate if the presence of sporozoites in the mosquito hemocel induces hemocyte proliferation. Mosquito hemocyte-like cells were stimulated with a recombinant form of the major surface antigen of malaria sporozoites, the circumsporozoite protein from *P. falciparum* (Pf-CS). Hemocyte-like cell division was determined by ^{3}H-Thymidine incorporation. This radioactive molecule is added to culture medium and taken up by cells that use it for DNA synthesis. Hence, levels of incorporated ^{3}H-thymidine correlate with cell division.

Sua 5.1* cell concentration was determined on a hematocytometer. Cells were diluted in medium to a final concentration of $2x10^{5}$cells/150µl, plated on a U-shaped 96-well plate, and incubated for 24h at 26ºC.

Cells were stimulated ON with different amounts of Pf-CS (0.5, 5 and 50ng/50µl of medium) and a solution of a combination of Gram + and Gram – heat killed bacteria (mixed bacteria – $4x10^{7}$cells/50µl of medium – 10bacteria/cell), as represented in Figure IV.2.1. Controls included medium alone and cells plus medium. All samples were analyzed in triplicates.

After ON stimulation, 50µl of a ^{3}H-Thymidine (Sigma) solution at 0.02µCi/µl were added to each well (1µCi/well). Cells were allowed to incorporate ^{3}H-Thymidine for 6h, after which cells were harvested from each well onto a piece of 3MM paper. Each paper was placed on a scintillation tube and covered with scintillation liquid. Incorporated radioactivity was measured on a scintillation β-counter (Beckman LS 6500, Beckman) and expressed as scintillations per minute (cpm). Three tubes with only scintillation liquid were used for blank reading. Five independent experiments were performed and data was analyzed for significant differences between controls and stimulation with a paired t test, using the GraphPad software (Prism).

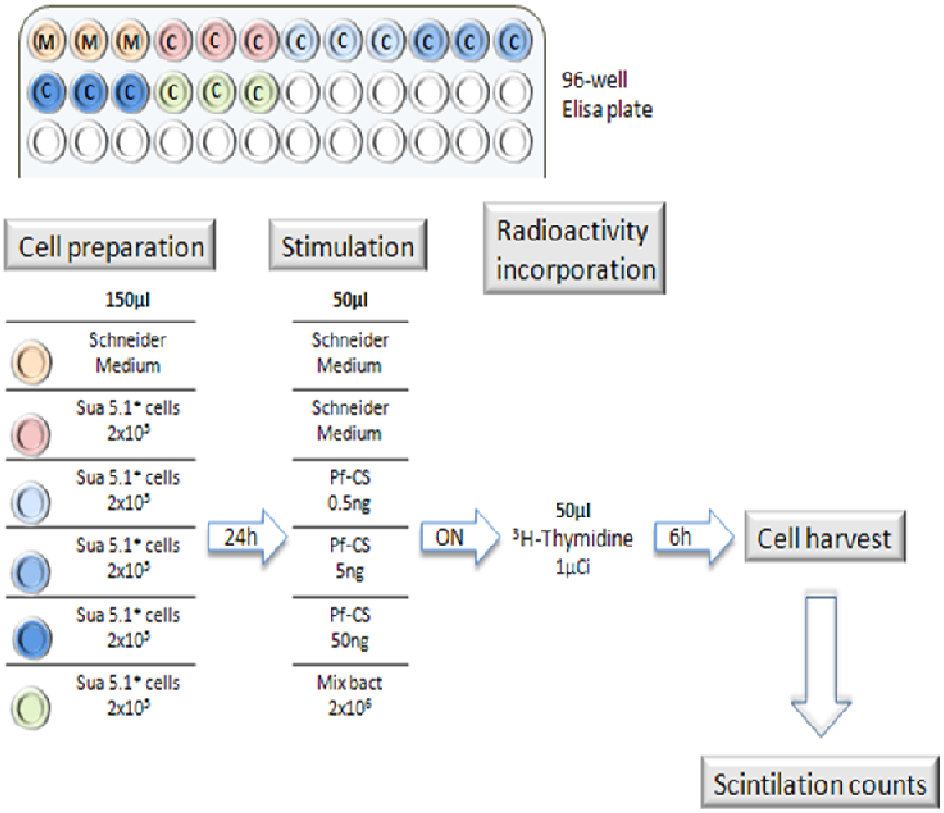


Figure IV.2.1 – Experimental design for hemocyte proliferation. Hemocyte-like Sua 5.1* cells from *An. gambiae* mosquitoes were plated on a U-shaped 96-well plate ($2x10^5$cell/well). Cells were stimulated ON with Pf-CS (0.5, 5 and 50ng) and mixed bacteria (**mix bact**) solution of heat killed *E. coli* and *M. luteus*. Cells were incubated with 1µCi of radioactive ^{3}H-Thymide for 6h, harvested and radioactivity was measured by counting scintillations per minute in each sample.

IV.3 - Results

We used an *in vitro* assay to test the proliferation of Sua 5.1* hemocyte-like cells from *A. gambiae* upon stimulation with Pf-CS and a mixture of heat killed Gram + and Gram – bacteria.

An endotoxin detection kit was used to detect LPS contamination in Pf-CS samples that could induce a response from the mosquito cells. LPS contamination is detected by the formation of a gel. Pf-CS samples remained in a liquid form as the negative control.

Endotoxin standards presented a gel-like form whose solidity increased in an endotoxin concentration-dependent fashion. No inhibitors were detected on protein samples, as Pf-CS plus endotoxin resulted in solid gel formation. This indicated that there was no LPS contamination on Pf-CS sample, thus a response of hemocyte-like cells to this sample was expected to be directed to Pf-CS protein.

Results of the proliferation assay are shown in Figure VI.3.1. There was a clear difference between scintillation counts in Medium and Medium + Sua 5.1* cells samples (P=0.0048), indicating that ^{3}H-thymidine was incorporated into the cells, thus validating the assay. Hemocyte number did not significantly differ between stimulated and control cells (P >0.05) with the exception of the 5ng Pf-CS treatment (P=0.0433). Also, a slight increase in hemocyte numbers was observed for cells stimulated with the highest dose of Pf-CS (50ng), which was variable among experiments, and thus, not significant.

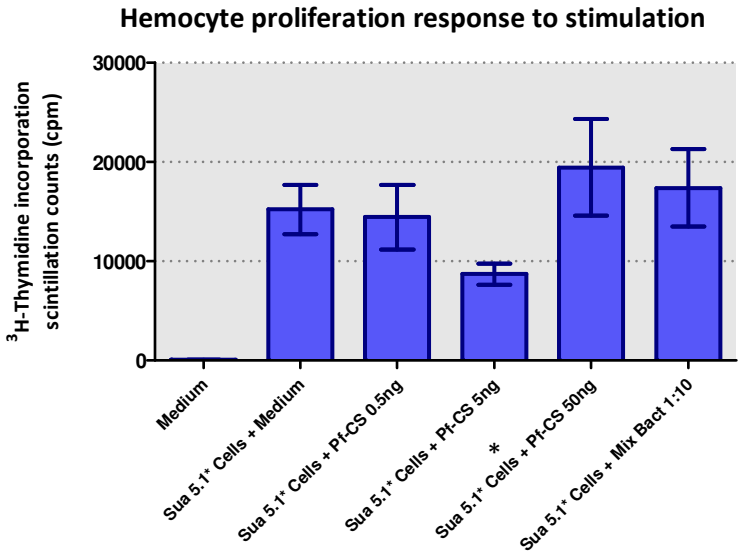


Figure IV.3.1 – Hemocyte proliferation. Sua 5.1* cells were stimulated ON with different amounts of Pf-CS (0.5, 5 and 50ng) or a mixture of heat killed Gram+ and Gram – bacteria (**Mix bact** – 10bacteria/cell). ^{3}H-Thymidine was allowed to incorporate into dividing cells for 6h and radioactivity was measured by scintillation counting in each sample.

IV.4 – Discussion

This assay intended to demonstrate if exposure of mosquito hemocyte-like cell lines to the major antigen of *Plasmodium* sporozoites (the circumsporozoite protein) induced cell proliferation. This would result from a cellular response to sporozoites, triggered in the mosquito hemolymph. If the mosquito would rely on cellular responses to restrain the vast numbers of sporozoites that invade the hemolymph and given that circulating hemocytes in adult mosquito's hemolymph are in scarce number, it is expected that the presence of the parasite would induce the proliferation of hemocytes.

Our assay failed to significantly show hemocyte proliferation upon contact with Pf-CS or heat killed bacteria, excepting for the 5ng Pf-CS dose that showed a significant decrease in cell number. Reduction of hemocyte numbers during a mosquito immune challenge may reflect microaggregation reactions, where hemocytes cease circulating and attach to microorganisms. The occurrence of these reactions was not studied. Nevertheless, our *in vitro* assay counts the total number of hemocytes, whether circulating or attached. Another explanation for the reduction observed would be hemocyte apoptosis after immune stimulation. The fact that no dose dependent response was observed may reflect a drawback of the assay. Even though assay optimization included different times of cell stimulation and ^{3}H-Thymidine incorporation (data not shown), it is possible that our assay did not cover the hemocyte proliferation induction window of time and that different doses of Pf-CS protein require different times for hemocyte response. Also, this cell line might not proliferate upon stimulation, as no major differences were observed after immune challenge with bacteria.

The circumsporozoite (CS) protein is the major antigen of *Plasmodium* sporozoites, and its protein synthesis starts when sporozoites are still developing inside the oocyst, covering the surface of the sporozoite. Upon oocyst rupture thousands of sporozoites are released into the hemocel, along with massive amounts of the CS protein (Kappe *et al.*, 2004). Thus it would be expected that mosquito PRRs would be able to detect and recognize this foreign protein. It is possible that other factors from the sporozoite

surface or released from the inside of the oocyst, induce hemocyte responses and proliferation. It would also be interesting to determine hemocyte numbers in mosquito hemolymph *in vivo* upon sporozoite invasion of the hemolymph. The major drawback of *in vivo* tests on hemolymph stages is that sporozoite egress from oocysts is not synchronized and may subsist for a few days. This makes a precise determination of hemocyte proliferation induction impossible.

Additionally, it would be important to evaluate if hemocyte differentiation occurs in response to sporozoite invasion, *ie*, if the response to the parasite requires a specific type of cell with particular immune abilities. The Sua 5.1* cell line is kept by selection of attached cells. Detached cells are eliminated each time the medium is replaced. As different hemocyte types have different attachment abilities, it is possible that our model does not include every hemocyte type, namely circulating cells.

This system was not appropriate to measure hemocyte proliferation, as no hemocyte number increase was detected upon bacterial challenge, indicating that this cell line may have no proliferation properties upon immune stimulation. Nevertheless, a time and dose-specific effect was observed after stimulation with Pf-CS, indicating that although not proliferating these cells may respond to the parasite antigen.

V – Mosquito hemolymph melanization

V.1 – Introduction

Melanization is an immune effector mechanism, through which insects fight microbial infections, and has been described for several insects, such as *M. sexta, D. melanogaster*, and *Ae. aegypti, An. gambiae, Ar. Subalbatus* mosquitoes (Christensen *et al.*, 2005; Jiravanichpaisal *et al.*, 2006 ; Kanost *et al.*, 2004 ; Ligoxygakis *et al.*, 2002).

Melanization consists in the deposition of a melanin layer over the microorganisms, to avoid spreading of infection and to allow a localized killing response, whether by melanization side-product (ROI and NOI) cytotoxicity or by another effector mechanism. Microbial recognition activates a protease cascade in the hemolymph that sequentially activate one another, through proteolysis. In the end, it leads to the cleavage of Prophenoloxidase (PPO), converting it to phenoloxidase (PO). This together with other enzymes converts tyrosine into melanin (Figure V.1.1). *An. gambiae* genome has nine genes that code for PO enzymes. Although six of these have been shown to be highly expressed after a blood meal, immune challenge does not lead to an increased expression of PPO. Instead, it is believed, that enzyme activation is induced at a post-transcriptional level by its cleavage, and is regulated by serpins and serine proteases (Muller et al, 1999).

Malaria vector mosquitoes are able to engage in melanization. *An. gambiae* mosquitoes were shown to melanize exogenous injected Sephadex beads and *Plasmodium* ookinetes (Collins *et al.*, 1986; Warr *et al.*, 2006). The *An. gambiae* L3-5 strain was genetically selected under laboratory conditions to be refractory to malaria. Indeed these mosquitoes are able to kill *P. berghei* and *P. falciparum* ookinetes, and these ookinetes show massive melanization (Collins et al, 1986). Melanization of *Plasmodium* ookinetes in these mosquitoes is known to be regulated by molecules such as TEP1, SRPN6 and some CLIP proteases (Abraham *et al.*, 2005; Blandin *et al.*, 2004; Volz *et al.*, 2006). In susceptible mosquitoes, that do not melanize *Plasmodium* ookinetes, melanization is negatively regulated by CTL4 and CTLMA2 (Osta et al, 2004). However these molecules do not seem to be involved in Sephadex bead melanization. The latter is however, regulated by TEP1 and LRIM1 (Warr et al, 2006).

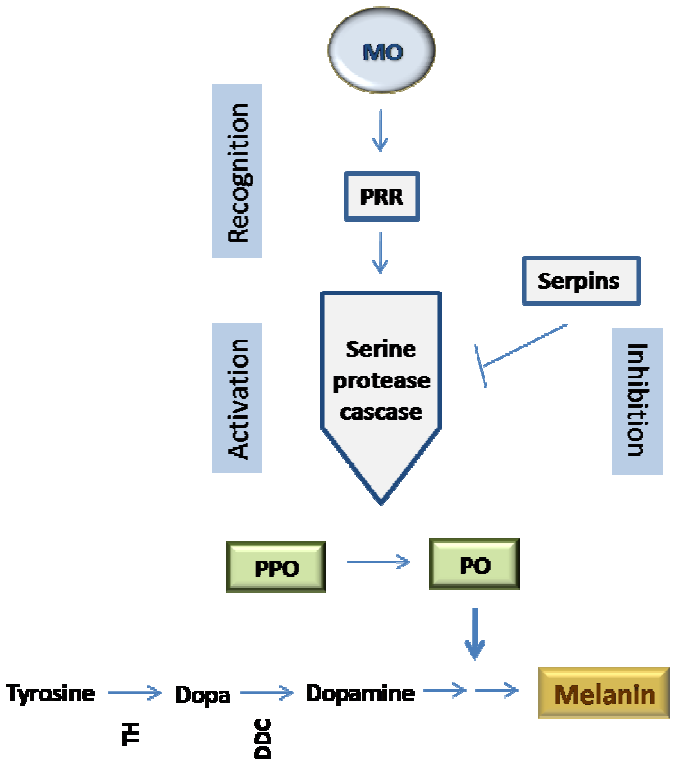


Figure V.1.1 – PPO activation and melanin synthesis. Microorganism (**MO**) recognition by pattern recognition receptors (**PRR**) leads to activation of a hemolymph serine protease cascade, negatively regulated by Serpins. Ultimately, serine proteases cleave Prophenoloxidases (**PPO**) into Phenoloxidases (**PO**), activating the enzyme. Active POs participate in the last steps of Tyrosine conversion into melanin. **TH**: Tyrosine Hydroxylase; **DCC**: Dopa Decarboxylase.

Albeit anopheline mosquitoes are able to mount melanization responses to *Plasmodium* ookinetes in the lab, it has rarely been observed in field mosquitoes. The implication of this mechanism on the control of malaria infection in the mosquito is not known. Experiments using *P. berghei* parasites indicate that melanization observed over *Plasmodium* ookinetes is operating in mosquitoes not as a killing mechanism but rather as a post-mortem event (Whiten et al, 2006).

These studies refer to the midgut stage of malaria infection in the mosquito. In the hemolymph stage, melanization was observed in *P. gallinaceum* sporozoites in *Ae. aegypti* (Hillyer *et al.*, 2003). It is not known yet if this response is universal for all malaria/vector models, nor if melanization may play a role in controlling sporozoite numbers in the hemolymph and thus parasite load in salivary glands.

V.2 – Methodology

V.2.1 – Mosquitoes and parasites

An. gambiae mosquitoes from the strains Suakoko and Yaoundé were maintained in a 12h light/dark cycle at 26ºC. Mosquitoes were fed on a 10% glucose solution.

P. berghei ANKA parasites were maintained in *Mus musculus* CD1 mice by blood passage.

V.2.2 – Melanization activation during mosquito infection

Based on the assumption that PPO activation into PO is the limiting step of melanin synthesis, melanization activation was determined by two assays that aimed at detecting PO activity: an enzymatic assay using a colorimetric substrate for PO (L-DOPA) and Western blot to evaluate PPO cleavage into PO.

V.2.2.1 – L-DOPA assay validation in melanizing mosquitoes

V.2.2.1.1 – Mosquito infection and hemolymph collection

An. gambiae Suakoko female mosquitoes were fed on mice either non-infected or *P. berghei* infected mice with a parasitemia of 10-20% and gametocyte exflagellation. Hemolymph was collected at D13 and D15pi by pricking the mosquito through the neck membrane with a glass needle inserted in a Nanojet II Auto-nanoliter injector (Drummond Scientific). Hemolymph was sucked out of the mosquito and dispensed onto a drop (20µl) of phosphate-saline buffer (PBS – Sigma) complemented with a cocktail of protease inhibitors (Roche).

An. stephensi female mosquitoes massively infected with *Microsporidia* spores were used as a positive control, as extensive spore melanization was microscopically observable in the mosquito hemolymph. The collection was performed the same way.

V.2.2.1.2 – L-DOPA assay

Hemolymph samples were centrifuged at 14 000 rpm for 5min to separate soluble proteins from hemocytes and cell debris. Supernatants were reserved as protein samples and pellets were discarded.

Protein quantity in hemolymph samples was determined by incubating 2µl of each sample with Bradford reagent (BioRad) for 15min on a flat-bottom 96-well plate. In the presence of protein this reagent turns blue and its color intensity is protein load-dependent. Sample absorbance was read on an Elisa reader (Awareness Technology Inc. at 630nm. A BSA standard curve, ranging from 2-14µg, was used for sample comparison.

To determine PO activity, 10µg of each sample were diluted in PBS plus protease inhibitors to a final volume of 20µl and incubated with 10µl of $CaCl_2$ and 150µl of a 3mg/ml L-DOPA (3,4-dihydroxy-L-phenylalanine) solution for 1h, on a flat-bottom 96-well plate. Sample absorbance was read at 492nm at 5, 15, 30, 45 and 60min post incubation.

V.2.2.2 – PPO activation during mosquito infection

To determine if melanization is activated in response to sporozoite invasion of the hemolymph (upon oocyst burst) PO activity in mosquito hemolymph was determined at several time-points of infection, starting when sporozoites were still developing in the oocyst and terminating when most sporozoites have invaded the salivary glands.

V.2.2.2.1 – Mosquito infection and hemolymph collection

An. gambiae female mosquitoes, from the Yaoundé strain, were fed on mice either non-infected or infected with *P. berghei* in the conditions described above.

Hemolymph was collected from 20 female mosquitoes as previously described at D7, D9 and D11-15pi.

Hemolymph samples were centrifuged at 14 000 rpm for 5min to separate soluble proteins from hemocytes and cell debris. Supernatants were kept for protein samples and pellets for DNA extraction. Three independent experiments were performed.

V.2.2.2.2 – PO activity determination

V.2.2.2.2.1 – L-DOPA assay

The L-DOPA assay was performed as described above. Data were evaluated for statistical significance by the Mann-Whitney test on the GraphPad software (Prism).

V.2.2.2.2.2 – Western blot

From the same samples, 2µg of hemolymph proteins were separated on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), along with 4µl of All Blue protein standard (BioRad), and transferred onto a nitrocellulose membrane.

Proteins were then incubated with a primary antibody directed against AgPO2 (*An. gambiae* Phenoloxidase 2) at a concentration of 1:10 000, followed by a secondary anti-rabbit IgG conjugated with horseradish peroxidase (HRP – Sigma) antidoby at a concentration of 1:10 000. The signal detection was performed with the ECL system (GE HEalthcare) on a Hybond ECL film (GE Health care).

AgPPO2 has a predicted molecular mass of 78kDa and is cleaved upon activation to yirld two peptides of predicted molecular masses of 72 and 6kDa, respectively.

V.2.2.2.3 – Sporozoite detection in collected hemolymph

Hemolymph pellets were submitted to DNA extraction and *Plasmodium* directed PCR to detect the presence of sporozoites.

DNA was extracted using Chlelx-100. Pellets were dissolved in 10µl of PBS, followed by 240µl of 100ºC heated 0.5% Chelex-100 (Sigma) and incubated at 100ºC for 10min. A tube with no pellet was included as a blank. Samples were centrifuged at 14 000rpm for 15min. Supernatants containing DNA were precipitated ON at -20ºC by incubation with 45µl of 3M acetic acid (pH5) and 1.0 ml of ice cold absolute ethanol. Samples were centrifuged at 14 000rpm for 15min and supernatants were discarded. DNA pellets were washed with 80% ice cold ethanol and centrifuged at 10 000 rpm for 10min. Supernatants were discarded and pellets left to dry ON at rt. DNA was dissolved in 10µl of MilliQ water.

Sporozoites in samples were detected by a Nested-PCR technique directed to the *Plasmodium* 18S rRNA, using genus specific primers. This technique is adequate for samples with low abundance of the desired DNA, and comprises two sequential PCR reactions: the first amplifies a product of ~1Kb of the original material and is hardly detected on an agarose gel; the second reaction uses the product generated on the first to amplify a product of ~300bp increasing sensitivity and specificity. PCR conditions and primer sequences are described in Table V.2.1.

One µl of each sample was used for the reaction. A positive control, containing DNA from *P. berghei* infected mosquito midguts was included in the reaction. One µl of the amplified product of the first reaction was used as a template for the second reaction. Amplified products were visualized on a 2% agarose gel, stained with ethidium bromide.

Table V.2.1

PCR conditions for *Plasmodium* detection

	Primer name	*Primer sequence*	*Ann. Temp.*	*Cycle rpts.*
Reaction 1	*rPLU1*	5' – TCAAAGATTAAGCCATGCAAGTGA – 3'	62ºC	30
	rPLU2	5' – ATCTAAGAATTTCACCTCTGACATCTG – 3'		
Reaction 2	*rPLU3*	5' – TTTTTATAAGGATAACTACGGAAAAGCTGT – 3'	64ºC	35
	rPLU4	5' – TACCCGTCATAGCCATGTTAGGCCAATACC – 3'		

Ann. Temp.: Annealing Temperature; **Cycle rpts**.: Cycle repeats

V.2.3 – Melanization activation by Pf-CS

V.2.3.1 – Mosquito injections

A. gambiae Yaoundé female mosquitoes, 3-5 days-old, were injected intratoraxically using a Nanojet II Auto-nanoliter injector (Drummond Scientific). Injected solutions included a recombinant form of *P. falciparum* circumsporozoite protein (Pf-CS – 10ng/µl – Sclavo Diagnostics) diluted in Schneider medium, BSA (10ng/µl – Sigma) diluted in Schneider medium, or with Schneider medium alone as a control. Hemolymph was collected as previously described at 6h, 24 and 48h post-injection.

V.2.3.2 – PO activity determination

PO activity was determined by the L-DOPA assay and Western blot, as described above. Three independent experiments were performed and data was evaluated for statistical significance by applying a Mann-Whitney test on the GraphPad software (Prism).

V.2.4 – Effect of PPO inhibition on mosquito infection

To test if melanization is necessary to control the number of sporozoites that effectively invade the salivary glands, infected female mosquitoes were injected with a chemical PO inhibitor. Sporozoite load was determined to evaluate differences.

V.2.4.1 – Mosquito infection

An. gambiae female mosquitoes, from the Yaoundé strain, were fed on mice infected with *P. berghei* using the conditions described above.

V.2.4.2 – PTU injections

At D6pi the infected mosquitoes were injected intratoraxically with 69nl of Schneider medium or a 1mM solution of *N*-Phenylthiourea (PTU), a chemical inhibitor of tyrosinases and melanin synthesis, in Schneider medium. Infection load on mosquito midgut was accessed at D12pi.

V.2.4.3 – Sporozoite load determination

Mosquitoes from both groups were dissected at D21pi and salivary glands were collected into a glass homogenizer, crushed to release sporozoites and mixed with 400µl of PBS. The number of sporozoites in the suspension was counted using a hematocytometer. Three independent experiments were performed and data were evaluated for statistical significance by applying a ratio t test on the GraphPad software (Prism).

Experimental design is schematized in Figure V.2.1.

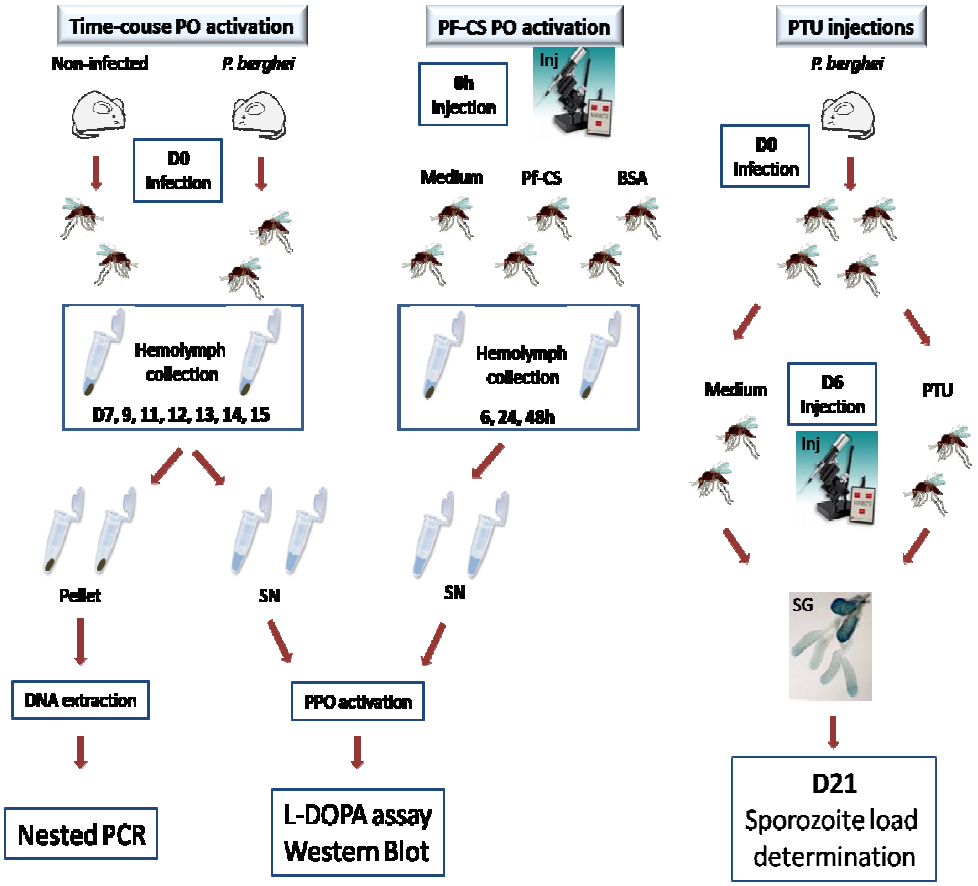


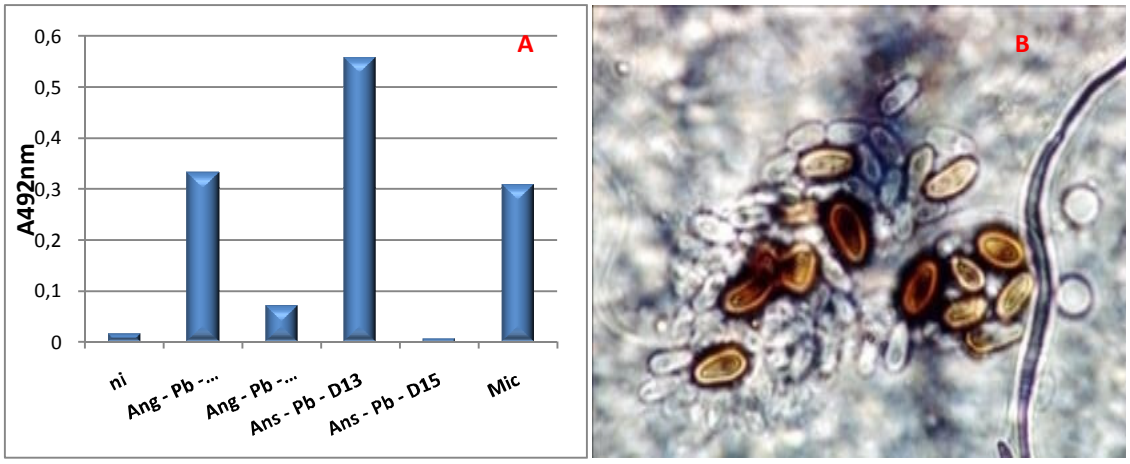
Figure V.2.1 – Experimental design to determine melanization role in the response to hemolymph sporozoites. PPO activation was accessed by L-DOPA assay and Western Blot in mosquitoes infected with *P. berghei* from D7 to D15, and in mosquitoes injected with Pf-CS and BSA. Effect of PPO inhibition was determined in *P. berghei* mosquitoes and injected with PTU at D6pi, by determining alterations in salivary gland sporozoite load. **Inj**: Injector; **SG**: Salivary Gland; **SN**: Supernatant.

V.3 – Results

V.3.1 – Melanization activation during mosquito infection

V.3.1.1 – L-DOPA assay validation in melanizing mosquitoes

By the time this work was initiated, L-DOPA assays were commonly used for immunity studies in *Drosophila*, but not in mosquitoes. Thus, it was necessary to validate the assay in mosquitoes. For this, we used hemolymph from *Microsporidia* infected *An. stephensi* mosquitoes, that showed melanized *Microsporidia* spores (Figure V.3.1 – B).



FigureV.3.1 – Melanization activation in the mosquito hemolymph. A: PPO activation by the L-DOPA assay, at 15 minutes after the addition of substrate in non-infected *An. gambiae* mosquitoes (**ni**), Pb infected mosquitoes 13 days (**Pb - D13**) and 15 (**Pb - D15**) post infection in *An. gambiae* (**Ang**) and *An. stephensi*(**Ans**) mosquitoes , and *Microsporidia* infected mosquitoes (**Mic**), measured by the absorbance at 492nm (**A492nm**). **B**: mosquito midgut infected with *Microsporidia*, showing melanization on the spores, observed by optical microscopy (1000x).

As expected, hemolymph of *Microsporidia* infected mosquitoes showed Absorbance at 492nm levels (reflecting PO activity) were distinctively higher than those of non-infected mosquitoes (Figure V.3.1 – A). At D13pi, PO activity levels in the hemolymph of *P. berghei* infected mosquitoes were similar to those of *Microsporidia* infected mosquitoes. At D15pi, however, levels observed were similar to those of non-infected mosquitoes, suggesting that PO activity may be regulated by the progression of infection (Figure V.3.1 – A).

V.3.1.2 – PPO activation during mosquito infection

PPO activation was followed during *P. berghei* infection in *An. gambiae* Yaoundé mosquitoes, starting at D7pi (when oocysts are still developing with incipient sporozoites inside) up to D15pi (when the majority of sporozoites have been released from the oocysts and invaded the salivary glands).

PO activity seemed to vary in non-infected mosquitoes according to time (Figure V.3.2 – A, white bars; B, grey line). PPO activity decreased between D7 and D12pi, peaked at D13pi, decreased at D14pi after which it remained steady. In *P. berghei* infected mosquitoes, PO activity followed the overall activation pattern observed in non-infected control mosquitoes (Figure V.3.2 – A, grey bars; B, red line). The only differences in PO activity between infected and non-infected mosquitoes were observed at D9, D12 and D15pi, when infected mosquitoes showed higher levels of PO activation than non-infected control mosquitoes, suggesting that at these time-points infection may induce melanization in mosquito hemolymph. Although not significant, the increase was observed in infected mosquitoes in all experiments performed. No differences were observed for the rest of the infection time-points studied.

Plasmodium identification by PCR in hemolymph samples (Figure V.3.1 – C) detected sporozoites starting at D9pi all through D15, suggesting that from D9 up to D15pi sporozoites are continuously being released from the midgut oocysts. However, no straight forward correlation was observed between sporozoite appearance in the hemolymph samples and PPO activation pattern: sporozoites were detected from D9pi in hemolymph samples and PO activation in infected mosquitoes was different from the non-infected ones at D9, D12 and D15pi. Although the slight differences in PO activation coincide in time with the appearance of sporozoites in the hemolymph, they are not maintained during the course of the infection even though sporozoites remain present.

As for Western blot, no differences were observed in hemolymph samples of infected and non-infected mosquitoes, correlating with similar pattern of PO activation observed in both sets of samples.

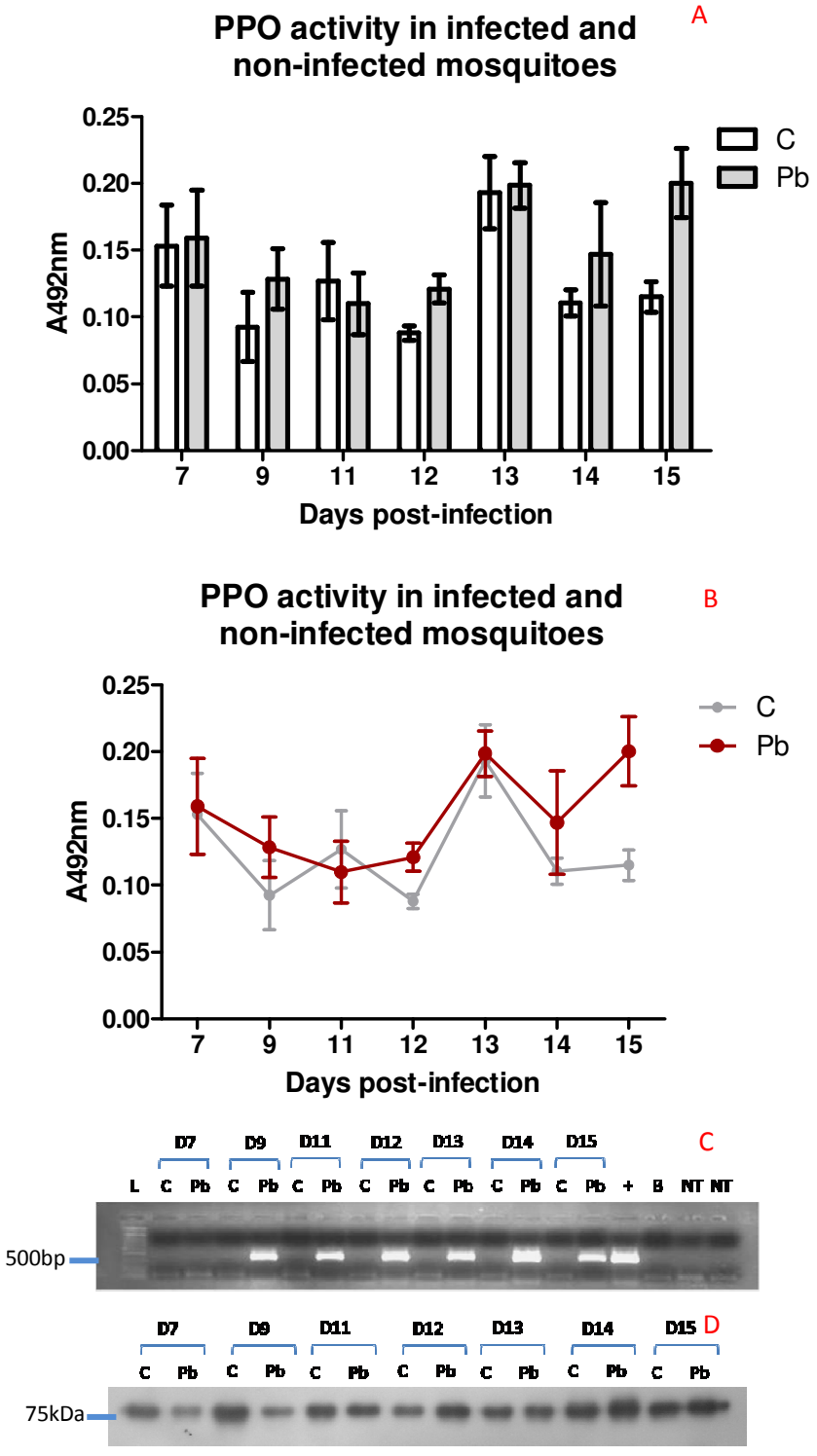


Figure V.3.2 – PPO activation during mosquito infection. **A**: Bar graph of PO activity in *P. berghei* infected (**Pb**) and control non-infected (**C**) mosquitoes from D7 to D15pi, bars represent the measured absorbance at 492nm (**A492nm**). **B**: Line graph of PO activity in *P. berghei* infected and control non-infected mosquitoes from D7 to D15pi, measured as absorbance at 492nm. **C**: *Plasmodium* detection in hemolymph samples of *P. berghei* infected and non-infected mosquitoes. **+**: positive control; **B**: Blank; **NT**: Non-template control. **D**: Western blot for AgPO2 of hemolymph samples of infected and non-infected

V.3.2 – Melanization activation by Pf-CS

A. gambiae female mosquitoes were injected with Schneider medium, Pf-CS or BSA. PO activity was measured at 6, 24 and 48h post-injection.

PO activity measured in injected mosquitoes is represented in Figure V.3.3 (A and B).

Overall, PO activity decreased at 24 and 48h, when compared to 6h post-injection, possibly reflecting PPO activation upon wounding which results from intrathoraxcial injection. PO activity decrease from 6h to 24h was observed in all experiments performed in Medium and BSA injected mosquitoes.

No differences in PO activity were observed between Pf-CS and BSA injected mosquitoes and medium injected mosquitoes. Only a slight increase was detected in the hemolymph of Pf-CS and BSA injected mosquitoes at 24h pi. However, it was neither significant nor consistent in the experiments performed.

Western blot for AgPO2 (Figure V.3.3 – C) showed no differences in band patterns between injections in the hemolymph of *An. gambiae* mosquitoes. All samples show the same band for AgPO2. The fact that both forms of the protein (PPO2: non-activated protein, 78kDa; PO2: activated protein72kDa) have similar molecular weights makes impossible to determine if the band detected represents AgPPO2 or AgPO2.

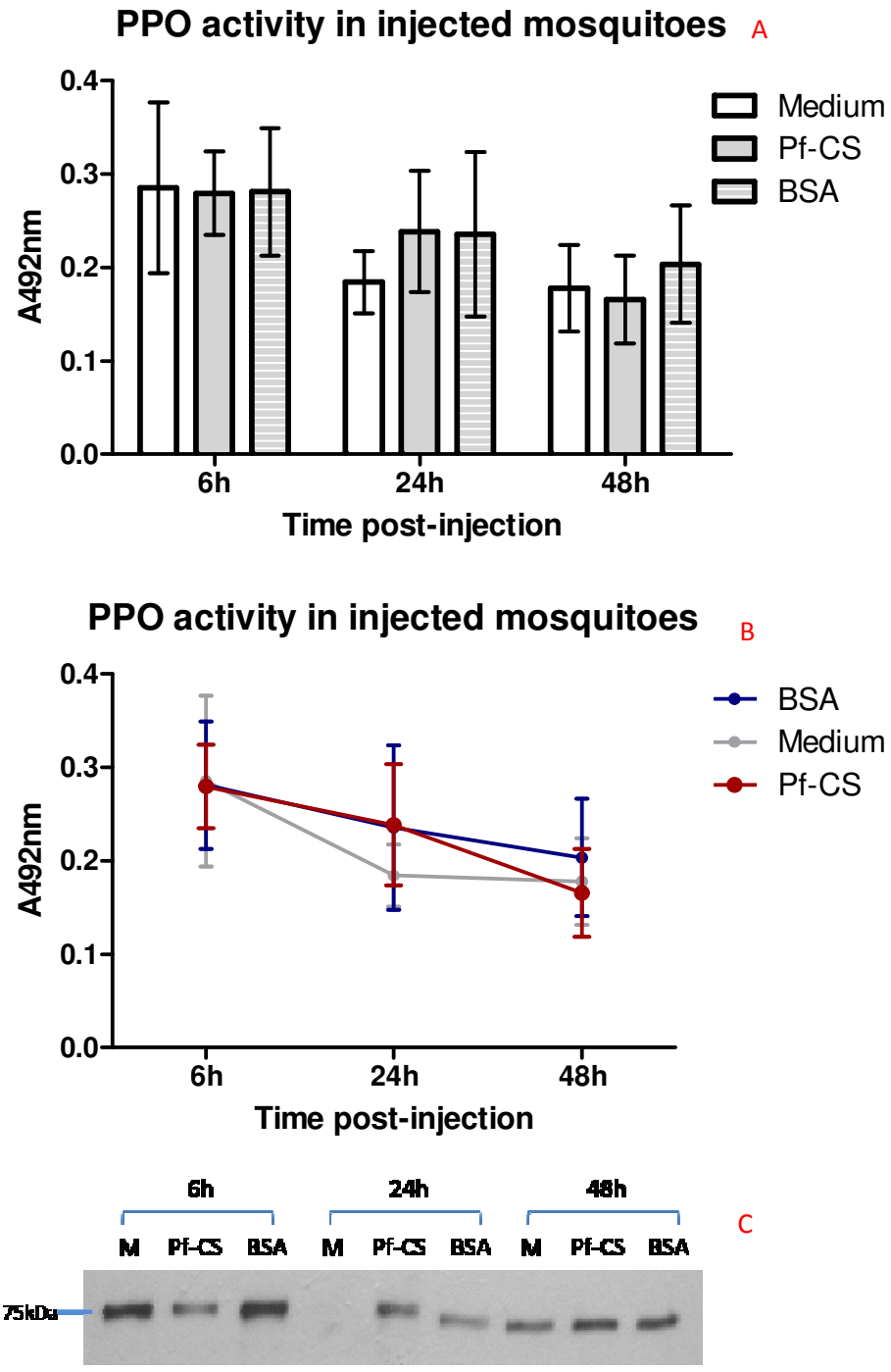


Figure V.3.3 – PPO activation in injected mosquitoes. A: Bar graph of PO activity in *An. gambiae* mosquitoes injected with Schneider medium (**M**), Pf-CS (10ng/µl) and BSA (10ng/µl) at 6, 24 and 48h post-injection, measured as absorbance at 492nm (**A492nm**). **B**: *An. gambiae* mosquitoes injected with Schneider medium, Pf-CS (10ng/µl) and BSA (10ng/µl) measured as absorbance at 492nm **C**: Western blot for AgPO2 of hemolymph samples of injected mosquitoes.

V.3.3 – Effect of PPO inhibition on mosquito infection

To evaluate the role of melanization in sporozoite number control in mosquito hemolymph, *P. berghei* infected *An. gambiae* mosquitoes were injected with PTU, a chemical inhibitor of PO. Injections were performed at a time in infection (D6) when oocysts were established beneath the basal lamina of the midgut epithelium and started to develop sporozoites inside.

The number of sporozoites per female in the salivary glands of mosquitoes injected with PTU was significantly higher (P=0.0099) than in medium alone injected mosquitoes. The increase in parasite numbers was consistent in all experiments performed (Figure V.3.4).

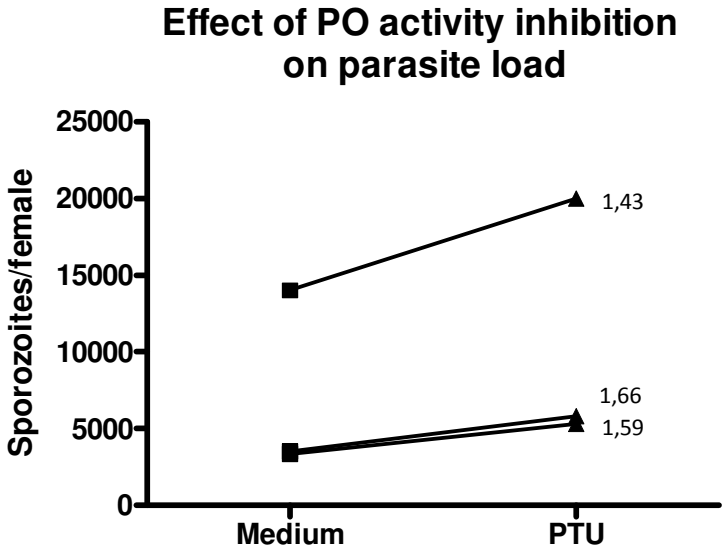


Figure V.3.4 – Effect of PO activity inhibition on parasite load. *P. berghei* infected mosquitoes were injected with Schneider medium or PTU (1mM). Sporozoite load on salivary glands was determined at D21pi. Each line represents an experiment performed. Values on the right indicate, for the correspondent experiment, the ratio of sporozoite number of PTU *vs.* Medium injected mosquitoes.

V.4 – Discussion

The hemolymph stage of malaria infection in the mosquito is known to represent one of the major bottlenecks for the sporogonic development, as only about 2% of the sporozoites produced in and released from the midgut oocysts efficiently invade the salivary glands. The mosquito is bound to respond to these parasites, as the hemolymph contains all the machinery to engage in immune responses, either humoral, by soluble proteins or cellular by circulating hemocytes. However, the type of response triggered towards sporozoites remains elusive.

Melanization is a known effector mechanism that insects use to control microbial infection. In *Ae. aegypti* mosquitoes it was shown to be part of the response to hemolymph sporozoites, as a small percentage of these were found to be melanized in the mosquito hemocel (Hillyer *et al.*, 2003). The work performed aimed at understanding if this mechanism represents a general response to malaria sporozoites by the mosquito vector.

Melanization was tested by an assay (L-DOPA assay) measuring PO enzyme activity and a Western blot to determine AgPPO2 cleavage and activation into AgPO2. L-DOPA assay was validated for mosquito hemolymph, using hemolymph samples from mosquitoes massively infected with *Microsporidia* and exhibiting observable spore melanization in the hemocel. As PO values of *Microsporidia* infected mosquitoes samples was distinctively higher than those of non-infected mosquitoes, the assay was considered valid to use in mosquito samples, as it is widely used in *Drosophila* immunity studies. The same experiment allowed observing differences in hemolymph samples of *P. berghei* infected mosquitoes collected at different time-points in infection. At D13pi *P. berghei* infected mosquitoes displayed PO activity levels similar to those of *Microsporidia* infected mosquitoes, while at D15pi it decrease close to non-infected mosquitoes PO activity values. The variation in time of PO activity observed in infected-mosquitoes could be an effect of the progression of malaria infection through the mosquito. The fact that sporozoites begin to be released into the hemolymph from D9-D11pi (depending on the experiment) suggests that at this time sporozoites would

be recognized and PO activity induced. At D15pi however, the majority of sporozoites have reach the salivary glands and few (if any) oocysts are still bursting would account for a decrease in PO activity at this time.

As such, we followed PO activity in *P. berghei*-infected and non-infected mosquitoes from D7pi, when sporozoites are still developing inside the oocysts, up to D15pi. PO activation followed the same pattern previously observed, as activity was lower in until D12pi, reached a peak at D13pi and decreased again, showing a low levels in D15pi. However, this pattern was observed in both *P. berghei* infected and non-infected mosquitoes. This indicated that PO activity varies in blood-fed mosquitoes, regardless of malaria infection. Nonetheless, the values observed are lower than the ones observed for Microsporidia infection, suggesting that a low level of PO activity may be maintained in blood fed mosquitoes. Melanization may be functioning for some other physiological process in mosquitoes. It is necessary to bear in mind that blood fed mosquitoes for these experiments were not allowed to lay eggs, and still carry them till the conclusion of the experiment. If this would account for some of the observed PO activity is not known. No significant differences were observed between infected and non-infected mosquitoes. However, a slight induction in PO activity in infected-mosquitoes was noticed at D9, D12 and D15pi, in all performed experiments. The fact that *Plasmodium* sporozoites are detected by PCR in hemolymph samples of infected-mosquitoes from D9pi onwards suggests that in fact mosquitoes recognize sporozoites and may trigger melanization towards them. The fact that PO remains active in non-infected mosquitoes may indicate that this activity is sufficient for the mosquito response to the parasite.

We have no indication of sporozoite numbers in the hemolymph samples, or even if the DNA detected by PCR belongs to live or dead parasites. Thus, no correlation between sporozoite load *vs.* PO activity was achieved, which might explain the slight PO increase in specific time-points of infection. Nevertheless, the fact that every three days PO activity seems to be slightly higher in infected mosquitoes, suggests that sporozoites may be release from the midgut in waves. Thus, parasite quantification at each time-point would be essential to understand PPO activation dynamic through

Plasmodium infection. It would also be interesting to analyze hemolymph samples from *P. berghei* infected *An. gambiae* mosquitoes by electron microscopy to attempt to visualize melanized sporozoites.

We also tried to determine PO activity in mosquitoes upon injection of a recombinant form of the major surface antigen of *P. falciparum* sporozoites, the circumsporozoite protein (Pf-CS). For all groups tested a higher PO activity was observed at 6hpi, possibly reflecting a wound healing procedure due to injection. Again no differences were found between controls and Pf-CS or BSA injected mosquitoes. This is consistent with the fact that increased melanization does not affect *P. falciparum* development (Michel *et al.*, 2006), even though this work was for midgut stages of the parasite.

Also, the mosquito may not recognize the protein itself, but the GPI anchor through which it attaches to the membrane. *Plasmodium* GPIs were found to have immunogenic properties of in mosquito responses and to be essential for sporogenesis (Arrighi *et al.,* 2009; Lim *et al.*, 2005; Wang *et al.*, 2005).

Although no significant differences in PO activity were found to be a result of *Plasmodium* infection in the mosquito, we attempted to determine if melanization has a role in the control of sporozoite numbers in the hemolymph. When a PO inhibitor was injected in *P. berghei* infected mosquitoes at a time-point when sporozoites are still developing in midgut oocysts and are not detected yet in the hemolymph, parasite load increases in the salivary glands. This suggests that PO activity is essential to control hemolymph infection by sporozoites. As opposed to midgut stages, where accumulating data indicates that melanization may have a post-mortem rather than a killing role in infection of malaria and bacteria (Schnitger et al, 2007; Whiten et al, 2006), in the hemolymph, it appears to be essential to control sporozoite numbers. Thus, even though the PO activity activation pattern does not strictly follow the presence of sporozoites in the hemolymph, melanization seems to be necessary to

help restrain infection. This may not be the only mechanism operating in this bottleneck, as the effect observed with PTU injections is not sufficient to account for the loss of sporozoites between midgut and salivary glands.

VI – Mosquito hemolymph proteome during infection

VI.1 – Introduction

Anopheles mosquitoes are known to respond to *Plasmodium* infection through the activation of immune responses. The work done so far in this field has focused mainly on the response to the midgut invasion by ookinetes. Only a few studies have been performed on salivary gland invasion and even less on the hemolymph response to sporozoites released from midgut oocysts. This step is bound to induce an important response, as hemolymph sustains systemic immunity. This fluid runs over the hemocel carrying immune responsive cells (hemocytes) and immune proteins secreted by hemocytes and the fat body. As these include recognition and effector molecules, it is likely that the thousands of parasites released into the hemocel are recognized by the mosquito immune system and that it mounts a response to clear the invasion. Although it is known that only about 2% of the sporozoites released from the midgut effectively invade the salivary glands the knowledge about the mechanisms responsible for this bottleneck is still scarce. Parasite number reduction in the hemolymph may be a result of physical barriers (reducing the success of reaching the salivary glands), immunity (parasite clearance), or of mechanisms involved in mosquito physiology.

The massive genomic data obtained to date by microarrays studies, comparing the transcriptome of *Plasmodium* infected and non-infected *An. gambiae* mosquitoes, has revealed that the expression of a large number of genes is altered due to the presence of the parasite. Genes affected belong to several functional classes, apart from immunity, such as cytoskeleton remodeling, apoptosis, redox metabolism and cell adhesion (Christophides *et al.*, 2002; Kumar *et al.*2003; Vlachou *et al.*, 2005). A lot of effort has been made to functionally characterize immune genes whose expression has been altered by parasite infection. However, only a few functional studies have attempted to evaluate the influence of molecules involved in mosquito physiological processes on infection. The gene RFABG (retinoid and fatty-acid binding glycoprotein) codes for a precursor of apolipophorin I and II, that account for the two major components of the insect lipid transporter. The expression of this gene is highly induced upon ookinete invasion and its KD leads to a reduction in oocyst number and

to a total inhibition of egg development (Vlachou *et al.*, 2005). This suggests that responses to pathogens may include the action of not only molecules previously described as immune related but also to physiological and general mechanisms operating in the mosquito.

Eicosanoid biosynthesis in particular, has been repeatedly implicated in the immune responses of several insects (reviewed by Stanley & Miller, 2006). Eicosanoids are oxygenated metabolites of arachidonic acid (AA) and other polyunsaturated fatty acids (PUFAs) that act mainly as signaling molecules, such as ovulatory pheromones and modulators of ion transport. Additionally, these molecules were found to modulate cellular immune responses in insects.

Eicosanoid biosynthesis from arachidonic acid is depicted in Figure VI.1.1. Membrane phospholipids are hydrolyzed by phospholipase A_2 (PLA_2) into AA that is subsequently metabolized into eicosanoids by three pathways: cyclooxygenases (COX) convert AA into prostaglandins, thromboxanes and prostacyclin; lipoxygenases (LOX) convert AA into leukotrienes, hydoxy fatty acids, lipoxins and Hepoxillins; and cytocrome P450 that forms hydroxy fatty acids and epoxy derivatives. Some of these molecules have a role in proinflammatory immune responses in mammals.

In insects, eicosanoid biosynthesis has been implicated in the response to bacteria, fungi and parasites, as the treatment with chemical inhibitors of enzymes involved in this process results in a compromised immune response. Dexamethasone (DEX), a PLA_2 inhibitor was shown to reduce the hemocyte microaggregation and nodulation response of *Manduca sexta* to the bacterium *Serratia marcescens*, which was reversed by AA treatment (Miller *et al.*, 1994). During bacterial infections, these molecules also mediate phagocytosis, cell spreading, prophenoloxidase (PPO) activation, and, in *Drosophila*, the IMD pathway activation (Downer *et al.*, 1997; Miller *et al.*, 1994; Yajima *et al.*, 2003). In *M. sexta* infected with *Metarhizium anisopliae* or *Beauveria bassiana* fungi, products of the LOX but not the COX pathway were found to reduce the nodulation response (Dean *et al.*, 2002; Lord *et al.*, 2002). As for parasite infections, DEX treatment of *Drosophila* larvae inhibits the encapsulation of *Leptopilina*

boulardi eggs (Carton *et al.*, 2002) and in the model *Rhodnius proxilus/Trypanosoma rangeli*, treatment with DEX, indomethacin (IN, a COX inhibitor) or Nordihydroguaiaretic acid (NDGA, a non-specific LOX inhibitor) totally abolishes the microaggregation response and greatly reduces PPO activation, while increases parasite numbers in the hemolymph. This is reversed by AA treatment, suggesting that eicosanoids resulting from both COX and LOX pathways modulate this response (Garcia *et al.*, 2004a).

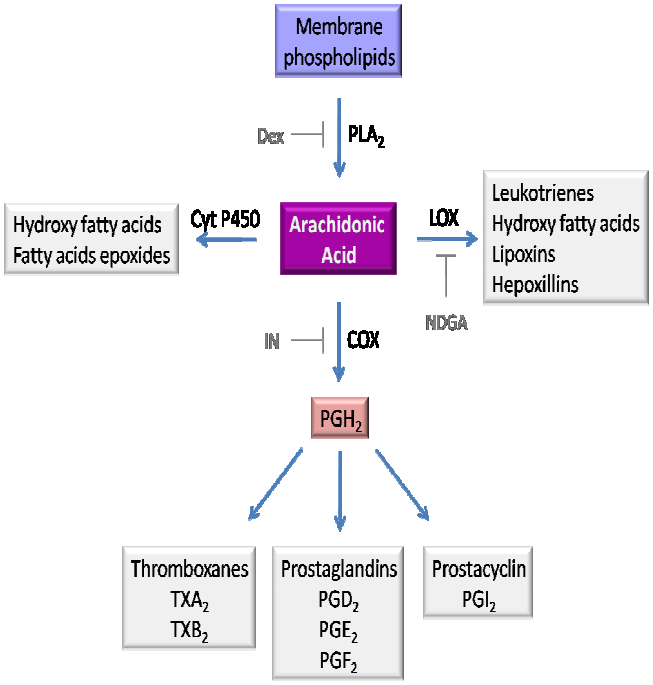


Figure VI.1.1 –Eicosanoid biosynthesis pathways from arachidonic acid. Phospholipase A_2 (**PLA_2**) hydrolyses membrane phospholipids into arachidonic acid. This is converted to several eicosanoids by 3 distinct pathways:
Cyclooxygenase (**COX**) pathway, in which AA is transformed into prostaglandin H_2 (**PGH_2**) that is used for the synthesis of Thromboxanes (as **TXA_2**, **TXB_2**), Prostaglandins (**PGD_2**, **PGE_2**, **PGF_2**) and Prostacyclin (**PGI_2**);
Lipoxygenase (**LOX**) pathway, that metabolizes AA into Leukotrienes, Hydro(preo)xy fatty acids, Lipoxins and Hepoxillins;
Cytocrome P450 (**Cyt P450**) pathway, in which AA is converted into Hydroxy fatty acids and epoxy derivatives.

Chemical eicosanoid biosynthesis inhibitors are displayed next to the respective enzyme inhibited: Dexamethasone (**DEX**) is a PLA_2 inhibitor; Indomethacin (**IN**) is a COX inhibitor, and Nordihydroguaiaretic acid (**NDGA**) in a non-specific LOX inhibitor.

An intriguing property of host immunosuppression by pathogens has been observed in two models of infection in insects. These include a bacterial and a parasite infections and both compromise the respective host's immunity by interfering with eicosanoids biosynthesis. The bacterium *Xenorahbdus neatophila* is able to inhibit PLA_2 activity of *Spodoptera exigua* and the subsequent eicosanoid biosynthesis. To account for this,

the injection of AA into *Spodoptera exigua larvae* reduces host mortality to 50%, while treatment with inhibitors potentiates bacterial mortality (Park & Kim, 2000; 2003). Also in *M. sexta*, infection with live *X. nematophila* bacteria produces about one fourth of nodules observed upon injection of heat-killed bacteria (Park *et al.*, 2003b). Moreover, in a protozoan infection by *Trypanosoma rangeli* in *Rhodnius prolixus,* AA treatment increases microaggregation reaction and reduces parasite numbers in the insect's hemolymph. *T. rangeli* was proposed to inhibit the release of AA in its host, although it is not known if the parasite has a direct action on PLA_2 (Garcia *et al.*, 2004b). These studies show that the interplay between parasites and hosts is complex and dynamic, revealing that physiological mechanisms other than immunity are also important in the response to infection.

In the present work we used a proteomic approach to determine proteins whose levels are altered due to sporozoite infection in the mosquito hemolymph. Identified proteins were analyzed to understand mechanisms involved in hemolymph response to the parasite and to unveil interactions between the host and the parasite.

VI.2 – Methodology

VI.2.1 – Mosquitoes and parasites

Anopheles gambiae mosquitoes from the Yaoundé strain (isolated from the Cameroon) were reared at 25ºC and 75% humidity, with a 12h light/dark cycle and maintained on a 10% glucose solution. At day 2-3 post emergence, mosquitoes were fed on *Mus musculus* CD1 mice infected either with:

i) *P. berghei* ANKA 2.34 - fully infective and transmissible clone; or

ii) *P. berghei* ANKA 2.33 – non-infective clone (carrying defective gametocytes).

Mosquitoes were allowed to feed for 10-20 minutes. To ensure effective infection only mice displaying 10-20% parasitemia and parasite male gametocyte exflagellation were used to feed mosquitoes. After blood feeding, mosquitoes were kept on a 10% glucose solution and non-infected females were removed.

The same procedure was followed for injection experiments, with the exception of naïve mice being used as controls, instead of *P. berghei* 2.33 infected mice. Three independent experiments were performed.

VI.2.2 – Hemolymph collection

To control infection in each experiment, 10 female mosquitoes were dissected at day13 post infection (D13pi) and the midguts were observed to determine oocyst number. Another 10 female mosquitoes were dissected and midguts were crushed within a glass homogenizer with 10µl of PBS. The resulting supernatant was then placed in a hematocytometer chamber to count the number of sporozoites.

Hemolymph was collected from female mosquitoes at D12-13pi, when the majority of sporozoites leave the midgut and invade the hemolymph. Collection was performed by pricking the mosquito through the neck membrane with a glass needle inserted in a Nanojet II Auto-nanoliter injector (Drummond Scientific). Hemolymph was sucked out of the mosquito and dispensed onto a drop (20µl) of suspension buffer prepared for two-dimensional electrophoresis (2DE) – see below. For each group 80-250 female mosquitoes were used to retrieve hemolymph. Samples were centrifuged at 10 000 rpm for 10min to separate proteins suspended in the collection buffer and cell debris from hemocytes. Pelleted debris's were kept at -80ºC for RNA extraction and protein suspensions were kept at -20ºC for 2DE.

The experiments performed to analyze gene expression and proteome of the hemolymph collected from *P. berghei* infected (2.34) *vs.* non-infected (2.33)

mosquitoes at D13pi are summarized in Figure VI.2.1. Hemolymph samples collected from mosquitoes in five independent experiments were used for both gene expression and proteome analysis.

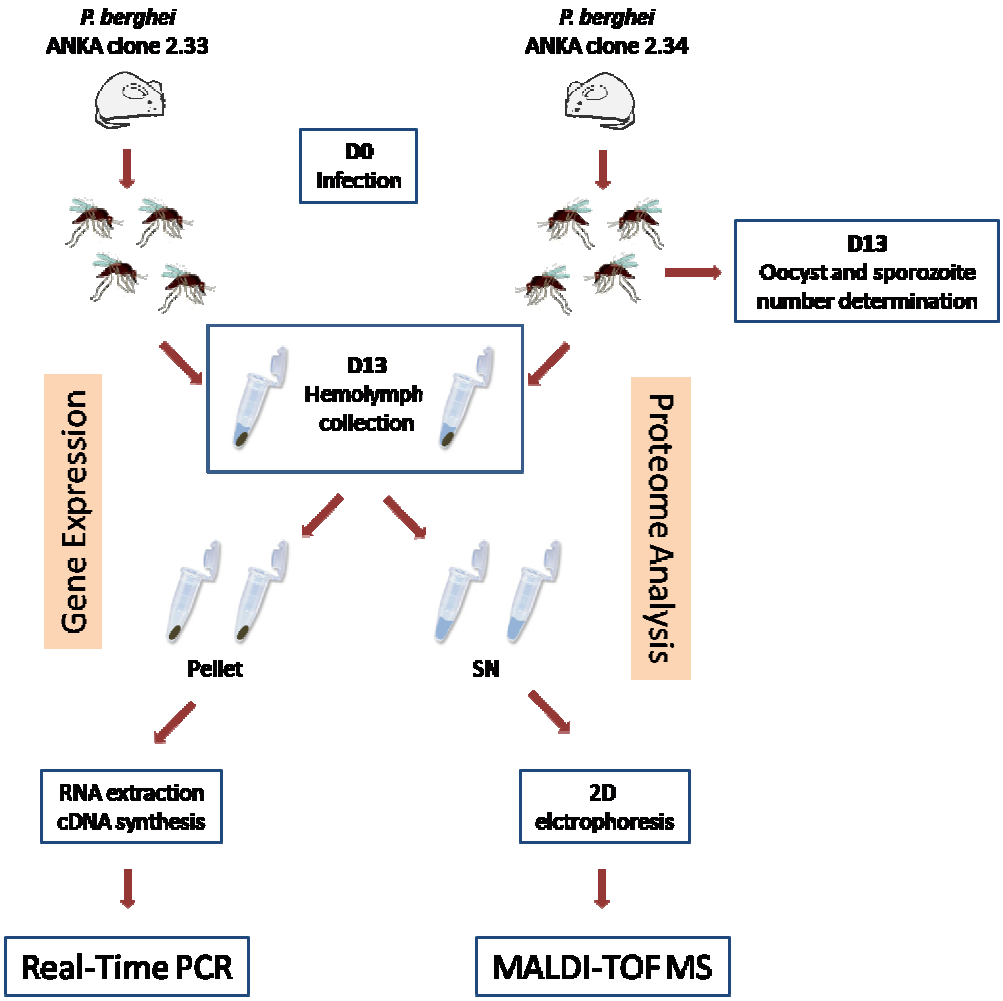


Figure VI.2.1 – Hemolymph gene expression and proteome analysis of infected *vs.* non-infected mosquitoes. Mice infected either with *P. berghei* ANKA clone 2.33 (non-infective to mosquitoes) or *P. berghei* ANKA clone 2.34 (infective to mosquitoes) were used to feed *An. gambiae Yaoundé* female mosquitoes. Hemolymph was collected at D13pi. Hemolymph pellets were used for gene expression analysis by RNA extraction, cDNA synthesis and Real-Time PCR. Hemolymph supernatants (**SN**) were used for proteome analysis by 2D electrophoresis and MALDI-TOF MS. At D13pi infection was monitored by determine oocyst and sporozoite number in the midgut of a few females infected with clone 2.34.

VI.2.3 – Hemolymph proteome analysis

The proteome of the hemolymph collected from non-infected mosquitoes (group 2.33) and infected mosquitoes (group 2.34) at D13pi was analyzed by two dimensional electrophoresis (2DE) and mass spectrometry (MS) to identify proteins whose levels are affected by sporozoite release into the hemolymph.

2DE allows a high resolution separation of proteins in a sample. This procedure includes two steps: firstly proteins are separated according to their isoelectric point (pI) – Isoelectric focusing (IEF) – and secondly according to their molecular mass – sodium dodecyl sulfate polycarylamide gel electrophoresis (SDS-PAGE). Separated proteins from different samples can then be compared to spots of specific proteins that exist in altered levels between samples, in this case between the hemolymph of non-infected (group 2.33) and infected mosquitoes (group 2.34). Proteins recovered from 2D gels are then analyzed and identified by MS.

VI.2.3.1 – Two dimensional electrophoresis

VI.2.3.1.1 – Sample preparation

Proteins were solubilized immediately upon hemolymph collection in a suspension buffer appropriated for 2DE composed of: 7M urea (Bio-Rad), 2M thiourea (Fluka/Sigma), 2% ASB-14 (Fluka/Sigma), 0.2% Bio-Lyte 3/10 Ampholyte (BioRad), 2% TritonX-100 (Sigma). Dithiothreitol (DTT) was added to the solution immediately prior to IEF to a final concentration of 20mM. Suspension buffer is prepared to assure the maximum protein unfolding and denaturation in order to obtain a better resolution in proteins spots separated by 2DE.

Samples were diluted in suspension buffer up to 120µl. Five µl per sample were used for total protein quantification using the RC-DC Protein Assay Kit II (Bio-Rad), according to manufacturer's instructions. Briefly, to 5µl of each sample were added 25µl of RC Reagent I. Mixture was vortexed and incubated 1min at rt. Next, 25µl of RC Reagent II

were added, and mixture was vortexed and centrifuged at 15000xg for 5min. The supernatant was replaced by 25.4µl of solution A' (composed of 250µl RC Reagent A plus 5µl of DC Reagent S). Samples were vortexed, sonicated for 4min at rt and vortexed again. To each sample 200µl of DC Reagent B were added. Samples were vortexed immediately and incubated for 15min and placed on a microplate to read their absorbance at 690nm on a spectrophotometer. Protein quantity of each sample was obtained by comparison with a standard curve composed of different loads of bovine serum albumin (BSA – Sigma) – 2 to 20 µg prepared by the same procedure.

VI.2.3.1.2 – Isoelectric focusing

IEF allows protein separation according to their isoelectric point (pI), the pH at which the protein global electric charge is null. When an electric field is applied on the sample, proteins migrate through an immobilized pH range until reach their respective pI. Once the charge is null proteins migration stops. IEF was performed using a Protean IEF Cell (BioRad) and 7cm immobilized pH gradient (IPG) strip, pH 5-8 or pH 3-10, non linear (NL) (BioRad).

Two IEF assays were performed each containing 8 samples:

Assay A: Experiments II, III, IV and V, groups 2.33 and 2.34;

Assay B: Experiments I, II, III and IV, groups 2.33 and 2.34.

Thirty µg of protein per sample were diluted in suspension buffer up to 125µl to which 0.5µl of bromofenol blue 0.5% and DTT was added to a final concentration of 20mM. Samples were loaded onto an IPG strip on a focalization tray and allowed to be incorporated into the strip by an active rehydration step (16h at 50V). Sample

dehydration and oxidation were prevented by covering both sample and strip with mineral oil.

IEF was performed as follows:

i) 15min at 250V – to eliminate salt excess,

ii) 2h gradient to 4000V in a slow ΔV,

iii) 4000V constant to reach a total of 11 000 Vh,

iv) 500V until program stops to avoid protein diffusion out of the strip.

VI.2.3.1.3 – Protein reduction/alkylation

This step is performed to saturate the gel with sodium dodecyl sulfate (SDS) and reduce the proteins with DTT. In a second phase, alkylation is achieved by replacing DTT by iodoacetamide that acts as a scavenger for the excess of DTT and alkylates proteins preventing their reoxidation, increasing spot sharpness and improving protein identification by MS.

Strips were incubated for

i) 15min in reduction equilibration buffer – 6M urea, 2% SDS, 375mM Tris-HCl pH 8.8, 20% glycerol, 130mM DTT,

ii) 15min in alkylation equilibration buffer – 6M urea, 2% SDS, 375mM Tris-HCl pH 8.8, 20% glycerol, 135mM iodoacetamide.

VI.2.3.1.4 – SDS-PAGE

SDS-PAGE was performed by placing the equilibrated IPG strips and a small paper 3MM soaked with 4µl of molecular mass ruler (Fermentas) on top of 12% polyacrylamide gels and sealing with 0.5% agarose. Gels were run on a Mini Protean III

apparatus (Bio-Rad) at 20V until the samples entered the acrlyamide and at 150V until the bromophenol blue left the gel.

VI.2.3.2 – Gel imaging and analysis

VI.2.3.2.1 – Gel staining

Proteins were fixed in the gels by incubating with fixation solution (45% methanol, 1% acetic acid) for 20min. Two staining protocols were followed: colloidal brilliant blue (CBB) staining and silver nitrate staining. CBB staining is compatible with most protein characterization methods such as MS analysis but is not the most sensitive. Silver staining is far more sensitive, but less reproducible and not compatible with all protein analysis methods. Either staining permits the visualization of the separated proteins that appear as spots in gels.

CBB staining was performed by:

i) incubating the gels with CBB solution – 34 % methanol, 17% ammonium sulfate, 0.5% acetic acid, 0.1% brilliant blue G250;

ii) decolorizing with hot milliQ water (55ºC) until obtaining a satisfying equalized relation between spot intensity and background.

Silver staining was performed to increase the number of spots obtained, as follows:

i) washing with miliQ water after CBB staining to remove all traces of blue;

ii) sensitizing with 0.05% (DTT) for 20min;

iii) staining with 0.1% silver nitrate for 20min;

iv) washing twice with milliQ water for 1min each time;

v) developing with 3% sodium carbonate and 0.02% formaldehyde until the desired coloration was obtained;

vi) incubating with 10% acetic acid for 10min to stop the reaction;

vii) washing with milliQ water twice for 5min each time.

VI.2.3.2.2 – Image analysis

Stained gels were scanned using a GS-800^{TM} Calibrated Densitometer (Bio-Rad) and the images were used for further analysis with the PDQuest 8.0 software (Bio-Rad) according to the manufactures instructions.

The PDQuest software allows for the comparison of protein spots present in the two sets of samples (hemolymph of non-infected mosquitoes (group 2.33) *vs*. infected mosquitoes (group 2.34)) to identify proteins that exist in altered levels in the two sets of samples. The software automatically detects proteins spots in gels, which is followed by a manual scrutiny to differentiate valid spots from artifacts.

Gels were analyzed simultaneously (both automatically by the program and manually) to identify identical spots existing in all gels - matching spots. One gel was then considered as the master gel, to which all others were compared for a quantitative analysis.

To avoid variability due to experimental factors, data resulting from the analysis of gels were normalized to the total density in gel image. Spots of interest were chosen based on quantitative (1.5 fold difference) and statistical (*t* test, $P<0.1$) analyses of groups 2.33 and 2.34, *ie*, non-infected *vs.* infected mosquitoes.

VI.2.3.3 – Protein identification by MS

Spots of interest were excised from the gel using a pipette tip cut and placed in a microtube. A piece of gel containing no spot was used as a blank control. Tubes were kept at -20ºC.

To perform protein identification, spots were submitted to a digestion with trypsin (tryptic digestion) and separated from the gel. The resulting peptides were sequenced by MS.

VI.2.3.3.1 – Tryptic digestion

As silver nitrate coloration is not the most compatible with MS peptide analysis, spots excised from gels colored with silver nitrate need a previous washing step to remove all traces of dye. Washing was performed by incubating the pieces of gel containing the spots of interest with a mixture of potassium ferricyanide and sodium thiosulfate (1:1 ratio) until coloration disappeared (1-2min), followed by a minimum of 5 washes with milliQ water.

1. Gel pieces were incubated with a buffer containing 50% acetonitrile (ACN - Merck) for 15min under agitation, to remove solvents, dyes and other contaminants present in the gel. Supernatants were removed and the step repeated.

   Gel pieces were dehydrated in 50µl of ACN for 5min under agitation. Supernatants were replaced by 50µ of NH_4HCO_3 buffer (Fluka – Sigma Aldrich) 25mM, pH 7.8 for 5min under agitation to rehydrate the samples.

   Another 50µl of ACN were added and samples were incubated for 5min under agitation. Supernatants were discarded and gel pieces were dried in a Speed-Vac (Savant) for a few minutes.

2. Gel pieces were submitted to reduction and alkylation to ensure protein denaturation.

   Samples were incubated with 50µl of NH_4HCO_3 buffer to which was added DTT 100mM for 45min at 56ºC. Supernatants were replaced by NH_4HCO_3 buffer with iodoacetamide 25mM for 30min in the dark. Supernatants were discarded.

3. Gel pieces were washed twice with a mixture of NH_4HCO_3 buffer and ACN (1:1 ratio) and once with pure ACN. Supernatants were discarded and gel pieces were dried in a Speed-Vac (Savant) for a few minutes.

4. Digestion was performed in the gel by adding 5µl of trypsin (Promega) - 15ng/µl NH_4HCO_3 buffer. After complete absorption, 2µl of NH_4HCO_3 buffer were added and samples were incubated ON at 37ºC.

   Peptides were excised from gel pieces by incubating with a solution composed of 70% ACN and 3% formic acid, sonicated for 10min and desalted and concentrated with a Zip Tip C_{18} microcone (Millipore) and eluting in 4µl of 50% ACN.

VI.2.3.3.2 – Mass Spectrometry analysis

Peptides resulting from tryptic digestion (tryptic fragments) were analyzed by MALDI-TOF (Matrix Assisted Laser Desorption Ionization – Time Of Flight) mass spectrometry. This technique allows determining the mass of the peptides present in a mixture to a subpicomolar concentration.

Samples were co-crystallized with a matrix (α-cyano hydroxycinnamic acid) and bombarded by a laser light. The acid nature of the matrix provides the protons that ionize the sample, creating monocharged ions ($[M+H^+]$) that are accelerated by a uniform electric field (19kV) with a retarded extraction (200ns). Ions traverse a field free flight tube in a speed that is inversely proportional to their mass, *ie*, smaller and more charged ions move faster. An internal calibration is used consisting of trypsin

autolysis resulting fragments. This analysis results in a list of masses of the different digested fragments for each spot.

To identify the proteins, mass values were compared with calculated values existing in proteomic and genomic databases by using informatics tools available in the internet, as Protein prospector (http://prospector.ucsf.edu) and Matrix Science (www.matrixscience.com). To this comparison a value (score) is attributed based on the number of proteolytical fragments obtained whose masses might correspond to the masses of the proteolytical fragments in the databases, and on the precision which the masses are determined. Known proteins (identified or reported as genes products) are then characterized based only on mass determination.

VI.2.4 – Hemolymph gene expression analysis

Hemolymph pellets, containing hemocyte cell debris, collected from infected (group 2.34) and non-infected (group 2.33) mosquitoes were used to determined the expression of the genes coding for the proteins identified by MS and other immune mosquito markers known to respond to malaria infection (AgCTL4, AgCLIPA2, AgPPO5 and AgSRPN2). To evaluate expression, RNAs extracted from the pellets, were used to synthesize cDNAs that were semi quantified by Real-Time PCR.

VI.2.4.1 – cDNA synthesis

RNA was extracted from pellets using Trizol® reagent (Invitrogen) according to the manufacturer's instructions, as described in section III.23.1.2.

Total RNA in samples was quantified in GeneQuant (GE Healthcare) using a 1:50 dilution of the RNA solution in DEPC treated water. In parallel, 2µl of the each RNA

solution were loaded into a denaturing 2% agarose gel to verify the integrity of RNA and confirm quantification.

Roughly the same amount of RNA was used for cDNA synthesis as follows:

i) RNA was diluted in 8µ of DEPC treated water to which was added 1µl of DNase buffer (Promega) and 1µl of DNase (Promega). Solutions were incubated at 37ºC for 30min.

ii) To the solution was added 18µl of the Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT – Promega) mix: 8µl of DEPC treated water, 6µl of MMLV-RT buffer, 1.5µl of dNTP's (Sigma) and 5µl of oligo d(T) (Roche). Samples were incubated at 65ºC for 5min and then at 37ºC for 10min. One µl of MMLV-RT enzyme was added to each sample and incubated at 37ºC for 50min followed by 75ºC for 5min. Samples were kept at -20ºC.

VI.2.4.2 – DNA standard curve preparation

DNA was extracted from *Anopheles gambiae Yaoundé* abdomens using a phenol-chloroform protocol to generate a standard curve to be used for quantification by Real-Time PCR.

i) Abdomens were incubated with 25µl of lysis buffer (Tris 40mM pH8.0; EDTA 80mM pH8.0; SDS 2% pH7.2) and 75µl of Pronase E (Sigma – Aldrich) 2mg/ml at 37ºC ON.

ii) To the tube was added 300µl of milliQ water and 500µl of phenol. The tube was vortexed and centrifuged at 14000rpm for 10min.

iii) The resulting aqueous phase was transferred into a new tube and mixed with 500µl of phenol:chloroform:isoamyl alcohol. Samples were centrifuged at 14000rpm for 10min.

iv) The resulting aqueous phase was transferred into a new tube and mixed with 45µl of sodium acetate 3M pH5.0 and 1.0ml of ice cold absolute ethanol. For DNA precipitation the solution was incubated at -80ºC for 5h.

v) The solution was centrifuged at 14000rpm for 15min. The supernatant was discarded and the pellet was washed with 800µl of ethanol 80%.

vi) The tube was centrifuged at 14000rpm for 10min and the supernatant discarded. The pellets dried ON at rt.

vii) DNA pellets were dissolved in 20µl of milliQ water.

viii) DNA was quantified in GeneQuant (GE Healthcare) using a 1:50 dilution.

ix) Serial dilutions of DNA were performed to generate a DNA standard curve composed of the following concentrations: 250, 25, 2.5, 0.25 and 0.025ng/µl.

VI.2.4.3 – Real-Time PCR

This technique provides a real-time monitoring of DNA amplification. This is possible by the use of a compound (SYBRGreen) that exhibits fluorescence when bound to double strand DNA (dsDNA). Thus, in each amplification-cycle the fluorescent signal increases proportionally to the number of dsDNA molecules. The more molecules of DNA present initially in the sample the lower the cycle to detect the signal. The use of a standard curve of known DNA concentrations allows the quantification of the same DNA present in each sample. To circumvent sample variation, results for each tested gene are normalized with the results obtained for an internal control, in this case, the mosquito ribosomal protein S7 (AgS7).

Primarily reactions were optimized for primer concentration and annealing temperature testing different concentrations and temperatures for each primer and the standard curve. The primers and the respective optimized conditions are listed in the Table VI.2.1.

The reaction was performed using iQ SYBR Green Supermix (Bio-Rad) that includes buffer, dNTPs, Taq enzyme, SYBR Green I, fluorescein and stabilizers, concentrated 2x. The reaction protocol included a standard PCR amplification protocol followed by a melting curve to determine for each sample the temperature at which half the molecules are denatured (Tm). As Tm is highly specific for each amplified product it is useful to verify contamination and primer dimmer formation. cDNA was used in a dilution of 1:10 of the initial solution for better results and placed in triplicate on a 96-well reaction plate. Reactions were performed on a final volume of 20µl, primer concentration varied as descbribed in Table VI.2.1. Cycle conditions included an initial step of denaturation at 95ºC for 10min, followed by 40 cycles of denaturation at 95ªC for15sec and 1min of annealing and extension at varying temperatures (see table VI.2.1).

Real Time amplification data for each gene was analyzed by the iCycler software (BioRad). Statistical data analysis was performed by applying a two-way ANOVA test using the GraphPad software (Prism).

Table VI.2.1

Primers used for Real Time PCR

Name	*NCBI gene ID*	*Primer Sequence*	*Stock Conc.*	*Final Conc.*	*Ann. Temp.*
AgS7	118785342	Fwd: 5' – GAGGTGGTCGGCAAGCGTATC – 3' Rev: 5' – CGGCGCTCGGCAATGAACAC – 3'	50µM	0,3µM	62ºC
AgCTL4	158294013	Fwd: 5' – TTGAATTGTTTGATGCCGTGTCCTA – 3' Rev: 5' – GGCGCTCGTTGGTATCCTTTATTGT – 3'	50µM	0,3µM	62ºC
AgCLIPA2	118793177	Fwd: 5' – CTTAACAACATTGCCGTGCTGGAG – 3' Rev: 5' – AGAACGCACCGACGCAGATAT – 3'	50µM	0,3µM	60ºC
AgPPO5	11877159	Fwd: 5' – CTCGGCACGGGGATAGATTTTGT – 3' Rev: 5' – CATGCTGCCCCCGTTATTGGTC – 3'	50µM	0,3µM	62ºC
AgSRPN2	158286630	Fwd: 5' – AGTCTGAGGGCGCGGTCATTACG – 3' Rev: 5' – GGGCCCGCCGCGAGTGCCATAGA – 3'	50µM	0,3µM	60ºC
AGAP001420	158302077	Fwd: 5' – TCCTTCGATTTCCAAACAGG – 3' Rev: 5' – TTCTGGTTCCACTCCGATTC – 3'	50µM	0,3µM	58ºC
AGAP002401	58383449	Fwd: 5' – AGCCGAGGAAGAGTTCAACA – 3' Rev: 5' – TCCAGTCTTCGAACATGCTG – 3'	50µM	0,9µM	60ºC
AGAP002518	158290877	Fwd: 5' – GAGGACCTCCTGCAGAACAG – 3' Rev: 5' – ATTTTTCACCACCTCGATGC – 3'	50µM	0,3µM	62ºC
AGAP004031	158298313	Fwd: 5' – CTGGCTACGTACCGAACGAT – 3' Rev: 5' – CGATCGTCTTCGAGTCCTTC – 3'	50µM	0,3µM	65ºC
AGAP005645	158294533	Fwd: 5' – GGTGCAGTGAAGACGGAAAT – 3' Rev: 5' – GATGAGCTCGTGCACCTGTA – 3'	50µM	0,3µM	62ºC
AGAP007593-A	158285381	Fwd: 5' – GTCCCTCGTGGAAATGAAAA – 3' Rev: 5' – GGCCTTCAACCATTTTGCTA – 3'	50µM	0,9µM	58ºC
AGAP007593-B	158285381	Fwd: 5' – ATTTACCGCCATCCACAAGA – 3' Rev: 5' – GGTTGCGTGAGAGGAGAAGT – 3'	50µM	0,3µM	62ºC
AGAP010130	158299169	Fwd: 5' – CGAGCAGAGTCCGAATTAGC – 3' Rev: 5' – ATCATCCCCACTTTCAGTGC – 3'	50µM	0,3µM	62ºC
AGAP011053	158285040	Fwd: 5' – GGACGTTTTCGCACAGGTAT – 3' Rev: 5' – GTGGCGATAACCCTCGTCTA – 3'	50µM	0,3µM	62ºC
AGAP0011833	158300868	Fwd: 5' – CTTTCCGGTCGAATTTTTCA – 3' Rev: 5' – ACGTCCAATGTTTGCCATTT – 3'	50µM	0,3µM	62ºC

Primers are described by **Name, NCBI identification, Sequence, stock concentration (conc.), Concentration (conc.) to use in reaction** and **Annealing temperature (Ann. Temp.). Fwd**: forward; **Rev**: reverse.

VI.2.5 – Eicosanoid biosynthesis impact on infection

Some of the proteins identified by 2D with altered levels in infected mosquitoes have a role or are involved in fatty-acid metabolism and eicosanoids biosynthesis. As such, mosquitoes were injected with either an inhibitor or a substrate for eicosanoids biosynthesis to evaluate their influence on *P. berghei* infection.

VI.2.5.1 – Solution preparation and toxicity

Indomethacin (IN –Fluka – Sigma) is a COX inhibitor that hinders prostaglandin (PG) synthesis and arachidonic acid (AA – Fluka – Sigma) is a COX and LOX substrate that promotes the synthesis of PG, leukotrienes and lipoxins (Figure VI.1.1). Both compounds were used to prepare solutions of 0.1, 1 and 10mg/ml in Schneider medium (Sigma) with 10% ethanol.

Solutions were injected into *Anopheles gambiae Yaoundé* female mosquitoes using a Nanojet II Auto-nanoliter injector (Drummond Scientific). The glass needle inserted in the injector was filled with mineral oil (Sigma). Some of the oil was discarded and replaced by a solution of IN, AA or Schneider medium with 10% ethanol that was used as a control (C). For each group, 20 female mosquitoes were injected intratoraxically with 69nl of the respective solution. Mosquitoes were allowed to recover and survival was followed for 7 days.

VI.2.5.2 – Mosquito infection and injections

To evaluate the influence of the compounds on infection, 3-5 five days old *A. gambiae Yaoundé* female mosquitoes were allowed to feed on mice infected with *P. berghei ANKA clone 2.34.*

At D7pi, when oocysts are still developing, infected mosquitoes (~100♀) were injected with either Schneider medium (control group – C_7), indomethacin 1mg/ml

(indomethacin group – IN_7) or AA 1mg/ml (AA group – AA_7). To control infection in injected mosquitoes, 10 female mosquitoes were dissected at D8pi and midguts used to count oocyst numbers.

Injections of *P. berghei* infected mosquitoes (non-injected yet) were performed also at D12pi, when the majority of oocysts are developed and sporozoites are starting to be released in to the hemolymph – groups C_{12}, IN_{12} and AA_{12}. In this case, infection was verified at D14pi.

VI.2.5.3 – Sporozoite load determination

The impact of IN and AA injection on malaria infection in mosquitoes was evaluated by comparing sporozoite load in salivary glands. For this, mosquitoes were dissected at D21pi and salivary glands were collected to a glass homogenizer where they were crushed to release sporozoites and mixed with 400µl of PBS. The number of sporozoites in the suspension was counted using a hematocytometer. Data was evaluated for statistical significance by applying a ratio t test on the GraphPad software (Prism).

Experiments performed to evaluate the impact of eicosanoids biosynthesis on *Plasmodium* infection in the mosquito are resumed in Figure VI.2.2.

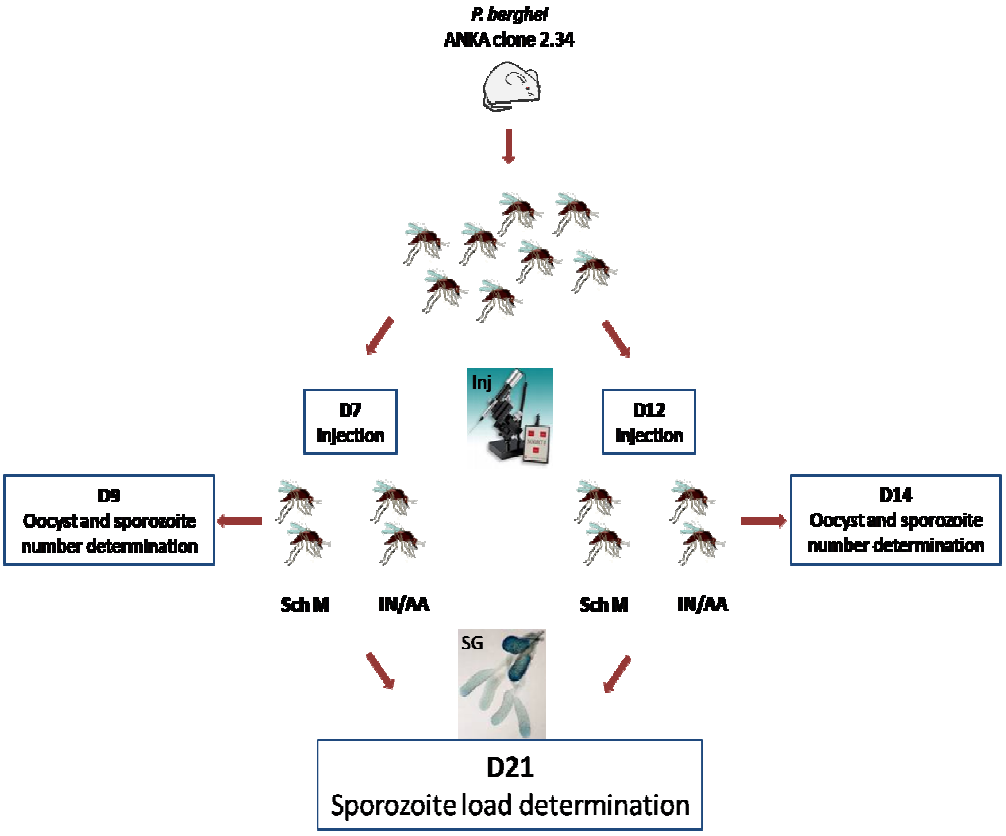


Figure VI.2.2 – Mosquito injections to evaluate eicosanoids biosynthesis impact on malaria infection. Female *An. gambiae Yaoundé* mosquitoes were fed on mice infected with *P. berghei* ANKA clone 2.34. One set of mosquitoes were injected with Schneider medium (**Sch M**) or indomethacin (**IN**)/AA at D7pi. At D9pi infection was monitored by determining oocyst number in the midgut of a few injected females. Injections were repeated on the other set of mosquitoes at D12pi. Infection was monitored at D14pi. Sporozoite load on salivary glands (**SG**) was determined at D21pi. **Inj**: injector.

VI.3 – Results

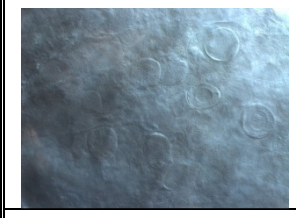
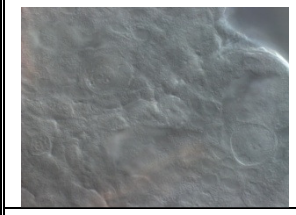
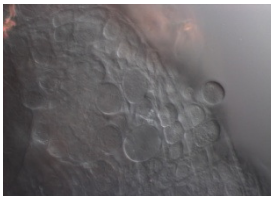
VI.3.1 – Mosquito infections

To monitor the infection in each experiment, midguts were dissected at D13pi from 20 female mosquitoes from group 2.34: 10 were used directly to determine oocyst load and 10 were crushed to determine sporozoite load. As *P. berghei* infection in the mosquito is asynchronous it is hard to predict at which exact timepoint and rate sporozoite are released from the midgut. A microscopical observation of infected midguts allowed a surveillance of matured oocysts and sporozoites while the number of sporozoites in the midguts indicated the presence of fully developed invasive forms ready to be released into the hemolymph. When both these criteria were fulfilled, it was assumed that in the majority of mosquitoes sporozoites had been or were being at that time released into the hemolymph. Table VI.3.1 summarizes the information about the infection status at D13pi of groups 2.34 in each experiment performed. A few mosquitoes belonging to group 2.33 were also dissected to confirm the non-existence of midgut infection.

Despite the differences in oocyst load, midguts carrying more than one oocyst displayed asynchronous infection, as some oocysts were still immature and developing while others were fully developed exhibiting thousands of sporozoites ready to release into the hemolymph. Also, in all experiments sporozoites were recovered from the dissected midguts of groups 2.34. This indicated that oocysts were ready to release sporozoites and that they were probably present at the mosquito hemocel at the time of hemolymph collection.

Table VI.3.1

Infection parameters at D13pi in *P. berghei* infected mosquitoes (groups 2.34)

Exp	*Infection D13pi – group 2.34*						*N mosq*	
	inf mdgt OM		Inf mdgt	ooc/ mdgt		N spz	2.33	2.34
	(200x)	(400x)	(%)	Avg	SD			
I	-	-	50	ND	ND	$1,5x10^5$	233	253
II			100	24,33	19,37	$2,9x10^5$	239	246
III			55	2,11	2,71	$6,6x10^4$	241	259
IV			100	9,1	7,98	$1,2x10^5$	252	257
V			100	40,11	30,49	$3,8x10^5$	80	78

Parameters included: Observation of infected midguts by optical microscopy (**inf mdgt OM**) using amplifications of 200 and 400x; percentage of infected midguts (**Inf Mdgt**); the average (**Avg**) number of oocysts per midgut (**ooc/mdgt**) and standard deviation (**SD**); the number of sporozoites in the midgut (**N spz**) and the number of mosquitoes (**N mosq**) used to collect hemolymph at D13pi in groups of non-infected (2-33) and *P. berghei* infected (2.34) mosquitoes. **Exp**: experiment.

VI.3.2 – Hemolymph proteome analysis

VI.3.2.1 – Two-dimensional electrophoresis

Two proteomic assays (A and B) were performed including the hemolymph protein samples of 4 experiments each.

In assay A, 30µg of hemolymph protein samples from experiments II-V (both groups 2.33 and 2.34) were loaded onto a 7cm IPG strip with a pH range of 3-10, NL. IEF was performed as described in the section VI.2.3.1.2. In figure VI.3.1 are displayed two 2D maps representative of this analysis, corresponding to groups 2.33 and 2.34 of experiment IV. All 8 maps analyzed (2 groups (2.33 and 2.34) x 4 replicates (experiments II-V)) are displayed in Annex 1.

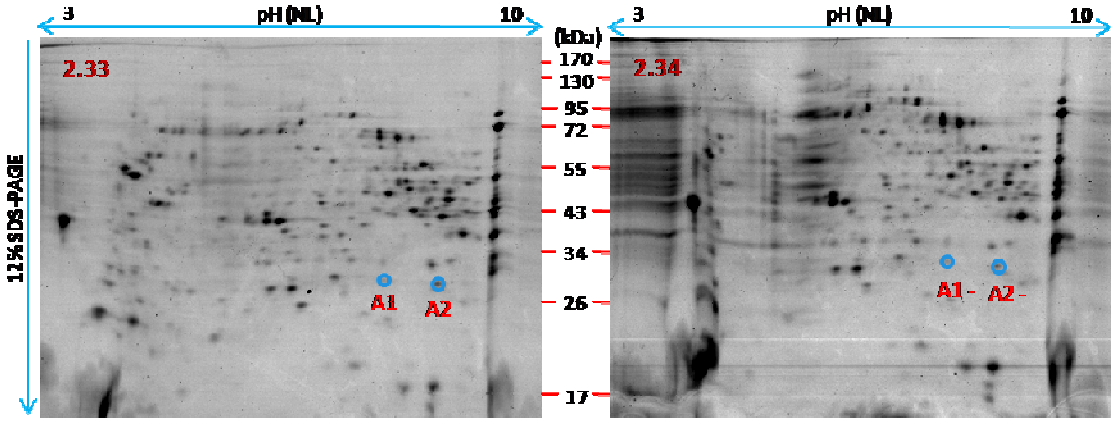


Figure VI.3.1 – 2D maps of CBB stained hemolymph proteins from malaria non-infected (2.33) and infected (2.34) mosquitoes. Maps shown in figure are representative of the first analysis and correspond to experiment IV. Hemolymph samples were collected at D13pi and submitted to 2DE (IEF on 7cm **pH3-10NL** strips followed by **12% SDS-PAGE**). Spots were analyzed by CBB staining and by PDQuest software analysis. Around 200 spots were detected in each gel. Spots in gels were analyzed qualitatively (fold difference >1.5) and quantitatively (t test, P<0.1). Spots **A1** and **A2** show down-regulation (-) in all 2.34 maps. Bands of the protein marker are represented in **kDa** in between maps.

The 2D maps obtained show reproducibility and resolution. This indicates that the buffer used for protein solubilization was adequate for these samples. However in the acidic area of the maps resolution is not as desirable reflecting a property of the detergent used (ASB-14) that hinders a correct solubilization of acidic proteins.

The 2D maps showed around 200 protein spots each, 69 of which matched all gels analyzed. The replicates were analyzed by the PDQuest software (BioRad). Spots were considered differentially regulated when the mean spot intensity in 2.34 replicates was 1.5-fold above or below the mean spot intensity in 2.33, and when applying a *t* test, $P<0.1$.

No protein induction or repression was observed, *ie* the appearance or disappearance of protein spots in 2D maps of infected mosquitoes (2.34) when comparing to 2D maps of non-infected mosquitoes (2.33). It is important to bear in mind that the 7cm gels used do not allow a spot resolution as high as that of a 17cm gel. The latter obliges that at least 100µg of protein are analyzed, allowing for the detection of spots corresponding to proteins with a low level of expression. As for differential regulation, no protein up-regulation was detected in 2.34 maps, corresponding to infected mosquitoes. Only two spots were identified as down-regulated in infected mosquitoes: spots A1 and A2.

The low number of spots retrieved from analysis A led us to make some changes in the analysis procedure. Alterations were made in pH range, narrowing the area to analyze, and by using a silver nitrate staining followed the CBB staining to allow the detection of more spots.

Two 2D maps representative of this assay are displayed in Figure VI.3.2, corresponding to groups 2.33 and 2.34 of experiment III. All 8 maps analyzed (2 groups (2.33 and 2.34) x 4 replicates (experiments I-IV)) are displayed in Annex 1.

Around 200 spots were detected in all maps analyzed, of which 130 had a match in all maps. Replicate gels were analyzed as previously described for assay A to identify differentially regulated spots in 2.34 maps – infected mosquitoes.

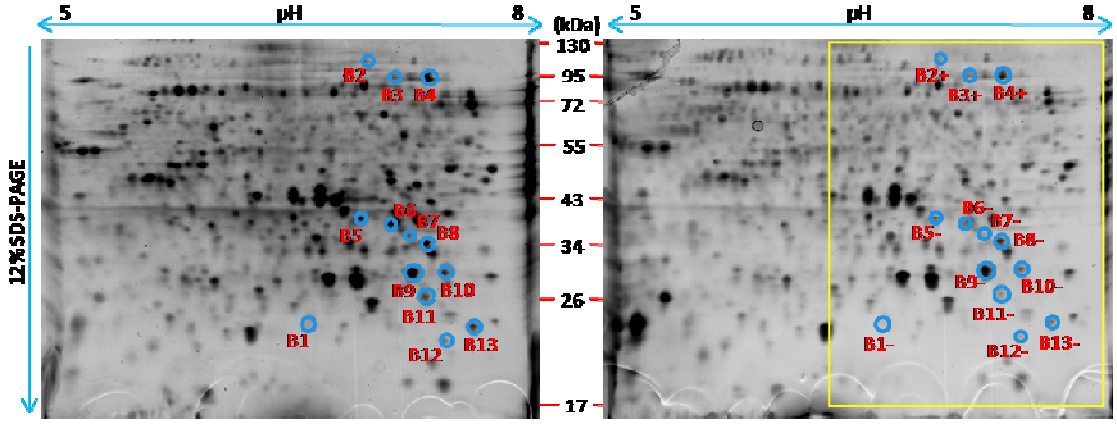


Figure VI.3.2 – 2D maps of silver nitrate stained hemolymph proteins from malaria non-infected (2.33) and infected (2.34) mosquitoes. Maps shown in figure are representative of analysis B and correspond to experiment III. Hemolymph samples were collected at D13pi and submitted to 2DE (IEF on 7cm **pH5-8** strips followed by **12% SDS-PAGE**). Spots were analyzed by silver nitrate staining and by PDQuest software analysis. Around 200 spots were detected in each gel. Spots in gels were analyzed qualitatively (fold difference >1.5) and quantitatively (t test, P<0,1). Spots **B5** to **B13** show down-regulation (-), while spots **B2** to **4** show up-regulation (**+**) in all 2.34 maps. Spot **B1** analysis was performed in three independent experiments, as experiment II was considered an outlier. Bands of the protein marker are represented in **kDa** in between maps. The region defined by the yellow rectangle is detailed in Figure VI.3.4.

As for assay A, no induction or repression was detected. Thirteen of the spots found to be differentially regulated in 2.34 maps when compared to 2.33 maps (in infected *vs.* non-infected mosquitoes). Three spots were up-regulated in infected mosquitoes (B2, B3 and B4) while ten were down-regulated (B1, B5-B13 – Figure VI.3.3 and Figure VI.3.4).

All spots showed the same up/down regulation in all experiments, except for spot B1 that showed up-regulation in experiment II and down regulation in the others. A thorough analysis of highlighted spots revealed a mismatch for spot B7, which was retrieved from analysis.

Differentially regulated spots in analysis B are shown in detail in Figure VI.3.3 that corresponds to the yellow box in Figure VI.3.2 and the differential regulation of each spot highlighted in both assays is summarized in Figure VI.3.4 Protein levels for each highlighted spot are displayed in Annex2.

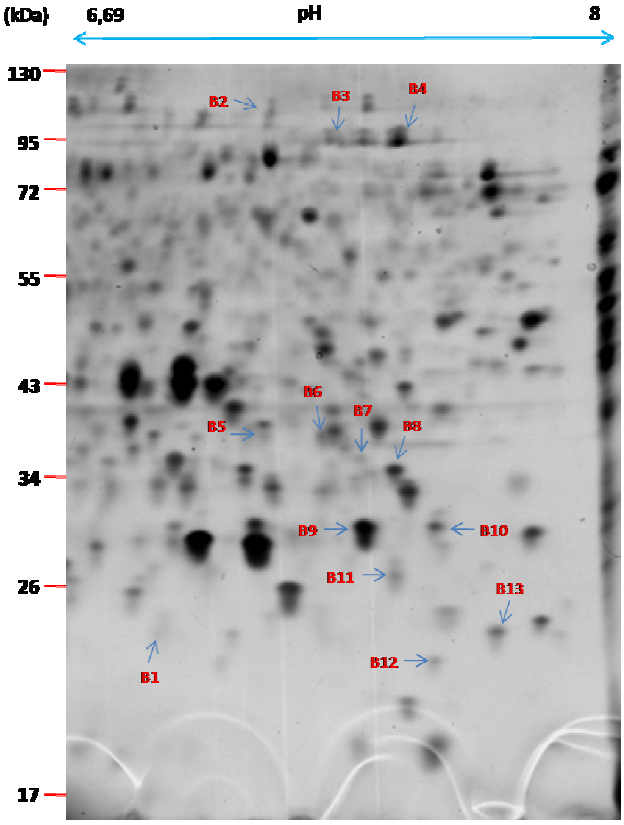
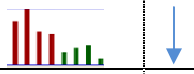
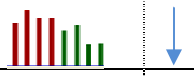
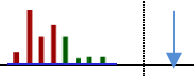
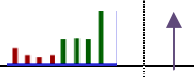
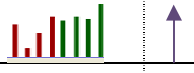
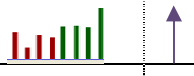
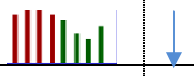
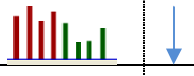
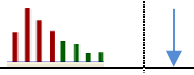
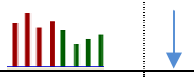
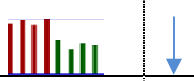
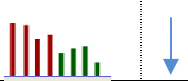
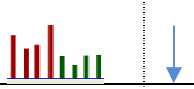
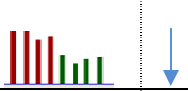
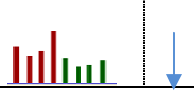


Figure VI.3.3 – Selected area from a 2D map of hemolymph proteins of malaria infected mosquitoes. Hemolymph was collected at D13pi from *P. berghei* infected female mosquitoes. Thirty micrograms of hemolymph protein sample was submitted to 2DE (IEF on 7cm pH5-8 IPG strips followed by 12% SDS-PAGE). This image illustrates in detail the area limited by the yellow box in figure VI.3.3, pH range: 6,69-8. Spots indicated were found to be differentially regulated regarding non-infected mosquitoes after a qualitative and quantitative analysis performed by the PDQuest software.

Figure VI.3.4 – Differential regulation of protein spots highlighted in assyas A and B. Protein spots found to be differentially regulated in the hemolymph of *P. berghei* infected mosquitoes at D13pi. The differential regulation for each spot is expressed graphically for each experiment. In graphs, red bars represent spot intensity/protein level in hemolymph samples of non-infected mosquitoes (groups 2.33) while green bars correspond to samples of *P. berghei* infected mosquitoes (groups 2.34). Bars are disposed sequentially according to experiment: for spots A1-A2 experiments II to V (analysis A) and for spots B1-B13 experiments I-IV (analysis B). Spot regulation is also characterized by trend (↑ - up-regulation; ↓ - down-regulation) and ratio of spot intensity between groups 2.34 and 2.33 (**Ratio 2.34/2.33**), represented by the mean (**M**) and the standard deviation (**SD**).

Spot	*Regulation*	*Ratio 2.34/2.33*	
		M	*SD*
A1		0,33	0,17
A2		0,59	0,20
B1		0,15	0,07
B2		3,81	2,07
B3		2,25	1,83
B4		2,01	0,86
B5		0,65	0,20
B6		0,57	0,22
B7		0,38	0,21
B8		0,66	0,18
B9		0,57	0,09
B10		0,52	0,20
B11		0,51	0,10
B12		0,51	0,09
B13		0,57	0,11

VI.3.2.2 – Protein identification by MS

Spots highlighted in analysis A and B as differentially regulated in infected mosquitoes (2.34) were removed from the gel and analyzed by MALDI-TOF. Table VI.3.2 shows the identification of the highlighted spots as retrieved from MS analysis. Peptides matched to the identified proteins by MALDI-TOF MS are displayed in detail in Annex3, along to the protein sequence itself.

Table VI.3.2

Protein spot identification by MALDI-TOF MS.

spot	*Identification Ensemble*	*Organism*	*MW (kDa)*	*pI*	*NCBI Accession number*	*Score[a] Mascot*
A1	AGAP010130	An. gambiae str. PEST	30,393	6,64	158299170	46
A2	AGAP011833	An. gambiae str. PEST	32,705	8,80	58394357	129
B3	AGAP002518	An. gambiae str. PEST	80,605	6,10	118782666	322
B4	AGAP002518	An. gambiae str. PEST	80,605	6,10	118782666	520
B5	AGAP011053	An. gambiae str. PEST	36,262	5,94	158285041	109
B6	AGAP007593	An. gambiae str. PEST	38,616	6,27	58378155	42
B8	AGAP004031	An. gambiae str. PEST	34,234	8,37	158298314	191
B9	AGAP001420	An. gambiae str. PEST	28,749	6,34	58396165	141
B10	AGAP002401	An. gambiae str. PEST	25,851	6,78	31207169	51
B11	AGAP005645	An. gambiae str. PEST	26,819	6,78	158294534	243

Spots retrieved in analysis A and B as differentially regulated in infected mosquitoes at D13pi are identified by: the Ensemble ID number, the organism to which the proteins belong to, the molecular weight (**MW**), given in kDa, the protein isoelectric point (**pI**), the NCBI accession number, and the Mascot scores ([a]) – scores higher than 54 indicate a positive identification ($p<0,05$), scores below indicate a probable identification. Spots B3 and B4 were identified as the same protein.

For spots B1, B7, B12 and B13, protein amount retrieved from gel was not sufficient to produce a result in MS analysis, even though spots were recollected in pools from more than one gel. Further analyses should be performed with an increased protein load.

Spots A1, B6 and B10 identification score was lower than desired for statistical significance ($P<0.05$), but provided a probable result.

Spot B6 reports to a gene that has 2 transcripts (AGAP007593-A and AGAP007593-B). The protein identified corresponds to the transcript AGAP007593-B.

Spot B2 is not represented in table VI.3.2 due to poor identification score. Yet, the best result matched a *P. chabaudi* protein (NCBI accession number – 70939505).

Spots B3 and B4 were identified as the same protein. These spots appear to have the same molecular weight (which corresponds to the one predicted by protein sequence analysis – 80kDa) but a different pI. This reflects a post-translational modification in the protein that affects its global charge. Also, both spots show a pI in the maps (above 7.0) higher than that predicted (6.10) from the sequence, indicating that more than one modification occurs in the protein. The fact that both spots showed an up-regulation in infected mosquitoes indicates that the infection does not lead to a change of one form into the other, that is, there is no preferential form of this protein that is up-regulated.

Spot B10 shows a molecular mass higher than predicted, also pointing to a post-translational modification.

As in assay A, a non linear pH range strip was used making it difficult to predict if the observed pIs correspond in fact to the predicted ones. As for analysis B, none of the spots identified seems to have the predicted pI – spots B5, B6, B9and B11 seem to have a higher pI while B8 seems to have a lower pI. This also points to protein modifications. The nature of the modifications was not accessed and requires further analysis.

To attribute a function to each protein, they were investigated in NCBI, Vectorbase and InterPro. As no functional data are available yet for any of the identified proteins, the functions provided are predicted based on protein aminoacid sequence and motifs present. Predicted protein functions are shown in Table VI.3.3. Proteins identified by MS are predicted to be involved in cellular processes such as fatty acid metabolism, redox metabolism, aminoacid synthesis, glycolysis and proton transport.

Table VI.3.3

Protein families of identified spots

spot	*ID*	*Protein family*	*Predicted Process*	*Reg.*
A1	AGAP010130	Enoyl-COA-Hydratase/isomerase	Fatty acid metabolism	↓
A2	AGAP011833	Enoyl-COA-Hydratase/isomerase	Fatty acid metabolism	↓
B3 **B4**	AGAP002518	Glutamate-5 kinase Aldehyde dehydrogenase Delta-l-pyrroline-5-carboxylate synthetase Aspartate/Glutamate/uridylate kinase Gamma-Glutamyl-phosphate reductase	Aminoacid biosynthesis	↑
B5	AGAP011053	Aldo/Keto reductase	Redox/Fatty acid metabolism	↓
B6	AGAP007593	Glycerol-3-phosphate dehydrogenase Glycerol-3-phosphate dehydrogenase, N terminal Glycerol-3-phosphate dehydrogenase, C terminal Potassium uptake protein TrKA	Redox metabolism Cation transport	↓
B8	AGAP004031	Electron transport flavoprotein, alpha subunit Electron transport flavoprotein, alpha subunit C terminal Electron transport flavoprotein, alpha/beta subunit C terminal	Electron transport Fatty acid metabolism	↓
B9	AGAP001420	Phosphoglycerate mutase Phosphoglycerate mutase 1 Phosphoglycerate/Bisphosphoglycerate mutase	Metabolism Glycolysis	↓
B10	AGAP002401	ATPase, V1/A1 complex, subnit E	Proton (H+) transport Energy	↓
B11	AGAP005645	Short-chain dehydrogenase/reductase SDR Glucose/Ribitol dehydrogenase Insect alcohol dehydrogenase 2,3-dihydro-2,3dihydro benzoate dehydrogenase Polyketide synthase, KR	Redox metabolism	↓

Protein identification/characterization according to MS. Table shows the sequence based prediction of protein family/function and the processes in which they may be involved, as described in Vectorbase. Regulation (**Reg**) in infected (2.34) *vs.* non-infected (2.33) mosquitoes is also shown.

VI.3.3 – Hemolymph gene expression analysis

The expression of the proteins identified by 2DE and MALDI-TOF MS as differentially regulated in *P. berghei* infected mosquitoes at D13pi was also evaluated at the transcriptional level. RNA from the pellets of the hemolymph samples collected was converted into cDNA and each gene was amplified by Real-Time PCR, using a DNA standard curve for quantification and AgS7 gene amplification for normalization.

This analysis also included some immune markers known to respond to infection: AgCLIPA2, AgCTK4, AgPPO5 and AgSRPN2.

Figure VI.3.5 shows an example of Real-Time amplification curves retrieved by the iCycler software (BioRad), in this case obtained for AgPPO5 gene amplification. The curves for all genes are displayed in Annex 4.

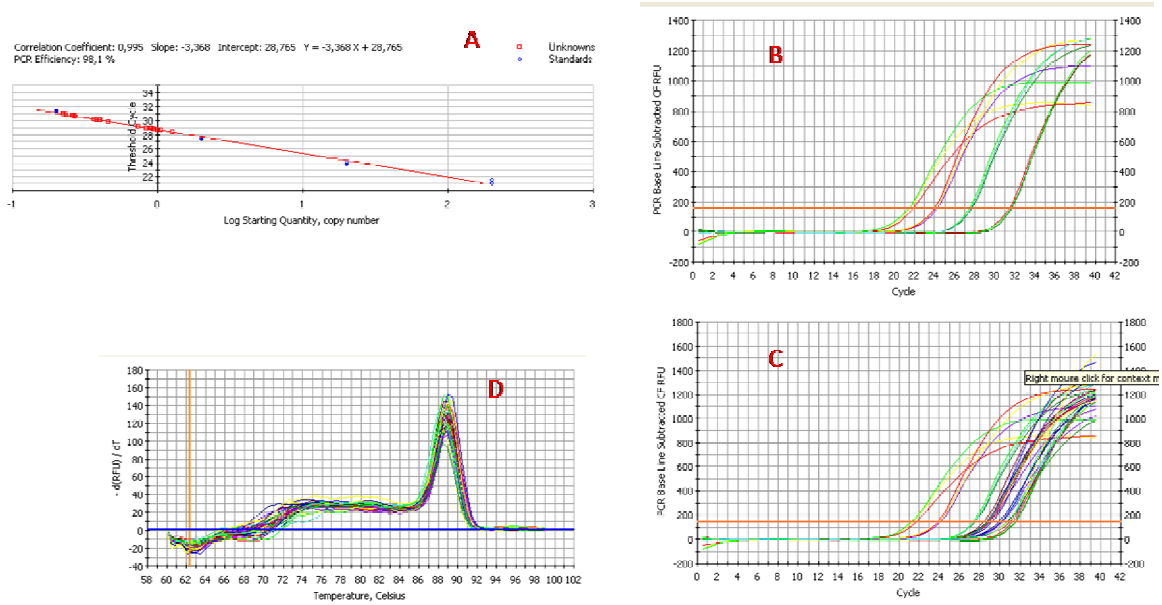


Figure VI.3.5 – Real-time amplification of AgPPO5, in standard mosquito DNA curve samples, non-infected and *P. berghei* infected mosquito hemolymph samples, collected at D13pi. A: DNA standard curve: includes points for all samples (blue – DNA standard curve samples; red – tested hemolymph samples), curve correlation coefficient and equation, and PCR efficiency. **B**: Amplification curves of the DNA standard curve samples. **C:** Amplification curves of all samples. **D:**Melt-curve for all samples.

Curves provided by the software included:

- Real-Time amplification curve for triplicates of each sample. Curves are built based on the fluorescence at the end of each cycle. In figure VI.3.4 are represented amplification curves for all samples (C) and for standard curve samples (B). The latter included DNA amounts of 200ng, 20ng, 2ng and 0.2ng.

- Standard curve and linear regression analysis (A). In this curve are included both DNA standard curve samples (blue dots) and hemolymph samples (red dots). Samples shown to have an mRNA amount lower than 0.2ng was considered not reliable and removed from analysis. Also in this graph there is information about the curve correlation coefficient (in this case 0,995), slope (-3,368) and PCR efficiency (98,1%).

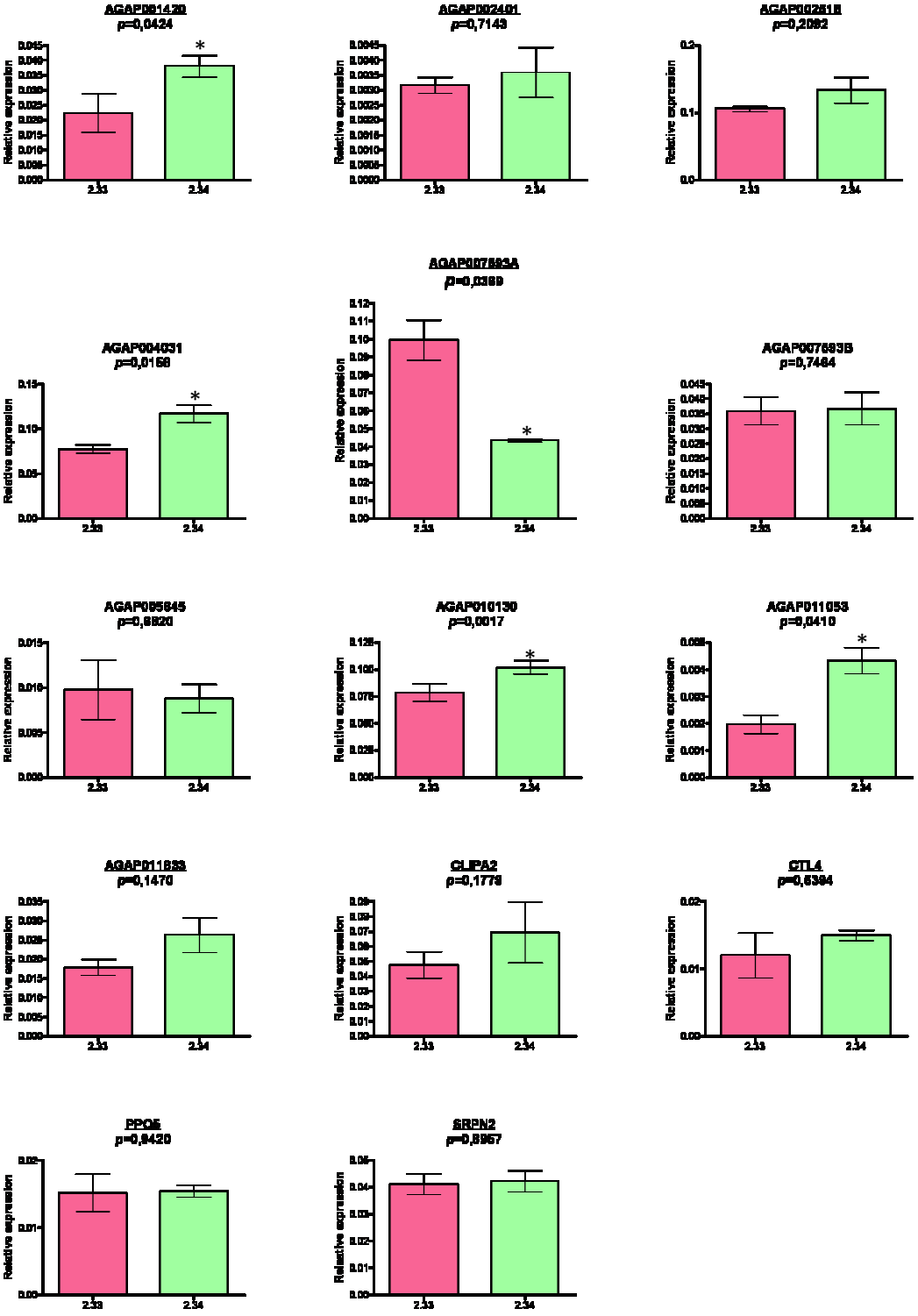
- Melting curve (D). As melting temperature (temperature at which half of the dsDNA molecule is denatured – the peak of the curve) is highly specific for each amplified product this curve allows to determine any contamination or primer dimmer in the amplification reaction.

Based on the DNA standard amplification curves and given that a threshold cycle number was determined for each reaction, iCycler software provided the mRNA amount (triplicate mean and standard deviation) present in each sample. Tables for each reaction are displayed on Annex4.

Relative expression for each gene tested is represented in Figure VI.3.6.

Significant difference in gene regulation between *P.* berghei infected (2.34) and non infected mosquitoes (2.33) was only observed for 5 of the genes tested. *AGAP001420*, *AGAP004031*, *AGAP010130* and *AGAP011053* genes showed up-regulation while *AGAP007593A* showed down-regulation in infected mosquitoes.

Although not significant, a slight up-regulation in infected mosquitoes was also observed for *AGAP002518*, *AGAP011833* and *CLIPA2* genes.



FigureVI.3.6 – Relative expression of Real-Time amplified genes in the hemolymph of non-infected and *P. berghei* infected *An. gambiae* mosquitoes, at D13pi. Red bars: Mean values of relative expression in non-infected (**2.33**) mosquitoes. Green bars: Mean values of relative expression in *P. berghei* infected (**2.34**) mosquitoes. Error bars represent standard deviation values. Differences in relative expression of 2.33 and 2.34 mosquitoes were analyzed for statistical significance using a two-way ANOVA approach. At least three independent experiments were used for analysis of each gene. Statistical significance (represented by *) was considered when P<0.05.

The infection effect on the levels of the proteins detected in the hemolymph was not always similar to that exerted on gene expression. Table VI.3.4 resumes the regulation of expression at the genomic and protein level of the tested spots/genes.

Table VI.3.4

Regulation of identified spots at genomic and protein levels in *P. berghei* infected *vs.* non-infected mosquitoes at D13pi

		Gene Regulation			Protein levels		
		Ratio 2.34/2.33			Ratio 2.34/2.33		
	Identification	Mean	SD		Mean	SD	
A1	AGAP010130	1.31*	0.11	↑	0.33	0.17	↓
A2	AGAP011833	1.48	0.38	=	0.59	0.20	↓
B3	AGAP002518	1.26	0.32	=	2.25	1.83	↑
B4	AGAP002518	1.26	0.32	=	2.01	0.86	↑
B5	AGAP011053	2.67*	1.81	↑	0.65	0.20	↓
B6	AGAP007593-	1.01	0.14	=	0.57	0.22	↓
B8	AGAP004031	1.51*	0.08	↑	0.66	0.19	↓
B9	AGAP001420	2.13*	1.21	↑	0.56	0.09	↓
B10	AGAP002401	1.20	0.71	=	0.52	0.20	↓
B11	AGAP005645	1.25	0.32	=	0.51	0.10	↓

Spots are identified by number in 2D maps and Ensemble ID. Genomic regulation and protein levels are characterized by mean and standard deviation (**SD**) of ratios of *P. berghei* infected (**2.34**) *vs.* non-infected mosquitoes (**2.33**) and by trend: ↑ - up-regulation, ↓ - down-regulation. ***** indicates statistical differences in gene expression between non-infected (2.33) and infected (2.34) groups.

VI.3.4 – Eicosanoid biosynthesis impact on infection

Some of the proteins identified by 2DE and MS as differentially regulated in *P. berghei* infected mosquitoes at D13pi were found to be involved in fatty acid metabolism (Table VI.3.3). This process is intimately related to eicosanoid biosynthesis, as fatty acids are used as substrates in this biosynthetic process that is in turn involved in processes such as immune responses. Thus, we hypothesised that eicosanoid biosynthesis is altered during mosquito infection and that it plays a role in the mosquito response to sporozoite invasion of the hemolymph. To test this hypothesis, *P. berghei* infected mosquitoes were injected either with an eicosanoid biosynthesis inhibitor (indomethacin - IN) or a substrate (arachidonic acid – AA – Figure VI.1.1).

VI.3.4.1 – Solutions toxicity

Before injecting indomethacin (IN) and arachidonic acid (AA) into the mosquitoes it was necessary to test for compound toxicity. Survival was determined by counting dead mosquitoes for 7 days. Some mosquitoes died in the first day, which was attributed to injection itself since there was no more mortality in the IN or AA groups than in controls (Figure VI.3.7). Solutions of 1mg/ml were chosen to perform the experiements.

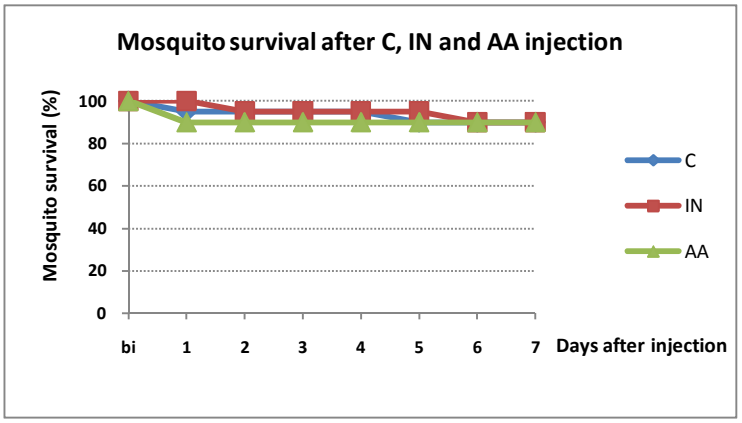


Figure VI.3.7 – Mosquito survival after medium, IN and AA injections. Mosquitoes were injected either with indomethacin (IN), arachidonic acid (AA) in Schneider medium with 6% ethanol. Control mosquitoes were injected with Schneider medium with 6% ethanol. Dead mosquitoes were counted in each group for 7 days. The graph represents the percentage of mosquito survival

VI.3.4.2 – Indomethacin and Arachidonic acid injections

To evaluate the impact of eicosanoid biosynthesis on infection, an inhibitor (IN) and a substrate (AA) were injected into *An. gambiae Yaoundé* mosquitoes infected with *P. berghei* at D7pi or D12pi.

Treatment of infected mosquitoes with the inhibitor resulted in a decreased number of sporozoites recovered from salivary glands when inhibition was perfomed at D7pi, while an increase was seen when inhibition occurred at D12pi (Figure VI.3.8-A). The opposite trend was observed when a eicosanoid biosynthesis substrate was injected rather than the inhibitor (Figure Vi.3.8-B). This is probably a result of of sporozoite maturation and the timming of their release into the hemolymph (Figure VI.3.8.-C).

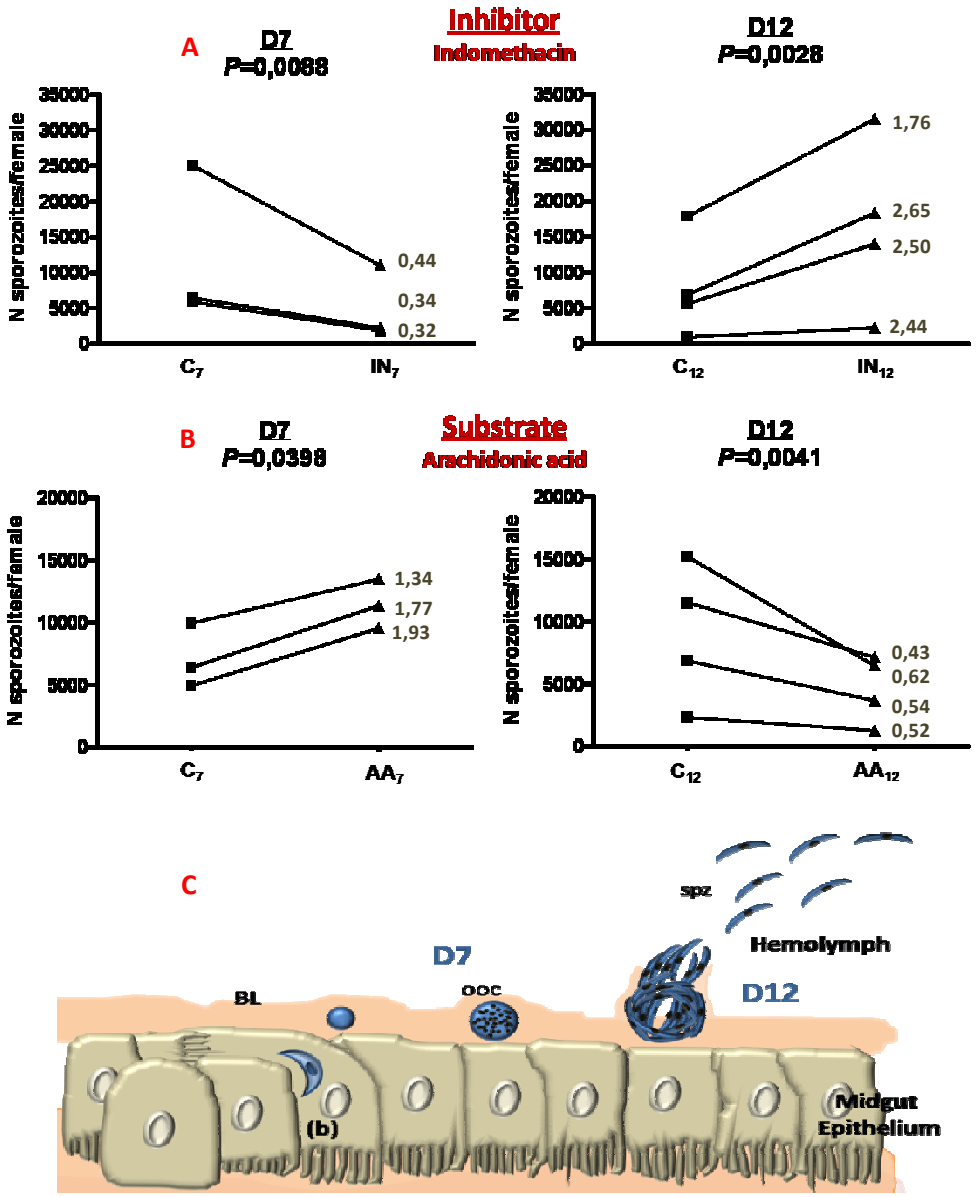


Figure VI.3.8 – The impact of eicosanoid biosynthesis on the outcome of *P. berghei* infection in *An. gambiae* mosquitoes. Infected mosquitoes were injected at D7 or D12pi with indomethacin (**IN**) or arachidonic acid (AA). Control mosquitoes were injected with 69nl of Schneider medium with 6% ethanol. Sporozoite load on salivary glands was determined at D21pi and analyzed by a ratio t test. Differences between IN/AA and control injected mosquitoes was considered significant when P<0.05.

A: Eicosanoid biosynthesis Inhibitor (**IN**) injections. Graphs represent the number of sporozoites per female at D21pi in control mosquitoes ($\mathbf{C_{7/12}}$) and in IN injected mosquitoes ($\mathbf{IN_{7/12}}$). Left panel: mosquitoes injected at D7pi. Right panel: mosquitoes injected at D12pi. Each curve represents an experiment performed. On the right of each curve is displayed the ratio of sporozoite number in IN *vs.* C injected mosquitoes.

B: Eicosanoid biosynthesis Substrate (**AA**) injections. Graphs represent the number of sporozoites per female at D21pi in control mosquitoes ($\mathbf{C_{7/12}}$) and in AA injected mosquitoes ($\mathbf{AA_{7/12}}$). Left panel: mosquitoes injected at D7pi. Right panel: mosquitoes injected at D12pi. Each curve represents an experiment performed. On the right of each curve is displayed the ratio of sporozoite number in AA *vs.* C injected mosquitoes.

C: Schematic representation of parasite infection in the mosquito. At D7 oocysts (**ooc**) are still maturing and sporozoites (**spz**) developing inside these. At D12 oocysts have matured and developed sporozoites start to be released into the hemolymph. **BL**: Basal Lamina.

VI.4 – Discussion

There is a deficit of data concerning the mosquito response to sporozoite hemolymph invasion that contrasts with the increasing amount of information on mosquito immune responses to midgut epithelium invasion by ookinetes. This led us to focus our attention to the hemolymph stage of parasite infection, as hemolymph is a crucial for immune responses. Thousands of parasites invade the hemolymph during sporozoite egress from the oocysts. Therefore, the mosquito is bound to respond to *Plasmodium* infection, contributing to the major sporozoite losses observed at this part of the parasite life cycle.

Hemolymph is a protein rich environment, carrying secreted proteins such as immune molecules and messengers, and identifying these proteins during infection will clarify the processes involved in the mosquito response to sporozoites.

VI.4.1 – Hemolymph proteome analysis

Protein spots were differentially regulated in the hemolymph of *P. berghei* infected mosquitoes when compared to those in the hemolymph of non-infected mosquitoes. Protein identification revealed that the differentially regulated protein spots were involved in cellular processes such as fatty acid metabolism, aminoacid synthesis, glycolysis and ion transport (Table VI.3.3). No immune-related proteins were detected as induced, repressed, up- or down-regulated in this study. This was unexpected, as one would expect that the mosquito response to sporozoite invasion of the hemolymph would induce the *de novo* synthesis of immune effector molecules. This may reflect the experimental procedure, namely the amount of protein loaded and consequent gel size, as a higher protein load on a 17cm gel would provide more protein spots to be analyzed and permit to detect spots corresponding to proteins existing at low concentrations. Also the detergent used (ASB-14) for protein sample

denaturation is not very effective in the acidic area, which also hinders the identification of additional protein spots.

At the same time, the nature of the identified protein spots indicates that mechanisms other than those usually related to immunity may be involved in mosquito response to the parasite. It is necessary to bear in mind that parasites depend on the mosquito biology to survive and that they use many mosquito proteins during their life cycle. A variation on the turn-over of proteins that are essential to parasite survival may be sufficient to hinder parasite survival and/or development. This strategy would account for a lower energy requirement from the mosquito, as exhaustive protein synthesis (as in most immune responses) demands a high amount of energy and can be harmful for the mosquito (Ahmed *et al.*, 2002; Demas *et al.*, 1997; Moret & Schmid-Hempel, 2000; Rivero & Ferguson, 2003; Tripet *et al.*, 2008). These results indicate that the general and basic mechanisms operating in mosquito biology may have a role in the control of infection, together with more specific immune responses, and that it is important to take them into account.

VI.4.2 – Hemolymph gene expression analysis

To further understand the differential regulation of the proteins identified by 2DE and MS, gene expression was studied by Real-Time PCR.

Although all the 10 protein spots identified were found to be differentially regulated in *P. berghei* infected mosquitoes (2.34) when compared to non-infected mosquitoes (2.33), only 4 of these (40%) were found to be differentially regulated at the genomic level (Figure VI.3.6).

Interestingly, no correlation was observed between the RNA transcription and the protein expression (Table VI.3.4). For 5 genes (AGAP011833, AGAP002518, AGAP007593-B, AGAP002401 and AGAP005645), no differential expression in infected

mosquitoes was detected at the mRNA level, even though proteins levels differ from infected and non infected mosquitoes. As for the genes that show differential regulation (AGAP010130. AGAP011053. AGAP004031 and AGAP001420) they indicate an up-regulation in infected mosquitoes at mRNA level while protein levels are down-regulated in infected mosquitoes.

At least two processes might explain these discrepancies: a) transcription regulation does not coincide in time with protein production and/or b) the origin of proteins that circulate in the hemolymph do not coincide with the cells used for profiling. The gene expression profiles were described for hemolymph circulating cells, and proteins in the hemolymph are probably mainly secreted by the fat body (Meister *et al.*, 1997; Meister, 2004; Samakovlis *et al.*, 1990; Shi & Paskewitz, 2006).

The gene corresponding to one of the proteins identified, AGAP007593-PB, was found to have two transcripts (A and B) that result from alternative splicing. Although the identified protein is known to correspond to transcript B, we decided to analyze also the expression of transcript A. It was interesting to realize that for one transcript there is no differential regulation in infected mosquitoes at the RNA level but a 43% down-regulation occurs at the protein level (transcript B), while for the other (transcript A) there is a 60% reduction in gene expression and no variation in protein levels detected by 2DE (Figure VI.3.6 and Table VI.3.4).

Gene AGAP002518 codes for a protein that showed two different spots in 2D maps, resulting from a post-translational modification (spots B3 and B4). The gene does not seem to be differentially regulated in infected mosquitoes, but both forms of the protein show a 2 fold increase in the same mosquitoes.

These results show that as important as gene expression regulation is, the resulting data have to be carefully analyzed, as direct correlation with protein concentration may not exist. As proteins are the effectors in biological mechanisms, immunity included, gene expression may not be sufficient to indicate a variation in protein levels at a specific point during infection.

VI.4.3 - Eicosanoid biosynthesis impact on infection

Fatty acids serve mainly energetic functions, existing in storage compartments in cells, but they may be converted into several products that function in numerous processes. One process that uses polyunsaturated fatty acids as substrate and is intimately related to the immune response is the eicosanoid biosynthesis (Calder, 2006). In vertebrates, eicosanoids mediate inflammation and immune responses, having roles in platelet aggregation, intensity and duration of pain and fever and blood pressure regulation. In insects, eicosanoid biosynthesis has also been linked to immune responses. Accumulating data suggests that eicosanoids have an important role in mediating responses to bacterial, fungal and parasite infections in six orders of insects (reviewed by Stanley & Miller, 2006).

We showed that eicosanoid biosynthesis inhibitor has an infection promoting action when sporozoites are released from oocysts and invade the hemolymph (Figure VI.3.8-A: right panel), suggesting that eicosanoids are necessary for the mosquito response to the parasite, which is in accordance with the eicosanoid effect on immunity described for several pathogen/insect models (Stanley & Miller, 2006). Interestingly, inhibition occurs when oocysts are still maturing and no sporozoites are observed within the oocyst, the number of salivary glands sporozoites decreases to one third of that found in control mosquitoes (Figure VI.3.8-A: left panel). This may reflect a role of mosquito synthesized eicosanoids in *Plasmodium* biology and development. The parasite has its own eicosanoid biosynthetic pathways that are different from those present in mammals, but it is not capable of *de novo* synthesis of fatty acids requiring arachidonic acid from its host (Holz, 1977). The implication of these on oocyst development has still to be unveiled.

As would be expected in a robust model, the injection of an eicosanoid biosynthesis substrate (arachidonic acid – AA) produced the opposite results obtained with the injection of an inhibitor (IN). The injection of AA at D12pi leads to a salivary gland sporozoite number reduction to half of that found in control mosquitoes (Figure VI.3.8-

B: right panel), confirming that eicosanoids are necessary to clear sporozoites from the mosquito hemolymph. Also, at D7pi, a 1.7fold increase is observed in parasite number in salivary glands of AA injected mosquitoes when compared to controls (Figure VI.3.8-B: left panel) corroborating that eicosanoids are at some point required for parasite development.

The fact that AA injections boost the mosquito hemolymph response to sporozoites suggests that eicosanoid biosynthesis in infected mosquitoes is not operating at a maximum level. One possible explanation would be an ability of the parasite to immunosuppress its mosquito host. This phenomenon was already observed in some pathogen/insect models, like in a bacterial infection of *Spodoptera exigua* and a *Trypanosome* infection of *R. proxilus* (Garcia *et al.*, 2004b; Park & Kim, 2003). *Trypanosome* is a parasite closely related to *Plasmodium* indicating that these protozoan parasites, albeit infecting not related hosts, share common ways to circumvent the host immune responses.

The ability of the parasite to evade and/or suppress the host's immune response has been extensively studied in the vertebrate host. In blood stages of its life cycle, *Plasmodium* has been reported to have both immune evasion and suppression behaviors. To evade the host's immunity, the parasite relies on antigen variation, differential erythrocyte invasion pathways and, in the case of *P. falciparum*, cytoadherence to blood vessels. As for immunosuppression, it seems to rely mainly on the malaria pigment (hemozoin) that results from hemoglobin digestion and accumulates in the infected red blood cells. *Plasmodium falciparum* hemozoin was shown to be responsible for the inhibition of human prostaglandin E_2 (PGE_2) gene expression, whose reduced levels would lead to a subsequent increase in TNFα levels accounting for the anemia (Keller *et al.*, 2004; 2006). Accordingly, it was reported that children with malaria anemia have lower levels of circulating PGE_2. At the same time, increased TNFα levels impair dentritic cells functions leading to a failure in T cells activation (Ocaña-Morgner *et al.*, 2003; Millington *et al.*, 2006, 2007; Wykes *et al.*, 2007).

In its mosquito vector, *Plasmodium* was also shown to have immune evasion and suppression behaviors. In late oocyst stages, parasites are believed to camouflage themselves by incorporating mosquito derived proteins into their surface and, in early stages, when ookinetes traverse the midgut epithelium it gets wrapped with mosquito plasmatic membrane (Vlachou *et al.*, 2004). Some mosquito proteins, as CTL4 and CTLMA2, were found to be protective for the parasite, possibly assisting it in immune evasion (Warr *et al.*, 2006). Further, CTL4 KD mosquitoes do not melanize Sephadex beads (Warr *et al.*, 2006), suggesting specificity of the protective effect and possible parasite manipulation of the immune system.

At the midgut stage the parasite also has the ability to immunosuppress the mosquito: *P. gallinaceum* infection reduces *Ae. aegypti* mosquito's ability to encapsulate Sephadex beads (Boete *et al.*, 2004). *P. falciparum* however, seems to have the opposite effect, as its infection increases the melanotic response to Sephadex beads (Lambrechts *et al.*, 2007). Nonetheless, *P. falciparum* infection was found to repress NOS gene expression, a gene involved in local epithelial responses (Tahar *et al.*, 2002). There is no indication about possible immune evasion and/or suppression mechanisms displayed by the parasite while traversing the mosquito hemolymph, a stage at which it is most vulnerable to immunity and that represents a significant bottleneck for the parasite. The fact that most of the protein spots highlighted in this work by 2DE were found to be down-regulated upon sporozoite infection of the hemolymph, may reflect an immunosuppression rather than an evasion mechanism by the parasite.

We therefore propose that the parasite is able to induce a reduction in the levels of mosquito proteins (such as those necessary for fatty acid metabolism) and to hinder the eicosanoid biosynthesis. An impaired ability to synthesize eicosanoids would compromise the mosquito immunity, protecting the parasite while it traverses the hemolymph to the salivary glands.

VII – General Discussion

Malaria eradication will integrate several measures, including transmission control. For this, it is vital to understand the interplay between parasite and vector. This work aimed at contributing to understanding this interaction. In particular, we attempted to evaluate the impact of antimalarials on mosquito immune response and the responses to sporozoites in the hemolymph.

We failed to show chloroquine effect on *Drosophila* immune responses as described for mosquitoes, indicating that this is model is not suitable for such a study. Antimalarials such as chloroquine are metabolized by cells. If the observed effects are due to the drug itself or one of its metabolites is not known, explaining why direct feeding of the drug to flies has no effect on *Drosophila* immune responses. Also, the fly and the mosquito have diverged about 250 million years ago, and through evolution have specialized their immune responses against natural pathogens, according to their life style. It is probable that different molecules and routes to signaling pathways activation have emerged to respond to particular pathogens. Even so, several molecules, in particular those involved in the intracellular signaling pathways and antimicrobial peptide synthesis are well conserved in both organisms, making *Drosophila* a useful model for mosquito immune responses.

Mosquito response to midgut invasion has been extensively studied and increasing amount of data have been produced. However, the response to other parasite stages, like sporozoite invasion of the hemolymph, has received little attention. This is surprising as it represents a major bottleneck in parasite development, as 90% of sporozoites are lost between midgut and salivary gland. In order to understand the mechanisms behind this reduction we focused our attention to this stage of parasite development in the mosquito. Previously, sporozoites were reported to be phagocysed by mosquito hemocytes and pericardial cells, when flowing along in the hemolymph. We have added knowledge about mechanisms such as melanization and eicosanoid biosynthesis to the mosquito response to the parasite at this stage. Our results point to several mechanisms being involved in the control of parasite load in the hemolymph. The mosquito seems to use several approaches to combat the infection. Cellular immune responses may play an important role, as mosquito hemocytes seem

to recognize the circumsporozoite protein of *P. falciparum* and, in the presence of this protein, a hemocyte number reduction is observed, even though it occurs in specific conditions (dosage and time of exposure). This points to the occurrence of cellular immune responses performed by the hemocytes, which is in accordance with the previously described sporozoite phagocytosis observed in both rodent and avian malarial infection. The hemocyte-like cells used in this work failed to proliferate upon different stimuli. Nevertheless, data from this and previous works suggest that hemocytes engage in cellular immune responses to *Plasmodium* sporozoites. Further analysis to determine morphological alterations in hemocytes and to evaluate different hemocyte sub-populations should give extra information about action of specific hemocyte types during the mosquito immune response to the parasite. Additionally, *in vivo* experiments would help to determine hemocyte proliferation and/or differentiation upon spoorozite invasion of the hemolymph. However, the asynchronicity of sporozoite release into the hemolymph makes it difficult to determine the exact moment of parasite recognition and cellular responses triggering. Other cellular immune responses, such as encapsulation and microaggregation should also be investigated in order to understand hemocyte dynamics towards sporozoites.

We also investigated humoral reactions such as melanization. Even though we failed to conclusively show induction of melanization upon sporozoite invasion in the hemolymph, as previously reported for an avian malaria model, we showed that this mechanism is effectively necessary to control parasite numbers in salivary glands, indicating that more that one response may me involved in parasite load control. Detailed microscopic studies should help to visualize melanized sporozoites, and parasite quantification would be helpful to correlate parasite numbers and response intensities.

Additionally, proteomic studies indicate that several molecules, besides the traditionally immune-related proteins, are affected by parasite presence. This coincides with microarrays studies that show altered expression in genes coding for proteins involved in processes such as immunity, apoptosis, detoxification, stress response and cytoskeleton remodeling. Our results show that even basic physiological

mechanisms like fatty acid metabolism, aminoacid biosynthesis and glycolysis may also play a role in immunity. However, we did not find any correlation between RNA expression and protein regulation. Even though our proteins are present in the hemolymph, their corresponding genes may be expressed in different cells, such as hemocytes or fat body. Nevertheless, many proteins known to engage in immune responses are processed and modified pos-translationally. Thus, it should be taken into account that gene expression may not correlate to protein regulation and, therefore immune responses should be analyzed primarily at the protein level. We also determined that eicosanoid biosynthesis has two opposite roles in parasite development: if, at the midgut stage of parasite development in oocysts eicosanoids seem to be necessary for sporozoite production, at the hemolymph stage these molecules seem to be necessary for the control of parasite load. At the same time, our results indicate that the parasite itself may engage in mechanisms to immunosuppress its vector, facilitating the transmission.

The mosquito seems to rely on not one but several mechanisms to control sporozoite load in the hemolymph, reducing parasite numbers in the salivary glands. These mechanisms involve both cellular and humoral immune responses, as well as mechanisms not usually related to immunity but to the mosquito biology and physiology. As sporozoites invade the hemolymph in massive quantities it should be expected that, depending on only one type of response would be deleterious and harmful for the mosquito that would be forced to produce large amounts of proteins and/or immune responsive cells. This is evidenced by the fact that each of the immune-related mechanisms studied here and elsewhere are not responsible for the complete elimination of hemolymph sporozoites. As such we propose that the mosquito sustains a basal level of constitutive immunity that can be upleveld to a certain extent by a larger number of parasites. At the same time, the parasite seems to be able to compromise the mosquito response, by immunosuppression. Overall, the interplay between mosquito and parasite seems to be highly complex, involving several factors and mechanisms from both organisms, the mosquito fighting for an

equilibrium between parasite load and the cost of engaging in several immune responses and the parasite attempting to avoid/suppress the mosquito immune responses in order to be able to achieve its transmission.

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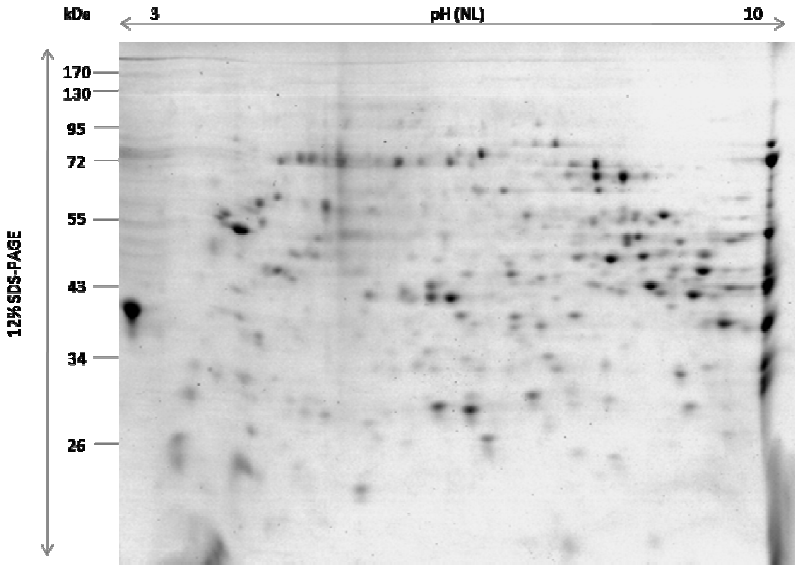
IX – Annexes

Annex 1

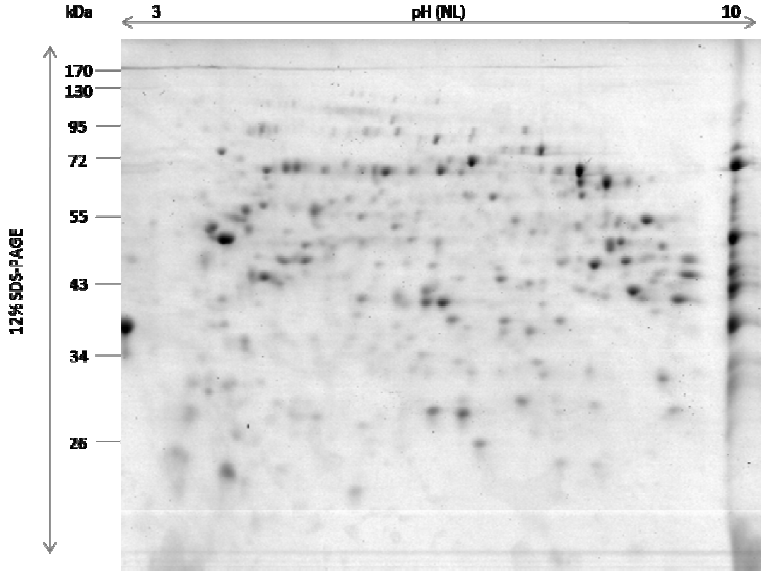
Assay A

2D maps obtained in analysis A of hemolymph protein samples (30µg) from *P. berghei* infected (2.34) and non-infected *An. gambiae* mosquitoes (2.33). Samples were separated by 2DE on a pH3-10NL IPG strip and on a 12% polyacrylamide SDS-PAGE and stained with CBB.

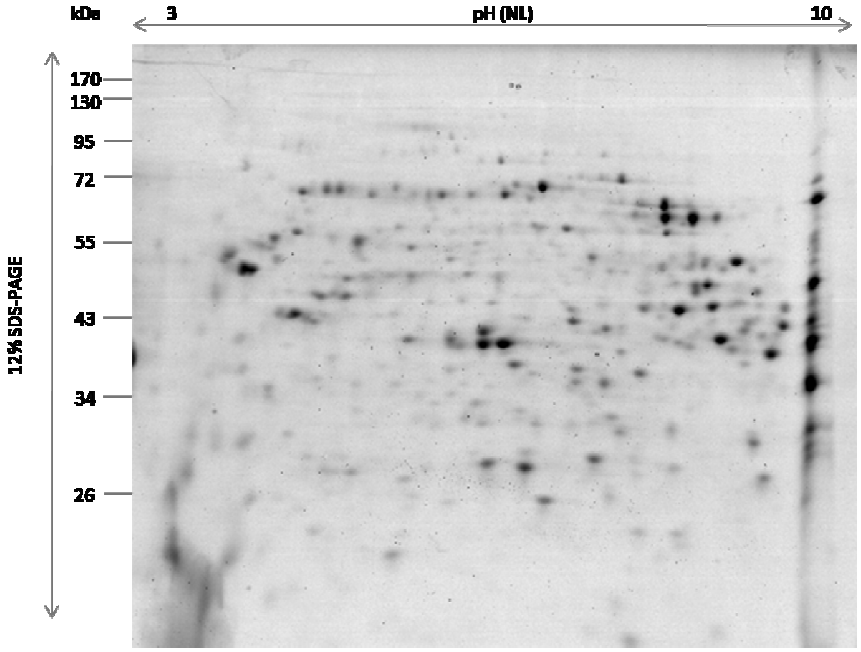
Experiment II – Hemolymph proteins of non-infected mosquitoes (2.33)



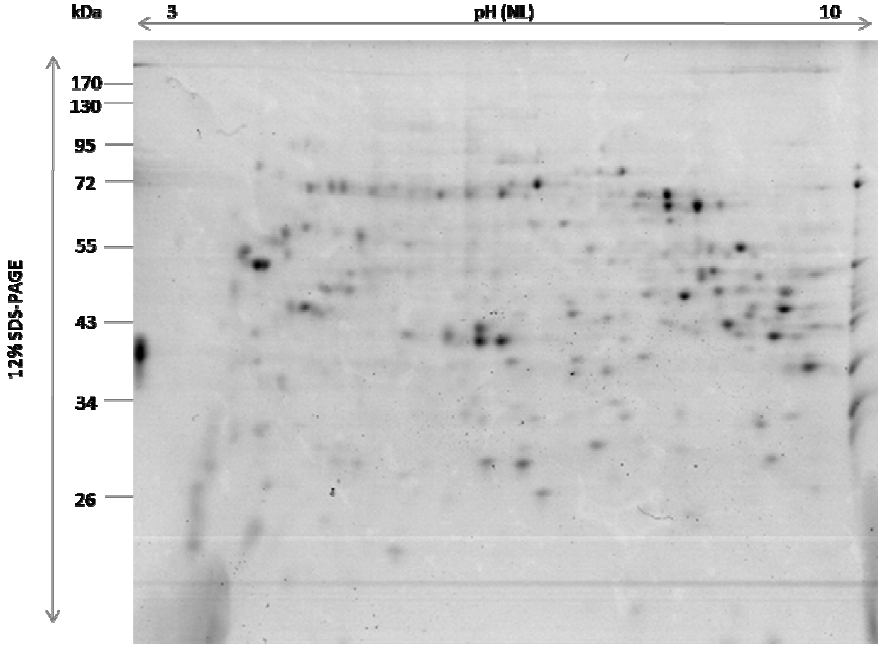
Hemolymph proteins of *P. berghei* infected mosquitoes (2.34)



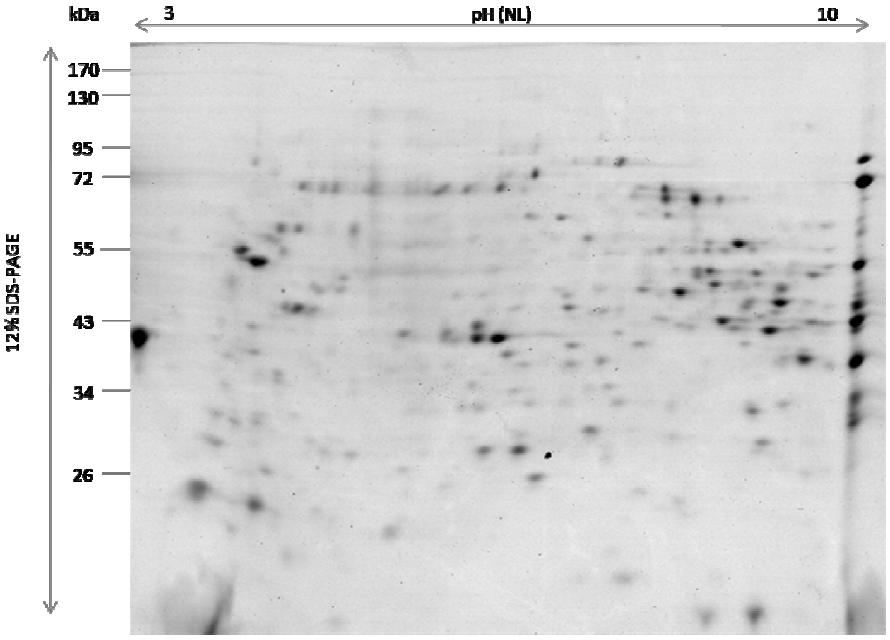
Experiment III – Hemolymph proteins of non-infected mosquitoes (2.33)



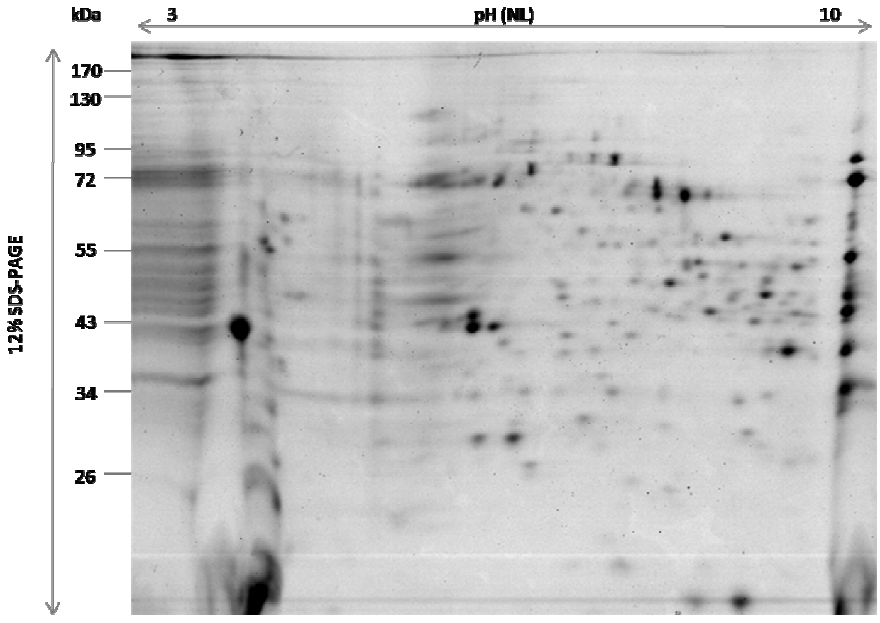
Hemolymph proteins of *P. berghei* infected mosquitoes (2.34)



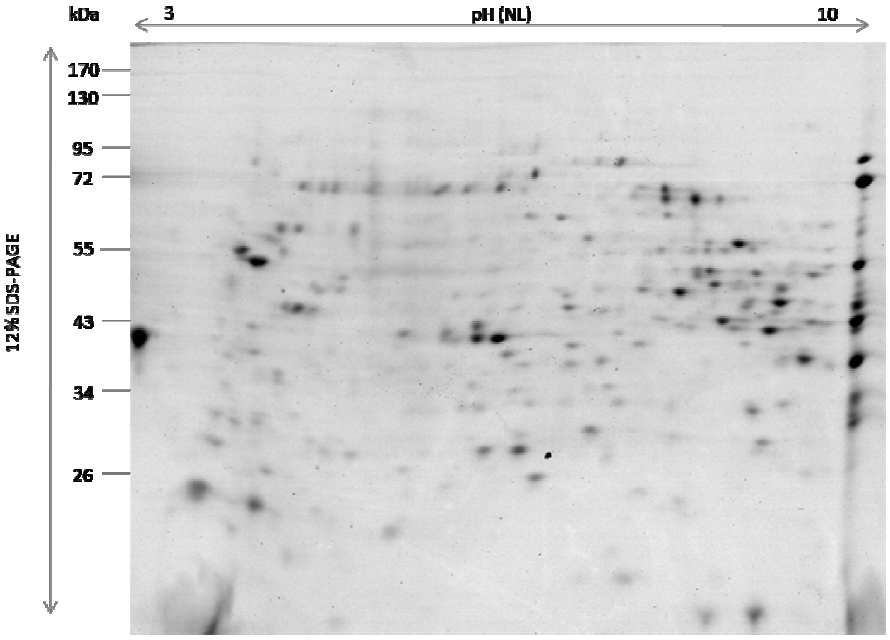
Experiment IV – Hemolymph proteins of non-infected mosquitoes (2.33)



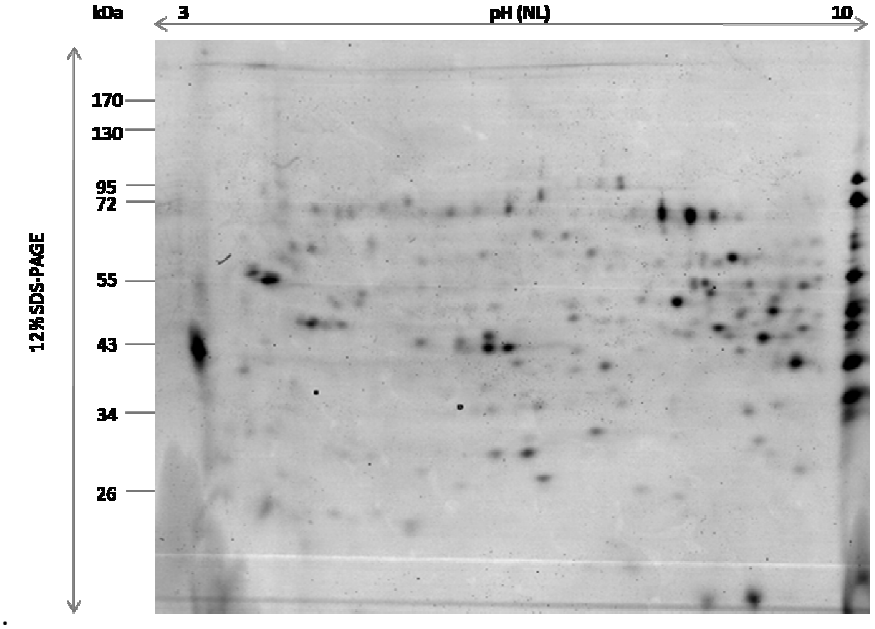
Hemolymph proteins of *P. berghei* infected mosquitoes (2.34)



Experiment V – Hemolymph proteins of non-infected mosquitoes (2.33)

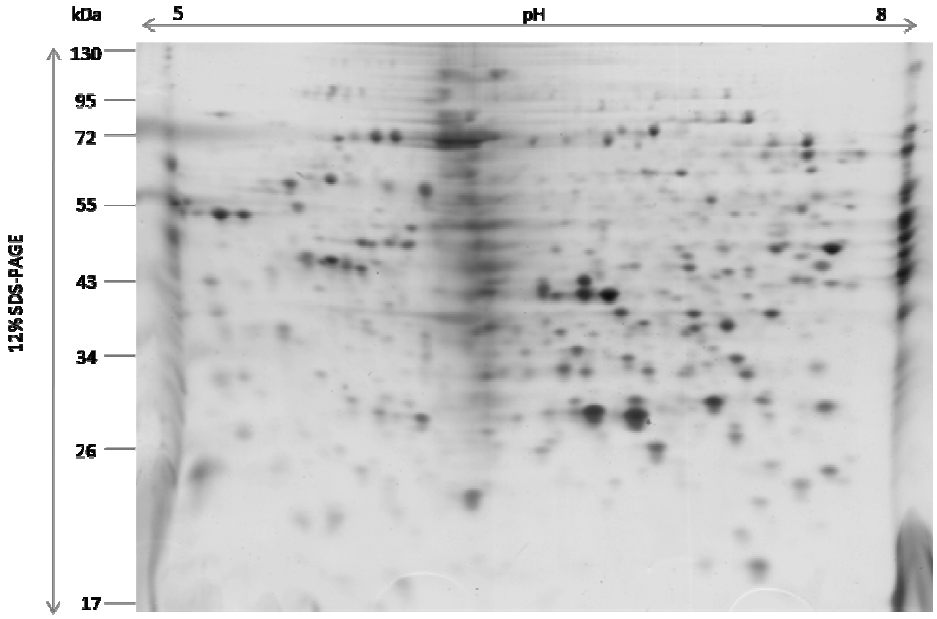


Hemolymph proteins of *P. berghei* infected mosquitoes (2.34)

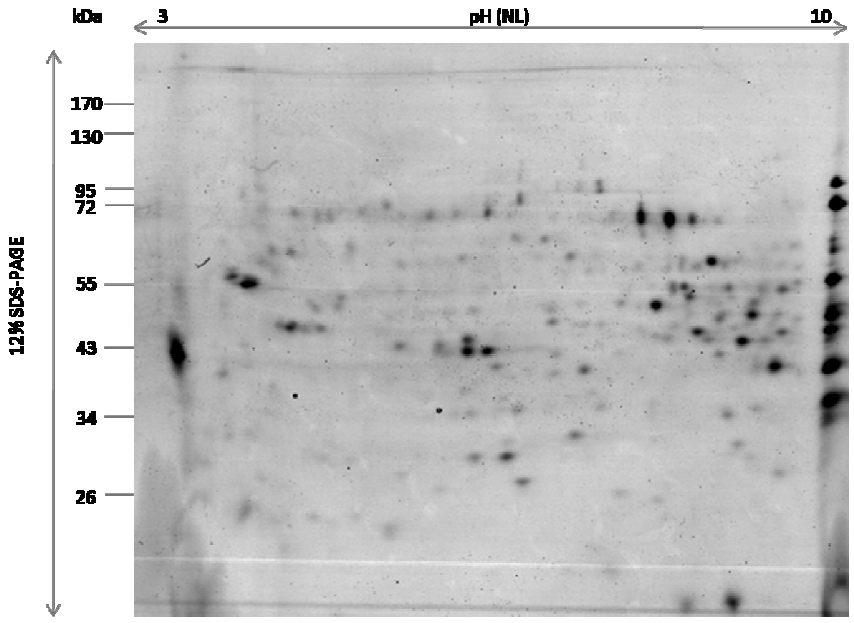


Assay B – 2D maps pbtained in analysis B of the hemolymph protein samples (30µg) of *P. berghei* infected (2.34) and non-infected *An. gambiae* mosquitoes (2.33). Samples were separated by 2DE on a pH5-8 IPG strip and on a 12% polyacrylamide SDS-PAGE and stained with Silver nitrate.

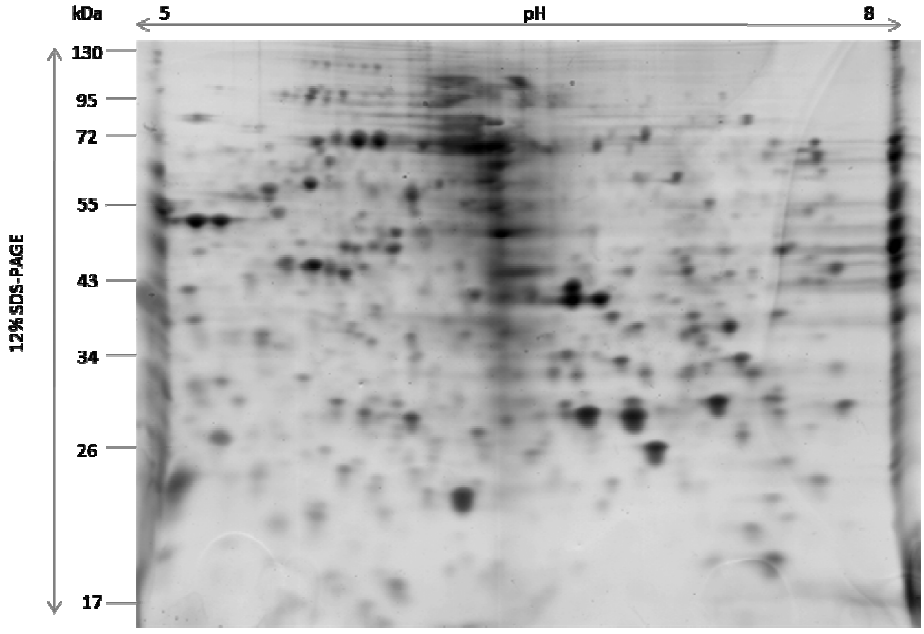
Experiment I – Hemolymph proteins of non-infected mosquitoes (2.33)



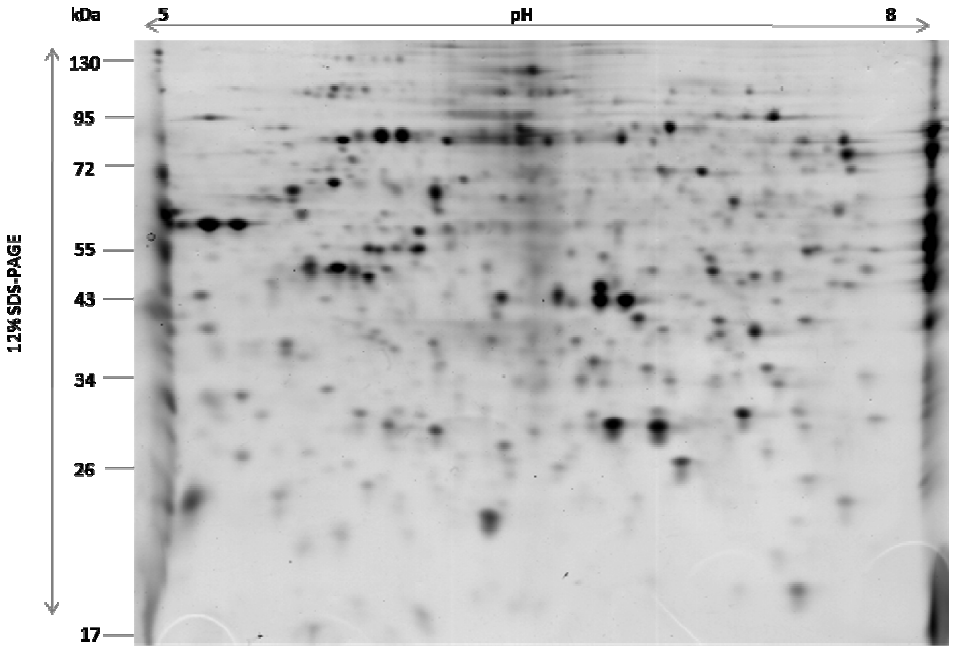
Hemolymph proteins of *P. berghei* infected mosquitoes (2.34)



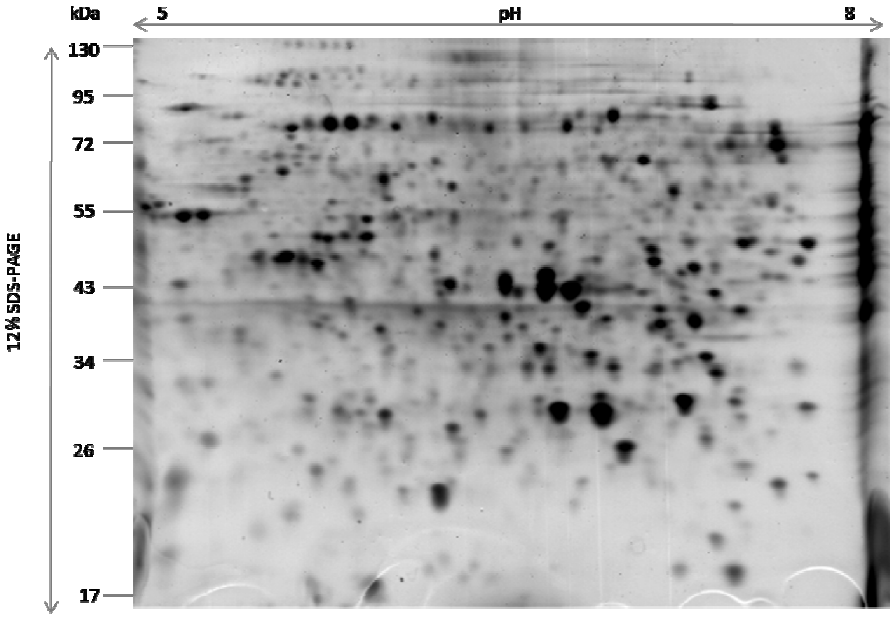
Experiment II – **H**emolymph proteins of non-infected mosquitoes (2.33)



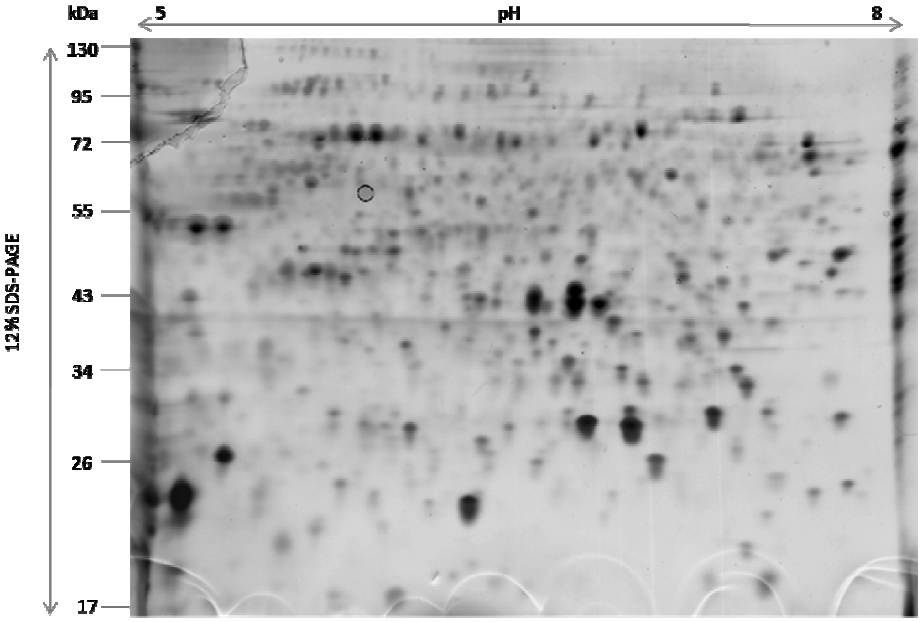
Hemolymph proteins of *P. berghei* infected mosquitoes (2.34)



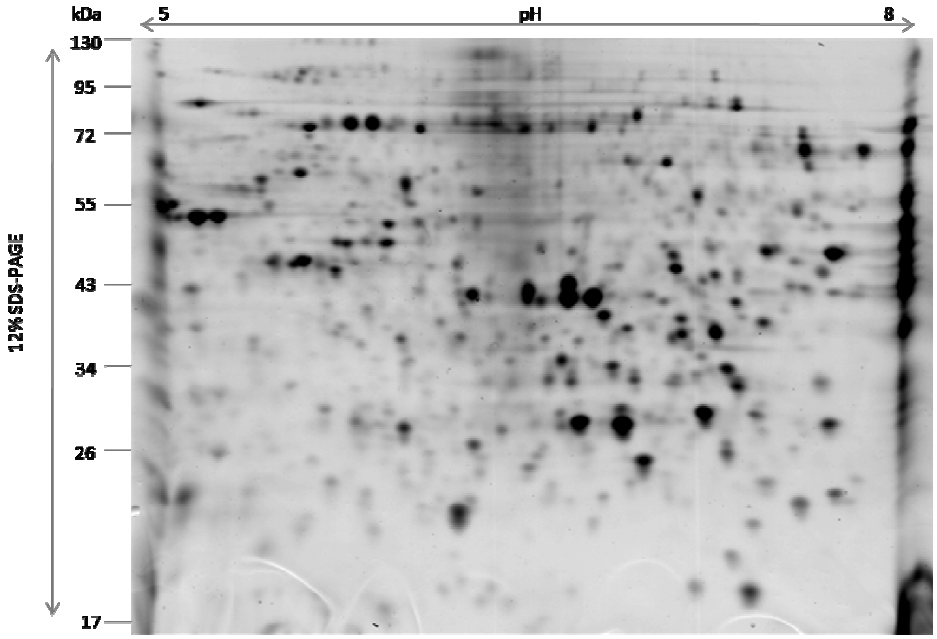
Experiment III – Hemolymph proteins of non-infected mosquitoes (2.33)



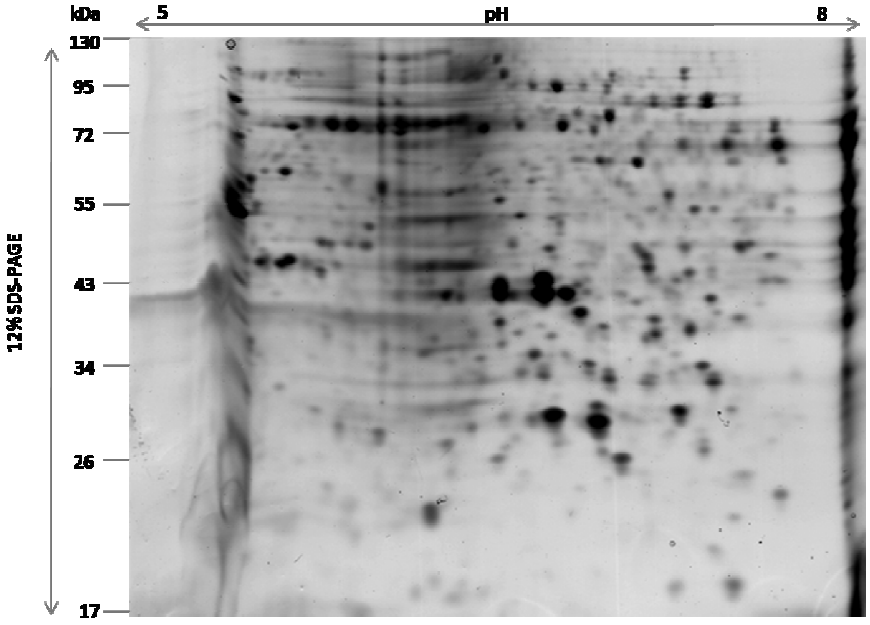
Hemolymph proteins of *P. berghei* infected mosquitoes (2.34)



Experiment IV – Hemolymph proteins of non-infected mosquitoes (2.33)

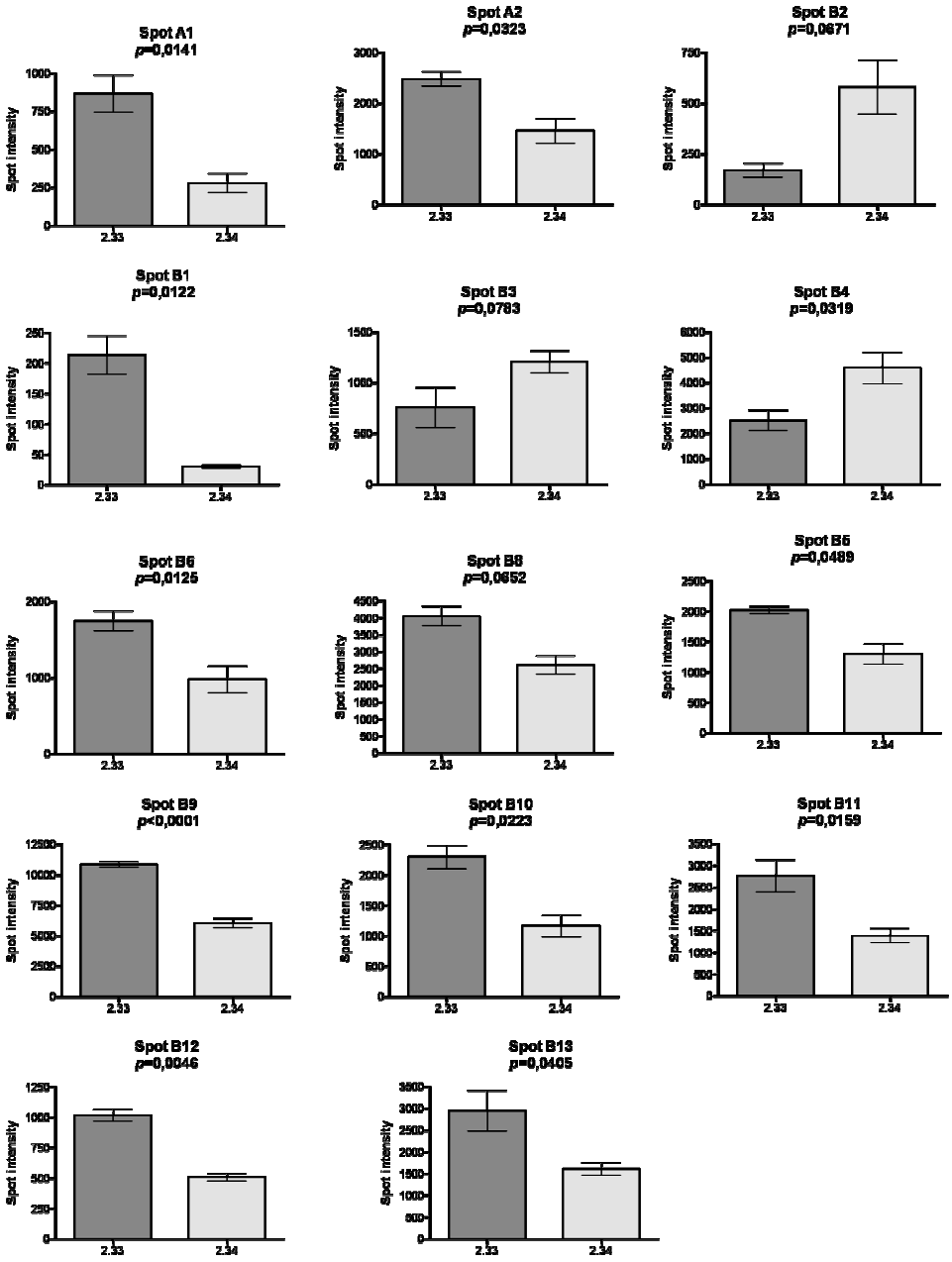


Hemolymph proteins of *P. berghei* infected mosquitoes (2.34)



Annex 2

Protein levels in the protein spots from the hemolymph of *P. berghei* infected (2.34) and non-infected (2.33) *An. gambiae* mosquitoes, collected at D13pi. Protein spots are differentially regulated in infected and non-infected mosquitoes by 2DE.



Annex 3

Mass spectrometry identification of protein spots. Peptides matched to the protein sequence are represented in underline. MS parameters include score, sequence coverage of the matching peptides, error and the number of matched peptides.

AGAP010130 – spot A1

>gi|158299170|ref|XP_554016.3| AGAP010130-PA [Anopheles gambiae str. PEST]
MLRVLQRSSRMVAPAARNFSSEAGNNKLVITEVDDKTGYATVTLNRPPVNSLSLELLRA
ISQTLDDLQNNKSRGMILTSSSNTVFSAGLDIMEMYKPNQERLRDFWSTLQDVWFKLYG
SPFPTAAAINGHAPAGGCLLSLCCEYRVMCPNYTIGLNETQLGIVAPTWFQASLRNTIS
RRESELALTLGKMYTTDEALKVGMIDEIAENKEKALEQAVAFLNRFRKISPMARAMTKQ
ALRSKDIVELEDNRSQDIDLFVYAVNQPAVQKGLEVYLESLKKKAQK

Socre: 46
Sequence coverage: 7%
Error: 183ppm
Peptides matched: 2

AGAP011833 – spot A2

>gi|58394357|ref|XP_320683.2| AGAP011833-PA [Anopheles gambiae str. PEST]
MANIGRLLASRVAGQLVRNVATNQQQQQANLLRFYSSAPKAYEFIKAELAGEKKNVAVI
TLNRPKALNALCNGLVAEISDALDRYEADDSIGAIVITGSEKAFAAGADIKEMQPNTYA
KCINTDFLANWTRVAKAQKPVIAAVNGYALGGGCELAMMCDIIYAGDKARFGQPEIALG
TIPGAGGSQRTTRAMGKSKAMEMCLTGNMITAEEAERSGLVSKVFPAEKLVEEAVKLGE
KISTFSPLIVRLCKEAVNASYEMSLNEGLRFERRHFHATFSTKDRLEGMTAFVEKRAPK
FSNE

Socre: 129
Sequence coverage: 28%
Error: 401ppm
Peptides matched: 3

AGAP011053 – Spot B5

>gi|158285041|ref|XP_308082.4| AGAP011053-PA [Anopheles gambiae str. PEST]
MAPKVPSVRLNNGLEMPVLGLGTYLATEEEGIAAVKMAIDEGYRHIDTAYFYQNENQVG
QAVRAKIAEGLIKREDVFIVTKVWNTYHAPEHVAEACQRSLDNLGLGYIDLFLIHWPMG
WKFCGWTGDDLLPMNANGKSIDSDVDYLDTWKAMERLVKEGKVKSIGVSNFNSEQLTRL
LANCEIKPVTNQVECNPGINQRKLIEFCRQHDIVITAYSPLGRPNMADPVVGTAGIPKH
ALDDPRVIAIGQKYGKSAGQVVLRYLVELGTLPIPKSSKLERIRQNIDIFDFSLTEEEI
KLMDGFNTGGRTVPFHFSSEHKYFPFKLEY

Socre: 109
Sequence coverage: 23%
Error: 432ppm
Peptides matched: 5

AGAP002518 – spot B3/B4

>gi|118782666|ref|XP_312421.3| AGAP002518-PA [Anopheles gambiae str. PEST]
ERKQATFSERNQLKYARRLVVKLGSAVITREDEHGLALGR**LASIVEQVAEYHVEGRECI**
MVTSGAVAFGKQR**LTQELIMSLSMRETLSPTDHT**RQDAGTLVEPRAAAAVGQSGLMSLY
DAMFAQYGIK**IAQVLVTEPDFYNEETR**KNLFSTLSELIHLNIVPIINTNDAVVPPMFIV
DQEVSATGKKRGIRIKDNDSLAALLAAEIHADLLILMSDVDGIYNKPPWEDGARLMHTY
TAGDKNLIKFGEKSKVGTGGMNSK**VMAATWALDRGVSVVICNGMQDKA**IKSILTGR**KVG**
TFFTESTAEKATPVEQIAENARNGSRVLQNLTAAER**AQAVNTLADLLISRQSQILEANA**
KDLDEAKKSGLAKPLLSRLSLTPSKLESLAKGLKQIADDSHRNVGRVVKRTKLADGLEL
KQVTVPIGVLLVIFESRPDSLPQVAALAMASGNGLLLKGGKEAAHSNRALMELVK**ESLA**
ATGASNAISLVSTREEISDLLSMDEHIDLIIPRGSSELVRSIQEKAQHIPVMGHAEGIC
HVYVDREADLDKALKIIRDSKCDYPAACNAMETLLIHEDLLQNSSFFTDVCNMLKREGV
KINSGPK**LNQMLTFGPPQAK**SLK**FEYGALECSIEVVK**NLEEAIDHVHTYGSGHTDVIVT
ENPTSATYFQSNVDSACVFHNASSRFADGFRFGLGAEVGISTARIHAR**GPVGVEGLLTT**
KWILSGVDHTASEFTDGSR**AWLHQSLPTDQ**

Socre: 322/520
Sequence coverage: 20%/29%
Error: 94ppm/134ppm
Peptides matched: 12/18

Note: two protein spots were identified as the same protein. Peptides derived from both spots are represented in underline in the protein sequence; one is also represented in bold to allow for differentiation.

AGAP007593-B – spot B6

>gi|58378155|ref|XP_308279.2| AGAP007593-PB [Anopheles gambiae str. PEST]
MSDKVRVCIVGSGNWGSAIAKIVGANAKRLATFEDRVTMYVYEEMIDGKKLTEIINTTH
ENVKYLPGHKLPENVVAVPDVVEAAKDADILIFVVPHQFIRGLGTQLLGKIKPTAVGLS
LIKGFDVAEGGGMELISHLITKHLKIPCSVLMGANLAGEVAEEKFCETTIGCRDMKIAQ
TLRDLFLTPNFRVVVVDDVDAVEICGALKNIVACGAGFVDGMGLGDNTKAAVIRLGLME
MIKFVDVFYPGSKLSTFFESCGVADLITTCYGGRNRKVSEAFVKTGKTIVELENEMLNG
QKLQGPITAEEVNFMLKSKGMEDKFPLFTAIHKICTGTVKPQGFLDCLRNHPEHM

Socre: 42
Sequence coverage: 37%
Error: 57ppm
Peptides matched: 9

AGAP004031 – spot B8

>gi|158298314|ref|XP_318487.3| AGAP004031-PA [Anopheles gambiae str. PEST]
ILRASAKFVNGINCFRQGFRRFQSTLVLAEHNNETLNPITANAVTAAKKLGGDVTVLVA
GTKVGPVSEAAAKLDGVKKVLVAEGDAYKGLLAESLTPLILATQEQLKFTHIVAGATAF
GKAVLPRIAAKLDVSPVSDIIGVQSADTFVRTIYAGNAIQTVKSKDPVKVITVRGTNFE
PTGAAGSAAAIEKAPEGDFASKTTEFVSQELTKSDRPSLTAAKIIVSGGRGMKSGDNFK
MLYDLADKWGAAVGASRAAVDAGYVPNDLQIGQTGKIVAPEVYVAIGISGAIQHLAGMK
DSKTIVAINKDPEAPIFQVADYGLVADLFKVVPEINEKC

Socre: 191
Sequence coverage: 34%
Error: 67ppm
Peptides matched: 8

AGAP001420 – spot B9

>gi|58396165|ref|XP_321710.2| AGAP001420-PA [Anopheles gambiae str. PEST]
MAAKYRIVMVRHGESEWNQKNLFCGWFDANLSDKGKEEALAAGKAVKEAGLKFDIAHTS
LLTRAQVTLDSILKESGQTSIPIQKTWRLNERHYGGLTGLNKSETAAKYGEEQVLIWRR
SFDVPPPNMEPDHAYYDAIVKDERYKDDPKPNEFPMAESLKLTIARTLPYWNDVIIPQL
KEGKNIIIAAHGNSLRGIVKHLDQMTDEAIMGLNLPTGIPFVYELDENLKPVVSMKFLG
DEETVRKAIESVANQGKAK

Socre: 141
Sequence coverage: 28%
Error: 316ppm
Peptides matched: 7

AGAP002401 – spot B10

>gi|31207169|ref|XP_312551.1| AGAP002401-PA [Anopheles gambiae str. PEST]
MALSDADVQKQIKHMMAFIEQEANEKAEEIDAKAEEEFNIEKGRLVQQQRLKIMEYYEK
KEKQVELQKKIQSSNMLNQARLKVLKVREDHVSNVLDECRRRLGEVTKDPARYGEILTA
LITQGLLQLMEAKVLIRGRQADAQVIQNVLPAAVELYKSKCGRDVVVTLDTENFLPADT
TGGVDLLAQSGRIKVANTLESRLELIAQQLVPEIRNALFGRNMNRKFND

Socre: 51
Sequence coverage: 12%
Error: 627ppm
Peptides matched: 3

AGAP005645 – spot B11

>gi|158294534|ref|XP_315663.4| AGAP005645-PA [Anopheles gambiae str. PEST]
MDRWNGKVAVVTGASSGIGAEIAKDLAKAGMITIGLARRVERVEQLKQQLPKEAANRLH
AMKCDVSIETDIERTFQRIADTYGGVDVLVNNAGIVRQNNLLDLGTAADLRAVLDTNVT
GLVLCSQWAYKSMVDRKVDGHIVHISSIAGHSVPNFPKLNIYPGTKHAVRAITETMRHE
MRDAGTKIKVTSVSPGAVKTEILDGVPIPEEMPLLEAEDISAAVLYAIGTPPHVQVHEL
IIKPVGEVM

Socre: 243
Sequence coverage: 39%
Error: 39ppm
Peptides matched: 7

Annex 4

Real-Time amplification of mosquito genes.

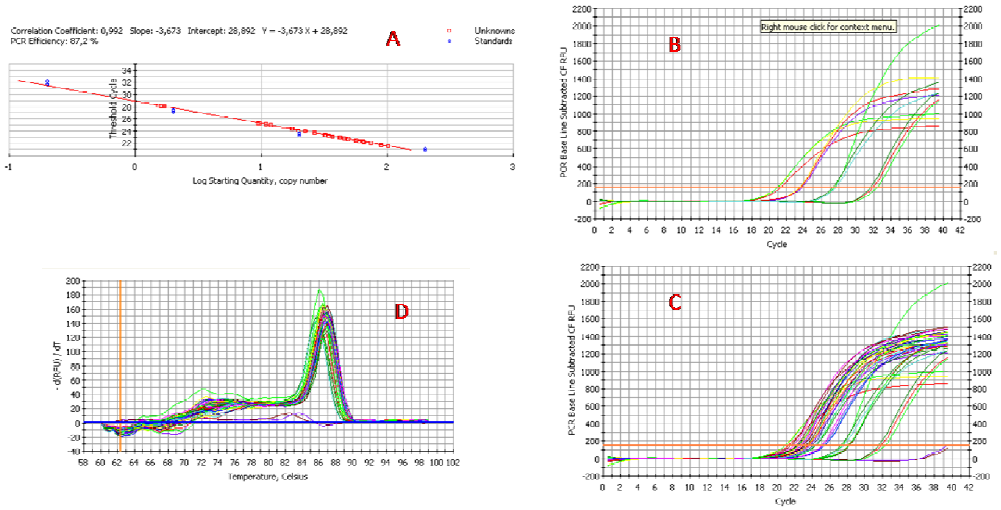
A - standard curve and linear regression analysis - DNA standard curve samples represented as blue dots, hemolymp samples represented by red dots.

B – amplification curves of DNA standard samples.

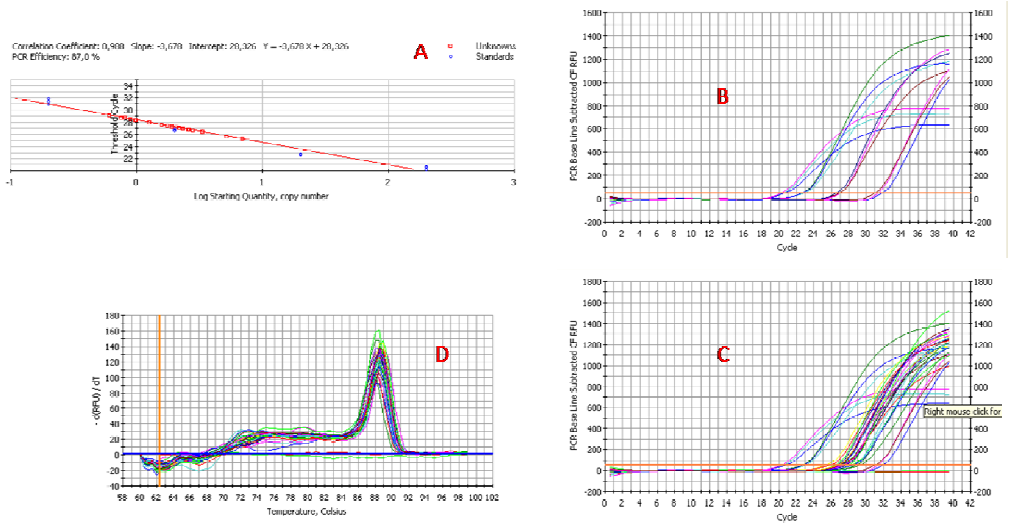
C – amplification curves of hemolymph samples.

D – melting curve.

AgS7



AgCLIPA2



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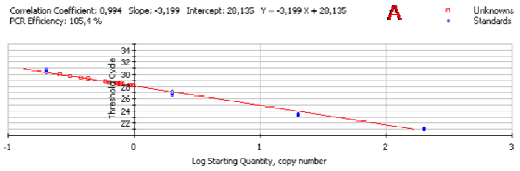

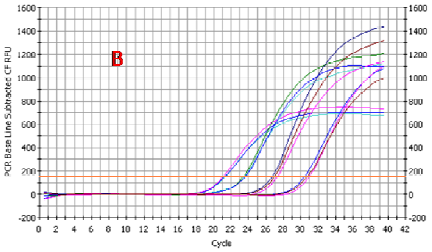

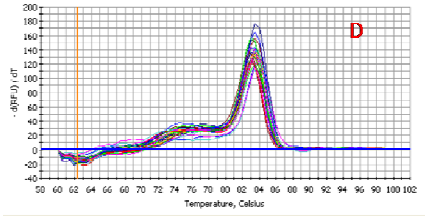

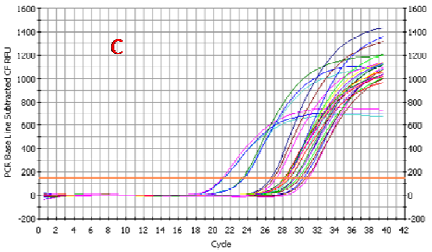

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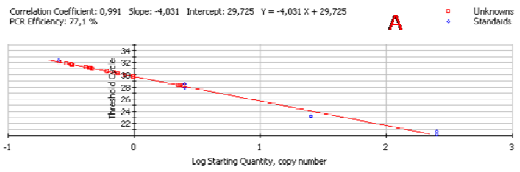

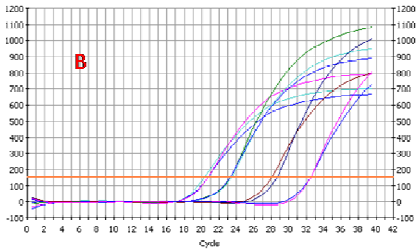

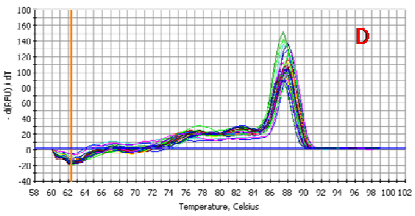

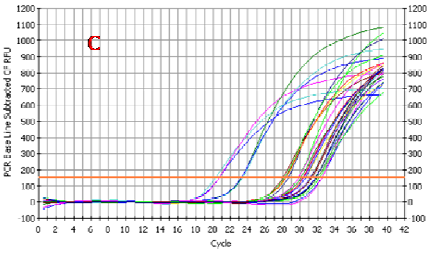

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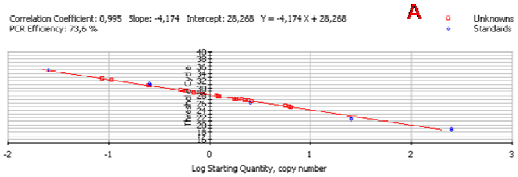

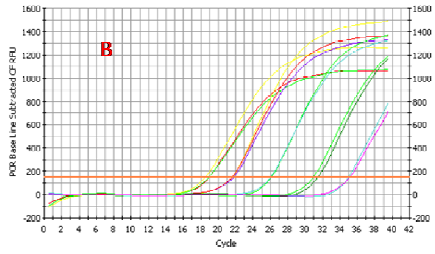

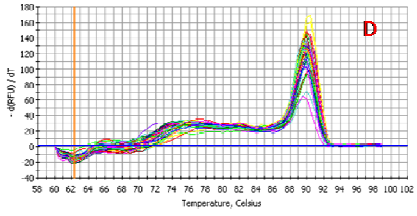

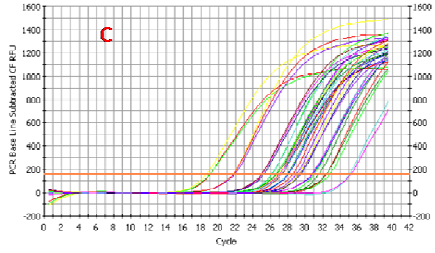

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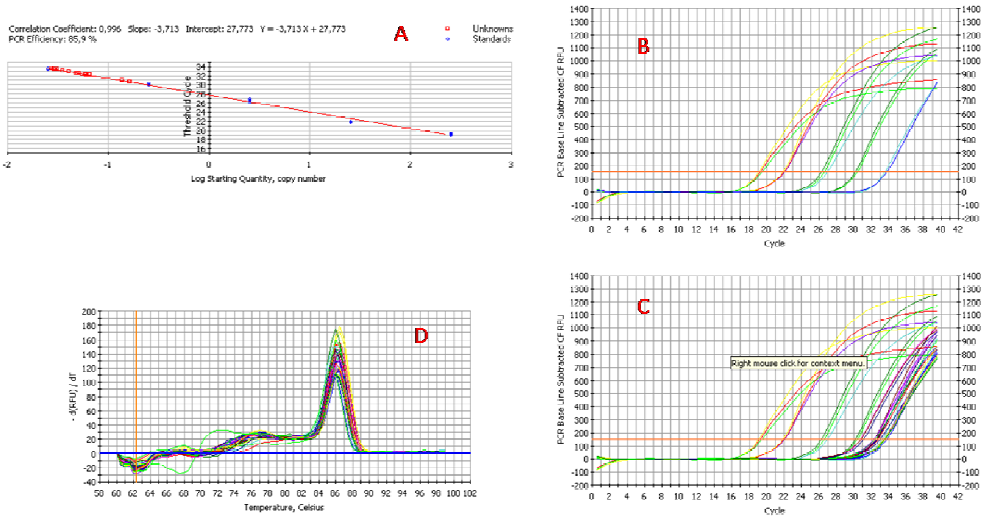

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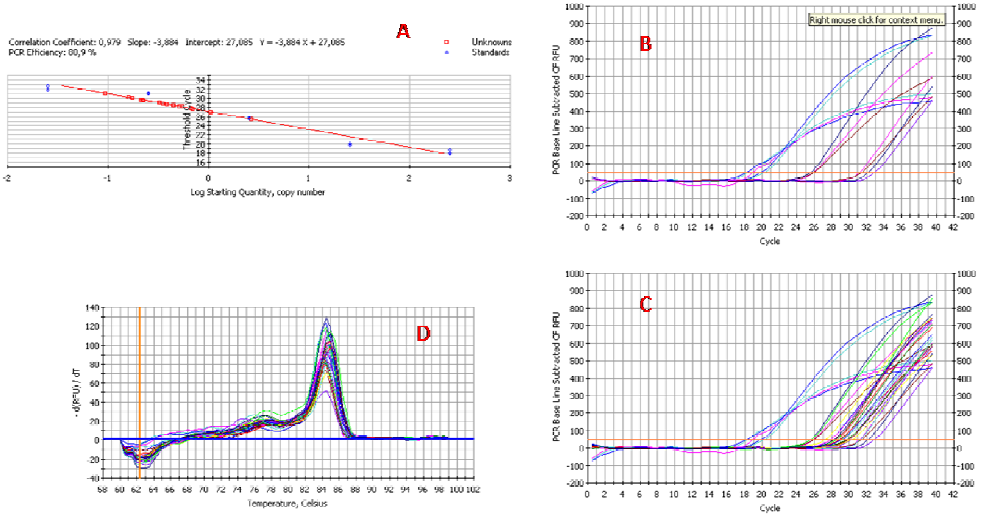

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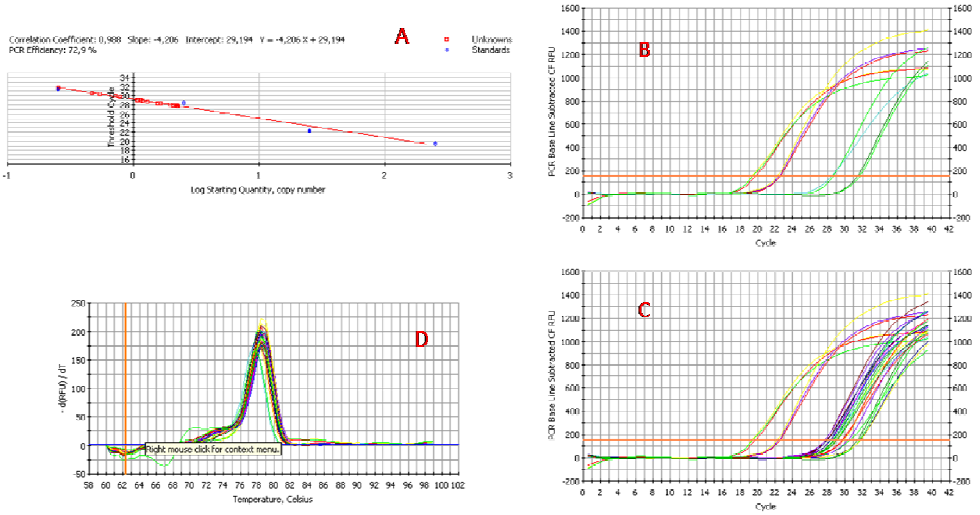

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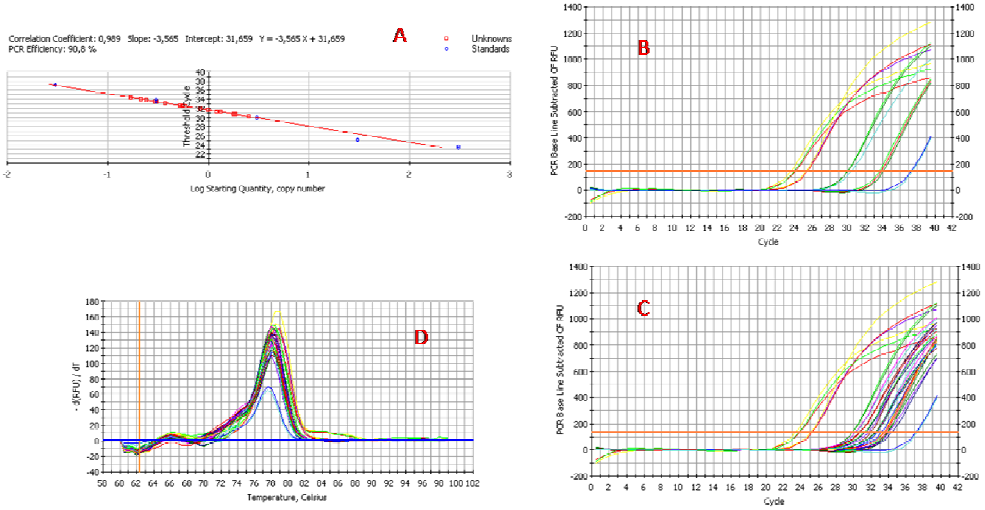

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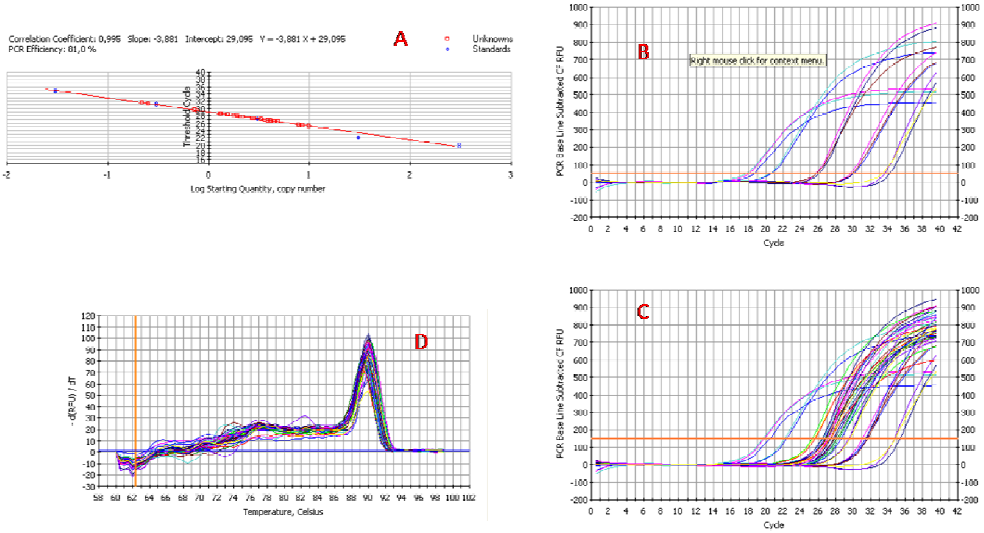

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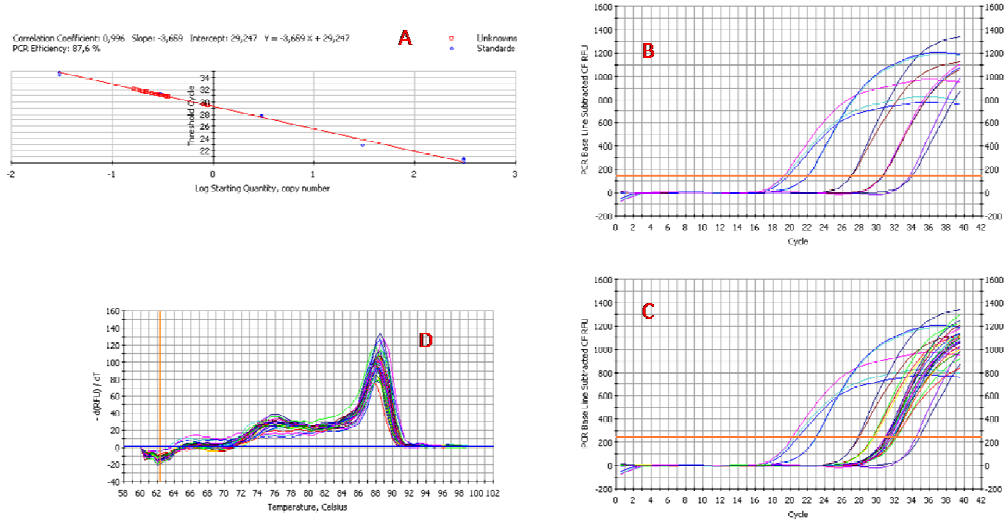

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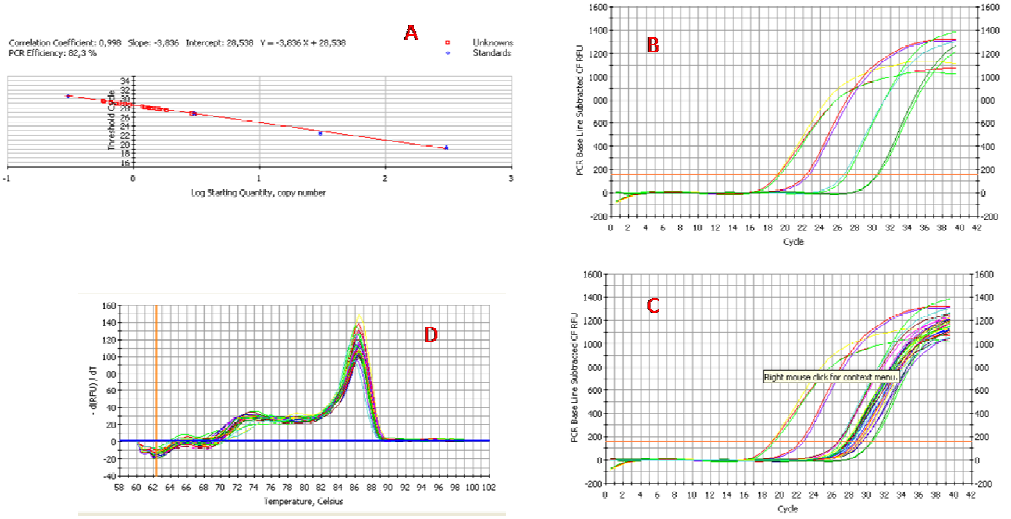

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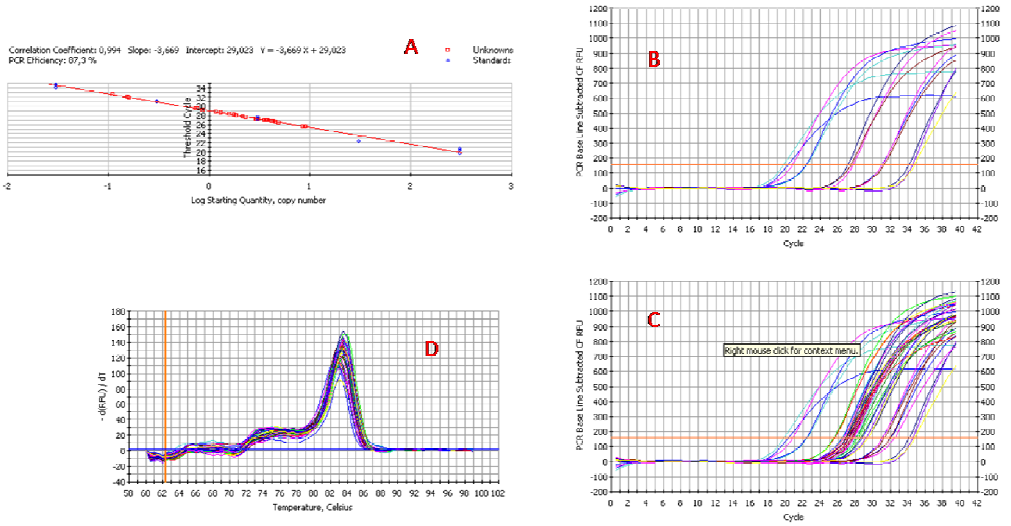

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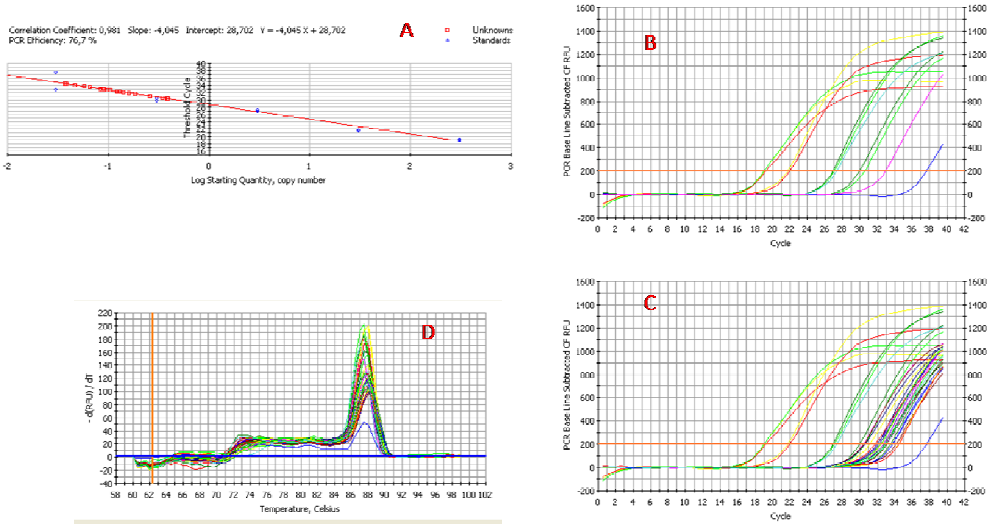

Annex 5

Composition of gels used to separate DNA, RNA and proteins, for *Plasmodium* detection, Northern blot and Western blot, respectively.

Standard agarose gel for DNA separation	1% agarose gel	**DNA sample**
TBE buffer (10X)	25ml	1µl
Agarose	2,5g	
dH_2O	225ml	
Total volume	**250ml**	

Denaturing agarose gel for RNA separation	1% agarose gel	**RNA sample prepration**	
MOPS buffer (10X)	30ml	RNA sample	20µg
Agarose	3g	Loading Buffer	8µl
MiliQ H_2O	216ml	Heat at 70ºC for 10min	
Heat to dissolve		Keep on ice	
Formaldeheyde	54ml		
Total volume	**300ml**		

SDS-PAGE for protein separation	Stacking gel (4%)	Resolving gel (10%)	Resolving gel (12%)
Stacking buffer (4x)	2,000ml	-	-
Resolving buffer (4x)	-	2,500ml	2,500ml
Acryl/Bisacrylamide 37:1 (40%)	0,800ml	2,500ml	3,000ml
MiliQ H_2O	5,140ml	4,895ml	4,395ml
APS (10%)	50µl	100µl	100µl
TEMED	10µl	5µl	5µl
Total volume	**8ml**	**10ml**	**10ml**

Sample preparation	
Protein sample	2µg
MiliQ H_2O	Up to 8µl
Laemmli buffer (5x)	2µl
Heat at 95ºC for 3min	
Short spin	
Keep on ice	